

## Original Article

# S100A11 influences the invasion and migration of lung cancer cells by the Notch signaling pathway

Chengyan Jin, Jindong Li, Donglei Shi, Peiyan Hua, Guangxin Zhang, Bin Wang

Department of Thoracic Surgery, The Second Hospital of Jilin University, Changchun 130041, China

Received May 11, 2017; Accepted November 3, 2017; Epub December 15, 2017; Published December 30, 2017

**Abstract:** Objective: To explore the expression of S100A11 in lung cancer tissues, its effects on the invasion and migration abilities of lung cancer cells and the relevant mechanism. Methods: the expression of S100A11 was detected in lung cancer and normal lung tissues by Western blotting; lentivirus silencing S100A11, GFP fluorescence and Western blotting were used to detect the LV3-S100A11 silencing efficiency and efficacy; the effect of S100A11 expression on invasion of lung cancer cells was detected by Transwell invasion assay; the effect of S100A11 expression on migration of lung cancer cells was detected by wound scratch assay; the expression of the proteins Notch1, NICD1 and Hes1 was detected by Western blotting; and the effects of silencing S100A11 on size and volume of lung cancer were detected in the subcutaneous tumor formation experiment in nude mice. Results: the expression of S100A11 increased significantly compared with that in normal lung tissues, and the high expression of S100A11 was related to the pathological type, differentiation degree and metastasis of local lymph nodes in lung cancer; LV3-S100A11 lentivirus could effectively inhibit the expression of S100A11; silencing S100A11 inhibited the invasion and migration abilities of the lung cancer A549 cells; silencing S100A11 could down-regulate the expression of Notch1, NICD1 and Hes1; and the tumor volume and weight of tumor-bearing mice decreased significantly in the LV3-S100A11 group as compared to the LV3-NC group. Conclusion: S100A11 influences the invasion and migration abilities of lung cancer cells by the Notch signaling pathway.

**Keywords:** S100A11, lung cancer, Notch, S100, A549, invasion, migration

## Introduction

Lung cancer is one of the malignancies with the highest morbidity and mortality in the world. Its morbidity and mortality are on the rise, especially in the developing countries like China. With aging of the population and increasing environmental pollution and effect of tobaccos, lung cancer poses an increasing burden to people [1]. Metastasis of lung cancer is the leading cause of death. With advances in surgical techniques, chemotherapy and molecular targeted therapy are increasingly important in the combined therapy for lung cancer, but the five-year survival rate of patients, especially those with advanced disease, is still very low [2]. Thus, it is particularly important to investigate the mechanism of lung cancer metastasis and identify the relevant molecular markers.

Tumor metastasis is a complex multi-step, multi-stage process, mainly including local infiltra-

tion, invasion in blood vessels, emigration from blood vessels, and settlement and proliferation in new sites, and the cytoskeletal changes, cell adhesion and changes in kinetic characteristics, epithelial-mesenchymal transition (EMT) and activation of various signaling pathways [3, 4]. It has been demonstrated in studies that calcium binding proteins play an important role in tumor invasion and metastasis [5, 6].

S100 protein family belongs to the calcium binding protein superfamily. At present, there are 21 members in the S100 protein family, they have the amino acid sequence of EF double helix structure in common, and when binding to calcium ions, the S100 proteins change in structure to expose the site binding to the target protein and exert the biological effects through interaction with the protein [7]. The dysregulation of S100 protein family expression is a common occurrence in many human

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cancers. Reports have shown that altered expression of ten family members contributes to the growth and metastasis of numerous tumors (S100A1-4, 6-11, S100P). Studies also indicated that silence of S100B and S100A9, are in clinical trials for melanoma and prostate cancer, respectively [8].

S100A11 is an important member of the S100 protein family, which is involved in a number of biological regulations, including enzymatic activity, inflammatory reaction, cell proliferation and apoptosis, etc. [9]. It was demonstrated that S100A11 played an essential role in tumor occurrence and progression [10]. Wang *et al.* [11] found out in immunohistochemistry staining of colon cancer tissues that the expression of S100A11 was significantly increased in the tissues and its expression level was closely related to stage of the disease, suggesting that S100A11 might be an indicator for evaluating the degree of invasion of colon cancer. The expression of S100A11 was also elevated dramatically in prostate cancer, breast cancer and ovarian cancer [12-14]. Report also indicated that knockdown of S100A11 expression restrained cell proliferation in lung adenocarcinoma cells A549 [15]. However, the relationship between S100A11 expression and lung cancer cell motility has not yet been reported. Therefore, in this study, we intended to investigate the expression of S100A11 in lung cancer and its effect on the invasion and migration abilities of lung cancer cells, and explore the related mechanisms.

### Materials and methods

#### *Tissues, cell lines and reagents*

Lung cancer tissues of 108 patients removed in thoracic surgery were collected in our hospital between June 2014 and July 2015, including those of 74 males and 34 females aged 36-72 years. There were squamous cell carcinoma in 38 patients and adenocarcinoma in 70 patients, with poor, moderate and well differentiation in 40, 39 and 29 patients respectively. In addition, the paracancerous tissues 2 cm from tumor margin and the normal lung tissues of 20 patients were used as control. After isolated from the body, the tissues were quickly put into liquid nitrogen for preservation. The lung cancer cells line A549 was purchased from ATCC, and the first antibodies S100A11, Notch1, NICD1 and Hes1 were

obtained from Abcam (ab180593, ab52627, ab8925 and ab108937). Transwell chambers were purchased from Millipore (US); S100A11-silencing and control lentiviruses were purchased from Shanghai GenePharma Co., Ltd.

#### *Cell culture, transfection and grouping*

The lung cancer cells A549 were put into the RPMI 1640 medium containing 10% fetal bovine serum for incubation at 37°C, 5% CO<sub>2</sub>. Relevant experiment was performed when the cells were at logarithmic growth phase.

One day before the experiment,  $5 \times 10^3$  A549 cells were inoculated in 96-well plates, to make the cell fusion degree at 40%~60%. According to the GenePharma lentivirus operating manual, the appropriate MOI (multiplicity of infection) of S100A11 lentivirus was determined using the gradients of 0, 10 and 100.

1) Two sterile EP tubes were prepared. 10  $\mu$ l of viruses at  $1 \times 10^8$  TU/ml were drawn to the first tube and mixed gently, producing no foams. Similarly, 10  $\mu$ l of viruses were drawn from the first tube to the second and mixed well, to get virus solutions at three different concentrations: stock solution, 10 $\times$  dilution, and 100 $\times$  dilution.

2) 10  $\mu$ l of virus solutions at three different gradients were added to three wells in each group, to calculate the MOI of three wells, which were 100, 10 and 1 respectively. The most appropriate MOI was determined to be 100.

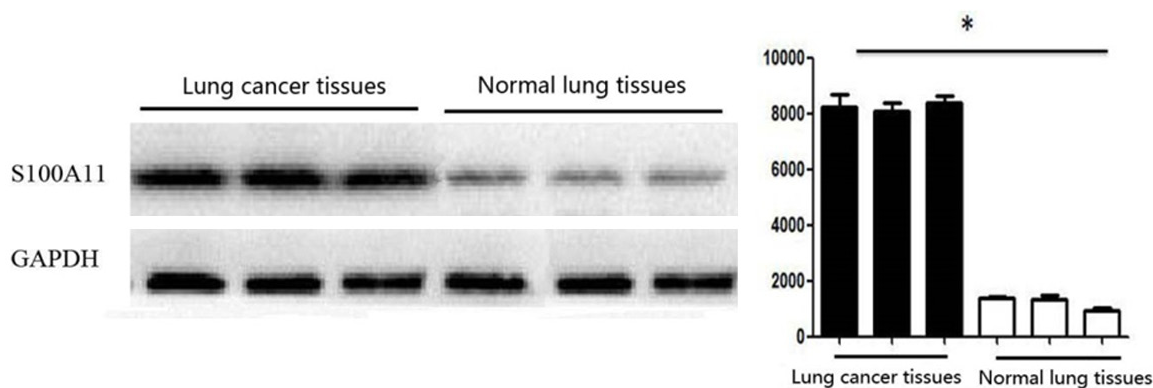
3) The experiment consisted of two groups: the silencing group, transfected with S100A11 silencing lentivirus (LV3-S100A11), and the control group, transfected with the negative control lentivirus (LV3-NC). 100-fold diluted virus stock solution was added to the LV3-S100A11 group, and 100-fold diluted negative control virus solution was added to the LV3-NC group. The expression of GFP fluorescence was observed 24 h later.

72 h after cell transfection, the proteins were extracted, with the concentrations determined by BCA method, and then loading buffer was added for protein denaturation. The proteins were stored at -20°C.

#### *Tissue protein extraction and preservation*

A tissue block of 100 mg in size was taken, cut into pieces and broken with an ultrasonic tis-

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**Figure 1.** The expression of S100A11 between lung cancer and normal lung tissues. \*P<0.05 compared with normal lung tissues.

**Table 1.** Comparison of the expression of S100A11 between lung cancer and normal lung tissues

Tissue samples	Number	High expression of S100A11	
		n	%
Lung cancer	108	98	90.7%*
Normal lung tissues	20	2	10%

\*P<0.05 compared with normal lung tissues.

sue instrument after 1 ml of lysis solution, 1  $\mu$ l of protease inhibitor, 1  $\mu$ l of PSMF and 1  $\mu$ l of phosphatase inhibitor were added. Then it was centrifuged at 12000 rpm for 20 min at 4°C, the supernatant was taken, with the protein concentrations determined by BCA method, and then loading buffer was added for protein denaturation. The proteins were stored at -20°C.

### *Expression of S100A11, Notch1, NICD1 and Hes1 detected by western blotting*

10% SDS-PAGE was prepared, and 20  $\mu$ g protein sample was added into each well for protein separation, then transferred to a PVDF membrane using the electric wet transfer method, sealed for 2 h with 5% skim milk, and the primary antibodies (S100A11, Notch1, NICD1 and Hes1) were diluted by 1:1000 TBST, overnight at 4°C; then 1:5000 dilution of goat anti-rabbit secondary antibody was added, incubated at room temperature for 2 h; and ECL was performed. The experiment was performed in triplicate.

### *Effects of silencing S100A11 on the invasion ability of the lung cancer cells detected by Transwell invasion assay*

All reagents and equipment were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. 50  $\mu$ l (0.2  $\mu$ g/ $\mu$ l) Matrigel was evenly applied to the inner membrane of the Transwell chambers, incubated for 15 min at 37°C to solidify the gel; when digested, centrifuged and counted, the cells were diluted with  $2.5 \times 10^4$ /mL serum-free medium to prepare cell suspension; the cell suspension was added to the upper Transwell chamber at 200  $\mu$ L each well, and 500  $\mu$ L of 10% FBS and medium were added to the lower Transwell chamber, placed in a 37°C incubator for culture; fixed with formalin, stained by crystal violet for 15 min, and then the cells on the inner membrane were wiped with a cotton swab, counted under a microscope, to count the cells that passed through the membrane under 4 high power fields ( $\times 40$ ). The experiment was performed in triplicate.

### *Effects of silencing S100A11 on the migration ability of the lung cancer cells detected by wound scratch assay*

The A549 cells were inoculated into the 6-well plate, and when cell confluence reached 90%, scratch from up to bottom using a 200  $\mu$ l sterile pipette tip, observe under a microscope, to measure the initial distance of scratch (0 time); at 24 h, 48 h and 72 h, the distances of scratch were measured respectively and photographed, to calculate the cell migration rate. The experiment was performed in triplicate.

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**Table 2.** Relationship between S100A11 expression and clinicopathological characteristics of lung cancer

Clinicopathological data	Number	High expression of S100A11	Low expression of S100A11	P value
Sex				0.741
Male	74	40	34	
Female	34	18	16	
Age (y)				0.692
≤60	63	31	32	
>60	45	24	21	
Pathological type				0.013
Squamous cell carcinoma	38	25	13	
Adenocarcinoma	70	61	9	
Differentiation				<0.001
Well	29	19	10	
Moderate	39	32	7	
Poor	40	39	1	
Tumor size				0.134
≤3 cm	43	33	10	
>3 cm	65	53	12	
Metastasis of local lymph nodes				0.001
No	56	34	22	
Yes	52	48	4	

0.05 indicated statistically significant difference.

### Results

*Significantly increased expression of S100A11 in lung cancer tissues*

The Western blotting results (**Figure 1** and **Table 1**) showed that compared to the normal lung tissues, the expression of S100A11 increased significantly in the lung cancer tissues, indicating that S100A11 is oncogenic and may be involved in occurrence and development of lung cancer.

*Relationship between S100A11 expression and clinicopathological characteristics of lung cancer*

### *Effect of silencing S100A11 on tumor growth in subcutaneous tumor formation experiment in nude mice*

Lung cancer cells in the logarithmic growth phase in the LV3-NC and LV3-S100A11 groups were taken and digested in culture flasks, with concentration adjusted to  $2 \times 10^8$  cells/ml. 0.1 ml of cell suspension was injected subcutaneously into each nude mouse (10 in total) by the left forelimb armpit to observe subcutaneous tumor growth daily. One week after inoculation, the tumor grew to about 5, 6 mm. The mice were divided into 2 groups, 5 in each group. The animals were sacrificed 28 days after inoculation of tumor cells. The tumor diameter (a) and the vertical orthogonal diameter (b) of tumor-bearing mice were measured in each group. The tumor size  $V$  ( $\text{mm}^3$ ) =  $a \times b^2 / 2$ ; and the weight was measured.

### *Statistical analysis*

The SPSS 22.0 software was used for statistical analysis, measurement data were expressed in  $(\bar{x} \pm s)$ , t-test was employed for comparison of means between groups, and  $P <$

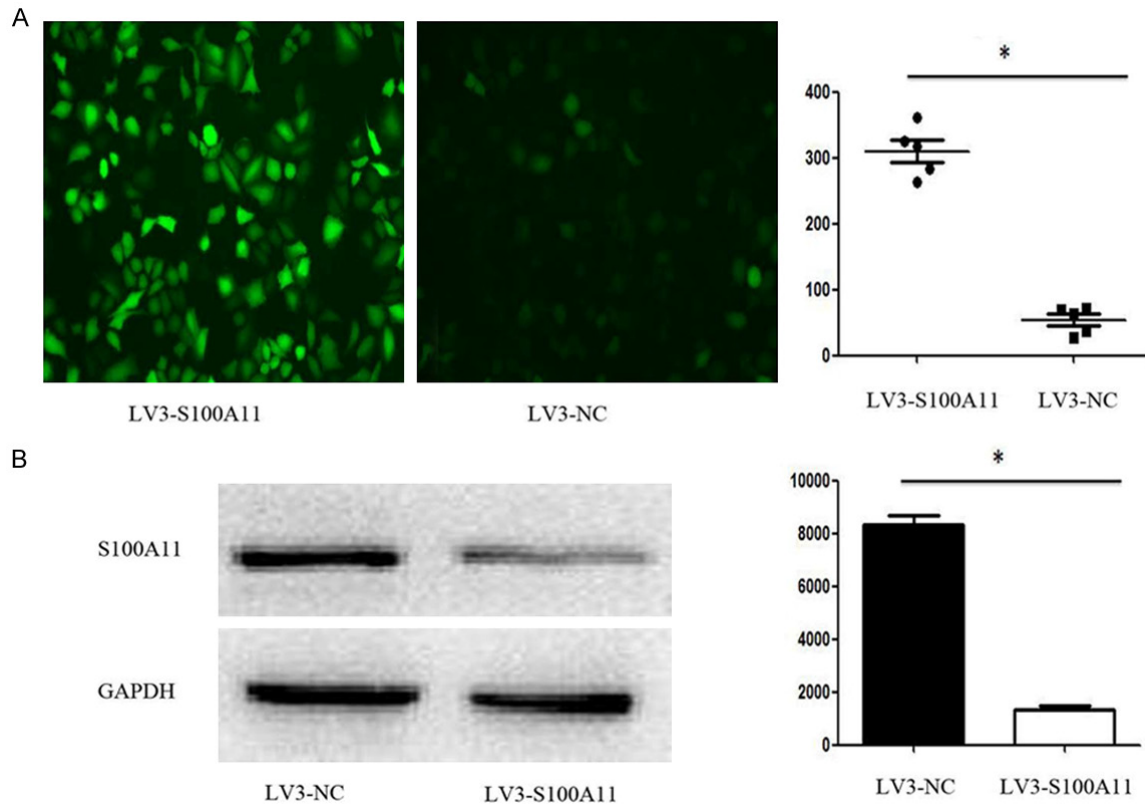
The expression of S100A11 was related to clinicopathological characteristics of lung cancer. In adenocarcinoma, the expression of S100A11 was significantly higher than that in squamous cell carcinoma (**Table 2**,  $P < 0.05$ ); it increased gradually with decrease in differentiation degree (**Table 2**,  $P < 0.05$ ); and it increased significantly in lung cancer tissues with metastasis of local lymph nodes (**Table 2**,  $P < 0.05$ ). The expression of S100A11 was irrelevant with sex, age and tumor size.

### *Expression of S100A11 decreased significantly after transfection of the A549 cells with LV3-S100A11*

24 h after transfection of the A549 cells with LV3-S100A11, GFP fluorescence showed (**Figure 2A**) that compared with the LV3-NC group, expression of green fluorescence in the LV3-S100A11 transfected cells was significantly increased [ $(308.9 \pm 14.6)$  vs.  $(48.7 \pm 4.03)$ ,  $P < 0.05$ ], suggesting that LV3-S100A11 could be well incorporated into the A549 cells.

The Western blotting results (**Figure 2B**) showed that compared with the LV3-NC group, the S100A11 expression level in the LV3-S100A11 group was significantly decreased

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**Figure 2.** A. Efficiency of transfection of S100A11 knocked down lentivirus into the lung cancer A549 cells detected by GFP fluorescence. B. Expression of S100A11 detected by Western blotting. Error bars represent standard error. \*P<0.05.

[(8125.8±51.6) vs. (1025.3±32.7), P<0.05], suggesting that S100A11 expression could be significantly reduced after LV3-S100A11 was transfected into the A549 cells.

### *Silencing S100A11 expression could inhibit the invasion ability of the A549 cells*

To determine whether silencing S100A11 could influence the invasion ability of the lung cancer A549 cells, changes in the ability before and after transfection were identified with Transwell chambers. The Transwell results showed (Figure 3) that in the LV3-S100A11 group, the number of cells passing through the Matrigel gel was 82.16±6.25, significantly less than that in the LV3-NC group (313.92±22.88), with statistically significant differences (P<0.05), indicating that silencing S100A11 expression could inhibit the invasion ability of the A549 cells.

### *Silencing S100A11 expression could inhibit the migration ability of the A549 cells*

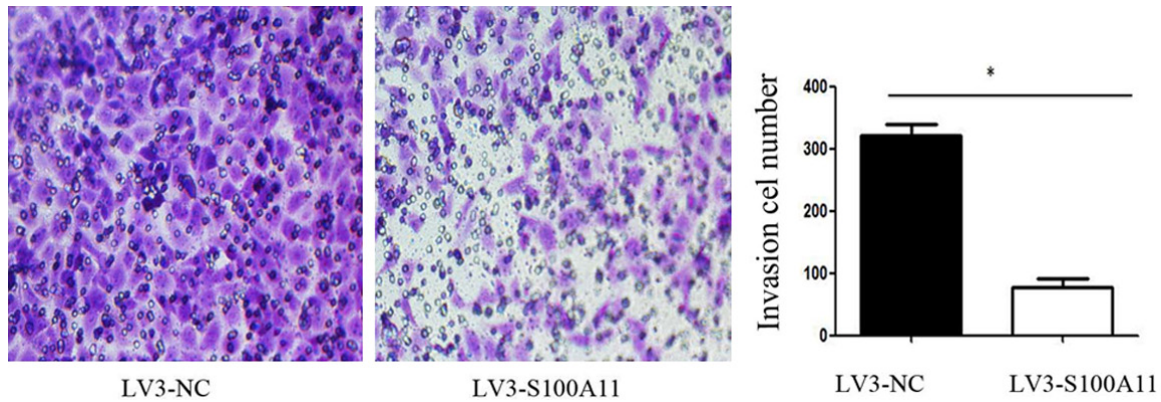
The width of scratches in any three parts of cells in each group was measured under a

microscope at the time points of 0 h, 24 h, 48 h and 72 h. The migration rate was calculated according to the formula: Migration rate =  $[D_{(t=24\text{ h}, 48\text{ h}, 72\text{ h})} - D_{(t=0\text{ h})}] / D_{(t=0\text{ h})}$ . Results of the wound scratch assay (Figure 4A and 4B) suggested that compared to the LV3-NC group, migration rate in the LV3-S100A11 group was significantly reduced at 24 h, 48 h and 72 h [24 h (0.19±0.03)% vs. (0.38±0.04)%, P<0.05; 48 h (0.38±0.04)% vs. (0.73±0.05)%, P<0.05; 72 h (0.44±0.04)% vs. (0.84±0.06)%, P<0.05], with statistically significant differences. It was revealed in the wound scratch assay that silencing S100A11 expression could inhibit the migration ability of the A549 cells.

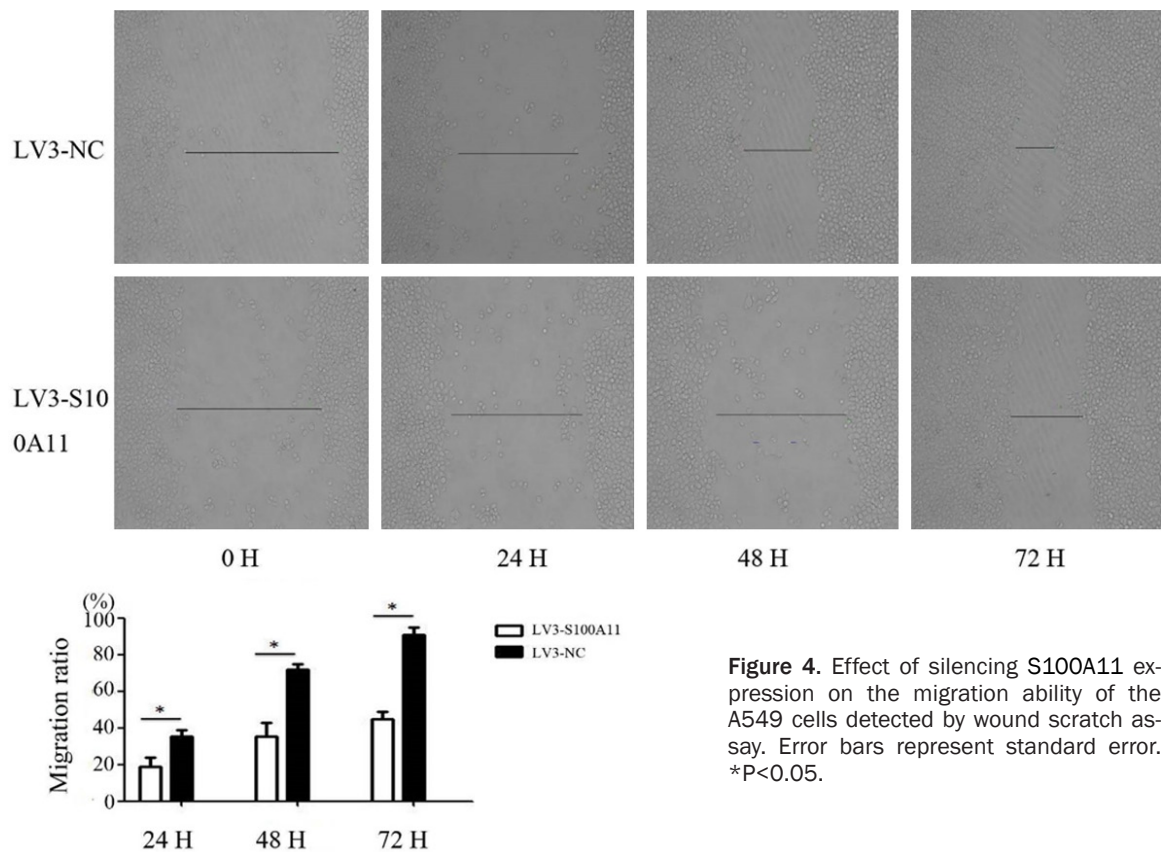
### *Silencing S100A11 expression could inhibit the expression of Notch1, NICD1 and Hes1*

It was demonstrated in the study that the Notch signaling pathway played an important part in tumor invasion and migration. When Notch was activated, enzymolysis occurred twice to form water soluble NICD, which bound to CSL, the DNA binding protein on the nucleus to form

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**Figure 3.** Effect of silencing S100A11 expression on the invasion ability of the A549 cells detected by Transwell invasion assay. Error bars represent standard error. \*P<0.05.



**Figure 4.** Effect of silencing S100A11 expression on the migration ability of the A549 cells detected by wound scratch assay. Error bars represent standard error. \*P<0.05.

a transcription factor and stimulate transcription of target genes like Hes1 [16].

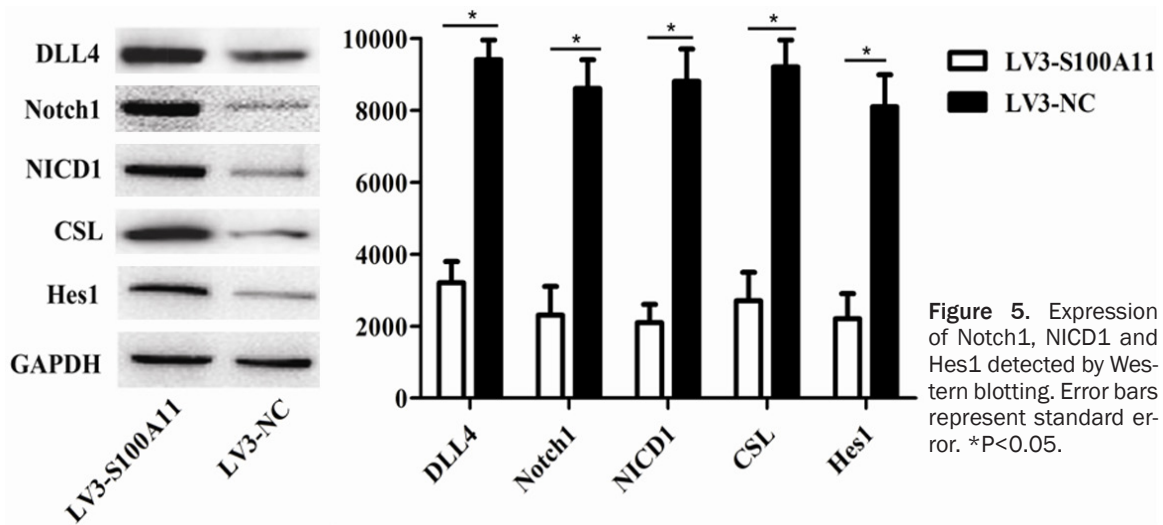
The Western blotting results (Figure 5) showed that compared to the LV3-NC group, the expression of Notch1, NICD1 and Hes1 decreased significantly in the LV3-S100A11 group [Notch1 (8214.3±88.4) vs. (2254.2±31.9); NICD1 (8237.1±81.3) vs. (2038.1±31.2); Hes1 (8025.3±92.1) vs. (2012.6±28.4), P<0.05], suggesting that silencing S100A11 expression

could down-regulate the expression of Notch1, NICD1 and Hes1. It indicated that S100A11 took effect by the Notch signaling pathway.

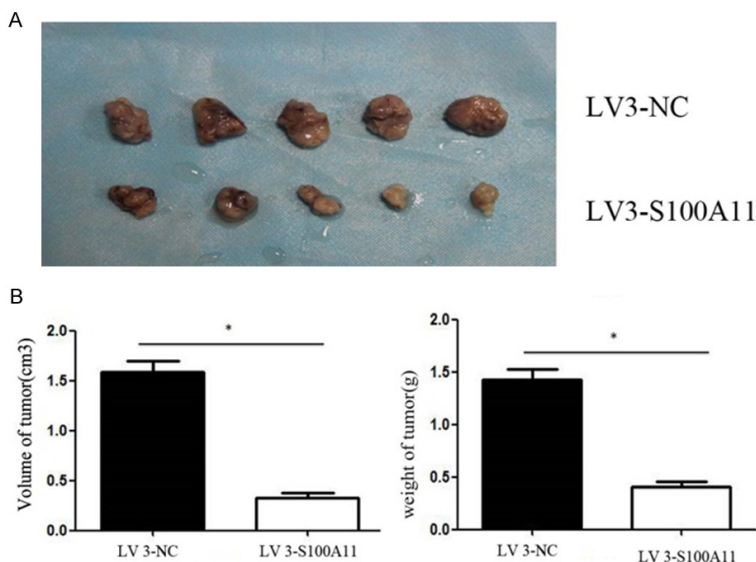
*Subcutaneous tumor formation experiment in nude mice indicated that silencing S100A11 could inhibit tumor growth*

The survival time of the tumor-bearing mice was 4-8 weeks, with a median of 6 weeks. The autopsy showed that tumor grew in the left

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**Figure 5.** Expression of Notch1, NICD1 and Hes1 detected by Western blotting. Error bars represent standard error. \*P<0.05.



**Figure 6.** Effect of S100A11 on tumor growth in *in vitro* experiment. A. Comparison of size of the nude mice in both groups. B. Comparison of tumor volume and weight in the nude mice in both groups.

armpit, and the tumor was gray, solid, round or oval, with nodular projection on the surface and fish-like sections; and the tumor formation rate was 100%.

Tumor growth in nude mice (**Figure 6A**): tumor size in the LV3-S100A11 group was significantly reduced compared with that in the LV3-NC group.

Comparison of tumor weight and volume (**Figure 6B**): compared to the LV3-NC group, the tumor volume and weight of the mice decreased significantly in the LV3-S100A11 group [vol-

ume (1.53±1.55) cm<sup>3</sup> vs. (0.26 ±0.02) cm<sup>3</sup>, P<0.05; weight (1.47±1.12) g vs. (0.36±0.02) g, P<0.05].

### Discussion

S100 is a family of calcium binding proteins with multiple genes. The family has 21 members, with the majority located at chromosome 1q21. The members are 25%~65% homologous in structure with similar physical characteristics. They are 100% soluble in ammonium sulfate solution, thus named S100 proteins [8]. S100A11 was originally discovered in smooth muscle cells of chicken gizzard, so it is also known as calmodulin [17].

The conformational change in the C-terminal of S100A11 is greater than that in the N-terminal, thus the C-terminal often becomes the target for genetic change [18]. S100A11 interacts with the target gene by transducing the calcium-dependent cell-mediated signals, to exert a variety of biological effects, participating in cell proliferation, differentiation and apoptosis, etc. [19].

In recent years, abnormal expression of S100A11 in tumors has been seen in many studies and S100A11 plays its tumor-promoting role in different tumors. Xiao *et al.* [20] performed immunohistochemical staining on pancreatic

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cancer tissues and found that the positive expression rate of S100A11 was significantly higher in the cancer tissues than that in the normal pancreatic tissues, and the high expression of S100A11 was associated with tissue differentiation, lymph node metastasis and poor prognosis. Similar studies on stomach cancer, breast cancer and colon cancer showed that S100A11 had a tumor-promoting effect [8, 11, 12]. In accordance with these reports, our research detected high expression of S100A11 in the lung cancer tissues from 108 patients. The expression level of S100A11 was closely related to pathological type, degree of differentiation and metastasis of local lymph nodes, suggesting that S100A11 plays a promoting role in occurrence and progression of lung cancer. Lentivirus infection is one of the commonly used methods in research on gene expression in recent years. In this study, in order to investigate the effects of S100A11 in the biological behaviors of lung cancer cells, the S100A11 silenced lentiviruses LV3-S100A11 and LV3-NC were used to transfect the lung cancer A549 cells, followed by Western blotting and GFP fluorescence to determine the transfection and silencing efficiency. Results showed that LV3-S100A11 could effectively down-regulate the expression of S100A11, which provided an ideal research platform for clarifying the effects of S100A11 in invasion and migration of lung cancer cells in the future.

Studies have shown that S100A11 is closely related to tumor invasion and migration. The study by Mori *et al.* [23] indicated that compared with gastric cancer tissues without lymph node metastasis, the expression of S100A11 was significantly increased in those with lymph node metastasis. S100A11 has been reported to associate with lung cancer cell viability. However, there are no research about the relationship between S100A11 and the motility of lung cancer. The results from the present study showed that compared with lung cancer tissues without lymph node metastasis, the expression of S100A11 was significantly increased in those with lymph node metastasis, consistent with the results obtained by other researchers, indicating a relationship between S100A11 and infiltration and metastasis of lung cancer. In order to further investigate this relationship, the Transwell chambers

and wound scratch assay were employed to detect the changes in the invasion and migration abilities of the A549 cells. It was shown that with down-regulation of S100A11, the invasion and migration abilities of the cells were significantly reduced. Our study help understand the effect of S100A11 on cell motility of lung cancer.

The Notch signaling pathway is closely related to the biological behaviors of tumor. It has been shown that the Notch signaling pathway is involved in invasion and migration of various tumors [24]. Bolós *et al.* [25] compared the expression levels of Notch-1 RNA and proteins between the highly invasive breast cancer cell line MCF-7/ADR and the poorly invasive breast cancer cell line MCF-7, and found that Notch-1 expression was significantly higher in MCF-7/ADR, suggesting that Notch-1 was positively correlated with invasion and migration of the cancer, and the Notch-1 signaling pathway was involved in these processes of breast cancer cells. Zhang *et al.* [26] found that the Notch signal activity in the highly invasive osteosarcoma cell lines was significantly higher than that in the normal cell lines, suggesting activation of the Notch signaling pathway in metastasis of osteosarcoma. In this study, by silencing the expression of S100A11, we further studied the gene levels of the Notch signaling pathway-related proteins Notch1, NICD1 and Hes1 and revealed that after S100A11 silencing, the expression levels of Notch1, NICD1 and Hes1 were significantly reduced, suggesting that S100A11 regulated the invasion and migration abilities of lung cancer cells through the Notch signaling pathway.

In order to further verify the tumor-promoting effect of S100A11 in lung cancer, the *in vivo* tumor formation experiment in nude mice was conducted and showed that after S100A11 silencing, the tumor volume and weight in the test group were significantly lower than the control group. The *in vivo* experiment verified again the tumor-promoting effect of S100A11.

By detecting the expression of S100A11 in lung cancer tissues and silencing S100A11 with lentivirus, it has been demonstrated that silencing S100A11 can inhibit the invasion and migration abilities of lung cancer cells through the Notch signaling pathway, suggesting that S100A11 may be involved in invasion and



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migration of the cells, and it may become a marker for predicting progression and prognosis of lung cancer and for monitoring the treatment effect. Considering the suppressing effect of S100A11 silence on lung cancer cell invasion and migration. We intend to investigate the cerebral metastasis and hepatic metastasis in lung cancer xenograft with S100A11 silence in the future study.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Bin Wang, Department of Thoracic Surgery, The Second Hospital of Jilin University, 218 Ziqiang Street, Changchun 130041, China. Tel: 13353111009; E-mail: wangbiniin@163.com

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