

Loganin inhibits Liver Cancer Cell Proliferation and Metastasis *via* Blocking Mitogen-Activated Protein Kinase Signaling Pathway

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Chen *et al.*: Action of Loganin on the Oncogenic Phenotypes of Liver Cancer Cells

To investigate the action of loganin on the oncogenic phenotypes of liver cancer cells. Huh7 cells were treated with loganin, or loganin and 20 $\mu\text{mol/l}$ mitogen-activated protein kinase signaling pathway inhibitor SP600125. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide and Transwell assays detects cell abilities. Western blot detects E-cadherin, N-cadherin, vimentin, phosphorylated-extracellular signal-regulated kinase, extracellular signal-regulated kinase, phosphorylated-p38, and p38 protein expressions. Cell survival rate, the number of migratory and invasive Huh7 cells were significantly reduced with the increasing dose of loganin. E-cadherin content was increased, and contents of N-cadherin, vimentin, phosphorylated-extracellular signal-regulated kinase, and phosphorylated-p38 proteins were decreased in cells with the increasing dose of loganin. SP600125 could enhance the effects mediated by loganin on Huh7 cells. Loganin could significantly restrain the proliferation and metastasis of liver cancer cells by inhibiting the mitogen-activated protein kinase pathway.

Key words: Loganin, mitogen-activated protein kinase, migration, invasion, epithelial–mesenchymal transition

Ranking as the 6th in incidence rate and the 3rd in mortality rate, liver cancer seriously threatens the human health and life^[1]. There have been great advances in treatment, such as radiotherapy, chemotherapy, hepatic artery chemoembolization, etc., however, late recurrence and poor 5 y prognosis are unfavorable factors affecting liver cancer treatment^[2,3].

Loganin is an iridoid glycoside isolated from *Cornus officinalis*, and has been identified to possess neuroprotective, anti-apoptotic, antioxidative, immune regulatory and anti-inflammatory properties^[4-6]. Modern pharmacological experiments have shown that loganin has significant functions in various diseases. For example, loganin could suppress sepsis-evoked lung damage by inducing M2 macrophage polarization and impairing Nod-Like Receptor Protein 3 (NLRP3) inflammasome^[7]. Loganin alleviated the pathologies and cognitive impairment, as well as molecular deficits in Alzheimer's disease mouse models^[8]. Li *et al.*^[9] showed that loganin inhibited

ischemia-reperfusion-evoked myocardial injury by reducing pyroptosis and myocardial infarct. Importantly, loganin impeded cell proliferation, cell cycle and induced death in liver cancer^[10]. However, the research mechanism of loganin on liver cancer is still unclear. MAPK cascades are significant pathways involving in modulating cell differentiation, survival, stress responses and Epithelial Mesenchymal Transition (EMT)^[11,12]. Moreover, the activation of Mitogen-Activated Protein Kinase (MAPK) pathway is tightly linked with liver cancer progression^[13]. Moreover, research results have shown that curcumin could inhibit liver cancer cell survival by inhibiting the MAPK pathway^[14]. Here, we hypothesized loganin might affect liver cancer tumorigenesis *via* MAPK pathway.

Hence, this study mainly explored the action of

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loganin on liver cancer cell growth and metastasis, and then probed whether the MAPK pathway was implicated in the modulatory action of loganin.

MATERIALS AND METHODS

Cells and reagents:

Huh7 cell lines, Fetal Bovine Serum (FBS), trypsin and Dulbecco's Modified Eagle Medium (DMEM) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) kit (Sigma-Aldrich, United States of America (USA)); loganin (Shanghai Yuanye Biotechnology Co., Ltd); Transwell and Matrigel (Corning Corporation, USA); Radio-Immunoprecipitation Assay (RIPA) lysis buffer and Bicinchoninic Acid (BCA) reagent kit (Shanghai Yanjing Biotechnology Co., Ltd); E-cadherin, vimentin, N-cadherin, phosphorylated Extracellular Regulated Kinase (pERK), ERK, phosphorylated (p)-p38, p38, and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) antibodies (CST, USA); Horseradish Peroxidase (HRP)-labeled Immunoglobulin G (IgG) antibodies (Santa Cruz, USA); Polyvinylidene Difluoride (PVDF) membrane and Enhanced Chemiluminescence (ECL) detection reagents (Millipore, USA).

Cell culture and treatment:

Huh7 cells were cultured in 10 % FBS contained DMEM with 5 % Carbon dioxide (CO₂) at 37°. For loganin treatment, per well was added with 0, 50, 100, 150 µg/ml of loganin and incubated with Huh7 cells for 48 h, namely Negative Control (NC), 50, 100, or 150 µg/ml group. In addition, Huh7 cells were co-treated with 150 µg/ml loganin and 20 µmol/l SP600125 (the inhibitor of MAPK pathway), namely loganin+SP600125 group, the cells treated with 150 µg/ml loganin were used as the control (loganin group).

MTT assay:

After indicated treatment, Huh7 cells were incubated with 20 µl (5 mg/ml) MTT for 4 h in a 96 well plate, followed by adding with 150 µl Dimethyl Sulfoxide (DMSO) into per well for 2 h. Lastly, Optical Density (OD) value at 490 nm was examined.

Transwell assay:

For cell migration experiment, 100 µl Huh7 cell supernatant (1×10⁶/ml) adjusting by complete culture medium without serum were inoculated

into the upper chamber of Transwell. 24 h later, migrated cells were observed and counted under an inverted microscope after crystal violet dyeing. For cell invasion experiment, the Matrigel was diluted with a complete culture solution without serum at 1:8, and 60 µl was taken and spread to the bottom of the upper chamber of Transwell. After drying, other steps were consistent with the above migration experiment.

Western blot:

The extracted protein samples were denatured by boiling water, and transferred to a PVDF membrane after separating. Then the antibody diluents were incubated overnight at 4° with membranes for 12 h. After secondary incubation (dilution 1:500) for 2 h at 37°, the protein bands were analyzed by ECL incubation, and processed by ImageJ software.

Statistical analysis:

The data were presented by $\bar{x} \pm s$. Analysis of Variance (ANOVA) or t-test was adopted for the comparison of multiple groups or two groups. $p < 0.05$ indicated significant difference.

RESULTS AND DISCUSSION

MTT assay showed that the survival rate, as well as migratory and invasive abilities of Huh7 cells were decreased after treating with 50, 100 or 150 µg/ml loganin ($p < 0.05$, Table 1).

Relative to the NC group, E-cadherin level was increased, while N-cadherin and vimentin levels were declined in Huh7 cells of 50, 100 or 150 µg/ml loganin group ($p < 0.05$, Table 2 and fig. 1), suggesting that loganin suppressed the EMT progression.

Relative to the NC group, p-ERK and phosphorylated (p)-p38 contents were decreased in Huh7 cells of 50, 100 or 150 µg/ml loganin group, while no changes were observed in ERK and p38 protein levels ($p < 0.05$, Table 3 and fig. 2), suggesting that loganin suppressed the activation of MAPK pathway.

Relative to the loganin group, p-ERK and p-p38 levels were decreased in Huh7 cells of loganin+SP600125 group, and levels of ERK and p38 protein between the two groups had no changes ($p < 0.05$, Table 4 and fig. 3).

The survival rate as well as migrated and invaded Huh7 cells in loganin+SP600125 group relative

to the loganin group were markedly decreased ($p<0.05$, Table 5).

Compared with the loganin group, E-cadherin

contents were rose, while contents of N-cadherin and vimentin were declined in Huh7 cells in loganin+SP600125 group ($p<0.05$, fig. 4 and Table 6).

TABLE 1: EFFECTS OF LOGANIN ON LIVER CANCER CELLS

Group	Survival rate (%)	Migration	Invasion
NC	100.55±7.35	125.61±9.46	112.34±9.27
50 µg/ml loganin	83.79±5.12*	107.78±8.67*	97.61±8.33*
100 µg/ml loganin	65.38±6.38*	90.44±7.32*	81.92±7.52*
150 µg/ml loganin	40.56±3.76*	67.69±4.85*	58.73±5.61*
F	176.351	91.018	77.816
p	0.000	0.000	0.000

Note: Relative to the NC group, * $p<0.05$

TABLE 2: EFFECTS OF LOGANIN ON THE EMT OF LIVER CANCER CELLS

Group	E-cadherin	N-cadherin	Vimentin
NC	0.23±0.03	0.74±0.07	0.68±0.06
50 µg/ml loganin	0.38±0.04*	0.59±0.06*	0.54±0.05*
100 µg/ml loganin	0.52±0.05*	0.45±0.05*	0.41±0.05*
150 µg/ml loganin	0.68±0.06*	0.30±0.03*	0.28±0.02*
F	154.988	107.496	117.967
p	0.000	0.000	0.000

Note: Relative to the NC group, * $p<0.05$

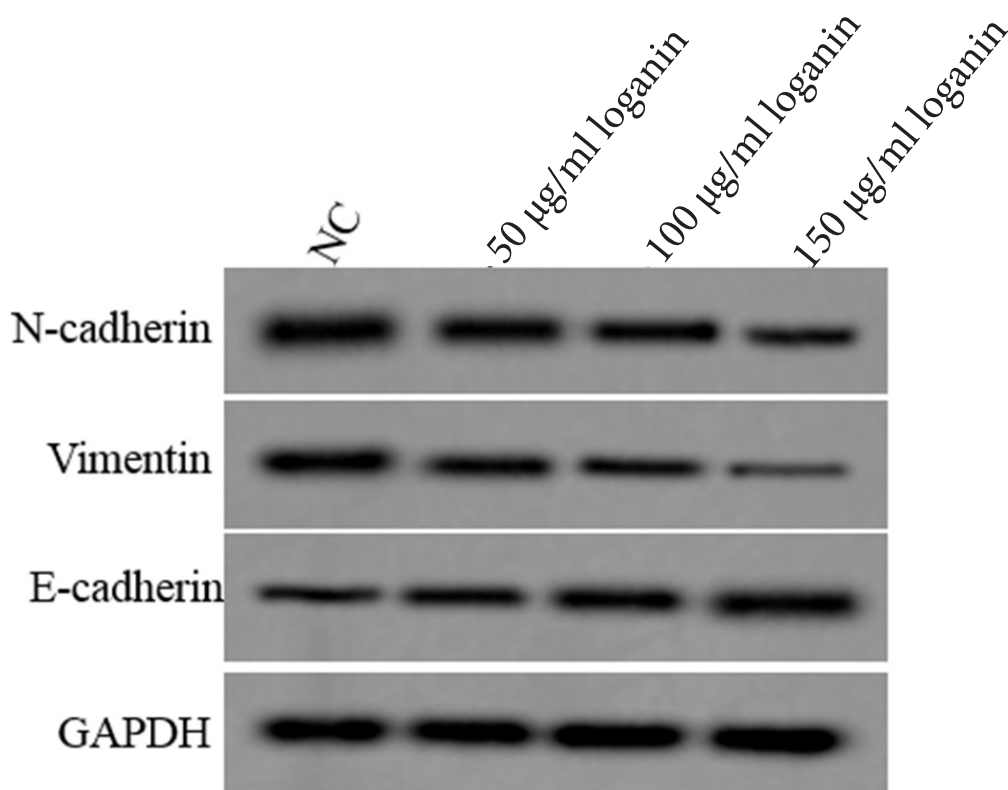
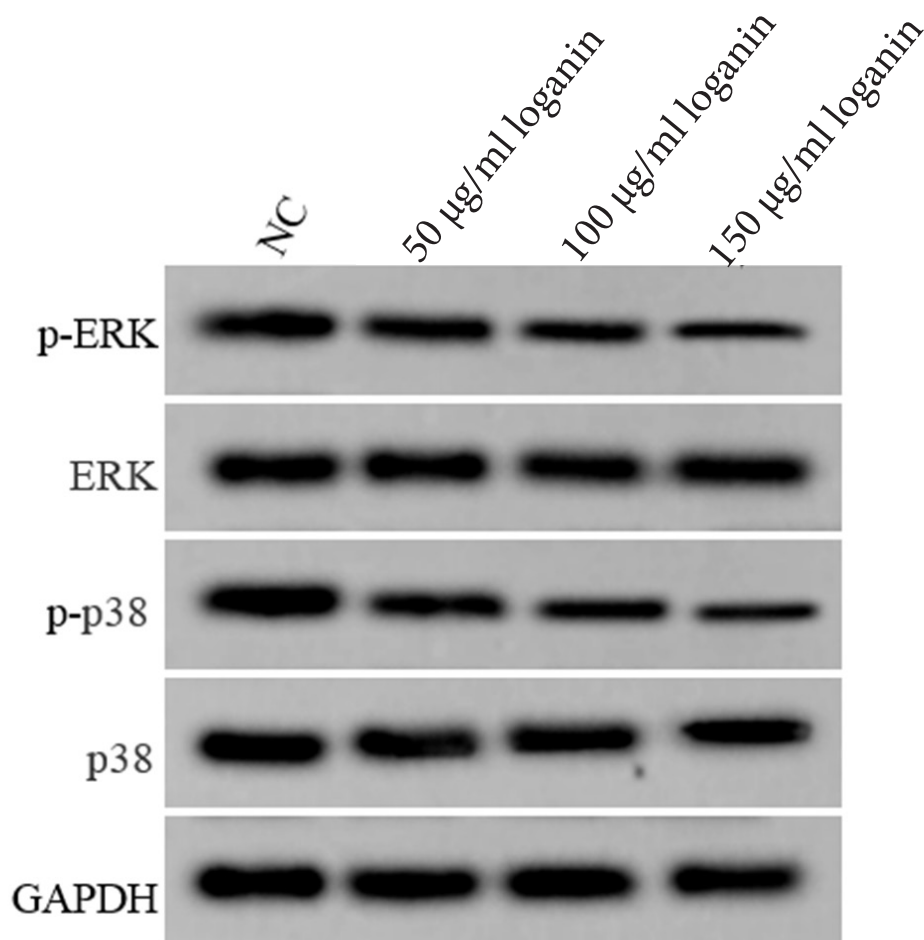


Fig. 1: Western blot for EMT-related proteins in liver cancer cells

TABLE 3: EFFECTS OF LOGANIN ON THE MAPK PATHWAY OF LIVER CANCER CELLS

Group	p-ERK/ERK	p-p38/p38
NC	0.86±0.07	0.78±0.05
50 µg/ml loganin	0.71±0.06*	0.63±0.06*
100 µg/ml loganin	0.56±0.05*	0.50±0.05*
150 µg/ml loganin	0.41±0.04*	0.32±0.03*
F	107.143	144.600
P	0.000	0.000

Note: Relative to the NC group, *p<0.05

**Fig. 2: Western blot for the MAPK pathway-related proteins****TABLE 4: SP600125 ENHANCED LOGANIN-EVOKED INACTIVATION OF THE MAPK PATHWAY IN LIVER CANCER CELLS**

Group	p-ERK/ERK	p-p38/p38
Loganin	0.43±0.04	0.35±0.03
Loganin+SP600125	0.28±0.04*	0.22±0.02*
t	7.955	10.817
p	0.000	0.000

Note: Relative to the loganin group, *p<0.05

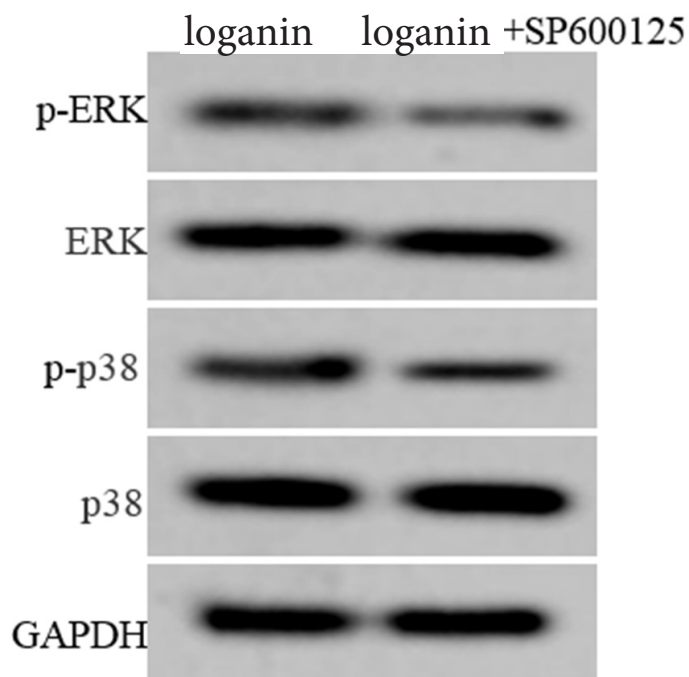


Fig. 3: SP600125 enhanced loganin-evoked inactivation of the MAPK pathway in liver cancer cells

TABLE 5: LOGANIN SUPPRESSED LIVER CANCER CELL ONCOGENIC PHENOTYPES BY BLOCKING MAPK PATHWAY

Group	Survival rate (%)	Migration	Invasion
Loganin	43.68±4.24	68.90±7.21	56.14±4.79
Loganin+SP600125	30.12±2.85*	46.35±4.33*	40.62±3.53*
t	7.963	8.044	7.825
p	0.000	0.000	0.000

Note: Relative to the loganin group, *p<0.05

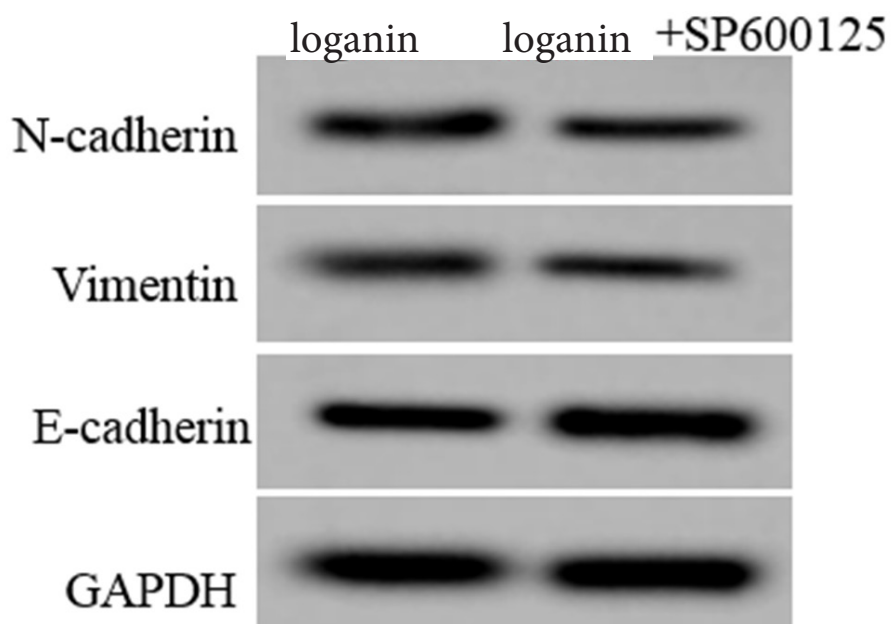


Fig. 4: Loganin suppressed liver cancer cell EMT by blocking MAPK pathway

TABLE 6: LOGANIN SUPPRESSED LIVER CANCER CELL EMT BY BLOCKING MAPK PATHWAY

Group	E-cadherin	N-cadherin	Vimentin
Loganin	0.65±0.06	0.33±0.04	0.30±0.03
Loganin+SP600125	0.83±0.06*	0.19±0.02*	0.17±0.02*
t	6.364	9.391	10.817
p	0.000	0.000	0.000

Note: Relative to the loganin group, *p<0.05

The pathogenesis of liver cancer is affected by many factors, such as staying up late for a long time, irregular diet, long-term alcohol and coffee drinking, genetic factors, etc., resulting in a trend of younger liver cancer patients in recent years^[15]. Moreover, liver cancer patients at advanced stage show poor prognosis and unsatisfactory survival rate, therefore, it is very important to find new treatment and diagnosis methods for liver cancer^[16]. At present, the main treatment methods of Western medicine have achieved certain therapeutic effects in clinical practice. However, there are problems such as high recurrence and metastasis rates and multiple adverse reactions after treatment. Traditional Chinese medicine combined with Western medicine treatment at different stages of liver cancer can achieve the goals of controlling tumor development, improving clinical efficacy, reducing adverse reactions, and preventing recurrence and metastasis. The therapeutic effect is quite good.

Loganin belongs to the iridoid glycoside class. It can enhance non-specific immune function, promote macrophage phagocytosis and delay senescence, it has good anticancer and anti-radiation effects, and is widely used in clinical medicine as a new anticancer drug. It has good anti-inflammatory and antibacterial effects, and is commonly used as a raw material for Chinese medicine. In addition, it also has antitussive, expectorant and other functions. Previous research results have shown that loganin has suppressing effects on cancer progression. For example, loganin declined B-Cell Lymphoma 2 (Bcl-2) level to induce the cell death of colorectal cancer^[17]. In gastric cancer, the growth and metastasis of cells were hindered by oganin^[18]. Loganin induced cycle arrest and repressed the proliferation in melanoma cells by ERK1/2 pathway^[19]. Loganin retrained the cell survival and mobility by blocking Protein

Kinase B (AKT)/mammalian Target of Rapamycin (mTOR) pathway in pancreatic cancer^[20]. However, the action of loganin on Hepatocellular Carcinoma (HCC) remains unclear. In our study, we discovered that loganin could suppress the proliferative, invasive and migratory capacities of live cancer cells, in addition, it was discovered that loganin reduced N-cadherin and vimentin level, and increased E-cadherin levels in cells, thus suppressing EMT process. In all, loganin retrained liver cancer cell proliferation and metastasis.

MAPKs including ERK1/2, JNK, SAPK, and p38 pathways, are closely related to the research on anti-tumor effects of traditional Chinese medicine^[21,22]. For example, tanshinone II could block the p38 signaling pathway to inhibit liver cancer cell movement and growth^[23]. Zhao *et al.*^[24] showed that a biflavonoid isolated from *Resina Draconis* exerted anti-tumor effects by activating the MAPK pathway to repress HCC cell survival and migration^[25]. Moreover, aconitine has inhibitory effects on HCC cell mobility and viability, and its mechanism was linked with p38 MAPK pathway. The above data indicates the implication of the MAPK pathway in liver cancer. In the present study, the treatment with different concentrations of loganin could reduce p-ERK and p-p38 proteins, and this reduction was enhanced by SP600125 (the inhibitor of MAPK pathway), indicating that loganin blocked the activation of MAPK pathways. Functionally, SP600125 boosted loganin-induced inhibition of cell proliferative, migratory and invasive capacities and EMT progression in live cancer, implying that loganin could affect liver cancer by MAPK pathway.

In summary, loganin suppressed cell proliferation and metastasis by blocking MAPK pathway. However, there were also shortcomings in this study. Further exploration is needed on the functions of loganin on liver cancer growth and

metastasis *in vivo* in order to provide reasonable theoretical support for the study of loganin.

Author's contributions:

Jiajun Chen and Yan Qu have contributed equally to this work.

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