LncRNA RGMB-AS1 Promotes Glioma Growth and Invasion Through miR-1200/HOXB2 Axis

Background: Dysfunction of long noncoding RNA (lncRNA) is associated with tumorigenesis of various malignancies, including glioma. LncRNA RGMB-AS1 (RGMB antisense RNA 1) has been reported to participate in initiation and progression of several cancers, such as lung cancer, hepatocellular carcinoma and laryngeal squamous cell carcinoma. Nevertheless, whether RGMB-AS1 regulates glioma development is not investigated. In this study, we aimed to determine its roles in glioma.

Methods: qRT-PCR and Western blotting were used to measure gene expression. CCK8 and colony formation assays were utilized to analyze proliferation. Transwell assay was used to determine cell migration and invasion. Luciferase reporter assay was used to validate the interactions among RGMB-AS1, miR-1200 and HOXB2.

Results: RGMB-AS1 was upregulated in glioma tissues and associated with glioma grade and patients’ prognosis. Moreover, RGMB-AS1 silencing significantly inhibited the proliferation, migration and invasion of glioma cells. RGMB-AS1 downregulation led to more tumor cells arrested in the quiescent state. Mechanistically, we found that RGMB-AS1 was a molecular sponge for miR-1200. MiR-1200 level was inhibited by RGMB-AS1. And RGMB-AS1 promoted HOXB2 expression via sponging miR-1200. Restoration of HOXB2 effectively rescued the abilities of proliferation, migration and invasion in RGMB-AS1-depleted glioma cells.

Conclusion: Collectively, our work clarified that RGMB-AS1/miR-1200/HOXB2 signaling exerts an essential role in regulating glioma progression.

Keywords: RGMB-AS1, miR-1200, HOXB2, glioma, progression

Introduction
Glioma is one of the most aggressive tumors in the central nervous system (CNS).1 Until today, it is still difficult to cure glioma and the 5-year survival time of glioma patients is under 15 months.2 Although current therapeutic strategies including surgery, radiotherapy and chemotherapy, have achieved advancement, outcomes of glioma patients remain unsatisfactory due to recurrence and metastasis.3 Thus, in-depth understanding the molecular mechanism of glioma development is urgently required.

Long noncoding RNAs (lncRNAs) have no ability to code proteins and are over 200 nucleotides in length.4 Numerous references have reported that lncRNAs play vital roles in various biological processes, such as development, immune response and cancer.5,6 LncRNA could regulate gene expression at the transcriptional or post-transcriptional level.7 Several evidences have indicated that dysregulation of lncRNAs is associated with tumorigenesis.8 LncRNA is involved in the regulation of malignant behaviors of cancer cells, such as proliferation and invasiveness.9,10 For
example, lncRNA POU3F3 promotes growth and invasion of cervical cancer via targeting miR-127-5p/FOXD1 axis.\textsuperscript{11} LINC01355 inhibited the proliferation and cell-cycle progression in breast cancer via inhibiting CCND1 transcription.\textsuperscript{12} Besides, LINC00339 increases proliferation and migration of hepatocellular carcinoma via regulating miR-1182/SKA1 signaling.\textsuperscript{13}

RGMB-AS1 was firstly identified as an upregulated lncRNA in non-small cell lung cancer.\textsuperscript{14} Subsequently, a study proved that RGMB-AS1 promotes growth and migration of lung cancer cells.\textsuperscript{15} Recently, studies also indicate that RGMB-AS1 plays oncogenic roles in hepatocellular carcinoma and laryngeal squamous cell carcinoma.\textsuperscript{16,17} However, its function in glioma remains undetermined. In this study, we found that RGMB-AS1 was upregulated in glioma tissues. Besides, RGMB-AS1 upregulation indicated clinical severity and poor prognosis. Loss-of-RGMB-AS1 induced suppression of proliferation, migration and invasion. In mechanism, we identified that RGMB-AS1 was a sponge for miR-1200 and promotes HOXB2 expression. Taken together, our study reveals that RGMB-AS1 exerts oncogenic roles in glioma through miR-1200/HOXB2 signaling.

**Materials and Methods**

**Clinical Samples**

57 glioma tissues and normal controls were collected from Zhuji People’s Hospital of Zhejiang Province and stored in the liquid nitrogen. No patient was treated by chemotherapy or radiotherapy before surgery. This study was approved by the Ethics Committee of Zhuji People’s Hospital of Zhejiang Province. All experiments using human samples were conducted in accordance with the Declaration of Helsinki. Written informed consent was achieved from each patient.

**Cell Culture and Transfection**

All glioma cell lines and normal human astrocyte cell line (NHA) were purchased from the Chinese Academy of Sciences cell bank (Shanghai, China). Cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS, Sciencell, LA, USA) and were incubated in an atmosphere containing 5% CO\textsubscript{2} at 37 °C.

**qRT-PCR**

Total RNA was isolated from tissues or cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA). qPCR was performed using TaqMan Non-coding RNA Assays and TaqMan miRNA Assays. Relative expression was normalized to GAPDH or U6 and calculated according to the $2^{-\Delta\Delta C_{t}}$ method. All samples were run in triplicate.

**Cell Proliferation Assay**

For Cell Counting Kit-8 (CCK8, Dojin, Japan) assay, 2000 cells were seeded in 96-well plates and cultured for indicated times. Then 10 μ CCK8 solution was added and incubated for 2 h. Then the absorbance at 450 nm was measured. For colony formation assay, 500 cells were seeded into 6-well plates and cultured for 14 days. Then colonies were fixed with methanol and stained with 0.1% crystal violet. Colony numbers were then counted.

**Cell Migration and Invasion Assays**

Transwell assay through a 24-well transwell chamber (8 μm core, Costar, Corning, NY) was performed to determine migration and invasion as described previously.\textsuperscript{18}

**Luciferase Reporter Assay**

RGMB-AS1 or HOXB2 3’-UTR sequences containing wild-type (WT) or mutant (MUT) predicted binding site with miR-1200 was inserted into pmirGLO Dual-Luciferase Vector (Promega). For luciferase reporter assay, the luciferase vectors and miR-1200 mimics or negative control (miR-NC) were co-transfected into glioma cells for 48h. Then the relative luciferase activity was determined by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and normalized to Renilla luciferase activity.

**RNA Immunoprecipitation**

RNA immunoprecipitation (RIP) assay was performed as previously reported.\textsuperscript{19}

**Statistical Analysis**

Results were displayed as the mean ± standard deviation (SD) from three independent experiments. Statistical analyses were performed using SPSS 22.0 software by the Student’s $t$ test, one-way ANOVA or Log rank test. Differences were considered statistically significant when $P < 0.05$.

**Results**

**RGMB-AS1 Was Upregulated in Glioma Tissues**

The expression of RGMB-AS1 in glioma tissues was analyzed by qRT-PCR. Results indicated that RGMB-AS1 was
upregulated in glioma tissues (Figure 1A). Furthermore, its level was positively correlated with pathological grades of glioma (Figure 1B). Similarly, RGMB-AS1 expression was elevated in glioma cell lines compared to NHA cells (Figure 1C). In addition, Kaplan-Meier survival analysis implied that RGMB-AS1 high expression predicted low overall survival rate (Figure 1D).

**RGMB-AS1 Accelerates Growth, Migration and Invasion of Glioma Cells**

To deeply investigate the roles of RGMB-AS1 in glioma, we knocked it down in U87 and LN229 cells (Figure 2A). The effects of RGMB-AS1 on malignant biological behaviors of glioma cells were then examined by CCK8, colony formation, flow cytometry and Transwell assay. RGMB-AS1 knockdown significantly inhibited the proliferation and colony formation compared to si-NC group (Figure 2B and C). Moreover, RGMB-AS1 downregulation increased the cell percent in G0/G1 phase (Figure 2D and E), suggesting RGMB-AS1 promoted cell-cycle progression. Additionally, reduction of RGMB-AS1 expression impairs the migration and invasion abilities of glioma cells (Figure 2F and G). Similarly, RGMB-AS1 overexpression promoted proliferation, migration and invasion of U87 cells (Figure 2H–J). Thus, above findings suggest that RGMB-AS1 is a novel oncogene in glioma.

**Regulatory Relationships Among RGMB-AS1, miR-1200 and HOXB2**

LncRNAs have been demonstrated to be miRNA sponges in tumor cells. Thus, we investigated whether RGMB-AS1 could be a molecular sponge for some miRNAs. Using bioinformatics database (miRDB), RGMB-AS1 was identified as a possible sponge for miR-1200 (Figure 3A). To confirm it, we constructed RGMB-AS1 wide-type (WT) and mutant (MUT) luciferase reporter vectors (Figure 3A). Results showed that RGMB-AS1-WT activity was reduced

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**Figure 1** RGMB-AS1 was upregulated in glioma tissues. (A) Relative expression of RGMB-AS1 in glioma tissues and normal tissues were analyzed by qRT-PCR. (B) Relative expression of RGMB-AS1 in different grades of glioma tissues. (C) Relative expression of RGMB-AS1 in glioma cell lines was determined. (D) Kaplan-Meier overall survival was analyzed according to RGMB-AS1 expression. *P<0.05.
Figure 2 RGMB-AS1 accelerates growth, migration and invasion of glioma cells. (A) RGMB-AS1 expression was determined in U87 and LN229 cells. si-NC: negative control siRNA. (B) CCK8 assay was performed using U87 and LN229 cells transfected with si-RGMB-AS1 or si-NC. (C) RGMB-AS1 knockdown decreases the number of colonies. (D and E) Cell-cycle analysis in U87 and LN229 cells after transfection with si-RGMB-AS1 or si-NC. (F and G) Transwell assay showed that RGMB-AS1 knockdown reduced the numbers of migration and invasion. (H) CCK8 assay was performed to analyze proliferation in U87 cells. (I and J) Transwell assay was performed to determine migration and invasion in U87 cells. *P<0.05.
after transfection with miR-1200 mimics (Figure 3B). In addition, RIP assay indicated that RGMB-AS1 and miR-1200 were both enriched by anti-Ago2 in U87 cell lysates (Figure 3C), suggesting RGMB-AS1 and miR-1200 were in an RNA-induced silencing complex (RISC). Of note, RGMB-AS1 knockdown promoted the level of miR-1200 (Figure 3D).

Next, the potential targets of miR-1200 were analyzed using TargetScan7 and miRDB. We identified HOXB2 as the potential target of miR-1200 (Figure 3E). We also constructed HOXB2 WT and MUT luciferase reporter vectors (Figure 3E). Luciferase reporter assay showed that miR-1200 mimics only inhibited the activity of HOXB2-WT reporter (Figure 3F). Moreover, HOXB2 expression was suppressed by miR-1200 mimics (Figure 3G and H). To determine whether HOXB2 expression was regulated by RGMB-AS1/miR-1200 axis, we administrated U87 and LN229 cells with miR-1200 inhibitors and/or RGMB-AS1. As shown, RGMB-AS1 knockdown suppressed HOXB2 expression while si-RGMB-AS1 plus miR-1200 inhibitors rescued HOXB2 expression, and vice versa (Figure 3I and J). Moreover, we found that there was a negative correlation between RGMB-AS1 and miR-1200 or between miR-1200 and HOXB2 in glioma tissues (Figure 3K). Therefore, RGMB-AS1 was

![Figure 3](image-url)  Regulatory relationships among RGMB-AS1, miR-1200 and HOXB2. (A) Binding site with miR-1200 in RGMB-AS1 was presented and the response element was mutated. (B) Luciferase reporter assay was conducted using RGMB-AS1 wide-type (WT) or mutant (MUT) reporter. (C) RIP assay showed that anti-Ago precipitated both RGMB-AS1 and miR-1200 in U87 cell lysates. (D) RGMB-AS1 knockdown promoted miR-1200 expression. (E) Binding site with miR-1200 in HOXB2 3’-UTR was presented and the response element was mutated. (F) Luciferase reporter assay was conducted using HOXB2 3’-UTR wide-type (WT) or mutant (MUT) reporter. (G and H) miR-1200 inhibited the mRNA and protein levels of HOXB2. (I and J) HOXB2 expression was analyzed after transfection with indicated plasmids. (K) Expression correlations among RGMB-AS1, miR-1200 and HOXB2 were analyzed in glioma tissues. *P<0.05.
a molecular sponge for miR-1200 and upregulated HOXB2 expression.

**RGMB-AS1 Promoted Glioma Progression Through HOXB2**

Interestingly, we found that HOXB2 expression was upregulated in glioma tissues through TCGA database and qRT-PCR results (Figure 4A and B). Moreover, TCGA result indicated that HOXB2 upregulation in glioma patients predicted a low survival rate (Figure 4C), suggesting HOXB2 was an oncogene in glioma and HOXB2 may be a downstream effector of RGMB-AS1/miR-1200 axis. To prove it, we conducted rescue assays. Results showed that HOXB2 restoration rescued the proliferation, migration and invasion of si-RGMB-AS1 transfected glioma cells (Figure 4D–F). Moreover, HOXB2 overexpression accelerated the proliferation, migration and invasion of glioma cells (Figure 4D–F). In conclusion, RGMB-AS1 promoted glioma progression through modulating miR-1200/HOXB2 axis.

**Discussion**

Increasing numbers of lncRNAs have been demonstrated to be biomarkers or therapeutic targets in glioma. They play vital functions in regulating glioma progression. For example, lncRNA HOTAIRM1 upregulation increases proliferation and metastasis of glioma via promoting HOXA1 expression. LncRNA GACAT3 promotes proliferation, migration and invasion of glioma via inhibiting miR-3127. LncRNA CASC9 promotes glioma cell growth and migration via regulating miR-519d/STAT3 pathway. And lncRNA ANRIL modulates growth and cell-cycle of glioma cells through sponging miR-203a. Our present work focused on illustrating the roles of RGMB-AS1 in glioma. Upregulation of RGMB-AS1 has been identified in several cancer types. RGMB-AS1 was upregulated in non-small cell lung cancer. Subsequently, a study proved that RGMB-AS1 promotes...
growth and migration of lung cancer cells. Recently, studies also indicate that RGMB-AS1 plays oncogenic roles in hepatocellular carcinoma and laryngeal squamous cell carcinoma. However, how it functions in glioma remains undetermined. We found that RGMB-AS1 was upregulated in glioma tissues and cell lines. Moreover RGMB-AS1 upregulation in glioma patients was associated with a low survival rate. Functionally, we showed that RGMB-AS1 downregulation successfully inhibited proliferation, migration and invasion of glioma cells. Our data demonstrated that RGMB-AS1 is a novel oncogene in glioma.

MicroRNAs (miRNAs) are a type of well-known ncRNAs and have less than 22 nucleotides in length. miRNAs are also very important molecules that regulate many biological processes in cancer. Large numbers of studies have demonstrated the pivot roles of miRNA in glioma. Mechanistically, miRNA could be regulated by lncRNAs, which releases their downstream target. In our research, we identified that miR-1200 was sponged by RGMB-AS1 through bioinformatics analysis. We demonstrated the direct interaction between RGMB-AS1 and miR-1200. Moreover, we showed that miR-1200 expression was increased after RGMB-AS1 downregulation in glioma cells. Furthermore, miR-1200 expression was negatively correlated with RGMB-AS1 in glioma tissues. miR-1200 is a poorly studied miRNA. Only one report showed that miR-1200 inhibited osteosarcoma pathogenesis. Whether miR-1200 plays a role in glioma is unclear. According to our study, we implied that miR-1200 is a tumor suppressor in glioma for the first time.

The possible downstream target of RGMB-AS1/miR-1200 axis was further identified by bioinformatics analysis. We identified that HOXB2 achieved the highest score. Previous study indicated that miR-1200 targets HOXB2 in osteosarcoma. Then through luciferase reporter assay, we demonstrated the direct interaction between miR-1200 and HOXB2 in glioma cells. Moreover, we showed that miR-1200 significantly inhibited the expression of HOXB2 in glioma cells. Interestingly, RGMB-AS1 knockdown suppressed HOXB2 expression in U87 and LN229 cells, which was abrogated by miR-1200 inhibitors. Thus, our findings identified a new signaling cascade of RGMB-AS1/miR-1200/HOXB2 axis. HOXB2 has been reported to participate in progression of several cancers. And a work also indicates that HOXB2 is an oncogene in glioma. Consistent with previous study, we also found that HOXB2 was upregulated in glioma tissues. Moreover, we found that HOXB2 is a potential prognostic biomarker. And rescue assay demonstrated that HOXB2 upregulation promoted proliferation, migration and invasion of glioma cells, further supporting its oncogenic roles in glioma.

In conclusion, our study demonstrated that RGMB-AS1 is a competing endogenous RNA (ceRNA) for miR-1200 to promote HOXB2 expression in glioma. And RGMB-AS1/miR-1200/HOXB2 axis accelerates glioma progression. However, the in vivo assay is required in the future to further confirm the physiological roles of RGMB-AS1/miR-1200/HOXB2 axis.

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Disclosure
The authors report no conflicts of interest in this work.

References


