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Original Article

BMP4 promotes metastasis of hepatocellular carcinoma by an induction of epithelial–mesenchymal transition via upregulating ID2

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ABSTRACT

The role of bone morphogenetic protein 4 (BMP4), a crucial epithelial–mesenchymal transition (EMT) mediator, in the progression of hepatocellular carcinoma (HCC) patients heretofore has not been elucidated. The present study analyzed BMP4 expression in tumors and paired non-tumorous liver tissue and its correlation with clinicopathological characteristics from two independent cohorts consisting of 420 HCC patients. Functional analysis of BMP4 was performed in Bel-7402 and HCCLM3 HCC cells, and in a murine HCC model. The downstream targets of BMP4 in HCC were screened and confirmed. The results indicated that BMP4 expression was significantly increased in HCC tissue and highly metastatic HCC cells. BMP4 expression was correlated with vein invasion, overall survival and recurrence-free survival of HCC. BMP4 promoted HCC EMT and metastasis *in vitro*, and consistently *in vivo*. BMP4 knockdown blocked EMT and tumor metastasis in nude mice. ID2 was up-regulated by recombinant human BMP4, resulting in HCC EMT. Knockdown of ID2 blocked BMP4-induced EMT. In conclusion, BMP4 promotes invasion and metastasis of HCC by an induction of EMT via up-regulating ID2. BMP4 may be a valuable prognostic factor and potential therapeutic target for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer mortality worldwide [1]. Surgical resection is the main treatment procedure for HCC, but the long-term prognosis remains less than ideal due to frequent tumor recurrence and metastasis after surgery [2,3]. Consequently, improved understanding of the molecular mechanisms underlying HCC metastasis is of much clinical and fundamental importance.

One of the key molecular mechanisms involved in distant metastasis of many cancers is epithelial-to-mesenchymal transition (EMT) of cancer cells [4,5], which is characterized by suppression of epithelial markers, up-regulation of mesenchymal markers and EMT-related transcription factors. EMT is a highly conserved cellular program that allows polarized, immotile epithelial cells to convert to motile mesenchymal cells [6]. This important process was initially recognized during several critical stages of embryonic development [7], and has more recently been believed to play important roles in invasion and metastasis of HCC [8,9]. Therefore, for the treatment of HCC, there exists significant clinical potential in targeting EMT-associated factors.

Signaling molecules that promote and regulate the primary EMT include the transforming growth factor-β (TGF-β) and bone

Abbreviations: AFP, alpha-fetoprotein; ANLT, adjacent non-tumorous liver tissue; BMP4, Bone morphogenetic protein 4; EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; ID2, the inhibitor of DNA binding 2; IHC, immunohistochemistry; MMP, matrix metalloproteinase; OS, overall survival; RFS, recurrence free survival; RFA, radiofrequency ablation; shRNA, short-hairpin RNA; siRNA, short interfering RNA; TACE, transarterial chemoembolization; TNM, tumor-node-metastasis.

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morphogenetic proteins (BMPs) [10–12]. BMPs are extracellular signaling molecules that belong to the TGF-β superfamily and are well-known for their indispensable roles in vertebrate development [13] and adult tissue formation, maintenance, remodeling and repair [14,15]. In recent years, important new information has been generated on the contribution of BMP family members, such as BMP4, in cancer pathogenesis. The functional effects of BMP4 include the control of cell proliferation, differentiation, apoptosis, angiogenesis, migration, invasion, and EMT [16]. BMP4 expression levels are commonly altered and have been linked to patient prognosis in some tumor types including HCC [17–19]. EMT is especially intriguing because it is typically associated with tumor metastasis and progression. BMP dimers transduce their signals through binding to and phosphorylation of type II and type I receptors. Subsequent intracellular signaling is mediated through activation of Smad1/5/8 interacting with the common Smad4, and subsequent translocation into the nucleus to regulate target gene expression [15]. Further investigation of the downstream mediators of BMP4 effects in cancer cells may allow dissection of BMP4-induced phenotypes and thereby suggest novel specific targeted therapies.

Although there is evidence suggesting possible BMP4 involvement in EMT of human malignancies, the relationship between BMP4 and EMT in HCC remains unknown. In the present study, we investigate the roles of BMP4-mediated EMT in HCC recurrence and metastasis, as well as evaluate the postoperative prognostic value of BMP4 expression in HCC patients.

Materials and methods

HCC patients samples

420 pairs of HCC and adjacent non-tumorous liver tissue (ANLT) were randomly collected from liver resections at Xiangya Hospital & the 3rd Xiangya Hospital, Central South University (Changsha, Hunan, China), and were divided into a training cohort (n = 212, December 2008–December 2011) and a validation cohort (n = 208, January 2004–November 2008). None of these subjects had received any kind of anticancer treatment prior to surgery, such as trans-arterial chemoembolization (TACE) or radiofrequency ablation (RFA). The samples were snap-frozen in liquid nitrogen for subsequent examination. All research protocols strictly complied with REMARK guidelines for reporting prognostic biomarkers in cancer [20]. Tumor differentiation was defined according to the criteria proposed by Edmondson [21]. All work on human specimens complied with the principles laid down in the Declaration of Helsinki. Prior informed consent was obtained from all recruited patients, and the study protocols were approved by the ethics committees of the two participating hospitals.

Postsurgical patient surveillance was carried out *via* telephone and return visits to the outpatient clinic. Patients were followed with regular surveillance for recurrence or metastasis by AFP level measurement, chest radiographs, abdominal ultrasonography, and computed tomography (CT) every 2–3 months in the first year, and at least every 5–6 months thereafter. A recurrence was suspected based on typical clinical signs, imaging appearance and/or an elevated AFP level. Once evidence of recurrence was confirmed, monthly AFP measurements, ultrasonography and/or CT scanning was performed. Hepatic angiography, bone scintigraphy, and/or magnetic resonance imaging (MRI) were performed when clinically indicated. Patient follow-up was terminated on 31 December 2015. Overall survival (OS) was defined as the time interval between HCC resection and death or the last observation. Recurrence-free-survival (RFS) was calculated from the surgery to the first radiological evidence of recurrence [22]. Patients that died with no sign of recurrence were censored [3]. The baseline demographics and clinic-pathological characteristics of the two cohorts of HCC patients are summarized in Table 1, which shows that they were well matched. More detailed information is provided in Supplementary Materials and Methods.

Quantitative RT-PCR (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA) was employed to extract total RNA from HCC cells and tissue as described in the manufacturer's manual. qRT-PCR was performed using SYBR Green fluorescent-based assay (TaKaRa Bio Inc., Otsu, Japan) on a ViiA™7 RT-PCR system (Applied Biosystems, Carlsbad, CA). Relative mRNA expression levels were calculated by the $2^{-\Delta Ct}$ (ΔCt = Ct (targeting gene)-Ct (GAPDH)) method and were normalized to GAPDH. The PCR primers are listed in Supplementary Table S1.

Immunohistochemistry (IHC)

The immunoreactivity was scored semi-quantitatively by two independent pathologists (Supplementary Materials and Methods). The primary antibodies are listed in Supplementary Table S2.

HCC cell lines

BMP4 expression was assessed in one liver cell line (L02) and eight HCC cell lines, including two lines with high invasiveness and proclivity for metastasis (HCCLM3 and MHCC97H) and PLC/PRF/5, Hep3B, HepG2, SMMC7721, Bel7402, and Huh7 cell lines with low invasiveness and metastatic proclivity. Bel7402 and HCCLM3 were chosen for subsequent experiments (Supplementary Materials and Methods).

Western blotting

The detailed procedure is described in the Supplementary Materials and Methods. The antibodies are listed in Supplementary Table S2.

Wound healing and transwell invasion assays

To test the cells' migration and invasion abilities, wound healing and transwell invasion assays were performed in triplicate (Supplementary Materials and Methods).

Immunofluorescence (IF) staining

To evaluate the protein expression and localization of an epithelial biomarker (E-cadherin) and a mesenchymal marker (vimentin) in HCC cells, IF staining images were captured using laser confocal microscopy (Supplementary Materials and Methods).

Cell morphology

Transmission electron microscopy was used to observe the morphological changes of EMT in HCC cells (Supplementary Materials and Methods).

Transfection of BMP4 short-hairpin (shRNA) lentivirus

A BMP4-shRNA lentiviral vector (GV118-BMP4) (GeneChem, Shanghai, China) containing four pairs of putative candidate BMP4 silencing sequences was constructed. The BMP4-shRNA sequences with the maximum of 77.5% knockdown efficiency were adopted to stably knockdown BMP4 expression in HCCLM3 and MHCC97H cells used in an orthotopic HCC metastasis model of nude mice (Supplementary Materials and Methods).

PCR array

RT^2 Profiler PCR Array for Human TGFβ/BMP Signaling Pathway (Qiagen, Valencia, CA) was used to profile the downstream targets of BMP4 in HCC cells (Supplementary Materials and Methods).

ID2-siRNA and transfections

The chemically synthetic siRNA sequences with >75% inhibition efficiency for ID2 expression were applied for transient transfection in HCC cells (Supplementary Materials and Methods).

Orthotopic HCC transplantation and metastasis model

1 mm^3 tumors cut from subcutaneous tumor, which originated from HCCLM3 and MHCC97H cells, respectively infected with BMP4-shRNA and shRNA-control lentivirus, were implanted into the left lobes of livers of male BALB/c mice. At the 35th day after tumor implantation, a 3D fluorescence molecular tomographic imaging system (FMT-4000, PerkinElmer, Boston, MA) was used to examine the orthotopic tumor sizes and matrix metalloproteinase-2 (MMP-2) and MMP-9 activities *in vivo*. Both intrahepatic and pulmonary metastatic nodules of HCC were observed.

All animal studies were conducted in the Animal Institute of Central South University, All animals received humane care. The protocols were approved by the Medical Experimental Animal Care Commission of Central South University according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The detailed procedure is described in the Supplementary Materials and Methods.

Gelatin zymography assay

HCCLM3 and MHCC97H were analyzed for MMP-2 and MMP-9 activities by a gelatin zymography assay [9]. Transparent bands of the gel with Coomassie blue background were scanned and sorted according to the presence of MMP-2 and MMP-9 enzymatic activities (Detailed in the Supplementary Materials and Methods).