Molecular impact of bone morphogenetic protein 7, on lung cancer cells and its clinical significance

YINAN LIU1,2, JINFENG CHEN1,2, YUE YANG1, LIJIAN ZHANG1 and WEN G. JIANG2

1Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Thoracic Surgery II, Peking University Cancer Hospital and Institute, Beijing 100142, P.R. China; 2Metastasis and Angiogenesis Research Group, Cardiff University School of Medicine, Heath Park, Cardiff, CF14 4XN, UK

Received January 9, 2012; Accepted February 15, 2012

DOI: 10.3892/ijmm.2012.948

Abstract. The aim of this study was to investigate the expression of bone morphogenetic protein 7 (BMP7), in human pulmonary cancer tissues/cells and to evaluate the cellular impact of bone morphogenetic proteins on pulmonary cancer cells. BMP7 expression was determined in human lung cancer cell lines. The invasiveness and growth of cells transfected with BMP7, in vitro, were evaluated using the in vitro invasion assay and in vitro tumour models. Cellular migration was analysed using wound assays. BMP7-positive tumours correlated with the absence of bone metastasis (P=0.040). In this analysis, we identified that 4 of 4 small cell lung cancer (SCLC) tissue specimens had no BMP7 expression, which illustrated that BMP7 may have no role in SCLC. BMP7 expression was not correlated with the overall survival time in lung cancer patients. Downregulation of BMP7 expression significantly inhibited the invasiveness of SPC-A1 cells (P<0.001) and forced-expression of BMP7 dramatically increased the motility of A549 cells. Overexpression of BMP7 in A549 cells and its knockdown in SPC-A1 cells did not significantly alter proliferation compared with the control cells (P>0.5 respectively). In conclusion, we have demonstrated that BMP7 has an important role in controlling lung cancer cell motility and invasiveness, without affecting the growth process, cell proliferation and cell apoptosis. A higher BMP7 expression may be an indicator for bone metastasis. The therapeutic role of BMP7 warrants further investigation.

Introduction

Lung cancer is one of the most fatal tumours worldwide. Distant metastasis is an important cause for the poor prog-

Correspondence to: Dr Jinfeng Chen, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Thoracic Surgery II, Peking University Cancer Hospital and Institute, Beijing 100142, P.R. China
E-mail: chengjinfeng123@163.com

Key words: bone morphogenetic protein 7, lung cancer, bone metastasis, apoptosis, cell migration, Tet-inducible expression
differing levels of BMP receptor expression. However, BMP7 was found to have an important role in controlling lung cancer cell motility. The most demonstrable effects of BMP7 on lung cancer cells were its inhibitory effects on matrix adhesion, motility and in vitro invasiveness by activating an intracellular signaling pathway involving alterations in crosstalk between SMAD/AR and the Wnt signaling pathways. In the present study, we investigated the biological function of BMP7 in lung cancer cells and explored the possible link between BMP7 and bone metastasis in patients with lung cancer.

Materials and methods

Immunohistochemistry. Seventy-five fixed and paraffin-embedded primary pulmonary cancer samples were acquired from the Department of Pathology, Peking University. Ethic approval was given by the local Research Ethics Committee. Sections (5 µm) were routinely processed, and stained with a mouse monoclonal anti-BMP7 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a concentration of 2 µg/ml, followed by the incubation with HRP-conjugated rabbit anti-mouse secondary antibody (Sigma-Aldrich, Poole, Dorset, UK). The visualization of antibody staining was developed using a DAB HRP Substrate-Chromogen 2 liquid component kit with haematoxylin counterstaining. For determination of BMP7 immunoreactivity, cytosolic staining of yellowish or brownish granules was graded as follows: 0, for background staining; 1, for negative staining; 2, for moderate staining; and 3, for strong staining. In addition, positive staining areas in the entire tissue section were graded as follows: 0, for <5%; 1, for 5-25%; 2, for 26-50%; 3, for 51-75%; and 4, for 76-100%. When combining these two parameters, 0-2 and >2 were considered negative or moderate and positive staining, respectively (Fig. 1).

Cell culture and supplements. A549 (BMP7-negative) and SPC-A1 (BMP7-positive) lung cancer cell lines were maintained under standard cell culture conditions in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich). Tissue culture media and supplements were obtained from Invitrogen (Pasley, UK).

Silencing of BMP7 expression by shRNA in SPC-A1 cells and BMP7 overexpression in A549 lung cancer cells. A recombinant human BMP7 inducible expression system (Tet-On system) was purchased from R&D Systems. Sequences of BMP7 shRNA (Table I) were inserted into the pGPU6/GFP/Neo vector. Pulmonary tumour SPC-A1 cells were cultured in a cell culture flask containing DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 4 mM L-glutamine. Lipofectamine™-mediated transfection was performed according to the manufacturer's instructions. Transfection efficiency was monitored by the expression of GFP protein. The target sequence of BMP7 shRNA is listed in Table I. The mRNA and protein levels of BMP7 were measured at 72 h post-infection.

We constructed a eukaryotic expression system for human BMP7. The full length BMP7 gene sequence was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the primers listed in Table I. The PCR product was T-A cloned into a pEF6/v5-His cloning vector (Invitrogen) which has an elongation factor α promoter (EF6) and the blasticidin resistance gene (for mammalian cells) and the ampicillin resistance gene (for prokaryotic cells). Plasmids were used to transfect A549 cell line with Lipofectamine. A549 cells with stable BMP7 expression were selected with blasticidin (5 µg/ml) for up to 2 weeks.

The BMP7 full length gene was inserted into the Tet-On system. The BMP7 gene containing vector was transfected together with pHelper1.0 and the lentiviral helper plasmid pHelper2.0 to generate the respective lentiviruses into 293T cells. Viral particles were collected from the cell culture media and used to transduce A549 cells. The mRNA and protein levels were measured after the induction with different concentrations of doxycycline (DOX).

RT-PCR. Total cellular RNA was extracted using the TRIzol reagent following the manufacturer's protocol, and genomic DNA was digested by DNase I. cDNAs were synthesized with 2 µg of total-RNA using the iScript cDNA synthesis kit (Bio-Rad, Hemel Hempstead, UK). Primers for BMP7 and GAPDH are listed in Table I. GAPDH was used as a loading control.

Table I. Primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>shRNA for BMP7</td>
<td>Sense</td>
<td>GATCCAAAAAAGGTTTGATCTTTTCAAGATCTCCTCTTTGAAGG ATCTTGGAAGATCAAACCA</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CACCCGTTTGGACTTTTCAAGATCTCCTTCAAGAGGATCTTTGG AAAGATCAAACCTTTTITGG</td>
</tr>
<tr>
<td>Full length BMP7</td>
<td>Forward</td>
<td>ATGCACGTGGCCTCCTGCTGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTAGTGGCCAGCCACAGGCCGCG</td>
</tr>
<tr>
<td>BMP7</td>
<td>Forward</td>
<td>GAATTTCTCCACCACCCAGCTACC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGATGTCAAACACCAGGCCAGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>AGGTCGGAGTCAACGGATTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTGATGGCATTGGACTTGTTG</td>
</tr>
</tbody>
</table>
Western blot analysis. Whole cell lysates and cytoplasmic proteins were prepared. Proteins were separated on SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat dried milk in TBST (15 mM Tris-HCl, pH 7.4, 0.9% NaCl, and 0.05% Tween-20, pH 7.4) followed by blot analysis with specified antibodies: mouse monoclonal anti-human BMP7 antibody (MAB3541, 1:5,000; R&D Systems); and rabbit anti-mouse secondary antibody (1:1,000). Immunoreactive bands were detected using SuperSignal West Femto Chemiluminescent Substrate. Experiments were performed at least twice with consistent results.

Cell migration assay. Transwell invasion assays were performed following the manufacturer's guidelines. Briefly, 5x10^4 cells in DMEM + 0.1% BSA were plated in 24-well plates with DMEM + 5% FBS as a chemoattractant. After 24 h, cells that had migrated through and adhered to the other side of the insert were fixed and stained with 0.5% (w/v) crystal violet. Invading cells on the bottom of the filters were imaged by fluorescence microscopy. Five high-power fields were counted per filter to score for invasion. The cell number was quantified with the ImageJ software.

Wound healing assay. Cells were cultured to confluence in a plate before scratching with a 200 µl pipette tip. Debris was removed by extensive washing with PBS and the cells were further incubated for another 48 h. The closure of the induced wound, through the migration of cells, was tracked and recorded over a 36-h period at five different locations under x100 magnification by a microscope and analyzed using the Metamorph version 7.0 software.

Colony formation assay. Cells were seeded into plates at a density of 500 cells/plate. Triplicate plates were set up for fortnight incubation periods. Following sufficient incubation, the plates were fixed in 4% formaldehyde and stained with 0.5% crystal violet, allowing for the detection of cell density by spectrophotometric analysis of the resulting solutions. Absorbance was measured using a Bio-Tek EL9 800 multiplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Flow cytometry analysis. To detect intact, necrotic and apoptotic cells, flow cytometry was performed. A549 and SPC-A1 cells were incubated for 72 h at 37°C in 5% CO_2. After 24 h of incubation, cells were harvested and assayed for apoptosis using the Annexin V-FITC apoptosis detection kit, Imgenex, according to manufacturer's instruction. Cells were analyzed in FACSCalibur Analyzer (Becton-Dickinson) using the CellQuest Pro software.

Statistical analysis. Statistical significance was determined by the Mann-Whitney test for continuous variables and by the χ^2 or Fisher's exact test or one-way ANOVA test for categorical variables. P-values <0.05 were considered statistically significant. Statistical tests were performed using the software SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical and prognostic analysis of BMP7 gene expression in lung cancer. Normal and tumour lung tissues had different profiles of BMP7 staining. Strong (Fig. 1A-D), weak (Fig. 1G and H) and negative (Fig. 1E and F) stainings were seen in human lung tumour tissues. The relationship between BMP7 expression and the status of bone metastasis was analyzed. All tumours from the five patients who had bone metastasis were negative for BMP7 (Table II). This was significantly different from tumours from patients who did not develop bone metastasis (P=0.040) (Table II), indicating that BMP7 may have some role in the bone metastasis process of lung cancer cells. We also observed that all 4 small cell lung cancer (SCLC) tissue specimens had no BMP7 expression, illustrating that BMP7 may have a less important role in SCLC. We also compared the expression status of BMP7 according to the main tumour types, tumor status, lymph node status and vessel status in the cohort. In the cohort of lymph node status, N0 tumours had a trend of low positive BMP7 expression, but this did not reach statistical significance (P=0.161).

Figure 1. BMP7 staining in human lung cancerous and paracancerous tissues. (A-D) Strong staining of BMP7 protein on pulmonary cancerous tissue specimens. (E and F) Negative expression of BMP7 protein on pulmonary cancerous tissue specimens and (G and H) moderate expression on paracancerous tissue. (Original magnification, x100 and x400).
BMP7 expression was not correlated with the overall survival time in lung cancer patients. The current cohort was monitored during a period of 48 months follow-up. After 48 months of follow-up, patients were analysed regarding the survival time based on the BMP7 expression level in cancer tissue. The mean (SD) survival time was 33.58±2.714 months. Cumulative survival curves were calculated using the Kaplan-Meier method. In patients with a low level of BMP7 expression (negative or moderate staining) in cancer tissues, the mean survival time was 32.184 (95% CI, 28.6-35.7) months. In patients with a high expression level of BMP7 (strong staining) in cancer tissues, the mean survival time was 30.1 (95% CI, 19.2-40.9) months. The expression status of BMP7 was not correlated with the overall survival time (P=0.374) (Fig. 2).

Modification of BMP7 expression by shRNA or inducible Tet-On system in lung cancer cells. To investigate the biological role of BMP7 gene in lung cancer cells, in vitro, we knocked down the BMP7 transcript in the human lung cancer cell line SPC-A1 by the shRNA constructed Pgpu6/GFP/Neo vector. As shown in Fig. 3, we designed four shRNAs targeting sites 1053, 888, 999 and 1198. RT-PCR and western blot analysis showed that shRNA designed for site 999 had the highest knockdown efficiency in comparison with the other sequences (Fig. 3B). So we chose this shRNA for further investigation.

In the contrary, overexpression of BMP7 was acquired in A549 cells which have low level of endogenous BMP expression (Fig. 3A). A549 cells were transfected with the BMP7 inducible expression system (Tet-On system) and successfully expressed BMP7 protein with treatment with different concentrations of DOX as shown by RT-PCR and western blot analysis showed that shRNA designed for site 999 had the highest knockdown efficiency in comparison with the other sequences (Fig. 3B). So we chose this shRNA for further investigation.

Table II. Clinicopathological information of the study cohort.

<table>
<thead>
<tr>
<th>Clinicopathological feature</th>
<th>Cases</th>
<th>BMP7 positive cases</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>31</td>
<td>7</td>
<td>0.543</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>36</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Other type</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tumor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1+T2</td>
<td>51</td>
<td>8</td>
<td>0.254</td>
</tr>
<tr>
<td>T3+T4</td>
<td>24</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>33</td>
<td>4</td>
<td>0.161</td>
</tr>
<tr>
<td>N1+N2</td>
<td>42</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Bone metastasis status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone metastasis</td>
<td>5</td>
<td>0</td>
<td>0.040</td>
</tr>
<tr>
<td>No bone metastasis</td>
<td>52</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Bone scan abnormity</td>
<td>18</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Vessel status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vessel carcinoma embolus</td>
<td>15</td>
<td>5</td>
<td>0.107</td>
</tr>
<tr>
<td>No vessel carcinoma embolus</td>
<td>60</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Expression of BMP7 in cancer tissue was not correlated with prognosis. Kaplan-Meier analysis of overall survival of lung cancer patients is not correlated with the expression status of BMP7 protein (P=0.05).
inhibited the invasiveness of SPC-A1 cells (Fig. 4) (P<0.001). Overexpression of BMP7 through transfection with a BMP7 expression plasmid significantly increased the invasiveness of A549 cells compared with control-transfected cells (P=0.003). Dox (10, 100 and 1000 ng/ml) induced BMP7 expression in a dose-dependent manner (Fig. 3Ca). In accordance, the levels of BMP7 expression were significantly correlated with the invasiveness of the A549 pulmonary cancer cells (P<0.001, respectively).

Effect of the BMP7 gene on lung cancer wounding closure ability in vitro. To investigate whether the expression of BMP7 could influence the motility of lung cancer cells, the A549 cells were transfected with BMP7 expression plasmid, and cell motility was monitored by the wound healing assay. Forced-expression of BMP7 dramatically increased the motility of the A549 cell line; and from an early time point of 6 h to the end of the experiments, a noticeable difference was seen compared with control cells (P<0.001). A statistically significant difference among BMP7 induced expression A549 cells became apparent approximately half way through the experiment at the 12-h time point (P<0.001). In contrast, BMP7 downregulation decreased motility of SPC-A1-999 pulmonary tumour cells compared to SPC-A1 cells, which indicated migration of BMP7-iRNA transfected cells. However, the results were not statistical different (P=0.12) (Fig. 5).
Effect of the BMP7 gene on lung cancer colony forming ability in vitro. The in vitro colony forming assay showed a significant higher rate of growth in BMP7 forced-expressing pulmonary A549 tumour cells, compared with A549\textsuperscript{wt} and A549-nc cells, which affected A549 colony formation (P<0.001). BMP7-induced expression in the A549 cell line resulted in a compared gradual increased capacity of this cell line to form colonies (P<0.001, respectively). In contrast to overexpression, BMP7 downregulation of orthotopically implanted SPC-A1-999 cells decreased the growth compared with SPC-A1-nc pulmonary cancer cells (P=0.001) (Fig. 6).

BMP7 expression does not affect lung cancer growth. Overexpression of BMP7 in A549 and under-expression in SPC-A1 cells did not significantly alter proliferation compared with the control cells (P>0.5, respectively) (data not shown). Assays were then carried out using flow cytometry with Annexin V antibodies to assess the apoptotic ratio. Overexpression of BMP7 in A549 cells and knocking down of endogenous BMP7 in SPC-A1 did not affect the proportion of apoptotic cells (P>0.5) (data not shown).

Discussion

BMP7 controls epithelial homeostasis by preserving the epithelial phenotype. New evidence suggests the importance of BMP7 in the development of an epithelial cancer and in metastatic behaviour. Aberrant BMP7 expression during tumour progression has been reported in a few cancer types, including colorectal cancer, breast cancer, melanoma, and prostate cancer (12,15,18,21-26). High levels of BMP7 have been detected in bone metastasis of prostate cancer, and breast carcinoma as they progress to a more aggressive phenotype (3,4,9,15). Upregulation of BMP7 expression in metastatic cells has been suggested to be a critical component of osteoblastic lesion development. BMP7 has been shown to have an influence on proliferation, migration and invasion, which in turn has a significant influence on the aggressiveness of pulmonary tumour cells (23). For example, Notting et al (10) have reported that BMP7 inhibits tumour growth of human uveal melanoma. Buijs et al (18) showed that loss of BMP7 expression during prostate cancer progression could contribute to the acquisition of an invasive phenotype. Similar results have been reported in breast cancer cells (17). However, exogenous BMP7 increases cell migration and the invasion process. We have recently demonstrated there was almost no BMP7 expression and different levels of BMP receptor expression in lung cancer cell lines (5).

In clinical analysis, we found there were no significant relationship between BMP7 expression status in primary pulmonary tumour and tumour stage, lymph node status, vessel carcinoma embolus status and most interestingly no correlation with the survival of the patients. However, we found a significant correlation between BMP7 expression in cancer and the bone metastasis status (P=0.04). This result thus suggest that BMP7 has some role in the process of lung...
cancer metastasis through the blood vessels, which may reflect cancer colony formation ability is important in this process. BMP7 protein may also be a useful tool in identifying the bone metastasis status.

Tissue invasion and metastasis are hallmarks of malignant tumours. Cancer cell metastasis to distant organs is the major cause of death in almost all forms of cancer. Metastasis is a multi-step process, depends not only on rapid proliferation of tumour cells, but also on other biological behaviors including motility, invasiveness, and metastatic potential (28,29). Tumour invasion is a critical step in tumour metastasis, and the understanding of this process may lead to appropriate therapies for treating cancer. BMP7 has been shown to be responsible for the increase in experimental endothelial cell metastasis and is further associated with metastatic disease in pulmonary tumour. Furthermore, it has also been shown to enhance cell invasion and migration in melanoma, and prostate as well as colon cancer cell lines, respectively (3,6,18). The same appears to be true for lung cancer cells as shown in the present study, in BMP7-transduced pulmonary tumour cells using in vitro scratch migration assays. Overexpression of BMP7 increased the cellular motility of A549 cells. A significant decrease in migrational rates was seen following downregulation of BMP7 in SPC-A1 999 cells. Collectively, the present in vitro study shows that BMP7 was positively associated with cell migration. This study also showed that upregulated expression of BMP7 was related to the invasion potentials of A549 cells, suggesting a role of BMP7 in lung cancer development and progression. In vitro overexpression of BMP7 in A549 cells caused increased colony formation, following culture on a Matrigel base, in comparison to A549 control cells. In keeping with this, a significant reduction in colony formation BMP7-downregulated and BMP7 induced expression cells were seen. Together, these data suggest that BMP7 may positively impact human pulmonary tumour growth in vitro, which may bring about a promotion in tumour development in vivo.
It has been demonstrated that BMP7 can regulate the invasion and motility of lung cancer cells, via activating certain intracellular signaling pathways (19,21,22), such as the SMAD pathway, without affecting the growth process. In keeping with this, data from the MTT cell proliferation assay showed no differences in cell viability and proliferation between control and A549 cells. BMP7 has been indicated to act as a mediator/regulator of cell survival and apoptosis in several tumour cell lines (3,6-8,13,25,26,27), including prostate cancer cells, colon cancer cells, breast cancer cells and melanoma cells and renal cancer cells. In the present study, our results from the apoptosis assay showed that BMP7 offers no significant protection against apoptosis in the tumour cells transfected with BMP7 in vitro. Meanwhile, downregulation of BMP7 detected in SPC-A1 in the study confirmed the role for BMP7 in the development of A549 by inhibiting apoptosis, indicating that the role of BMP7 in the regulation of apoptosis is tumour-type and cell-type dependent.

In conclusion, we have demonstrated that BMP7 has an important role in controlling lung cancer cell motility and invasiveness, without affecting the growth process, cell proliferation and cell apoptosis in this cell type. A higher BMP7 expression may be an indicator for bone metastasis. The therapeutic role of BMP7 warrants further investigations.

Acknowledgements

This study was supported by the National Nature Science Foundation of China (30872550) and the Beijing Institute for Cancer Research Grant 08-04. The Cancer Research Wales and the Cancer Research UK are acknowledged for their support. Y.L. is a recipient of the Albert Hung China Medical Scholarship of Cardiff University.

References


