

Group IIa secretory phospholipase A2 (sPLA2IIa) and progression in patients with lung cancer

M. WANG^{1,2}, F.-Y. HAO³, J.-G. WANG³, W. XIAO¹

¹Respiratory Medicine, Oilu Hospital, Shandong University, Jinan, China

²Respiratory Medicine, the Affiliated Hospital of QingDao University, QingDao, China

³Pathology, the Affiliated Hospital of QingDao University, QingDao, China

Abstract. – OBJECTIVE: Group IIa secretory phospholipase A2 (sPLA2 IIa) plays a role in the malignant potential of several epithelial cancers. It is overexpressed in many cancer specimens and its elevated levels are correlated with high tumor grade and metastasis. Here, we evaluate the clinical significance of sPLA2 IIa in lung adenocarcinoma and the role of sPLA2 IIa in the process of cancer cell invasion and metastasis.

PATIENTS AND METHODS: Immunohistochemistry was used to investigate sPLA2 IIa in surgically resected lung adenocarcinoma of 180 patients and its correlation with survival. We overexpressed sPLA2 IIa in a lung adenocarcinoma cell line with very low sPLA2 IIa levels and investigated the *in vitro* and *in vivo* effects of sPLA2 IIa expression.

RESULTS: High expression of sPLA2 IIa in lung cancer tissue was significantly associated with clinical stage, metastasis, postoperative relapse and shorter patient survival. The overexpression of sPLA2 IIa enhanced xenograft tumor growth and invasion *in vitro*.

CONCLUSIONS: sPLA2 IIa expression can predict the clinical outcome of lung adenocarcinoma patients. sPLA2 IIa is a novel invasion-promoting gene in lung adenocarcinoma.

Key Words:

Lung adenocarcinoma, Group IIa secretory phospholipase A2, Immunohistochemistry, Prognosis.

highly sophisticated process of metastasis². Current clinical means cannot accurately identify those patients who will develop metastasis. To develop effective new strategies for the prediction, diagnosis, and treatment of metastasis of lung cancer, molecular mechanisms controlling metastasis must be identified³.

Phospholipase A2 (PLA2) enzymes release arachidonic acid from cell membranes, initiating downstream production of tumor-promoting eicosanoids. Group IIa secretory phospholipase A2 (sPLA2IIa), a subgroup of PLA2s, has recently been identified as a potential biomarker for small and large intestine cancer⁴ and prostate cancer⁵. In a cohort of patients with solitary pulmonary nodules, patients with lung cancer had higher plasma levels of sPLA2IIa than patients with a benign nodule. In addition, histologic analysis demonstrates overexpression of sPLA2IIa mRNA by RT-PCR assay in lung tumors compared with normal lung tissue⁶. From a functional perspective, sPLA2IIa has been shown to regulate lung cancer cell growth and invasion⁶. RNA interference of sPLA2IIa decreases tumor growth in a xenograft model of lung cancer⁷. The aim of this study was to evaluate the clinical significance of sPLA2IIa expression by immunohistochemistry assay in lung adenocarcinoma patients and evaluate the role of sPLA2IIa overexpression on cancer cell invasion and metastasis.

Introduction

Lung cancer is the leading cause of cancer death worldwide. Metastasis is the most common cause of death in lung cancer patients and is a major obstacle to the successful treatment. The spread of tumor cells from a primary tumor to the secondary sites within the body is a complicated process involving cell proliferation and migration, degradation of basement membrane, invasion, adhesion, and angiogenesis (1). A variety of positive and negative factors are involved in this

Patients and Methods

Patients and Tissue Collection

Surgically resected lung adenocarcinoma tissues (n= 180) and specimens of normal lung adjacent to the tumor tissues (n= 50) were obtained from People's hospital of Linyi, Shandong from the 1st January 2004 to the 31st December 2013.

None of the patients had been treated with any preoperative chemotherapy or radiotherapy. The pathologic stages were determined according to the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification system for malignant tumors. To further explore, the extent of differentiation and histological type was determined according to the World Health Organization (WHO) classification for non-small cell lung cancer (NSCLC). After surgical resection, all patients received standard therapies according to the 2004 NCCN Clinical Practice Guidelines in Oncology for NSCLC. Written informed consent was obtained from each patient, and Institutional Review Board approval of this study was obtained from People's hospital of Linyi, Shandong.

Immunohistochemistry

Formalin-fixed paraffin-embedded specimens were cut into 4 μ m-thick sequential sections. After dewaxing in xylene and rehydrating stepwise in ethanol, antigen retrieval was carried out using 0.01 mol/L of citrate buffer (pH 6.0) for 2 min in an autoclave. Immunostaining was performed by the streptavidin-peroxidase (S-P) method (Ultra-sensitive™ MaiXin, Fuzhou, China). Hydrogen peroxide (3%) was applied to block endogenous peroxidase activity, and normal goat serum was used to reduce nonspecific binding. Then sections were incubated with rabbit anti-sPLA2IIa (ab23 705, 1:200; Abcam, Cambridge, MA, USA) antibody (1:200 dilution) overnight at 4°C. The peroxidase reaction was developed with DAB (Maixin Biotechnology, Fuzhou, China), and sections were counterstained with hematoxylin, dehydrated through alcohol, and mounted using a standard procedure.

Semiquantitative Immunohistochemical Scoring

All tissue samples were assessed in a consecutive analysis to ensure maximal internal consistency. The analysis was assessed according to both the percentage of positive cells of cytoplasmic reactivity. Each histological section was examined at X40 to identify areas of maximum tumour staining. At X200, cells were analyzed from five areas of maximum tumour staining from each case and the average percentage of positive cells was recorded. In the statistical analysis, < 10% staining were considered negative; $\geq$ 10% and above were considered positive staining.

Cell Culture

A549 cell line was purchased from ATCC, and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 units/ml streptomycin, and 100 units/ml penicillin in a humidified 5% v/v CO₂ atmosphere.

Subcloning of Human sPLA2-IIa cDNA and Construction of Expression Plasmids

The full coding region of human sPLA2-IIa was amplified by PCR using primers 5'-ACA-GATCCAGGGAGCATTCA-3' and 5'-CTCA-GAGGACTCCAGAGTTG-3' from cDNA of human lung adenocarcinoma. The PCR product was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Isolated DNA sequences were determined using a cycle sequencing procedure.

Gene Transfection

pcDNA3.1-sPLA2-IIa or pcDNA3.1 plasmid was transfected into 70% confluent A549 cells with Lipofect 2000 method (Invitrogen). Gentamicin (G418; Invitrogen) was added to 500 μ g/mL for the selection of stable transfectants. High-sPLA2-IIa overexpression clone were selected. The mock vector (pcDNA3.1)-transfected cells were used in bulk for the control.

Cell Growth Inhibition Studies by MTT Assay

The Mock, control and sPLA2-IIa transfectant were seeded at a density of 1×10^3 /well in 96-well culture dishes. After 24 h, the cells were incubated with MTT (0.5 mg/ml; Sigma, St Louis, MO, USA) at 37°C for 4 h and then with DMSO at room temperature for 1 h. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan, Durham, NC, USA) at 495 nm.

In Vitro Cell Invasion and Migration Assay

In vitro Matrigel invasion assays were done using 6.5 mm Costar transwell chambers (8- μ m pore size; Corning, NY, USA). The Transwell filters were coated with appropriate Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). After the Matrigel solidified at 37°C, 1×10^5 cells were seeded onto the Matrigel. After 24-hour incubation, the filter was gently removed from the chamber and the noninvasive cells on the upper surface were removed by wiping with a cotton

swab. The cells that invaded the Matrigel and attached to the lower surface of the filter were fixed with methanol and stained with Giemsa solution. The number of cells attached to the lower surface of the polycarbonate filter was counted at $\times 400$ magnification under a light microscope. Each type of cell was assayed in triplicate.

Cell motility was assessed using a scratch wound assay. The cells were seeded into six-well tissue culture dishes at a concentration of 1×10^6 and cultured in medium containing 10% fetal bovine serum (FBS) to nearly confluent cell monolayers, which were then carefully wounded using a cell scraper. Any cellular debris was removed by washing with phosphate buffered saline (PBS). After making wounds, the cultures were incubated at 37°C and photographed immediately ($t = 0$) and 24 hours later. Migration was evaluated by the number of cell migration into the cell-free zone. The experiments were repeated in quadruplicate wells thrice.

Western Blot

Western blotting was performed using the following antibodies and dilutions: 1: 1,000 sPLA2-IIa and 1:1,000 β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total proteins were extracted from NSCLC tumor tissues and transfected cultured cells and then qualified using a protein extraction kit and the BCA Protein Assay reagent (Thermo Scientific, Rockford, IL, USA). The proteins were separated by SDS-PAGE and visualized by immunoblotting.

Tumorigenicity in Severe Combined Immunodeficient Mice

Six-week old SCID mice were housed in an isolator and ad libitum fed with autoclaved food. For tumor growth in animals, cancer cells were trypsinized, washed, centrifuged, and resuspended in Hank's Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA, USA).

A total volume of 0.2 mL containing 5×10^6 cells (sPLA2-IIa transfectant) was s.c. injected on the back side of each animal. A total of 18 mice (three groups: Mock, control and sPLA2-IIa groups) were studied. Injected mice were examined every 7 days for tumor appearance and tumor volumes were estimated from the length (a) and width (b) of the tumors using the formula: $V = ab^2 / 2$. After 49 days, animals were sacrificed, and tumors were confirmed by histologic examination. For histologic examination, tissues were fixed in PBS/10% formalin and embedded in

paraffin. From each paraffin block, three consecutive sections were cut, which were stained with H&E, anti-sPLA2-IIa antibody. Immunohistochemical staining was carried out as above.

Statistical Analysis

Where appropriate, the data are presented as the mean $\pm$ SD. All statistical analyses were done with SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA). Continuous data were compared using Student's t test. A standard chi-square test was performed to assess the association between sPLA2IIa expression and clinicopathological parameters, except for age, which was assessed by Student's t -test. Survival curves were estimated by the Kaplan-Meier method and compared with the log-rank test. Multivariate analysis was performed using the Cox regression model (backward, stepwise) to assess whether a factor was an independent predictor of disease-free survival (DFS). Hazard ratios (HRs) with 95% confidence intervals (CIs) were estimated. A two-tailed p -value of < 0.05 was considered statistically significant.

Results

sPLA2-IIa Expression and Clinicopathological Parameters

sPLA2-IIa was detected in cytoplasm by immunohistochemical staining. Positive sPLA2-IIa expression was 40% (72/180) in lung cancer tissues (Figure 1A), which was significantly higher than that in corresponding nontumorous lung tissues 0% (0/50) (Figure 1B). The relationship between sPLA2-IIa expression and different clinicopathological factors was shown in Table I. The overexpression of sPLA2-IIa in tumors of stage III-IV 66.6% (48/72) was higher than that in stage I-II 22.2% (24/108, $p < 0.05$). It was also higher in cases with lymphatic metastasis 59.7% (37/62) than in cases without lymphatic metastasis 29.6% (35/118) ($p < 0.05$). There were no differences in age, gender, and differentiation between the two groups (Table I).

Expression of sPLA2-IIa was Associated with Poor Prognosis of Lung Adenocarcinoma Patients

The median duration to postoperative recurrence was shorter in the sPLA2-IIa positive expression group (10.4 months; 95% confidence interval, 8.2-12.9 months) than in the sPLA2-IIa negative expression group (25.0 months; 95%

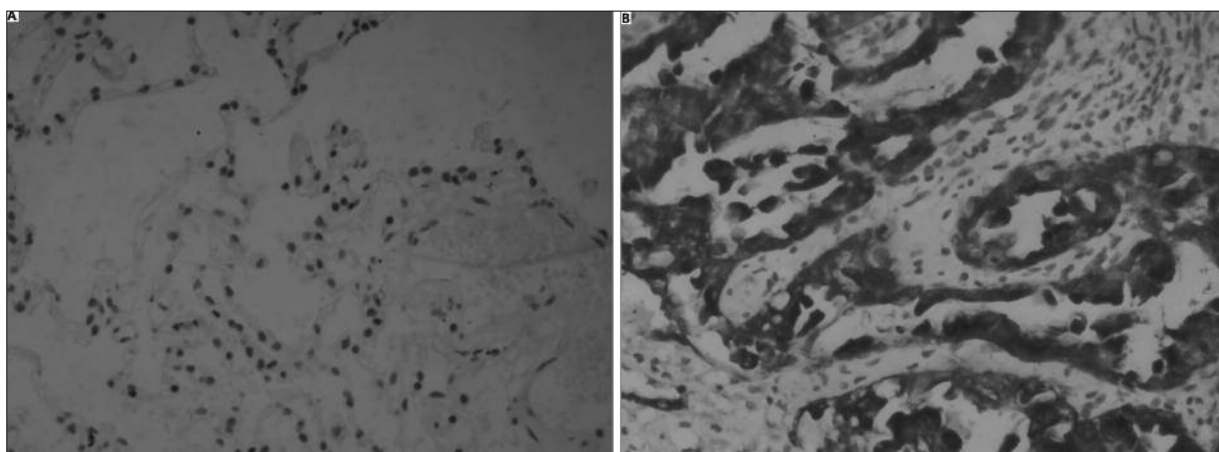


Figure 1. Immunohistochemical staining of sPLA2-IIa in non-tumorous tissues and NSCLCs. **A**, sPLA2-IIa was negative expressed in non-tumorous lung tissues. **B**, sPLA2-IIa was strongly expressed cancer tissues ($\times 200$).

confidence interval, 0-48.4 months; $p = 0.023$, log-rank test; Figure 2A). The sPLA2-IIa positive expression (median survival, 13.2 months; 95% confidence interval, 4.2-23.8 months) had a significantly shorter survival than the sPLA2-IIa positive expression (median survival, 39.7 months; $p < 0.001$, log-rank test; Figure 2B).

Univariate analysis showed that sPLA2-IIa expression, disease stage, tumor status, nodal status, adjuvant chemotherapy and/or radiotherapy, and age were prognostic factors for relapse and survival. Multivariate analysis using the Cox re-

gression model, sPLA2-IIa expression ($p = 0.01$), stage of disease ($p = 0.02$), and age ($p = 0.01$) were the significant prognostic factors for survival, whereas sPLA2-IIa expression ($p = 0.001$) and stage of disease ($p < 0.001$) were significant factors for predicting recurrence.

Overexpression of sPLA2-IIa Promotes In Vitro Carcinoma Cells Invasion

sPLA2-IIa-transfected A549 clones expressed higher levels of sPLA2-IIa protein (Figure 3A) than the control clone (mock). We used an *in vit-*

Table I. Clinicopathologic characteristics of tumors with positive and negative sPLA2-IIa expression.

Features	Cases (n)	sPLA2-IIa expression		p value
		Positive (+)	Negative (-)	
Sex				NS
Male	129	44	85	
Female	51	28	23	
Age (y)				NS
< 60	77	25	52	
≥ 60	103	47	56	
Stage				0.025
I-II	108	24	84	
III-IV	72	48	24	
Differentiation				0.068
Well	40	14	26	
Moderate	88	23	65	
Poor	52	35	17	
Nodal status				0.001
N0	118	35	83	
N1-2	62	37	25	
Adjuvant chemotherapy and/or radiotherapy		NS.		
Positive	74	27	47	
Negative	106	45	61	

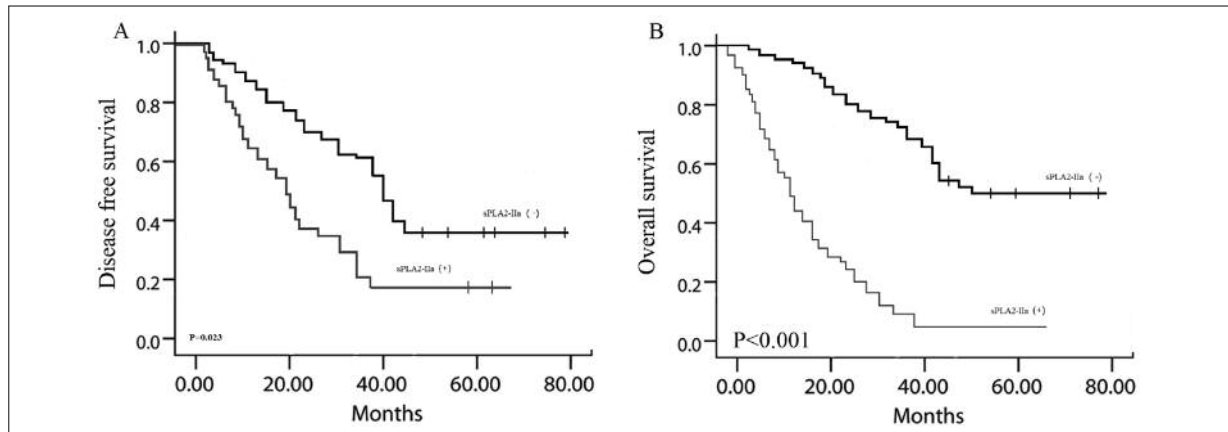


Figure 2. Kaplan-Meier survival plots of disease-free survival (**A**) and overall survival (**B**) for lung adenocarcinoma patients grouped according to sPLA2-IIa expression. There was a significant difference in disease-free survival ($p = 0.02$) and overall survival ($p < 0.001$) between patients with positive and negative expression of sPLA2-IIa.

in vitro-reconstituted basement membrane invasion assay to investigate whether sPLA2-IIa expression affected the invasive activity of A549 cells. After a 24-hour incubation, we noted a statistically significant increase in the invasive activity of sPLA2-IIa-transfected A549 clones ($p = 0.02$) than the control clone (Figure 3B).

Expression of sPLA2-IIa Does Not Affect Cancer Cell Migration and Proliferation In Vitro

To examine whether the sPLA2-IIa invasion promoting potential is associated with its promoting on the cell motility, the effects of sPLA2-IIa on the migration capability of cells was ana-

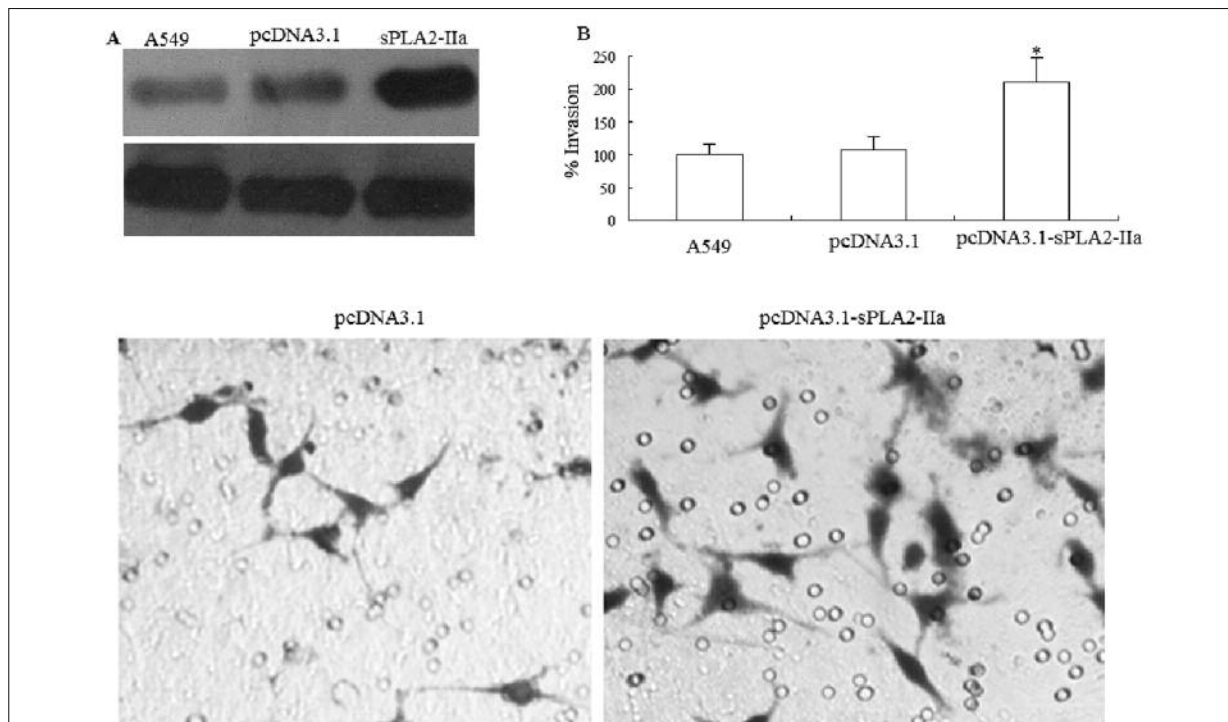


Figure 3. Overexpression of sPLA2-IIa promotes *in vitro* carcinoma cells invasion. **A**, Western blot revealed that sPLA2-IIa-transfected A549 clone had higher expression of sPLA2-IIa than mock transfected and parental A549. **B**, sPLA2-IIa had increased *in vitro* invasion activity compared with the mock transfected and parental A549. Vs control or mock, * $p < 0.05$.

lyzed. In the standard scratch wound assay, confluent monolayers of mock, sPLA2-IIa transfected cells were scratch wounded using a cell scraper. We noted that there was no significant difference in the migration capability of these cells (data not shown). There was no significant difference in the cell proliferation rates between mock and sPLA2-IIa transfected cells as shown by MTT assay (data not shown).

sPLA2-IIa Promotes *In Vivo* Tumor Growth

We next investigated the effect of sPLA2-IIa expression on tumorigenicity *in vivo*. Overexpression of sPLA2-IIa resulted in a marked increase of the growth of tumors formed by lung cancer cell in SCID mice (Figure 4A). Our results showed that mock transfectant failed to develop tumors in mice (6 of 6) 4 weeks after inoculation, whereas sPLA2-IIa transfected developed tumors (6 of 6) larger than 1 cm³ within 4 weeks. After 7 weeks, the tumor sizes from the mock transfectants were < 1 cm³, whereas the tumor sizes from the sPLA2-IIa transfected reached 2.8 cm³. It is evident that sPLA2-IIa expression promotes the tumorigenicity *in vivo*. However, no lung or liver metastasis by microscopic examinations was noted while SCID mice were scarified on week 7.

Discussion

The present study is the first to systematically assess the expression of group IIA PLA2 in human lung adenocarcinoma, as related to clinical data and disease outcome. In our series, normal

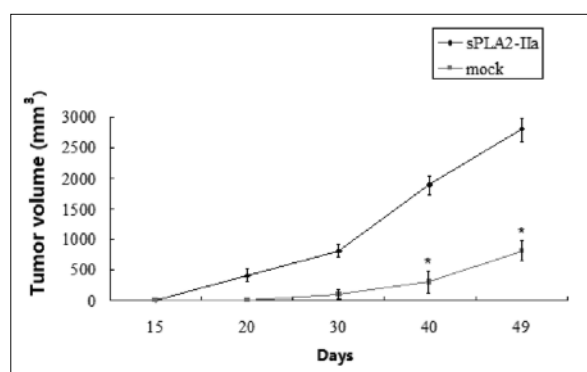


Figure 4. sPLA2-IIa promoted *in vivo* tumor growth. Tumor development in SCID mice of sPLA2-IIa was much faster and larger than the mock transfectant. Bars, SD. Vs mock, * $p < 0.05$.

lung tissue did not show any expression of sPLA2-IIa. However, 40% of the 180 carcinomas showed positive epithelial cytoplasmic sPLA2-IIa expression.

Tumor biomarkers with prognostic and predictive (theragnostic) power will aid in the selection of optimal pharmacologic therapies. In our study, interesting correlations were found between the sPLA2-IIa expression and some important clinicopathological parameters. First, a positive association was shown between sPLA2-IIa and tumor stage, high expression of sPLA2-IIa being more common among high stage tumors. Second, positive association was shown between sPLA2-IIa and nodal status, high expression of sPLA2-IIa being more common among metastatic tumors. We also found high sPLA2-IIa expression in lung adenocarcinoma patients can predict shortened survival. Results of sPLA2-IIa protein expression can predict overall survival of patients with lung adenocarcinoma in this study are consistent with reports of sPLA2-IIa expression was associated with adverse survival in patients with prostate cancer⁵. This suggests the role of sPLA2-IIa as a biological factor that might affect the behavior of the tumor cell population.

Indeed, the role of sPLA2-IIa in the pathophysiology of premalignant cells and the enzyme's possible role in carcinogenesis, when premalignant cells evolve to malignant phenotype, are unclear. It has been reported that sPLA2-IIa inhibitors inhibits growth in human endothelial cells *in vivo*⁵, whereas inhibition of secretory (group IIA) PLA2 in rat intestinal and mouse colon cancer cell lines induces proliferation by an unknown mechanism⁸. In lung cancer cells, sPLA2-IIa inhibition suppresses lung cancer growth *in vitro* and *in vivo*⁷. In the present study, we found sPLA2-IIa overexpression did not affect cell proliferation *in vitro*, but promoted growth *in vivo*. Thus, the biological role of sPLA2-IIa in lung carcinogenesis may relate to different mechanisms leading to increased tumor growth.

Invasion is the early process of metastasis for cancer cells to go through the basement membrane and into the stroma. Invasion is one of the markers of the cellular malignancy and poor prognosis of cancer⁹. Previous reports showed that in addition to being a prognostic biomarker, sPLA2-IIa plays an intimate functional role in inhibiting gastric cancer progression¹⁰. In the present study, overexpression of sPLA2-IIa in lung cancer cells can promote cancer cell invasiveness *in vitro* and tumor growth *in vivo*.

Conclusions

The application of large-scale gene expression analysis to cancer studies has made identification of the differentially expressed genes responsible for invasion a practical approach. sPLA2-IIa is a metastasis-promoting gene, which has significant value to predict overall survival of patients with lung adenocarcinoma. Disclosing the genes regulated by sPLA2-IIa will provide further clues to its biological roles and more generally will contribute to the understanding of the mechanisms underlying the invasion of lung cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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