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Original Research Article

Inhibitory Effect of Polysaccharides from *Scutellaria barbata* D. Don on Invasion and Metastasis of 95-D Cells Lines via Regulation of C-MET and E-CAD Expressions

Xiao-Kun Yang[1,2], Ya-Dong Yang[1], Shu-Qian Tang[1], Ling Xu[1], Gui-Hong Yang[1], Qiao-Yu Xu[1], Hui Tang[1] and Jin-Jin Wu[1*]

[1] Department of Dermatology, Research Institute of Wound Healing and Tissue Engineering of Chongqing, Daping Hospital, The Third Military Medical University, Chongqing, 400042, [2]Department of Emergency, General Hospital of Chengdu Military Command Area, Chengdu 610083, Sichuan Province, PR China

For correspondence: wujjtmmu@126.com; **Tel: +86 023-68757599 ; **Fax :** +86 023-68757599*

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Abstract

Purpose: *To investigate the inhibitory effect of polysaccharides from Scutellaria barbata (PSB) on invasion and metastasis of lung cancer, and study the possible mechanism.*
Methods: *PSB was extracted with water and by alcohol precipitation, and purified by DEAE-52 column chromatography. A highly invasive and metastatic lung carcinoma cell, 95-D cell line, was used for the study. Cell adhesion and invasion assays in vitro were performed to evaluate the anti-invasive and anti-metastatic effects of PSB (50 - 200 μg/ml) on 95-D cell. Immunocytochemical staining and Western blot techniques were employed to study the regulatory effects of PSB on the expression of C-MET and E-CAD.*
Results: *The results indicate that PSB significantly inhibited cell invasion and migration of 95-D in a concentration-dependent manner ($p < 0.05$). The adhesion of 95-D cells to fibronectin was also inhibited by PSB ($p < 0.05$). The expression of C-MET and E-CAD in 95-D cells treated with PSB were significantly down-regulated and up-regulated, respectivelt ($p < 0.05$).*
Conclusion: *Treatment with PSB can significantly inhibit the invasion and metastasis of 95-D cells in vitro, probably through the regulation of C-MET and E-CAD.*

Keywords: *Polysaccharide, Scutellaria barbata, 95-D cell lines, Invasion, Metastasis*

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INTRODUCTION

Lung carcinoma is one of the leading causes of cancer deaths in both men and women worldwide [1-2]. Lung cancers are classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC constitutes approximately 75 % of lung cancer cases. It is well known that lung cancers are associated with a substantial risk of invasion and metastasis [3-4]. Tobacco smoke is the predominant etiologic risk factor for lung cancers. In addition, polycyclic aromatic hydrocarbons are reported to be the major causative agents for development of lung cancers among cigarette smoke components [5]. Although improvements have been made in diagnosis and treatment of lung cancers, it remains an aggressive cancer with a poor prognosis, and tissue invasion and metastasis are one of the primary causes of the mortality of lung cancers patients [6]. In recent years, more and more people have begun to recognize traditional Chinese medicine (TCM) as a potential source of new anti-cancer drugs [7, 8] and some studies have isolated novel

compounds with therapeutic activities from TCMs [9, 10].

Scutellaria barbata D. Don, a perennial herb belonging to the family Lamiaceae, is widely distributed throughout China and Korea. It has been traditionally used in folk medicine as anti-inflammatory and anti-tumor agents [11, 12]. *S. barbata* is known to contain a large number of polysaccharides, alkaloids, flavones, organic acid, and neo-clerodane diterpenoids [13-15]. However, there has been no report regarding the inhibitory effect of PSB on invasion and metastasis of lung cancers thus far. The present investigation is aimed ay achieving this as well as elucidate the possible mechanism involved.

EXPERIMENTAL

Plant material

Scutellaria barbata was purchased from the Hehuachi Market of Traditional Chinese Herbs and identified by Professor Yong Chen of Chengdu Academy of the Chinese Materia Medica (Chengdu, China). A voucher specimen of this herb (S-2011-0901) was kept in the institutional herbarium.

Cell lines

The highly invasive and metastatic lung carcinoma cell line, human 95-D Human cells was obtained from Chinese Academy of Science Cellbank (CAS, Shanghai, China). Cells were cultured in RPMI 1640 medium supplemented with 15 %(v/v) heat-inactivated fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified and 5 % CO_2 atmosphere.

Chemicals

Silica-gel was purchased from Qingdao Haiyang Chemical Co, Ltd. (Qingdao, China), DEAE-52 was purchased from Whatman Ltd. (Maidstone, UK). The RPMI 1640 media and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, USA). Transwell well culture chambers were purchased from Corning (New York, USA). Matrigel was obtained from Collaborative Research Inc. (Bedford, USA). Fibronectin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, USA). All other chemicals used in this study were of analytical reagent grade.

Isolation of polysaccharides from *S. barbata*

PSB was prepared by the method described previously by Song *et al* [16] with minor modification. In brief, the crude polysaccharides were extracted by the traditional technique of water extraction and alcohol precipitation. The dried and crushed *S. barbata* was extracted four times (each extraction period lasting 2 h) with deionized water by decoction at 90 °C. After filtration, the resulting extract was mixed with 4 volumes of dehydrated ethanol (ethanol final concentration, 80 %) and kept overnight at 4 °C in a refrigerator. Thereafter, the mixture was centrifuged at 4000 rpm/min for 10 min, washed 4 times with dehydrated ethanol, and the precipitate was collected as crude PSB. Subsequently, DEAE-52 column chromatography was used to purify the crude PSB with NaCl solution (0~2 mol/l).

MTT assay

The viability of cells was monitored after various concentrations of PSB treatment. MTT assay was carried out using standard protocol and optical density (OD) was read at 570 nm according to the method of previous reported [17]. Assays were performed in triplicate on three independent experiments.

In vitro invasion assay

The invasive activity of the 95-D cells was evaluated in a Transwell cell culture chamber using a method described previously [18]. Cell invasion assays were performed using 8.0 μm pore size Transwell well culture chambers which were coated with 500 μg/ml of Matrigel. The coated filters were washed thoroughly in PBS and dried immediately before use. Ten percent FBS-RPMI 1640 was placed in the lower chamber, and 95-D cells (2 ×10^5/chamber) in RPMI 1640 were placed in the upper chamber. The PSB solution was added to the upper chamber and incubated for 12 h at 37 °C in 5 % CO_2. The number of the invaded cells through Matrigel-coated PVPF filter was measured by counting cells stained with 0.2 % crystal violet solution.

Wound-healing assay

Wound-healing assay was carried out using the method described previously [19]. 95-D cells were cultured in 6-well plates, and a sterile pipette (200 μL) was used to scratch the cells to form a wound. Cells were washed once with cold 1× PBS buffer, and treated with different

concentrations of PSB for 48 h. Migration of the cells was evaluated at 48 h.

Cell adhesion assay

Cell attachment assay was carried out in 96-well plates according the method described previously [20]. The wells were precoated with 50 µl of 5 µg /ml fibronectin overnight at room temperature and blocked with 0.2 ml of RPMI 1640/well containing 3 % BSA for 1 h at 37 °C. They were then re-suspended in RPMI 1640 containing 0.1 % BSA, added (5×10^5 /ml, 0.2 ml/well) to each well and the PSB was added. This suspension was incubated at 37 °C for 1 h. The wells were washed twice with warm PBS to remove the unattached cells, and the attached cells were then stained with a 0.2 % crystal violet aqueous solution in 20 % methanol for 10 min. Once stained, the cells were dissolved in 200 µl of a 1 % sodium dodecyl sulfate (SDS) solution, and the optical density was measured at 560 nm using a microplate reader.

Western blot test

C-MET and E-CAD expressions were examined by western blotting. The 95-D cells were treated with different concentrations of PSB for 72 h. Then, cells were harvested in lysis buffer after treated with different concentrations of PSB and homogenized by sonification. Then, equal amounts of protein (40 µg) were separated by sodium dodecyl sulfate/polyacrylamidegel electrophoresis (SDS/PAGE) on 8 % gels, blotted on polyvinylidene difluoride (PVDF), and probed with anti-E Cadherin and anti-C Met rabbit monoclonal antibody (Abcam Biotechnology, UK) and subsequently with goat-anti-rabbit (HRP), and detected by chemiluminescence. To measure protein loading, antibodies directed against β-actin were used.

Immuno-cytochemical staining

Immuno-cytochemical staining assay was performed according to the method described on the commercial kits to examine the expressions of C-MET and E-CAD (Abcam Biotechnology, UK).

Statistical analysis

All the results are expressed as Mean ± SD. The statistical significance of differences was analyzed using SPSS software (SPSS for Windows 16.0, USA). The significance of the mean difference was determined by one-way ANOVA, followed by a LSD-t test for multi group comparisons. Probability values (p) < 0.05 were considered significant.

RESULTS

Cytotoxic effect of PSB on the 95-D cells

MTT assay was carried out to select a suitable concentration to investigate the inhibitory effect of PSB on cell invasion and metastasis. As shown in Fig. 1, PSB at the concentrations > 300 µg /ml showed slight effect on the survival of cells. On the other hand, PSB had no inhibitory effect on the growth of 95-D cells after 24 h incubation at concentrations of 50 to 200 mg/ml, which was used in the present study.

In vitro invasion and wound healing

PSB effectively inhibited the cell invasion of 95-D in a dose-dependent manner, and an approximately 60 % reduction was achieved at 200 µg /ml (Fig. 2a). In addition, wound healing rates in the 95-D cells treated with different doses of PSB were reduced, in a dose-dependent manner (Fig 2b).

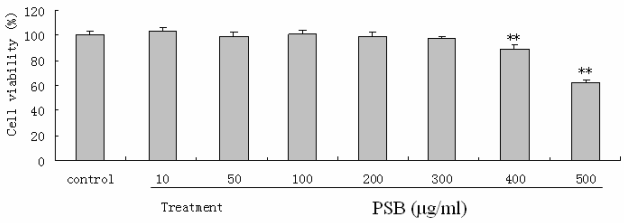

Figure 1: Viability of 95-D cells treated with PSB at different concentrations. Data are mean ± SD (n = 3); *p < 0.05, **p < 0.01.

Cell adhesion

The PSB inhibited the adhesion of 95-D cells to the fibronectin in a concentration-dependent manner, and an approximately 50 % reduction was achieved at 200 µg /ml (Fig 3).

Effect of PSB on expressions of C-MET and E-CAD

Expressions of C-MET of the 95-D cells treated with PSB were down-regulated, in a concentration-dependent manner, compared with control group (Figs 4 and 5). However, the expressions of E-CAD in the 95-D cells treated with PSB were significantly up-regulated, in a concentration-dependent manner, compared to control group (Figs 4 and 6).

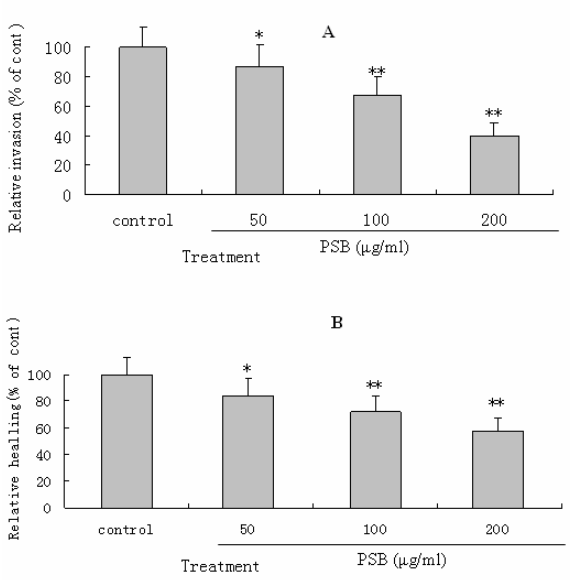

Figure 2: *In vitro* invasion *and wound healing*: (A) 95-D cells treated with different concentrations of PSB for 12 h, and cells invaded at the lower surface counted; (B) 95-D cells treated with different concentrations of PSB for 24 h, and migration of the cells evaluated after 48 h. Data are expressed as % of control, and as mean ± SD (n = 3); *$p < 0.05$, **$p < 0.01$

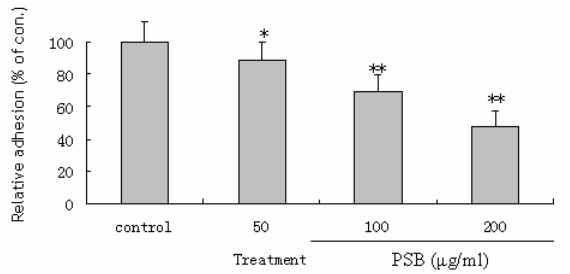

Figure 3: Cell adhesion following treatment. Cell adhesion is expressed as % of control, and as mean ± SD (n = 3); *$p < 0.05$, **$p < 0.01$

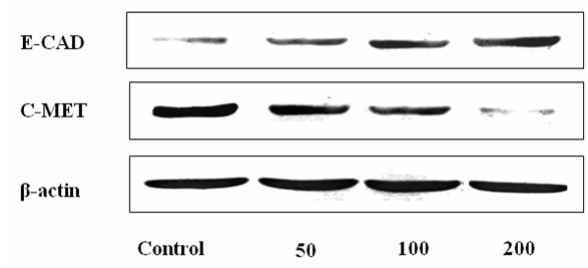

Figure 4: Effect of PSB on expression of C-MET and E-CAD.

DISCUSSION

Traditional Chinese medicines (TCM) have been used for more than millennium in China to prevent and alleviate a wide variety of diseases. Some anti-invasive and anti-metastatic agents for treatment of cancer have been derived from TCM [21]. In the present study, we report the inhibitory effect of PSB on invasion and metastasis of lung cancers cell lines for the first time.

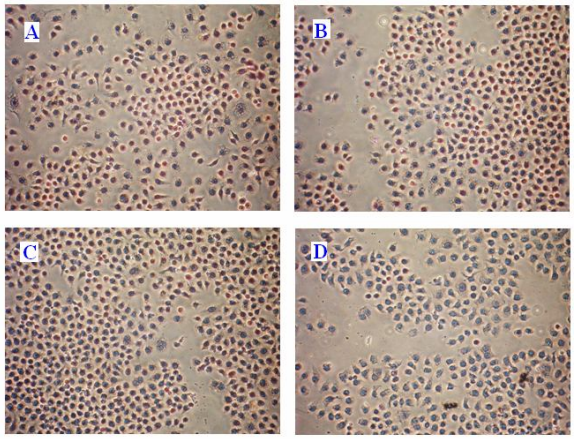

Figure 5: Expression of C-MET as observed by immunocytochemical staining; 95-D cells were treated with different concentrations of PSB for 72 h. The analyzed sections stained for anti-C Met rabbit monoclonal antibody (brown). A, B, C and D represent control, and PSB concentrations of 50, 100, 200 μg/ml, respectively (Magnification, ×200)

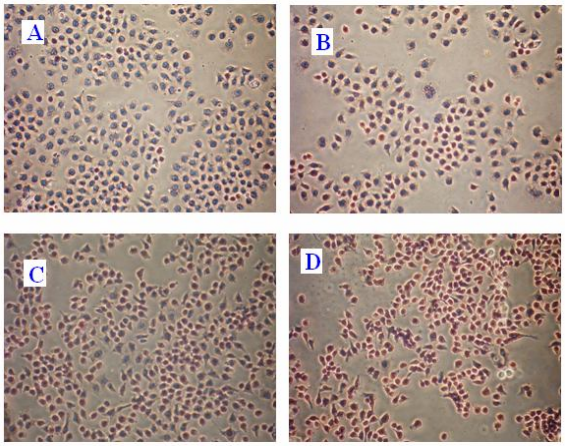

Figure 6: Expression of E-CAD as observed by immunocytochemical staining. The 95-D cells were treated with different concentrations of PSB for 72 h. The analyzed sections stained for anti-E Cadherin rabbit monoclonal antibody (brown). A, B, C and D represent control, and PSB concentrations of 50, 100, 200 μg/ml, respectively (Magnification, ×200)

Tumor cells invasion to the extracellular matrix (ECM) is an important step in the process of tumor metastasis [22], and the ECM were composited with basement membrane (BM) and intercellular substance. This results of our present study demonstrated that PSB inhibits the invasion of 95-D cells to the BM *in vitro*. In addition, tumor cell adhesion to the ECM is another key step in metastasis formation of tumor. Previous studies have demonstrated that alterations in the adhesive properties of tumor cells correlate with progression to tumor malignancy; additionally, the increasing

evidences now indicate that an aberrant tumor cell adhesion is causally involved in tumor progression and metastasis [22, 22]. In our present study, we demonstrated that the PSB significantly inhibited the adhesion of 95-D cells to the fibronectin, in a concentration-dependent manner. Additionally, the results of MTT assay suggested that the inhibitions of 95-D cell adhesion, invasion and migration are not due to the cytotoxicity of PSB.

Tumor invasion and metastasis are the hallmarks of tumor malignancy, frequently coincide with the loss of E-cadherin (E-CAD). E-CAD is a calcium-dependent membrane glycoprotein that has important effects on the maintenance of cell-cell adhesion, preservation of epithelial tissue polarity, and structural integrity [22,20,24]. E-CAD expression is commonly down-regulated in a wide variety of human malignancies, such as lung cancers, skin cancer, oral squamous carcinoma, and reduced E-CAD expression has been shown to be an indicator of unfavorable prognosis in malignancies [25].

C-MET is a transmembrane tyrosine kinase receptor that mediates the oncogenic activities of the hepatocyte growth factor (HGF). Overexpression of C-MET has been shown to contribute to progression and dissemination of several malignancies including lung cancers [25, 26]. Previous studies have indicated that the inverse correlation between the expression of HGF/C-MET and membrane E-CAD expression in tumors [26,27], suggesting that these proteins may be useful markers of malignant transformation. In the results of our present study, we found that the expressions of C-MET by 95-D cells treated with PSB were down-regulated, while, expressions of E-CAD of 95-D cells treated with PSB were significantly up-regulated. Therefore, inhibition of 95-D cell adhesion and invasion is possibly due to the regulation of C-MET and E-CAD expressions by PSB.

CONCLUSION

Treatment with PSB can significantly inhibit the invasion and metastasis of 95-D cells lines *in vitro*, due probably to the regulation of C-MET and E-CAD by PSB. Consequently, PSB might be useful for the treatment of lung cancers.

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