SNHG7 Facilitates Glioblastoma Progression by Functioning as a Molecular Sponge for MicroRNA-449b-5p and Thereby Increasing MYCN Expression

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Abstract
Background and Aims: Long noncoding RNA (small nucleolar RNA host gene 7) has been reported to be involved in multiple malignancies and acts as an oncogene. However, the potential mechanism of small nucleolar RNA host gene 7 in glioblastoma is rarely known. In this study, we attempted to elucidate the biological effects of small nucleolar RNA host gene 7 and the possible molecular mechanism in glioblastoma. Methods: The expression level of small nucleolar RNA host gene 7 in glioblastoma tissues and corresponding tumor cell lines was evaluated by using quantitative real-time polymerase chain reaction. Bioinformatics analyses and dual-luciferase reporter gene assay were conducted to verify the correlation among small nucleolar RNA host gene 7, miR-449b-5p, and MYCN. The role of small nucleolar RNA host gene 7 on cell viability, migration, and invasion was measured. Results: Small nucleolar RNA host gene 7 expression was markedly increased in glioblastoma tumor tissue. Small nucleolar RNA host gene 7 can sponge miR-449b-5p and negatively regulate miR-449b-5p expression. MiR-449b-5p was remarkably repressed in glioblastoma tissues. Reduction of miR-449b-5p reversed the repressive effects of small nucleolar RNA host gene 7 knockdown on cellular behaviors in glioblastoma. In addition, miR-449b-5p can directly bind with MYCN. Compared with normal samples, MYCN expression was increased. The MYCN expression was negatively related to miR-449b-5p expression while positively related to small nucleolar RNA host gene 7 expression. Rescue experiments revealed that MYCN overexpression reversed the repressive role of small nucleolar RNA host gene 7 knockdown on viability, migration, and invasion of U251 cells. Conclusion: In summary, our results demonstrated that small nucleolar RNA host gene 7 regulates glioblastoma proliferation, migration, and invasion via regulating miR-449b-5p and its target gene MYCN, thereby providing a potential therapeutic target for glioblastoma.

Keywords
GBM, SNHG7, miR-449b-5p, MYCN, progression

Abbreviations
CCK-8, Cell Counting Kit 8; ceRNA, competitive endogenous RNA; GBM, glioblastoma; lncRNAs, long noncoding RNAs; miR inhibitor, si-NC + miR-449b-5p inhibitor; miRNAs, microRNAs; MUT, MYCN mutant; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction; SNHG7, small nucleolar RNA host gene 7; WT, wild type; 3'-UTR, 3'-untranslated region.

Received: February 25, 2020; Revised: June 1, 2020; Accepted: June 19, 2020.

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**Introduction**

Glioblastoma (GBM) is identified as a most frequent and lethal primary brain cancer.\(^1\) Although treatment such as surgical resection and chemoradiotherapy greatly advances, GBM inevitably recurs around the tumor removal cavity.\(^2,3\) According to statistics, the survival time of most patients with GBM who had undergone standard treatment still is not more than 2 years.\(^4,5\) Thus, advancing our understanding of GBM pathogenesis and identifying the efficacious and unique GBM targets are important for GBM treatment.

Competitive endogenous RNA (ceRNA), initially proposed by Salmena and his co-workers, is composed of RNA with protein-coding capacity, including long noncoding RNAs (lncRNAs), transfer RNA, and ribosomal RNA.\(^6,7\) Recently, increasing evidences have validated that lncRNAs exert promotion or suppressive effects to modulate various biological processes of different types of tumors as key ceRNAs.\(^8,9\) This suggests that lncRNAs might serve as prospective therapeutic targets to predict the prognosis of patients with GBM and participate in the GBM treatment. For instance, LOXL1 antisense RNA 1 (LOXL1-AS1) could impair mesenchymal features of GBM through nuclear factor $\kappa$B pathway.\(^10\) Highly regulation of HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1) can increase the expression of homeobox A1 (HOXA1) and sequester G9a/ enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2)/DNA methyltransferase (Dnmts) in GBM, thereby contributing to the growth and invasion of tumor.\(^11\) Zhang et al illustrated that LINC01446 could accelerate the development of GBM via regulating miR-489-3p/tumor protein translationally-controlled 1 (TPT1) axis.\(^12\) Small nucleolar RNA host gene 7 (SNHG7), located at chromosome 9q34.3, has been proven to be overexpressed in various types of tumors.\(^13-15\) Despite SNHG7 has been confirmed as an oncogene molecule for GBM progression through mediating miR-5095/catenin beta 1 (CTNNB1) axis,\(^16\) the potential mechanism of SNHG7 in GBM has not been clearly explained yet.

MicroRNAs (miRNAs) have been previously classified as small short RNA molecules, encoding none proteins. Numerous publications have expounded that miRNAs hold crucial capability in tumorigenesis through targeting the 3'-untranslated region (3'-UTR) of key genes and being sponged by lncRNAs.\(^17\) Yin et al have elucidated that miR-449b-5p hinders cell growth, invasion, and migration via targeting TPD52 in nasopharyngeal carcinoma.\(^18\) Breast cancer cell viability and invasion are blocked by miR-449b-5p.\(^19\) Additionally, miR-449b-5p functions as a sponge of NEAT1 in glioma.\(^20\)

MYCN, belongs to the MYC family with 63 kDa in length and was firstly discovered as a paralog of $c$-MYC by Schwab et al in 1983.\(^21\) It is located downstream of multiple signaling pathways and participates in the processes of cell growth, cycle, and apoptosis.\(^22,23\) Accumulating evidences have demonstrated that MYCN plays an essential role during the progression of neuroblastoma.\(^24\) The exogenous expression of MYCN contributes to the proliferation and tumorigenic potential of neuroblastoma cells without MYCN amplification.\(^25\) Notably, MYCN is a direct target of miR-34a and MYCN overexpression could ameliorate GBM development.\(^26\) However, its relationship with miR-449b-5p in GBM has not explored.

Hence, in the current study, we investigated the function of SNHG7 in regulating the malignant progression of GBM and the possible mechanism. We found that SNHG7 knockdown suppressed GBM cell proliferation, migration, and invasion in vitro by elevating miR-449b-5p expression and reducing MYCN (a target of miR-449b-5p) expression.

**Materials and Methods**

**Collection of GBM Tissues**

The cancerous tissues and noncancerous tissues of 53 patients with GBM were enrolled from our hospital from January 2016 to January 2019. All clinical samples were quickly stored in liquid nitrogen before the exploration began. This investigation was approved by the ethics committees of our hospital (No. 2016013), and informed consent has been signed by all of the participating patients with GBM. Before the surgery, these patients underwent no radiotherapy or chemotherapy. Postoperative follow-up was performed by outpatient or telephone, every half a year.

**Cell Lines and Culture**

Human normal astrocytes NHA, U87, U251, T98G, and LN229 cell lines were purchased from the American Type Culture Collection and cultivated in the Dulbecco modified Eagle medium containing 10% fetal bovine serum (Gibco) at 37°C with 5% CO$_2$. Conventional passages were performed when the cell adherence was over 90%.

**Transfection**

MiR-449b-5p mimics/inhibitor (20 nM), mimics/inhibitor negative control (NC, 20 nM), si-SNHG7-1 (20 nM), si-SNHG7-2 (20 nM), si-NC (20 nM), pcDNA3.1-SNHG7 (1 μg), pcDNA3.1-MYC (1 μg), and pcDNA3.1-empty vector (pcDNA3.1-NC, 1 μg) were all synthesized by GenePharma (Shanghai, China), and then transfected into cells using Lipo-fectamine 3000 (Invitrogen) on the basis of the manufacturer guidelines.

**Luciferase Activity Assay**

The fragment of SNHG7 3'-UTR or MYCN 3'-UTR carrying the wild type (WT) or mutated (MUT) binding sites for miR-449b-5p was cloned into the pMIR-reporter vector. In order to verify the associations among SNHG7, MYCN, and miR-449b-5p, GBM cells were cotransfected with above agents and miR-449b-5p mimic (20 nmol), mimic NC (20 nmol), or miR-449b-5p mimics (20 nmol) + pcDNA3.1-SNHG7 (1 μg). At 48 hours after transfection, the relative luciferase activity was assessed by a dual-luciferase reporter gene assay system (Promega).
Analysis of CCK-8

The proliferative ability of U251 and U87 cells was detected by Cell Counting Kit 8 (CCK-8) assay. Briefly, transfected cells (2 x 10^3 cells per well) were incubated into the 96-well plate to incubate for specific time at 37 °C with 5% CO2. After 0, 24, 48, and 72 hours of incubation, 10 μL of CCK-8 solution was added into each well, and corresponding cells were continuously cultured for 1.5 hours. At last, the optical density values were assessed under a microplate reader (Bio-Rad) with the wavelength of 450 nm.

Scratch Assay

After transfection for 24 hours, GBM cells were seeded into 6-well plates with the density of 5 x 10^5 cells/well to incubate until 80% confluency. Then, the scratch was generated with a 200 μL pipette tip and cultured for 24 hours. The healing distance was captured by a microscope. The migration distance = (width at 0 hour − width at 24 hours)/width at 0 hour x 100%.

Cell Invasion Analysis

Transwell assay was utilized to measure the cells invasive ability. Firstly, Matrigel (100 μL; diluted 1:6 in serum-free medium) was added into the transwell chamber, which was next inserted in the 24-well plates. Cell suspension (200 μL, 2.0 x 10^5 cells/mL) was placed into the top chamber, while the basolateral chamber was fulfilled with 500 μL culture medium. After 48 hours of incubation, transwell chambers were removed and the invading cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Images of penetrating cells were photographed, and invaded cells were counted with 6 randomly selected fields.

qRT-PCR

Total RNA was extracted from GBM tissues and cells by miR-Neasy Mini Kit (Qiagen), and the complementary DNA was reverse transcribed using Primerscript RT reagent (Takara, Japan). Real-time polymerase chain reaction was conducted on ABI7500 system with SYBR Premix Ex Taq Reagent (Takara, Japan). Real-time polymerase chain reaction was conducted reverse transcribed using Primescript RT reagent (Takara, Japan). Real-time polymerase chain reaction was conducted qRT-PCR, we found that SNHG7 expression was significantly upregulated in GBM tissue samples compared with noncancerous tissues (P < .001). We also assessed the SNHG7 expression in grade I +II and grade III + IV GBM samples and found that advanced patients had the higher expression of SNHG7 than early patients (Figure 1B). Furthermore, the recruited patients were divided into high SNHG7 expression group and low SNHG7 expression group according to the median of SNHG7 expression level. The chi-square test was applied to evaluate the relationship between SNHG7 and clinical characteristics. As shown in Table 1, SNHG7 expression was positively related with World Health Organization Grade. We further examined the expression levels of SNHG7 in 4 GBM cell lines and normal cell line human normal astrocyte (NHA) using qRT-PCR method. The data revealed that SNHG7 was dramatically increased in all GBM cell lines in comparison with NHA cell line (Figure 1C). U251 and U87 cell lines were selected for further investigation due to the higher expression of SNHG7 than other GBM cell lines.

SNHG7 Sponges MiR-449b-5p in GBM Cells

StarBase prediction tool was employed to explore the binding site of SNHG7 and miR-449b-5p, and its sequence is shown in Figure 2A. In order to verify the association between SNHG7 and miR-449b-5p, we first used small interfering RNA strategy to knockdown the expression of SNHG7. On the basis of qRT-PCR, we found that SNHG7 expression was significantly inhibited after transfection with si-SNHG7-1 and si-SNHG7-2 in U251 and U87 cells (Figure 2B). si-SNHG7-1 was selected to be used for the following experiments owing to its higher transfection efficiency. Knockdown of SNHG7 significantly increased miR-449b-5p expression in U251 and U87 cells (Figure 2C). Next, luciferase reporter assay was conducted to validate the association between SNHG7 and miR-449b-5p.
The transfection of miR-449b-5p mimics and inhibitor was utilized to increase or decrease the expression of miR-449b-5p in GBM cells (Figure 2D). Cotransfection of miR-449b-5p mimics and SNHG7 WT significantly attenuated the luciferase activity, while GBM cells cotransfected with miR-449b-5p mimics and SNHG7 MUT revealed no change in luciferase activity (Figure 2E). Importantly, miR-449b-5p was markedly reduced in GBM tissue samples compared with normal samples, as described by qRT-PCR (Figure 2F). Pearson correlation analysis elucidated that there was a negative correlation between SNHG7 and miR-449b-5p expression (Figure 2G).

The GBM cells (U251, L229, U87, and T98G) showed lower expression levels of miR-449b-5p compared to NHA cells (Figure 2H). These findings implied that SNHG7 directly targeted miR-449b-5p as a ceRNA.

**Reduction of MiR-449b-5p Can Reverse the Repressive Effects of si-SNHG7 on GBM Cells**

To investigate the possible functions of SNHG7 and miR-449b-5p in GBM, CCK-8 analysis, scratch assay, and transwell experiments were performed using U251 and U87 cells after various treatments. The proliferative ability of GBM cells was remarkably inhibited after SNHG7 knockdown; whereas the transfection of miR-449b-5p inhibitor increased GBM cell viability. Furthermore, cotransfection of si-SNHG7-1 and miR-449b-5p inhibitor indicated that SNHG7-knockdown-induced inhibition of cell proliferation could be relieved because of the knockdown of miR-449b-5p (Figure 3A). As shown in Figure 3B, the wound healing distance of si-SNHG7-1 + inhibitor NC group was less than that in the si-NC + inhibitor NC

### Table 1. Relationship Between SNHG7 Expression and Clinical Characteristics of Patients With GBM.

| Characteristics   | Low (n = 25) | High (n = 28) | P value |
|-------------------|--------------|---------------|---------|
| Age               |              |               | .685    |
| <50 years         | 26           | 13            |         |
| ≥50 years         | 27           | 13            |         |
| Gender            |              |               | .637    |
| Males             | 23           | 13            |         |
| Females           | 30           | 15            |         |
| Diameter          |              |               | .833    |
| <5 cm             | 22           | 10            |         |
| ≥5 cm             | 31           | 15            |         |
| Resection degree  |              |               | .610    |
| Total resection   | 21           | 12            |         |
| Subtotal resection| 32           | 16            |         |
| WHO Grade         |              |               | .028*   |
| I + II            | 17           | 12            |         |
| III + IV          | 36           | 13            |         |

Abbreviation: GBM, glioblastoma; SNHG7, small nucleolar RNA host gene 7; WHO, World Health Organization.

*P < .05.

The transfection of miR-449b-5p mimics and inhibitor was utilized to increase or decrease the expression of miR-449b-5p in GBM cells (Figure 2D). Cotransfection of miR-449b-5p mimics and SNHG7 WT significantly attenuated the luciferase activity, while GBM cells cotransfected with miR-449b-5p mimics and SNHG7 MUT revealed no change in luciferase activity (Figure 2E). Importantly, miR-449b-5p was markedly reduced in GBM tissue samples compared with normal samples, as described by qRT-PCR (Figure 2F). Pearson correlation analysis elucidated that there was a negative correlation between SNHG7 and miR-449b-5p expression (Figure 2G). The GBM cells (U251, L229, U87, and T98G) showed lower expression levels of miR-449b-5p compared to NHA cells (Figure 2H). These findings implied that SNHG7 directly targeted miR-449b-5p as a ceRNA.

**Reduction of MiR-449b-5p Can Reverse the Repressive Effects of si-SNHG7 on GBM Cells**

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Figure 2. Small nucleolar RNA host gene 7 directly targeted miR-449b-5p in GBM. A, The binding site between SNHG7 and miR-449b-5p. B, Transfection efficacy of si-SNHG7-1 and si-SNHG7-2 using qRT-PCR analysis. The SNHG7 expression was relative to blank group. C, The expression level of miR-449b-5p after SNHG7 knockdown was detected by qRT-PCR assay. The miR-449b-5p expression was relative to blank group. D, Abnormal expression of miR-449b-5p was assessed using qRT-PCR method in U251 and U87 cells after different transfections. MiR-449b-5p expression was relative to blank. E, Luciferase reporter assay confirmed the association between SNHG7 and miR-449b-5p. Luciferase activity was relative to that of mimics NC group. F, Analysis of collected patients with GBM showed that miR-449b-5p expression was reduced in GBM tissues compared with normal samples. G, Pearson correlation analysis of SNHG7 and miR-449b-5p expression in GBM tissue. H, MiR-449b-5p expression in GBM cells compared with normal cell line NHA. Data were expressed as the mean ± SD and were analyzed using 1-way ANOVA with Tukey multiple comparisons test. **P < .01. ANOVA indicates analysis of variance; GBM, glioblastoma; SNHG7, small nucleolar RNA host gene 7; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation.
group; on the contrary, the si-NC + miR-449b-5p inhibitor (miR inhibitor) group had greater migration distance compared with the si-NC + inhibitor NC group. By contrast to si-SNHG7-1 + inhibitor NC group, the addition of miR inhibitor elevated migratory capability of GBM cells. As expected, transwell invasion analysis revealed that less U251 and U87 cells in the si-SNHG7-1 + inhibitor NC group invaded to the bottom chamber, while increasing number of invaded GBM cells was exhibited in si-NC + miR inhibitor group. The suppression induced by si-SNHG7-1 on GBM cell invasion was reversed by miR...
inhibitor (Figure 3C). To sum up, these data suggested that downregulation of SNHG7 might serve as a suppressor in the GBM development, and miR-449b-5p inhibitor could alleviate its effects.

MYCN is a Directly Target of MiR-449b-5p

Through accessing to online StarBase, we discovered that MYCN was a putative target gene of miR-449b-5p (Figure 4A). Luciferase reporter gene analysis revealed that luciferase activity of MYCN WT group was significantly attenuated after transfection with miR-449b-5p mimics, which was partially reversed by overexpression of SNHG7. However, the MYCN MUT group exhibited none change of luciferase activity after indicated transfections (Figure 4B). Knockdown of miR-449b-5p can increase the expression of SNHG7 and this increasing effect could be inversed due to the interference of MYCN (Figure 4C). Notably, MYCN was increased in GBM tissues compared with normal controls (Figure 4D), which showed the consistent expressional pattern with SNHG7. Correlation analyses displayed that MYCN expression was inversely proportional to miR-449b-5p expression, while directly proportional to the expression of SNHG7 (Figure 4E and F). Collectively, all above results demonstrated that MYCN might bind to miR-449b-5p in GBM.

Up-regulation of MYCN Aggravates GBM Cellular Behaviors

In order to evaluate the underlying mechanism of MYCN and SNHG7 in GBM, we transfected pcDNA-MYC and si-SNHG7 to adjust the expression of MYCN in U251 and U87...
cells. In Figure 5A, qRT-PCR assay revealed that knockdown of SNHG7 decreased the expression of MYCN that increased by the transfection of pcDNA3.1-MYCN. U251 cell viability was elevated due to highly expression of MYCN, which was overturned by si-SNHG7 (Figure 5B). Moreover, the migration and invasion of U251 cells showed the similar tendency with cell proliferation (Figure 5C and D).

**Discussion**

Glioblastoma has been identified as a highly infiltrative brain tumor and difficult to cure.27 Currently, emerging reports have demonstrated the significance of lncRNAs in the tumorigenesis. Using the sequencing technology, a large number of cancer-related lncRNAs have been found.28 However, more investigations are needed owing to the unknown biological influence of lncRNAs as well as potential mechanism for tumor treatment. On the basis of previous reports, lncRNAs have been assigned to cancer-promoting molecules or anticancer factors because of the aberrant expression levels in different tumors.29 Among which, SNHG7 has been identified as a promoting factor in tumorigenesis. Small nucleolar RNA host gene 7 promotes the cell viability, invasion, and migration of hepatocellular carcinoma by modulating miR-122-5p and RPL4.30 The progression of Figure 5. Small nucleolar RNA host gene 7 can enforce the promoting effects of MYCN on GBM progression. A, Relative expression of MYCN was investigated using qRT-PCR assay in U251 and U87 cells with different agents. MYCN expression was relative to si-NC + pcDNA-NC group. B, U251 cell proliferation was explored by CCK-8 assay. Data were relative to si-NC + pcDNA-NC group. C, Scratch analysis was used to examine the migratory ability of U251 cells. Data were relative to si-NC + pcDNA-NC group (bar = 100 μm, × 200). D, Transwell invasion assay was conducted for invasive potential detection. Data were relative to si-NC + pcDNA-NC group (bar = 100 μm, × 400). *P < .05, **P < .01, ***P < .001. CCK-8 indicates Cell Counting Kit 8; GBM, glioblastoma; qRT-PCR, quantitative real-time polymerase chain reaction.
neuroblastoma is modulated by the SNHG7-miR-653-5p-STAT2 feedback loop. A prior literature has confirmed that SNHG7 is an oncogenic factor in GBM through inhibiting miR-5095. Our present study validated the promoting effect of SNHG7 in GBM.

Our data exhibited that SNHG7 was remarkably increased in GBM tissue samples and cell lines. Functional in vitro experiments, including CCK-8, scratch and transwell assays were performed and revealed that silencing SNHG7 prominently facilitated to the attenuated proliferation, migration, and invasion of U251 and U87 cells. In a word, these findings determined that knockdown of SNHG7 inhibited cellular malignant behaviors in GBM.

Accumulating publications have suggested that lncRNAs and miRNAs could cross-modulate each other through competing for common miRNA response elements. Many lncRNAs have been identified as ceRNAs of specific miRNAs implicated in GBM carcinogenesis. For example, AC016405.3, a novel lncRNA, plays a suppressive role via regulating of TET2 by miR-19a-5p sponging in GBM. The suppression of lncRNA MALAT1/miR-199a/ZHX1 feedback loop can alleviate GBM proliferation and metastasis. Chai and Xie have illustrated that LINC01579, serving as a ceRNA of miR-139-5p, played a promoting role in GBM through upregulating EIF4G2. Small nucleolar RNA host gene 7, a well-characterized lncRNA, was observed to exert important roles in multiple neoplasms by modulating different miRNAs and key genes. Furthermore, Ren et al determined that SNHG7 could elevate the progression of GBM by regulating miR-5095/CTNNB1 axis. In this work, bioinformatics analysis and luciferase reporter assay showed that SNHG7 can sponge miR-449b-5p and there was a negative correlation between SNHG7 and miR-449b-5p expression. MiR-449b-5p inhibitor promoted GBM cell viability, migration, and invasion, and can reverse the inhibitory effects of SNHG7 knockdown on proliferation, migration, and invasion in GBM cells. These results determined that SNHG7 might act as a sponge of miR-449b-5p in GBM. Subsequently, MYCN was identified as a directly target of miR-449b-5p in accordance with StarBase prediction analysis. A previous study has suggested that MYCN is situated downstream of diverse signaling pathways. Targeting MYCN has been identified as a therapeutic avenue for the treatment of GBM.

In summary, our present study highlights the significance of SNHG7 to GBM progression, which exerts impacts through mediating miR-449b-5p/MYCN axis and indicates that SNHG7/miR-449b-5p/MYCN network might be a potential therapeutic therapy for GBM.

Authors' Note
Yaogang Chen was mainly responsible for conceptualization, data analysis, and interpretation; Shaoyong Yuan was responsible for supervision, management, and approval of the implementation of the study; Huqing Xu was responsible for investigation and confirmation of methodology; Bo Guan was mainly responsible for software management and visualization. Tie ying Ning checked the experimental process and provided technical support and was a major contributor in writing the manuscript. All authors were involved in writing and revising, and reading and approving the manuscript. The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request. This investigation was approved by the ethics committees of the Central Hospital of Qingdao (No. 2016013), and informed consent has been signed by all of the participating patients with GBM. All patients agreed to publish the study.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

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