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Original Paper

Ligustrazine Inhibits Growth, Migration and Invasion of Medulloblastoma Daoy Cells by Up-Regulation of miR-211

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Key Words
Ligustrazine • Medulloblastoma • miR-211 • PI3K/AKT pathway • mTOR pathway

Abstract
Background/Aims: Ligustrazine (LSZ) has been identified as an antitumor agent against some types of cancers. Nevertheless, its ability to inhibit growth, migration and invasion of medulloblastoma cells is still unclear. This study aimed to explore the effect of LSZ on Daoy cells. ***Methods:*** The effects of LSZ on viability, proliferation, apoptosis, migration, and invasion of Daoy cells were analyzed by CCK-8, BrdU, flow cytometry and Transwell assays, respectively. The effect of LSZ on miR-211 expression was analyzed by qRT-PCR. miR-211 inhibitor transfection was performed to suppress miR-211 expression. The effects of LSZ on apoptosis-related factors, MMP-2, MMP-9, and Vimentin (Vim), as well as main factors of PI3K/AKT and mTOR pathways were analyzed by Western blot. ***Results:*** LSZ inhibited viability but promoted apoptosis of Daoy cells. Additionally, the proliferative, migratory and invasive abilities of Daoy cells were decreased by LSZ. Meanwhile, LSZ promoted the activations of Caspase-3 and Caspase-9, increased Bax level, decreased Bcl-2 level, as well as inhibited the expressions of MMP-2, MMP-9 and Vim. Additionally, we found that LSZ enhanced miR-211 expression and exerted its anti-medulloblastoma effect by up-regulation of miR-211. Furthermore, LSZ inhibited PI3K/AKT and mTOR signaling pathways by up-regulating miR-211. ***Conclusion:*** LSZ suppressed medulloblastoma Daoy cells by up-regulating miR-211 and further modulating the activations of PI3K/AKT and mTOR signaling pathways.

Introduction

Medulloblastoma is first discovered in 1925 by Bailey and Cushing [1], which is one of the most malignant neuroepithelial neoplasms of human central nervous system. The incidence of medulloblastoma is high, accounting for high percentage of intracranial tumors and intracranial neuroepithelial neoplasms. Medulloblastoma occurs mainly in children under 14 years old but may also occur in adults [2]. Medulloblastoma is the most malignant glioma

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in the skull due to the rapid growth, unsatisfactory surgical removal, and high dissemination through cerebrospinal fluid.

Ligustrazine (LSZ), an alkaloid isolated from the rhizoma of *Ligusticum wallichii* (a traditional Chinese herb), which has been found to inhibit inflammation, possess anti-fibrosis activity, and modulate immune system [3, 4]. Multiple functions of LSZ have been widely reported. For example, LSZ is an effective blocker of vasoconstriction, used to scavenge oxygen free radicals, reduce cell apoptosis in tubules, and attenuate ischemia-reperfusion injury [5, 6]. Moreover, LSZ has the therapeutic effect on diabetic nephropathy by improving renal function and reducing urine protein in patients [7]. Recently, some studies have demonstrated the significant antitumor property of this compound against several malignancies, including melanoma [8], breast cancer [9] and prostate cancer [10]. However, the effect of LSZ on medulloblastoma has not been investigated. Thus, we explored the anti-tumor effect of LSZ on medulloblastoma in the present study.

microRNAs (miRNAs) are known to be involved in the activities of some Chinese medicine [11]. It was reported that LSZ alleviated apoptosis of neurons in injured spinal cord by down-regulating miR-214-3p [12]. In another study, LSZ was shown to make miR-21 overexpression and improve recovery of function in spinal cord injury model [13]. Therefore, several miRNAs might be linked with the action of LSZ. miR-211 was found to modulate colorectal cancer cell growth [14], development of melanoma [15], and metastasis of breast cancer [16]. Studies also reported that miR-211 down-regulation was associated with glioma poor prognosis [17], and it governed apoptosis, chemo-sensitivity and radio-sensitivity of glioma cells [18]. However, the role of miR-211 in LSZ-affected medulloblastoma cells has not been reported. Herein, we studied the effect of LSZ on medulloblastoma cell line Daoy and the role of miR-211 in modulation of function of LSZ was also explored.

Materials and Methods

Cell culture and treatment

Human medulloblastoma cell line Daoy, provided by the American Type Culture Collection (ATCC, Manassas, VA, USA) was incubated in improved minimum essential medium (IMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) (37°C, 5% CO_2). Daoy cells were treated by LSZ (W323713, natural, ≥98%, Sigma-Aldrich) for 24 h through incubating cells in culture medium added with water-LSZ solution.

Cell Counting Kit-8 (CCK-8) assay

Daoy cell suspension was seeded in 96-well plate (100 μL/well). Then the plate was placed in the incubator (37°C, 5% CO_2) for pre-culture. 10 μL of CCK-8 solution (Beyotime, Shanghai, China) was carefully added to each well and ensure no bubbles generated. After added with CCK-8, the culture plate was incubated for 2 h. The absorbance at 450 nm was detected by microplate reader (Bio-Rad, Hercules, CA, USA) and changes of Daoy cell viability were observed.

Proliferation assay

Bromodeoxyuridine (BrdU) is stably integrated into DNA, which can be used for assessment of cell proliferation. BrdU (Sigma-Aldrich) was added to the cultured cells obtaining a concentration of 50 μM. After incubation of 1 h, cells were counted by microscope from five visual fields.

Apoptosis assay

Daoy cell suspension was adjusted to 1×10^6 cells/mL. 1 mL of Daoy cell suspension was centrifuged at 4°C for 10 min; and then cells were re-suspended in 1 mL cold PBS and centrifuged, which were repeated for three times. Daoy cells were re-suspended in 200 μL binding buffer added with 10 μL Annexin V-FITC (Beyotime) and 5 μL PI (Beyotime). After incubation for 15 min at 4°C, cell suspension was added with 300 μL binding buffer and analyzed by flow cytometer within 1 h.

Migration and invasion assays

Cells in the logarithmic phase were washed with PBS and serum-free medium. Cells were then incubated in serum-free medium and diluted into 2 × 10^5 cells/mL. 600 μL of complete medium was added to the lower compartment of ThinCert™ cell culture inserts (24-well, 8.0-μM pore membranes, Greiner bio-one, Switzerland), and 100 μL of Daoy cell suspension was added to the upper chamber. After incubation for 24 h, medium in the upper chamber was removed, and cells were fixed with methanol. Cells on the upper surface of the filter were removed with a cotton swab. Cells on the lower side of the filter were stained with 2% Giemsa (Sigma-Aldrich) and counted. For determining cell invasion, the melted matrigel (BD, New Jersey, NY, USA) was diluted with serum-free medium to 1 mg/mL. The diluted matrigel solution was vertically added on the upper chamber and it was dried to gelatineous. Afterwards, cell invasion was evaluated according to the methods used in cell migration assay.

Transfection assay

miR-211 inhibitor and the negative control (NC) synthesized by GenePharma Co. (Shanghai, China) were transfected into Daoy cells by using lipofectamine 3000 reagent (Invitrogen) following the relevant specifications.

qRT-PCR analysis

Total RNA from Daoy cells was extracted using miRNeasy Mini kits (Qiagen, Crawley, UK). miR-211 was reverse transcribed to cDNA using gene-specific RT primers with the miRCURY LNA™ Universal RT microRNA PCR/Universal cDNA Synthesis Kit II (Exiqon, Woburn, MA, USA). miR-211 expression was determined using the miScript SYBR Green PCR Kit (Qiagen) with the Stratagene Mx3000P real-time PCR system (Stratagene, La Jolla, California). The relative quantification of miR-211 was normalized to the U6 snRNA and was calculated by $2^{-\Delta\Delta Ct}$ method [19].

Western blot assay

Daoy cells were harvested after different treatment and lysed using lysis buffer containing Tris-HCl (pH 7.5, 25 mM), NaCl (137 mM), KCl (2.7 mM), 1% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich) for 30 min at 4°C. The primary antibodies were rabbit polyclonal anti-Caspase-3 (ab4051), cleaved-Caspase-3 (ab13847), Caspase-9 (ab25758), cleaved-Caspase-9 (ab25758), Bcl-2 (ab59348), Bax (ab53154), MMP-2 (ab37150), MMP-9 (ab73734), Vimentin (Vim; ab137321), PI3K (ab1678), p-PI3K (ab182651), p-AKT (ab131443), mTOR (ab2732), p-mTOR (ab84400), S6K (ab9366), p-S6K(ab2571), and GADPH (ab8227, Abcam, San Francisco, USA) used at 1:1000, as well as rabbit monoclonal anti-AKT (ab179463, Abcam) used at 1:1000. The second antibody of goat anti-rabbit IgG conjugated with HRP (ab205718) was used at a dilution of 1:1000. β-actin (ab8227, Abcam) was used as an internal control. The representative image from one of three independent experiments was shown and the quantified band intensity obtained using Image-J software (Bio-Rad, Shanghai, China) was analyzed.

Statistical analysis

Assays were performed for three times and obtained data was shown as mean ± standard deviation (SD). Two-way analysis of variance was conducted by Graphpad statistical software (GraphPad Software Inc, San Diego, California, USA) and a P-value of <0.05 was considered significant difference.

Results

LSZ inhibited viability, proliferation but promoted apoptosis of Daoy cells

The chemical name of LSZ is 4-methyl-pyrazine (tetramethylpyrazine). As shown in Fig. 1A, the chemical formula of LSZ is $C_8H_{12}N_2$ and the molecular weight is 136.2. The anti-cancer property of LSZ was ever reported [20]; and herein, the activity of LSZ in medulloblastoma Daoy cells was explored. LSZ with 1, 1.5, and 2 mM concentrations significantly inhibited Daoy cells viability ($P<0.05$, $P<0.01$, and $P<0.001$, respectively, Fig. 1B). The middle concentration of LSZ, 1.5 mM was used for further investigation. The results in Fig. 1C displayed that LSZ significantly decreased BrdU-labeled cells ($P<0.01$), suggesting proliferation of Daoy cells

was inhibited by LSZ. Additionally, LSZ increased apoptotic cell rate of Daoy cells (P<0.01, Fig. 1D); and also facilitated activated form of Caspasee-3 and Caspase-9, decreased Bcl-2 expression, and increased Bax expression (Fig. 1E). Thus, we concluded that LSZ treatment induced suppressive effects on viability and proliferation, as well as induced promoting effect on apoptosis in Daoy cells.

LSZ inhibited migration and invasion of Daoy cells

Fig. 2A demonstrated that LSZ significantly inhibited migration of Daoy cells (P<0.01). The effect of LSZ on MMP-2 and MMP-9 expressions were next analyzed, and we found that LSZ induced down-regulation of them (both P<0.01, Fig. 2B). LSZ produced the similar effect on invasion of Daoy cells. The invasion of Daoy cells was significantly suppressed by LSZ (P<0.05, Fig. 2C) and Vim expression was significantly reduced (P<0.05, Fig. 2D). These data implied that LSZ resulted in suppression on migration and invasion of Daoy cells.

LSZ inhibited cell proliferation and promoted apoptosis of Daoy cells through regulation of miR-211

During the research on action mechanism of LSZ, it was found that miR-211 expression was stimulated by LSZ (P<0.05, Fig. 3A). Therefore, the role of miR-211 was explored in LSZ-treated Daoy cells; and then miR-211 inhibitor was transfected in Daoy cells to evaluate the effect of miR-211 repression on function of LSZ. Results showed that miR-211 expression in miR-211 inhibitor-transfected cells was declined (P<0.01, Fig. 3B). Compared with LSZ+NC treatment group, viability of cells treated by LSZ+miR-211 inhibitor was significantly increased (P<0.05, Fig. 3C) and apoptosis was significantly decreased (P<0.05, Fig. 3D). The effects of LSZ on Caspase-3/9, Bcl-2, and Bax expressions were partially reversed by miR-211 inhibition (Fig. 3E). Above results hinted that LSZ inhibited cell growth of Daoy cells through regulation of miR-211.

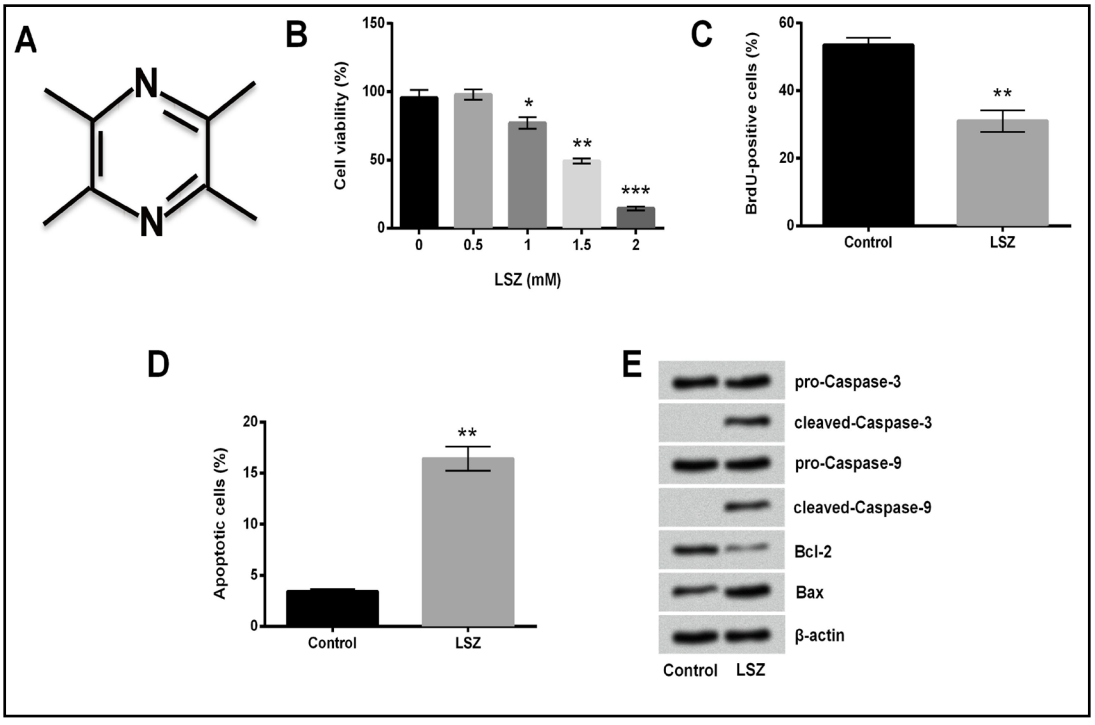

Fig. 1. LSZ suppressed growth of Daoy cells. (A) The molecular structure of LSZ; (B) effect of different doses of LSZ on viability of Daoy cells was determined by CCK-8 assay; effects of LSZ with concentration of 1.5 mM on (C) proliferation, (D) apoptosis, and (E) expressions of apoptosis-associated proteins were examined by BrdU, flow cytometry and Western blot assays, respectively. * P<0.05, ** P<0.01, *** P<0.001.

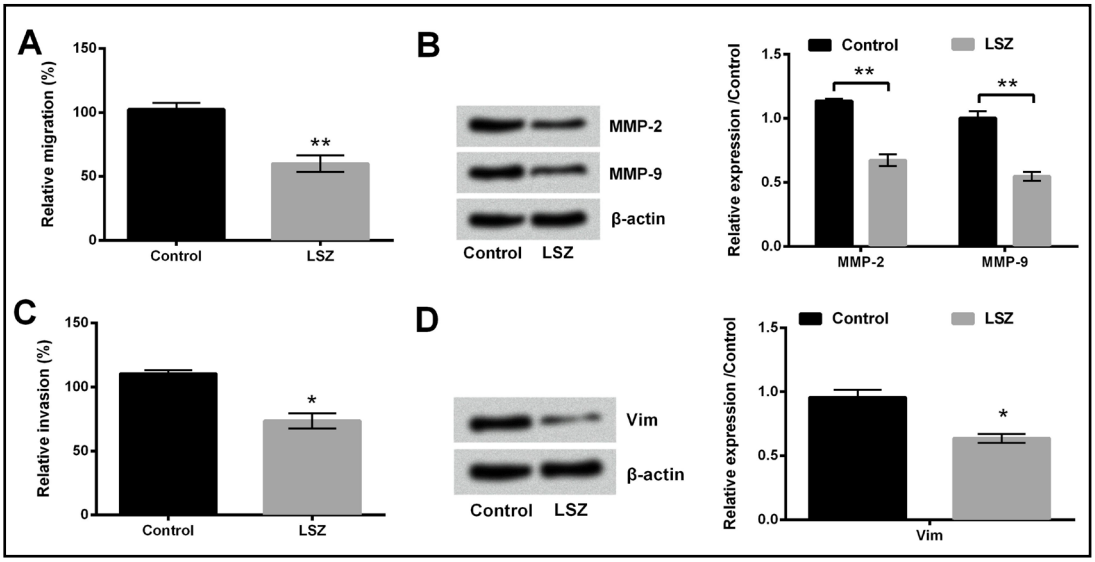

Fig. 2. LSZ suppressed migration and invasion of Daoy cells. Effects of LSZ on (A) migratory capacity and (B) MMP-2/9 expressions were detected by Transwell and Western blot assays; effects of LSZ on (C) invasive capacity and (D) Vim expression were assessed by Transwell and Western blot assays. * P<0.05, ** P<0.01.

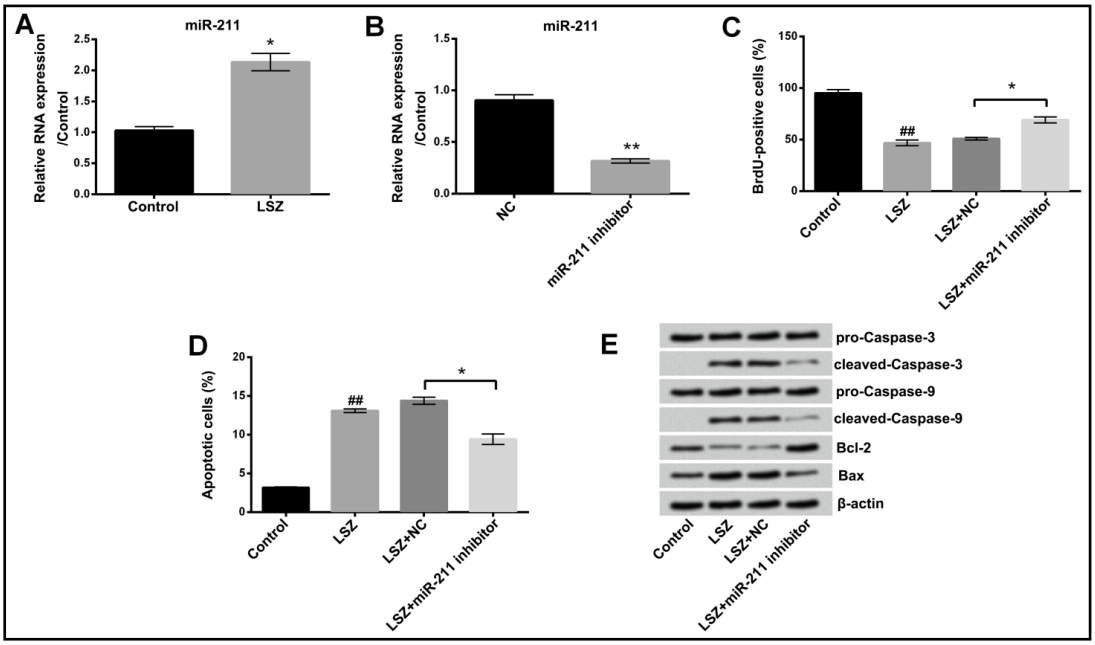

Fig. 3. Suppressing miR-211 impaired the effects of LSZ on cell growth of Daoy cells. (A) Effect of LSZ on miR-211 expression was analyzed by qRT-PCR assay; Daoy cells were transfected with miR-211 inhibitor and NC, and (B) relative expression level of miR-211 in these transfected cells was determined by qRT-PCR assay; after treatment with LSZ, (C) proliferation, (D) apoptosis, and (E) apoptosis-associated factors were detected by BrdU, flow cytometry and Western blot assays, respectively. ## P<0.01: LSZ group vs Control group; * P<0.05, ** P<0.01: LSZ+miR-211 inhibitor group vs LSZ+NC group.

LSZ inhibited migration and invasion of Daoy cells through regulation of miR-211

Effect of miR-211 on LSZ affected cell migration and invasion in Daoy cells were investigated. As shown in Fig. 4A-4C, LSZ-induced inhibitory effects on migration and MMP-2, MMP-9 expression in Daoy cells were abrogated by miR-211 inhibitor treatment (both

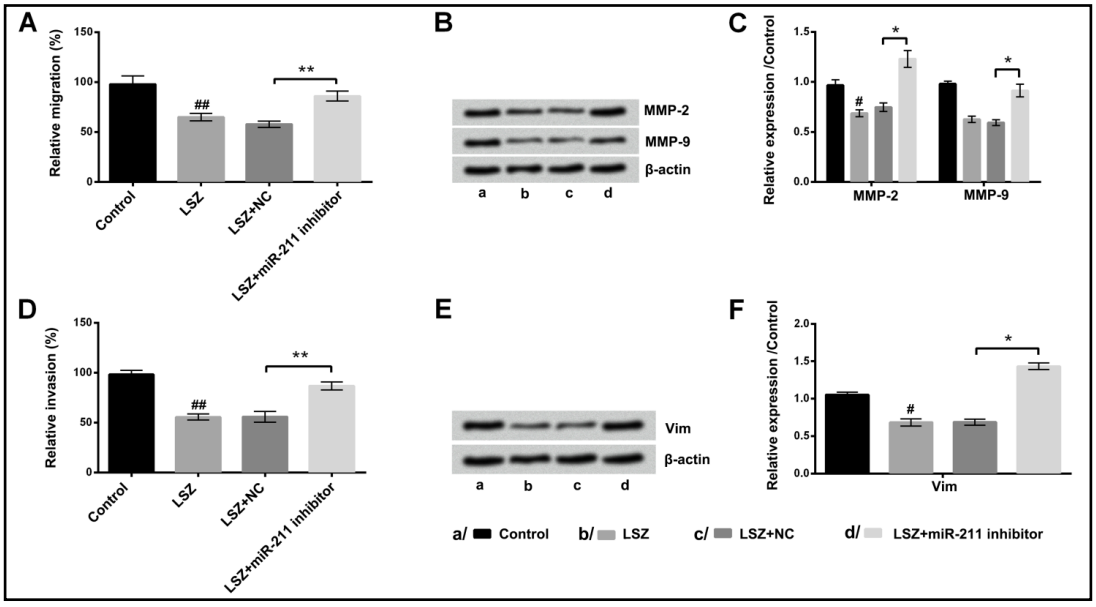

Fig. 4. Suppressing miR-211 impaired the effects of LSZ on cell migration and invasion of Daoy cells. Daoy cells were transfected with miR-211 inhibitor and NC, as well as treated with LSZ, (A) migratory capacity, (B and C) MMP2/9 expressions, (D) invasive capacity, and (E and F) Vim expression were assessed by Transwell and Western blot assays.# P<0.05, ## P<0.01: LSZ group vs Control group; * P<0.05, ** P<0.01: LSZ+miR-211 inhibitor group vs LSZ+NC group.

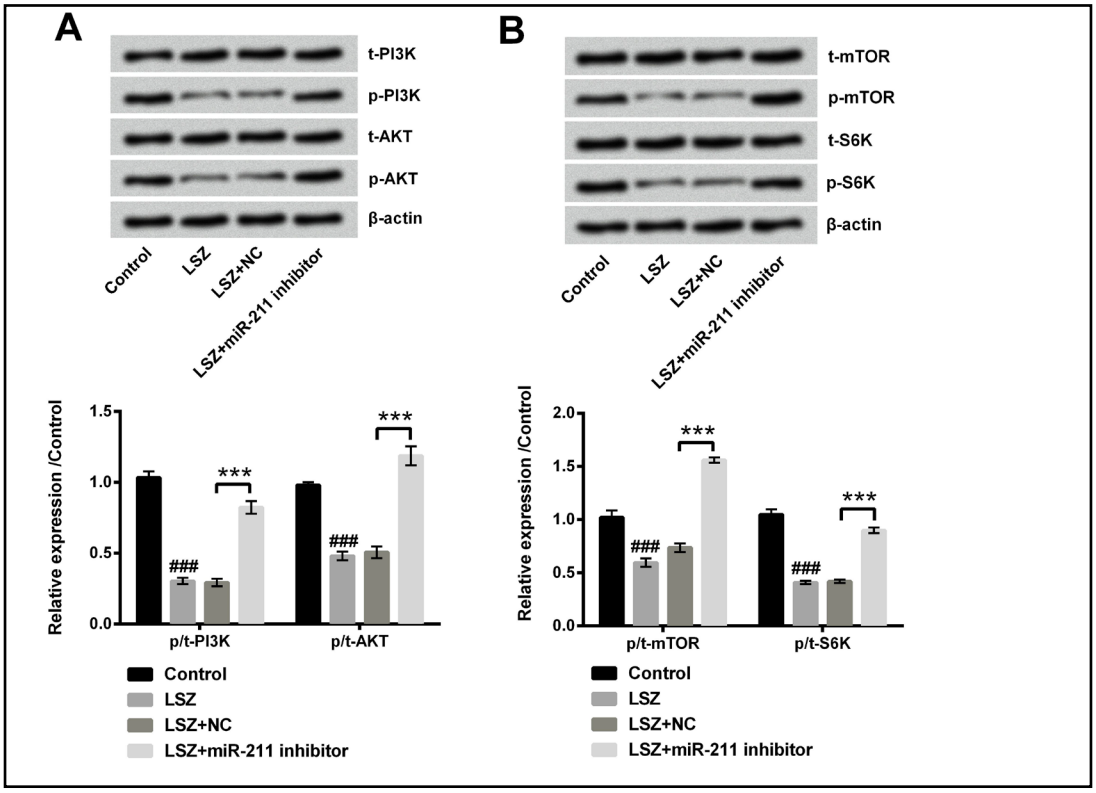

Fig. 5. miR-211-modulated PI3K/AKT and mTOR pathways involved in the effects of LSZ on Daoy cells. (A) Effect of LSZ and miR-211 on PI3K/AKT signaling pathway, and (B) effect of LSZ and miR-211 on mTOR signaling pathway were examined by Western blot assay. ### P<0.001: LSZ group vs Control group; *** P<0.001: LSZ+miR-211 inhibitor group vs LSZ+NC group.

P<0.01). Additionally, Cell invasion and the protein level of Vim were all significantly up-regulated by LSZ+miR-211 inhibitor treatment compared to LSZ+NC treatment (all P<0.05, Fig. 4D-4F). These data indicate that LSZ inhibited migration and invasion of Daoy cells through regulation of miR-211.

LSZ repressed Daoy cells via inhibiting PI3K/AKT and mTOR pathways

As shown in Fig. 5A and 5B, LSZ significantly decreased phosphorylated PI3K and AKT (both P<0.001) and also decreased phosphorylated mTOR and S6K (both P<0.001), suggesting that LSZ blocked PI3K/AKT singling and mTOR signaling, which might explain the anti-medulloblastoma effect of LSZ. Besides, we also found that miR-211 modulated both pathways. Because phosphorylated PI3K, AKT, mTOR and S6K in cells of LSZ+miR-211 inhibitor group were all significantly increased relative to LSZ+NC group (all P<0.001). Thus we speculated that LSZ repressed Daoy cells might via inhibiting PI3K/AKT and mTOR pathways which were modulated by miR-211.

Discussion

Actually, the tumor-suppressive effect of LSZ was proved by previous studies. However, the functions of LSZ in medulloblastoma cells have not been investigated. In the present study, we observed that LSZ significantly inhibited proliferation, induced apoptosis and suppressed migration and invasion of Daoy cells. Additionally, miR-211 was up-regulated by LSZ, and inhibition of miR-211 reversed the regulatory effect of LSZ on Daoy cells. Besides, miR-211 overexpression-modulated activations of PI3K/AKT and mTOR signaling pathways might be involved in the property of LSZ.

The cytoprotective effect of LSZ was widely reported in existing researches. Evidence from Shao *et al*. demonstrated that LSZ protected neurons from apoptosis and death induced by oxygen-glucose deprivation [21]. Wang *et al*. uncovered that LSZ played the anti-inflammatory activity in treating traumatic brain injury [22]. In addition, many reports considered that LSZ was an effective cure for the treatment of cancers. For instances, one study proven that LSZ inhibited viability and proliferation, promoted apoptosis, and decreased abilities of invasion and migration in renal cell carcinoma cells. LSZ also inhibited epithelial-mesenchymal transition progression by altering levels of E-cadherin, Vim and fibronectin [23]. Additionally, the cancer-suppressive activity of LSZ was reported in prostate cancer cells via inhibition of proliferative, migratory and invasive abilities, and down-regulation of forkhead box M1 (FoxM1) [24]. Furthermore, LSZ decreased the proliferative cells and induced G1-phase arrest of retinoblastoma cells via modulating C-X-C chemokine receptor type 4 (CXCR4) [25]. The similar results were also demonstrated in our study, which showed the viability-/proliferation-inhibiting effect, apoptosis-promoting effect, and migration-/invasion-inhibiting effect of LSZ on Daoy cells.

The action mechanism of LSZ in Daoy cells was explored by analyzing the expression of miR-211, and by investigating the role of miR-211 in Daoy cell progression after LSZ treatment. The experiment results revealed that miR-211 expression was enhanced in LSZ-treated Daoy cells, indicating that miR-211 might be linked with the activity of LSZ in Daoy cells. Therefore, miR-211 inhibitor was transfected into Daoy cells and the influence of miR-211 suppression on the effects of LSZ was investigated. Results indicated that miR-211 suppression impaired LSZ-induced inhibitions of cell growth, migration and invasion. Activated caspase-3/9 and Bax were up-regulated and Bcl-2 was down-regulated in LSZ-treated group, whereas all of them showed the opposite changes in LSZ and miR-211 inhibitor co-treated group. Additionally, we found that MMP-2/9 and Vim expressions were decreased in LSZ-treated group, but further increased after knocking down miR-211. Comprehensive above results, we inferred that miR-211 might act as a tumor suppressor in Daoy cells and LSZ repressed their growth, migration and invasion by up-regulating miR-211.

The antitumor role of miR-211 *in vitro* and *in vivo* was proved by abundant studies [16, 26, 27]. In Wang *et al*. research, miR-211 was found to arrest cell cycle, suppress proliferation and invasion of thyroid tumor cells [26]. Chen *et al*. uncovered the antitumor effect of miR-211-5p on breast cancer cells through suppression of cell proliferation, invasion, migration and metastasis by targeting SET binding protein 1 (SETBP1) [16]. Interesting study demonstrated that miR-211-5p suppressed metastasis of renal cancer cells via down-regulation of Snail expression [27]. Thus, our data was supported by these previous studies. Besides, it was found that miR-211 might target oncoproteins, like MMP-9, and suppress glioblastoma multiforme cells [18], which was consistent with our data that miR-211 suppression stimulated MMP-9 expression.

Recent study have stated that miR-211 could modulate inflammatory diseases through regulating PI3K/AKT pathway [28]. Further, LSZ has been proven to be an important regulator of the PI3K/AKT pathway [29, 30]. Previous study reported that blocked PI3K/AKT signaling pathway could suppress medulloblastoma growth [31]. It is generally known that mTOR is also an important signal transduction pathway. Recent study demonstrated that LSZ inhibited prostate cancer by regulating mTOR signaling pathway [10], and mTOR signaling pathway was closely associated with the pathogenesis of medulloblastoma [32]. Based on these previous studies, we analyzed the effects of LSZ and miR-211 on PI3K/AKT and mTOR signaling pathways. Our findings showed that LSZ inhibited PI3K/AKT and mTOR pathways, and miR-211 suppression reversed the inhibitory effect of LSZ on these two signaling pathways, indicating that LSZ exerted the antitumor effect on medulloblastoma might through blocking PI3K/AKT and mTOR pathways by up-regulating miR-211.

Conclusion

Taken together, this study revealed the anti-medulloblastoma effect of LSZ on Daoy cells. Up-regulation of miR-211 enforced by LSZ might contribute to the anti-cancer activity of LSZ. Additionally, LSZ inhibited miR-211-modulated activations of PI3K/AKT and mTOR signaling pathways in Daoy cells. Our findings might provide new therapeutic strategies and guidance for the clinical treatment of medulloblastoma patients. Further studies are still need to be confirmed the effect of LSZ on other cell lines and *in vivo*.

Disclosure Statement

The authors declare that they have no competing interests.

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