Ropivacaine represses the proliferation, invasion, and migration of glioblastoma via modulating the microRNA-21-5p/KAT8 regulatory NSL complex subunit 2 axis

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ABSTRACT
Ropivacaine (Rop) is available to suppress the growth of glioblastoma (GBM), while its mechanism has not been completely elaborated. In this study, we explore the latent mechanism of Rop repressing GBM's growth via mediating the microRNA (miR)-21-5p/KAT8 regulatory NSL complex subunit 2 (KANSL2) axis. MiR-21-5p was declined in GBM, while KANSL2 was elevated. Clinical association studies manifested miR-21-5p was distinctly linked to the tumor size and grade of GBM. Rop constrained GBM cell proliferation, invasion, and migration but boosted apoptosis. Elevated miR-21-5p strengthened Rop's action, while augmented KANSL2 weakened Rop's role. Furthermore, the impact of silencing miR-21-5p on GBM was turned around via declining KANSL2 in Rop-treated GBM cells. KANSL2 was the target gene of miR-21-5p. In short, Rop exerted an anti-tumor impact on GBM via mediating the miR-21-5p/KANSL2 axis, which offered novel viewpoints for the later adoption of Rop as GBM drugs.

1 Introduction
Glioblastoma (GBM) is a popular and extremely aggressive primary malignant tumor in the brain [1,2]. Approximately 52% brain tumors are GBM [3]. The molecular mechanism of the pathogenesis of GBM has not yet been completely figured out. The present treatment for GBM crucially counts on surgery [4], chemotherapy [5] radiotherapy [6], and electric field therapy [7]. These measures are adopted to rescue the lives of patients, but the prognosis of GBM is still unpleasing and the majority of patients relapse after resection [8]. As reported, approximately 90% patients diagnosed with GBM will die within 3 years [9]. Consequently, it is crucial to further explore the pathogenesis of GBM and seek more imperative treatment methods.
Ropivacaine (Rop), a long-acting amide levorotatory local anesthetic, has been broadly adopted in anesthesia or local analgesia [10]. Recently, multiple researches have reported diversified local anesthetics like bupivacaine, Rop, etc. [11]. Manifest remarkable effects in the cure of cancer, mainly presenting in restraining cancer proliferation [12], invasion [13], migrate [14] and transfer [15], etc. The present study elucidated how Rop constrains the growth and self-renewal of GBM stem cells via repressing zinc finger Asp-His-His-Cys-type palmitoyltransferase 15 (ZDHHC15)-mediated palmitoylation of GP130 [16]. Nevertheless, the latent molecular mechanism of Rop repressing GBM remains to be further figured out.

MicroRNA (miRNA), an endogenous non-coding RNA has been discovered to exert a tremendous action in modulating cancer cell proliferation [17], migration [18], apoptosis [19]. MiR-21-5p, belonging to the miRNA family, has been testified to be aberrant in multiple cancers covering colon cancer [20], lung cancer [21], stomach cancer [22] and ovarian cancer [23]. Foregoing researches have elucidated miR-21-5p is declined in GBM, and elevated miR-21-5p is available to repress migration and invasion of GBM and boost apoptosis in vitro [24]. Additionally, declined miR-21-5p were also discovered in GBM external vesicles [25]. Nevertheless, the downstream target genes of miR-21-5p modulating GBM’s growth has hardly been reported, and its action on the GBM should be further explored.

In this research, the latent molecular mechanism of Rop suppressing GBM’s growth was explored. Furthermore, the miR-21-5p/KAT8 regulatory NSL complex subunit 2 (KANSL2) pathway is the crucial approach for Rop to constrain GBM.

2 Materials and methods
2.1 Clinical samples

From 2015 to 2018, a collection of 49 pairs of tumor and para-cancerous normal tissues was from patients undergoing GBM resection at Xiangya Hospital of Central South University. Grading of GBM was in the light of the pathological diagnostic criteria of World Health Organization (WHO). No recipient of the patient had any local or systemic treatment prior to surgery. This research accorded with the regulations of the Ministry of Health of the People’s Republic of China and Declaration of Helsinki on Ethical Principles of Medical Research Involving the Human Body. All patients offered informed consent on the grounds of the plan of the Ethics Committee of Xiangya Hospital of Central South University. Collections of all tissue samples and freeze in liquid nitrogen were immediately performed, and storing was conducted until RNA was extracted.

2.2 Cell culture

GBM U87, U373, and U251 cells line and human astrocyte nano-hydroxyapatite (NHA) cell lines were applied (all Cell resource center, SIBS, CAS, Shanghai, China). The culture of the cells was in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, MA, USA) covering 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Invitrogen, CA, USA). Detachment of the cells in the logarithmic growth phase was with 0.25% trypsin (Thermo Fisher HyClone, Utah, USA) for cell passage and further experiments. In order to determine the optimal inhibitory concentration of Rop on GBM cell lines, GBM cell lines were treated with different concentrations of Rop (0.5, 1, 5, 10 μmol/L) for 12, 24, 48 h, and cell viability was detected by CCK-8 [26].

2.3 Cell grouping

Division of the cells was into the following groups, in which introduction of cells was with 5 μmol/L Rop and/or transfection with the corresponding plasmids except for the mimic-NC, the miR-21-5p-mimic, and the Control. Treatment of cells in the control was with normal saline. There were the Rop, the Rop + mimic-negative control (NC) (mimic-NC), the Rop + miR-21-5p-mimic (miR-21-5p-mimic), the Rop + oe-NC/KANSL2 (oe-NC/KANSL2), the Rop + miR-21-5p-inhibitor + si-NC/KANSL2 (miR-21-5p-inhibitor and si-NC
V-FITC and PBS, CellQuest density The Transwell chamber, coater's Propidium Iodide (PI) Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection Kit (Shanghai, China) in the light of the manufacturer's instructions [27]. After 48 h of incubation, the rinse of U87, U373, and U251 cells was with PBS, the resuspension was in the binding buffer, and then the double staining was with Annexin V-FITC and PI. Analysis of the apoptosis rate was via adopting the flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and CellQuest software (BD Biosciences).

2.6 Transwell examination of cell invasion

The seeding of the transfected cells was into the chamber of Transwell (2.5 × 10⁴ cells/well), and resuspension of the cells in the chamber was in serum-free medium, while the bottom of the chamber was medium covering 20% serum. Precoating of the matrigel layer is placed into the inner chamber to simulate the extracellular matrix [28]. After incubation, taking of the cells was from the chamber, and fixation of the cells in the lower Wells was via the mixture of formaldehyde and acetic acid, and staining was with crystal violet. Ultimately, a count of the number of invaded cells was conducted.

2.7 Scratch test detection of cell migration

The electroblot of the cells (2 × 10⁵/well) was onto a 12-well plate, and the culture was in Dulbecco's Modified Eagle Medium with 10% FBS. Once the confluence of the cells reached 100%, scratch of the monolayer cells was with a 200 mL tip, and rinse of the wells was with serum-free medium for 3 times; Observation of the wound was conducted and pictures were taken. Then culture of the cells was in serum-free medium [29]. After 24 h, observation of the wound and pictures was taken again. Scratch healing rate = 100%-(0 h scratch width-24 h scratch width)/0 h scratch width × 100%.

2.8 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Extraction of RNA was from tissues or cells adopting TRIzol reagent (Thermo Fisher Scientific, Wilmington, DE, USA). Subsequently, tests of the purity and concentration of RNA were performed via exerting a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and synthesis of a complementary DNA (cDNA) was via adopting the Universal cDNA Synthesis Kit (Roche, Basel, Switzerland) in the light of the method of the kit. RT-qPCR was implemented on the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA) exerting Power SYBR Green Master Mix (located in Takara, Dalian, China). The standardization of adoption of B-ACTIN and U6 was for the messenger RNA (mRNA) and miRNA, respectively. Calculation of the relative expressions of miRNA and mRNA was on the grounds of the method of 2⁻ΔΔCt [30]. The primer sequence is manifested in Table 1.


### 2.9 Western blot

The Western blot was implemented as previously described [31]. After extracting total protein from tissues and cells with Radio-Immunoprecipitation assay lysis buffer (Beyotime, Shanghai, China), sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed, and then electroblot of the protein was onto polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Block of the membrane was with 5% skim milk, and then incubation was with the following primary antibodies: KANSL2 (1:1000, HPA038497, MilliporeSiGBMa) and β-actin (1:1000; ab181602, Abcam). Subsequently, the incubation of the membrane was with horseradish peroxidase-labeled secondary antibodies (1:2000; Beyotime). Visualization of the protein was via exerting an electrochemiluminescence kit (Promega, Madison, Wisconsin, USA).

### 2.10 The luciferase activity assay

Insertion of the KANSL2 3’untranslated region fragment covering the miR-21-5p target site was into the pmirGLO dual-luciferase miRNA target vector (Promega), and then into the KANSL2-wild type (WT) reporter vector. Construction of the KANSL2 mutant (MUT) reporter vector covering mutation-binding sites was conducted. Co-transfection of WT/ MUT reporter gene and miR-493-5p mimic or negative control miRNA was into U87, U373, and U251 in the cells exerting LipofectamineTM 2000 (Invitrogen). After 48 h, inspection of the luciferase activity was via adopting the luciferase assay kit (Promega), and adoption of Renilla luciferase activity was as a control.

### 2.11 Data analysis

Manifestation of the data was in mean ± standard deviation (SD) (n = 3). Determination of the statistical significance of the two-group differences was via exerting Student’s t-test, and analysis and manifestation of the data were via exerting Prism software 8.0 (GraphPad Software, USA). * P < 0.05 was considered to be statistically dramatic. Calculation of the significance of each group was via adopting one-way analysis of variance, and correction of the variance was via exerting Tukey’s test.

### 3 Results

#### 3.1 Rop restrains the proliferation, migration, and invasion of GBM

To explore the impact of Rop on GBM, GBM cells were treated with different concentrations of Rop. As presented in Figure 1(a), the inhibitory effect of Rop on the cell viability of three GBM cell lines was dose- and time-dependent. Since 5 μmol/L Rop had the highest inhibitory effect, 5 μmol/L Rop was selected for subsequent experiments. The results manifested that Rop distinctly repressed the proliferation of U87 and U251 cells (Figure 1(b)). Additionally, cell apoptosis was examined via flow cytometry and Hoechst. As presented in Figure 1(c), the apoptosis rates of U87 and U251 cells were distinctly increased after Rop treatment. Subsequently, cell invasion and migration were examined via Transwell and cell scratch assay. As presented in Figure 1(d-e), the invasion and migration of U87 and U251 cells were distinctly constrained after Rop treatment. These results clarify that

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**Table 1. RT-qPCR primer sequences.**

| Primer sequences (5’ – 3’) |
|---------------------------|
| β-actin                   |
| F: 5’- CTCCATCTCTGGCCTGCTGT-3’ |
| R: 5’-GCTGTCACCTCAGCCGTCC-3’ |
| U6                        |
| F: 5’-CTGGTCGGCCGACACATATACT-3’ |
| R: 5’-AGGCTTCAGAATTGCGGTCG-3’ |
| MiR-21-5p                 |
| F: 5’-ACACTCAAGCTGGGTTAGCTATACGACTGA-3’ |
| R: 5’-CTCAACTGGGGTGCTGGAGTCCCGCAATTTCAGTTTCAATC-3’ |
| KANSL2                    |
| F: 5’-TCCCAATGCTGCCCACAAGGCGC-3’ |
| R: 5’-GTTTCTTGCTCCCCATCCTCA-3’ |

Note: F, forward; R, reverse.
Rop can distinctly restrain the proliferation,
invasion, and migration of GBM but boost apoptosis.

3.2 Augmented miR-21-5p restrains the proliferation, migration, and invasion of GBM

The latent mechanism of Rop in restraining GBM is figured out. MiR-21-5p has been testified to be a tumor suppressor gene with decline in multiple cancers. A speculation that miR-21-5p was provided with the analogous action in GBM is manifested. Consequently, an inspection of miR-21-5p in GBM patients and cell lines was conducted. As presented in Figure 2(a), miR-21-5p was declined in GBM patients and U87, U373, and U251 cells. Additionally, miR-21-5p was distinctly linked with tumor size and grade of GBM (Table 2). Subsequently, miR-21-5p in U87, U373, and U251 cells was elevated (Figure 2(b)), and inspection of the influence of elevated miR-21-5p on the biological progression of GBM was conducted. As presented in Figure 2(c-d), after elevating miR-21-5p, U87, U373, and U251 cell proliferation was critically repressed, but cell apoptosis rate was distinctly augmented. Additionally, the overexpression of miR-21-5p also reduced the invasion and migration ability of U87 and U251 cells (Figure 2(e-f)). The results manifested miR-21-5p was declined in GBM, and elevated miR-21-5p was available to constrain GBM’s growth.

### Table 2. Association of miR-21-5p and clinicopathological characteristics of patients in human glioma cells.

| Characteristic          | Groups | Cases | The elevated (n = 24) | The declined (n = 25) | P     |
|------------------------|--------|-------|-----------------------|-----------------------|-------|
| Age (years)            | < 45   | 30    | 17                    | 13                    | 0.1762|
|                        | ≥ 45   | 19    | 7                     | 12                    |       |
| Gender                 | Male   | 21    | 10                    | 11                    | 0.8690|
|                        | Female | 28    | 14                    | 14                    |       |
| Tumor size (cm)        | < 3    | 31    | 21                    | 10                    | 0.0006*|
|                        | ≥ 3    | 18    | 3                     | 15                    |       |
| Family history of cancer | Yes   | 16    | 6                     | 10                    | 0.2630|
|                        | No     | 33    | 18                    | 15                    |       |
| WHO’s Grade            | I + II | 11    | 2                     | 9                     | 0.0203*|
|                        | III + IV | 38   | 22                    | 16                    |       |

3.3 MiR-21-5p exerts the crucial action in Rop repressing GBM’S growth

For examination of whether miR-21-5p implicated in Rop restraining GBM’S growth, miR-21-5p-mimic was transfected in Rop-treated U87, U373, and U251 cells (Figure 3(a)). It turned out (Figure 3(b-c)) the proliferation ability of the U87, U373, and U251 cell was lower, but its apoptosis rate was higher in the Rop + miR-21-5p-mimic vs. the Rop + mimic-NC. Additionally, the invasion and migration abilities of U87 and U251 cells in the Rop + miR-21-5p-mimic were distinctly lower than those in the Rop + mimic-NC (Figure 3(d-e)). In brief, miR-21-5p was the critical gene in Rop constraining GBM.

3.4 KANSL2 is the target gene of miR-21-5p

The downstream target genes of miR-21-5p were explored. MiR-21-5p frequently exerted the action in diseases via modulating downstream target genes. Foregoing research studies have elaborated KANSL2 is elevated in GBM and mediates cell self-renewal [20]. It is consistent with this research (Figure 4(a)). In order to explore whether miR-21-5p modulated KANSL2, the impact of elevated miR-21-5p on KANSL2 was examined. As manifested in Figure 4(b), after augmenting miR-21-5p, KANSL2 in U87, U373, and U251 cells was distinctly declined. Consequently, it has been speculated that KANSL2 is the latent target gene of miR-21-5p. Subsequently, through the prediction of the website https://cm.jefferson.edu/, it was discovered that miR-21-5p and KANSL2 had latent-binding sites (Figure 4(c)). Additionally, the targeting association between miR-21-5p and KANSL2 was further verified. As manifested in Figure 4(d), WT KANSL2 can distinctly reduce the luciferase activity of the miR-21-5p-mimic, while the MUT one exerted no distinctive influence on it. To sum up KANSL2 was the target gene of miR-21-5p in GBM.

3.5 KANSL2 participates in Rop constraining GBM

To explore whether KANSL2 was implicated in Rop repressing GBM, oe-KANSL2 was transfected
into Rop-treated U87, U373, and U251 cells (Figure 5(a)). It turned out (Figure 5(b-c)) compared with the Rop + oe-NC, the proliferation of the U87, U373, and U251 cell was critically increased, and the apoptosis was distinctly reduced in the Rop + oe-KANSL2. Additionally, the over-expression of KANSL2 also distinctly increased the invasion and migration ability of the Rop-treated cells (Figure 5(d-e)). In short, KANSL2 participated in Rop restraining GBM.

### 3.6 Rop constrains GBM’s growth via modulating the miR-21-5p/KANSL2 axis

MiR-21-5p-inhibitor and si-KANSL2 was co-transfected into Rop-treated U87, U373, and U251 cells to figure out whether the miR-21-5p/KANSL2 axis was the crucial target for Rop-repressing GBM growth. As manifested in Figure 6(a), after co-transfection with si-KANSL2, KANSL2 in U87, U373, and U251 cells was distinctly declined. Additionally, after transfection of miR-21-5p-inhibitor, proliferation, migration, and invasion of U87, U373, and U251 cell were distinctly augmented, and the apoptosis was distinctly reduced. After co-transfection with si-KANSL2, these actions were distinctly reversed (Figure 6(b-e)). In short, Rop repressed proliferation, migration, and invasion of the GBM but boosted apoptosis via modulating the miR-21-5p/KANSL2 axis.

### 4 Discussion

As numerous drugs are adopted in the cure of GBM, exploring the latent mechanism of GBM drugs exerts a crucial role in clinical applications [32]. In this research, Rop treatment was made
available to distinctly restrain the biological process of GBM cells in vitro. Additionally, Rop impacted the GBM cell growth via mediating the miR-21-5p/KANSL2 signaling pathway. It is of critical significance for seeking novel therapeutic targets.

Recently, the application of local anesthetics to cancer therapy has been discovered to be an imperative measure [33,34]. For instance, Rop constrains the proliferation and migration of colorectal cancer via silencing Integrin beta-1 and influencing its downstream pathways [35]. Rop boosts liver cancer cell apoptosis via disrupting mitochondrial function and the caspase-3 pathway’s activation [36]. Additionally, Rop is available to stimulate oxidative stress and cell apoptosis of gliomas and repress cancer cell proliferation via controlling the circular RNA (circ) SCAF11/miR-145-5p axis [37]. The present research has manifested that Rop represses cervical cancer cell
growth via declining miR-96 to modulate the MEG2/pSTAT3 axis [38]. In this research, Rop was available to constrain GBM cell proliferation, invasion, and migration and boost apoptosis, illuminating that Rop was provided with a superior latent in preventing neurological tumors’ deterioration. Additionally, this study discovered 5 μmol/L Rop had the best inhibitory effect on GBM cell viability in vitro. Nevertheless, the dosage of Rop in clinical treatment needs to be further explored. It is worth noting that numerous studies have affirmed that Rop as a neuroanesthetic can damage neurons [39,40]. Consequently, the use of Rop in the clinical treatment of GBM might damage normal surrounding neurons.

The action of miRNA in tumors has been extensively reported. Numerous researches have elucidated miRNAs exerts a role as a regulator in GBM’s pathogenesis [41]. Presently, Yu K et al. have clarified that 309 miRNAs are maladjusted in GBM tissues, and MIR155HG/miR-129-5p/complement Component 18s is the latent marker and cure target for GBM [42]. Additionally, Luo C et al. have maintained miR-640 boosts GBM cell proliferation and adhesion via targeting SLIT1 [43]. Studies have also illuminated circPARP4 accelerates GBM’s progress via sponging fucosyltransferase 4 [44]. Foregoing studies have elaborated miR-21-5p is aberrant in GBM extracellular vesicles [45] and is available to negatively mediate p21 in the p53 network [46]. Meanwhile, in this research, augmented miR-21-5p is available to constrain the proliferation, migration, and invasion of GBM but boost apoptosis, which is consistent with foregoing studies [47]. Additionally, elevated miR-21-5p is available to further strengthen Rop’s suppression on GBM.

KAT8 of KANSL2 gene encoding, a member of the KANSL protein family, modulates the
NSL complex subunit 2 protein [48] belonging to the KAT8/MOF-NSL complex [47], and it has been discovered to be associated with cancer [49] and neurodevelopmental disorders [50]. Antecedent researches have elucidated that KANSL2 is elevated in GBM, which accelerates tumorigenesis via mediating GBM’s cancer stem cell-like characteristics [51]. Additionally, the discovery that KANSL2 modulates cholangiocarcinoma cell invasion is manifested. In this research, elevated KANSL2 was made available to turn around Rop’s suppression on GBM, and miR-21-5p targeted KANSL2 on GBM. It turned out KANSL2 was the crucial protein for Rop to constrain GBM. Furthermore, the results of this research intensified KANSL2’s cognition as a GBM proto-oncogene. Nevertheless, it is of necessity to further verify Rop’s action in mediating miR-21-5p/KANSL2 in animal models and clinics. Additionally, further determination of the molecular targets and latent pathways modulated via KANSL2 in GBM should be implemented.

5 Conclusion
In brief, Rop restrained the proliferation, invasion, and migration of GBM, but boosted apoptosis in vitro via mediating the miR-21-5p/KANSL2 pathway. The results illuminated and replenished the latent molecular mechanism of Rop modulating GBM’s biological process, offering novel data support for Rop’s clinical application as a drug to cure GBM.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
The author(s) reported that there is no funding associated with the work featured in this article.

Figure 6. Rop constrains GBM’s growth via mediating the miR-21-5p/KANSL2 axis. A: RT-qPCR and Western blot tests of KANSL2 in Rop-treated U87, U373, and U251 cells; B: CCK-8 examination of Rop-treated U87, U373, and U251 cell proliferation treated with Rop; C: Flow cytometry test of Rop-treated U87, U373, and U251 cell apoptosis; D: Transwell examination of Rop-treated U87, U373, and U251 cell invasion; E: Cell scratch inspection of Rop-treated U87, U373, and U251 cell migration; A-E, the influence of co-transfection of miR-21-5p-inhibitor and si-KANSL2 on them. Manifestation of values was in mean ± SD (N = 3).
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