Decreased BMP-7 and p-Smad1/5/8 expression, and increased levels of gremlin in hepatocellular carcinoma

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Abstract. The aim of the present study was to investigate the expression of bone morphogenetic protein-7 (BMP-7), gremlin, and p-Smad1/5/8 in carcinomatous and para-carcinoma tissue specimens from patients with hepatocellular carcinoma (HCC). The association of serum BMP-7 levels with clinicopathological parameters was examined to assess its relevance as a clinical biomarker for HCC. A total of 27 patients with HCC and 7 healthy subjects were included. Gene expression levels of BMP-7 and p-Smad1/5/8 were examined by reverse transcription-quantitative polymerase chain reaction. Immunohistochemical and western blot analysis were performed to determine the protein expression of target genes. The serum levels of BMP-7 were assessed by enzyme linked immunosorbent assay. The mRNA and protein expression of BMP-7 and gremlin were significantly down- and upregulated in HCC tumor tissues, respectively, compared with para-carcinoma tissues (P<0.05). The association of BMP-7 and gremlin expression with the differentiation status of HCC was also analyzed. There was a relatively higher level of BMP-7 and a lower level of gremlin expression in tumor tissues from patients with poorly differentiated HCC when compared with poorly or moderately differentiated HCC (BMP-7, F=42.29, P<0.01; gremlin, F=37.93, P<0.01). Furthermore, the level of BMP-7 and p-Smad1/5/8 was decreased in patients with advanced stages of HCC, when compared with stage I HCC. The findings from the present study suggest that the BMP-7/p-Smad signaling pathway may be involved in the pathogenesis of HCC. The serum levels of BMP-7 may serve as a potential biomarker for HCC.

Introduction

Liver cancer is a highly prevalent malignancy worldwide, and is associated with high mortality. According to Global Cancer Statistics (2012), liver cancer accounts for 521,000 and 224,500 mortalities among men and women, respectively (1). Hepatocellular carcinoma (HCC) accounts for >70% of all primary liver cancer (2). Due to its asymptomatic nature, patients with HCC are typically diagnosed at an advanced stage (3). Hence, there is an urgent requirement to identify biomarkers for early detection of HCC to improve patient prognosis (3).

Bone morphogenetic proteins (BMPs), which are homodimeric members of the transforming growth factor-β (TGF-β) superfamily, have been implicated in the pathogenesis of human malignancies (4). To date, >20 human BMPs have been identified, among which BMP-7 levels have been demonstrated to be closely associated with HCC (1). The anti-tumorigenic potential of BMP-7 has been detected in several human malignancies, including breast cancer and glioblastoma (5-7). Hou et al (8) indicated that BMP-7 attenuates liver fibrosis, which may develop into HCC. Furthermore, inhibition of BMP-7 signaling was revealed to facilitate invasive growth of HCC cells (9). The BMP family members mediate signal transduction by binding to their receptors, which triggers the phosphorylation of Smad proteins 1, 5, and 8 (Smad1/5/8) (10,11). However, the potential association between BMP-7 and Smad signaling pathway in HCC has not yet been fully elucidated.

Gremlin, an endogenous antagonist of BMP-7, inhibits the binding of BMP-7 to its receptor, thereby, inhibiting Smad phosphorylation and the downstream signaling cascade (12). Gremlin protein, secreted by fibroblasts has been revealed to be a biomarker of liver fibrosis (13). Gremlin promotes stem cell maturation and proliferation via inhibition of the BMP signaling pathway, which in turn accelerates hepatic fibrosis and carcinogenesis (14). These findings indicate that the BMP signaling pathway may serve as an endogenous self-defense mechanism against carcinogenesis and progression of HCC.

In the present study, it was hypothesized that the BMP-7 and Smad signaling pathway may serve a pivotal role in the prevention of HCC. Therefore, HCC samples with varied tumor differentiation status and at different clinical stages were included. Expression levels of BMP-7, gremlin, and Smad1/5/8
in carcinomatous and their adjacent non-carcinomatous (normal) tissues were assessed. The findings from the present study indicate the clinical significance of these indices in predicting the disease status of HCC.

Materials and methods

Reagents. Rabbit anti-human anti-BMP-7 antibody was obtained from Abcam, Cambridge, UK (cat. no. ab56023). Rabbit anti-human anti-gremlin antibody was purchased from Wuhan Boster Biological Technology, Ltd., Wuhan, China (cat. no. BA2287). Rabbit anti-human anti-p-Smad1/5/8 antibody was purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA (cat. no. sc-12353-R). The 3,3’-diaminobenzidine tetrahydrochloride (DAB) kit was obtained from ZSGB-Bio, Beijing, China. RNAlater solution, TIANScript first-strand cDNA synthesis kit, and SuperReal PreMix Plus SYBR Green were obtained from Tiangen Biotech Co., Ltd., Beijing, China. RIPA lysis buffer and phenylmethylsulfonyl fluoride (PMSF) were obtained from Beyotime Institute of Biotechnology, (Haimen, China).

Patients. A total of 27 patients with HCC who underwent curative surgical resection at the Department of General Surgery, The Affiliated Hospital of Xuzhou Medical University, (Xuzhou, China), between November 2014 and August 2015, were included in the present study. Inclusion criteria are as follows: i) Diagnosis of HCC according to the diagnostic criteria recommended by the Professional Committee of Chinese Anti-Cancer Association of Liver Cancer in 2001 (15); ii) not received surgical, interventional or radiation therapy prior to sample collection. Tumor-node metastasis (TNM) staging was performed based on the criteria of the American Joint Committee on Cancer (AJCC) (16). Pathological examination was conducted and tumor differentiation was recorded. Baseline demographic and clinical characteristics of patients are listed in Table I. In addition, a total of 7 healthy subjects were included in the present study. Written informed consent was obtained from all subjects prior to their enrollment in the study. The study was approved by the Ethics Committee at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China).

Sample collection. A total of ~100 mg tumor tissues or para-carcinoma tissues (3 cm away from the tumor margin) were collected following curative surgical resection for polymerase chain reaction (PCR) analysis. The tissues were washed in phosphate buffer solution (PBS). The samples to be used for RNA extraction were incubated in 1 ml RNAlater solution at 4°C overnight and stored at -80°C until further use. For western blot analysis, samples were grinded into pieces, immediately immersed in liquid nitrogen (-196°C), followed by long-term storage at -80°C. For histological examination, tissue samples were fixed in 4% paraformaldehyde solution for 24 h at room temperature. In addition, fasting venous blood samples were collected from patients with HCC prior to operation and from healthy subjects. The samples were incubated at room temperature for 1-2 h, followed by centrifugation at 2,200 x g for 5 min at room temperature. The serum was separated and stored at -80°C until use.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A total of ~100 mg of the tissue samples were grinded, and the total RNA was extracted using TRIzol reagent according to the manufacturer’s protocol (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA (1 µg) was reverse transcribed using TIANScript first-strand cDNA synthesis kit, and SuperReal PreMix Plus SYBR Green was obtained from Tiangen Biotech Co., Ltd., Beijing, China. RIPA lysis buffer and phenylmethylsulfonyl fluoride (PMSF) were obtained from Beyotime Institute of Biotechnology, (Haimen, China).

Table I. Demographic and clinical characteristics of subjects.

| Parameters          | Patients with HCC (n=27) | Healthy subjects (n=7) |
|---------------------|--------------------------|------------------------|
| Sex (%)             |                          |                        |
| Male                | 21 (78)                  | 5 (71)                 |
| Female              | 6 (22)                   | 2 (29)                 |
| Age, years          | 57±11                    | 54±14                  |
| TNM staging (%)     |                          |                        |
| I                   | 3 (11)                   | -                      |
| II                  | 12 (45)                  | -                      |
| III A               | 9 (33)                   | -                      |
| III B               | 3 (11)                   | -                      |
| Differentiation (%) |                          |                        |
| Poor                | 4 (15)                   | -                      |
| Moderate            | 15 (56)                  | -                      |
| High                | 8 (29)                   | -                      |

HCC, hepatocellular carcinoma; TNM, tumor-node metastasis.

Immunohistochemistry. Tissue samples collected after curative surgical resection were embedded in paraffin, and the sections (thickness, 4 µm) were prepared using an automatic tissue processor (RM2235; Leica, Germany). Subsequently, the sections were deparaffinized in xylene (twice, each for 10 min), and rehydrated in an ethanol series (100% for 5 min, 90% for 2 min, 70% for 2 min and distilled water for 2 min).
The sections were heated in 0.01 M citrate buffer (pH 6.0) in a microwave. After three washes in PBS, the samples were incubated with 3% hydrogen peroxide at room temperature, to block the endogenous peroxidase activity. After three washes with PBS (5 for each time) at room temperature, the sections were incubated with 100 µl primary antibodies, including rabbit anti-BMP-7 (1:100) and rabbit anti-gremlin (1:200) at 4°C overnight as described previously. Immunostaining was performed using rabbit ultra-sensitive two-step kit according to manufacturer's protocol (ZSGB-Bio, Beijing, China). The following day, the samples were washed three times in PBS and probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody, pre-diluted from the supplier, for 20 min at 37°C. After PBS washes, the immunoreactivity was assessed by DAB staining for 3 min at room temperature. Nuclei were counterstained with hematoxylin. Subsequently, the sections were washed with running tap water for 10 min, followed by dehydration through 95% ethanol for 5 min and 100% ethanol for 5 min. After two washes of xylene (each for 5 min), the samples were mounted with neutral balsam.

Imaging. Immunoreactivity was analyzed under a phase contrast microscope (BX51; Olympus Corporation, Tokyo, Japan) at x400 magnification. BMP-7 positive cells were identified by a brown-yellow stained cytosol or nuclei. Gremlin-positive cells were defined as those with brown-yellow-stained cell membrane, cytosol or nuclei. A total of five fields of interest were randomly selected from each sample for imaging in order to minimize bias as described previously (18), and the average integral optical density (OD) per area was calculated by Image-Pro Plus software (version 6.0, Media Cybernetics, Inc., Rockville, MD, USA).

Western blotting. A total of ~100 mg of tissue samples were lysed in 1 ml RIPA lysis buffer, which contained 10 µl PMSF and 7 µl protease inhibitor. Following centrifugation at 1,452 x g for 10 min at 4°C, the supernatant was collected. For the determination of protein concentration, bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology) was used. Equal amounts of total protein (80 µg) per well were separated by 10% SDS-PAGE electrophoresis. After electrophoresis, the proteins were transferred on to PVDF membranes, blocked with 5% non-fat dry milk dissolved in PBS for 3 h at room temperature (RT) and probed with primary antibodies, including anti-BMP-7 (1:500 dilution) and anti-p-Smad1/5/8 (1:500) antibodies, at 4°C overnight. Following four washes with TBST (each for 5-10 min), the membranes were incubated with goat anti-rabbit HRP-conjugated secondary antibodies (1:4,000) for 2 h at room temperature. After washes with TBST, immunoreactivity was assessed using an enhanced chemiluminescence assay. The immunobands were exposed to X-ray films and scanned. The density of bands was analyzed by Quantity One software 4.62 patch (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ELISA for cytokine analysis. The serum BMP-7 level was examined by ELISA using a human BMP-7 ELISA kit according to manufacturer's protocol (RayBiotech Inc., GA, USA). The absorbance was measured at 450 nm using a CliniBio128 microplate reader (Asys Hitech GmbH, Eugendorf, Austria). Statistical analysis. All data are expressed as the mean ± standard deviation. The differences between the groups were assessed with unpaired Student's t-test, and multiple group comparisons were performed using one-way analysis of variance following Student-Newman-Keuls post-hoc test. All statistical tests were two-tailed with α=0.05. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of BMP-7 and gremlin in carcinomas and non-carcinomas of HCC. To investigate the involvement of BMP-7 during carcinogenesis and progression of HCC, the mRNA levels of BMP-7 and its endogenous antagonist gremlin were determined in carcinoma and non-carcinoma tissues of HCC by RT-qPCR. BMP-7 mRNA expression was significantly downregulated in tumor tissues, compared with para-carcinoma tissues (P<0.05; Fig. 1). By contrast, the mRNA expression of gremlin in carcinoma tissues was significantly increased compared with non-carcinoma tissues (P<0.05; Fig. 1). Immunohistochemical analysis revealed that BMP-7 was predominantly expressed in the nucleus of tumor cells (Fig. 2A). The protein expression of BMP-7 was greatly reduced in carcinoma tissues compared with the non-carcinoma tissues (t=11.808, P<0.01; Fig. 2A and B). Gremlin exhibited a different intracellular distribution pattern compared with BMP-7, as gremlin expression was predominantly detected in the cell membrane and cytosol (Fig. 2). Furthermore, by contrast to BMP-7, gremlin protein was significantly increased in carcinoma tissues compared with non-carcinoma tissues (t=-6.182; P<0.01; Fig. 2C-E). These results indicate that BMP-7 mRNA and protein expression levels were reduced in HCC tissues, and that the level of BMP-7 was negatively associated with that of gremlin.

Expression of BMP-7 and gremlin in HCC tissues at various stages of differentiation. Expression levels of BMP-7 and gremlin in HCC tissues with poor, moderate, and high differentiation status were compared. It was observed that patients with highly differentiated HCC had a relatively higher BMP-7 level but a lower level of gremlin expression (BMP-7, F=42.29, P<0.01; gremlin, F=37.93, P<0.01; Fig. 3). The data of BMP-7 and gremlin in para-carcinoma tissues are presented in Fig. 2. The results from the present study indicate that BMP-7 may be used to predict the tumor differentiation status.

Expression of BMP-7, gremlin and p-Smad1/5/8 in carcinoma tissues of HCC at different clinical stages. To elucidate the role of BMP-7 and gremlin in the regulation of HCC development, gene expression levels in carcinoma tissues at various clinical stages (I, II, IIIA and IIIB) were examined. BMP-7 mRNA expression was gradually reduced with increasing clinical stage (F=18.45, P<0.01), while the level of gremlin was elevated in advanced staged HCC tissue samples (F=18.45, P<0.01; Fig. 4). Western blot analysis indicates that BMP-7 protein expression was downregulated in carcinoma tissues compared with non-carcinoma tissues (Fig. 5A and B), which is consistent with the results of PCR and histological analysis (Figs. 1 and 2). Furthermore, BMP-7 levels were decreased with advanced disease progression, since a lower level of BMP-7 expression was detected in samples from patients with stage
IIIB HCC compared with stage I HCC samples (Fig. 5A-C). In addition, the expression pattern of p-Smad1/5/8 was similar to that of BMP-7. The level of p-Smad1/5/8 was downregulated in carcinoma tissues, and it was significantly downregulated in advanced stage HCC (P<0.01; Fig. 5C).

Serum levels of BMP-7. To investigate the potential use of serum BMP-7 levels for the prediction of HCC progression, serum BMP-7 levels in patients with HCC and healthy subjects were compared. Serum BMP-7 levels were relatively high in healthy subjects (45.41±5.73 ng/ml), whereas the levels of BMP-7 in patients with HCC were decreased and the level of BMP-7 decreased to varying degrees depending on the stage of the disease. The reduction in serum BMP-7 levels was observed to be associated with disease progression since patients with more advanced stages of HCC generally exhibited lower serum level of BMP-7 (stage I, 38.35±5.81; stage II, 28.24±2.82; stage IIIA, 20.79±3.72; stage IIIB, 10.40±2.15; F=74.467; P<0.01; Fig. 6).

Discussion

HCC is a severe hepatic disorder associated with a high mortality rate (19). Patient prognosis is typically poor as the majority of cases are diagnosed at an advanced stage (19). Understanding the molecular mechanisms that underlie hepatocarcinogenesis may help facilitate therapeutic decision-making in clinical settings. In the present study, it was demonstrated that the BMP-7-Smad signaling pathway may have an anti-tumorigenic function in the progression of HCC.

In the present study, it was observed that there was a lower level of BMP-7 expression in HCC tumor tissues compared with non-carcinoma tissues. BMP-7 expression appeared to be directly associated with the extent of tumor differentiation and was inversely associated with the clinical stage. Therefore, these results indicate that BMP-7 may act as a tumor suppressor, and that the loss of BMP-7 may accelerate the progression of HCC.

Consistent with the findings in the present study, Yang et al (20) demonstrated that BMP-7 was able to block the development of hepatic fibrosis via inhibition of TGF-β1 production and increase in gremlin generation. Similarly, Wang et al (21) reported that BMP-7 mediated the prevention of liver fibrosis and hepatic stellate cell activation in mice injected with carbon tetrachloride (21). In addition, exogenous administration of BMP-7 was demonstrated to suppress hepatic fibrosis in rodents via regulation of the Smad signaling pathway (22).
In the present study, protein expression of p-Smad1/5/8 was observed to be downregulated in HCC tissues compared with non-carcinoma tissues. The reduced level of p-Smad1/5/8 was associated with advanced stages of HCC, which indicates that BMP-7/Smad signaling pathway may act as a self-defense mechanism against carcinogenesis and progression of HCC. BMP-7/Smad signals may have anti-fibrogenic and anti-hepatocarcinogenic effect during the pathogenesis of HCC (8,9,14,23). By contrast, Li et al (24) reported that the mRNA and protein expression of BMP-7 was upregulated in HCC cells compared with normal hepatic cells. Furthermore, increased BMP-7 levels were associated with poor prognosis in patients with HCC, which implies that BMP-7 may serve as an oncogene for HCC. This discrepancy may have been caused by the small sample size. Therefore, future studies are required which should include larger sample sizes of patients with HCC. Although the molecular mechanisms involved in BMP-7/Smad-regulated HCC development remain unclear, it is possible that BMP-7 counteracts the actions of TGF-β, which is known to contribute to epithelial-mesenchymal transition (EMT) of hepatocytes (25). In addition, efforts should be made to investigate the regulatory role of BMP-7/Smad signaling pathway in the EMT process in HCC using cell culture and rodent models.

In summary, the present study demonstrated that the levels of BMP-7 and p-Smad1/5/8 are decreased, while the level of gremlin is increased in HCC, compared with non-carcinoma tissues. These findings suggest a potential involvement of BMP-7/Smad signaling pathway in the carcinogenesis and progression of HCC. In addition, serum BMP-7 levels may have potential diagnostic value as a marker for hepatic carcinogenesis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
LW prepared samples, performed experiments and was a major contributor in writing the manuscript. QD contributed to conception and design. LZ, YP and XY performed analysis and interpretation of data. ZS and YQ participated in acquisition of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Written informed consent was obtained from all subjects prior to their enrollment in the present study. The present study was approved by the Ethics Committee at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China).

Consent for publication
Patients provided written consent for the publication of the present study.

Competing interests
The authors declare that they have no competing interests.

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