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Short Communication

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Differential expression of bone morphogenetic protein 5 in human lung squamous cell carcinoma and adenocarcinoma

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Abstract

Bone morphogenetic proteins (BMPs) play important roles in tumor cell proliferation, metastasis, and invasion. However, the expression patterns of BMPs in patients with non-small-cell lung cancer (NSCLC) and their correlations with NSCLC pathogenesis have not been examined yet. In this study, the mRNA levels of BMP family members in NSCLC tissues were analyzed and results showed that the mRNA levels of *BMP5* and *BMP7* were significantly down-regulated and up-regulated, respectively, in tumor tissues compared with those in the corresponding noncancerous tissues. Interestingly, the mRNA level of *BMP5* was significantly higher in lung adenocarcinoma tissues than that in lung squamous cell carcinoma tissues. Furthermore, results from immunohistochemistry analysis confirmed stronger expression of BMP5 protein in lung adenocarcinoma than in lung squamous cell carcinoma. Our findings suggested that BMP5 might be a potential prognostic biomarker or therapeutic target for patients with NSCLC.

Key words: non-small-cell lung cancer, adenocarcinoma, squamous cell carcinoma, bone morphogenetic protein, prognostic biomarker

Introduction

Lung cancer is the leading cause of cancer mortality, resulting in more than 2 million deaths worldwide each year [1]. Non-small-cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma, large-cell lung cancer, and mixed carcinoma, accounts for ~85% of all lung cancers [2]. Although surgical resection is widely applied for patients with all types of NSCLC, different strategies such as radiotherapy and multimodal neoadjuvant chemotherapy are adopted to treat distinct subtypes of NSCLC after operations [2]. For example, paclitaxel and docetaxel are preferentially used to treat squamous cell carcinoma, while pemetrexed disodium is a major choice for adenocarcinoma. In addition, chemotherapy could serve as a primary choice for patients whose conditions do not allow surgical operations. Under such circumstances, it is quite important to characterize the subtypes of NSCLC before designing appropriate treatment strategies. Previous studies have demonstrated that follistatin and the family with sequence similarity 83, member B (FAM83B) are candidate biomarkers for lung adenocarcinoma and

squamous cell carcinoma, respectively [3,4]. Whether there exist additional potential prognostic biomarkers for different subtypes of NSCLC is of great interest.

Bone morphogenetic proteins (BMPs) are secreted extracellular matrix-associated proteins that belong to the transforming growth factor β (TGF-β) superfamily and are originally identified by their presence in bone-inductive extracts of demineralized bone [5,6]. So far, ~20 BMP family members have been identified in humans, most of which are widely expressed in various organs or tissues [7]. BMPs exist as pro-protein dimers in the cytoplasm and are cleaved into mature forms before being secreted into the extracellular matrix. The mature BMPs bind to the type I serine-threonine kinase receptors (BMPRIs) that recruit type II receptors (BMPRIIs), leading to the phosphorylation and activation of BMPRIs by BMPRIIs. Activated BMPRIs trigger different signaling pathways that result in activation of SMAD1/5/8 and MAPKs and subsequent expression of a large number of downstream genes [5,8].

Although BMPs are first known to trigger bone formation from mesenchymal stem cells in culture [6], it has been documented that BMPs are also involved in tumorigenesis [9]. For example, BMP2 is expressed as a sensitive marker in various human cancers, including glioma, ovarian, salivary adenocarcinoma, and pancreatic cancer, whereas BMP7 is most frequently expressed in breast cancer and up-regulated in melanoma and metastases of malignant melanoma [10–15]. In addition, it has been shown that BMP2 is highly expressed in NSCLC and small-cell lung carcinoma (SCLC) cell lines to promote cell migration and invasion, correlating with poor survival of patients with advanced NSCLC [16,17], while the expression of BMP3 and BMP6 is down-regulated in NSCLC tissues and cell lines, which is due to aberrant DNA hypermethylation in the promoters of *BMP3* and *BMP6* genes and is associated with lung tumor development [18,19]. It is currently unclear whether other BMPs are associated with NSCLC progress.

In this study, the expressions of BMP3, 4, 5, 6, 7, 10, and 13 in NSCLC tissues and the corresponding normal tissues were examined by quantitative real-time PCR analysis. Our results showed that the expression of *BMP5* and *BMP7* was down-regulated and up-regulated in the tumor tissues, respectively. Interestingly, the mRNA and protein levels of BMP5 were significantly higher in lung adenocarcinoma than those in squamous cell carcinoma, indicating that BMP5 could serve as a potential prognostic biomarker or therapeutic target for NSCLC.

Materials and Methods

Human tumor samples

Fresh specimens of NSCLC and corresponding noncancerous tissues and paraffin-embedded tumor samples of NSCLC were used in this study. Three cohorts of human NSCLC samples (73 samples in total) were collected in the Department of Thoracic Surgery of Tongji Hospital. For initial screening (cohort 1), a total of 14 specimens of tumor tissues and noncancerous tissues were collected from patients with NSCLC (7 adenocarcinoma and 7 squamous cell carcinoma) undergoing surgery from July 2013 to September 2013. Cohort 2 included 34 specimens of tumor tissues and noncancerous tissues (17 adenocarcinoma and 17 squamous cell carcinoma) which were collected from March to May of 2014. Cohort 3 contained 25 paraffin-embedded NSCLC samples (13 adenocarcinoma and 12 squamous cell carcinoma) which were collected from October to December of 2012. Noncancerous tissues were excised at least 5 cm away from the tumor border. Tumor and noncancerous tissues were immersed in Trizol reagent (Invitrogen, Carlsbad, USA) immediately after isolation, snap-frozen in liquid nitrogen and stored at −80°C until RNA preparation. For cohort 3, total RNA from each paraffin-embedded sample was isolated using a nucleic acid isolation kit (Life Technology, Waltham, USA). Clinicopathological data were collected, including patient age, gender, smoking condition, histological type, tumor differentiation, TNM stage, and lymph nodal status. The present study was approved by the Institutional Review Committee of Tongji Hospital. Informed consent was obtained from all patients.

RNA isolation and quantitative RT-PCR

Trizol reagent was used to extract total RNA from specimens according to the manufacturer's manual. M-MLV Reverse Transcriptase (Invitrogen) and oligo(dT) (Invitrogen) were used to synthesize the first-strand cDNAs from equivalent RNA based on manufacturer's protocol. The expression of *BMP* genes was analyzed with quantitative RT-PCR (qPCR). The reactions were performed in CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, USA) using SsoAdvanced SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions for 40 cycles as follow: denaturation at 95°C for 20 s, annealing and extension at 60°C for 30 s. The expression levels of target genes were quantified after normalization to the endogenous control *GAPDH*. The sequences of forward and reverse primers of *BMPs* and *GAPDH* listed in **Table 1** (Invitrogen) were used.

Tissue array and immunohistochemistry analysis

Tumor samples (from cohorts 2 and 3) embedded into paraffin blocks were sectioned (5 μm) for H&E staining to select the punching area. A manual tissue puncher was used to punch the samples out of the paraffin blocks. The punched tissues were 1.5 mm in diameter and the length ranged from 4 to 6 mm. The punched tissues were embedded in a new paraffin to form a tissue array. This array was sectioned (5 μm) and stained with H&E to verify the histology. The immunohistochemistry analysis was performed on the 5 μm sections. The sections were placed on polylysine-coated slides, deparaffinized in xylene, rehydrated through graded ethanol, quenched for endogenous peroxidase activity in 3% hydrogen peroxide, and processed for antigen retrieval by microwave heating for 7 min in 10 mM citrate buffer (pH 6.0). The anti-BMP5 antibody (sc-73747; Santa Cruz Biotechnology, Santa Cruz, USA) was diluted 1:100 in phosphate-buffered saline containing 1% bovine serum albumin and incubated at room temperature for over 6 h. Immunostaining was performed using the Maixin_Bio Detection kit peroxidase/diaminobenzidine (DAB) rabbit/mouse (Kit-9710, DAB-0031; Maixi_Bio, Fuzhou, China), which resulted in a brown-colored precipitate at the antigen site. Subsequently, sections were counterstained with hematoxylin (Zymed Laboratories, San Francisco, USA) for 5 min and coverslipped. Pictures were acquired using a HistoFAXS system. The intensity of DAB staining in each sample was quantified with the HistoQuest software.

Statistical analysis

All statistical analyses were carried out using SPSS Statistics version 17.0 package (IBM SPSS Software; Armonk, New York, USA) and GraphPad Prism version 5.0.1 (GraphPad Software, San Diego, USA) for Windows. Data are presented as the mean ± SEM. Statistical analyses between two samples were performed by paired *t*-test (tumor vs. the corresponding noncancerous tissues) or unpaired *t*-test (adenocarcinoma vs. squamous cell carcinoma). $P < 0.05$ were considered significant difference.

Table 1. The sequences of forward and reverse primers

Target genes	Forward primers	Reverse primers
BMP3	5′-TCGGAATTGCGCCAGGAGAT-3′	5′-CCCCACAGCTCTCACTATACTC-3′
BMP4	5′-TGAGTATCCTGAGCGCCCGG-3′	5′-TCGTTCTCAGGGATGCTGCT-3′
BMP5	5′-TGGCAGGACTGGATTATAGCA-3′	5′-CAAGGCTTTGGTACGTGGTC-3′
BMP6	5′-CTGGGATGGCAGGACTGGAT-3′	5′-GGGGACATACTCGGGGTTCA-3′
BMP7	5′-GTCAGCTTCCGAGACCTGGG-3′	5′-GTTGATGAAGTGGACCAGCGT-3′
BMP10	5′-TTGCAACAGATCGGACCTCC-3′	5′-TGGACACATTGAAGAGGAGGGG-3′
BMP13	5′-CGCTGAGAAGCTGGGCATCA-3′	5′-CGCCCACCAGCTCTTCTTTG-3′
GAPDH	5′-GAGTCAACGGATTTGGTCGT-3′	5′-GACAAGCTTCCCGTTCTCAG-3′

Results

The mRNA levels of *BMP* genes in NSCLC tissues and noncancerous tissues

To characterize the expression patterns of *BMP* genes in NSCLC tumors, 14 pairs of NSCLC and the adjacent noncancerous tissues (cohort 1) were collected and the mRNA levels of *BMP* genes, including *BMP3*, *4*, *5*, *6*, *7*, *10*, and *13* were examined by qPCR analysis. Interestingly, we observed that the mRNA levels of *BMP4* (0.279 vs. 1.551, $P = 0.030$), *BMP5* (2.629 vs. 93.703, $P < 0.001$), and *BMP6* (0.104 vs. 0.566, $P = 0.010$) were significantly down-regulated in NSCLC tissues compared with noncancerous tissues, while *BMP7* mRNA level was significantly increased in NSCLC tissues (0.616 vs. 0.060, $P = 0.036$) (**Fig. 1**). In contrast, there was no difference of mRNA expressions of *BMP3* (0.390 vs. 0.303, $P = 0.750$), *BMP10* (0.008 vs. 0.013, $P = 0.388$) and *BMP13* (0.009 vs. 0.016, $P = 0.403$) between NSCLC and noncancerous tissues (**Fig. 1**). To further confirm these results, qPCR analysis with another cohort of samples was performed, which contained 34 paired NSCLC and noncancerous tissues (cohort 2). Results suggested that the mRNA levels of *BMP5* (2.72 vs. 9.92, $P = 0.014$) and *BMP7* (0.118 vs. 0.0375, $P = 0.035$) were significantly down-regulated and up-regulated in tumor tissues compared with noncancerous tissues, respectively (**Fig. 2**), indicating that the expression of *BMP5* and *BMP7* is associated with NSCLC.

BMP5 is differentially expressed in adenocarcinoma and squamous cell carcinoma

To investigate whether the expression patterns of *BMP5* and *BMP7* were correlated with the clinical characteristics of NSCLC patients, we compared the *BMP5* and *BMP7* mRNA levels in NSCLC tissues according to age, gender, smoking condition, histological type, tumor differentiation, TNM stage, and lymph node metastasis. Results showed that NSCLC tumors from both cohorts 1 and 2 in adenocarcinoma and female patients were associated with relatively higher *BMP5* mRNA levels. In contrast, the mRNA levels of *BMP7* were not significantly associated with any of the clinical features (**Table 2**). To substantiate these results, we obtained 25 paraffin-embedded NSCLC blocks (cohort 3) and performed qPCR analysis. Our results showed that the mRNA levels of *BMP5* were significantly higher in female patients than those in male patients, and also higher in adenocarcinoma than in squamous cell carcinoma (**Table 2**). These data suggested that BMP5 is differentially expressed in patients with adenocarcinoma or squamous cell carcinoma.

The expression of BMP5 protein in adenocarcinoma is higher than that in squamous cell carcinoma

Results from qPCR analysis suggested that the mRNA level of *BMP5* in adenocarcinoma tissues was higher than that in squamous cell carcinoma tissues (**Table 2**). To substantiate this conclusion, we made a tissue array with cohorts 2 and 3 samples and performed immunohistochemistry analysis with the BMP5 antibody. The results indicated that the expression level of BMP5 protein was substantially higher in adenocarcinoma tissues than that in squamous cell carcinoma tissues (**Fig. 3**). Taken together, we identified that BMP5 was highly expressed in adenocarcinoma when compared with squamous cell carcinoma, and it could be served as a biomarker for diagnosis.

Discussion

Lung cancer is one of the leading causes of cancer-related mortality worldwide, and NSCLC accounts for nearly 85% of lung cancer cases [1]. Although multimodal chemotherapies and radiotherapy have greatly improved the disease-free survival of NSCLC patients, the overall outcome remains unsatisfactory because of the toxicity and less effectiveness on relapse or metastasis of these therapies. More than a dozen of gene mutations have been identified as tumor-promoting factors for NSCLC, including EGFR, ALK, PI3K, K-Ras, and PTEN [20]. Various drugs such as Gefitinib and Afatinib that target the tumor-driving factors have been used to treat NSCLC, which improved the survival of NSCLC patients [21,22]. NSCLC is divided into different subtypes according to the histological features, each of which may be treated with different therapeutic strategies. Thus, there is an urgent need to understand the molecular mechanism in NSCLC tumorigenesis in order to identify effective prognostic biomarkers and potential therapeutic targets.

BMPs are secreted proteins that play important roles in the development of lung carcinoma [9,23]. It has been reported that BMP2 is over-expressed in all subtypes of NSCLC and contributes to tumor cell proliferation and migration [17,24]. In this study, we examined the mRNA levels of *BMP3*, *BMP4*, *BMP5*, *BMP6*, *BMP7*, *BMP10*, and *BMP13* in the NSCLC tissues and the corresponding noncancerous tissues by qPCR. Our results showed that the mRNA levels of *BMP5* and *BMP7* were significantly down-regulated and up-regulated in tumor tissues, respectively, compared with noncancerous tissues.

It has been shown that BMP7 elicits both growth stimulatory and inhibitory effects on tumor cells [25–28]. In this study, we found that the mRNA level of *BMP7* was significantly higher in NSCLC tissues than that in adjacent noncancerous tissues, indicating that BMP7 might be positively associated with NSCLC. In this context, several reports have demonstrated that BMP7 promotes cell invasiveness and motility of tumor cells [27]. BMP7 is over-expressed in highly bone-metastasized breast cancer cells and prostate cancer cells [27,29]. However, we did not observe any associations between the lymph node metastasis and the mRNA level of *BMP7*. There is evidence showing that BMP7 inhibits TGF-β-induced epithelial-to-mesenchymal transition and suppresses the progression of cholangiocarcinoma [28].

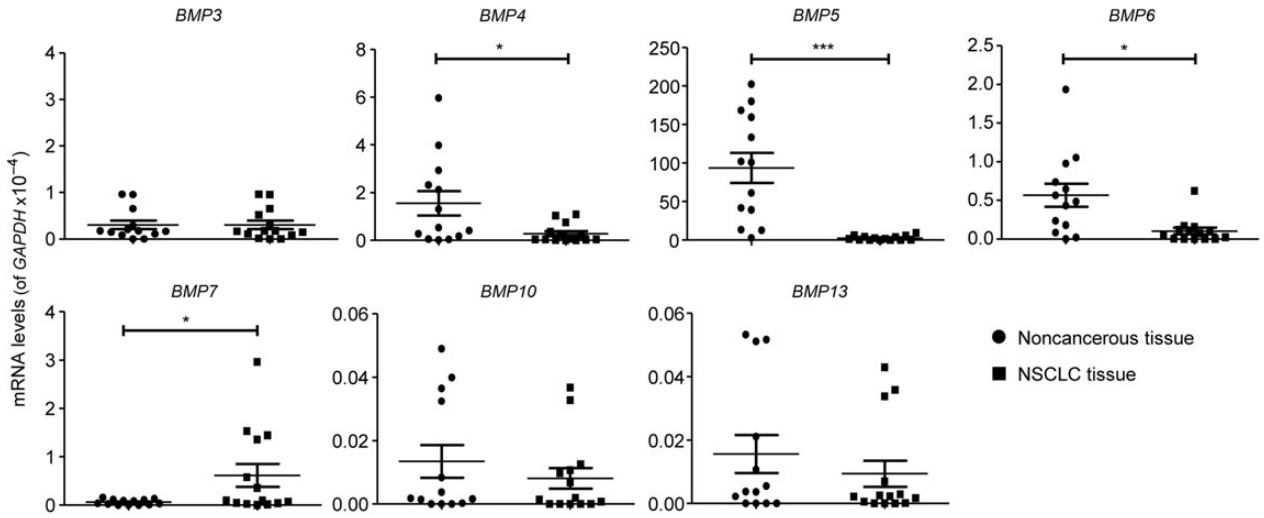

Figure 1. The mRNA levels of BMPs in NSCLC and noncancerous tissues (cohort 1) Data are shown as the mean ± SEM. $*P < 0.05$, $***P < 0.001$.

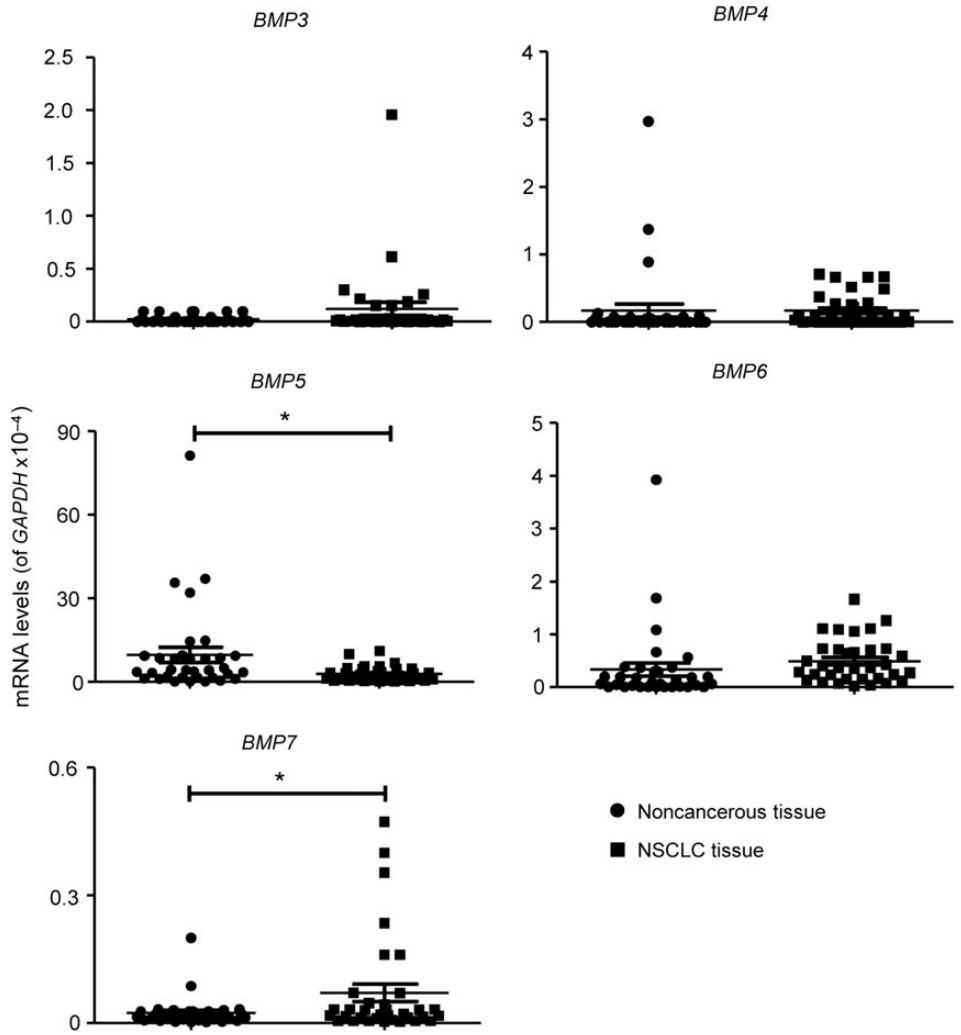

Figure 2. The mRNA levels of *BMP3, 4, 5, 6,* and *7* in NSCLC and noncancerous tissues (cohort 2) Data are shown as the mean ± SEM. $*P < 0.05$.

Table 2. Correlation between clinicopathological characteristics and *BMP5* and *BMP7* mRNA levels

Characteristics	Cohort 1					Cohort 2					Cohort 3		
	Quantity	*BMP5*		*BMP7*		Quantity	*BMP5*		*BMP7*		Quantity	*BMP5*	
		mRNA level	*P*-Value	mRNA level	*P*-Value		mRNA level	*P*-Value	mRNA level	*P*-Value		mRNA level	*P*-Value
Age			0.097		0.32			0.58		0.11			0.38
<60	5	4.74 ± 1.51		0.327 ± 0.280		20	3.12 ± 1.13		0.0721 ± 0.0239		13	3.97 ± 2.44	
≥60	9	1.46 ± 0.673		0.776 ± 0.334		14	2.15 ± 1.28		0.184 ± 0.0733		12	1.54 ± 0.916	
Gender			0.010*		0.84			0.049*		0.086			0.0062**
Male	11	1.86 ± 0.848		0.643 ± 0.280		24	1.88 ± 0.827		0.156 ± 0.0462		20	1.06 ± 0.561	
Female	3	5.46 ± 0.694		0.517 ± 0.031		10	4.75 ± 1.97		0.0276 ± 0.00624		5	9.79 ± 5.72	
Smoking condition			0.010*		0.84			0.13		0.056			0.0062**
Smoker	11	1.86 ± 0.848		0.643 ± 0.280		22	2.02 ± 0.897		0.166 ± 0.0498		20	1.06 ± 0.561	
Nonsmoker	3	5.46 ± 0.694		0.517 ± 0.508		12	4.02 ± 1.72		0.0291 ± 0.00554		5	9.79 ± 5.72	
Histological type			0.011*		0.15			0.020*		0.072			0.031*
Adenocarcinoma	7	4.63 ± 1.12		0.262 ± 0.212		17	4.64 ± 1.55		0.0569 ± 0.0335		13	5.19 ± 2.43	
Squamous cell carcinoma	7	0.625 ± 0.292		0.969 ± 0.396		17	0.802 ± 0.229		0.179 ± 0.0564		12	0.212 ± 0.0623	
Tumor differentiation			0.69		0.53			0.69		0.066			0.61
Well-moderate	8	2.32 ± 0.821		0.465 ± 0.224		22	2.47 ± 0.989		0.0607 ± 0.0162		14	3.42 ± 2.01	
Poor	6	3.05 ± 1.56		0.817 ± 0.483		12	3.18 ± 1.59		0.210 ± 0.0804		11	2.02 ± 1.74	
TNM stage			0.387		0.61			0.14		0.89			0.57
I–II	6	1.85 ± 0.977		0.783 ± 0.499		23	3.59 ± 1.20		0.115 ± 0.0412		13	2.05 ± 1.48	
III–IV	8	3.22 ± 1.18		0.490 ± 0.210		11	0.910 ± 0.311		0.125 ± 0.0632		12	3.62 ± 2.34	
Lymph node metastasis			0.357		0.92			0.11		0.86			0.81
N0	9	1.94 ± 0.713		0.634 ± 0.333		22	3.73 ± 1.24		0.114 ± 0.0432		14	2.50 ± 1.50	
N1–3	5	3.88 ± 1.79		0.583 ± 0.334		12	0.873 ± 0.289		0.126 ± 0.0574		11	3.18 ± 2.46	

*$P < 0.05$; **$P < 0.01$.

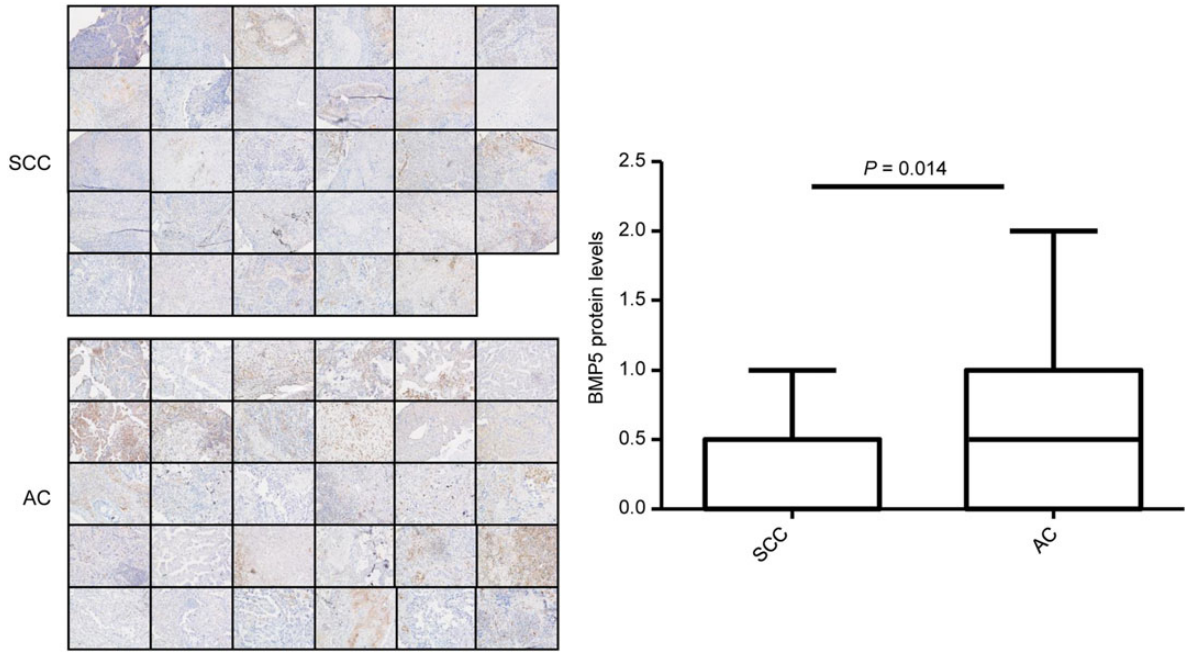

Figure 3. Immunohistochemistry analysis of BMP5 in squamous cell carcinoma (SCC) and adenocarcinoma (AC) tissues The intensity of the DAB staining was quantified (right).

Thus, it is possible that BMP7 regulates development and metastasis of various tumors through distinct mechanisms. We also observed that the mRNA level of *BMP7* was higher in squamous cell carcinoma than that in adenocarcinoma. However, the difference is not statistically significant. Therefore, more studies are required to confirm the expression of *BMP7* in NSCLC tissues, as well as its effect on tumorigenesis and metastasis of NSCLC.

It has been demonstrated that TGF-β-induced epithelial-to-mesenchymal transition is mediated by Blimp-1-dependent repression of BMP5 [30]. Consistent with this notion, lower *BMP5* mRNA level is detected in breast tumor vs. normal tissue, and correlated with cancer recurrence [30]. We also observed that the expression of *BMP5* was lower in NSCLC tissues than in adjacent noncancerous tissues. It is possible that the lower expression of *BMP5* in NSCLC tissues contributes to the development of lung carcinoma through a similar mechanism. Adenocarcinoma and squamous cell carcinoma are two major subtypes of NSCLC. Adenocarcinoma is the predominant histological type of lung cancer among females and nonsmokers, while squamous cell carcinoma is mostly found in males and smokers [31]. Interestingly, we found that the expression of BMP5 was significantly higher in adenocarcinoma and female patients than that in squamous cell carcinoma and male patients. Smoking is considered as a risk factor for lung tumor. In this study, all female patients were nonsmokers and diagnosed as adenocarcinoma, and almost all the male patients were smokers. It remains unclear whether BMP5 is differentially expressed between male smokers and nonsmokers. EGFR and K-Ras mutations are frequently found in adenocarcinoma, whereas simultaneous inactivation of LKB1 and PTEN in mice results in development of squamous cell carcinoma [32–34]. Further investigations are needed to determine whether the differential expression of BMP5 is linked to the mutations in patients and mouse models.

In summary, we have demonstrated that higher BMP5 expression is associated with female patients and lung adenocarcinoma, although the overall expression of BMP5 is inhibited in NSCLC tissues compared with adjacent normal tissues. These findings suggest that BMP5 may be a biomarker of diagnosis of NSCLC and a drug target for the treatment of NSCLC.

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