Oncogenic miR-132 sustains proliferation and self-renewal potential by inhibition of polypyrimidine tract-binding protein 2 in glioblastoma cells

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Abstract. Glioblastoma multiforme (GBM) is the leading type of brain tumor, exhibiting unlimited proliferation and invasion potential. The present study indicated that a high expression level of miR-132 was detected in the neural subtype of GBM and predicted an unfavorable prognosis for patients from The Cancer Genome Atlas cohort (n=526). Cox hazard regression analysis demonstrated miR-132 as an independent prognostic indicator for GBM patients. Further in vitro experiments indicated that miR-132 promoted the proliferation and sphere formation of U87 cells. Unsupervised hierarchical clustering analysis was performed to compare differently expressed genes between two Gene Expression Omnibus (GEO) datasets and Gene Ontology analysis was applied to evaluate the significant signaling pathways modulated by miR-132 in GBM cells within a genetic bioinformatic lab, the Gene-Cloud of Biotechnology Information. By combining the results based on GEO datasets and the miRNA bioinformatic prediction, polypyrimidine tract-binding protein 2 (PTBP2), a brain tissue-specific post-transcriptional protein, was identified as a potential downstream target of miR-132 in GBM. Thus, miR-132 overexpression in GBM cells predicted an unfavorable outcome for patients, and sustained the proliferation and self-renewal abilities of GBM cells in an miR-132/PTBP2 signaling pathway.

Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive and lethal type of malignant tumor of the central nervous system (CNS) (1-3), which is characterized by its fast growth and unlimited self-renewal potential. Despite development of therapeutic strategies for GBM, the overall survival of patients improved marginally, with a 5-year survival rate of just 9% (2). Therefore, more precise prognostic predictors and more effective therapeutic approaches are urgently required for patients.

MicroRNAs (miRNAs) are single-stranded non-coding RNAs with 19-23 oligonucleotides, which bind to the 3'-untranslated region of target genes, induce degradation and partake in virtually all biopathological steps. Increasing evidence indicates that miRNAs regulate the diverse biological steps of carcinogenesis and progression in cancer (4). Additionally, dysregulation of miRNA exhibits oncogeneic and tumor suppressor properties (4,5). Human microRNA-132 (has-miR-132), located in ch.17, is aberrantly expressed in gastric cancer, chronic lymphocytic leukemia, hepatocellular carcinoma and colorectal cancer (CRC) (6-9). Furthermore, as a central nervous system-specific miRNA, miR-132 displays vital roles in neurogenesis, neuron stem cell differentiation and development (10-13). Its dysregulation results in various types of brain-associated disease, including Huntington's disease, Parkinson's disease and schizophrenia (14,15). Previously, miR-132 was detected to be highly expressed in glioma, serving as a biomarker of a poor prognosis in patients (16). However, the functions of miR-132 in GBM stemness are complex and require further exploration.

In the present study, the potential bias from sample size was minimized by enrolling the GBM specimens from The Cancer Genome Atlas (TCGA) Research Network and investigated the clinical significance of miR-132. A high level of miR-132 was identified to be significantly correlated with neural subtype of GBM and a poor outcome for patients. Furthermore, a Gene-Cloud of Biotechnology Information (GCBI) bioinformatics analysis was performed to investigate the GEO datasets and the results revealed that miR-132 fuels proliferation and
self-renewal potential potentially by targeting polypyrimidine tract-binding protein 2 (PTBP2) in GBM cells.

Materials and methods

Cell culture and sphere culture. The U87 GBM cell line was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Gibco Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS). The conditional culture medium, Gibco DMEM/F12 (Thermo Fisher Scientific, Inc.) was supplemented with Invitrogen B27 (1X; Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor and 20 ng/ml epidermal growth factor (both from PeproTech, Inc., Rocky Hill, NJ, USA). First generation U87-neuropheres were observed in all wells of a 6-well plate 72 h later. All cultures were maintained at 37°C in an atmosphere of 5% CO₂.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The miRNA was extracted with RNAiso for small RNA (Takara Bio, Inc., Otsu, Japan) and miR-132 was examined using a TaqMan microRNA Assay (Thermo Fisher Scientific, Inc.) with U6 serving as the internal control. Total mRNA extraction was performed using TRIzol (Takara Bio, Inc.) from U87 cells and fresh glioma specimens. The fresh specimens were obtained from resected samples from glioma patients in the Chongqing Cancer Hospital (Chongqing, China). Expression levels of PTBP2 and GAPDH were measured using an RT-PCR kit (cat no. RR055A; Takara Bio, Inc.) according to the manufacturer’s instructions and a CFX 96 system (Bio-Rad Laboratories, Inc., CA, USA). Each sample was examined in triplicate and analyzed according to the 2^ΔΔCq method (17) and GAPDH served as the internal control in the assay. The PCR reaction was run as follows: 95°C for 30 sec, 39 cycles of 95°C for 5 sec and 60°C for 30 sec. The primer sequences for qPCR were as follows: Forward, 5’-TGGATTTGCTTGCATTGC-3’ and reverse, 5’-TCAAGTATGGATCGTC-3’ for PTBP2; forward, 5’-GGCGCTGTGCTC-3’ and reverse, 5’-ATGGAGCTGTC-3’ for GAPDH.

miRNA reagent transfection. The miR-132 mimic and control reagents were obtained from Guangzhou Ribobio Co., Ltd. (Guangzhou, China). U87 cells with 75% confluence were transfected with Invitrogen Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm.

Sphere formation assay and colony formation assay. Both sphere formation assay and colony formation assay were performed to evaluate the self-renewal ability of U87 cells. According to our previous study (18), different numbers (20, 50 or 100) of cells were seeded into a 96-well plate. Serum-free medium (25 µl) was added to each well every 2 days. Plates were incubated for 2 weeks at 37°C in an atmosphere of 5% CO₂ until neurospheres formed and the number of spheroid cells was counted for statistical analysis.

Bioinformatics analysis. TCGA (http://cancergenome.nih.gov) Research Network, a huge tumor profiling data set for a very large collection of tumor types, was mined in order to evaluate the predictive value of miR-132 in GBM specimens (n=526). A comprehensive bioinformatics analysis approach (GCBI; https://www.gcbi.com.cn/gcblib/html/index), which was deeply integrated with the Affymetrix Gene Chip in GEO database, was used to enrich the dataset for genes, including the heat map analysis, volcano map analysis, gene ontology (GO) analysis and pathway analysis (19,20). In order to compare the different expression genes between groups of neurosphere cell lines with miR-132 transfection (GCS24468, GCS24458 and GCS24463) or control transfection (GCS24456, GCS24465 and GCS24464) (19), heat mapping was performed. To further analyze the functions of different expression genes on the basis of biological processes and molecular function, GO analysis was performed. In addition, pathway analysis was used to establish the significant pathway of differential genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/), BioCarta’s pathways (www.biocarta.com/gene/index.asp) and Reactome pathway databases (www.reactome.org). In order to establish the downstream targets of miR-132, five renowned miRNA prediction databases (TargetScan, www.targetscan.org/vert_71; miRanda, www.microrna.org/microrna/home.do; miRDB, www.mirdb.org; miRWalk, zmft.unm.edu/index.html; RNA22, cm.jefferson.edu/rna22) were bioinformatically scanned as previously described (18,21).

Patient specimens. Newly diagnosed GBM patients who had received no previous treatment from 01/2015 to 01/2016 were enrolled in the present study. A total of 13 fresh GBM specimens were obtained from these patients straight after surgery at the Department of Neurosurgery, The Chongqing Cancer
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Hospital (Chongqing, China). Specimens were pre-mixed with liquid nitrogen at -80˚C and ground. Following TRIzol (Takara Bio, Inc.) was added, the samples were prepared for the extraction of miRNA/mRNA following the related protocol. Written informed consent was obtained from each patient according to the national regulations of clinical samples and the study was approved by the Ethics Board of The Chongqing Cancer Hospital.

Statistical analysis. All statistical analyses were performed using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). The expression levels of miR-132 in different subtypes of GBM were compared using Student’s t-test, and the χ² test was used to analyze the correlation between miR-132 and the GBM subtypes. X-tile software (version 3.6.1; Yale University, New Haven, CT, USA) was used to determine the cutoff value of miR-132 according to previously reported instructions (18). Kaplan-Meier survival curve and the log-rank test were performed to compare the overall survival (OS) in patient groups. COX’s proportional hazard regression model was established for multivariate analysis of the prognostic value of each factors. \( P<0.05 \) was considered to indicate a statistically significant difference.

Results

miR-132 is correlated with molecular subtypes and predicts patient outcomes. It has been reported that miR-132 was over-expressed in a cohort of 43 patients with glioma and correlated with unfavorable clinical outcomes (16). In order to minimize the system bias caused by sample scale, a GBM cohort \((n=526)\) was included, which was obtained from TCGA database to analyze the expression level of miR-132. GBM is divided into four molecular subtypes according to gene expression patterns; classical, mesenchymal, neural and proneural (22). The results indicated that miR-132 was markedly higher in the neural subtype \((8.625\pm0.077; \ n=84)\) than that in the three other subtypes \[8.625\pm0.055 \text{ for classical subtype } (n=138), \ 8.132\pm0.051 \text{ for mesenchymal subtype } (n=154) \text{ and } 8.183\pm0.056 \text{ for proneural subtype } (n=125; \ P<0.01)\] as presented in Figure 1A.

To examine the association between miR-132 expression and GBM patient outcome, a meaningful approach to classify the cutoff value \((8.0)\) for the miR-132 expression level was set using X-tile software \((P=0.0463; \text{Fig. 1B})\). Results revealed that >80% of patients with the neural subtype of GBM demonstrated a higher expression level of miR-132, as compared with patients with the classical subtype \((63.04\%)\), the mesenchymal...
miR-132 fuels proliferation, as well as self-renewal of U87 cells. miR-132 was reported to inhibit U87 cell invasion and metastasis (23), indicating its anticancer potential. Furthermore, the data demonstrated that miR-132 acted as an oncogenic miRNA during GBM progression. miR-132 expression levels were identified to be higher in U87 sphere, as compared with U87-monolayer cells (P<0.01; Fig. 2A). The miR-132 mimic was transfected into the U87 cells to elevate the expression level of endogenous miR-132 (Fig. 2A). Following pre-treatment of the U87 cells with miR-132 mimic, the proliferation ability was significantly enhanced at 48 and 72 h, as compared with U87 cells in the control group (P<0.01; Fig. 2B). To further investigate the effect of miR-132 on self-renewal in U87 cells, two representative assays (sphere formation and colony formation assays) were performed. The sphere formation assay indicated that miR-132 overexpression significantly enhanced the sphere formation potential of the cells when compared with the control group, which was treated with PBS (P<0.01; Fig. 2C and D). For the colony formation assay, no significant increase/decrease was observed within the groups of U87 with or without miR-132 treatment (P>0.05; Fig. 2E and F). These results demonstrated that a high expression level of miR-132 promotes proliferation, as well as self-renewal potential of U87 cells. Microarray based bioinformatics analysis revealed the role of miR-132 in GBM cells. To further elucidate the mechanisms underlying the method by which miR-132 promotes GBM proliferation and maintains the sphere formation properties of U87 cells, a genetic bioinformatics database, the GCBI was searched, which provides a web-lab with bioinformatics approaches to manage numerous microarray results (19). The Affymetrix Gene Chip was obtained (control group vs. miR-132 transfected group) from the GEO database and run in the GCBI web-lab. Following the unsupervised hierarchical clustering analysis, differently expressed genes were selected according to their P-value threshold and represented in a heat map, as well as in a volcano map (Fig. 3A and B). The results revealed that 3,121 genes were overexpressed and 2,742 genes were downregulated following miR-132 overexpression in GBM cells (data not shown). To elucidate the miR-132-associated biological processes and underlying mechanisms, GO analysis was applied and the results demonstrated that significant GO pathways were screened according to their P-values. The representative top 15 significant GO pathways are presented in Fig. 3C. An additional method to identify the associated signaling pathways is evaluation by their enrichment score (24). The representative top 15 enriched signaling pathways are presented in Fig. 3D. To investigate the pathway interactions in miR-132 overexpressed GBM cells, the pathway interaction-based network was also applied. The results indicated that following miR-132 transfection in GBM cells, only gliomas, the ErbB signaling pathway, the adherens junction, proteasomes, CRC, pancreatic cancer, chronic myeloid leukemia, thyroid cancer and the extracellular matrix-receptor (ECM-receptor) interaction were definitely upregulated (as demonstrated by red spheres; Fig. 4). Furthermore, other signaling pathways/functional pathways are represented by yellow spheres, which indicates that these pathways may be upregulated by certain pathways but downregulated by some other signaling pathways (Fig. 4). Taken together, the results demonstrated that miR-132 transfection in GBM cells significantly altered a great number of genes and induced activation of various downstream signals, leading to sustained proliferation and sphere formation. However, the detailed mechanisms require further investigation.

PTBP2 was the downstream target of miR-132 in GBM cells. The GCBI successfully revealed 2,743 genes, which were downregulated in miR-132 transfected GBM cells. miRWalk was used to predict the miR-132 target genes (21). A total of 38 genes were commonly predicted in at least five of the six miRNA prediction databases (miRanda, miRDB, miRWalk, PICTAR5, RNA22 and Targetscan). Notably, PTBP2 was the only common gene of the 38 predicted genes and the 2,743

| Factor      | Univariate analysis | Multivariate analysis |
|-------------|---------------------|-----------------------|
|             | P-value             | HR (95% CI)           | P-value             | HR (95% CI)           |
| Gender      | 0.237               | 0.885 (0.724-1.083)    | 0.280               | 0.894 (0.731-1.095)    |
| Subtypes    | 0.113               | 0.934 (0.858-1.016)    | 0.088               | 0.929 (0.853-1.011)    |
| miR-132     | 0.013               | 1.205 (1.041-1.395)    | 0.013               | 1.204 (1.040-1.395)    |

HR, hazard ratio; CI, confidence index.
downregulated genes (Fig. 5A). To further elucidate the miR-132/PTBP2 regulation mechanism, 13 fresh GBM specimens were obtained from GBM patients and the expression levels of miR-132 and PTBP2 were examined. Subsequently, a linear regression model demonstrated that miR-132 and PTBP2 were negatively correlated in the GBM specimens ($R^2=0.5393$, $P<0.01$; Fig. 5B). Thus, these results indicate that PTBP2 was a putative downstream target of miR-132 in GBM cells.

**Discussion**

Despite the availability of surgical treatment and chemoradiotherapy, patients with GBM continue to experience unfavorable outcomes (2,3). A notably malignant behavior of GBM is its unlimited proliferation potential, which leads to recurrence following surgery. Increasing evidence demonstrates that miR-132 is essential during tumorigenesis and progression. While, the role of miR-132 remains unclear, it may serve as either an oncogene or tumor suppressor depending on the tumor type (8,9,16,25).

In ovarian cancer cells, miR-132 suppresses cell proliferation, invasion and migration by targeting E2F transcription factor 5 (25). Downregulated miR-132 was detected in CRC specimens and associated with a poor prognosis in patients (8). In addition, miR-132 inhibits proliferation of hepatic carcinoma cells by targeting yes-associated protein (6). Furthermore, miR-132 expression levels were

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**Figure 2.** Expression of miR-132 in glioblastoma multiforme cells and its effect on proliferation and self-renewal of GBM cells. (A) Expression level of miR-132 in U87 cells and U87 neurospheres with or without miR-132 mimic transfection analyzed by quantitative polymerase chain reaction. (B) A Cell Counting Kit-8 assay evaluating the impact of miR-132 on proliferation of U87 cells. (C and D) Neurosphere formation assay was conducted to evaluate the influence of miR-132 on sphere formation ability of U87 cells. Magnification, x400. (E and F) Colony formation assay was used to compare the clones formed by U87 cells that were transfected with miR-132 mimics or that were untreated. *$P<0.05$ and **$P<0.01$.
significantly increased in gastric cancer (GC) specimens and resulted in enhanced GC cell growth, which was mediated by the suppression of Forkhead box protein O1 (7). In human glioma tissues, high expression levels of miR-132 were widely detected and positively correlated with the WHO glioma grade (16), which indicated an oncogenic role in glioma cells. However, miR-132 was reported to inhibit the invasion and metastasis of U87, which indicates a tumor suppressive function (23). Therefore, a comprehensive evaluation of miR-132 is required to evaluate its expression in GBM specimens. A total of 526 GBM specimens were obtained from the TCGA database to minimize the system bias resulting from sample size, and revealed miR-132 as a promising and potential independent prognostic indicator for GBM patients. The clinical functions of miR-132 in GBM cells were quickly verified in vitro with an interesting result, which indicated that miR-132 may serve as an oncogenic miRNA during GBM progression.

The identified molecular subtypes may underlie differences in patient sensitivity to therapy and prognosis (22,26). Notably, miR-132 was significantly elevated in the neural subtype of GBM specimens. According to previous reports, neural subtype is not sensitive to concurrent chemoradiotherapy or temozolomide (26). In addition, according to the classification approach used by Phillips et al (26), the neural subtype belongs to a proliferative subclass, which has a much shorter median survival time when compared with proneural and mesenchymal subclasses (26). The present study demonstrated that miR-132 promoted the proliferation and self-renewal potential of U87 cells, which is consistent with a previous study (26).

To further elucidate the underlying mechanisms of how miR-132 regulates proliferation and self-renewal of U87 cells, the GEO microarray database was searched and the miRWalk web tool was used to predict miR-132 target genes. PTBP2 was identified as the only common gene within the downregulated gene pool in the GEO microarray and miRWalk prediction gene pool.

PTBP2 belongs to the polypyrimidine tract binding (PTB) proteins, is primarily detected in brain tissues and regulates tissue-specific post-transcriptional functions during neuron development and pathological processes (27). In osteosarcoma, the combination of PTBP2/PTB-associated splicing factor inhibited cell proliferation, migration, invasion and the epithelial-mesenchymal transition processes (28). However, its function in the CNS is quite specific and different. A previous study revealed that the PTBP2 level induced various splicing programs including the differentiation of neuron stem cell, early differentiating neuron splicing, and synaptic

Figure 3. Bioinformatics analysis of miR-132 based on GEO glioblastoma multiforme datasets. Unsupervised hierarchical clustering analysis was performed to compare differently expressed genes between two GEO datasets (control vs. miR-132 transfection). The result is presented as (A) a heat map and (B) a volcano map (C) Representative top 15 significant GO signaling pathways. (D) Top 15 enriched signal pathways. GEO, Gene Expression Omnibus; GO, gene ontology; GnRH, gonadotropin-releasing hormone; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3 kinase; HCM, hypertrophic cardiomyopathy; TGF-β, transforming growth factor β; SNARE, soluble NSF attachment protein receptor.
PTBP2 was demonstrated to promote proliferation and migration in the human glioma cell lines, U251 and LN229 (30). However, in another glioma cell, T98 G, the expression level of PTBP2 was lower than that of healthy brain tissues (31). In the present study, PTBP2 was downregulated in glioma stem cells and served as a tumor suppressor, which was revealed as a promising downstream target of miR-132 during GBM progression.

In conclusion, the results indicate that the miR-132/PTBP2 signaling pathway may sustain U87 cell proliferation and self-renewal, and elucidate the potential role exerted by miRNAs in GBM. In addition to highlighting the ability of miRNAs, the present study demonstrated the complexity of the underlying mechanisms regulating GBM progression. There are certain limitations in the present study. The fresh sample size was small and needs to be increased to be of statistical value. The only cell model used in the present study was U87, to verify the results and conclusions, more cells need to be used in the future. Detailed underlying mechanisms require further investigation, which will be the focus of future studies by the authors.

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