Bone morphogenetic protein and activin membrane-bound inhibitor overexpression inhibits gastric tumor cell invasion via the transforming growth factor-β/epithelial-mesenchymal transition signaling pathway

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Abstract. Gastric carcinoma is one of the most common human malignancies and remains the second leading cause of cancer-associated mortality worldwide. Gastric carcinoma is characterized by early-stage metastasis and is typically diagnosed in the advanced stage. Previous results have indicated that bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) overexpression has been demonstrated to inhibit growth and metastasis of gastric cancer cells. However, the molecular mechanisms of the BAMBI-mediated signaling pathway in the progression of gastric cancer are poorly understood. In the present study, to assess whether BAMBI overexpression inhibited the growth and aggressiveness of gastric carcinoma cells through regulation of transforming growth factor (TGF)-β/epithelial-mesenchymal transition (EMT) signaling pathway, the growth and metastasis of gastric carcinoma cells were analyzed following BAMBI overexpression and knockdown *in vitro* and *in vivo*. Molecular changes in the TGF-β/EMT signaling pathway were studied in gastric carcinoma cells following BAMBI overexpression and knockdown. DNA methylation of the gene regions encoding the TGF-β/EMT signaling pathway was investigated in gastric carcinoma cells. Tumor growth in tumor-bearing mice was analyzed after mice were subjected to endogenous overexpression of BAMBI. Results indicated that BAMBI overexpression significantly inhibited gastric carcinoma cell growth and

aggressiveness, whereas knockdown of BAMBI significantly promoted its growth and metastasis compared with the control (P<0.01). The TGF-β/EMT signaling pathway was downregulated in BAMBI-overexpressed gastric carcinoma cells; however, signaling was promoted following BAMBI knockdown. In addition, it was observed that BAMBI overexpression significantly downregulated the DNA methylation of the gene regions encoding the TGF-β/EMT signaling pathway (P<0.01). Furthermore, RNA interference-mediated BAMBI overexpression also promoted apoptosis in gastric cancer cells and significantly inhibited growth of gastric tumors in murine xenografts (P<0.01). In conclusion, the present findings suggest that BAMBI overexpression inhibited the TGF-β/EMT signaling pathway and suppressed the invasiveness of gastric tumors, suggesting BAMBI may be a potential target for the treatment of gastric carcinoma via regulation of the TGF-β/EMT signaling pathway.

Introduction

Gastric carcinoma is one of the most prevalent types of human cancer and remains the second leading cause of cancer-associated mortality worldwide (1). Gastric signet ring cell carcinoma is typically associated with diffuse, infiltrating myositis that increases the difficulty of clinical diagnosis and treatments for patients with suspected gastric cancer (2). Furthermore, gastric cancer has a higher morbidity and mortality rate than other carcinomas concerning the digestive system (3,4). In addition, previous studies have demonstrated that apoptotic resistance of gastric cancer is inevitable in cancer progression (5-7). Furthermore, previous findings have suggested that target therapies for advanced gastric cancer are more efficient compared with alternative treatments for patients with gastric cancer (8,9). Therefore, the investigation into efficient target molecules has attracted more interest in researchers and clinicians in the field of cancer research and clinical therapy.

Acidosis is a key physiological and pathological feature that is commonly associated with gastric cancer (10).

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Previous studies have demonstrated that acidosis induces a hypoxia-inducible factor (HIF)-1α signaling pathway chain reaction in the gastric cancer cells (11,12). In addition, previous studies have also indicated that acidosis induces the activation of transforming growth factor (TGF)-β and epithelial-mesenchymal transition (EMT) signaling pathways, which contribute to the growth, invasion and apoptotic resistance of gastric carcinoma cells (13,14). Furthermore, a previous study indicated that the association of low PH, tumor-associated macrophages, microvessel density, vascular endothelial growth factor and matrix metalloproteinases (MMPs) in human gastric cancer affect survival (15). These reports suggest that the TGF-β/EMT signaling pathway is crucial for growth, migration and invasion of gastric carcinoma cells.

Bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) is a pseudo-receptor of mothers against decapentaplegic homolog (SMAD)7 and is homologous to TGF-β receptor type 1 (TGFβR1), but lacks the functional domain of active kinase (16,17). BAMBI is similar to TGF-βRI and participates in the regulation of the TGF-β-mediated signaling pathway in various cancer cells (18). Although loss of BAMBI has been identified in a large number of cancer tissues, including non-small cell lung cancer, bladder cancer and colorectal cancer, a limited number of studies have explored its function in gastric cancer (18-20). In addition, Pils *et al* (21) previously reported BAMBI is overexpressed in ovarian cancer and co-translocates with SMADs into the nucleus upon TGF-β treatment. Notably, BAMBI overexpression is beneficial for suppressing metastasis of gastric cancer cells by inhibiting β-catenin and TGF-β (22). These findings suggest that BAMBI may be associated with the progression of gastric cancer.

In the present study, it was speculated that BAMBI expression levels may be associated with the growth and aggressiveness of gastric carcinoma cells by regulating the TGF-β/EMT signaling pathway. Although, previous studies have indicated the role of BAMBI in bladder cancer, non-small-cell lung cancer, ovarian cancer and gastric cancer (16,18-23), to the best of our knowledge the present study is the first to have comprehensively investigated BAMBI-mediated TGF-β/EMT processes in gastric carcinoma cells *in vitro* and *in vivo* and suggest BAMBI may be a promising therapeutic target for the treatment of gastric cancer.

Materials and methods

Ethics statement. The present study was implemented legitimately according to the Guide for the Care and Use of Laboratory Animals of the Affiliated Tumor Hospital of Guangxi Medical University (Nanning, China) (24). The present study was performed in accordance with the Ethics of Animal Experiments Defense Research (25) and approved by the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University.

Cell culture and reagents. Gastric tumor cell lines HGC-27 and BGC-823, and human gastric mucosa epithelial cells GES-1 were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). All tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). GES-1 cells were cultured in Eagle's minimal essential medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal calf serum. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C. Intracellular pH was analyzed as previously described (26).

Apoptosis assay. Apoptosis of gastric tumor cells was assessed using flow cytometry. BGC-823 cells ($1x10^6$/well) were cultured in 6-well plates with BAMBI (2.0 mg/ml) for 24 h at 37˚C. Subsequently, cells were harvested via trypsinization, washed in cold PBS and adjusted to $1x10^6$ cells/ml with PBS. Following double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide using the FITC Annexin V Apoptosis Detection kit I (BestBio, Shanghai, China) for 2 h at 37˚C according to manufacturer's protocol, cells were analyzed using a FACScan flow cytometer equipped with Cell Quest software 1.2 (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol in order to detect the apoptotic rate of BGC-823 cells. All experiments were performed in triplicate.

siRNA transfection. Knockdown of BAMBI was performed via transfection of specific small interfering (si)RNA designed by siDirect2.0 (version 2.0; sidirect2.rnai.jp/). All siRNAs were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.), including siRNA-BAMBI (Si-BAMBI; gene accession no. WO 2005116204-A/725618; sense, 5'-GCAAGCAGA GCUCAGUAAUTT-3' and antisense, 5'-AUUACUGAGCUC UGCUUGCTT-3') or siRNA-mimic (control; sense, 5'-GCA AGCAGAGCUCAGUAAUTT-3' and antisense, 5'-ACGUGA CACGUUCGGAGAATT-3'). BGC-823 cells ($1x10^7$) were transfected with 100 pmol Si-BAMBI or Si-vector using a Cell Line Nucleofector kit L (Lonza, Slough, UK) according to the manufacturer's protocol (27). Cells were used further analysis after 72 h transfection. BAMBI knockdown BGC-823 cells were treated with 2 mg/ml TGFβ (Si-BAMBI-TGFβ) for 12 h at 37˚C for further analysis.

Endogenous BAMBI overexpression. BAMBI gene was cloned into PMD-18-T vector (Takara Biotechnology Co., Ltd., Dalian, China) and sequenced to identify its sequence according to previous report (28). BAMBI gene was subsequently cloned into eukaryotic expression vector pCMVp-NEO-BAN (pBAMBI; Takara Biotechnology Co., Ltd.) to generate BAMBI-overexpressed BGC-823 cells. Subsequently, pBAMBI (1.0 µg) or an empty vector (pvector; 1.0 µg) was transfected into cultured BGC-823 cells ($5x10^6$) using Lipofectamine® 2000 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Stable BAMBI-overexpression BGC-823 cells were selected using a G418 screening system (29). Cells were incubated with TGF-β (2 mg/ml) for 12 h at 37˚C and used for further analysis after 72 h transfection.

Cell invasion and migration assays. BGC-823 cells ($1x10^6$) were transfected with Si-vector, Si-BAMBI, pvector (control)

or pBAMBI to analyze the efficacy of BAMBI. For the migration assay, transfected BGC-823 cells were incubated for 96 h at 37°C using a Transwell insert (BD Biosciences, San Jose, CA, USA) instead of a Matrigel Invasion Chamber. For the invasion assay, Si-vector, Si-BAMBI, control or pBAMBI-treated cells were suspended at a density of $1x10^5$ in 500 μl serum-free DMEM in the upper chamber and 500 μl serum-free DMEM with 5% FBS in the lower chamber of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's protocol. Cells were then stained with 0.1% crystal violet dye (Sigma-Aldrich; Merck KGaA) for 20 min at 37°C. Tumor cell invasion and migration were counted in at least three random fields of view using a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

DNA methylation analysis. Methylation analysis of HOPE®-fixed, paraffin-embedded (HOPE® Fixative system I; Polysciences, Inc., Warrington, PA, USA) BAMBI-overexpressed or pvector-overexpressed BGC-823 cells was performed according to the manufacturer's protocol as previously described (30). DNA methylation was measured using the EZ DNA Methylation kit (ZymoResearch, Irvine, CA, USA) and the Infinium HumanMethylation450k BeadChip (HM450KBC; Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. Bisulfite pyrosequencing of the selected loci was performed as indicated in a previous study to determine the DNA methylation state (31).

Western blot analysis. Total protein from BGC-823 or BAMBI-overexpressed BGC-823 cells ($1x10^7$) was extracted using radioimmunoprecipitation assay lysis buffer containing phenylme thanesulfonylfluoride (Sigma-Aldrich; Merck KGaA). Protein concentration was measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 30 μg protein lysates were subjected to 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes as previously described (32). The following antibodies were blocked in 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) for 1 h at 37°C and subsequently used to incubate protein samples for 1 h at room temperature: Monoclonal goat anti-mouse BAMBI (1:500; cat. no. AF2387; R&D Systems, Inc., Minneapolis, MN, USA), TGF-β, (1:1,000; cat. no. AF532; R&D Systems, Inc.) SNAI1 (1:500; cat. no. PAB29288; Abnova Corporation, Taipei, Taiwan), α-actin-2 (ACTA2; 1:200; cat. no. MA515806; Invitrogen; Thermo Fisher Scientific, Inc.), vimentin (VIM; 1:500; cat. no. PAB24865; Abnova Corporation), twist-related protein 1 (TWIST1; 1:500; cat. no. DR1088100UG; EMD Millipore), MMP9 (1:500; cat. no. PAB19095; Abnova Corporation), SRY-box 4 (SOX4; 1:500; cat. no. PAB14092; Abnova Corporation), N-cadherin (1:200; cat. no. NBP238856; Novus Biologicals), collagen-I (CT-I, 1:200; cat. no. NB6004080.01MG; Novus Biologicals, Littleton, CO, USA) IPO-38 (cat. no. ab1045), Ki68 (cat. no. ab15580), Smad6 (cat. no. ab214009), Smad7 (cat. no. ab216428), pMAPK (cat. no. ab32047), pSmad2 (cat. no. ab53100), β-actin (cat. no. ab8227) (all 1:1,000; Abcam, Cambridge, MA, USA) and fibronectin (FIB; 1:500; cat. no. P1H11; R&D Systems, Inc.) for 12 h at 4°C. Samples were subsequently incubated with HRP-conjugated polyclonal anti-rabbit IgG antibody (1:10,000; cat. no. PV-6001; R&D

Systems, Inc.) for 1 h at room temperature. Signals were visualized by using an enhanced chemiluminescence western blot analysis detection reagent (EMD Millipore). The density of the bands was analyzed using Quantity One software (version 4.6; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Animal study. A total of 20 female C57BL/6 nude mice (specific-pathogen-free; body weight, 30-35 g; age, 6 weeks) were purchased from Slack Experimental Animals Co., Ltd. (Shanghai, China). All mice were housed in a 12-h light/dark cycle, temperature-controlled facility at 23±1°C with a relative humidity of 50±5%. All mice were free access to food and water. Mice were divided into two groups (n=10/group). A total volume of 200 μl BGC-823 cells ($5x10^5$; control group) or Si-BAMBI-BGC-823 cells ($5x10^5$; experimental group) was administered by subcutaneous injection into C57BL/6 nude mice. Tumor diameters were recorded every 2 days and tumor volume was calculated using the formula: 0.52x smallest diameter^2 x largest diameter. The experimental mice were sacrificed when tumors reached 10 mm. On day 25, all mice in each group were sacrificed for further analysis.

Histological immunostaining. Tumors tissues were isolated from gastric carcinoma xenograft mice and were fixed using 10% formaldehyde for 2 h at 37°C and embedded in paraffin. Tumor samples were cut into sections (4-μm-thick) and antigen retrieval was performed as described previously (33). Tumor sections were blocked in 5% BSA for 1 h at 37°C and incubated with the following primary antibodies: Rabbit anti-mouse ki67 (1:1,000; cat. no. 652401), HIF (1:500; cat. both no. 580809; Biolegend, Inc., San Diego, CA, USA), TWIST1 (1:500; cat. no. 573208; Biolegend, Inc.), MMP9 (1:500; cat. no. PAB19095), SOX4 (1:500; cat. no. PAB14092; both Abnova Corporation), N-cadherin (1:200; cat. no. NBP238856), CT-I (1:200; cat. no. NB6004080.01MG; both Novus Biologicals) and FIB (1:500; cat. no. P1H11; R&D Systems, Inc.) for 12 h at 4°C. Subsequently, tumor sections were incubated with HRP-conjugated anti-IgG (1:10,000; cat. no. ab6721, Abcam) for 24 h at 4°C. The results were visualized using a LumiGLO chemiluminescence system (Cell Signaling Technology, Inc., Danvers, MA, USA). Images were obtained using a fluorescent microscope (BZ-9000; Keyence Corporation, Osaka, Japan) at magnification, x40.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. For apoptotic analysis of tumor cells, the DeadEnd Colorimetric TUNEL System from Promega Corporation (Madison, WI, USA) was used to determine the number of apoptotic cells in tumor sections. Tumor tissues from xenogeneic mice were fixed with 4% paraformaldehyde solution for 12 h at 4°C. Serial 5-μm thick sections were prepared and stained with TUNEL (5%) for 1 h at 37°C. Biotinylated nucleotides were used to incorporate at the 3'-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase for 2 h at 37°C. Horseradish peroxidase (HRP)-conjugated streptavidin (1:2,000; cat. no. PV-6001; OriGene Technologies, Inc., Beijing, China) was then bound to these biotinylated nucleotides for 2 h at 37°C. Peroxidase activity was calculated using the liquid 3,3'-diaminobenzidine substrate chromogen system (Dako; Agilent Technologies, Inc.,

Santa Clara, CA, USA). The percentage of TUNEL-positive tumor cells in xylene was analyzed by counting $1x10^3$ cells from six random selected fields of view in this assay.

Statistical analysis. Data are presented as the mean ± standard error of the mean and were analyzed using GraphPad Prism software 19.0 (GraphPad Software, Inc., La Jolla, CA, USA). Analysis on two experimental groups was performed using a two-tailed Student's t-test. Multiple comparisons (in more than two groups) were analyzed using one-way analysis of variance followed by Kruskal-Wallis test or Dunn's post-test. Bisulfite pyrosequencing and HM450KBC analysis was used to analyze similar methylation patterns of loci located in the BAMBI gene region using a Pearson correlation coefficient. $P<0.05$, was considered to indicate a statistically significant difference.

Results

Analysis of BAMBI expression levels its function in gastric cancer cells. Previous studies have indicated that BAMBI is downregulated in gastric cancer cells (22,23). In order to analyze the function of BAMBI in gastric cancer cells, BAMBI expression levels in BGC-823 and HGC-27 gastric cancer cells were compared with normal GES-1 gastric cells (control). BAMBI expression levels were significantly downregulated in BGC-823 cells compared with GES-1 cells ($P<0.01$; Fig. 1A). The effects of BAMBI on the growth and aggressiveness on gastric cancer cells were also assessed. BAMBI overexpression resulted in a 7.4-fold increase in expression level whereas BAMBI inhibition resulted in a 9.1-fold decrease in BAMBI expression level in BGC-823 cells compared with the control ($P<0.01$; Fig. 1B and C). As indicated in Fig. 1D, endogenous BAMBI overexpression significantly inhibited BGC-823 cell growth, whereas endogenous inhibition of BAMBI expression significantly promoted the growth of BGC-823 cells compared with the control and Si-vector, respectively ($P<0.01$). In addition, the migratory ability of BGC-823 cells was significantly inhibited by the treatment of pBAMBI, whereas BAMBI inhibition significantly increased cell migration compared with the control and Si-vector, respectively ($P<0.01$; Fig. 1E). Furthermore, the invasive ability of BGC-823 cells was also significantly suppressed following endogenous overexpression of BAMBI, whereas the invasive characteristics were significantly enhanced following knockdown of BAMBI compared with the control and Si-vector, respectively ($P<0.01$; Fig. 1F). These results suggest that BAMBI expression may be associated with the growth, migration and invasion of gastric cancer cells.

BAMBI overexpression downregulates EMT marker expression levels mediated by TGF-β in gastric cancer. The signaling pathway mediated by BAMBI was further analyzed in gastric carcinoma cells. TGF-β expression and differential expression levels of EMT markers in gastric cancer cells following overexpression and knockdown of BAMBI were examined in the present study. As indicated in Fig. 2A, BAMBI overexpression markedly inhibited proliferation marker IPO-38 and Ki67 expression levels in BGC-823 cells compared with the control. Notably, BAMBI overexpression significantly increased the intracellular pH in

BGC-823 cells compared with the control ($P<0.01$; Fig. 2B). As indicated in Fig. 2C, the protein expression levels of EMT markers TWIST1, MMP9 and SOX4 were also markedly downregulated in BAMBI-overexpressed BGC-823 cells compared with the control. In addition, EMT transcription factors SNAI1, ACTA2 and VIM exhibited reduced levels of protein expression in BGC-823 cells following BAMBI overexpression compared with the control (Fig. 2D). Furthermore, it was observed that the protein expression levels of TGF-β and its regulatory molecules (N-cadherin, CT-I and FIB) were inhibited in BAMBI-overexpressed BGC-823 cells compared with the control (Fig. 2E). Notably, it was also demonstrated that the protein expression levels of TGF-β, N-cadherin, CT-I and FIB were increased following BAMBI knockdown in BGC-823 cells compared with the control (Fig. 2F). However, TGF-β treatment reversed the inhibitory effects of BAMBI overexpression on EMT signaling pathway markers TWIST1, SOX4, MMP9, SNAI1, ACTA2 and VIM (Fig. 2G). Notably, BAMBI silencing promoted these expression levels in BGC-823 cells compared with the control (Fig. 2H). These results suggest that BAMBI overexpression downregulates EMT marker protein expression via TGF-β in gastric cancer.

BAMBI overexpression regulates methylation patterns of TGF-β signaling pathway members in gastric cancer cells. A previous study has indicated that changes in methylated modifications are observed during carcinogenesis and growth, migration and invasion in gastric tumors (34). To determine the influence of BAMBI on the methylation patterns of TGF-β signaling pathway members and mediators in gastric cancer, gastric cells were analyzed using Ilumina's BeadChip technology. Data indicated that BAMBI overexpression significantly decreased the DNA methylation of genes for molecules associated with TGF-β-signal transduction in BGC-823 cells compared with the control ($P<0.01$; Fig. 3A). Furthermore, BAMBI overexpression decreased BAMBI methylation based on epigenetic modifications of the CpG loci located in the BAMBI gene region compared with the control (Fig. 3B and C). Bisulfite pyrosequencing and HumanMethylation450K BeadChip analysis indicated a similar methylation patterns of loci located in the BAMBI gene region with a Pearson correlation coefficient of r=0.926 following BAMBI overexpression (Fig. 3D). In addition, it was demonstrated that BAMBI overexpression decreased the BAMBI-induced upregulation of TGF-β target genes Smad6 and Smad7, whereas knockdown of TGF-β induced the upregulation of TGF-β target genes (Fig. 3E and F). It was also indicated that BAMBI overexpression abolished TGF-β-induced phosphorylation of mitogen-activated protein kinase (MAPK) and Smad2, whereas knockdown of BAMBI (Si-BAMBI-TGFβ) decreased the protein expression levels of pMAPK and pSmad2 in BGC-823 cells (Fig. 3G and H). These findings suggest that the BAMBI overexpression decreased the signal molecular expression levels following TGF-β-stimulation.

BAMBI overexpression inhibits tumor growth in BGC-823-bearing mice. In order to investigate the efficacy of BAMBI on the progression of gastric carcinoma,
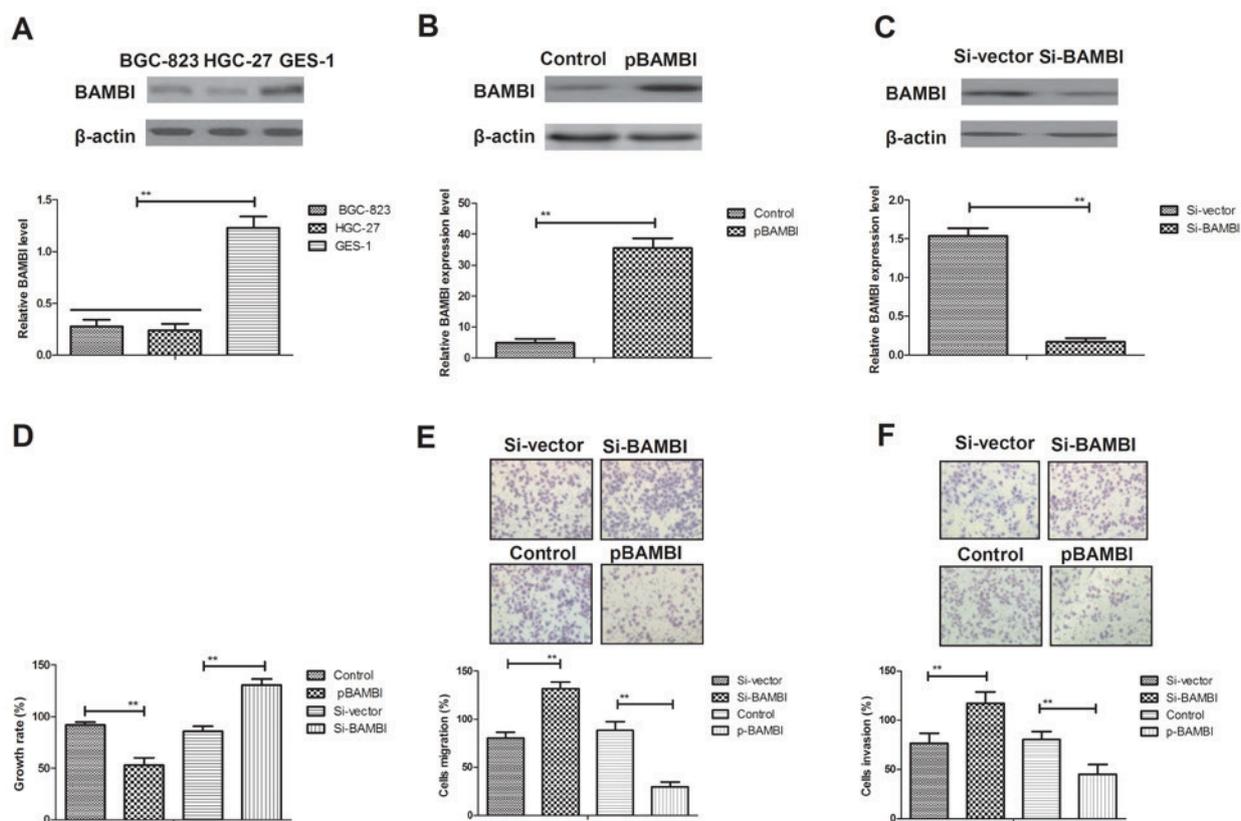
Figure 1. Effect of BAMBI expression on growth and aggressiveness in gastric cancer cells. (A) Protein expression levels of BAMBI in gastric cancer and normal gastric cells was assessed. Protein expression levels of BAMBI following transfection of (B) pBAMBI or (C) Si-BAMBI were determined. Endogenous BAMBI overexpression inhibited BGC-823 (D) cell growth, (E) migration and (F) invasion. Magnification, x40. One-way analysis of variance or two-tailed Student's t-test were performed. Control, pvector. $^{**}P<0.01$ as indicated. BAMBI, bone morphogenetic protein and activin membrane-bound inhibitor; Si, small interfering RNA; pBAMBI, overexpressed BAMBI.
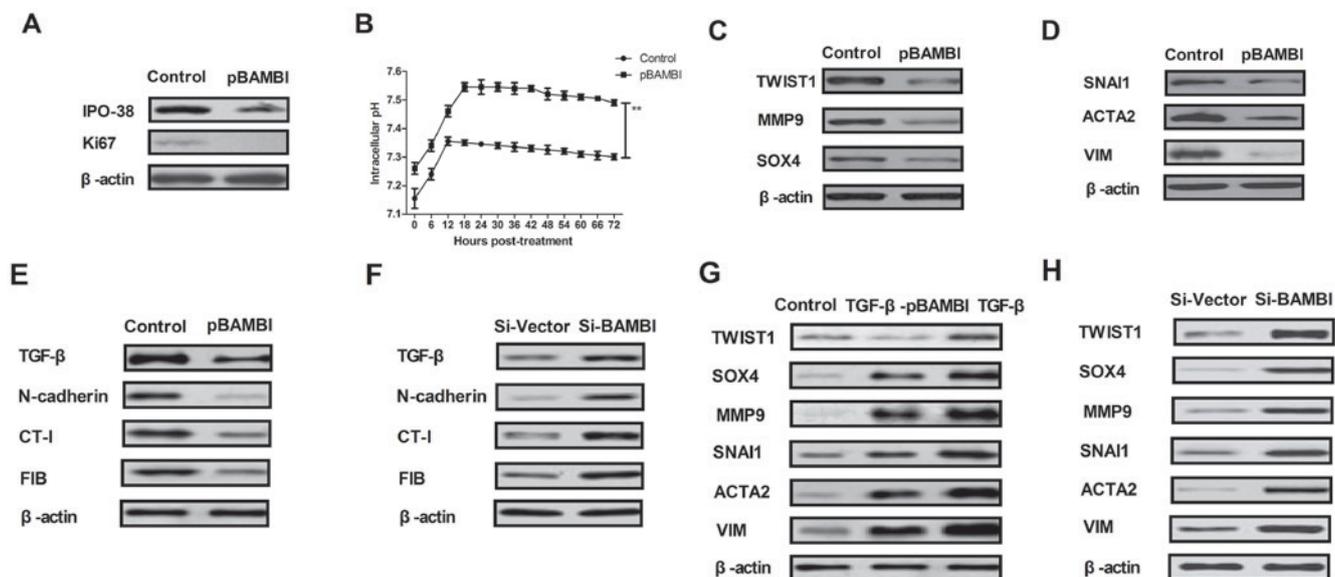

Figure 2. BAMBI regulated the growth and aggressiveness of gastric cancer cells through the TGF-β/EMT signaling pathway. (A) Proliferation marker IPO-38 and Ki67 protein expression levels in BGC-823 cells following BAMBI overexpression were assessed. (B) Intracellular pH changes in BAMBI-overexpressed BGC-823 cells were analyzed. (C) EMT marker TWIST1, MMP9 and SOX4 protein expression levels in BGC-823 cells following transfection with BAMBI were indicated. (D) EMT transcription factor SNAI1, ACTA2 and VIM protein expression levels in BGC-823 cells following transfection with BAMBI were determined. Effects of BAMBI (E) overexpression or (F) knockdown on TGF-β and its regulatory molecules expression levels of N-cadherin, CT-I and FIB in BGC-823 cells. Effects of BAMBI (G) overexpression or (H) knockdown on TGF-β-induced EMT signal pathway in BGC-823 cells. One-way analysis of variance or two-tailed Student's t-test were performed. $^{**}P<0.01$ as indicated. Control, pvector. BAMBI, bone morphogenetic protein and activin membrane-bound inhibitor; TGF-β, transforming growth factor-β; EMT, epithelial-mesenchymal transition; Si, small interfering RNA; pBAMBI, overexpressed BAMBI; TWIST1, twist-related protein 1; MMP9, matrix metallopeptidase; SOX4, SRY-box 4; ACTA2, α-actin-2; VIM, vimentin; FIB, fibronectin; CT-I; collagen-I.
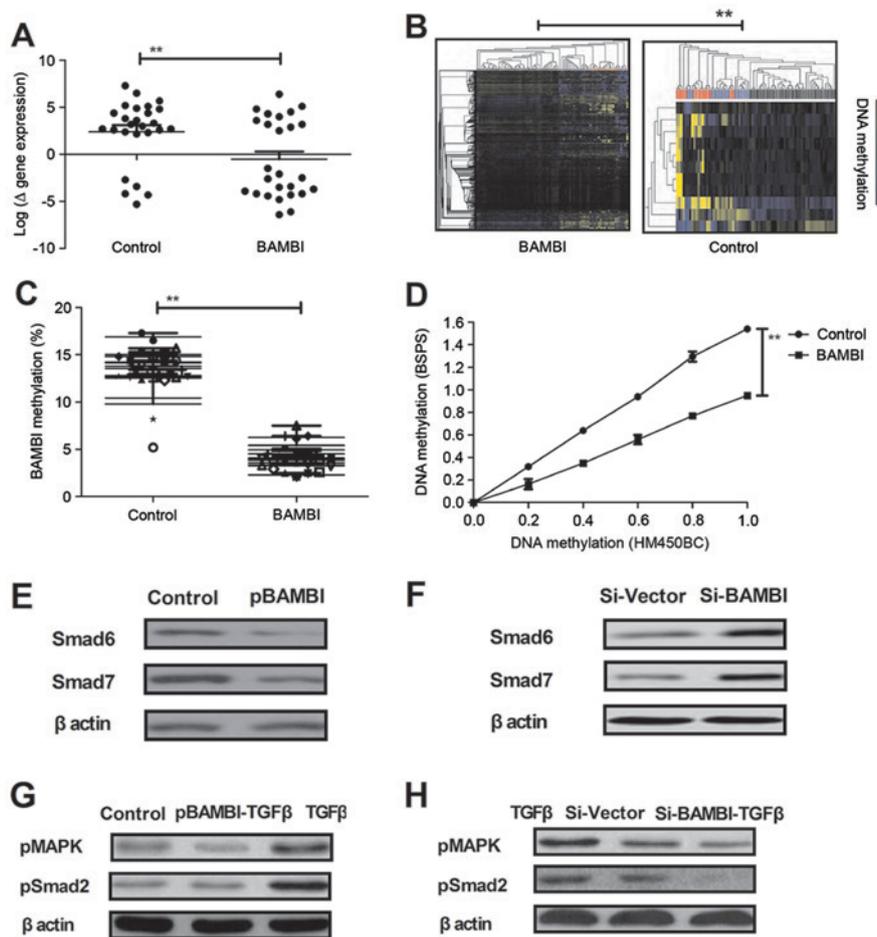
Figure 3. Analysis of the association between BAMBI and DNA methylation. (A) DNA methylation analysis of TGF-β signaling pathway genes in gastric cancer cells were assessed following transfection with BAMBI. (B) Expression levels of BAMBI mRNA negatively correlated with increased methylation in BGC-823 cells (change from grey-blue-yellow indicated methylation was increased). (C) Percent of epigenetic modifications of the CpG loci located between the control and BAMBI group. (D) Scatter plot of DNA methylation values determined by HumanMethylation450K BeadChip analysis and bisulfite pyrosequencing. Pearson correlation coefficient, r=0.926. Effects of (E) BAMBI overexpression or (F) knockdown on the protein expression levels of Smad6 and Smad7 in BGC-823 cells were determined using western blot analysis. Effects of (G) BAMBI overexpression or (H) knockdown on TGF-β-induced phosphorylation of MAPK and Smad2 in BGC-823 cells were also assessed. One-way analysis of variance or two-tailed Student's t-test revealed a significant effect. Control, pvector. **P<0.01 as indicated. BAMBI, bone morphogenetic protein and activin membrane-bound inhibitor; TGF-β, transforming growth factor-β; Si, small interfering RNA; pBAMBI, overexpressed BAMBI; MAPK, mitogen-activated protein kinase; p, phosphorylated; Smad, mothers against decapentaplegic homolog.

BGC-823-bearing C57BL/6 nude mice were established in the present study. BAMBI-overexpressed BGC-823 cells significantly suppressed tumor formation compared with PBS in experimental mice (P<0.01; Fig. 4A). Intracellular pH was significantly increased in the tumors induced with BAMBI-overexpressed BGC-823 cells compared with PBS in mice (P<0.01; Fig. 4B). In addition, the expression levels of key regulatory factors in the TGF-β/EMT signaling pathway were analyzed. As indicated in Fig. 4C, ki67 and HIF expression levels were markedly downregulated in tumors induced with BAMBI-overexpressed BGC-823 cells compared with PBS. In addition, EMT signaling pathway mediators TWIST1, MMP9 and SOX4 were markedly downregulated in tumors induced with overexpressed BAMBI compared with PBS (Fig. 4D). Furthermore, it was also observed that the expression levels of TGF-β signaling pathway mediators N-cadherin, CT-I and FIB were markedly downregulated in tumors treated with overexpressed BAMBI cells compared with PBS (Fig. 4E). Additionally, the apoptotic rate in tumor cells was significantly upregulated following BAMBI overexpression compared with PBS in tissues from the *in vivo* model (P<0.01;

Fig. 4F). These results suggest that BAMBI overexpression may inhibit the formation of gastric tumors by regulating the activation of the TGF-β/EMT signaling pathway *in vivo*, which contributes to inhibition of gastric tumor growth.

Discussion

Gastric cancer is an important healthcare problem that is difficult to treat due to apoptotic resistance, as gastric tumor cells typically exist in the hydrochloric acid gastric juice, which is a more acidic environment compared with other human cancer types and further inhibits the efficacy of anticancer drugs (35,36). Previous reports have demonstrated that TGF-β and EMT signaling pathways are correlated with the malignancy of gastric carcinoma and are responsible for its growth, migration and metastasis (13,37-39). The aim of the present study was to investigate the expression and function of BAMBI in gastric carcinoma and explore the association between BAMBI and gastric tumor formation. In addition, the mechanism associated with the BAMBI-mediated signaling
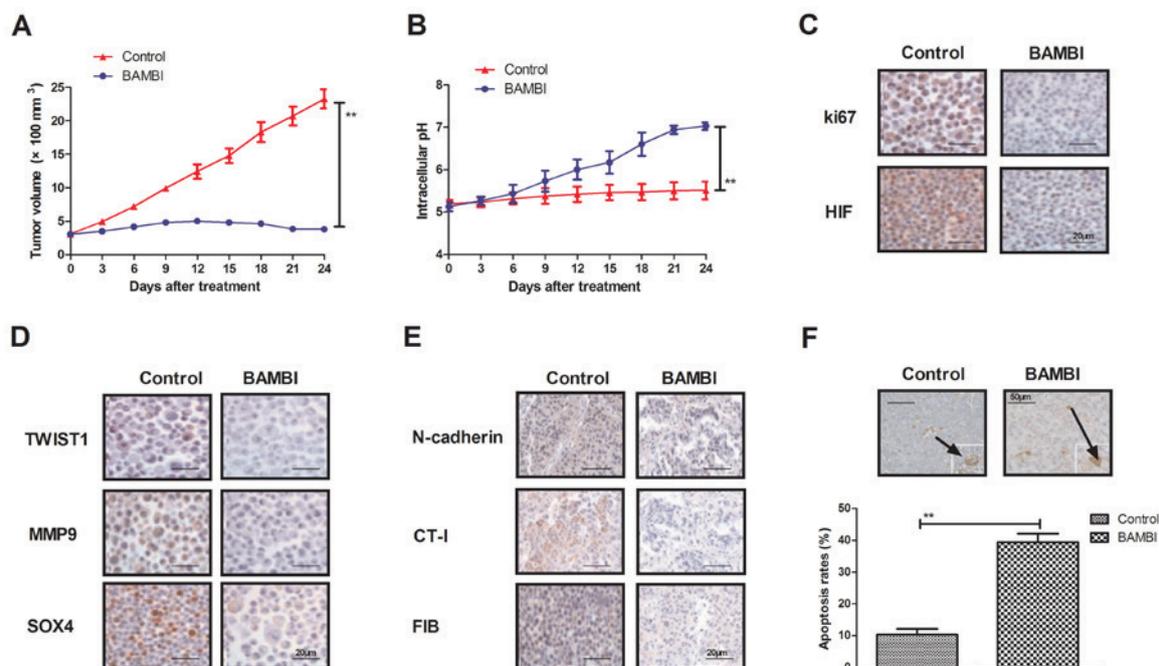
Figure 4. *In vivo* effects of BAMBI overexpression on gastric tumor progression. (A) Overexpression of BAMBI inhibited gastric cancer tissues growth. (B) Endogenic BAMBI overexpression improved intracellular pH in gastric tumors from xenograft mice. (C) Protein expression levels of ki67 and HIF in BAMBI overexpressed tumors were assessed. (D) TWIST1, MMP9 and SOX4 protein expression levels involved in the EMT signaling pathway in tumors were overexpressed BAMBI. (E) Protein expression levels of N-cadherin, CT-I and FIB were also assessed in BAMBI-overexpressed gastric tumors. (F) Apoptosis rate in gastric tumors transfected with BAMBI *in vivo* were indicated. White arrows indicate the apoptotic cells. One-way analysis of variance or two-tailed Student's t-test revealed a significant effect. **P<0.01 as indicated. BAMBI, bone morphogenetic protein and activin membrane-bound inhibitor; TGF-β, transforming growth factor-β; Si, small interfering; pBAMBI, overexpressed BAMBI; TWIST1, twist-related protein 1; MMP9, matrix metallopeptidase; SOX4, SRY-box 4; ACTA2, α-actin-2; VIM, vimentin; FIB, fibronectin; CT-I; collagen-I; HIF, hypoxia-inducible factor.

pathway in gastric cancer cells was investigated *in vitro* and *in vivo*. It was speculated that BAMBI overexpression relieved the intracellular acidity by regulating the TGF-β signaling pathway in gastric cancer cells. The present data indicated that BAMBI serves a critical role in pH regulation and the growth of gastric carcinoma cells. Furthermore, the present results demonstrated that BAMBI downregulation drives the invasiveness of gastric cancer via the TGF-β/EMT signaling pathway.

Previous studies have suggested that TGF-β is a pleiotropic cytokine during the inflammatory response and tissue homeostasis, which regulates differentiation, proliferation, survival and apoptosis in tumor cells (40,41). Fu *et al* (42) indicated that TGF-β promotes migration and metastasis of gastric cancer cells by regulating ERK and JNK signaling pathways. Notably, TGF-β serves a dual function in the process of carcinogenesis by suppressing apoptosis and regulating proliferation via the EMT signaling pathway (43,44). The EMT signaling pathway is a vital process in tumor cell progression and metastasis that is stimulated by the TGF-β signaling pathway, which ultimately leads to complex biochemical reaction processes in tumor cells (45,46). TGF-β regulates the EMT signaling pathway by binding to ligands TGFβR1 and TGF-β2 (47). A previous study revealed the molecular mechanisms of the EMT signaling pathway are associated with Ras-induced signaling, which regulates the EMT process in human tumor cells (48). In the present analysis, it was indicated that TGF-β regulated BAMBI-mediated growth and invasion of gastric carcinoma through the regulation of EMT signaling.

The EMT signaling pathway is associated with the progression of gastric cancer (13). The present data indicated that the TGF-β signaling pathway may be targeted via TGF-β in BAMBI-overexpressed gastric cells. Distinct upregulation in the DNA methylation of the gene regions encoding the TGF-β signaling pathway components was observed in BAMBI-overexpressed gastric cells and resulted in a marked reduction of TGF-β-induced growth, migration and invasion. However, DNA methylation was decreased in the present study. In addition, BAMBI overexpression abolished TGF-β-induced phosphorylation of MAPK and Smad2 and knockdown of BAMBI decreased the protein expression levels of pMAPK and pSmad2 in BGC-823 cells. Furthermore, endogenous BAMBI overexpression significantly promoted apoptosis in gastric cancer tissues and markedly inhibited the growth of gastric tumors in murine xenografts. Therefore, these results suggest that BAMBI overexpression in gastric tumor regression may be dependent on the inhibition of TGF-β-induced signaling at receptor level.

Although the association between progression and TGF-β signaling has been investigated in gastric cells, a limited number of studies have evaluated the association of BAMBI and TGF-β in cancer cells (49). It was observed that lower expression of BAMBI was induced by lower PH, sensitized gastric cancer cells to TGF-β-induced aggression and promoted EMT-dependent malignant processes. A previous study has identified that BAMBI may inhibit TGF-β signaling in colorectal tumor cells (50). Similarly, the present results supported this hypothesis in xenograft mice and demonstrated

that overexpression of BAMBI in gastric cancer cells resulted in the significant inhibition of gastric tumor growth.

In conclusion, the present findings suggest that gastric cancer epigenetic overexpression of BAMBI results in reduced TGF-β/EMT signaling, which contributes to the inhibition of tumor growth and EMT-mediated signaling (51). Notably, epigenetic activating of BAMBI results in inactivation of the TGF-β/EMT signaling pathway *in vitro* and *in vivo*, which may enhance the therapeutic effects of BAMBI in the treatment of gastric cancer. These findings suggest that BAMBI overexpression may suppress the growth and invasiveness of gastric tumors through regulation of the TGF-β/EMT signaling pathway, suggesting that BAMBI may be a potential target for the treatment of patients with gastric carcinoma. However, future studies are required to investigate other gastric carcinoma cell lines in order to fully elucidate the therapeutic effects of BAMBI.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

CLY performed data analysis and wrote the manuscript. YL designed the study and revised the manuscript. RL, ZHL and YQL performed experiments and collected data. XLL and JZY performed data analysis.

Ethics approval and consent to participate

The present study was implemented legitimately according to the Guide for the Care and Use of Laboratory Animals of the Affiliated Tumor Hospital of Guangxi Medical University (Nanning, China). The present study was performed in accordance with the Ethics of Animal Experiments Defense Research and approved by the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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