Exosomal circRNA BTG2 derived from RBP-J overexpressed-macrophages inhibits glioma progression via miR-25-3p/PTEN

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Macrophage-derived exosomes (Mφ-Exos) are involved in tumor progression, but its role in glioma is not fully understood. RBP-J is related to macrophage activation. In this study, we assess the role of exosomes derived from RBP-J-overexpressed macrophages (RBP-J OE Mφ-Exos)) in glioma. The circular RNA (circRNA) profiles in RBP-J OE Mφ-Exos and THP-1-like macrophages (WT Mφ)-Exos were evaluated using circRNA microarray. Then the functions of Mφ-Exo-circRNA in glioma cells were assessed via CCK-8, EdU, Transwell invasion, and nude mouse assays. Besides, luciferase reporter assay, RNA immunoprecipitation, and Pearson’s correlation analysis were adopted to confirm interactions. We found that circRNA BTG (circBTG2) is upregulated in RBP-J OE Mφ-Exos compared to WT Mφ-Exos. RBP-J OE Mφ-Exos co-culture and circBTG2 overexpression inhibited proliferation and invasion of glioma cells, whereas circBTG2 knockdown promotes tumor growth in vivo. The effects of RBP-J OE Mφ-Exos on glioma cells can be reversed by the circBTG2 knockdown. In conclusion, Exo-circBTG2 secreted from RBP-J OE Mφ inhibits tumor progression through the circBTG2/miR-25-3p/PTEN pathway, and circBTG2 is probably a diagnostic biomarker and potential target for glioma therapy.

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INTRODUCTION

Gliomas are primary malignancy commonly seen in the nervous system [1, 2], which are featured with heterogeneous genetic molecular aberrations [3]. Glioblastoma multiforme (GBM) is the most aggressive type with repeated relapse. The median survival rate of GBM patients is only 14.6 months even after standard advanced surgery and chemoradiotherapy with temozolomide [4, 5]. Thus, top priority should be given to the probation of the possible molecular mechanisms by which gliomas progress, so as to improve the therapy of gliomas, especially GBM.

Increasing evidence unveils that the exosomes (Exos) mediate the interactions between macrophages and cancer cells [6–8]. M2 Macrophage-derived exosomes (M2-Exos) were found to boost cancer cells to migrate and invade [9], and tumor-associated M2-Exos facilitate gastric cancer cells to migrate via transfer of functional Apolipoprotein E [10]. Besides, Mφ-Exo-miR-501-3p contributes to progression of PDAC via the TGF-β signaling pathway mediated by TGFBR3 [11]. Downregulated IncRNA SBF2-AS1 in M2 Mφ-Exos raises miR-122-5p to restrict XIAP, thus curbing PC development [12]. M2 bone marrow-derived Mφ-Exos elevate miR-21 to accelerate immune escape of gliomas via modulating PEG3 [13].

Circular RNAs (circRNAs) have also been found in Exos [14, 15], and they are thought to modulate the expression of genes and miRNAs [16]. Exo-circRNAs can promote malignant phenotype of peripheral cells in cholangiocarcinoma [17]. Recent studies have unveiled the involvement of circRNAs in glioma progression by competitive sponging miRNAs [18, 19]. For instance, circ_0037655 is able to boost gliomas to progress via controlling miR-214/PI3K signal transduction [20]. However, whether Mφ-Exo-circRNAs can regulate the progression of gliomas is unclear.

The Notch pathway is involved in several core cellular processes, such as proliferation and tumor development [21, 22], and it is also believed to be responsible for the activation and differentiation of macrophages [23–25]. The recombination signal-binding protein-Jk (RBP-J) is a transcriptional regulator that is often used as a marker for the activation of Notch signaling [26]. Notch intracellular domains are released by Notch ligands and translocate to the nucleus where they bind to RBP-J [27]. Loss of the Notch effector RBP-J promotes tumorigenesis [28]. Moreover, Notch-RBP-J signal transduction regulates the transcription factor IRF8 to facilitate inflammatory macrophage polarization [29].

In the current research, we probed the impacts of RBP-J OE Mφ-Exos (exosomes derived from RBP-J-overexpressed macrophages) on glioma cell proliferation and invasion and compared them with Exos from THP-1-like macrophages (WT Mφ-Exos) [30]. To further understand the regulatory mechanism of RBP-J OE Mφ-Exos in gliomas, we also determined the differentially regulated circRNAs when RBP-J was upregulated in Mφ-Exos. In addition, we identified the miRNA binding partners of the circRNA and their targets. This study aimed at identifying pathways that are uniquely expressed in glioma progression to understand the mechanisms of Mφ-Exo-circRNAs, and determining diagnostic biomarkers and potential therapeutic targets.
RESULTS

RBP-J is lowly expressed in the macrophages from the glioma tissues and related to the prognosis of glioma patients

Glioma tissues (n = 40) and para-tumor tissues (n = 40) were obtained from 40 patients. The expression of RBP-J in the macrophages from the glioma tissue samples and adjacent normal tissue samples was detected using qRT-PCR. Results showed that RBP-J is lowly expressed in the macrophages from the glioma tissues (Fig. 1A). The correlation between RBP-J expression in the macrophages of glioma tissue samples and clinicopathological parameters of glioma patients is shown in Table 1. The median expression level of RBP-J in the macrophages of glioma tissues was taken as the cutoff value for high and low levels. We found that higher RBP-J expression was associated with lower glioma Grade (P < 0.05), but there was no association between the expression level of RBP-J and other clinicopathological parameters in glioma such as age and gender (P > 0.05). In addition, the survival rate of glioma patients with highly expressed RBP-J in the macrophages of glioma tissues was better than that of patients with lower RBP-J expression in the macrophages of glioma tissues (Fig. 1B).

RBP-J OE Mφ inhibit proliferation and invasion of glioma cells through extracellular vesicles

To further investigate the effects of the RBP-J OE Mφ on the proliferation and migration of glioma cells, we cocultured U87 MG cells with Mφ for 5 days and measured cell proliferation and migration with CCK-8 and wound healing assays. As demonstrated in Fig. 1C, D, RBP-J OE Mφ significantly inhibited cell proliferation and migration of U87 MG cells compared with WT Mφ. We wonder whether Mφ play a role through direct contact or indirect contact. Thus, we co-culture Mφ and U87 cells in separate spaces, allowing only the medium to contact each other. Then we found that RBP-J OE co-culture with Mφ could also significantly inhibited cell proliferation and migration of U87 MG cells (Fig. 1C, D). This means that Mφ play a role through indirect contact. Nevertheless, whether it is through extracellular vesicles or soluble small molecules still needs to be further explored. We extracted extracellular vesicles and supernatant of Mφ respectively. As shown in Fig. 1C, D, we found that Mφ play a role through extracellular vesicles.
RBP-J OE Mφ-Exos inhibit proliferation and invasion of glioma cells

WT Mφ-Exos and RBP-J OE Mφ-Exos were isolated by ultracentrifugation and characterized by TEM and NTA. To confirm the successful isolation of Exos, the expression levels of CD63 and TSG101 were determined by Western blotting.

Cell proliferation in glioma cell lines U87 MG and U373 MG treated with WT Mφ-Exo or RBP-J OE Mφ-Exo for 3 h. Cell invasion was assessed by CCK-8 and EdU assays. A Transwell invasion assay was performed to indicate cell invasion (bar = 100 μm). All experiments were performed three times. *P < 0.05 and **P < 0.01 for statistical differences.
these results confirm that the overexpression of RBP-J in Exos can suppress glioma cells to proliferate and invade.

Expression profiles of circRNAs in RBP-J OE Mφ-Exos

We wonder whether RBP-J OE Mφ-Exos could influence the expression of RBP-J in glioma cells. We performed qRT-PCR to detect the expression of RBP-J in the WT Mφ-Exos treated U87 MG cells, and RBP-J OE Mφ-Exos treated U87 MG cells. Results showed that there is no significant difference of RBP-J level between WT Mφ-Exos treated U87 MG cells and RBP-J OE Mφ-Exos treated U87 MG cells (Fig. 3A). This result means that RBP-J OE Mφ-Exos can’t influence the expression of RBP-J in U87 MG cells. Thus, we wonder whether there are different expressions of circRNAs between WT Mφ-Exos and RBP-J OE Mφ-Exos. The circRNA profiles in RBP-J OE Mφ-Exos and WT Mφ-Exos were evaluated using a circRNA microarray technique. 39 circRNAs were differentially expressed (P < 0.05 and log2FC > 2.0 or < −2.0) in RBP-J OE Mφ-Exos and the controls (Fig. 3B). Among them, 25 circRNAs dramatically rose up and 14 ones evidently declined. circBTG2 with the most obvious rising trend was selected and validated to be present in RBP-J OE Mφ-Exos and WT Mφ-Exos by qRT-PCR (Fig. 3C). In the meantime, it was unveiled that circBTG2 was expressed in the RBP-J OE Mφ at a notably higher level relative to that in the WT Mφ cells (Fig. 3D). Compared with those in the producer cells, the levels of circBTG2 are enriched by approximately 4 folds in the RBP-J OE Mφ-Exos and WT Mφ-Exos (Fig. 3E). This result means that circBTG2 is enriched in exosomes. According to the circBase (http://www.circbase.org), circBTG2 (chr1:203274663-203278729) was derived from BTG2. Mφ-Exo-circBTG2 inhibits glioma cells to proliferate and invade

Since the circRNA BTG2 level was the highest in RBP-J OE Mφ-Exos, to remove its expression from Exos, the siRNA of circBTG2 was transfected into Mφ cells for 48 h, after which Exos were collected (Fig. 3F, G). Next, glioma cell proliferation and invasion were investigated by coculturing cells with Mφ-Exos. The inhibitory effects of RBP-J OE Mφ-Exos on the proliferation and invasion of glioma cells (U87 MG and U373 MG) were eliminated when circBTG2 was knocked down in Mφ (Fig. 4A–C). This would be
expected if there was an association between the expression of RBP-J and circBTG2.

To continuously figure out the biological role of circBTG2 in glioma cells, U87 MG and U373 MG cells underwent transfection with a circBTG2 overexpression vector (Supplementary Fig. S1A). The results unveiled that circBTG2 overexpression significantly inhibited glioma cells to proliferate and invade (Supplementary Fig. S1B–D). As with the overexpression of RBP-J, the overexpression of circBTG2 inhibited proliferation and invasion of glioma cells.

circBTG2 acts as a sponge for miR-25-3p

For discovering more about the specific regulation of circBTG2, we performed bioinformatics prediction (starBase). Bioinformatics analysis predicted that circBTG2 and miR-25-3p possessed complementary binding sites (Fig. 5A). Then we carried out a dual-luciferase experiment in 293 T cells to confirm this interaction by mutating the predicted binding site in circBTG2. It was unveiled that the luciferase activity was reduced only in presence of WT circBTG2 and miR-25-3p mimics in 293 T cells (Fig. 5B), which was further validated using an Ago2 RIP assay. Ago2 significantly enriched RNA levels of both circBTG2 and miR-25-3p (Fig. 5C). Besides, levels of circBTG2 and miR-25-3p were also analyzed in glioma and matched para-carcinoma tissues, and the results further substantiated that glioma tissues exhibited a lower circBTG2 level (Fig. 5D) and a higher miR-25-3p level (Fig. 5E). Besides, Pearson’s analysis confirmed a negative interrelation between circBTG2 and miR-25-3p in glioma and matched para-carcinoma tissues (Fig. 5F). This indicates that circBTG2 may compete to bind to miR-25-3p as a sponge and prevent it from regulating other pathways.
circBTG2 represses glioma cells to proliferate and invade via the miR-25-3p/PTEN pathway

We next probed the potential binding sites of miR-25-3p. Target prediction and assessment were implemented using starBase (http://starbase.sysu.edu.cn) and miRDB (http://mirdb.org), which identified that miR-25-3p probably interacts with PTEN, a tumor suppressor gene implicated in several cancers [31, 32]. Later, we mutated two potential miR-25-3p target sites in PTEN (Fig. 6A) and performed a luciferase reporter experiment, which ascertained that miR-25-3p overexpression in HEK293T cells dramatically weakened the luciferase activity of PTEN at both target sites (Fig. 6B). Thereafter, we examined the transfection efficiency of miR-25-3p mimics and inhibitor (Fig. 6C), and the relative expression level of miR-25-3p was measured in U87 MG and U373 MG cells transfected with miR-25-3p mimics or inhibitor (Fig. 6D, E). Overexpression of circBTG2 upregulated PTEN whereas miR-25-3p mimics transfection reversed it in U87 MG cells (Fig. 6F). Relative to the matched para-carcinoma tissues, PTEN was expressed at a lower level in glioma tissues (Fig. 6G). In glioma tissues, PTEN was negatively correlated with miR-25-3p, but positively correlated with circBTG2 expression (Fig. 6H, I). Further, circBTG2 overexpression inhibited cells to proliferate and invade whereas miR-25-3p mimics transfection reversed it in U87 MG and U373 MG cells (Supplementary Fig. S2A–C). It can be assumed that circBTG2 inhibits proliferation and invasion in glioma cells by sponging miR-25-3p and upregulating PTEN expression.

RBP-J OE Mφ-Exo inhibits tumor growth through the circBTG2/miR-25-3p/PTEN pathway in vivo

For proving the effect of Mφ-Exo-circBTG2 on the modulation of glioma growth in vivo, U87 MG cells undergoing transfection with sh-circRNA or sh-NC or coculture with WT Mφ-Exos, RBP-J OE Mφ-Exos or (RBP-J OE + sh-circRNA) Mφ-Exo were subcutaneously injected into nude mice. Tumors cultured with RBP-J OE Mφ-Exos were significantly smaller, whereas those undergoing sh-circRNA transfection were significantly larger. The greatest differences in the tumor volume and weight were observed in the tumors between RBP-J OE Mφ-Exo group and sh-circRNA group (Fig. 7A–C). What’s more, the inhibitory effects of RBP-J OE Mφ-Exos on the tumor growth in vivo were eliminated when circRNA BTG2 was knocked down in Mφ (Fig. 7A–C). The relative expression of circBTG2 was the highest in the RBP-J OE Mφ-Exo group and the lowest in sh-circRNA group (Fig. 7D). While the relative expression of miR-25-3p was the lowest in the RBP-J OE Mφ-Exo group and the highest in sh-circRNA group (Fig. 7E). Meanwhile, protein levels of PTEN were the highest in RBP-J OE Mφ-Exo group and lowest in sh-circRNA group (Fig. 7F). These results signify that RBP-J OE Mφ-Exos might inhibit tumor growth through a circBTG2/miR-25-3p/PTEN pathway in xenograft tumor models.

DISCUSSION

Macrophages are abundant in the glioma tumoral environment and associated with chronic inflammation [33, 34]. Moreover, the
The macrophage environment is heterogeneous with the progression of tumors dependent on alternatively polarized M2 macrophages and tumorigenic immune responses dependent on M1-polarized macrophages [35, 36]. Therefore, improving the understanding of macrophage regulation in the tumoral environment is important in developing effective therapies for gliomas. Notch-RBP-J signaling is believed to regulate TLR-induced inflammatory macrophage polarization by the indirect regulation of M1-specific genes [29].

In this study, we examined whether RBP-J overexpression in macrophages would influence glioma cells. We found that RBP-J OE Mφ-Exos could curb glioma cells to proliferate and invade. Furthermore, we probed their interrelations by investigating the differentially regulated circRNAs in Mφ-Exos with upregulated RBP-J. Using the circRNA microarray technique, we discovered that 39 Exo-circRNAs were differentially regulated in WT Mφ-Exos with RBP-J overexpression, of which 25 were upregulated and 14 were downregulated. Later, we selected the Exos with the highest circBTG2 expression for further analysis. Then we unveiled that the inhibitory effects of RBP-J OE Mφ-Exos on the proliferation and invasion of glioma cells (U87 MG and U373 MG) were eliminated when circBTG2 was knocked down in Exos. Meanwhile, circBTG2 overexpression dramatically repressed glioma cells to proliferate and invade. These associations required further investigation, so we searched for miRNAs that may interact with circBTG2.

Fig. 6 circRNA BTG2/miR-25-3p axis is critical for PTEN expression. A Bioinformatics analysis revealed the predicted binding sites between PTEN and miR-25-3p. B Luciferase reporter assay demonstrated miR-25-3p mimics significantly decreased the luciferase activity of PTEN-WT in HEK293T cells. C The transfection efficiency of miR-25-3p mimics and inhibitor in U87 MG and U373 MG cells. D, E The mRNA (D) and protein (E) level of PTEN was detected through qRT-PCR and western blotting after transfection with miR-25-3p mimics and inhibitor in U87 MG and U373 MG cells. F circRNA BTG2 overexpression upregulated PTEN, this effect can be reversed by co-transfection with miR-25-3p mimics in U87 MG cells. G The expression levels of PTEN in 40 glioma tissues and matched para-carcinoma normal tissues was determined by qRT-PCR. H Expression levels of PTEN negatively correlated with miR-25-3p in glioma tissues. I Expression levels of PTEN positively correlated with circRNA BTG2 in glioma tissues. **P < 0.01 and ***P < 0.001 for statistical differences.
The public database (starBase) predicted that circBTG2 may interact with miR-25-3p, which was validated via luciferase or RIP assays. Our studies proved that the overexpression of circBTG2 could reduce miR-25-3p level. Then, a negative correlation between miR-25-3p and circBTG2 in glioma and matched para-carcinoma tissues was confirmed by Pearson’s analysis. Thus, we deduced that circBTG2 may repress miR-25-3p to prevent it from interacting in other pathways. StarBase revealed that miR-25-3p interacted with PTEN, a well-known tumor suppressor gene \([31, 32]\). Mutation or deletion of PTEN via complete loss of its locus on chromosome 10q is found in a multifold of GBMs \([37, 38]\) and correlated with poor prognosis in diverse glioma subtypes \([39, 40]\). Furthermore, PTEN loss dramatically enhances gliomagenesis in quantities of murine model systems \([41–43]\). In the current research, we found that circBTG2 regulated PTEN expression in glioma cells, and the overexpression of circBTG2 upregulated PTEN expression and inhibited glioma cell proliferation and invasion (Fig. 8). The above results indicate that RBP-J OE Mφ-Exos probably play a potential regulation role in the glioma progression and circBTG2 could be a biomarker for glioma diagnosis and potential target for glioma therapy.

**MATERIALS AND METHODS**

**Cell culture and clinical specimens**

Human monocytic cell line THP-1 and glioma cell lines (U87 MG and U373 MG) were purchased from ATCC and maintained according to ATCC guidelines. THP-1 cells were cultured in RPMI-1640 medium provided by Gibco (Shanghai, China), and glioma cells were cultured in Dulbecco’s Modified Eagle medium (DMEM, Gibco, China) with 10% heat-inactivated fetal bovine serum (FBS) from Thermo Fisher Scientific (Shanghai, China), 100 U/mL penicillin, and 100 µg/mL streptomycin from HyClone Laboratories (Beijing, China) at 37 °C in a moist incubator with 5% CO2 and used in the exponential growth phase.

Forty paired glioma tissues and para-tumor tissues were obtained from patients receiving surgery at The First people’s Hospital of Yancheng between 2015 and 2018. They were diagnosed by histopathology and received no treatment prior to the operation. Besides, all participants signed informed consent in written form before the research. This research gained the approval of the Ethics Committees of The First people’s Hospital of Yancheng and was conducted as per the Helsinki Declaration.
Macrophage extraction
The tissue single cell suspension (2*10^8/mL) was obtained and mixed into the separation solution (Sangon Biotech, Shanghai, China). The mixture was centrifuged for 20 min (1500 r/min) and the milky white macrophage layer was then collected. The macrophage layer was added into a test tube containing 5 mL of cell washing solution. After mixing, the macrophage layer was centrifuged (1800 r/min) and washed twice.

Isolation of Exos derived from THP-1 Mφ cells with or without the overexpression of RBP-J
To obtain WT Mφ and RBP-J OE Mφ, THP-1 cells underwent transfection with the pCMV6 empty vector or pCMV6 overexpressing RBP-J (OriGene, Rockville, MD, USA) and seeded at 1 × 10^6 cells/well in a six-well culture plate. Cellular debris were discarded from the collected culture medium that was centrifuged at 3000 × g for 15 min. Then Exoquick exosome precipitation solution (System Biosciences, CA, U.S.A.) was utilized for exosome separation [44–46].

Nanoparticle tracking analysis (NTA)
We measured the exosome particle size and concentration using NTA with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software ZetaView 8.04.02. Isolated exosome samples were appropriately diluted using 1X PBS buffer (Biological Industries, Israel) to measure the particle size and concentration. NTA measurement was recorded and analyzed at 11 positions. The ZetaView system was calibrated using 110 nm polystyrene particles. The temperature was maintained at around 23 °C and 37 °C.

Transmission electron microscopy assay
Exos for transmission electron microscopy (TEM) were prepared as mentioned above.32 Briefly, Exos were first fixed in 2.5% glutaraldehyde (pH 7.2) at 4 °C, then washed in PBS, embedded in 10% gelatin and fixed in 1% osmium tetroxide for 60 min at indoor temperature. Next, the embedded Exos were cut into 1 mm-thick blocks and dehydrated with gradient alcohol. The alcohol was then replaced with gradient mixture of Quetol-812 epoxy resin and propylene oxide. Afterward, samples were embedded in Quetol-812 epoxy resin, polymerized at a temperature gradient, and cut into ultrathin sections using a Leica UC6 ultramicrotome. Finally, subsequent to dying by uranyl acetate and lead citrate, a transmission electron microscope was utilized for section observation.

Exosome labeling
Exosomes from 1.5 × 10^6 cells were suspended in 100 μl of PBS with 1 ml of mixed PKH67 (Sigma, in Diluent C). After 4 min of incubation at room temperature, 2 ml of 0.5% bovine serum albumin (BSA) was added to terminate exosome labeling, and dyed exosomes were isolated using Exoquick exosome precipitation solution. Exosomes were suspended in 9.6 ml of basal medium, and 250 μl was added to the subconfluent layer of U87 MG cells. After incubation for 3 h at 37 °C, cells were washed and fixed at room temperature. To stain the nuclei, 4′,6-diamidino-2-phenylindole (DAPI, Sigma) was added for 10 min, and the stained cells were observed with a fluorescence microscope (Zeiss, LSM700B, Germany).

Microarray analysis
The isolation and quantification of the total RNAs were independently implemented using Trizol reagent provided by Life Technologies (Shanghai, China) and NanoDrop ND-1000. To enrich circRNAs and remove linear RNAs,
RNA extraction and quantitative real-time PCR (qRT-PCR)

The reverse transcription of mRNAs and circRNAs into cDNAs was implemented using a reverse transcription kit from Takara (Japan), which were synthesized using the miRNA 1st-strand DNA synthesis kit (Sangon Biotech, China). Next, cDNAs were subjected to RT-PCR on a QuantaStudio™ DX system (Applied Biosystems, Singapore) under the following conditions: denaturation at 95 °C for 30 s and (denaturation at 95 °C for 5 s, at 60 °C for 10 s and at 72 °C for 30 s) × 40 cycles. Afterward, we utilized 2^ΔΔCT to quantify mRNAs and circRNAs by normalizing to GAPDH [47] and to determine the relative expression subsequent to the normalization of miRNA expression to small nuclear U6. Each experiment was separately performed in triplicate. All PCR primers were listed in Table 2.

Cell transfection

CircRNA BTG anti-proliferation factor 2 (BTG2) (circBTG2) overexpression plasmid (p-circRNA) and its mimic pcDNA3.1, small interfering RNAs (siRNAs) targeting circRNAs and nonspecific negative control oligos (si-NC), miR-25-3p mimics, inhibitor and the negative control (NC), and the lentivirus containing circBTG2 were bought from GeneChem (Shanghai, China). Detailed sequences were depicted in Table 2. U87 MG and U373 MG cell lines underwent inoculation in six-well plates at 24 h prior to transfection with pcDNA3.1, p-circRNA, si-NC, si-circRNA, and miR-25-3p mimics or inhibitor under 50–60% cell confluence using Lipofectamine 3000 (Invitrogen) as per the guideline of the manufacturer. Later, the effects of knockdown or overexpression were examined by qRT-PCR using the RNAs that were extracted after 48 h transfection. For Exo treatment, after 48-h transfection, for 48 h after transfection. For Exo treatment, 60 °C for 10 s and at 72 °C for 30 s) × 40 cycles. Afterward, we utilized 2^-ΔΔCt to quantify mRNAs and circRNAs by normalizing to GAPDH [47] and to determine the relative expression subsequent to the normalization of miRNA expression to small nuclear U6. Each experiment was separately performed in triplicate. All PCR primers were listed in Table 2.

Cell proliferation assays

Approximately 1.0 × 10^4 transfected U87 MG and U373 MG cells were cultured in 96-well plates, and then underwent 1 h incubation with CCK-8 reagent (Beyotime, Beijing, China). The absorbance at 450 nm was recorded using an Infinite M200 multimode microplate reader (Tecan, Shanghai, China). After approximately 48 h transfection, the 5-ethyl-2-deoxyuridine (EdU) essay kit provided by Ribo (Guangzhou, China) was utilized to examine the proliferation of U87 MG and U373 MG cells. Specifically, cells were grown in culture medium containing EdU (Invitrogen) solution at indoor temperature. Later, we incubated the membrane with primary antibodies against TSG101 (1:1000, ab125011, Abcam, Shanghai, China) and GAPDH (1:1000, ab8245, Abcam, Shanghai, China) and GAPDH in 10 min at 20,000 rpm for 10 min at 4 °C. The cell lysates were incubated nightlong at 4 °C, and then incubated with magnetic beads for a further hour. Proteinase K was then added for sample incubation at 55 °C for another hour. In the end, RNA extraction reagent (Solarbio, Beijing, China) was used to obtain the RNAs, and specific genes were detected and measured using qRT-PCR.

Western blot analysis

Cell lysis was performed in RIPA buffer (Beyotime, Nantong, China) containing protease and phosphatase inhibitors (Beyotime). A BCA Protein Assay kit (Beyotime) was utilized to identify protein concentration, and the samples (40 µg proteins per lane) underwent SDS-PAGE with 10% gel for separation. Next, proteins were electrotransferred onto a PVDF membrane (Beyotime) that was sealed by 5% BSA (Beyotime) for 1 h at indoor temperature. Later, we incubated the membrane with primary antibodies against TSG101 (1:1000, ab125011, Abcam, Shanghai, China), CD63 (1:1000, ab217345, Abcam, Shanghai, China), PTEN (1:1000, ab267787, Abcam, Shanghai, China), and GAPDH (1:1000, ab62425, Abcam, Shanghai, China) at 4 °C nightlong. The membranes were then probed with HRP-labeled secondary antibodies (Beyotime, Nantong, China) at indoor temperature for 1 h and signals were detected by chemiluminescence.

Xenograft nude mouse model

6–8-week-old adult male BALB/C nude mice (n = 3/group) were commercially provided by Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and reserved in a SPF environment with a LD (12:12) cycle. All animal studies obtained the approval of the Institutional Animal Care and Use Committee of The First people’s Hospital of Yancheng and implemented in line with institutional and national guidelines. U87 MG cells undergoing stable sh-NC or sh-circRNA transfection, or WT Mq Exo, RBP-J OE Mq Exo or RBP-J OE sh-circRNA (5 µg/ml) treatment were hypodermically injected into the nude mice (1 × 10^6 cells per mouse) on the right upper back. Later, we utilized a caliper to determine the growth of tumor every 7 days for 35 days, and calculate its volume based on the formula: volume = (length × width^2)/2. Five weeks later, we intraperitoneally injected overdose pentobarbital (>120 mg/kg body weight) to kill all the mice so that they were unable to spontaneously breathe. Afterward, the xenograft tumor tissues were sampled for subsequent analyses.

Statistical assessment

GraphPad Prism 6.0 software provided by GraphPad Inc. (San Diego, CA, USA) was utilized to statistically evaluate data. Experimental results were presented as mean ± standard deviation (SD). The statistically significant differences between tumor tissues and paracarcinoma tissues were determined using paired Student’s-t test. Besides, the statistically significant differences between other two groups were detected using Mann–Whitney U-test or unpaired Student’s-t test in light of conditions. Furthermore, the comparisons among different groups (multigroup comparisons) were implemented by one-way ANOVA and the post hoc Bonferroni test. Lastly, Pearson’s correlation coefficient was determined to test associations among circBTG2, miR-25-3p, and PTEN. Log-rank test and Kaplan–Meier method were used to assess survival rates. Data concerning the association of RBP-J expression with clinicopathological features of glioma were analyzed by Fisher’s exact test. P < 0.05 signified statistically significant differences.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

vector from Promega Corp. (Beijing, China). 293 T cells seeded into 24-well plates underwent co-transfection with 50 nM miR-25-3p mimics or a NC and 80 ng WT or MUT plasmids using Lipofectamine 2000 (Invitrogen) and the 80 ng of plasmids were later added with 5 ng of pRL-SV40. Lastly, luciferase intensity was determined using the Dual-Luciferase Reporter Assay Kit from Promega (Beijing, China) and a microplate reader.

RNA binding protein immunoprecipitation (RIP) assay

We carried out the RIP assay using a Magna RIP Kit from Millipore (Hongkong, China) as per the guideline of the manufacturer. Specifically, cells (2 × 10^5) were lysed with the lysis buffer provided in the kit and the lysate was separately put into two tubes (one with anti-Argonaut2 (Ago2) antibody and the other with a nonspecific anti-IgG antibody (Millipore)). The cell lysates were incubated nightlong at 4 °C, and then incubated with magnetic beads for a further hour. Proteinase K was then added for sample incubation at 55 °C for another hour. In the end, RNA extraction reagent (Solarbio, Beijing, China) was used to obtain the RNAs, and specific genes were detected and measured using qRT-PCR.

Cell invasion assays

For invasion assays, the lower chambers were precoated with 100 µL of Matrigel (BD Bioscience, San Jose, CA, USA) for 30 min before the addition of medium to the chambers. The glioma cells (2 × 10^5 cells/mL) were resuspended in DMEM medium. The upper chamber contained 100 µL of cell suspension medium, and 600 µL of complete medium was added to the bottom chamber. After incubating at 37 °C with 5% CO2 for 24 h, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. The cells that passed through the filter were photographed and counted by inverted fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany) in four randomly selected fields.

Luciferase reporter assay

Sequences of WT or MUT circBTG2 or the full length of the 3'-UTR of PTEN with WT or MUT putative binding sites were interposed into the pmirGLO
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research conception and design, and revised the manuscript. JG supervised experiments and contributed to analytic tools. All authors read and approve the final version of the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
The studies involving human participants were reviewed and approved by the Ethics Committee of The First people's Hospital of Yancheng. The participants provided their written informed consent to participate in this study. All animal studies obtained the approval of the Institutional Animal Care and Use Committee of The First people's Hospital of Yancheng and implemented in line with institutional and national guidelines (No. 2015-007).

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