1 **Total saponins isolated from *Radix et Rhizoma Leonticis* suppresses**
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3 **tumor cells growth by regulation of PI3K/Akt/mTOR and p38**
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5 **MAPK pathways**
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Abstract: Both PI3K/AKT/mTOR and mitogen activated protein kinase (MAPK) signaling cascades played an important role in tumorigenesis, a more complete understanding of these signaling pathways allowed the development of new therapeutic strategies. Total saponins isolated from *Radix et Rhizoma Leonticis* (RLTS) was recognized with anticancer properties. In a murine hepatocellular carcinoma H22 cell-bearing mouse model, RLTS exhibited significant inhibitory effect on tumor growth. Here, we investigated the role of RLTS on the PI3K/AKT/mTOR signaling pathway and MAPK pathways in liver and lung cancer cells. Results obtained showed RLTS inhibited cell proliferation and induced cell apoptosis in vitro, which attributed to the inhibition on the activation of PI3K/AKT/mTOR cascade and its related signaling molecules, such as activated VEGFR and NF- κ B, and activation of p38 MAPK in tumor cells. Additional, RLTS inhibited cell migration and downregulated proteins that mediated metastasis including CXCR4, MMP2 and MMP9. Overall, these findings suggested that RLTS interfered with multiple signaling cascades involved in tumorigenesis and had potential in cancer therapy.

Key words: Total saponins, hepatocellular carcinoma, lung cancer, PI3K/Akt/mTOR, p38 MAPK

1. Introduction

The phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB/AKT)-mammalian target of rapamycin (mTOR) kinase cascade (PI3K/AKT/mTOR) is a signal transduction pathway activated in the majority of human cancers. It is involved in the regulation of multiple cellular functions including cell proliferation, survival, differentiation, adhesion, motility and invasion (Zhang *et al.*, 2013; Gil, 2014; Slomovitz and Coleman, 2012). The pathway is switched on through activation of

membrane receptors, including tyrosine-kinase receptors (RTKs), such as VEGFR and PDGFR (Slomovitz and Coleman, 2012; Trinh XB *et al.*, 2009; Zhang *et al.*, 2003). Also, activation of this pathway in tumor cells can increase VEGF secretion by both HIF-1 dependent or independent mechanisms, and regulate angiogenesis by modulating expression of nitric oxide and angiopoietins (Karar and Maity, 2011). The potential of PI3K/AKT/mTOR pathway inhibition to enhance clinical response of cancer therapy is currently the focus of many preclinical and clinical studies. Many agents have been developed targeting AKT and/or mTOR signaling in tumor cells, and these drugs have effects on tumor cell proliferation and survival. Besides the PI3K/AKT/mTOR pathways, mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases that play an important role in the regulation of many cellular processes including cell growth and proliferation, differentiation, and apoptosis. ERk1/2 MAPKs, Jun N-terminal kinase (JNK) and p38 MAPK pathways are three major mammalian MAPK pathways (Wagner and Nebreda, 2009). Especially, p38 MAPK is a tumor suppressor nuclear protein, and regulate cell cycle progression at different transition points by both transcription dependent and transcription-independent mechanisms (Bradham and McClay, 2006).

Radix et Rhizoma Leonticis (“Hong Mao Qi” in Chinese) is a plant that widely distributes in China. Previous studies demonstrated that taspine isolated from the roots of *Radix et Rhizoma Leonticis* had many pharmacological actions such as bacteriostasis, antibiosis, antiviral, anti-inflammatory, anti-ulcer and anti-cancer effects (Zhang *et al.*, 2008). Saponins are a class of chemical compounds found in nature plants, which are considered as bioactive constituents in many plants due to their various pharmacological effects. Total saponins were isolated successfully from *Radix et Rhizoma Leonticis* (RLTS) in our laboratory. In this study, we characterized

the anti-cancer effects of RLTS in vivo and assessed PI3K/AKT/ mTOR signaling
decades and p38 MAPK involved in anti-proliferative effects of RLTS in cancer cells.

2 Materials and methods

2.1 Animals and cell culture

4-6 weeks Kunming SPF mice (body wt. 18–22 g) provided by Animal Research
Center of Xi'an Jiao tong University (Xi'an, China) were used for all experiments.
The methods were carried out in accordance with the approved guidelines of the
regional authorities according to Xi'an Jiao tong University animal-care regulations.
Hepatocellular carcinoma cell lines including HepG2, BEL-7402 and SMMC-7721,
and lung cancer cell lines including A549, NCI-H1299 and NCI-H460 were obtained
from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences.
BEL-7402, SMMC-7721, A549, NCI-H1299 and NCI-H460 cells were maintained in
RPMI1640 medium (Sigma-Aldrich, USA) supplemented with 10% (v/v) fetal bovine
serum (FBS) (Hyclone, China), HepG2 cells and L-02 cells were cultured in
Dulbecco's Minimal Essential Medium (DMEM) (Sigma-Aldrich, USA)
supplemented with 10% (v/v) FBS. All cell lines were incubated at 37°C in a 5% CO₂
incubator with saturated humidity.

2.2 Extraction and purification of RLTS

Radix et Rhizoma Leonticis used in the study was purchased from the TCM Store
(Xi'an, P.R. China) and was grounded to 40 mesh. The raw material was extracted
once with 8 volumes of 90% ethanol for 2 h and then 80% ethanol by refluxing for 2 h
twice. The ethanol water solution was condensed to an extractum. The extractum was
percolated twice with 12 volumes of 1% HCl for 30 h. The merged supernatant was
concentrated, the pH value was adjusted to 3–5. The percolate was obtained by
treating the concentrate with LSD001 ion exchange resin, and was chromatographed

over D101 resin column eluted with 95% ethanol. Total saponins (RLTS) was gotten by evaporating the eluent under reduced pressure and dried in vacuum conditions overnight. RLTS was dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentrations before use. The concentration of DMSO was kept below 0.1% (v/v) in the treated groups. 0.1% (v/v) DMSO was used as a vehicle control throughout the study.

2.3 Animal experiments

Mice housed under aseptic and ventilated condition were inoculated by subcutaneous injection into the oter of mice with H22 cells resuspended in 5% saline. Tumor size was measured on alternate days by using a vernier caliper and calculated by $\text{length} \times \text{width}^2 \times 0.5$ (in cm^3). Body weights were monitored weekly as an indicator of overall health. Treatment began when tumors were palpable and no less than 0.1 cm^3 in volume. The mice were randomly assigned to control and treatment groups (n = 8 per group). Animals were administrated orally with RLTS daily at doses of 100 mg/kg while control animals received equivalent volumes of dissolvant. After a total of 14 days of treatment, mice were sacrificed and tumor tissue was removed and weighed. The anticancer activity *in vivo* was expressed as an inhibitory rate calculated by the formula: $[(A - B)/A] \times 100\%$, where A and B were the mean tumor weights of the control and treatment groups, respectively.

2.4 Cell viability assay

HepG2, BEL-7402, SMMC-7721, A549, NCI-H1299, NCI-H460 and L-02 cells were cultured in 96-well plates, and fresh medium with or without RLTS, was added for 48 h. Cell proliferation reagent MTT (Sigma-Aldrich, USA) was added and incubated at 37°C and 5% CO_2 for 4 h. Absorbance was then measured at 490 nm with a microplate reader (Bio-RAD instruments, USA).

2.5 Colony formation assay

SMMC-7721 and NCI-H460 cells were cultured in 6-well plates and fresh medium with or without RLTS for 10-15 days. Colonies with cell numbers of >50 cells per colony were counted after staining with 0.2% crystal violet (Beijing Chemical Works, China) solution. All the experiments were performed in triplicate wells in three independent experiments.

2.6 Wound healing assay

SMMC-7721 and NCI-H460 cells were planted into 6-well plate and allowed to grow to 70% confluency in complete medium. Cells were then serum starved for 24 h, and cell monolayers were scratched with a pipette tip. Wounded monolayers were then washed several times with serum-free medium to remove floating cells and photographed in microscope. Cells were incubated in medium in the absence or presence of RLTS for 24 h, cell migrating into the wound surface and the average distance of migrating cells was determined under an inverted microscope.

2.7 Analyses of the cell apoptosis

NCI-H460 cells were plated in culture flask and incubated for 24 h after the medium was changed to serum-free medium. Cells were treated with RLTS for 48 h, and then trypsinized, collected, washed in PBS, and incubated in 5 μ L Annexin V-FITC, 10 μ L PI for 15 min away from light. Samples were analyzed on the flow cytometer (BD Biosciences, USA).

2.8 Immunoblotting analysis

SMMC-7721 and NCI-H460 cells were plated in culture flask and treated with RLTS at different concentrations for 48 h. Cells were then harvested and lysed in RIPA Lysis Buffer (Applygen Technologies, China) supplemented with protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets (Roche, Switzerland). The

cell lysates were centrifuged at $12,000 \times g$ at 4°C for 10 minutes. Protein concentration of the supernatants was determined by a protein assay kit (Bio-Rad Laboratories, USA). Equivalent amount of protein was resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in TBS containing 0.05% Tween-20 (TBST) and 5% nonfat powdered milk and then probed with different primary and secondary antibodies. Proteins were detected by using enhanced chemiluminescence reagents (Thermo, USA). The primary antibodies included anti-VEGFR, anti-p-VEGFR, anti-p38 MAPK, anti-p-p38 MAPK, anti-ERK1/2, anti-p-ERK1/2, anti-AKT, anti-p-AKT, anti-NF- κ B, anti-p-NF- κ B (Cell Signaling Technology, USA), anti-CXCR4, anti-MMP2, anti-MMP9 (Epitomics, USA), anti-mTOR, anti-p-mTOR (Proteintech, USA), and anti-GAPDH (Proteintech, USA) antibodies.

2.9 Statistical analysis

All values were represented as means $\pm$ standard error of the mean (SEM). Statistical significance of the data compared with the untreated control was determined using the Student unpaired t-test. Results were considered statistically significant if the P value was less than 0.05.

3 Results

3.1 RLTS suppressed the growth of mouse tumor *in vivo*

The effect of RLTS on tumor growth *in vivo* was assessed by mouse liver cancer cells H22 transplantation tumor models. Compared with the untreated group, treatment with RLTS resulted in significant reduction of tumor weight (The mean tumor weight were 0.53 g in the untreated group and 0.21 g in the treated group, respectively), inhibiting tumor growth at a rate of 60.38% at 100 mg/kg (Table. 1). No significant body weight loss and any other abnormalities were observed in the RLTS-treated mice.

3.2 RLTS inhibited proliferation and colony formation both in lung and liver cancer cells

The anti-tumor activity of RLTS on lung and liver cancer cells was examined by MTT assay. As shown in Figure 1A and B, stimulation of A549, NCI-H1299, NCI-H460 lung cancer cells with 40, 80, 160, 320, 640, 1280 $\mu\text{g/mL}$ RLTS and BEL-7402, HepG2, SMMC-7721 liver cancer cells with 25, 50, 100, 200, 400, 800 $\mu\text{g/mL}$ RLTS for 48 h, RLTS inhibited these cells viability in a concentration dependent manner. The 50%-growth inhibitory concentrations (IC_{50}) of RLTS at 48 h was 134, 162, 76, 159, 406 and 61 $\mu\text{g/mL}$, respectively, whereas it was considerably less active in L-02 cells (IC_{50} was 790 $\mu\text{g/mL}$). In addition, the colony formation assay exhibited that RLTS significantly decreased numbers of colonies of NCI-H460 and SMMC-7721 cells in comparison with the control (Figure 1C). These findings indicated that RLTS had potential anti-tumor properties in both lung and liver cancer in vitro.

3.3 RLTS inhibited the migration of NCI-H460 and SMMC-7721 cells

Scratch motility (wound-healing) assay was performed to investigate the effect of RLTS on cell migration. Confluent monolayers of cells were scratched to form wound and cultured in the absence or presence of various concentrations of RLTS for 24 h. as shown in Figure 2, RLTS treated cells moved slowly compared to control cells, suggested that treatment of RLTS suppressed migration of both NCI-H460 and SMMC-7721 cells.

3.4 RLTS induced cell apoptosis in tumor cells

To further demonstrate the anti-cancer effects of RLTS, we investigated the effect of RLTS on cell apoptosis, RLTS treated NCI-H460 cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. We observed an increase in the percentage of apoptotic cells in the treatment groups (Figure 3). The percentage of

apoptotic NCI-H460 cells resulting from treatment with 0, 17.5, 35 and 70 $\mu\text{g/mL}$ of RLTS for 48 h was 5.82%, 5.30%, 9.10%, and 29.83%, respectively.

3.5 RLTS inhibited PI3K/AKT/mTOR signaling cascades

In order to investigate the role of RLTS in mediating PI3K/AKT/mTOR activation in tumor cells, the expression of AKT, p-AKT, mTOR and p-mTOR in NCI-H460 and SMMC-7721 cells were analyzed by western blotting. As shown in Figure 4, significantly activation of AKT and mTOR was suppressed in the two cells upon RLTS treatment. Furthermore, activated Akt can subsequently regulate the NF- κB pathway, we also found p-NF- κB p65 was decreased in both NCI-H460 and SMMC-7721 cells. Our data clearly indicated that the inhibition of PI3K/AKT/mTOR and AKT/NF- κB signaling cascade by RLTS led to the suppression of cell proliferation in NCI-460 and SMMC-7721 cells.

3.6 RLTS regulated p38 activation in tumor cells

We next performed western blot analysis to determine whether RLTS regulated the activity of MAPK cascades including ERK1/2 MAPK, and p38 MAPK in tumor cells. As shown in Figure 5, RLTS substantially induced the activation of p38 MAPK, and had no significant effect on the phosphorylation of ERK1/2 in NCI-460 and SMMC-7721 cells.

3.7 RLTS suppressed activated VEGFR in tumor cells

The PI3K/Akt cascade could be activated by receptor tyrosine kinases, we next examined the effects of RLTS on the activated VEGFR to investigate whether RLTS suppressed AKT activation by downregulation of VEGFR activity. We found that RLTS suppressed the expression of p-VEGFR in NCI-460 and SMMC-7721 cells (Figure 6).

3.8 RLTS downregulated expression of proteins involved in metastasis

Additional, we examined the effects of RLTS on the expression of proteins involved in metastasis. These results clearly demonstrated that RLTS also suppressed proteins linked with migration and metastasis, including CXCR4, MMP-2 and MMP-9 (Figure 7).

4 Discussion

It had been documented that several triterpenoid saponins had been found in *Radix et Rhizoma Leonticis*, whose aglycone included oleanolic acid, echinocystic acid, hederagenin and cauloside etc. (Strigina LI *et al.*, 1970; Strigina LI *et al.*, 1974; Strigina LI *et al.*, 1975; Strigina LI *et al.*, 1976; Chetyrina and Kalinovskii, 1979; Yang *et al.*, 2013). Even though RLTS has been known to exert anti-bacteria, analgesic effect and anti-inflammatory effects (Yang *et al.*, 2013), the activity and mechanism of action of RLTS on cancer has not yet been elucidated. The aim of our study was to investigate the effects of RLTS on the PI3K/AKT/mTOR and MAPKs signaling and cellular responses. Here, we found that RLTS not only exerted significant anti-proliferative and pro-apoptotic effects, but also regulated PI3K/AKT/mTOR and p38 MAPK activation in human hepatocellular carcinoma and lung cancer cells.

In our experiment *in vivo*, murine hepatocellular carcinoma H22 cell-bearing mice model indicated that RLTS effectively inhibited tumor growth through oral administration. So, we hypothesized it could exhibit potent inhibitory effects in human cancer cells, *in vitro* experiment showed that RLTS exhibited obvious inhibition on proliferation and colony formation of human hepatocellular carcinoma SMMC-7721 cells and lung cancer NCI-H460 cells but was considerably less active in normal liver cells. RLTS also had potential to suppress cancer cell migration and induce cell apoptosis.

1 The phosphatidylinositol 3-kinase (PI3K) /AKT/mammalian target of rapamycin
2 (mTOR) pathway has been reported to be a promising target due to its frequent
3 dysregulation in lung and hepatocellular carcinoma (Fumarola *et al.*, 2014; Grabinski
4 *et al.*, 2012). This is major antiapoptotic/prosurvival pathways that control cell
5 proliferation, survival and malignant transformation (Nissim, 2005; Polivka and Janku,
6 2014; Karar and Maity; 2011). Our findings more specifically indicated that AKT
7 activation was abrogated by RLTS, which further indicated the possibility that RLTS
8 could interfere with mTOR. As was expected, RLTS suppressed activated mTOR in
9 NCI-460 and SMMC-7721 cells.

10 NF- κ B is a major sensor of cell stress, which is known to regulate the expression of
11 various cell survival, proliferative, metastatic, and angiogenic gene products
12 (DiDonato *et al.*, 2012; Luedde and Schwabe, 2011). NF- κ B is not a molecule within
13 the core PI3K/Akt/mTOR pathway, but it is downstream of Akt. Activation through
14 Akt is important for many tumorigenic functions of NF- κ B. Since, both mTOR and
15 NF- κ B are downstream effector Akt, it appears that the signaling through Akt can
16 potentially proceed in two mutually exclusive independent directions, either through
17 mTOR or through NF- κ B (Romashkova and Makarov, 1999; Salminen and
18 Kaarniranta, 2010). We speculated NF- κ B perhaps was suppressed as AKT decrease.
19 Consistent with our hypothesis, NF- κ B activation was downregulated in RLTS treated
20 cells. p38 MAPK has recently gained attention as a tumor suppressor, and p38
21 activation can induce cell apoptosis and suppress oncogenesis (Bradham and McClay,
22 2006). The induction of p38 MAPK activation by RLTS could contribute to the
23 suppression of tumorigenesis in upon RLTS treatment in tumor cells. VEGFR was the
24 upstream molecule of AKT, and we found that VEGFR protein activity in cancer cells
25 were decreased after treatment with RLTS.

Metastases, rather than primary tumors, are responsible for most cancer deaths, cancer cells metastasis was a complex multistep process involving cell adhesion, motility (migration) and invasion. Hence, interruption of one or more of these steps is one approach for anti-metastatic therapy (Nam and Shon, 2009). We observed that RLTS could suppress cell migration by wound-healing assays. The expression and activity of MMPs against matrix macromolecules have been linked to the development of malignant phenotypes and the promotion of cell metastasis (Vihinen *et al.*, 2005). MMP2 and MMP9, two major MMPs, play important roles in cancer cell invasion and metastasis (Yodkeeree *et al.*, 2009; Mook *et al.*, 2004). CXCR4, the chemokine receptor belonging to the large superfamily of G protein-coupled receptors, was also identified to play a crucial role in cancer metastasis (Furusato *et al.*, 2010). MMP2, MMP9 and CXCR4 activity was strongly decreased after treatment of cancer cells with RLTS, which dissected the alterations and function of MMPs and CXCR4 in RLTS-inhibited cell metastasis.

Overall, our results indicated that RLTS could inhibit transplantation tumor growth *in vivo*, induce apoptosis and proliferation inhibition by regulating PI3K/AKT/mTOR signaling cascades and p38 MAPK activation. RLTS also suppressed cell migration through the down-modulation of MMP2, MMP9 and CXCR4 that mediate tumor cell proliferation and metastasis. However, further studies on the orthotopic human cancer and metastasis to other organs in animals are needed to validate these findings for the therapeutic use of this agent in humans.

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Figure legends

Figure 1 RLTS suppressed tumor cell proliferation and colony formation. A and B, Effect of RLTS on proliferation of hepatocellular carcinoma cells and lung cancers cells. Cells were treated by RLTS at indicated concentrations for 48 h. The cell proliferation was measured using the MTT assay. Data were represented as the means $\pm$ SEM from five repeated experiments. C, Effect on colony formation of SMMC-7721 and NCI-H460 cells by RLTS. Data were represented as the means $\pm$ SEM from three repeated experiments.

Figure 2 RLTS inhibited migration of tumor cells. A and B, Photographs of wound of cells treated with RLTS. Confluent monolayers of SMMC-7721 (A) and NCI-H460 (B) cells were scarred and pretreatment with RLTS for 24 h, the migrating cells into the wound surface were photographed. C, quantification of A and B. The results shown were representative of three independent experiments. Data were expressed as mean $\pm$ SEM. *P < 0.05, **P < 0.01 compared with untreated control cells.

Figure 3 RLTS induced cell apoptosis in tumor cells. NCI-H460 cells were treated with indicated concentrations of RLTS for 48 h and the cells were incubated with an Annexin V-FITC and PI and then analyzed by a flow cytometry.

Figure 4 RLTS suppressed PI3K/AKT/mTOR signaling pathway in tumor cells. A, SMMC-7721 and NCI-H460 cells were treated with various concentrations of RLTS for 48 h. Then equal amounts of lysates were analyzed by western blotting using antibodies against p-AKT, AKT, p-mTOR, mTOR, p-NF- κ B, and NF- κ B. B and C, quantification of A. The results shown were representative of three independent experiments. Data were expressed as mean $\pm$ SEM. *P < 0.05, **P < 0.01 compared with untreated control cells.

Figure 5 RLTS suppressed p38 MAPK signaling in tumor cells. A, SMMC-7721 and NCI-H460 cells were treated with various indicated concentrations of RLTS for 48 h. Then equal amounts of lysates were analyzed by western blotting using antibodies against p-ERK1/2, ERK1/2, p-p38 MAPK, and p38 MAPK. B and C, quantification of A. The results shown were representative of three independent experiments. Data were expressed as mean $\pm$ SEM. *P < 0.05, **P < 0.01

compared with untreated control cells.

Figure 6 RLTS inhibited activated VEGFR expression in cancer cells. A, SMMC-7721 and NCI-H460 cells were treated with the indicated concentrations of RLTS for 48 h, then equal amounts of lysates were analyzed by western blotting using individual antibodies.. B, quantification of A. The results shown were representative of three independent experiments. *P < 0.05 compared with untreated control cells.

Figure 7 RLTS downregulated expression of proteins related to metastasis. A, western blot analysis of CXCR4, MMP-2 and MMP-9 protein expression in NCI-H460 cells after treatment with RLTS at indicated concentrations for 48 h. B, quantification of A. The results shown were representative of three independent experiments. Data were expressed as mean $\pm$ SEM. *P < 0.05, **P < 0.01 compared with untreated control cells.

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

A

B

Table 1 *In vivo* inhibitory effect of RLTS on H22 cells

Treatment	Control	RLTS
Dose (mg/kg)	—	100
Initial tumor volume (mm ³)	105.45 ± 21.01	101.18 ± 16.68
Final tumor weight (g)	0.53 ± 0.43	0.21 ± 0.18
Tumor growth inhibition (g/g, %)	—	60.38
Final body weight (g)	28.96 ± 1.93	27.87 ± 1.28

Values are mean ± standard error of the mean (SEM).