Ligustrazine eases lung cancer by regulating PTEN and Wnt/β-catenin pathway

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Background: Lung cancer (LC) is the most common malignant tumor in the world. Ligustrazine (LSZ), an alkaloid, is an active ingredient extracted from chuanxiong Hort. Earlier studies suggested that LSZ plays a crucial role in lung diseases. The study aimed to investigate the detailed mechanism of LSZ on LC.

Methods: Protein expression was determined by qPCR, western blotting and immunohistochemistry. Cell viability was determined by CCK8 assay. Invasiveness was detected by Transwell assay. Cell growth was determined by clone formation assay. Male BALB/c nude mice were randomly divided into 2 groups (n=10): LSZ group and Control group.

Results: Results showed that PTEN is low-expressed in lung tumor tissues and LC cells (H1650, A549, H1299, and PC-9). Appropriate doses of LSZ could significantly promote the expression of PTEN in LC cells. Besides, LSZ inhibits invasion and proliferation of H1299 cells. However, knockdown PTEN counteracts the inhibitory effect. Animal experiments suggest that LSZ inhibits tumor formation *in vivo*. Mechanistically, western blotting shows that LSZ treatment significantly decreased the levels of Wnt and β-catenin, while increasing the levels of PTEN and GSK-3β.

Conclusions: Collectively, these results demonstrate that LSZ ameliorates LC by down-regulating PTEN level and blocking Wnt/β-catenin signaling pathway.

Keywords: Lung cancer (LC); ligustrazine (LSZ); phosphatase and tensin homolog (PTEN); Wnt/β-catenin

Introduction

Lung cancer (LC) is the most common malignant tumor in the world, which causes over a million deaths per year (1,2). In recent years, the morbidity and mortality of LC have increased sharply (3). At present, surgery and chemotherapy are the primary treatment of LC, but there are still problems with drug resistance and easy recurrence (4-6). Over the past 10 years, the 5-year survival rate for patients with advanced LC has not exceeded 20%. With the development of targeted molecular biology, targeted therapy has become a promising method for the treatment

of LC. Therefore, the search for new targeted drugs plays a key role in improving the survival rate of patients with advanced LC.

Ligustrazine (LSZ), an alkaloid, is an active ingredient extracted from the Chinese herbal medicine Ligusticum chuanxiong Hort (7). Numerous studies have suggested that LSZ exhibits a variety of biological activities such as anti-depression (8), anti-inflammatory (9), cardioprotective (10) anti-cancer (11,12), and neuroprotective (13). In recent years, pieces of evidence also indicated that LSZ exhibited potent anticancer effects in melanoma (14) and breast cancer (15). Zou *et al.* found that LSZ-based analogs

repressed proliferation, motility, and heteroadhesion of HCT-116 cells by inactivating Wnt/β-catenin pathway via inhibiting the Akt and GSK-3β phosphorylation (16). In lung disease, LSZ can alleviate acute lung injury and induce apoptosis in LC cells (17,18).

Phosphatase and tensin homolog (PTEN) is a newly discovered tumor suppressor gene, which is the most popular tumor suppressor gene after p53 and plays a vital role in various cancer cells (19). Studies have shown that PTEN has little effect on the protein product of the gene, but it has a strong catalytic effect on the phosphorylation of the substrate (20). In human malignancies such as breast cancer (21) and liver cancer (22), PTEN exhibits low expression and dysfunction. Similarly, in LC, the inactivation of PETN induces the movement of non-small cell LC cells (23). Besides, the level of PTEN can be inhibited by MIR, thereby preventing the progression of non-small cell LC (24).

The Wnt/β-catenin signaling pathway is a canonical Wnt signaling pathway that plays a vital role in the progression of LC (25,26). It affects various physiological processes in cells such as cell cycle, proliferation, invasion, migration, apoptosis, and angiogenesis (27-29). Abnormal expression of molecules in Wnt/β-catenin signaling plays a vital role in the development of LC, such as glycogen synthase kinase-3β and β-catenin (30). Recently, Curcumin, Garcinol, and Qiyusanlong have inhibition of LC by regulating the Wnt pathway (31-33). However, the roles and molecular mechanism of LSZ in LC is still unclear. In the study, we investigated the effects of different concentrations of LSZ on the expression of PTEN protein in LC cells. The results show that LSZ can regulate the malignant biological behavior of LC cells by regulating Wnt/β-catenin signaling pathway.

Methods

Samples collection

From March 2015 to May 2016, 30 patients with LC and 40 patients with the adjacent normal group were admitted to our hospital. All patients did not receive chemotherapy or radiation before surgery. Two pathologists confirmed them after pathological staging. The tumor tissue is removed and quickly placed in the RNA preservation solution.

Cell lines and reagents

Human lung fibroblasts (MRC-5) and human LC cells (H1650, A549, H1299, and PC-9) were purchased from the Cell Research Institute of Wuhan University (Wuhan, China) and cultured in RPMI 1640 medium supplemental with 5% fetal bovine serum at a constant temperature. (Hyclone Corporation) Transwell chamber was bought from Millipore, Inc., and matrigel was bought from Bio-Rad (Bio-Rad, Madrid, Spain). Lentivirus kit and retrovirus LV3-PTEN were bought from Shanghai Gemma Biology Co., Ltd.

Quantitative real-time reverse transcription PCR (RT-qPCR)

Total RNAs are extracted from cells using RNAprep pure Cell / Bacteria Kit. (Tiangen Biotech. Beijing, China) RT-qPCR was conducted by FastKing One-Step RT-qPCR Kit (Probe) (Tiangen Biotech. Beijing, China) and ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The primers of GAPDH were 5'-GTACAATGCACGCCTAGCCG-3' (sense) and 5'-CATTCGACTCGACTAATGCC-3' (antisense); PTEN, 5'-GCATTTACGCAACTCGACG-3' (sense) and 5'-CCTGCTACTAGACATAGGCT -3' (antisense). GAPDH was used as an internal reference. Fold the equation $2^{-\Delta\Delta Ct}$ calculated changes.

Cytotoxic effect

Cell viability was measured by CCK-8 assay according to the manufacturer's protocol. MRC-5 cells were treated with LSZ (0, 10, 20, 40, 60, 80, 100 and 120 mg/kg) and then incubated in 10% CCK-8 at 37 ℃ for 3 h. Cell viability was measured, and the absorbance was determined at 570 nm with multifunctional microplate reader SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA).

Cell infection

H1299 cells in the logarithmic growth phase were inoculated on the 6-well culture plate until the fusion degree was 40%. Then, cells were infected with lentivirus holding LV3-NC or LV3-PTEN, respectively. The experiment was randomly divided into two groups: the LV3-NC group and the LV3-PTEN group. The cells were evenly laid into a 6-well plate and incubated in an incubator at 37 ℃.

Immunohistochemistry

The tissue sections were deparaffinized with xylene and

rehydrated in gradient ethanol. After repairing with 1×
citrate, the parts were placed in a 3% aqueous hydrogen
peroxide solution and incubated for 10 minutes to remove
endogenous peroxidase. Next, the sections were blocked
with a blocking solution for 1 hour at room temperature
and then incubated with the primary antibody (#9188, 1:125,
Cell Signaling Technology, USA) at 4 ℃ overnight. After
washing with TBST, the sections were incubated with the
secondary antibody (#8114, Cell Signaling Technology,
USA) for 30 minutes at room temperature, and stained with
SignalStain® DAB. The images were seen under a light
microscope.

Western blotting

The total protein in cells and tissues is extracted with cell
lysate and cryopreserved for use. After separating by SDS-
PAGE, the proteins (25 μg) were transferred to the PVDF
membrane. Then, the membrane was incubated with
primary antibodies PTEN (#9188, 1:1000, CST, USA),
GAPDH (#5174, 1:1,000, CST, USA), β-catenin (#8480,
1:1,000, CST, USA), Wnt (#2391, 1:1,000, CST, USA) and
GSK-3β (#12456, 1:1,000, CST, USA) overnight at 4 °C.
The next day, after washing with TBST, the membrane
was incubated with a second antibody (#7074, CST,
USA) for 1 hour. The ECL developer was developed and
photographed.

Transwell assay

H1299 cells were diluted to $1×10^6$ cells/mL. Two hundred
and fifty μL medium (fetal bovine serum) and 25 μL cell
suspension were added to the upper chamber covered with
Matrigel and cultured in 37 ℃ incubators for 24 hours.
The upper room was wiped with aseptic cotton swabs,
stained with crystal violet for 10 minutes, washed with
PBS for 5 minutes, then observed under a microscope and
photographed.

Cloning formation assays

Cloning formation assays were used to detect the ability
of clone formation of H1299 cells. Briefly, LSZ-treated
cells were resuspended in a single cell suspension and
cultured in RPMI 1640 medium supplemental with 5%
FBS at a constant temperature. The colonies were fixed
with paraformaldehyde and stained with crystal violet.

LC xenografts

Male BALB/c nude mice (4–6 weeks old) were housed
under specific pathogen-free conditions. All animal
experiments were performed following the NIH Guide for
the Care and Use of Laboratory Animals and were approved
by The First Affiliated Hospital of Hebei North University.
H1299 cells ($2×10^8$/mL) were injected subcutaneously into
the right-back flanks of the mice. After successful modeling,
the mice were randomly divided into 2 groups (n=10):
LSZ group; Mice were injected intraperitoneally (i.p.) with
70 mg/kg LSZ; Control group, mice were subjected to the
same volume of physiological saline daily. After treatment
for 6 weeks, the mice were sacrificed. Tumors were taken
out, and volume and weight were measured.

Statistical analysis

SPSS 13.0 software was used for statistical analysis. Data
were expressed as mean ± standard deviation, and one-way
ANOVA analyzed the data. The variance inequality was
treated with the Kruskal-Wallis method. The difference was
considered statistically significant at $P<0.05$.

Results

Low expression of PTEN and the promoting effect of LSZ on PTEN in LC

The results of the immunohistochemical analysis showed
that the expression of PTEN in LC tissue was significantly
lower than that in normal tissues, indicating that PTEN
was low expressed in LC tissues (*Figure 1A*). RT-qPCR
and Western blotting showed that PTEN in LC cell lines
(H1650, A549, H1299, and PC-9) was down-regulated
compared with lung fibroblasts (MRC-5). Among them, the
change of PTEN in H1299 cells was the most significant.
Therefore, H1299 cells were selected as the research
object in the follow-up experiment (*Figure 1B,C*). Besides,
cytotoxicity experiments showed that when the dose was lower
than 60 mg/kg, LSZ had no apparent toxic effect on MRC-5.
However, LSZ inhibited the growth of MRC-5 cells in varying
degrees when it was higher than 80 mg/kg. RT-PCR and
Western blotting were implemented again to investigate the
effect of LSZ on PTEN in H1299 cells (*Figure 1D*). The
results showed that LSZ promoted the expression of PTEN
in a dose-dependent manner in a certain dose range. When
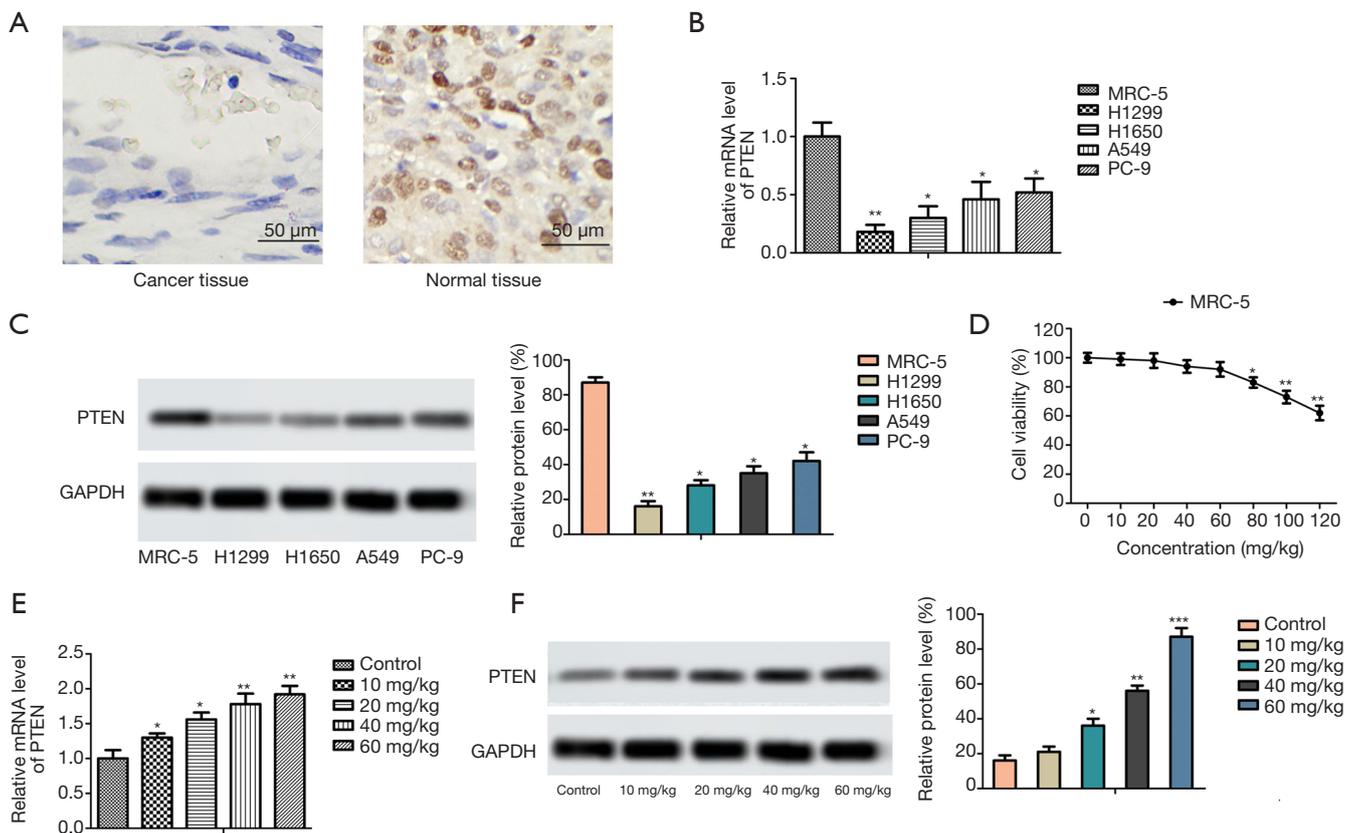the dose exceeded 60 mg/kg, the level of PTEN began to

Figure 1 Low expression of PTEN and the promoting effect of LSZ on PTEN in LC. A. The expression of PTEN was detected by IHC in cancer tissue and normal tissue. B. The mRNA level of PTEN was detected by RT-qPCR in human lung fibroblasts (MRC-5) and human LC cells (H1650, A549, H1299, and PC-9). (*, P<0.05; **, P<0.01 *vs.* MRC-5 cells) C. The protein level of PTEN was detected by western blotting in MRC-5, H1650, A549, H1299, and PC-9 cells. (*, P<0.05; **, P<0.01 *vs.* MRC-5 cells) D. Cytotoxic effect of LSZ on MRC-5 was detected by CCK8 assay. (*, P<0.05; **, P<0.01; ***, P<0.001 *vs.* control group) E. The mRNA level of PTEN was detected by western blotting in H1299 cells treated with different doses of LSZ (0, 10, 20, 40, 60, 80, 100, and 120 mg/kg). F. The protein level of PTEN was detected by western blotting in H1299 cells treated with different doses of LSZ (0, 10, 20, 40, and 60 mg/kg). (*, P<0.05; **, P<0.01; ***, P<0.001 *vs.* control group).

decrease (*Figure 1E,F*). These results show that PTEN was low expressed in LC, while LSZ promoted the expression of PTEN in LC.

LSZ inhibits invasion and proliferation of LC cells

Transwell and clone formation assay was implemented to investigate the effect of LSZ on the invasion and proliferation of H1299 cells. As shown in *Figure 2*, LSZ treatment significantly inhibited the invasion and proliferation of H1299 cells compared with the control group. These results suggest that LSZ inhibits the growth and motility of LC cells to a certain extent.

Knockdown PTEN counteracts the inhibitory effect of LSZ on the invasion and proliferation of LC cells

Cells, knockdown experiments, Transwell, and clone formation assays were performed to investigate the effects of PTEN and LSZ on invasion and proliferation of H1299. As shown in *Figure 3A,B*, the level of PTEN was significantly reduced in the LV3-PTEN group compared to the LV3-NC group. After infecting H1299 cells with a lentivirus holding LV3-PTEN, a PTEN-deficient H1299 cell line was selected. Normal H1299 cells and PTEN-deficient H1299 cells were treated with LSZ, and invasive ability and proliferative ability were measured by Transwell and clone formation assays, respectively. The results showed
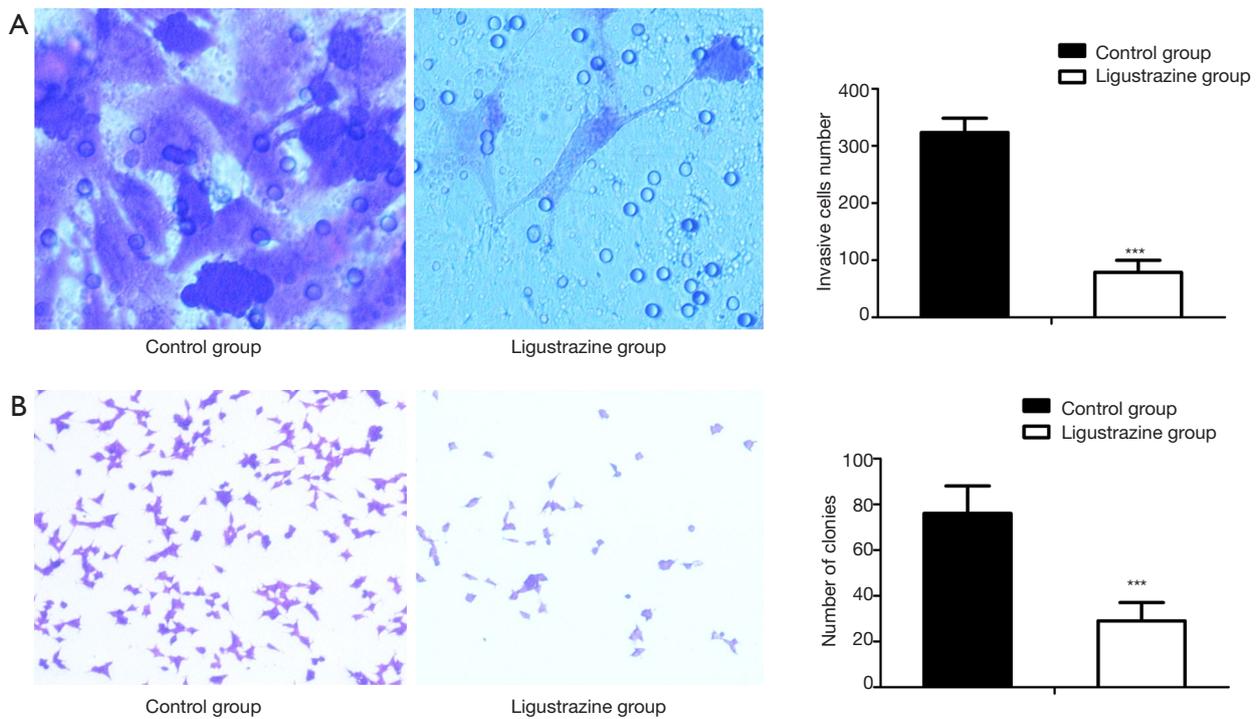
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Figure 2 LSZ inhibits invasion and proliferation of LC cells. A. The invasive ability of H1299 cells treated with 500 mg/L LSZ was detected by the Transwell assay. B. Proliferative ability of H1299 cells treated with 60 mg/kg LSZ was detected by clone formation assay. Magnification ×400 (***, P<0.001 *vs.* control group). LSZ, ligustrazine; LC, lung cancer.

that compared with normal H1299 cells, cell invasion, and proliferation of PTEN-deficient H1299 cells were significantly inhibited (*Figure 3C,D*). These results show that knockdown PTEN counteracts the inhibitory effect of LSZ on the invasion and proliferation of LC cells

LSZ inhibits tumor formation by increasing PTEN levels and blocking the Wnt/β-catenin pathway in vivo

Tumor growth in nude mice after 6 weeks is shown in *Figure 4A*. The results showed that the tumors in the LSZ group were significantly smaller than the control group. Tumor weight and volume were shown in *Figures 4B,C*; the weight and volume of the tumor were significantly reduced after LSZ treatment. In terms of mechanism, Western blotting showed that LSZ treatment significantly reduced the levels of Wnt and β-catenin, while increased the level of GSK-3β, indicating that LSZ inhibited the activation of Wnt/ β-catenin pathway by increasing the level of GSK-3β. Also, western blotting and immunohistochemical analysis showed that the expression of PTEN in tumor tissues treated with LSZ was significantly higher than that in the

control group without LSZ treatment. These results show that LSZ inhibits tumor formation by increasing PTEN levels and blocking the Wnt/β-catenin pathway *in vivo*.

Discussion

LC is one of the malignant tumors with the highest morbidity and mortality. The high metastasis of LC cells leads to a meager early diagnosis rate and easy to deteriorate. The recurrence rate of advanced LC is high, while the survival rate is low with a poor prognosis (34). At present, the early diagnosis, prognosis, and treatment of patients with LC are a hot clinical study. Abnormal PTEN mutations can lead to some tumor gene mutations in the occurrence and development of tumors, resulting in the inactivation of tumor suppressor genes (35). Therefore, we hope to study the relationship between PTEN diagnosis and treatment of LC.

PTEN is a recently discovered tumor suppressor gene, found on chromosome 10q23.3. The transcript is about 515 kb (36). The phosphatase domain of PTEN exists in tyrosine and bispecific phosphatase (37). PTEN
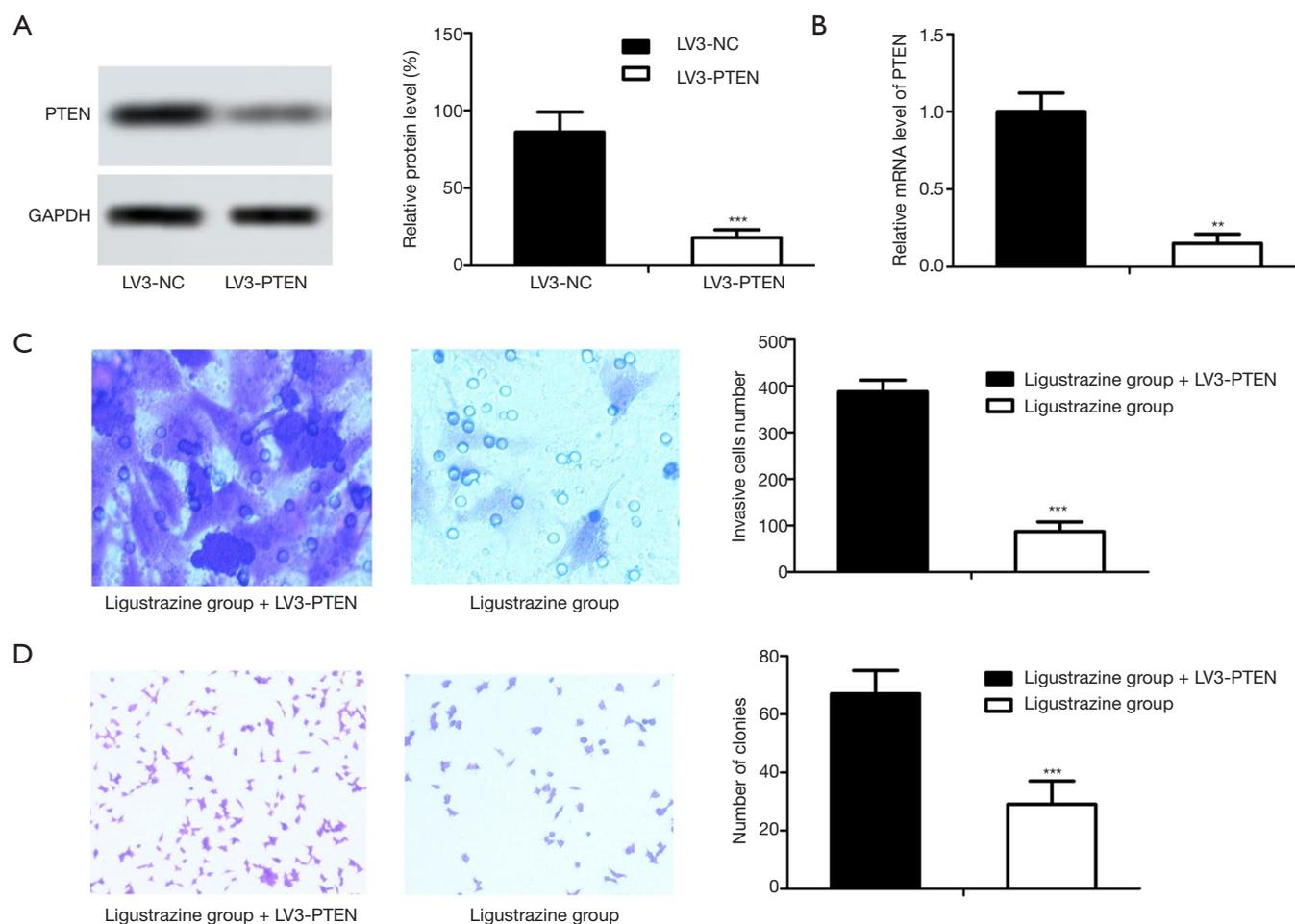
Figure 3 Knockdown PTEN counteracts the inhibitory effect of LSZ on the invasion and proliferation of LC cells. (A) The protein level of PTEN was detected by western blotting in H1299 cells infected with lentivirus holding LV3-NC or LV3-PTEN. (***, P<0.001 *vs.* LV3-NC group). (B) The mRNA level of PTEN was detected by RT-qPCR in H1299 cells infected with lentivirus holding LV3-NC or LV3-PTEN. (**, P<0.01 *vs.* LV3-NC group). (C,D) Normal H1299 cells and PTEN-deficient H1299 cells were treated with LSZ. (B) Invasive ability was measured by the Transwell assay. (C) Proliferative ability was measured by the clone formation assay. Magnification ×400 (***, P<0.001 *vs.* control group). LSZ, ligustrazine; LC, lung cancer.

may play an essential role in tumor development and progression through dephosphorylation (38). Li *et al.* found that microRNA-374b promotes LC cell motility and proliferation by targeting PTEN and downregulating PTEN expression via modulating the PI3K/AKT pathway (39). Liu *et al.* found that PTEN was significantly decreased in NSCLC cell lines. Knockdown PTEN by shRNA significantly increased cell viability, while overexpression of PTEN significantly inhibited the growth and apoptosis of NSCLC cells (40). In this study, it was found that the levels of PTEN in tumor tissues and LC cells of patients with LC were decreased, while LSZ treatment

could significantly increase the level of PTEN, but also inhibit the invasion, proliferation and tumor growth of LC cells. These results suggest that PTEN plays a crucial role in the pathogenesis of LC.

LSZ is an effective alkaloid isolated and purified from *Chuanxiong*. It has a wide range of anti-platelet and de-polymerized platelet coagulation function and has a good alleviation effect on tissue and organ ischemia and hypoxia (41). Feng *et al.* found that LSZ can inhibit autophagy and apoptosis of liver cancer cells, thereby affecting the metastasis and spread of liver cancer cells and inhibiting the development of liver cancer (42). Li and
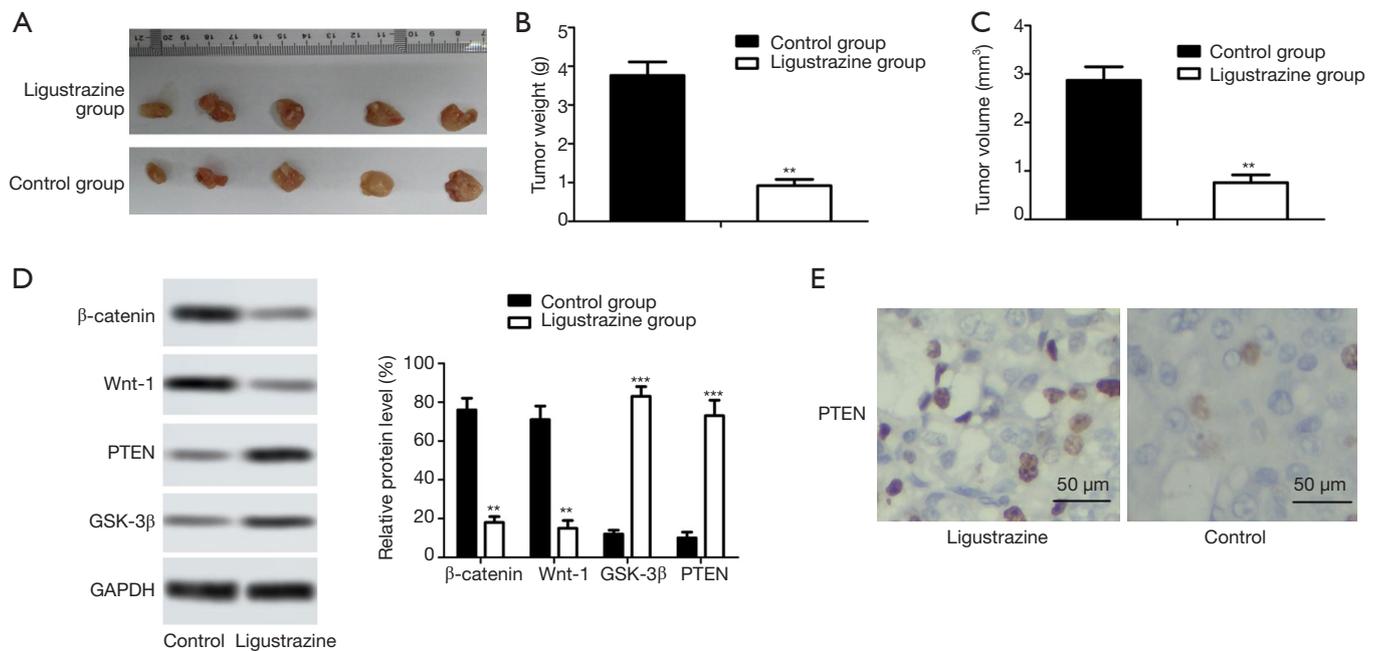
Figure 4 LSZ inhibits tumor formation by increasing PTEN levels and blocking the Wnt/β-catenin pathway *in vivo*. The mice were randomly divided into 2 groups (n=10): LSZ group; mice were injected intraperitoneally (i.p.) with 70 mg/kg LSZ; Control group, mice were subjected to the same volume of physiological saline daily. (A) Tumor growth; (B) tumor weight; (C) tumor volume; (D) the protein levels of PTEN, GSK-3β, Wnt, and β-catenin were detected by western blotting in the control group and LSZ group. (**, P<0.01; ***, P<0.001 *vs.* control group); (E) the expression level of PTEN was detected by IHC in tumor tissues from the control group and the LSZ group. LSZ, ligustrazine; LC, lung cancer.

other studies have shown that LSZ can effectively alleviate the proliferation of glioma in glioma (43). It is reported that LSZ exhibited therapeutic effects on sepsis-induced acute lung injury in rats (17). Besides, LSZ induces cycle arrest and apoptosis of LC cells (18). Jia *et al.* also found that LSZ suppressed proliferation, migration, and capillary tube formation of HMEC-1 cells by blocking the BMP/Smad/Id-1 pathway *in vitro* and *in vivo* (44). Consistent with these results, this study found that LSZ has a certain promoting effect on the expression of PTEN in LC cells. Increased PTEN further inhibits invasion and proliferation of LC cells. Also, animal experiments have shown that LSZ inhibits tumor formation by down-regulating Wnt/β-catenin pathway in *vivo*.

Wnt signaling pathway has irreplaceable physiological functions *in vivo*. Wnt signaling pathway can not only regulate the formation of the brain and nervous system but also closely related to the self-renewal and differentiation regulation of various stem cells (45). Wnt signaling can modulate the biological behavior of malignant cells. Studies have shown that the Wnt signaling pathway in malignant

tumor cells such as rectal cancer, colon cancer, and ovarian cancer is activated, which has certain reference value for the diagnosis and prognosis of cancer (46-48). Wang *et al.* found that curcumin inhibited the proliferation of A549 cells by inhibiting the Wnt pathway and reduced oxidative stress damage (31). Huang *et al.* found that Garcinol inhibited the phenotype of human non-small cell LC stem cells by inhibiting the Wnt/β-catenin/STAT3 axis signaling pathway (32). Similarly, this study found that LSZ significantly inhibited the activation of the Wnt/β-catenin pathway in tumor tissues by up-regulating the level of GSK-3β, thereby inhibiting tumor formation in mice.

In conclusion, current studies have explored the effects of LSZ and PTEN on the formation of LC. The results showed that the level of PTEN was significantly down-regulated in LC tissues or cells, while LSZ treatment promoted the expression of PTEN in LC cells in a dose-dependent manner. The increase of PTEN further inhibited the proliferation and invasion of LC cells. Animal experiments show that LSZ treatment can inhibit the formation of tumors in mice by up-regulating the

levels of PTEN and GSK-3β and inactivating Wnt/β-catenin pathway. These results suggest that LSZ and PTEN are of great significance in the treatment and prognosis of LC.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.03.26). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All studies mentioned in this manuscript were approved by Institutional Review Board of The First Affiliated Hospital of Hebei North University and individual informed consent was waived. All animal experiments were performed following the NIH Guide for the Care and Use of Laboratory Animals.

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