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Original Research Article

Ophiopogonin B suppresses the growth and epithelial-mesenchymal transition in laryngeal cancer cells by inhibiting FAK/AKT signaling pathway

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Abstract

Purpose: *To investigate the effect of ophiopogonin B on laryngeal cancer cells, and whether it is related to epithelial mesenchymal transition (EMT).*
Methods: *Human laryngeal cancer cells (AMC-HN-8) were used as tool cells in vitro. Cell growth was characterized by cell viability and proliferation while cell apoptosis was analyzed using Annexin V/PI staining. Cell invasion and migration were assessed Transwell assay.*
Results: *Ophiopogonin B inhibited the viability and proliferation of AMC-HN-8 cells at a concentration > 10 μM. Cell apoptosis was enhanced after treatment with ophiopogonin B. The fraction of apoptotic cells for 5, 10 and 20 μM groups were 6.25, 16.16, 28.3 and 39 %, respectively. Transwell assay data showed that ophiopogonin B inhibited the invasion and migration of laryngeal cancer cells. In addition, the expression of N-cad and snail (EMT inducer) was inhibited, while the expression of E-cad was enhanced. These results also indicate that ophiopogonin B inhibited the migration and EMT of laryngeal cancer cells. Besides, ophiopogonin B inhibited the phosphorylation of FAK and AKT.*
Conclusion: *These results indicate that ophiopogonin B suppresses the growth, migration and EMT in laryngeal cancer cell by inhibiting FAK/AKT signaling pathway. These results provide some ideas for improved treatment of laryngeal cancer.*

Keywords: *Ophiopogonin B, Laryngeal cancer, Epithelial-mesenchymal transition (EMT), FAK/AKT signaling pathway*

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INTRODUCTION

Laryngeal cancer is one of the most common malignant otorhinolaryngologic tumors and it has with an increasing incidence of about 25% each year [1]. Epithelial-to-mesenchymal transition (EMT) is a crucial event in cancer invasion and metastasis [2]. Epithelial-mesenchymal transition (EMT) is a complex process characterized by the epithelial cells losing their typical features and acquiring a mesenchymal-like phenotype [3]. The invasion and metastasis are accompanied by changed expression of EMT-related proteins (vimentin, E-cadherin, N-cadherin).

Recent evidence indicates that EMT and epithelial reversion (mesenchymal-epithelial transition, MET) were dynamically inter-converted through variable intermediate-hybrid states [4]. In a sense, EMT is reversible. For example, van Kampen reported that silencing CDH1 repressors contributed to the non-invasive epithelial phenotype of PANC-1 cells [5]. Therefore, the EMT may be a potential therapeutic target in cancer therapy, including laryngeal cancer.

Despite the use of comprehensive treatments, such as surgical resection and chemoradiotherapy, the overall survival of laryngeal cancer patients has declined, with the survival of advanced laryngeal cancer less than 40 % [6]. Many Chinese herbal medicine and active components have been used to treat cancer for centuries [7].Ophiopogonin B, one of the main bioactive components of Radix *Ophiopogonjaponicas*, exerts anticancer effects on many tumors such as gastric and lung cancers [8,9]. Many reports indicate that Ophiopogonin B regulated multiple cancer-related signaling, including JNK/c-Jun, PI3K/AKT and ERK signaling pathways [10,11]. Moreover, Hu *et al* reported that Ophiopogonin B inhibited EMT in human lung cancer cells via the regulation of lincRNA [9]. However, the effects of ophiopogonin B on laryngeal cancer and the underlying mechanisms have not been elucidated. This aim of this research was to investigate the effect of ophiopogonin B, and its possible relationship with EMT.

EXPERIMENTAL

Cell culture

Human laryngeal cancer cells (AMC-HN-8), purchased from ATCC (Manassas, VA), were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C with 5 % CO_2.

Evaluation of cell growth

Cell growth was characterized by MTT assay based on cell viability while cell proliferation was assessed by EdU staining. Human laryngeal cancer cells (AMC-HN-8) cells were treated with ophiopogonin B at gradient concentration (0, 5, 10, and 20 μM). Then, the cells were incubated with MTT solution (0.5 mg/mL) for another 4 h at 37°C, and replaced by 150 μL of DMSO to dissolve formazan. Absorbance at 490 nm was measured on a microplate reader.

Cell proliferation was measured using Click-iT™ EdU Assay (Invitrogen) according to the manufactory suggestion. Four hours to the end of drug treatment, the cells were briefly incubated with 10 μM of EdU. Then, the cells were fixed and incubated with reaction cocktail, blocked with 200 μLBSA solution and incubated with 100 μL UltraRed reaction mixture for 15 min in the dark. The red fluorescence indicated the proliferation of the cells, and visualized under a fluorescence microscope.

Determination of apoptosis

Cell apoptosis was analyzed with Annexin V/PI staining using flow cytometry. Cells were collected and labelled using a commercial kit according to the manufacturer protocol, and analyzed with a FACScan flow cytometer. Living cells (Annexin V-FITC$^-$/PI$^-$, Q4), early apoptotic cells (Annexin V-FITC$^+$/PI$^-$, Q3), late apoptotic cells (Annexin V-FITC$^+$/PI$^+$, Q2), and necrotic cells (Annexin V-FITC$^-$/PI$^+$, Q1) were enumerated. The results were analyzed using FlowJo software.

Cell invasion and migration assay

Cell invasion ability was analyzed with polyethylene terpthalate filters (8 μM)as reported earlier[12]. The AMC-HN-8 cells were loaded in the upper chamber of Transwell and allowed to invade through matrigel-coated filters for 16 h. The cells which moved to the lower sides were stained with crystal violet, and counted under three randomly selected fields.

Cell migration was analyzed by Transwell assay using nuclepore filters (8 nm pore size, coated with type IV collagen). The cells were then added to the upper chambers in a serum-free medium, while a complete medium was added to the lower chamber as a chemoattractant. After 24 h, the cells that migrated to the lower side were stained with crystal violet. The migrated cells were then observed and photographed under an inverted microscope.

Western blot

Total protein was extracted with RIPA lysis and quantified using BCA method. Equal amounts of each protein sample were separated via gel electrophoresis and transferred to a PVDF membrane. After blocking with 5 % nonfat dry milk solution, the membrane was incubated with primary antibodies at 4 ℃ overnight and secondary antibodies at room temperature 2 h. The protein expression was detected by Quantity-one software using an ECL substrate, and normalized to β-actin as an internal control. The primary antibodies used were FAK (CST,

#3285S, 1:1000), p-FAK (CST, #8556S, 1:1000), Akt (CST, #9272S, 1:1000), p-Akt (CST, #9271S, 1:1000), E-cadherin (CST, #3195, 1:1000), N-cadherin (CST, #13116, 1:1000), snail (CST, #3879, 1:1000), β-actin (CST, #3700, 1:2000).

Statistical analysis

All data are shown as mean ± SD. Data were analyzed with Student's t-test for inter-group difference and One-Way ANOVA for multi-group difference. GraphPad Prism 5.0 was used for statistical analysis. Values of $p<0.05$ was considered as statistically significant.

RESULTS

Ophiopogonin B inhibited the growth of laryngeal cancer cells

The growth of laryngeal cancer cells (AMC-NH-8) were characterized with cell viability (MTT) and proliferation (Edu staining). The chemical structure of ophiopogonin B were shown in Figure 1 A. After treatment with gradient ophiopogonin B, cell viability was inhibited over 10μM ($p < 0.01$ vs. control) (Figure 1 B). The number of proliferated cells (red fluorescence) decreased in the 10-μM and 20-μM group (Figure 1 C), which was in accordance with cell viability. These results indicated that Ophiopogonin B inhibited the growth of AMC-NH-8 cells.

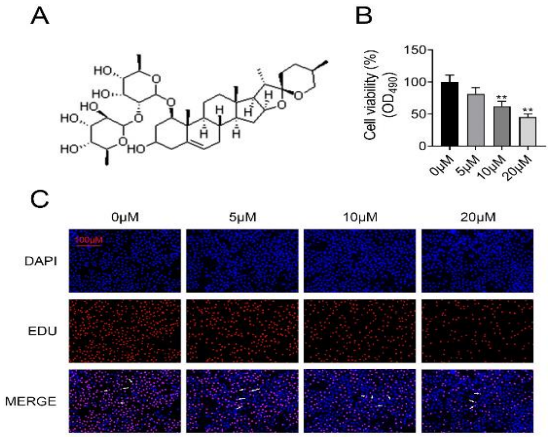

Figure 1: Ophiopogonin B inhibited the growth of laryngeal cancer cells. A. Chemical structure of ophiopogonin B. B. Cell viability of AMC-HN-8 treated with various concentrations of ophiopogonin B (0, 5, 10 and 20 μM) and assessed using MTT. C. Cell proliferation of AMC-HN-8 analyzed using EdU staining; Red fluorescence indicated the proliferating cells $^{**}p< 0.01$ vs. 0-μM group

Ophiopogonin B promoted the apoptosis of laryngeal cancer cells

Cell apoptosis was analyzed using Annexin V/PI staining. The fraction of apoptotic cells were enumerated as early apoptotic and late apoptotic cells (Q2+Q3). As shown in Figure 2, the amount of apoptotic cells in control group was 6.25 %, but after treatment with ophiopogonin B, the apoptotic cells increased significantly in a dose-dependent manner. The fractions of apoptotic cells for the 5, 10 and 20 μM groups were 16.16, 28.3 and 39 %, respectively. These results demonstrated that ophiopogonin B promoted the apoptosis of AMC-NH-8 cells.

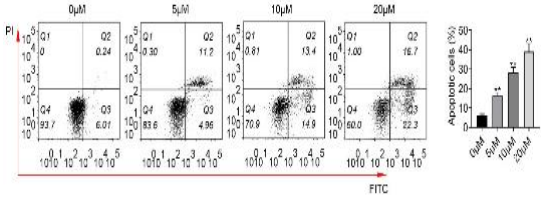

Figure 2: Ophiopogonin B promoted the apoptosis of laryngeal cancer cells. Representative spot figures of cell apoptosis analyzed using Annexin V/PI staining. Q1: necrotic cells (Annexin V-FITC$^-$/PI$^+$); Q2: late apoptotic cells (Annexin V-FITC$^+$/PI$^+$); Q3: early apoptotic cells (Annexin V-FITC$^+$/PI$^-$); Q4: living cells (Annexin V-FITC$^-$/PI$^-$)

Ophiopogonin B inhibited the migration and EMT of laryngeal cancer cells

To study the effects of ophiopogonin B on migratory and invasive properties of AMC-NH-8, Transwell migration and invasion assays were performed. As shown in Figure 3 A (migration) and Figure 3 B (invasion), the number of cells that migrated to, or invaded the lower side decreased significantly in a dose-dependent manner after treatment with ophiopogonin B. In particular, the number of migrated cells reduced by more than 50 % following treatment with 5 μM ophiopogonin B. In order to investigate the effects of ophiopogonin B on EMT, the expressions of N-Cadherin (N-cad), E-Cadherin (E-cad) and snail were analyzed using Western blot. As shown in Figure 3 C, ophiopogonin B inhibited the expression of N-cad and snail (an EMT inducer), but enhanced the expression of E-cad. These results indicated that ophiopogonin B inhibited the migration and EMT of laryngeal cancer cells.

Ophiopogonin B inhibited FAK/AKT signaling pathway

Since FAK-AKT signaling plays a critical role in cancer cell adhesion, migration and invasion,

further investigation was carried out on the phosphorylation of FAK at Y397 and Akt at S473. As shown in Figure 4, the phosphorylation of FAK and AKT were down-regulated after treatment with ophiopogonin B in a dose-dependent manner, confirming that ophiopogonin B partly inhibited EMT via the regulation of FAK/AKT signaling pathway.

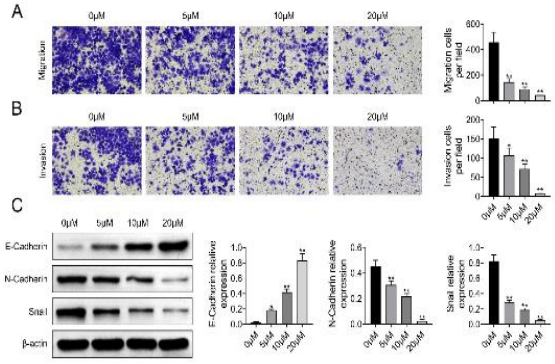

Figure 3: Ophiopogonin B inhibited the migration and EMT of laryngeal cancer cells. A. Representative imagesof migrated cells analyzed by Transwell migration assay. B. Representative images of invaded cells analyzed by transwell invasion assay. Cells were counted after staining with crystal violet. C. The expression of EMT related proteins analyzed using Western blot. *$P < 0.05$, **$p < 0.01$ vs. 0-μM group

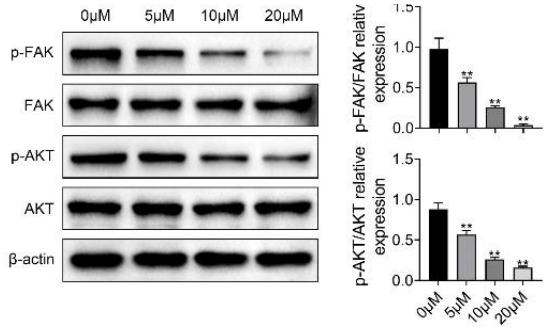

Figure 4: Ophiopogonin B inhibited the FAK/AKT signaling pathway. The expression of FAK/AKT related proteins as Western blot; **$p < 0.01$ vs. 0-μM group

DISCUSSION

Epithelial-to-mesenchymal transition (EMT) is a self-regulated physiological process that takes place during tissue repair. However, in abnormal conditions, it may lead to fibrosis, angiogenesis or cancer [4]. EMT is a critical process during cancer development. During EMT, cancer cells lose adhesion, acquire mesenchymal-like phenotype, become anchorage-independent growth types, and finally lead to migration, invasion, immunosuppression and drug resistance [13].A critical characteristic of EMT is the up-regulation of N-cad and the down-regulation of E-cad, which was called “cadherin switch” [14].

Mezi *et al* confirmed that EMT was an additional adverse feature in laryngeal cancer. They evaluated EMT in 50 patients with laryngeal carcinoma and found that cytoplasmic E-cad was associated with the low survival while N-cad and vimentin were associated with poor differentiation and tumor relapse [15]. Many natural plant extract and compounds such as resveratrol, oxymatrine and ligustrazine, are reported to possess the ability to regulate the pathological EMT through various cellular signal transduction pathways [16]. The present research indicates that ophiopogonin B inhibits the growth, invasion, migration and EMT of laryngeal cancer cells, which is in accordance with previous reports which showed that it suppressed EMT in lung adenocarcinoma cells *in vitro* [9]. Snail, a vital transcription factor, regulates EMT by inhibiting E-cadherin and activating genes associated with the mesenchymal phenotype [17]. The present study also demonstrated that ophiopogonin B decreased the expression of Snail, which further confirmed that it inhibited EMT.

Focal adhesion kinase (FAK), a cytoplasmic protein tyrosine kinase that mediates the signal transduction of integrins and other surface receptors, is a key regulator of cell adhesion, migration, proliferation and survival [18]. Canonical FAK signaling is activated through phosphorylation by a broad range of growth factors and chemokines, which further activate three major signaling pathways, including the PI3K-AKT pathway [19].

It has been reported that during laryngeal cancer, FAK and AKT are activated, and related with increased cell motility, cell survival and metastasis [20]. Previous studies reported that increased FAK/AKT signaling facilitated the progression of EMT [21]. Researchers confirmed that the deactivation of FAK could suppress the EMT of some cancer cells. For example, Nonpanya *et al* reported that ephemeranthol A (isolated from *Dendrobium infundibulum*) suppressed EMT and FAK/AKT signaling in non-small cell lung cancer cells H460 [22].This study shows also that ophiopogonin B inhibited the phosphorylation of FAK and AKT, which demonstrated that ophiopogonin B regulated the activation of FAK/AKT signaling pathway.

CONCLUSION

These results indicate that ophiopogonin B suppresses the growth, migration and EMT in laryngeal cancer cell by inhibiting FAK/AKT

signaling pathway. Thus, ophiopogonin B may be a inhibitor of laryngeal cancer progression, and thus can potentially be utilized for the development of suitable anticancer therapies.

DECLARATIONS

Conflict of Interest

There is no conflict of interest to disclose.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Rong Jie Hu designed the study and supervised the data collection; Chun Wu and Xiaoxiao Song analyzed and interpreted the data; and Ling Guo prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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