Abstract. Background: Lung cancer is the leading cause of cancer-related death in the world, with metastasis being the main reason for the mortality. As a member of the bone morphogenetic protein (BMP) family, BMP7 has important biological functions in malignant tumours. As yet, however, there is little knowledge of the role of BMP7 and its cellular function in lung cancer. This study aimed to investigate the biological impact of BMP7 on lung cancer cells and the expression pattern of the molecule in clinical lung carcinomas. Materials and Methods: The expression pattern of BMP7 and its receptors was examined in different lung cancer cell lines and normal cells. The biological function of BMP7 in lung cancer cells was further evaluated. The impact of BMP7 on the downstream signalling, namely the activation of SMAD1 status, was assessed. BMP7 expression level was assessed in a cohort of human lung cancer samples (tumour, n=70; matched normal tissues, n=70), in association with patient clinical variables, using quantitative analysis of BMP7. Results: In vitro, it was found that recombinant human (rh)BMP7 significantly reduced cellular motility of lung cancer cell line, SK-MES1, and its adhesion to Matrigel (p<0.05). rhBMP7 was also able to significantly reduce invasion of lung cancer cells (p<0.05). We found that rhBMP7 had little effect on actual growth of the lung cells. In addition, BMP7 was able to regulate the phosphorylation of SMAD1, a downstream signalling intermediate of the BMP7 signalling pathway. The study further revealed that reduced levels of BMP7 in lung cancer tissues significantly correlated with lymph node metastasis for lung cancer patients (p<0.05). Conclusion: BMP7 protein is a pivotal regulator of cell motility in lung cancer with little impact on the growth of tumour cells. BMP7 expression is linked to lymph node involvement in patients with lung cancer. These results indicate that BMP7 plays a key role in regulating the progression of lung cancer.

Lung cancer is the most prevalent type of cancer and is also one of the principal causes of death in the world (1). There has been little improvement in 5-year survival over the last 30 years (2). Distant metastasis, which is regulated by a complex network of molecules and cellular behaviours, is an important cause for the poor prognosis of lung cancer patients. Of the factors related to metastasis, bone morphogenetic proteins (BMPs), crucial in embryonic development including lung development and airway branching (3, 4), have been recently shown to regulate the aggressiveness of cancer cells. BMPs are essential in a variety of processes of vertebrate embryonic development and the epithelial–mesenchymal interactions. They are multifunctional signal molecules and belong to the transforming growth factor-β (TGF-β) superfamily. BMPs were first identified as being regulators of bone forming in extraskeletal sites (5, 6). BMPs exert biological functions by interaction with a heteromeric complex of membrane receptors, type I and type II BMP receptors. BMP proteins bind the type-I serine kinase receptors (BMPRIA/BMPIRIB), leading to the recruitment of the BMP II receptors, which in turn activates the transcription factors, SMAD (homolog of mothers against decapentaplegic, Drosophila) 1, 5 or 8 (7), known as the SMAD-dependent pathway. BMPs can stimulate epithelial branching in...
embryonic lung (8-10), and their abnormal expression can lead to abnormal lung development (11). The role of BMPs in cancer has also been reported in recent years. Overexpression of a specific BMP inhibitor, noggin, inhibits the formation of the osteoblastic aspect of the bone lesions and the tumour growth (12). BMPs are also regulators during angiogenesis, by such mechanism as activating vascular endothelial growth factor (VEGF) promoter which can result in the formation of skeletal metastases (13).

In lung cancer, expression of the BMP family members has also been reported. For example, BMP2 is overexpressed in virtually all types of lung cancer, including non-small cell lung cancer (NSCLC) and small cell lung cancer (14, 15). BMP2 protein can stimulate migration, invasion and proliferation of lung cancer cells (14). This is in contrast to its effect on neuroblastoma-derived cell lines, in which p27kip1 expression is up-regulated and growth arrest and differentiation are induced by BMP2 (16). Thus, there may be a contrasting response to any given BMP, depending on the tumour and cell type. Interestingly, the status of K-Ras mutation is a factor determining the response of the cells to BMP3b and BMP6 (17). In NSCLC, BMP3b promoter is frequently methylated, resulting in silencing of BMP3b expression during tumour development (18, 19). BMP4, on the other hand, is able to induce senescence of A549 lung adenocarcinoma cell line (20). This leads to less telomerase and invasive activity, low degree of extracellular signal-regulated kinase (ERK) activation, reduced VEGF and BCL2 expression. Finally, BMP4 pathway signalling can negatively regulate the growth of lung cancer cells (20).

Studies on BMP7 in lung cancer have not been reported. However, BMP7 has been shown to play a role in other tumour types. BMP7 is strongly expressed in breast cancer and malignant melanoma (21-23). BMP7 regulates the proliferation and apoptosis of mammary epithelial cells, by acting as a LIM domain-only 4 (LMO4) responsive gene (24). In gastric cancer, however, BMP7 promoter has been shown to be methylated (25), suggesting a role in the carcinogenic process. In prostate cancer, BMP7 exposure can modulate the biological behaviour in a cell type-specific manner (26). It can up-regulate survivin activity, restore the starvation-induced suppression of c-jun NH-2 terminal kinase (JNK) activity, and affect prostate cancer cell apoptosis (27).

In the present study, we investigated the expression characteristics of BMP7 in lung cancer cells and tissues. Furthermore, we also investigate the biological impact of BMP7 protein on lung cancer cells.

Materials and Methods

Cell lines, materials and human lung specimens. Human lung cancer A549, SK-MES1, CorL77, CorL88, CorL25, and CorL47 cell lines (European Collection of Animal Cell Culture, Salisbury, UK) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotics (100 U penicillin and 50 μg streptomycin per ml). Recombinant human BMP7 (rhBMP7) protein was purchased from Sigma-Aldrich (Poole, Dorset, UK). Goat anti-human phospho-SMAD1 antibody (P-SMAD1), anti-SMAD1, and mouse anti-human GAPDH antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Product (Bedford, MA, USA). Other kits and reagents were obtained from Sigma-Aldrich, Poole, UK, unless otherwise stated.

Fresh frozen lung tissues, matched normal and tumour pairs from the same patients (n=70 pairs) were obtained from patients who attended Beijing Cancer Hospital from January 2004 to August 2007, under Ethical Committee approval. None of the patients received any neoadjuvant therapy prior to surgery. These tissues were collected immediately after surgical resection at the Beijing Cancer Hospital and were stored at the Tissue Bank of Peking University Oncology School. Clinico-pathological characteristics of the tumours were defined according to the TNM criteria of UICC (28). Clinico-pathologic factors, including age, sex, histological type of tumours, TNM stage, and lymph node metastasis were recorded and stored in the patients’ database and are shown in Table 1.

RNA isolation and RT-PCR. RNA was obtained using Total RNA Isolation Reagent (ABgene™) according to manufacturer’s instructions (Abgene, Surrey, UK). Total RNA was converted to cDNA using DuraScript™, a commercial RT kit from Sigma-Aldrich. PCR was carried out using a REDTaq™ ReadyMix PCR reaction mix (primers listed in Table II). Cycling conditions were 94°C for 5 minutes, followed by 36 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 40 seconds. This was followed by final extension for 10 minutes at 72°C. Products were visualized on 2% agarose gel stained with ethidium bromide.

In vitro cell growth assay. This was based on a previously reported method (29). SK-MES1 cells (3,000 cell per well) were seeded in a 96-well plate with the 10% FCS and cells were incubated with or without rhBMP7 protein at a concentration from 2.5 ng/ml to 100 ng/ml. The relative cell number was determined at 48, 72 and 96 hours. Crystal violet was used to stain cells and absorbance was determined at a wavelength of 540 nm using an ELx800 spectrophotometer (Bio-tek, Wolf Laboratories, York, UK). The absorbance measured represents the cell number.

Cell matrix adhesion assay. This was based on a method we previously reported (30, 31). A total of 40,000 cells were added to each well of a 96-well plate pre-coated with Matrigel at 5 μg per well. After 40 minutes of incubation, non-adherent cells were washed off using balanced salt solution (BSS) buffer. The remaining cells were fixed with 4% formalin and stained with 0.5% crystal violet. The number of adherent cells was then counted.

In vitro motility assay using cytodex-2 beads. The protocol described by Rosen et al. (32) was followed. Cells (1×10⁴) were incubated with 100 μl Cytodex-2 beads (Pharmacia, Piscataway, New Jersey) in 10 ml DMEM overnight. After washing, 100 μl beads per cell were transferred into each well of a 24-well plate, in the presence or absence of BMP7 (2.5-100 ng/ml). After 24 hours incubation, floating cells and beads were washed with BSS. The adherent cells were fixed in 4% formalin for 5 minutes and then stained with crystal violet for counting. Three independent experiments were performed.
In vitro invasion assay. Transwell inserts with an 8 μm pore size were coated with 50 μg Matrigel™ and air dried. Matrigel was rehydrated before use. A total of 20,000 cells were added to each well with or without rhBMP7. After 96 hour’s incubation, matrix proteins and non-invading cells were removed by cotton swabs. Cells that had migrated through the matrix and pores were fixed, stained in crystal violet and counted.

Immunocytochemical staining for P-SMAD1 protein. To determine the phosphorylation status of SMAD1, cells were first serum starved for 4 hours, followed by one hour’s exposure to rhBMP7 protein (5 ng/ml, 10 ng/ml, 50 ng/ml) in serum-free medium. Cells were fixed with 4% formaldehyde and then permeabilized with 0.1% Triton X-100 for 5 minutes. After blocking with horse serum for 60 min, the cells were probed with anti-P-SMAD1 antibody for 1 hour, followed by extensive washing. Horseradish peroxidase-conjugated anti-mouse antibody was then added for 1 hour and visualized using the Vectastain™ ABC system (Nottingham, UK). Slides were mounted with Sterilyte mounting media.

Western blotting and immunoprecipitation in detecting SMAD-1 and p-SMAD1. SK-MES1 cells were treated with different concentrations of rhBMP7 protein (5-100 ng/ml) then pelleted and extracted in HCMF buffer (160 mM NaCl, 0.6 mM Na2HPO4, 0.1% w/v glucose and 0.01 M HEPES, pH 7.4) containing 1.5% Triton X-100, 2 mM CaCl2, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 30 min. Protein concentration in cell lysates was quantified using as DC Protein Assay kit (Bio-Rad™, Hemmal Hamstead, England, UK) and an ELx800 spectrophotometer. Equal amount of proteins prepared in a loading buffer were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose sheets. Proteins were probed with the respective primary and peroxidase-conjugated secondary antibodies. For immunoprecipitation experiments, cell lysate was collected. Equal amounts of cell lysate were incubated with anti-SMAD1 IgG (4 μg/ml) to precipitate SMAD1. The immune complexes subsequently formed were then precipitated by incubating with protein A/G-agarose conjugate for 1 hour and subsequent centrifugation at 13,000g for 15 minutes. After washing, samples were boiled in SDS-PAGE sample buffer for 5 minutes prior to separation using SDS-PAGE. The membrane was probed with an anti-phosphoserine/threonine antibody (Sigma-Aldrich) and subsequently with a peroxidase-conjugated secondary antibody (1:1000). Protein
bands were visualized by a Supersignal™ Western Dura system and documented using a gel documentation system (UVITech, Cambridge, United Kingdom). The band densities on the photographic film were analysed with a densitometer and are shown as relative values.

BMP7 expression level in lung cancer and normal lung tissue assessed by real time RT-PCR. Levels of BMP7 transcript in cDNA samples prepared from the fresh frozen lung tissues were determined using real-time quantitative PCR based on the Amplifluor™ technology, modified from a method reported previously (33). PCR primers were designed using Beacon Design software but to the reverse primer an additional sequence, known as the Z sequence (5'-actgaacctgaccgtaca-3'), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added (Table II). The reaction was carried out using the following conditions: Hot-start Q-master mix (Abgene, Surrey, England, UK), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence, 10 pmol of FAM tagged probe (Intergen Inc.) and cDNA. The reaction was carried out using an iCyclerIQ (BioRad) which is equipped with an optic unit that allows real time detection of 96 reactions under the following conditions: 94˚C for 12 minutes and 60 cycles of 94˚C for 15 seconds, 55˚C for 60 seconds and 72˚C for 20 seconds. The level of BMP7 transcript was generated from a standard that was simultaneously amplified with the samples. The expression level of BMP7 gene was then normalized against cytokeratin 19 (CK19) expression already measured in these specimens, to correct for varying amounts of epithelial tissue between samples (34).

Statistical analysis. One-way ANVOA was used to determine the difference in BMP7 mRNA expression level observed between the group of cancer tissues and the normal tissues. The samples were grouped according to the tissue stage, lymph node stage and bone metastasis. Differences were considered statistically significant at p<0.05. Statistical tests were performed using SPSS software version 12.0.0 (SPSS Inc., Chicago, IL, USA).

Results

Expression characteristic of BMP7 gene in different cell lines. Figure 1 shows the expression status of BMP7 and the receptor transcripts in the cell lines tested. None of the lung cell lines showed a signal for BMP7. SK-MES1 was positive for BMPR1B, but negative for BMPR2. Normal lung tissues displayed a stronger signal for BMP7 than lung tumour tissues (Figure 1 bottom).

Effect of exogenous BMP7 on adhesion, motility and invasion ability of lung cancer cells in vitro. SK-MES1 cells showed significant change in cell motility and adhesion, depending on BMP7 concentration using the in vitro cell matrix adhesion technique. The number of adherent cells for the
SK-MES1 cells when treated with rhBMP7 5 ng/ml and 10 ng/ml was similar to the control, 35.75±6.77 and 35.67±6.02, respectively vs. 38.5±7.18 (p>0.05, Figure 2A). When the concentration of BMP7 was increased to 50 ng/ml, the number of adherent cells was significantly reduced (26.5±6.24) compared with no treatment (p<0.001, Figure 2A). The number of motile cells when treated with rhBMP7 was only significantly reduced when BMP7 was used at 50 ng/ml (11.11±4.14), p<0.001 vs. control, Figure 3B). In the invasion assay, BMP7 significantly reduced the numbers of invading cells at 10 ng/ml (22.3±16.36) and 50 ng/ml (22.2±15.58), in comparison with control cells (41.65±18.51) (p<0.05, Figure 2C).

It is interesting to observe that rhBMP7 had no effect on the growth of lung cancer cells (Figure 2D).

**Phosphorylation of SMAD1 in response to BMP7.** To assess the phosphorylation status of SMAD1, we utilized immunocytochemical and immunoprecipitation method. As shown in Figure 3A, there was almost no staining of p-SMAD1 in control cells and the cells exposed to lower concentrations of rhBMP7. A strong staining of p-SMAD1 was seen in the cytoplasm of cells treated with 50 ng/ml of rhBMP7 (Figure 3D). Western blotting also revealed an increased in p-SMAD-1 after treatment with rhBMP7 (Figure 3E/F).

**BMP7 gene transcript expression in lung cancer.** BMP7 transcripts in lung cancer specimens (tumour, n=70; matched paired normal tissue, n=70) were quantified using real-time quantitative PCR (shown in Figure 4. as BMP7 transcript copies/μl). There was no significant difference in transcript expression between normal lung tissues and lung cancer tissues (p=0.074) (Figure 4A). In addition, there were no differences in different T stage tumours (p=0.770) (Figure 4B). Tumours from patients with and without bone metastasis also failed to show any difference in BMP7 transcript levels (Figure 4C).

Perhaps the most interesting finding is the relationship between BMP7 and nodal involvement. In the cohort, 29 tumours were without nodal involvement (N0), 16 were at N1 stage and 25 at N2 (N1+N2=41). N0 tumours had significantly higher levels of BMP7 transcript compared with tumours with N1 lymph node and N2 lymph node metastasis (p=0.031) (Figure 4D). When N1 and N2 were combined, tumours with no lymph node metastasis (N0) had significantly high levels of BMP7 compared with tumours with lymph node metastasis (p=0.008) (Figure 4E).

After 48 months’ follow-up, patients were analysed regarding the survival time based on BMP7 expression level. The mean time of follow-up for the cohort (n=70) was 18.49 months (range 1-48 months). The mean (SD) survival time was 18.49±10.07 months. Cumulative survival curves were
calculated using the Kaplan-Meier method. In the patients with a low level expression of BMP7, the mean survival time was 35.37 (95%CI: 27.71-43.04) months. In the patients with a high expression level of BMP7, the mean survival time was 28.90 (95%CI: 24.15-33.65) months. The levels of BMP7 mRNA expression were not correlated with cumulative survival time (p=0.5107, Figure 4F).

Discussion

Aberrant expression of BMP7 has been reported in a few cancer types, including colorectal cancer, breast cancer, melanoma and prostate cancer. BMP7 has also shown an influence on proliferation, migration and invasion, and has been indicated as a useful clinical marker (35-38). The present study has demonstrated that BMP7 also has a significant influence on the aggressiveness of lung cancer cells. Lung cancer cell lines had almost no BMP7 expression and different levels of BMP receptor expression.

A few studies have shown a possible inhibitory effect of BMP7 on cell growth, for example, Notting et al. showed that BMP7 inhibits tumour growth of human uveal melanoma (39). In the present study, we did not find any effect of exogenous rhBMP7 on the growth of the lung cancer cell line, indicating that the impact of BMP7 on cell growth is cell type dependent. However, the most demonstrable effects of BMP7 on lung cancer cells are the inhibitory effects on matrix adhesion, motility and in vitro invasiveness, an effect seen at a relatively higher concentration (50 ng/ml). Transforming growth factor beta (TGF-ß) is a well characterized inducer of epithelial-mesenchymal transition (EMT). As members of the TGF-ß superfamily, BMPs can affect cancer cell adherence and migration which have important roles in cancer pathogenesis. Tada et al. reported that BMP2 can reduce microfilament organisation restoration in A549 lung cancer cells and reduce cancer cell migration (40). In breast cancer cells, however, exogenous BMP7 increases cell migration and invasion process (41).

BMP signalling requires serine-threonine kinase receptors, BMPRIA, BMPRIB and BMPRII, to transmit their signals (42). These receptors phosphorylate intracellular SMADs (43), which are transcription factors for gene transcription (44).
Here, we report that BMP7 is able to induce SMAD1 phosphorylation. This is interesting, given that SK-MES1 cells expressed only BMPR1B, but not BMPR2. This suggests that BMP7 requires other type II BMP receptors.

Our results on the expression pattern of BMP7 in clinical lung cancer is also interesting. The study has shown that node positive tumours have significantly lower BMP7 levels in comparison with node-negative tumours. In contrast, levels of BMP7 transcripts do not have a clear correlation with tumour staging, nor with the long-term survival of the patients. This result probably reflects the possibility that BMP7 does not influence the growth of lung cancer. Buijs et al. reported that BMP7 can affect bone metastasis formation in breast and prostate cancer animal models (45, 46), a link not demonstrated in lung cancer as shown here.

In summary, we have demonstrated that BMP7 has an important role in controlling lung cancer cell motility. It can regulate the invasion, motility and adhesion of lung cancer cells by activating the intracellular signalling pathway through the phosphorylation of SMAD1 without affecting the growth process. A lack of BMP7 expression is a good indicator of lymph node involvement. BMP7 may have a therapeutic role in lung cancer, an area which warrants further investigations.

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Conflict of Interest

None identified.

References


