A subpopulation of cancer stem cells is recognized as the cause of tumorigenesis and spreading. To investigate the effects of casticin (5,3″-dihydroxy-3,6,7,4″-tetramethoxyflavone), derived from *Fructus Viticis Simplicifoliae*, on lung cancer stem cells, we isolated and identified a subpopulation of lung cancer stem-like cells (LCSLCs) from non-small-cell lung carcinoma A549 cells with the features including self-renewal capacity and high invasiveness in vitro, elevated tumorigenic activity in vivo, and high expression of stemness markers CD133, CD44, and aldehyde dehydrogenase 1 (ALDH1), using serum-free suspension sphere-forming culture method. We then found that casticin could suppress the proliferation of LCSLCs in a concentration-dependent manner with an IC₅₀ value of 0.4 μmol/L, being much stronger than that in parental A549 cells. In addition, casticin could suppress the self-renewal and invasion of LCSLCs concomitant with decreased CD133, CD44, and ALDH1 protein expression and reduced MMP-9 activity. Further experiments showed that casticin suppressed self-renewal and invasion at least partly through down-regulation of Akt phosphorylation. In conclusion, casticin suppressed the characteristics of LCSLCs, suggesting that casticin may be a candidate compound for curing lung cancer via eliminating cancer stem cells.

**Keywords** lung cancer; casticin; cancer stem cells; pAkt; therapeutic action

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**Introduction**

Lung cancer is the most common cause of cancer deaths worldwide for its high incidence and mortality. Globally, more than one million new lung cancer cases were reported each year [1,2]. Lung cancer is generally subdivided into small cell lung cancer and non-small cell lung cancer (NSCLC). The 5-year survival for NSCLC is less than 9% in developing countries [2], and no more than 15% in USA [3]. So it is necessary to explore the novel therapeutic approaches for improving life quality of patients with lung cancer including NSCLC.

Recently, more and more researchers are focusing on cancer stem cells (CSCs), trying to find new breakthrough point for curing cancer. The CSCs possess self-renewal capacity and differentiation potential, and can reconstruct the phenotypic and histologic heterogeneity of its parent tumor when transplanted in vivo [4]. The CSC theory presumes that the tumor carcinogenesis, recurrence, and resistance are caused by the existence of a small subpopulation of CSCs. Recent studies are mainly involved in characterizing CSCs from different tumor cell lines or solid tumor tissues including glioblastoma, melanoma, breast cancer, lung cancer, etc., and their crucial signal molecules [5–8].

CSCs can be enriched or identified using cell surface markers via flow cytometry and immunomagnetic beads sorting system [9,10], or sphere-forming assay [4,11] in selective serum-free medium. In NSCLC, CSCs are commonly characterized as a subpopulation of cells expressing CD133 [10], or aldehyde dehydrogenase (ALDH) [12,13] or as a side population [14] of cells capable of effluxing Hoechst dye. Identification of CSCs in tumor cells is important, but studies on this area are just the beginning. What is more important is to find the effective therapeutic strategies, including screening of novel drugs targeting CSCs.

Casticin (5,3″-dihydroxy-3,6,7,4″-tetramethoxyflavone) is a main active ingredient of *Fructus Viticis Simplicifoliae*, which is often used as the traditional Chinese medicine for the treatment of certain types of cancer. Studies have shown that casticin possesses extensive anticancer bioactivity for cancers including lung cancer [15], cervical cancer [16], leukemia [17,18], colon cancer [19], hepatocellular carcinoma...
cells [20], and so on [21,22]. We have found that casticin could inhibit self-renewal of glioma stem-like cells [23]. However, the effects of casticin on lung cancer stem-like cells (LCSLCs) and its mechanisms remain elusive.

In this study, we enriched and identified LCSLCs from NSCLC A549 cells, and found that casticin could suppress the self-renewal and invasion of LCSLCs, accompanied with a reduced CD133, CD44, and ALDH expression. The underlying mechanisms were associated with the decrease of phosphorylation Akt, which was reported to play a crucial role in the maintenance of CSC features.

**Materials and Methods**

**Cell culture**

Human lung cancer A549 cell line was purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China), cultured in RPMI-1640 (HyClone, Logan, USA), supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂.

**Sphere-forming and self-renewal assay**

To obtain sphere cultures, cells were plated at a density of $5 \times 10^3$ cells/well in 6-well ultra-low plates (Corning, Acton, USA) containing serum-free medium DMEM/F12 (Gibco, Carlsbad, USA), supplemented with commercial hormone mix B27 (Gibco), 20 ng/ml EGF (PeproTech, Rocky Hill, USA), 10 ng/ml bFGF (PeproTech), 0.4% bovine serum albumin (Gibco), 4 μg/ml insulin (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C. After being cultured for 6 days, the lung cancer spheres were collected, dissociated into single cell suspension and resuspended in fresh medium for serial subcultivation every 6 days.

To investigate the self-renewal capacity of lung cancer sphere-forming cells (SFCs), single cell suspension prepared from the lung cancer sphere was diluted to 500 cells/ml. Two microliters of the single cell suspension was plated in 96-well ultra-low plates containing 150 μl serum-free medium per well. Wells containing no cells or more than one cell were excluded, and those with one cell were marked and monitored daily under a microscope (Nikon Eclipse TE2000-S, Nikon, Japan) for 6 days and the colonies were counted. To investigate the effects of casticin on self-renewal of SFCs, single cell suspension of SFCs was plated at a density of $2 \times 10^3$ cells/ml in 6-well ultra-low plates. Different concentrations of casticin (1.0, 5.0, and 10.0 μM) were added to medium. After being cultured for 6 days, the colonies were counted under a microscope.

**MTT [3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay**

The SFCs or parental cells were plated at 5000 cells/well in 96-well ultra-low plates. The cells were first incubated with casticin at the indicated concentrations for 48 h, and then incubated with 5 mg/ml MTT for 4 h. Lastly, 100 μl of dimethyl sulfoxide (DMSO) was added to each well, and the absorbance at 570 nm ($A_{570}$) each well was detected on a plate reader (Bio-Tek EXL-800, Winooski, USA). The relative cell proliferation inhibition rate $= (1—\text{experimental group } A_{570\text{mean}}/\text{control group } A_{570\text{mean}}) \times 100\%$. IC₅₀ (50% inhibiting concentration) was calculated by Prism (GraphPad Software, San Diego, USA).

**Matrigel invasion assay**

Two thousands cells incubated in serum-free medium were added on the top of the transwell chamber coated with fresh Matrigel (Gibco). The medium supplemented with 10% FBS, as the chemical inducing agent, was added to the bottom of the transwell chamber. After incubation for 48 h, the cells on top of the chamber were scraped off using a cotton swab. And the cells in the bottom of the chamber were fixed with methanol and stained with Wright staining solution. The cells invading through the membrane were counted under an optical microscope.

**Tumorigenicity assay**

The SFCs or parental cells were injected subcutaneously into the back of 4-week-old BALB/C-nude mice (supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China). The mice were reared for 2 months, and the tumor growth and tumorigenic time were examined visually. Tumor volumes were calculated in accordance with the formula: $V = L \times W^2 \times 0.5$. At the end of experiment, the mice were sacrificed under deep anesthesia with pentobarbital. The tumors were then dissected, weighed and fixed with 10% neutral formalin. Paraffin sections were prepared following routine procedures for H&E staining.

**MMP-9 activity assay**

The SFCs were plated at a density of $1 \times 10^6$ cells/well in 6-well ultra-low plates containing serum-free medium. After 24 h incubation, the medium was replaced with serum-free medium containing different concentrations of casticin (1.0, 5.0, 10.0 μM), cultured for additional 72 h. Then the medium was collected and subject to MMP-9 activity assay using human active MMP-9 fluorokine E kit (R&D Systems, Minneapolis, USA) according to manufacturer’s instructions. The activity unit was defined as ng/ml/10⁶ cells.

**Western blot analysis**

The cells were lysed using lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 0.2% NP-40, 10% glycerol, 1 M β-Me, 1 μg/ml aprotinin, 0.5 μg/ml aprotinin...
aldehyde peptide, 0.1 mM Na₃VO₄, 0.5 mM 4 NPP, 0.5 mM NaF, and protease inhibitors), and the total cell extracts were prepared. The protein content was measured using the Bradford method. The proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. After being blocked with 5% non-fat milk in phosphate buffered saline containing Tween-20 (PBST), the PVDF membrane was incubated using the following mouse anti-human primary monoclonal antibodies: anti-pAkt (Ser473) (1:500; Cell signaling, Beverly, USA), anti-Akt (1:2000; cell signaling), anti-CD44 (1:500; cell signaling), anti-CD133 (1:500; cell signaling), anti-ALDH1 (1:500; cell signaling), and anti-β-actin (1:200; Sigma, St. Louis, USA), with slight shaking at 4°C overnight. Then the membrane was incubated with peroxidase-labeled secondary antibody for 1 h, and the signal was detected using an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK) according to manufactures’ protocols.

Statistical analysis
Data were presented as the mean ± standard deviation. To assess the statistical significance of differences, student’s t test was performed. P < 0.05 was regarded as statistically significant.

Results
Lung cancer SFCs derived from A549 cell lines have the self-renewal capability
In order to enrich LCSLCs from human lung cancer A549 cell line, stem cell conditioned medium suspension culture method was used. The results showed that A549 cells could form non-adherent sphere which is called lung cancer SFCs (Fig. 1A2). The results suggested that LCSLCs exist in human lung cancer A549 cells.

We next performed the SFCs subculture and tumor sphere formation test. It was found that the single cell of SFC from lung cancer A549 was able to form a new sphere (Fig. 1B), suggesting that the SFCs from A549 cells have the ability of self-renewal.

Lung cancer SFCs overexpress stem cell markers
Furthermore, we investigated the expressions of CSC biomarker CD44, CD133, and aldehyde dehydrogenase 1 (ALDH1) in SFCs using western blotting. The results demonstrated that, compared with human lung cancer parental cells, the CD44, CD133, and ALDH1 protein expression levels were apparently increased in their SFCs (Fig. 1C).

Lung cancer SFCs possess higher tumorigenicity
In addition, the xenograft tumor model in nude mouse was used to investigate the tumorigenicity of A549 cells and their SFCs in Balb/c-nu immunodeficient mice. The results showed that 1 × 10⁶ A549 SFCs were enough to form tumors in vivo (Fig. 1D), and the tumors have similar immunohistochemical characteristics with its parental cell xenograft tumor (Fig. 1E). However, in the same model, at least 1 × 10⁸ A549 parental cells were required to induce stable tumor formation (Table 1). Our data indicate that the SFCs from A549 cell line have higher tumorigenicity in vivo than parental cells.

The above data confirmed that SFCs from human lung cancer A549 cell line cells possessed characteristics of CSC. Therefore, we can, at least, deduce that the lung cancer SFCs prepared by us belong to LCSLCs, and the LCSCs were possibly enriched in them.

Casticin inhibits the self-renewal capability of LCSLCs
The unlimited proliferation potential is an important feature of CSCs [24]. Genistein is a natural polyphenolic compound that can preferentially inhibit the proliferative activity of the pancreatic CSCs than parent cells [23]. Our results...
showed that casticin (0.1, 1, 3, 10, 30 μM) could inhibit the proliferation of human lung cancer SFCs in a dose-dependent manner (Fig. 2A), and the IC_{50} of casticin on A549 cells and their SFCs were 14.3 and 0.4 μmol/L, respectively, suggesting that casticin is able to preferentially suppress the proliferation activity of LCSLCs.

Furthermore, casticin (1, 5, 10 μM) could reduce the size and number of SFCs from A549 cells in a dose-dependent manner (Fig. 2B,C), suggesting that casticin can suppress the self-renewal of LCSLCs.

### Casticin decreases invasion of LCSLCs
In addition, in view of the important role of CSCs in cancer early metastasis, the Transwell invasion assay was used to investigate the effects of casticin on invasion of LCSLCs. The results showed that casticin (1, 5, 10 μM) could inhibit the invasion of SFCs from A549 cells in a dose-dependent manner (Fig. 2D,E). It is reported that the MMP-9 is highly expressed in LCSLCs from A549 cells than in parent cells [25]. In this study, the MMP-9 activity in SFCs from A549 cells was found to be decreased gradually with the increase of casticin concentration (Fig. 2F). These data indicate that casticin can suppress the invasion of LCSLCs, possibly via reducing MMP-9 activity.

### Casticin reduces CD133, CD44, and ALDH1 expression in LCSLCs
Western blot analysis showed that casticin (1, 5, 10 μM) reduced CD133, CD44, and ALDH1 protein expression in SFC from A549 cells, in a dose-dependent manner (Fig. 3A). It suggests that casticin can decrease CSC marker expression in LCSLCs.

### Casticin inhibits the self-renewal of LCSLCs via down-regulation of pAkt
Western blot analysis showed that the phosphorylation levels of Akt in SFCs from A549 cells were higher than those in A549 cells (Fig. 3B). Casticin could decrease phosphorylated Akt (p-Akt) expression in the SFCs in a dose-dependent manner (Fig. 3C). Akt inhibitor LY294002 (5, 10, 20 μM) could effectively reduce phosphorylation of Akt.

**Table 1. Xenotransplantation of human lung cancer A549 cells and its SFCs into Balb/c- nu immunodeficient mice**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Inoculum size</th>
<th>Tumor incidence</th>
<th>Latency period (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549 cells</td>
<td>1 × 10^4</td>
<td>0/4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1 × 10^5</td>
<td>0/4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1 × 10^6</td>
<td>2/4</td>
<td>31</td>
</tr>
<tr>
<td>SFCs</td>
<td>2 × 10^3</td>
<td>2/4</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>1 × 10^4</td>
<td>4/4</td>
<td>19</td>
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<tr>
<td></td>
<td>1 × 10^5</td>
<td>4/4</td>
<td>11</td>
</tr>
</tbody>
</table>

**Figure 2. Effects of casticin on LCSLCs**  Casticin inhibited proliferation (A) and reduced the size (B) and number (C) of SFC from A549 cells. Casticin suppressed the invasion of SFC from A549 cells (D) and (E). Casticin decreased MMP-9 activity (F). *P < 0.05 vs. 0.01% DMSO. PC, Parental cell.
Akt and the expression of stem cell markers CD44, CD133, and ALDH1 in SFCs from A549 cells (Fig. 3D), and significantly decrease the number of SFCs (Fig. 3E). Moreover, LY294002 could augment the down-regulation of p-Akt (Fig. 3F) and the number reduction of SFCs (Fig. 3G) induced by casticin. These results suggest that Akt plays an important role in maintaining stem cell features of LCSLCs, and the mechanisms underlying casticin suppressing LCSLCs are partly via the down-regulation of pAkt.

**Discussion**

We isolated the SFC subpopulation from human NCLSC A549 cells using a sphere-forming enrichment culture method with selective serum-free medium. The results showed that the SFCs could proliferate and form a new sphere, confirming that the SFC subpopulation possessed self-renewal capacity. Then we further discovered that the expression levels of CSC markers CD133, CD44, and ALDH1 in SFCs were much higher than that in parental cells. CD133, CD44, and ALDH1 are important CSC markers in sorting and identification of various types of CSCs, especially in lung cancer.

CD133 often serves as a stemness biomarker for CD133\(^+\) CSCs. CD133\(^+\) cells are related to the maintenance, metastasis and drug-resistance of lung cancer [9,26,27]. CD133\(^+\) cells from primary NSCLC tissue have higher tumorigenic potential in vivo than the CD133\(^-\) counterpart [28]. CD44 is also an important stemness biomarker in lung cancer. Recent studies have shown that CD44\(^+\) cells from NSCLC have differentiation potential in vivo, express the pluripotency genes OCT4/POU5F1, NANOG, and SOX2, and are more resistant to cisplatin treatment than CD44\(^-\) cells [29]. It has been demonstrated that the CD133\(^+\)/CD44\(^+\) cell subpopulation in A549 cells possesses the continuous proliferative capacity and differentiation potential [30]. ALDH1 has been reported to be over-expressed in NSCLC stem-like cells [12], and the ALDH1A1\(^+\) lung cancer cells are more resistant to EGFR tyrosine kinase inhibitor gefitinib than the ALDH1A1\(^-\) cells [13].

Our results showed that the sorted SFCs from A549 cells expressing higher levels of CD133, CD44, and ALDH1, had
stronger tumorigenic activity in vivo than the parental cells, which indicated that the SFCs, obtained from A549 cells via sphere-forming enrichment culture method, possess CSC characteristics and the LCSLCs are successfully enriched in it. This provided a substantial basis for the subsequent investigation of casticin on LCSLCs.

We further found that casticin could preferentially suppress the proliferation activity of LCSLCs, inhibit self-renewal, and invasion of LCSLCs, decrease MMP-9 activity, CD133, CD44, and ALDH1 expressions, and Akt phosphorylation. It has been confirmed that LCSLCs are more invasive than the parental cells [14]. MMP-9 is a crucial molecule in the process of invasion and metastasis of cancer [31]. Reduction of MMP-9 activity may contribute to inhibition of invasiveness of LCSLCs [25]. The Akt inhibitor LY294002 could suppress self-renewal and expression of stemness biomarkers CD133, CD44, and ALDH1 in LCSLCs, indicating that Akt may play an important role in maintaining stemness features of LCSLCs. Our results were in accordance with the findings obtained by Singh et al. They reveal that Akt inhibition could decrease self-renewal of LCSLCs from NSCLC via down-regulation of expression of Sox2 [32]. Many studies have shown that Akt pathway is critical for cancer stem-like cell maintenance in various types of cancers, and the molecular mechanisms may be correlated with phosphatase and tensin homolog deleted on chromosome ten (PTEN), singal transducer and activator of transcription 3 (Stat3), extracellular signal-regulated kinases1/2 (ERK1/2), ALDH1, Twist and so on [33–38]. In addition, it has been demonstrated that the inhibition of Akt pathway could suppress the expression of CD44 [36], and decrease MMP-9 activity, resulting in repression of cancer metastasis [39]. Our results also showed that Akt inhibitor could augment the inhibitory effects of casticin on LCSLCs, suggesting that other signaling pathways may also take part in this process. It is well known that Wnt, Notch, Hedgehog, TGF-β, etc. signaling pathways are also involved in proliferation and maintenance of CSCs [40]. Whether these important signaling pathways are involved needs further study.

In conclusion, our studies showed that the serum-free suspension sphere-forming culture method was efficient for defining and isolating the enriched stem-like cell population in NSCLC. The sorted SFCs from A549 possessed stemness features including self-renewal capacity, high invasiveness, elevated tumorigenic activity in vivo, and high expression of stemness markers CD133, CD44, and ALDH1, characterized as LCSLCs. Further studies demonstrated that casticin could suppress proliferation, self-renewal, and invasion of LCSLCs partly through down-regulation of pAkt, and Akt was confirmed to be a crucial signaling molecule in maintaining LCSLCs. The activity of casticin in suppressing proliferation of LCSLCs was much stronger than that in A549 parental cells. These data suggest that casticin is a candidate compound for curing lung cancer via eliminating CSCs.

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