Original Article
Amygdalin-mediated inhibition of non-small cell lung cancer cell invasion *in vitro*

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**Abstract:** Lung cancer is a common malignant tumor claiming the highest fatality worldwide for a long period of time. Unfortunately, most of the current treatment methods are still based on the characteristics of cancer cells in the primary lesion and the prognosis is often much poorer in patients with metastatic cancers. Amygdalin, a natural product of glycosides and lots of evidence shows that amygdalin can inhibit the proliferation of some kinds of cancer cells. In this study, we first obtained the highly metastatic NSCLC cell lines H1299/M and PA/M and further treated these cells with amygdalin. We found that the in vitro proliferability of H1299/M and PA/M was inhibited, but such inhibition required higher concentration of amygdalin. When lower concentration of amygdalin was used for the experiments, we observed that the in vitro invasive and migration capacities of H1299/M and PA/M were significantly inhibited. These results strongly suggested that amygdalin was likely to have anti-metastatic NSCLC effect. This study offers information of the role of amygdalin that may be useful as a therapeutic target in lung tumors.

**Keywords:** Lung cancer, amygdalin, invasion

**Introduction**

Metastasis is the main cause of mortality in patients with cancer, accounting for approximately 90% of the total number of deaths [12, 20]. Lung cancer is a common malignant tumor claiming the highest fatality worldwide for a long period of time [13]. Lung cancer cells often metastases to the brain, bones, liver, adrenal glands and other organs [1]. These organs are important organs of the body in which cloning and growth of the cancer cells cause great harms and threaten human life. Currently, metastatic cancers are still treated with surgery, chemotherapy, radiation therapy, targeted therapy, biological therapy, combined therapies and other means, but these methods are difficult to effectively treat metastatic tumors. The main reason is that these metastatic cancer cells not only possess biological characteristics different from the cancer cells in the primary lesions but also manifest higher malignancy. Unfortunately, most of the current treatment methods are still based on the characteristics of cancer cells in the primary lesion [6]. Therefore, the prognosis is often much poorer in patients with metastatic cancers than those without metastasis. It is highly necessary to develop drugs that can inhibit and target at metastatic cancers.

Amygdalin, a natural product of glycosides, was originally isolated from the seeds of sweet almond. Subsequently, this natural product is also found in almonds, blackberries, apples and other plants [11]. In the 1970s, amygdalin was one of the most common non-conventional anti-cancer drugs in Western countries. According to the statistics, as of 1978, there are totally more than 70,000 patients with cancers in the United States have been treated with amygdalin [5]. However, the anti-tumor efficacy and mechanisms of action of amygdalin have remained controversial since then. Some recent studies provide positive evidence to resolve the controversy. The evidence shows that amygdalin can inhibit the proliferation of liver cancer [19], cervical cancer [3], bladder cancer [9] and other cancer cells and/or promote their apoptosis. Makarević et al [10] also found that amygdalin could inhibit the in vitro adhesion and invasion of bladder cancer cells. Accordingly, this study was designed to investigate the inhibition of amygdalin on metastatic
non-small cell lung cancer (NSCLC) and preliminarily explores its molecular mechanisms of action. These studies can not only help us better understand the anti-tumor effects and mechanisms of amygdalin but also provide references for chemical modification of amygdalin and improvement of its anti-tumor activity.

**Materials and methods**

**Cell culture and induction of highly metastatic cell line**

The cells were maintained in RPMI-1640 containing 10% fetal bovine serum and cultured in an incubator under 37°C, 5% CO₂ and saturated humidity condition. The cells were digested with 0.25% trypsin-EDTA for passaging. Cells in logarithmic growth phase were used in all experiments. In order to screen highly metastatic gastric cancer cell lines, H1299 and PA were cultured in the upper chamber of Transwell (Corning) coated with Matrigel (BD Biosciences). In 24 h, the cells migrating to the lower chamber were collected for cloning culture. The above procedures were repeated 10 times to screen out the highly metastatic cell lines H1299/M and PA/M.

**MTS cell proliferation assay**

Cells in logarithmic growth phase, 5×10⁴ cells/ml, were seeded in 96-well microplates, 100 μl/well, and cultured overnight to allow cell adhesion. Different concentrations of amygdalin were added to continue the culture for 48 h. After removing the medium, MTS was added in accordance with the instructions to continue the culture for 4 h. Finally, the OD was determined at 490 nm wavelength with a microplate reader to represent the number of cells. The inhibition rate of the drug on cells was calculated using the formula as follows: inhibition rate = (1 - experimental group OD/control group OD)×100%.

**Western blotting assay**

Cells were treated with 2.5 and 5 mg/ml amygdalin for 48 h, and then total RNA was extracted from various groups using Trizol method. Real-Time PCR kit was used to carry out reverse transcription; the obtained cDNA was used to determine the mRNA level of MMP-2/9, CD44, integrin β1, integrin β4, ILK, FAK, p-FAK, E-cadherin and β-catenin, and the phosphorylation level of Akt and RICTOR were determined.

**In vitro invasion and migration assay**

The in vitro invasibility was assessed using the CultureCoat® 96-Well BME-Coated Cell Invasion Optimization Assay Kit supplied by R & D Systems. The cells starving in serum-free medium for 16 h were seeded at 25000 cells/well in the upper chamber of Transwell, in the meantime, 2.5 and 5 mg/ml amygdalin was added to continue the culture for 48 h. Then, the number of invasion cells was analyzed in accordance with the kit instructions. The wound-healing assay was used to assess the in vitro migratability of cancer cells. Specifically, the cells were cultured in 6-well plate until single-layer confluency. After starving in serum-free medium overnight, the 200 μl pipette tip was used to make scorings in the cell layer. Then, 2.5 and 5 mg/ml amygdalin was added to continue the culture for 48 h. The distance between the scorings was observed and measured.
AAG TAA GGA AAT ACA GTT TGG-3', downstream primer sequence: 5'-CCA CAT ACA CAC ACC AAA CAT CAT CCA-3'; β-catenin upstream primer sequence: 5'-CCT ATG CAG GGG TGG TCA AC-3', downstream primer sequence: 5'-CGA CCT GGA AAA CGC CAT CA-3'; MMP2 upstream primer sequence: 5'-TAC TGA GTG GCC GTG TTT GC-3', downstream primer sequence: 5'-AGG GAG CAG AGA TTC GGA CTT-3'; MMP9 upstream primer sequence: 5'-GGG CTT AGA TCA TTC CTC AGT G-3', downstream primer sequence: 5'-GGG CTG GTA AGA TCA TTC CTG CTG C-3', downstream primer sequence: 5'-GCC ATT CAC GTC GTC AT-3'; CD44 upstream primer sequence: 5'-ACC CCA ACT CCA TCT GTG C-3', downstream primer sequence: 5'-GGG ACA TAG CGG GTG-3'; GAPDH upstream primer sequence: 5'-CTTAGATTTGCTCGATTGG-3', downstream primer sequence 5'-GAAGATGGT-GATGGGATT-3'.

Statistical analysis

The experimental data were expressed as mean ± standard deviation. SPSS13.0 software was used for analysis. One-way ANOVA was used for comparison. P<0.05 indicated that the difference had statistical significance.

Results

Potential of proliferation, migration, invasion of H1299, H1299/M, PA and PA/M in vitro

After the in vitro screening, the highly metastatic cell lines H1299/M and PA/M were successfully obtained. As shown in Figure 1A-E, both the in vitro invasion assay and wound-healing assay showed that the invasibility of H1299/M and PA/M were significantly higher than the...
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Parent cell lines H1299 and PA.MTS cell proliferation assay showed that high-concentration amygdalin inhibited the in vitro proliferation of H1299/M and PA/M cells; its IC₅₀ was 12.2 mg/ml. The inhibition rate of 2.5 and 5 mg/ml amygdalin on cell proliferation was 15.6% and 25.1%, respectively, exhibiting a weaker inhibitory effect (Figure 1F). Therefore, these two drug concentrations were selected for the subsequent invasion and migration experiments.

Low-concentration amygdalin inhibits the in vitro invasion and migration

As described above, the inhibitor concentrations used for the in vitro invasion and migration assays were 2.5 and 5 mg/ml, respectively. Within the experimental time, selecting these drug concentrations would not significantly inhibited cell growth. In 48 h, the number of invasive cells in inhibitor-treated group significantly reduced. The wound-healing assay showed that the gap between the scorings was smaller in inhibitor-treated group than in control group in 48 h (Figure 2).

Low-concentration amygdalin down-regulates the expression of cell integrin β1, integrin β4, ILK, FAK, p-FAK and β-catenin while up-regulates the expression of E-cadherin

After the cells have been treated with 5 mg/ml amygdalin for 48 h, the relevant protein expression was determined. WB assay result showed that the expression levels of integrin β1, integrin β4, ILK, FAK, and β-catenin, which were proteins with metastasis-promoting action, in highly metastatic cancer cells significantly decreased after inhibitor treatment when compared with control group. Among them, the down-regulation of amygdalin affected FAK phosphorylation (p-FAK) more than FAK expression. In contrast, the expression of E-cadherin protein capable of inhibiting metastasis was significantly up-regulated. See details in Figure 3B and 3C. Real-time-PCR study showed that

Figure 2. Effects of amygdalin on the metastatic capacities of H1299/M and PA/M cell lines. A. Representative wound-healing assay images for H1299/M cell migration pretreatment with different amygdalin concentration. B. Representative transwell assay images for H1299/M cell invasion pretreatment with different amygdalin concentration. C. Representative wound-healing assay images for PA/M cell migration pretreatment with different amygdalin concentration. D. Representative transwell assay images for PA/M cell invasion pretreatment with different amygdalin concentration. E/F. The relative cells invasion and migration of H1299/M and PA/M cells treatment with different amygdalin concentration. *P<0.05; **P<0.01, compared with the control.
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A

H1299/M

Relative gene expression

Integrin β1 Integrin β4 ILK FAK E-cadherin β-catenin MMP2 MMP9 CD44

- 2.5 mg 5 mg

amygdalin

H1299/M

B

CD44 MMP2 MMP9 E-cadherin ILK p-FAK FAK Integrin β4 Integrin β1 β-action

2.5 mg 5 mg

C

amygdalin

PA/M

- 2.5 mg 5 mg

PA/M

CD44 MMP2 MMP9 E-cadherin ILK p-FAK FAK Integrin β4 Integrin β1 β-action

D

Relative gene expression

Integrin β1 Integrin β4 ILK FAK E-cadherin β-catenin MMP2 MMP9 CD44

- 2.5 mg 5 mg

PA/M

E

amygdalin

- 2.5 mg 5 mg

H1299/M p-RICTOR RICTOR p-Akt Akt

F

amygdalin

PA/M

- 2.5 mg 5 mg

p-RICTOR RICTOR p-Akt Akt
the regulation of all these protein expressions occurred at transcriptional level as shown in Figure 3A and 3D.

Low-concentration amygdalin inhibits the phosphorylation Akt and RICTOR

Western blot results showed that amygdalin had no significant impact on Akt and RICTOR expression. The determination of Akt and RICTOR phosphorylation level showed that amygdalin significantly reduced the phosphorylation level of these two proteins in highly metastatic cells, suggesting that amygdalin was able to regulate the activity of Akt and RICTOR signaling pathways (Figure 3E and 3F).

Discussion

Although studies have shown that amygdalin has anti-tumor proliferation and metastasis effects, studies of the effect of amygdalin on lung cancer invasion and metastasis are few. In this study, we first obtained the highly metastatic NSCLC cell lines H1299/M and PA/M and further treated these cells with amygdalin. We found that the in vitro proliferability of H1299/M and PA/M was inhibited, but such inhibition required higher concentration of amygdalin. When lower concentration of amygdalin was used for the experiments, we observed that the in vitro invasibility (infiltrating Matrigel) and migrationality of H1299/M and PA/M were significantly inhibited. These results strongly suggested that amygdalin was likely to have anti-metastatic NSCLC effect.

The interaction between tumors and extracellular matrix is a key part for successful metastasis. Under normal circumstances, the cells interact with the extracellular matrix or the surrounding cells through membrane-associated proteins such as integrin and E-cadherin to immobilize the cells in a particular environment. Such interaction is a dynamic process to ensure cell movement in response to the normal physiological needs, but such movement is strictly controlled under normal physiological conditions. In contrast, the tumor cells often spontaneously acquire the moving ability and break away from normal physiological regulation. Integrin is a protein containing multi-subunits including α and β-subunits, also known as heterodimer protein. Wherein, the two subtypes, namely β1 and β4, are often related to tumor metastasis [4, 14, 16]. We observed in Western blotting and real-time-PCR assays that amygdalin was able to down-regulate β1 and β4 expression level in H1299/M and PA/M cells, which was consistent with the results that amygdalin inhibit in vitro metastasis of H1299/M and PA/M cells. E-cadherin is another type of protein mediating intercellular interactions. In contrast with integrin β1 and β4, E-cadherin plays a negative role in tumor cell migration. Because the loss of E-cadherin function causes the cancer cells to break free from constrains of the surrounding cells, making it easier to penetrate through the basal membrane and invade the surrounding tissues. Clinically, the loss of its function or under-expression is generally related to cancer progression and metastasis [15]. Both Western blotting and real-time-PCR assay results showed that the intracellular E-cadherin expression level increased after treatment with amygdalin. In addition, the functions of integrin and E-cadherin should not be simply regarded as a protein used to connect the extracellular matrix with the surround cells because they also transmit signals into the cells. Integrin β1 and β4 can activate FAK to further release β-catenin; the latter can migrate into the nucleus to mediate the expression of tumor growth and migration-related proteins [18, 20]. Integrin β1 can activate ILK to initiate the downstream signaling pathways such as Akt-mTOR pathway and mediate cell proliferation, adhesion and metastasis functions [8, 17]. Our study showed that ILK, FAK (especially p-FAK) and β-catenin levels decreased significantly with the down-regulation of integrin β1 and β4 expression. RICTOR as a subunit of mTOR plays an important role in the Akt-mTOR signaling pathway; its phosphorylation level is positively regulated by Akt [2, 7].
After having observed that amygdalin was able to inhibit ILK expression level, we further found that the phosphorylation level of Akt and RICTOR significantly decreased although their expression levels were not significantly affected. These results further confirmed that amygdalin was able to inhibit tumor cell metastasis by regulating integrin expression and its downstream signaling pathways.

In summary, this study showed that amygdalin inhibited the in vitro proliferation, invasion and migration of NSCLC. The mechanisms may be related to the more extensive regulation of gene expression by regulating integrin and E-cadherin expression, and the downstream key signaling pathways such as Akt-mTOR.

Disclosure of conflict of interest

None.

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