Molecular mechanism of m6A methylation of circDCL1 mediated by RNA methyltransferase METTL3 in the malignant proliferation of glioma cells

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Research Article

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Abstract

Glioma is an intracranial malignant tumor and remains largely incurable. circular RNAs are prominent modulators in glioma progression. This study investigated the function of circular RNA DLC1 (circDCL1) in the malignant proliferation of glioma cells. circDCL1 expression in glioma tissues and cells was determined using RT-qPCR. The effect of circDCL1 on the malignant proliferation of glioma cells was analyzed using CCK-8, colony formation, and EdU staining assays. METTL3, miR-671-5p, and CTNNBIP1 expressions were determined. N⁶ methyladenosine (m⁶A) level of circDCL1 was analyzed using MeRIP. The binding relationship between miR-671-5p and circDCL1 or CTNNBIP1 was verified using RNA pull-down and dual-luciferase assays. A xenograft tumor model was established in nude mice to verify the effect of METTL3-mediated circDCL1 on glioma in vivo. circDCL1 was poorly expressed in glioma. circDCL1 overexpression suppressed glioma cell proliferation, while circDCL1 silencing showed an opposite trend. Mechanically, METTL3-mediated m⁶A modification enhanced circDCL1 stability and upregulated circDCL1 expression in glioma. circDCL1 upregulated CTNNBIP1 transcription by competitively binding to miR-671-5p. METTL3 overexpression repressed the malignant proliferation of glioma via circDCL1/miR-671-5p/CTNNBIP1 in vivo. Collectively, METTL3-mediated m⁶A modification upregulated circDCL1 expression, and circDCL1 promoted CTNNBIP1 transcription by sponging miR-671-5p, thus repressing the malignant proliferation of glioma.

Introduction

Glioma is a heterogeneous group of primary malignancies of the central nervous system, which is commonly graded I-IV according to histology and the degree of tumor invasion to surrounding tissues [1]. The most prevalent histological types of glioma in adults include astrocytomas, glioblastoma multiform, oligodendrogliomas, and mixed glioma [2]. Glioma is notorious for its rapid cell proliferation and angiogenesis, and particularly the tumors cells are resistant to conventional treatments, resulting in poor outcomes and a high incidence of relapse [3]. In addition to rapid proliferation, wide invasion, and drug resistance, the dismal outcomes of glioma patients also stem from the deficient knowledge of molecular pathogenesis and the lack of timely and effective diagnostic and therapeutic monitoring strategies [4]. Consequently, investigations on the precise molecular mechanism of glioma malignant proliferation and potent therapeutic targets have aroused extensive concerns.

circular RNAs (circRNAs), a novel class of covalently linked single-stranded RNAs with a closed loop structure, are primarily generated by back-splicing of transcription products [5]. Current evidence has unveiled that circRNAs are abundantly expressed in the brain tissues compared with other tissues, indicating their critical roles in various brain diseases [6]. circRNAs have differential expressions in glioma and participate in the modulation of glioma occurrence and development [7]. circDLC1 is derived from exons 14, 15, and 16 of the DLC1 gene, and circDLC1 overexpression is demonstrated to repress the proliferation of hepatoma cells [8]. Nevertheless, the exact role of circDLC1 in glioma remains unknown.

Recently, N⁶-methyladenosine (m⁶A) modification is emphasized for its abundant enrichment and critical biological functions in circRNAs [9]. m⁶A modification emerges as methylation at the N⁶ position of adenosine, representing the most prevalent internal modification in eukaryotes mRNA [10]. The aberrant alternation of m⁶A level affects RNA maturation, transcription, translation, and metabolism, which disrupts gene expression and vital cellular processes, and ultimately leads to the initiation and progression of multiple tumors, including glioma [11]. Methyltransferase-like 3 (METTL3) is identified as one of the "writers" for m⁶A methylation that participate in regulating various cellular biological processes [12]. Notably, METTL3 is poorly expressed in glioma tissues, and METTL3 deficiency facilitates glioma cell proliferation [13]. METTL3 is also implicated in the biogenesis and function of circRNAs by mediating m⁶A modification [9]. However, the mechanism of METTL3-mediated m⁶A modification on circDCL1 in glioma has not been investigated before.

Mechanistically, circRNAs can modulate the expression of genes implicated in tumorigenesis and progression by serving as a competitive endogenous RNA (ceRNA) of microRNA (miRNA) [14]. miRNAs are a class of small endogenous non-coding RNAs (18-25 nt in length) that modulate gene expression at the post-transcriptional level [15]. miRNA expression profile affects the functions of genes implicated in gliomagenesis, tumor growth, therapeutic response, and prognosis which is accepted as a prospective therapeutic tool against malignant glioma [16]. Therefore, this study probed into the effect of METTL3-mediated m⁶A methylation of circDCL1 on the malignant proliferation of glioma cells and the downstream ceRNA mechanism of circDCL1, hoping to shed light on glioma treatment.

Materials And Methods

Ethics statement

This study was approved by the Ethical Committee of The Second Hospital of Shanxi Medical University and in accordance with *Helsinki Declaration*. All the animal experiments were implemented based on the *Guide for the Care and Use of Laboratory Animals* [17].

Clinical sample collection

We collected the tumor tissues of 40 patients (at an average age of 54.35 ± 9.52 ; 21 males and 19 females) with primary glioma and corresponding adjacent tissues in The Second Hospital of Shanxi Medical University from June 2016 to June 2018 and stored in liquid nitrogen at -80°C. None patients received chemotherapy or radiotherapy before. The histological features of collected tissues were independently diagnosed by 2 pathologists.

Cell culture

Human glioma cell lines (T98G, LN229, A172, and LN18) and healthy glioma cell line (HEB) were obtained from American Type Culture Collection (Manassas, Virginia, USA) and cultured in RPMI-1640 medium (HyClone, South Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (Invitrogen,

Carlsbad, CA, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO₂.

Cell treatment

Three siRNAs specifically targeting circDCL1, circDCL1 pcDNA 3.1, METTL3 pcDNA 3.1, three siRNAs specifically targeting catenin beta interacting protein 1 (CTNNBIP1), and their negative controls (NCs) were designed and synthesized by GenePharma (Shanghai, China). Finally, these vectors were transfected into A172 or LN229 cells using Lipofectamine 3000 (Thermo Fisher Scientific).

Cell counting kit-8 (CCK-8) assay

A172 and LN229 cells were seeded into 96-well plates (2000 cells/well) and cultured for 0, 24, 48, and 72 h. Each well was added with 10 µL CCK-8 reagent (Beyotime, Beijing, China) for 2-h incubation at 37°C. The absorbance at the wavelength of 450 nm was measured using a microplate reader (Bio-Rad, Philadelphia, PA, USA).

Colony formation assay

A172 or LN229 cells (200-300 cells) were seeded into the petri dish in humidified air at 37°C with 5% CO₂ for 10 d. After the removal of the complete medium, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 20 min. Colony cells were counted under an optical microscope (Nikon, Tokyo, Japan).

5-ethynyl-2'-deoxyuridine (EdU) staining

A172 and LN229 cells were seeded into 96-well plates (20000 cells) and cultured in the medium (100 µL/well, ThermoFisher) containing 50 µM EdU for 2 h (5% CO₂, 37°C). EdU binding detection kit (Ribobio, Guangzhou, Guangdong, China) was used for proliferation evaluation. Briefly, the cells were treated with 4% paraformaldehyde for 30 min and stained with 100 µL Apollo dye solution (Ribobio) for 30 min. The nuclei were stained with DAPI (ThermoFisher) for 30 min, followed by observation under the fluorescence microscope (Olympus, Tokyo, Japan). The final results are presented as a percentage.

Actinomycin D treatment

A172 or LN229 cells were seeded into 24-well plates (5×10^5 cells/well). After 24 h, the cells were treated with 1 µg/mL actinomycin D for 0, 4, 8, 12, 24, 36, 48, and 72 h. The relative RNA expressions of circDCL1 and DLC1 were analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

RNase R treatment assay

The total RNA of A172 and LN229 cells was extracted using TRIzol reagent (Invitrogen). Then, 3 µg total RNA was incubated with 10 U RNase R (20 U/µL, Epicentre, Madison, WI, USA) at 37°C for 30 min and at 75°C for 10 min to deactivate the RNase R, followed by RT-qPCR.

m⁶A level detection

The m6A level in total RNA was measured using m6A RNA methylation quantitative kit (ab185912, Abcam Inc., Cambridge, MA, USA). Firstly, RNA was coated in the detection well at 37°C for 90 min, then added with capture antibody (50 µL), detection antibody (50 µL), and enhancer solution (50 µL) respectively, and incubated at room temperature for 60 min. Finally, the chromogenic solution was added for signal detection and absorbance measurement. The m6A level was measured using the colorimetry method. The absorbance of each well at 450 nm was read and calculated according to the standard curve. The final results were displayed as relative values.

m⁶A RNA immunoprecipitation (MeRIP) assay

MeRIP was performed using Magna MeRIP m6A kit (Millipore, Billerica, MA, USA) to detect the m6A level of circDCL1. Briefly, 3 µg anti-m6A antibody (ab208577, Abcam) was incubated with protein A/G magnetic beads at 4°C overnight. Then, the conjugate was incubated with RNA in the IP buffer containing RNase inhibitor and protease inhibitor. RNA was isolated and detected using RT-qPCR.

Nuclear/cytosol fractionation assay

After rinsing with pre-cooled phosphate-buffered saline (PBS), 1×10^6 A172 or LN229 cells were resuspended in 1 mL RLN buffer (50 mM Tris-HCl pH 7.4, 0.14 M NaCl, 1.5 mM MgCl₂, 0.5% IGEPAL CA-630, and 1 mM DTT), incubated on ice for 5 min, and then homogenized. The cytoplasm was the supernatant after centrifugation. The precipitate was resuspended in 1 mL RSB buffer (0.25 M sucrose, 10 mM Tris-HCl pH7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride), homogenized, and centrifuged to remove the residual cytoplasm. The preliminary nucleus was resuspended in 3 mL 2 M RSB (2 M sucrose, 10 mM Tris-HCl pH7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, and 0.5 mM PMSF) and centrifuged at 3000 g for 45 min. The isolated circDCL1 was subjected to RT-qPCR analysis, with U6 and GAPDH as nuclear and cytoplasmic controls, respectively [18].

Dual-luciferase assay

The binding site of circDCL1 and miR-671-5p was predicted on the Starbase database, and the binding site of miR-671-5p and CTNNBIP1 was predicted on the Targetscan database. Wild-type (WT) circDCL1 or CTNNBIP1 containing miR-671-5p binding sequence was synthesized and inserted into the pmirGLO vector (Promega, Madison, WI, USA) and named circDCL1 WT or CTNNBIP1 WT. Mutant-type (MUT) circDCL1 or CTNNBIP1 containing miR-671-5p mutant binding sequence was synthesized and inserted into the pmirGLO vector, named circDCL1 MUT or CTNNBIP1 MUT. The constructed plasmids were co-transfected with miR-671-5p mimic or mimic NC into A172 and LN229 cells using Lipofectamine 3000, respectively. The luciferase activity was measured after 48 h using a luciferase detection kit (Promega).

RNA pull-down assay

RNA pull-down assay was performed to explore the interaction between miR-671-5p and circDCL1 or CTNNBIP1 in A172 and LN229 cells. miR-671-5p and miR-NC were biotinylated using the biotin RNA Labeling Mix (Roche, Indianapolis, IN, USA) and transcribed using T7/SP6 RN polymerase (Roche). Then, biotin-labeled miR-671-5p (bio-miR-671-5p) and bio-miR-NC were treated with RNase free DNase I

(Promega) and RNeasy Mini kit (Qiagen, Redwood City, CA, USA). Next, M-280 streptavidin magnetic beads were incubated with bio-miR-671-5p or bio-miR-NC at room temperature for 4 h, and then with A172 and LN229 cell lysates. The RNA binding to bio-miR-671-5p or bio-miR-NC was extracted with TRIzol reagent for RT-qPCR.

Xenograft tumor experiments

Male BALB/c nude mice (aged 6 weeks old; weighing 20 g) purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) [SYXK (Beijing) 2017-0033] were raised in a standard animal room and maintained under a 12-h light/dark cycle, with food and water provided ad libitum. LN229 cells were infected with the lentiviral overexpression vector of METTL3 (LV-oe-METTL3) or NC (LV-oe-NC) obtained from Genechem (Shanghai, China). Afterward, puromycin was used to screen the stably expressed cells. LN229 cells (5×10^5) were injected into the posterior flank of mice subcutaneously and tumor growth was observed. The tumor size was measured every 3 days and the tumor volume was calculated: volume (mm^3) = (length $\times$ width²)/2. The mice were sacrificed by intraperitoneal injection of 1% pentobarbital sodium (150 mg/kg) 21 days after injection. The tumors were removed and weighed. The tumor tissue was fixed with 4% paraformaldehyde and embedded in paraffin for immunohistochemistry. The expression of cytokines in tumor homogenate was detected using RT-qPCR.

Immunohistochemistry

The tumor tissue of mice was fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm sections. After dewaxing and dehydration, the expressions of METTL3 and Ki-67 were analyzed using immunohistochemistry. In short, the sections were incubated with METTL3 antibody (ab195352, Abcam) and Ki67 antibody (ab15580, Abcam) at 4°C overnight. After PBS washing, the sections were incubated with the secondary antibody (ab6721, Abcam) at 37°C for 20 min. After 2,4-diaminobutyric acid development (10 min) and hematoxylin counterstaining (3 min), the sections were dehydrated with gradient alcohol, cleared with xylene, and sealed with neutral resin, following observation under the microscope. Five visual fields were randomly selected from the section, and the positive rate of each visual field was calculated [19].

RT-qPCR

Total RNA was extracted from glioma tissues, transplanted tumor tissues, and cells using the TRIzol reagent (Invitrogen) and 2 μg RNA was reverse transcribed into cDNA using SuperScript RT kit (Invitrogen). RT-qPCR was performed using SYBR Premix ExTaqTM (Takara, Kyoto, Japan) and Applied Biosystems 7300 system (Applied Biosystems, Inc., Carlsbad, CA, USA). PCR primers are shown in Table 1. The relative expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [20], with GAPDH and U6 as internal references [21].

Table 1
PCR primer sequences

Name	Sequences (5'-3')
circDLC1	F: TATGGTGAGGGTCATGAAGGA
	R: ACCAAATTACGAAGTGGTCGC
METTL3	F: TGGGGGTATGAACGGGTAGA
	R: TGGTTGAAGCCTTGGGGATT
DLC1	F: AGCTTTTCCAGGTTCCCGAG
	R: AGGGGCTTCAGCTCTTGTTT
miR-671-5p	F: ATGAGTAGGAAGCCCTGGAG
	R: TCAACTGGTGTCGTGGAGTC
CTNNBIP1	F: TATGCAGGGGTGGTCAACAG
	R: GACCTGGAAAACGCCATCAC
GAPDH	F: ATGGTTTACATGTTCCAATATGA
	R: TTAATCCTTGGAGGCCATGTGG
U6	F: TCGCTTCGGCAGCACATATACT
	R: GCTTCACGAATTTGCGTGTCATC

Western blot

Total protein was extracted from glioma tissues and cells using radio-immunoprecipitation assay buffer. The extract was centrifuged at 4°C and 12000 g for 15 min to collect the supernatant and the protein concentration was determined using bicinchoninic acid kits (Beyotime). The protein was separated using 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% skim milk for 1 h and incubated with the primary antibodies METTL3 (ab195352, 1:1000, Abcam) and GAPDH (ab9485, 1:2000, Abcam) at 4°C overnight. Following tris-buffered saline-tween buffer washing, the membranes were incubated with the secondary antibody (ab6721, 1:2000, Abcam) at 37°C for 1 h. NIH Image J (National Institutes of Health, Bethesda, Maryland, USA) was used to analyze the gray value, with GAPDH as internal reference.

Bioinformatics analysis

The downstream miRNAs of circDCL1 were predicted through the Starbase database (<http://starbase.sysu.edu.cn/index.php>) [22]. The downstream genes of miR-671-5p were predicted through the Starbase database, Targetscan database (http://www.targetscan.org/vert_71/) [23], RNA22 v2 database (<https://cm.jefferson.edu/rna22/Precomputed/>) [24], and miRDB database (<http://mirdb.org/index.html>) [25].

Statistical analysis

Data analysis and map plotting were performed using the SPSS 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). The data complied with the assumption of normality and homogeneity of variance. Data are expressed as mean $\pm$ standard deviation. The *t* test was adopted for comparisons between two groups. One-way or two-way analysis of variance (ANOVA) was employed for the comparisons among multiple groups, following Tukey's multiple comparison test. A value of $p < 0.01$ indicated a significant difference.

Results

circDCL1 was poorly expressed in glioma tissues and cells

circRNA has become a research hotspot in the field of glioma molecular biology with the characteristics of high content and good stability [26]. circDCL1 is poorly expressed in hepatocellular carcinoma [8], but its role in glioma is unclear. Therefore, we detected circDCL1 expression in collected glioma tissues and adjacent tissues. The results exhibited that circDCL1 expression was reduced in glioma tissues ($p < 0.01$, Fig. 1A). Then, circDCL1 expression in human glioma cell lines (T98G, LN229, A172, and LN18) and healthy glioma cell line (HEB) was detected, and circDCL1 was also poorly expressed in glioma cells ($p < 0.01$, Fig. 1B). In addition, circDCL1 level had no significant change after actinomycin D treatment, but linear DCL1 level was decreased notably after actinomycin D treatment ($p < 0.01$, Fig. 1C), and RNase R treatment assay showed that circDCL1 expression did not change significantly, but linear DCL1 expression was reduced significantly after RNase R incubation, indicating that circDCL1 was more stable than linear DCL1 ($p < 0.01$, Fig. 1D). Briefly, the above results demonstrated that circDCL1 was poorly expressed in glioma tissues and cells.

Overexpression of circDCL1 suppressed malignant proliferation of glioma cells

To explore the effect of circDCL1 on the malignant proliferation of glioma cells, we intervened in the expression of circDCL1. circDCL1 pcDNA 3.1 (pc-circDCL1) was transfected into LN229 cells with relatively low circDCL1 expression and circDCL1 expression in LN229 cells was successfully upregulated ($p < 0.01$, Fig. 2A); circDCL1 siRNA (si-circDCL1) was transfected into A172 cells with relatively high circDCL1 expression and circDCL1 expression in A172 cells was successfully downregulated ($p < 0.01$, Fig. 2A), and si-circDCL1#1 with the best intervention efficiency was selected for subsequent detection. CCK-8 and colony formation assays showed that circDCL1 overexpression decreased glioma cell proliferation and circDCL1 silencing enhanced glioma cell proliferation ($p < 0.01$, Fig. 2B-C). EdU assay results further confirmed this conclusion ($p < 0.01$, Fig. 2D). Briefly, overexpression of circDCL1 suppressed the malignant proliferation of glioma cells.

METTL3-mediated m⁶A modification enhances circDCL1 stability and upregulates circDCL1 expression

m⁶A RNA methylation greatly affects RNA metabolism and plays a key role in cancer through a variety of mechanisms [27, 28]. m⁶A modification exists in circDCL1 [8]. METTL3, as an m⁶A modifying enzyme, is poorly expressed in glioma [28, 13]. We speculated that METTL3 regulated circDCL1 expression through m⁶A modification. RT-qPCR results exhibited that METTL3 expression was reduced in glioma tissues and cells ($p < 0.01$, Fig. 3A-B), and Western blot results confirmed the same trend ($p < 0.05$, Fig. 3C-D). m⁶A quantitative analysis showed that the m⁶A level in glioma tissues and cells was consistent with the trend of METTL3 expression ($p < 0.01$, Fig. 3E-F). MeRIP showed that the m⁶A level of circDCL1 was decreased in glioma tissues and cells ($p < 0.01$, Fig. 3G-H). In 40 cases of glioma tissues, METTL3 expression was positively correlated with circDCL1 expression ($p < 0.01$, Fig. 3I). To verify the regulatory effect of METTL3 on circDCL1, we transfected METTL3 pcDNA 3.1 (pc-METTL3) into glioma cells and METTL3 expression in cells was elevated significantly ($p < 0.05$, Fig. 3J-K). With the increase of METTL3 expression, m⁶A level in cells, circDCL1 expression, and m⁶A level of circDCL1 were also increased ($p < 0.01$, Fig. 3L-N). In addition, the half-life of circDCL1 was prolonged after overexpression of METTL3 ($p < 0.01$, Fig. 3O). Briefly, METTL3-mediated m⁶A modification upregulated circDCL1 expression by increasing the stability of circDCL1.

Overexpression of METTL3 annulled the promoting effect of circDCL1 silencing on the malignant proliferation of glioma cells

To verify the role of METTL3 in the regulation of malignant proliferation of glioma cells by circDCL1, we designed a combined experiment: A172 cells were treated with METTL3 overexpression and circDCL1 silencing. Compared with circDCL1 silencing alone, the combined treatment of METTL3 overexpression + circDCL1 silencing increased circDCL1 expression in glioma cells ($p < 0.01$, Fig. 4A) and decreased glioma cell proliferation ($p < 0.01$, Fig. 4B-C). EdU staining showed that compared with the si-circDCL1 + pc-NC group, the cells in the si-circDCL1 + pc-METTL3 group had enhanced proliferation ($p < 0.01$, Fig. 4D). Briefly, overexpression of METTL3 annulled the promoting effect of circDCL1 silencing on the malignant proliferation of glioma cells.

circDCL1 promoted CTNNBIP1 transcription by competitively binding to miR-671-5p

Next, we further explored the downstream mechanism of circDCL1. Firstly, nuclear/cytosol fractionation assay demonstrated that circDCL1 was mainly localized in the cytoplasm of glioma cells (Fig. 5A). Then, the downstream miRNAs of circDCL1 were predicted, in which miR-671-5p was highly expressed in glioma [29, 30]. The target genes of miR-671-5p were predicted and intersected (Fig. 5B), in which

CTNNBIP1 was poorly expressed in glioma [31, 32]. Therefore, we speculated that the miR-671-5p/CTNNBIP1 axis was a potential downstream mechanism of circDCL1. To verify our hypothesis, we identified the binding sites between circDCL1 and miR-671-5p, and miR-671-5p and CTNNBIP1 through the database (Fig. 5C), and designed a dual-luciferase assay. The results confirmed that there were binding relationships between circDCL1 and miR-671-5p, and miR-671-5p and CTNNBIP1 ($p < 0.01$, Fig. 5D). Meanwhile, RNA pull-down assay further emphasized the binding relationship between miR-671-5p and circDCL1 or CTNNBIP1 ($p < 0.01$, Fig. 5E). RT-qPCR results exhibited that miR-671-5p expression was elevated, while CTNNBIP1 expression was reduced in glioma tissues and cells ($p < 0.05$, Fig. 5F-G). circDCL1 overexpression decreased miR-671-5p expression and circDCL1 silencing increased miR-671-5p expression, while miR-671-5p expression was decreased after the combined treatment of METTL3 overexpression and circDCL1 silencing; the expression trend of CTNNBIP1 was opposite to that of miR-671-5p ($p < 0.01$, Fig. 5H). In 40 cases of glioma tissues, miR-671-5p expression was negatively correlated with circDCL1 expression, and CTNNBIP1 expression was positively correlated with circDCL1 expression ($p < 0.01$, Fig. 5I). Briefly, circDCL1 promoted CTNNBIP1 transcription by competitively binding to miR-671-5p.

CTNNBIP1 silencing attenuated the inhibitory effect of circDCL1 overexpression on malignant proliferation of glioma cells

Then, we verified the regulatory effect of CTNNBIP1 on glioma cells. After three CTNNBIP1 siRNAs (si-CTNNBIP1) were transfected into LN229 cells, CTNNBIP1 mRNA expression in LN229 cells was decreased ($p < 0.01$, Fig. 6A). We chose si-CTNNBIP1#2 and pc-circDCL1 to treat LN229 cells for a combined experiment. Our results showed that the combined treatment of si-CTNNBIP1#2 and pc-circDCL1 significantly promoted the proliferation of glioma cells ($p < 0.05$, Fig. 6B-D). Briefly, CTNNBIP1 silencing attenuated the inhibitory effect of circDCL1 overexpression on the malignant proliferation of glioma cells.

METTL3 suppressed the malignant proliferation of glioma cells via the circDCL1/miR-671-5p/CTNNBIP1 axis in vivo

Finally, we verified the regulation of METTL3-mediated circDCL1 on the malignant proliferation of glioma cells *in vivo*. Overexpression of METTL3 suppressed the tumor growth, manifested as reduced tumor volume and weight ($p < 0.01$, Fig. 7A-B), and decreased positive rate of Ki67 ($p < 0.01$, Fig. 7C). Compared with the LV-oe-NC group, the LV-oe-METTL3 group showed increased METTL3 expression ($p < 0.01$, Fig. 7D-E), elevated m6A level and circDCL1 expression ($p < 0.01$, Fig. 7F-G), reduced miR-671-5p expression ($p < 0.01$, Fig. 7H), and enhanced CTNNBIP1 expression ($p < 0.01$, Fig. 7I). Briefly, METTL3 suppressed the malignant proliferation of glioma cells via the circDCL1/miR-671-5p/CTNNBIP1 axis *in vivo*.

Discussion

Glioma presents a challenging clinical situation that causes notable mortality and morbidity [1]. circRNAs make a significant contribution to the regulation of cell cycle, angiogenesis, metastasis, and drug resistance of glioma cells [33]. This study elucidated that METTL3-mediated circDCL1 promoted CTNNBIP1 transcription by sponging miR-671-5p, thus repressing the malignant proliferation of glioma cells (Fig. 8).

It has been revealed that patients with lower circDLC1 expression exhibit a more advanced hepatocellular carcinoma stage, indicating that circDLC1 is correlated with hepatocellular carcinoma progression [8]. Nevertheless, the role of circDLC1 in glioma remained unknown. Herein, we collected 40 pairs of glioma tissues and adjacent tissues and cultured human glioma cell lines and healthy glioma cell lines, and found that circDLC1 was poorly expressed in glioma tissues and cells. Unlike linear RNAs, the circular structure of circRNAs contributes to their stability in tissues and ensures their capacity to participate in various biological events [34]. Consistently, we found that circDLC1 level did not change notably after actinomycin D or RNase R treatment, but linear DLC1 level was reduced significantly, indicating that circDLC1 was more stable than linear DLC1. To investigate the effect of circDLC1 on the malignant proliferation of glioma cells, we intervened in circDLC1 expression in glioma cells. pc-circDLC1 was transfected into LN229 cells with relatively low circDLC1 expression and si-circDLC1 was transfected into A172 cells with relatively high circDLC1 expression. Our results exhibited that circDLC1 overexpression suppressed glioma cell proliferation, while circDLC1 silencing showed an opposite trend. Briefly, we were the first to demonstrate that circDLC1 expression was reduced in glioma tissues and cells, and circDLC1 overexpression suppressed the malignant proliferation of glioma cells.

The biogenesis and functions of circRNAs can be mediated by m⁶A modification and m⁶A mediated-circRNAs are implicated in a variety of cellular and physiological processes, which may confer potential therapeutic targets for human malignancies [35]. circDLC1 has been identified as a circRNA regulated by m⁶A modification in hepatocellular carcinoma pathogenesis [8]. The reduction of m⁶A level in glioma tissues can be partially attributed to the decreased METTL3 level [28]. METTL3 is a well-studied RNA methyltransferase that executes an m⁶A-dependent modification of circRNAs implicated in carcinogenesis [36, 37]. Therefore, we speculated that METTL3 regulated circDLC1 expression in glioma through m⁶A modification. Our results exhibited that the m⁶A level, METTL3 expression, and the m⁶A level of circDLC1 were reduced in glioma tissues and cells. METTL3 expression showed a positive correlation with circDLC1 in glioma tissues. Then, we transfected METTL3 pcDNA 3.1 into glioma cells. With the increase of METTL3 expression, the m⁶A level, circDLC1 expression, and the m⁶A level of circDLC1 were elevated in glioma cells. Moreover, the upregulation of METTL3 prolonged the half-life period of circDLC1. Consensus m⁶A motifs are abundant in circRNAs and METTL3 enhances m⁶A-driven RNA translation [38]. We demonstrated that METTL3-mediated m⁶A modification increased circDCL1 expression by enhancing the stability of circDCL1.

Thereafter, we designed a combined experiment by treating A172 cells with METTL3 overexpression and circDCL1 silencing. Our results exhibited that the combined treatment of METTL3 overexpression and circDCL1 silencing reduced glioma cell proliferation. METTL3 expression is dramatically downregulated in glioma tissues and METTL3 downregulation underlies a potential mechanism of glioma tumorigenesis [13]. METTL3 knockdown dramatically facilitates glioblastoma stem cell growth, self-renewal, and tumor progression [39]. Similarly, we showed that METTL3 overexpression annulled the promoting effect of circDCL1 silencing on the malignant proliferation of glioma cells.

Concerning the downstream regulatory mechanism of circRNAs, it has been confirmed that circRNAs modulate the expression of target genes by functioning as a miRNA sponge [14]. Our results confirmed that circDCL1 was mainly expressed in the cytoplasm. circDCL1 was predicted to have a binding site with miR-671-5p. miR-671-5p is encoded by a gene localized at 7q36.1, which is amplified in glioblastoma multiforme [30]. miR-671-5p is highly expressed in glioblastoma cells, and facilitates the proliferation and reduces the apoptosis of glioblastoma cells [29]. Subsequently, the target genes of miR-671-5p were predicted, among which we focused on CTNNBIP1. CTNNBIP1 is accepted as a tumor suppressor that regulates the β -catenin signaling and is demonstrated to be diminished in glioma [32]. Dual-luciferase and RNA pull-down assays verified the binding relationships between miR-671-5p and circDCL1 or CTNNBIP1. We demonstrated that miR-671-5p expression was elevated and CTNNBIP1 expression was declined in glioma tissues and cells. circDCL1 upregulation reduced miR-671-5p expression, while circDCL1 downregulation enhanced miR-671-5p expression. miR-671-5p expression was negatively correlated with circDCL1 expression, and CTNNBIP1 expression was positively correlated with circDCL1 expression. All these confirmed that circDCL1 promoted CTNNBIP1 transcription by competitively binding to miR-671-5p.

Furthermore, the regulatory role of CTNNBIP1 in glioma cells was verified. si-CTNNBIP1 was transfected into LN229 cells overexpressing circDCL1, and the results exhibited that the combined treatment of CTNNBIP1 silencing and circDCL1 overexpression promoted glioma cell proliferation. CTNNBIP1 downregulation is related to unfavorable prognosis, high histological grade, and advanced glioma progression [31]. Consistently, our results demonstrated that CTNNBIP1 silencing attenuated the inhibitory effect of circDCL1 overexpression on the malignant proliferation of glioma cells. Finally, we verified the regulation of METTL3-mediated circDCL1 on the malignant proliferation of glioma cells *in vivo*. The reduction of METTL3-mediated m⁶A modification results in the *in vivo* growth of glioma cells [13]. Our results exhibited that METTL3 overexpression repressed tumor growth, elevated m⁶A level, and circDCL1 expression, reduced miR-671-5p expression, and enhanced CTNNBIP1 expression. Briefly, METTL3 suppressed the malignant proliferation of glioma cells via the circDCL1/miR-671-5p/CTNNBIP1 axis *in vivo*.

Conclusion

To sum up, METTL3-mediated m⁶A modification upregulated circDCL1 expression by enhancing the stability of circDCL1, thereby promoting the competitive binding of circDCL1 and miR-671-5p, facilitating

the transcription of CTNNBIP1, and eventually repressing the malignant proliferation of glioma cells. This study first reported circDCL1 expression patterns in glioma and elucidated its effect on the malignant proliferation of glioma cells. However, this study only explored the role of the miR-671-5p/CTNNBIP1 axis downstream of circDCL1, and there are still many miRNAs downstream of circDCL1 to be explored. Whether circDCL1 is also affected by other m⁶A modifying enzymes needs to be further studied. In addition, we only detected the effect of the change of CTNNBIP1 transcription level on the malignant proliferation of glioma cells, and the effect of the change of CTNNBIP1 protein level is not clear. In the future, we will verify the effect of CTNNBIP1 protein level on the malignant proliferation of glioma cells and explore other possible mechanisms of circDCL1.

Abbreviations

Abbreviations	Full name
circRNAs	circular RNAs
m ⁶ A	N ⁶ -methyladenosine
METTL3	Methyltransferase-like 3
ceRNA	competitive endogenous RNA
miRNAs	microRNAs
CTNNBIP1	catenin beta interacting protein 1
NC	negative control
PBS	phosphate-buffered saline
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
CCK-8	cell counting kit-8
EdU	5-ethynyl-2'-deoxyuridine
MeRIP	m ⁶ A RNA immunoprecipitation
ANOVA	analysis of variance

Declarations

Author Contribution

Quansheng Wu and Laizhao Chen have given substantial contributions to the conception and the design of the manuscript, Xiaofeng Yin, Wenbo Zhao and Wenli Xu contributed to acquisition, analysis and interpretation of the data. All authors have participated to drafting the manuscript, Quansheng Wu revised it critically. All authors read and approved the final version of the manuscript.

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Availability of Data and Materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent to Participate

All patients had consented to participate.

Consent for Publication

The consent to publish this manuscript has been obtained from all authors.

Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Informed Consent

All patients had signed the written informed consent.

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Figures

Figure 1

circDLC1 was poorly expressed in glioma tissues and cells. A: circDLC1 expression in 40 pairs of glioma tissues and adjacent tissues was determined using RT-qPCR. B: circDLC1 expression in human glioma cell lines and normal glioma cell line was detected using RT-qPCR. C: After actinomycin D treatment, the expressions of circDLC1 and linear DLC1 in cells were detected using RT-qPCR. D: After RNase R treatment, the expressions of circDLC1 and linear DLC1 in cells were detected using RT-qPCR. N = 40. The cell experiment was repeated 3 times independently. Data in panels B-D are presented as mean $\pm$ standard deviation. Data in panel A were analyzed using paired t-test. Data in panel B were analyzed using one-way ANOVA, and data in panels C-D were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$.

Figure 2

Overexpression of circDCL1 suppressed malignant proliferation of glioma cells. circDLC1 pcDNA 3.1 (pc-circDLC1) was transfected into LN229 cells, with NC pcDNA 3.1 (pc-NC) as control, circDLC1 siRNA (si-circDLC1) was transfected into A172 cells, with NC siRNA (si-NC) as control. A: circDLC1 expression in cells was detected using RT-qPCR. B-D: Cell proliferation in each group was measured using CCK-8 assay (B), colony formation assay (C), and EdU staining (D). The cell experiment was repeated 3 times independently. Data are presented as mean $\pm$ standard deviation. The comparisons between two groups

in panels A/C-D were analyzed using t-test. The comparisons among multiple groups in panel A were analyzed using one-way ANOVA, and the comparisons among multiple groups in panel B were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test, $^{**}p < 0.01$.

Figure 3

METTL3-mediated m⁶A modification enhances circDCL1 stability and upregulates circDCL1 expression. A-D: METTL3 expression in glioma tissues and cells was detected using RT-qPCR and Western blot. E-F: m⁶A quantitative analysis of m⁶A level in tumor tissues and cells. G-H: MeRIP analysis of m⁶A level of circDCL1 in tumor tissues and cells. I: Pearson correlation analysis of the correlation between METTL3 and circDCL1 in 40 cases of glioma. METTL3 pcDNA 3.1 (pc-METTL3) was transfected into LN229 and A172 cells respectively, with NC pcDNA 3.1 (pc-NC) as NC. J-K: METTL3 expression in cells was detected using RT-qPCR and Western blot. L: m⁶A quantitative analysis of m⁶A level in cells. M: circDCL1 expression in cells was detected using RT-qPCR. N: MeRIP analysis of m⁶A level of circDCL1 in cells. O: After actinomycin D treatment, circDCL1 expression in cells was detected using RT-qPCR. N = 40. The cell experiment was repeated 3 times independently. Data in panels B-D/F/H/J-O are presented as mean ± standard deviation. The comparisons between two groups in panels A/E/G were analyzed using paired t-test, and the comparisons between two groups in panels C/J-N were analyzed using t-test. The comparisons among multiple groups in panels B/D/F/H were analyzed using one-way ANOVA, and the comparisons among multiple groups in panel O were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$.

Figure 4

Overexpression of METTL3 annulled the promoting effect of circDCL1 silencing on malignant proliferation of glioma cells. A172 cells were treated with METTL3 overexpression and circDCL1 silencing. A: circDCL1 expression in cells was detected using RT-qPCR. B-D: Cell proliferation in each group was measured using CCK-8 assay (B), colony formation assay (C), and EdU staining (D). The cell experiment was repeated 3 times independently. Data are presented as mean $\pm$ standard deviation. Data in panels A/C-D were analyzed using paired t-test. Data in panel B were analyzed using one-way ANOVA, and data in panel B were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test, ** $p < 0.01$.

Figure 5

circDLC1 promoted CTNNBIP1 transcription by competitively binding to miR-671-5p. A: The location of circDLC1 in LN229 and A172 cells was analyzed using nuclear/cytosol fractionation assay. B: The downstream target genes of miR-671-5p were predicted through the Starbase, TargetsCan, RNA22 v2, and miRDB databases and intersected. C: The binding sites of miR-671-5p with circDLC1 or CTNNBIP1 in the Starbase and TargetsCan database. D-E: The binding relationship between miR-671-5p and circDLC1 or CTNNBIP1 was verified using dual-luciferase assay and RNA pull-down assay. F-H: miR-671-5p and

CTNNBIP1 expressions in glioma tissues and cells were determined using RT-qPCR. I: Pearson correlation analysis of the correlation between miR-671-5p or CTNNBIP1 and circDCL1 in 40 cases of glioma tissues. N = 40. The cell experiment was repeated 3 times independently. Data in panels A/D-E/G-H are presented as mean $\pm$ standard deviation. Data in panels E-F were analyzed using t-test. Data in panels G-H were analyzed using one-way ANOVA, and data in panel D were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$.

Figure 6

CTNNBIP1 silencing attenuated the inhibitory effect of circDCL1 overexpression on malignant proliferation of glioma cells. Three CTNNBIP1 siRNAs (si-CTNNBIP1) were transfected into LN229 cells, with NC siRNA (si-NC) as control. A: CTNNBIP1 mRNA expression was determined using RT-qPCR. B-D: Cell proliferation in each group was measured using CCK-8 assay (B), colony formation assay (C), and

EdU staining (D). The cell experiment was repeated 3 times independently. Data are presented as mean $\pm$ standard deviation. Data in panels A/C-D were analyzed using one-way ANOVA, and data in panel B were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$.

Figure 7

METTL3 suppressed the malignant proliferation of glioma cells via the circDCL1/miR-671-5p/CTNNBIP1 axis *in vivo*. LN229 cells stably overexpressing METTL3 (LV-oe-METTL3) were used to establish the xenograft tumor model in nude mice. A: Tumor volume. B: The representative images of the stripped tumors and the weight of the tumors after the nude mice were euthanized on the 21st day. C: Ki67-positive rate in tumor tissues was detected using immunohistochemistry. D-E: METTL3 expression in tumor tissues was determined using RT-qPCR and immunohistochemistry. F: m⁶A quantitative analysis of m⁶A level in tumor tissues. G-I: circDCL1, miR-671-5p, and CTNNBIP1 expressions in tumor tissues were determined using RT-qPCR. N = 6. The cell experiment was repeated 3 times independently. Data in panels A-C/E are presented as mean $\pm$ standard deviation. Data in panels B-I were analyzed using t-test. Data in panel A were analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test, ^{**} $p < 0.01$.

Figure 8

Molecular mechanism of circDCL1 regulating malignant proliferation of glioma cells. METTL3-mediated m⁶A modification can increase the stability of circDCL1, upregulate the expression of circDCL1 in glioma cells, and promote the competitive binding of circDCL1 and miR-671-5p, thereby upregulating the transcription of CTNNBIP1 and then inhibiting the malignant proliferation of glioma cells.