

Expression of bone morphogenetic protein 6 in non-small cell lung cancer and its significance

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Abstract. The present study investigated the expression and clinical significance of bone morphogenetic protein 6 (BMP-6) in patients with non-small cell lung cancer (NSCLC). The tumor and adjacent normal lung tissues were harvested from 65 patients with NSCLC. BMP-6 mRNA expression was measured by reverse transcription-quantitative polymerase chain reaction, while protein expression was measured using immunohistochemistry or an ELISA. Cell viability was determined using Cell Counting Kit-8. The association of BMP-6 mRNA expression with the prognosis of patients with NSCLC was analyzed using the Kaplan-Meier plotter database. BMP-6 mRNA expression in NSCLC tumor tissues was significantly reduced, compared with the adjacent normal lung tissues ($P < 0.001$), yet no significant differences were observed between patients with different clinicopathological features ($P > 0.05$). The expression level of BMP-6 protein in NSCLC tumor tissues was significantly reduced, compared with the adjacent normal lung tissues ($P < 0.05$). Analysis with the Kaplan-Meier plotter database revealed that patients with NSCLC with low BMP-6 mRNA expression had a reduced overall survival rate ($P < 0.01$). The active BMP6 protein significantly inhibited cell proliferation in H460, H1299, A549 and H520 cells. In conclusion, BMP-6 is a tumor suppressor in lung cancer and loss of BMP-6 expression is significantly associated with a poor prognosis in patients with NSCLC.

Introduction

Lung cancer was reported as one of the most common malignancy types in the United States in 2016 (1). Non-small cell

lung cancer (NSCLC) is the most common tissue subtype of lung cancer, accounting for ~80% of all lung cancer cases (2). Approximately 75% of patients were diagnosed at the middle and late clinical stage in Australia, Canada, Denmark, Norway, Sweden and the UK, which contributed to the low 5-year survival rate (3). Therefore, it is important to identify biomarkers for the early diagnosis, predicting the prognosis, and identifying a targeting therapy of NSCLC.

Bone morphogenetic protein 6 (BMP-6) belongs to the transforming growth factor- β superfamily, which is not only involved in the growth and development of normal tissues, but is also associated with the development of various tumor types, including colorectal cancer, salivary adenocarcinoma, breast cancer, liver cancer and prostate cancer (4). An *in vitro* study demonstrated that BMP-6 was inactivated in lung cancer cells (5). However, the expression of BMP-6 in the tumor tissues of NSCLC and its clinical significance have not been well documented. In the present study, reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR) and immunohistochemistry were performed to measure the expression of BMP-6 mRNA and protein in NSCLC tumor and tumor adjacent normal lung tissues, while the Kaplan-Meier plotter database was used to analyze the prognostic value of BMP-6 mRNA in patients with NSCLC.

Materials and methods

Specimens. A total of 65 NSCLC tumor tissues and their adjacent normal lung tissues were collected between January 2016 and January 2017 at The Second Xiangya Hospital (Changsha, China) by surgical resection for RT-qPCR analysis. The patients included 41 males and 24 females with a mean age of 57.6 years (range, 18-74 years). A total of 73 paraffin-embedded NSCLC and paired adjacent normal lung tissues were provided by the Department of Pathology of The Second Xiangya Hospital and used for immunohistochemistry. Tissues were obtained from 43 male and 30 female patients with a mean age of 58.2 years (range, 19-75 years). The inclusion criteria were as follows: Definitive diagnosis of NSCLC, aged between 18 and 75 years, stage I to stage III tumor, and Tumor Node Metastasis classification (6) data were available. The pathological data, including tumor type, tumor differentiation degree, tumor size, lymph node metastasis and clinical stage, were also collected. The

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protocol of this study was reviewed by the Ethics Committee of Human Study of the Second Xiangya Hospital (Changsha, China). This approved study was performed in accordance with the ethical standards of the Declaration of Helsinki (as revised in Brazil 2013). Written informed consent was obtained from the subjects for use of their tissue.

RT-qPCR. The total RNA was extracted from tissues using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. cDNA was synthesized using RevertAid[™] First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-qPCR was performed using SYBR[®] Green Master mix for Real-Time RT-PCR kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. The BMP-6 gene was amplified using forward primer, 5'-CCCTTCATGGTG GCTTTCTT-3' and reverse primer, 5'-GAGCGATTACGA CTCTGGTTCTGTTGTC-3'. The BMP-6 was amplified at the following thermocycling conditions: 94°C, 1 min, followed by 28 cycles of 94°C, 30 sec; 59°C, 40 sec; and 72°C, 2 min. β -actin was amplified using forward primer, 5'-GCACCACAC CTTCTACAATGAG-3' and reverse primer, 5'-GATAGCACA GCCTGGATAGCA-3', as an internal control. Each sample was amplified 3 times and the raw data were averaged for mean. Relative gene expression was quantified using the $2^{-\Delta\Delta Cq}$ in log-10 scale method (7). The normalized log10 value of BMP-6 expression was used for statistical analysis.

Immunohistochemistry. The tissues were fixed in 10% formalin solution for 48 h at room temperature and then paraffin-embedded. Tissues were sectioned at 5 μ m. Immunohistochemical staining was performed using a Two-step Immunohistochemical Staining kit (OriGene Technologies, Inc., Beijing, China) according to the manufacturer's protocols. After dewaxing, rehydration in descending alcohol series (100-70%), antigen repair at 98°C for 20 min in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0), and blocking in 10% donkey serum (Sigma-Aldrich; Merck KGaA, Darmstadt, German) in tris-buffer for 2 h at room temperature, sections were incubated with rabbit anti-human BMP-6 monoclonal antibody (dilution, 1:50; cat. no. ab101056; Abcam, Cambridge, UK) overnight at 4°C. Following rinsing with PBS three times for 30 sec each, the sections were incubated with horseradish peroxidase-conjugated goat-anti rabbit secondary antibody (dilution, 1:1,000 dilution; cat. no. ab6721; Abcam) for 2 h at room temperature. After the slices were subject to 3,3'-diaminobenzidine treatment for 5 min at room temperature, hematoxylin staining for 30 sec at room temperature and dehydration, the slices were then sealed and observed under a light microscope. The normal prostate tissue slices provided with the kit were used as a positive control and PBS instead of the primary antibody was used as the negative control. The staining was scored by two experienced pathologists in the Department of Pathology of The Second Xiangya Hospital. The brown granules indicated positive staining of BMP-6. The whole slice was observed with a magnification of x40 to determine the tumor infiltration edge. A total of 10 randomly selected fields of view were selected under a high magnification (x400) and 100 tumor cells were counted for each field. The intensity of staining was scored as 1 for negative, 2 for positive and 3 for

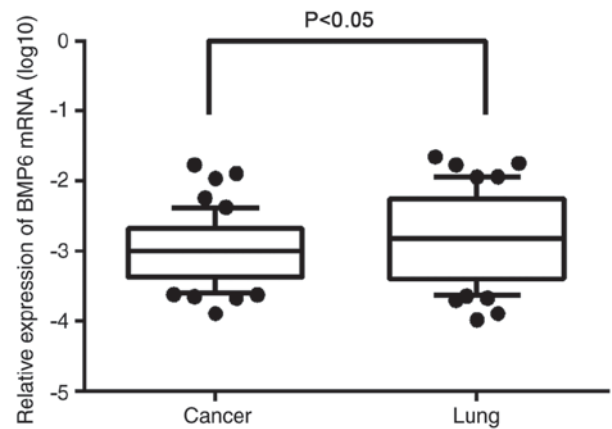


Figure 1. BMP6 mRNA expression in NSCLC tissues and normal lung tissues. mRNA expression of BMP6 in 65 NSCLC and adjacent normal lung tissues was measured by reverse transcription-quantitative polymerase chain reaction (n=65). BMP6, bone morphogenetic protein 6; NSCLC, non-small cell lung cancer; cancer, NSCLC tissues; lung, normal lung tissues.

strong positive. The percentage of positively stained cells (the score was 2 or 3) was also calculated. A tissue was defined as negative staining (-) when the tissue was scored 1 or <10% cells were scored 2 or 3, weakly positive staining (+) when 10-30% cells were scored 2 or 3, positive staining (++) when 31-50% cells were scored 2 or 3 and strongly positive staining (+++) when >50% cells were scored 2 or 3.

Survival analysis. The Kaplan-Meier plotter online database (<http://kmplot.com/analysis/index.php?p=service&cancer=lung#>) was computationally used to analyze the association between BMP-6 mRNA expression and overall survival of patients with NSCLC. The Kaplan-Meier survival curve, hazard ratio (HR), and log-rank P-value were obtained. The high expression group was defined when the BMP-6 mRNA expression in the patient was higher or equal to the median mRNA expression of all NSCLC samples. The low expression group was the patients with BMP-6 mRNA expression lower than the median.

Cell culture. The human lung cancer cell lines including H125, A549, H23, H460, H520, H1299, PC9 and Human Bronchial Epithelial Cell (HBE) were purchased from the Shanghai Institute for Biological Sciences (<http://english.sibs.cas.cn/rs/fs/>). Cells were cultured in Dulbecco's modified Eagle medium or RPMI-1640 medium with 10% fetal calf serum. (Thermo Fisher Scientific Inc.) in 5% CO₂ at 37°C.

Cell proliferation assay. H460, H1299, A549, and H520 cells (1x10⁴) were seeded in 96-well plates overnight and treated without (control), or with 5 or 50 ng/ml active BMP-6 recombinant protein (GeneTex, Inc., Irvine, CA, USA) for 48 h. Cell viability was determined using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA), according to the manufacturer's protocol. The plates were read at microplate reader at 450 nm and the optical density value was normalized with the control.

ELISA of BMP-6 concentration in cell supernatant. The supernatant of BMP-6 in the culture of all tested cells was

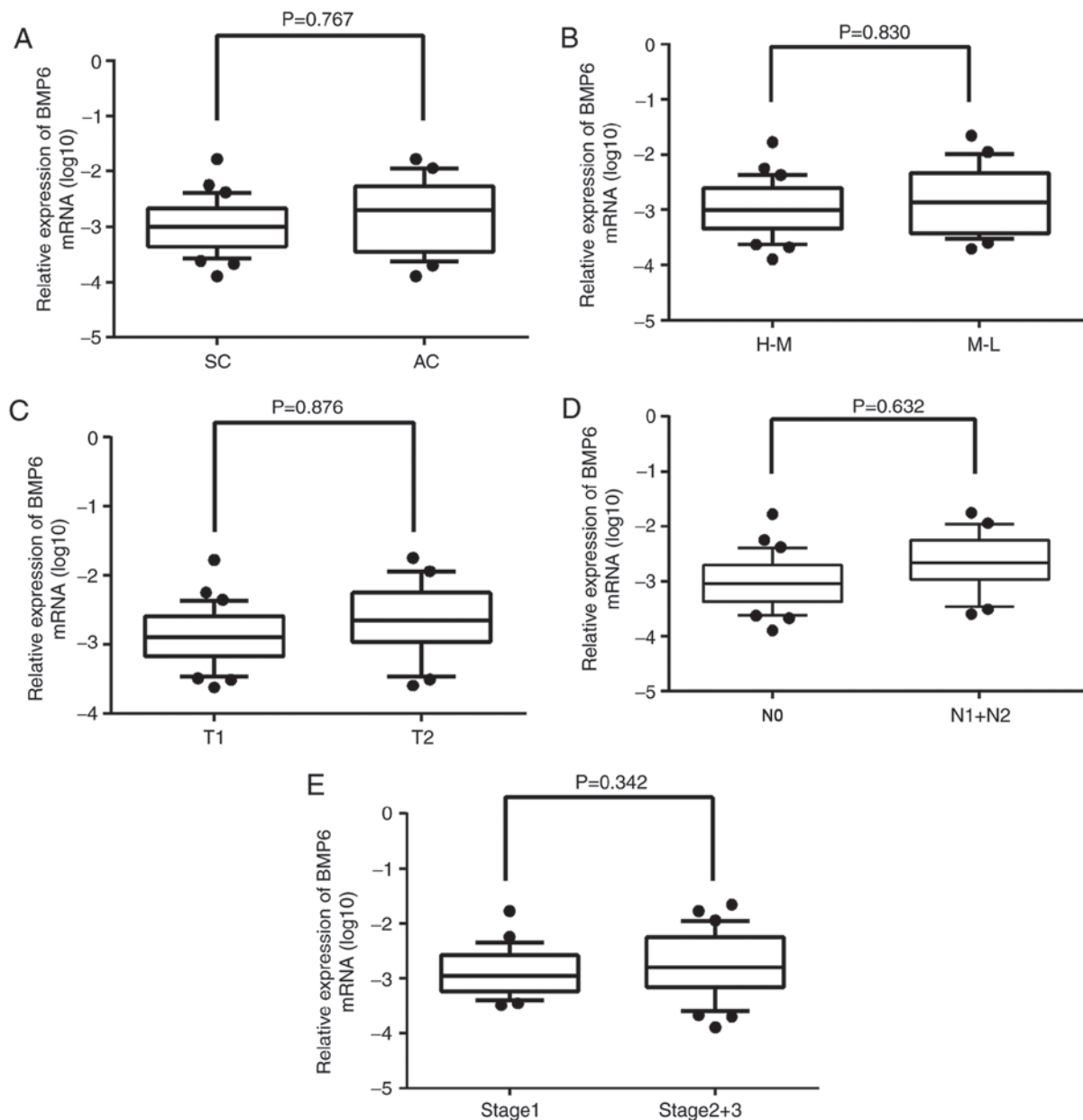


Figure 2. Comparison of BMP6 mRNA expression levels in patients with non-small cell lung cancer with different pathological features. (A) Comparison of BMP6 mRNA expression between patients with SC and AC. (B) Comparison of BMP6 mRNA expression between patients with high to middle tumor differentiation and middle to low differentiation. (C) Comparison of BMP6 mRNA expression between patients with tumor <2 cm (longest diameter) (T1) and tumor being 2-5 cm (T2). (D) Comparison of BMP6 mRNA expression between patients without lymph node metastasis (N0) and patients with 1-3 (N1) and 4-9 (N2) axillary (underarm) lymph node metastasis (N1). (E) Comparison of BMP6 mRNA expression between patients with stage I and stage II/III disease. BMP6, bone morphogenetic protein 6; SC, squamous lung cancer; AC, adenocarcinomas; H-M, high to middle differentiation; M-L, middle to low differentiation; T1, tumor is <2 cm or less (longest diameter); T2, tumor is 2-5 cm; N0, cancer not spread; N1, cancer has spread to 1-3 axillary (underarm) lymph nodes; N2, cancer has spread to 4-9 lymph nodes (n=73).

measured using a Human BMP-6 ELISA kit (cat. no. ab99984; Abcam), according to the manufacturer's protocol.

Statistical methods. The data were analysed using SPSS 18.00 (SPSS, Inc., Chicago, IL, USA) and were presented as the mean \pm standard error of the mean. The Mann-Whitney U and Kruskal-Wallis test were used to analyze the data for two groups and multiple groups, respectively. The Nemenyi test was used for a post-hoc test for multiple comparisons. While the Pearson χ^2 test was used to analyze the data of immunohistochemistry. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of BMP-6 mRNA in NSCLC and its associations with clinicopathological features. The expression of BMP-6 mRNA in NSCLC and adjacent tissues was detected by RT-qPCR. The level of BMP-6 mRNA in NSCLC tissues was significantly reduced, compared with adjacent normal lung tissues ($P < 0.05$; Fig. 1). There were no significant differences in BMP-6 mRNA expression between NSCLC patients with different tumor types, tumor differentiations, tumor size, clinical stage, and with or without lymph node metastasis ($P > 0.05$; Fig. 2).

Table I. BMP-6 protein expression in NSCLC and adjacent tissues.

Tissue type	Case no.	BMP-6 expression				Pos rate (%)	Neg rate (%)
		-	+	++	+++		
Adjacent lung tissues	73	8	16	35	14	89.04 ^a	10.96
NSCLC	73	54	19	0	0	26.03	73.97
χ^2			0.34	46.04	15.49		59.32
P-value			0.56	<0.001	<0.001		<0.01

^aP<0.05. Pos, positive; Neg, negative; BMP-6, bone morphogenetic protein 6; NSCLC, non-small cell lung cancer; -, negative staining, when the tissue was scored negative or <10% cells were scored positive or strong positive; +, weakly positive staining, when 10-30% cells were scored positive or strong positive; ++, positive staining, when 31-50% cells were scored positive or strong positive; +++, strongly positive staining, when >50% cells were scored positive or strong positive.

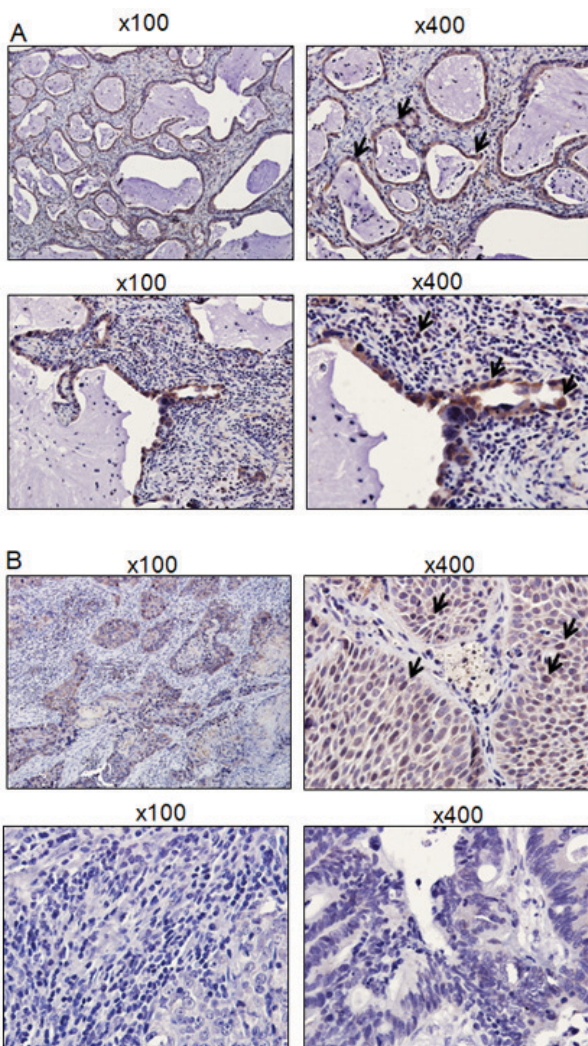


Figure 3. Bone morphogenetic protein 6 expression in NSCLC and adjacent lung tissues. (A) Adjacent normal lung and (B) NSCLC tissues with magnification, x100 and x400. Brown granules represent a positive staining and are indicated by arrows. NSCLC, non-small cell lung cancer.

Expression of BMP-6 protein in NSCLC tissues. Immunohistochemical staining of BMP-6 expression in 73 NSCLC and adjacent normal lung tissues revealed that BMP-6 was positively expressed in a number of lung cancer

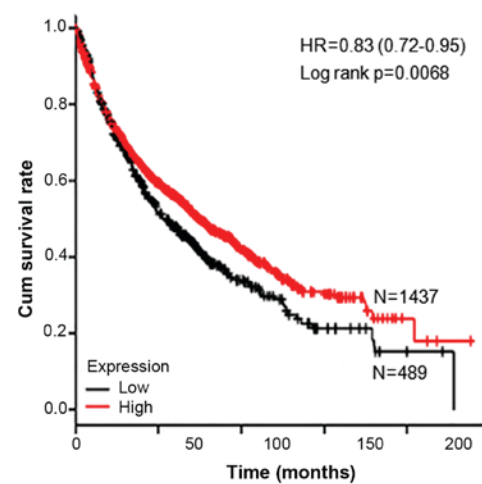


Figure 4. The association between the prognosis of patients with non-small cell lung cancer and the BMP-6 mRNA level. High and low expression means higher or equal, and lower than the median BMP-6 mRNA expression in all samples, respectively. BPM6, bone morphogenetic protein 6.

tissues and negatively expressed in the majority of tumor tissues (Fig. 3). The positive rate of BMP-6 expression in 73 lung cancer tissues was 26.03% (19/73). In normal lung tissue, BMP-6 expression was positively expressed in the majority of the samples. The positive rate of BMP-6 expression in 73 adjacent normal lung tissues was 89.04% (65/73). The expression of BMP-6 was significantly reduced in NSCLC tissues, compared with adjacent lung tissues ($\chi^2=59.32$; P<0.001; Table I).

Association of BMP-6 mRNA levels with the prognosis of patients with NSCLC. The association of BMP-6 mRNA expression with the overall survival was analyzed using the Kaplan-Meier plotter online database. The database contained a total of 1,926 cases of NSCLC with available data for BMP-6 mRNA expression and overall survival rate. The online analysis using the Kaplan-Meier plotter database calculated a HR value of 0.83, and log-rank P-value of 0.0068, indicating that BMP-6 mRNA level can function as a predictive factor, and that reduced BMP-6 mRNA expression (lower than the median) is associated with a poor prognosis of patients. The Kaplan-Meier survival curve was presented in Fig. 4.

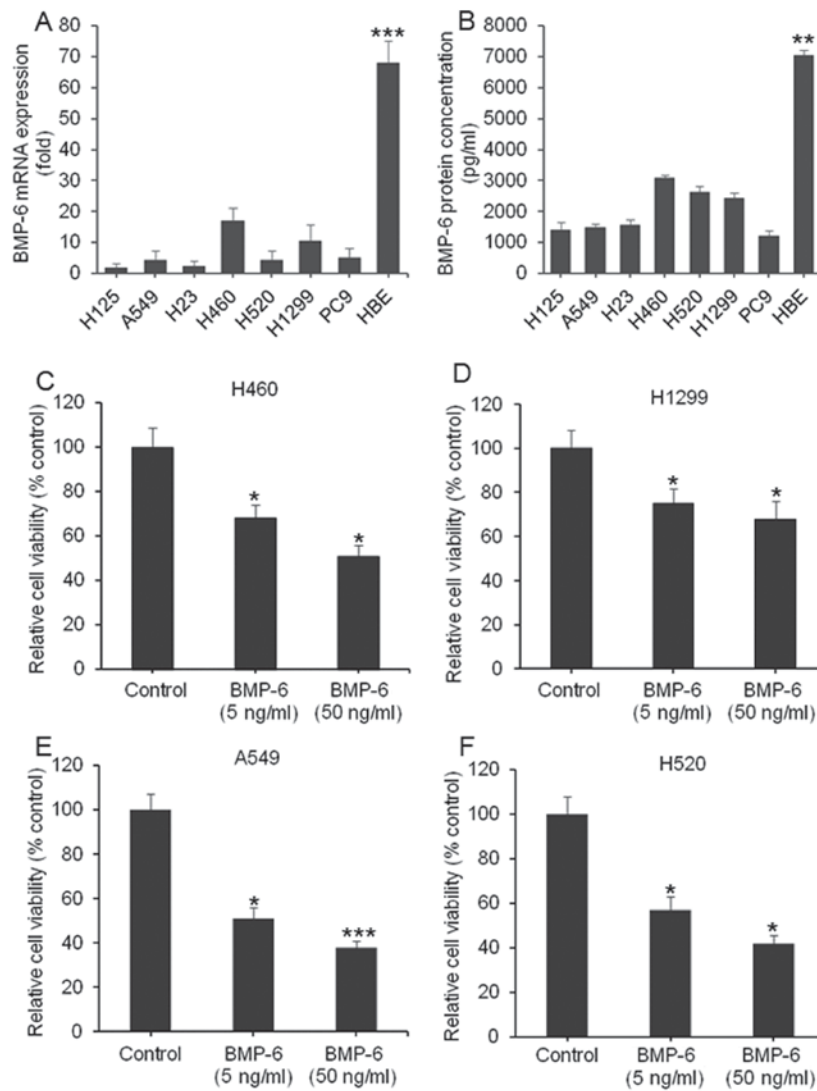


Figure 5. BMP-6 expression and effect in human lung cancer cells. (A) The expression of BMP-6 mRNA in H125, A549, H23, H460, H520, H1299 and PC9 lung cancer, and HBE cells was measured by reverse transcription-quantitative polymerase chain reaction. ***P<0.001 vs. all other groups. n=4. (B) The concentration of BMP-6 protein in the supernatant of cultured cells was measured by ELISA. **P<0.01 vs. all other groups. n=4. BMP-6 recombinant protein significantly inhibited cell proliferation in (C) H460, (D) H1299, (E) A549 and (F) H520 cells. BMP6, bone morphogenetic protein 6; HBE, human bronchial epithelial. *P<0.05 and ***P<0.001 vs. control group. n=4.

BMP-6 inhibits the proliferation of human lung cancer cells. The expression of BMP-6 mRNA in cells (Fig. 5A) and the concentration of BMP-6 protein in the supernatant of cultured cells (Fig. 5B) were measured. The mRNA and protein expression of BMP-6 were significantly decreased in lung cancer cells, compared with that in HBE cells. To further validate the role of BMP6 in lung cancer cells, the H460, H1299, A549 and H520 cells were treated with active BMP6 recombinant protein for 48 h, where it was revealed that 5 or 50 ng/ml BMP-6 protein significantly inhibited cell proliferation in H460 (Fig. 5C), H1299 (Fig. 5D), A549 (Fig. 5E) and H520 (Fig. 5F) cells, compared with the control group. These data indicate that BMP-6 is a suppressor in human lung cancer cells.

Discussion

Previous studies reported that BMPs may promote lung cancer growth or have an anti-cancer effect. For example, BMPs can regulate the secretion of various cytokines to enhance

proliferation and differentiation of lung cancer cells (8). In contrast, BMPs may also exhibit an inhibitory role in lung cancer. For instance, an immunohistochemical study in 35 lung squamous cell carcinoma samples revealed that the expression of BMP4 in the tumor tissues was decreased (9). BMP-6 is a multifunctional growth and differentiation regulatory factor, which is not only involved in tissue formation and development, and is also associated with tumor progression (10,11). A previous study detected a high BMP-6 expression in prostate and breast cancer, and salivary adenocarcinoma (12). Conversely, other studies demonstrated that BMP-6 inhibited the growth of breast cancer, plasmacytoma, renal cell carcinoma, adrenocortical cancer, skin cancer and myeloma (13-18). BMP-6 has been revealed to be inactivated in lung cancer cells (5). In the present study, RT-qPCR revealed that the expression of BMP-6 mRNA in NSCLC tissues was significantly reduced, compared with adjacent normal lung tissues. The results of immunohistochemical staining demonstrated that BMP-6 protein expression in NSCLC tissues was

significantly reduced, compared with adjacent normal lung tissues. The active BMP-6 recombinant protein significantly inhibited cell proliferation in cultured lung cancer cells. Collectively, these data indicate that BMP-6 is a suppressor in human lung cancer cells.

Previous studies reported that the BMP-6 gene was hypermethylated in lung cancer (19), breast cancer (20), malignant lymphoma (21), malignant pleural mesothelioma (22) and adult T-cell leukemia (23). Hypermethylation may inhibit gene transcription and reduce the protein expression, and can result in the inactivation of tumor suppressor genes (24).

Although the present study did not measure the methylation of the BMP6 gene, the mRNA and protein expression of BMP-6 gene was identified to be significantly decreased in the tumor tissues of NSCLC and seven lung cancer cells, compared with adjacent normal lung tissues and human bronchial epithelial cells, respectively. Furthermore, the BMP-6 protein significantly inhibited cell proliferation in the 4 examined lung cancer cell lines. The present study indicates that the loss of BMP-6 expression may be a crucial factor associated with tumor growth in NSCLC.

The Kaplan-Meier plotter database is a prognostic associated online analytical database. This database contains 10,188 tumor samples (4,142 cases of breast cancer, 1,648 cases of ovarian cancer, 2,437 cases of lung cancer and 1,065 cases of gastric cancer) and can analyze the association of 54,675 genes with the prognosis of these patients with cancer (25,26). The present study first used the Kaplan Meier plotter database to analyze the prognostic value of the BMP-6 gene in patients with NSCLC. The results demonstrated that low mRNA expression of the BMP-6 gene was associated with a poor prognosis in patients with NSCLC. However, data regarding the mRNA level require further verification at the protein level, although via ELISA, the BMP-6 protein was significantly reduced in 73 NSCLC tissues, compared with adjacent normal tissues.

In conclusion, BMP-6 expression is reduced in NSCLC tumor tissues indicating that it serves an inhibitory role in the development of the disease and is a predictive factor of poor prognosis in patients with NSCLC.

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Availability of data and materials

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

Authors' contributions

WX conducted the experiments. LW collected the patients' clinical data and performed statistical analysis. FY designed the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of this study was reviewed by the Ethics Committee of Human Study of the Second Xiangya Hospital. This approved study was performed in accordance with the ethical standards of the Declaration of Helsinki (as revised in Brazil 2013). Written informed consent was obtained from the subjects for use of their tissue.

Patient consent for publication

Not applicable.

Competing interests

All authors declared that they have no competing interests.

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