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Putative Dual Roles of Bone Morphogenetic Protein 8B (BMP8B) in Disease Progression of Gastric Cancer

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Abstract. *Background/Aim: Increased expression of bone morphogenetic protein 8B (BMP8B) in bone marrow and primary tumors of patients with gastric cancer (GC) is associated with disease progression and poor prognosis. However, a reduced expression has also been seen in GCs due to histone acetylation. This study aimed to evaluate BMP8B transcript levels in a large GC cohort and its impact on cellular functions. Materials and Methods: BMP8B transcripts were determined in 319 gastric tumors and compared with 182 adjacent normal tissues using real time PCR, with a further analysis conducted in the TCGA database. Kaplan-Meier plotter analysis was performed to evaluate the correlation between BMP8B and prognosis of the disease. BMP8B knockdown model was employed to determine the effect of BMP8B on the function of GC cells (HGC27). Results: BMP8B mRNA levels were significantly up-regulated in the GC tissues compared with adjacent normal tissues in both TCGA database and our own database from Beijing Cancer Hospital, and high BMP8B expression was associated with poor prognosis. BMP8B is most likely to be involved in the differentiation of GC. Poorly differentiated GC samples presented a significantly reduced BMP8B expression in relation to well-differentiated and moderately differentiated GC. BMP8B knockdown inhibited proliferation of GC cells, while promoted invasion and migration of cancer cells. Conclusion: BMP8B was reduced in GCs, whereas higher BMP8B expression was associated with poor prognosis. BMP8B knockdown inhibited proliferation of GC cells, and promoted invasion and migration. Our results suggest that BMP8B plays dual roles in GC.*

During the past few decades, the incidence and mortality of gastric cancer (GC) have shown a significant decline, but it remains the fourth leading cause of cancer-related death worldwide because of its poor prognosis (1). Only less than 20% of the newly diagnosed cases are stage I or II for whom surgery is the main treatment. For unresectable locally advanced or metastatic gastric cancer, chemotherapy is the backbone of palliative treatment. Although chemotherapy in GC has progressed in recent years (2, 3), patients with GC have poor prognosis (4). An improved understanding of the molecular mechanisms of GC and the exploration of new therapeutic targets may improve the outcome for patients.

Bone morphogenetic proteins (BMPs) are signalling molecules that belong to the transforming growth factor β (TGF-β) superfamily. They directly activate at least two signal transduction pathways: one is the SMADs pathway (5, 6) and the other is the MAPKs pathway (7-9). BMPs were originally identified as molecules that can induce ectopic bone and cartilage formation, and then several studies revealed that they are involved in several biological processes, including organogenesis, cell proliferation,

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differentiation, migration, immune response, angiogenesis, and apoptosis (10). BMPs are detected in many types of tumors, such as bone, odontogenic, colorectal cancer and maxillofacial tumors (11-14), and correlated with the development and metastasis (15-21). However, most of the studies were carried out on breast and prostate cancer. For instance, by up-regulating the expressions of MMP-1 and CXCR4, BMP4 may involve in the progression of invasion and migration of breast cancer cells (16). BMP-10 can suppress the growth of prostate cancer cells by inducing apoptosis *via* a Smad independent pathway in which XIAP and ERK1/2 are involved, and it can also prevent prostate cancer cell migration and invasiveness (17).

In the field of GC, research has shown that BMPs play an important role in regulating the homeostasis of the gastric epithelium and tumorigenesis through their ability to control the biological functions of the parietal cells (22-25). Inhibition of BMP signaling in the gastric mucosa leads to severe abnormalities in the proliferation, maturation, and differentiation of several lineages of gastric epithelial cells, and further formation of metaplasia, atypical hyperplasia, and tumors (22, 26). Moreover, some studies suggested that BMPs could regulate the growth and metastasis of GC. BMP-2 inhibits the growth of GC cells (27, 28). BMP-4 expression rate was inversely related to the prevalence of lymph node metastasis and tumor invasiveness (29).

Recently, two studies suggested that BMP8B may also be involved in the progression of GC. Mima *et al*. (30) reported that high BMP8B mRNA expression in the primary tumor was significantly associated with a shorter cancer-specific survival following a curative resection (p=0.007). And the multivariate analysis revealed that the prognostic power of BMP8B mRNA expression in the tumor was independent of other standard prognostic markers [hazard ratio (HR)=2.066; 95% confidence interval (CI)=1.132-3.772; p=0.018]. Wisnieski (31) demonstrated that BMP8B expression was reduced in GC compared to nontumor samples (p<0.01), and reduced BMP8B expression was associated with poorly differentiated GC (p=0.02). However, there is no research on the effect of BMP8B on the function of GC cells and its molecular mechanism.

In the present study, we aimed to examine the expression of BMP8B in GC compared to normal tissues, and its relationship with clinicopathological factors of patients. Moreover, we established a BMP8B knockdown model to determine its effect of on the function of GC cell lines.

Materials and Methods

Tumor samples from patients with gastric cancer. Primary tumor samples (n=319) together with paired adjacent normal tissues (n=182) were taken from patients with GC immediately after the surgery in Beijing Cancer Hospital. The tissues were kept at –80˚C until RNA extraction. All the patients had signed a written informed consent at the Beijing Cancer Hospital. The protocols and procedures of the tissue collection were approved by Peking University Cancer Hospital Research Ethics Committee. The pathological diagnoses and clinicopathological factors of patients were collected.

Analysis of BMP8B expression in human gastric cancer tissues using gene expression array data. We analysed the expression of BMP8B in GC tissues (n=274) compared to normal gastric tissues (n=33) in the TCGA database and its relationship with the clinicopathological parameters. In addition, TCGA database was analyzed to evaluate the correlation between BMP8B and key genes relevant to the hallmarks of cancer including proliferation, cell cycle, matrix metalloproteinases, and stemness. Heatmaps and scatter plots were used for presenting the results. Kaplan-Meier (KM) plotter analysis was also performed to evaluate the prognostic value of BMP8B in GC. The best cut-off was automatically selected and the most efficient and specific probe set for BMP8B, as recommended by KMplot, was employed.

Cell lines. HGC27 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell line was routinely cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, Poole, Dorset, UK) supplemented with 10% foetal bovine serum (FBS; HyClone™ , Cytiva, Marlborough, MA, USA) at 37˚C under a 5% CO2 and 95% air.

RNA extraction and Reverse transcription-PCR (RT-PCR). Total RNA was isolated from tissues and cell lines using TRI Reagent kit (Sigma-Aldrich, Inc., Poole, Dorset, UK), according to the manufacturer's instructions. The RNA concentration and quality were measured using an Implen Nanophotometer (Implen GmbH, München, Germany). Reverse transcription was performed using the GoScriptTM Reverse Transcription System (Promega, Southampton, UK), followed by PCR or quantitative real-time PCR (q-PCR). Cycling conditions for PCR were 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s. GAPDH was used as a control.

Real-time quantitative PCR (q-PCR). Q-PCR for BMP8B and GAPDH were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with the following conditions: 94˚C for 2 min and then 40 cycles of 94˚C for 15 s and 55˚C for 1 min. The primers were as follows: BMP8B forward: CTGGTTGCTGAAGCGTCACAAG, reverse: AGT GACCACGAAAGGCTGTTGG; GAPDH forward: TGCACCACC AACTGCTTAGC, reverse: GGCATGGACTGTGGTCATGAG.

Western blot. The DC protein assay kit (Bio-Rad Laboratories, Hemel-Hempstead, UK) was used for determining the protein concentration. Proteins were then loaded and separated in SDS-PAGE and transferred onto PVDF membranes. Then, proteins were probed with either an antibody against BMP8B (ab230553, Abcam, Cambridge, UK) or GAPDH (sc-47724, Santa Cruz Biotechnology, Dallas, TX, USA) and corresponding secondary antibody. Protein bands were visualized using the Supersignal™ West Dura system (Pierce Biotechnology, Inc., Rockford, IL, USA), and photographed using an UVITech imager (UVITech, Inc., Cambridge, UK).

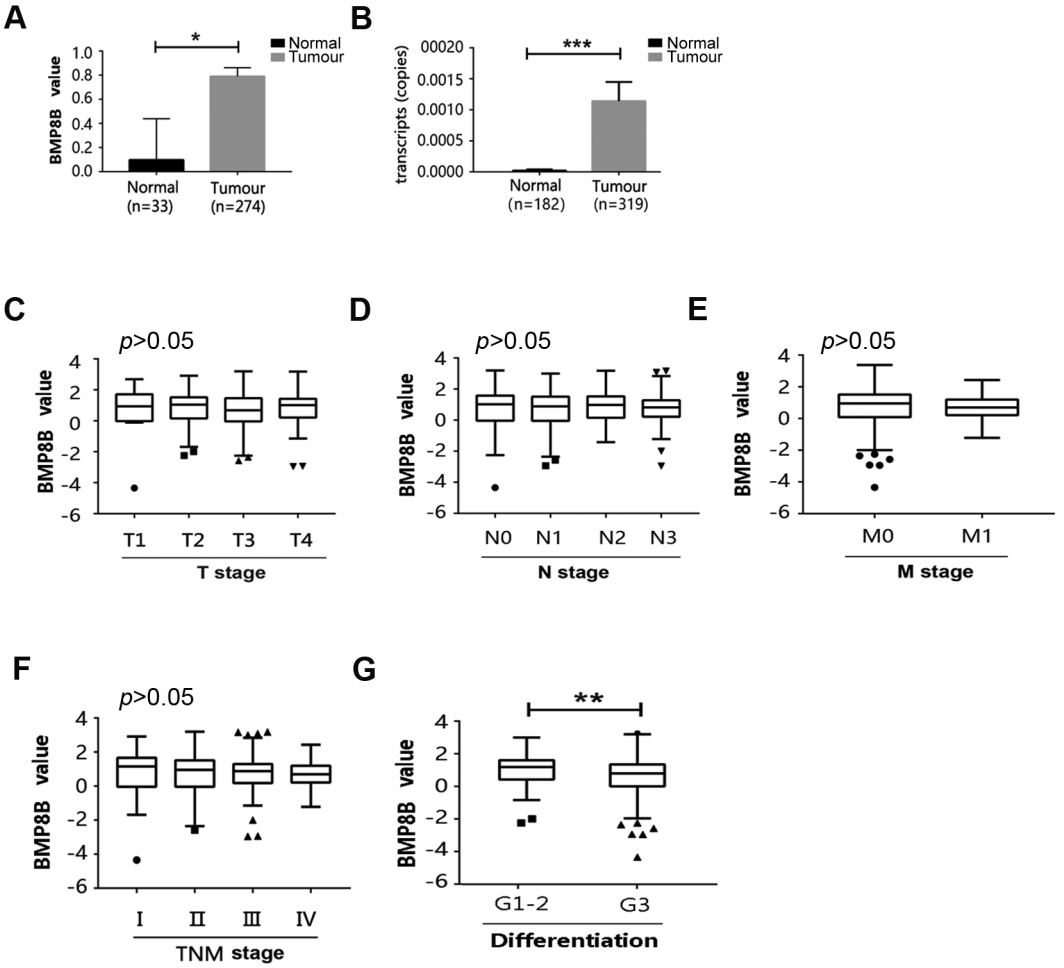

Figure 1. *BMP8B expression in gastric cancer (GC) and its clinical relevance. (A) Expression of BMP8B transcripts in GCs (n=274) and normal tissues (n=33) in the TCGA GC cohort. (B) Expression of BMP8B transcripts in GCs (n=319) and normal tissues (n=182) in the Beijing Cancer Hospital cohort. Correlation between BMP8B mRNA expression and T stage (n=265) (C), N stage (n=262) (D), M stage (n=261) (E), TNM stage (n=245) (F) of GC in the TCGA cohort. (G) Correlation between BMP8B mRNA expression and differentiation (n=269). *p<0.05, **p<0.01; ***p<0.001.*

BMP8B knockdown. Lentiviral vectors carrying either BMP8B shRNA (GACCCTCACAACCACGTACAT) or scramble shRNA (CCTAAGGTTAAGTCGCCCTCG) were purchased from Cyagen Biosciences (Santa Clara, CA, USA). After a packaging of lentiviral particles with pMD2G and pSPAX2 plasmid vectors, HGC27 cells were transduced to establish BMP8B knockdown cells. G418 (500 μg/ml) was used for the selection. Q-PCR and western blot were employed to verify the expression of BMP8B in the transduced cells.

Cell proliferation assay. Cells were plated into 96-well plates (3,000 cells/well) and incubated at 37˚C with 5% CO_2. Cells were then fixed in 4% formaldehyde at days 1, 3, and 5 after plating, and then stained with 0.1% crystal violet. Following washing, staining was extracted with 10% (v/v) acetic acid and the absorbance was read at a wavelength of 540 nm using a spectrophotometer (BIO-TEK, ELx800, Wolf Laboratories, York, UK).

Cell adhesion assay. 30,000 cells were seeded into each well of 96-well plates previously coated with Matrigel (BD Biosciences, Oxford, UK). Non-adherent cells were washed off with PBS buffer after 40 min of incubation, and adhered cells were then fixed with 4% formalin and stained with 0.5% crystal violet. Absorbance was measured at 540 nm after the staining was dissolved with acetic acid (10%).

Cell invasion assay. Transwell inserts with an 8.0 μm pore size membranes (Greiner Bio-One Ltd., Stonehouse, UK) were coated with 50 μg Matrigel airdried. After rehydration, 30,000 cells were added to each well of 24-well plates and incubated for 72 h at 37˚C. Cells that had invaded through the matrix to the other side of the insert were fixed with formalin (4%) and stained with crystal violet (0.5%).

Wound healing assays. Cells were seeded in six-well plates (2×10^6 per well) and allowed to adhere overnight. The layer of cells was then

Table I. *The expression of BMP8B transcripts in gastric cancer.*

Category		No.	Mean±SD	*p*-Value
Tumor	Tumor	319	0.00114±0.00031	0.0004
	Normal	182	0.00002±0.00002	
Sex	Male	227	0.00135±0.00039	0.25
	Female	92	0.00064±0.00047	
Location	Cardia	65	0.00141±0.00085	0.95 (*vs*. Polorus)
	Fundus	21	0.00027±0.0002	0.0.056 (*vs*. Polorus)
	Corpus	61	0.00059±0.00034	0.19 (*vs*. Polorus)
	Pylorus	130	0.00148±0.00059	
Differentiation	Diff-H	1	1.20E-22(*-*)	
	Diff-HM	6	0.00084±0.00055	0.85 (*vs*. Diff-L)
	Diff-M	61	0.00237±0.00107	0.15 (*vs*. Diff-L)
	Diff-ML	82	0.00112±0.00067	0.59 (*vs*. Diff-L)
	Diff-L	136	0.00071±0.00035	
T stage	T1	16	0.00046±0.00038	0.16 (*vs*. T4)
	T2	26	0.00221±0.00178	0.59 (*vs*. T4)
	T3	40	0.00017±0.00007	0.0068 (*vs*. T4)
	T4	229	0.00123±0.00038	
	T1+T2	42	0.00155±0.00111	0.68 (*vs*. T3+T4)
	T3+T4	269	0.00107±0.00032	
N stage	N0	70	0.00112±0.00067	
	N1	48	0.00032±0.00012	0.24 (*vs*. N0)
	N2	63	0.00193±0.00097	0.50 (*vs*. N0)
	N3	132	0.00111±0.00047	0.99 (*vs*. N0)
	N1+N2+N3	243	0.00117±0.00036	0.95 (*vs*. N0)
M stage	M0	278	0.00106±0.00032	0.58
	M1	40	0.00172±0.00114	
TNM stage	I	25	0.00254±0.00186	
	II	60	0.00032±0.00011	0.24 (*vs*. I)
	III	216	0.00125±0.0004	0.50 (*vs*. I)
	IV	9	0.00009±0.00009	0.20 (*vs*. I)
	II+III+IV	285	0.00101±0.00031	0.42 (*vs*. I)
His	Adeno	235	0.00144±0.00042	
	Islet	5	0.00023±0.00023	0.013 (*vs*. Adeno)
	Mixed	48	0.00019±0.00016	0.0057 (*vs*. Adeno)

Numbers in each subgroup represent the number of samples that have both gene levels and clinical information.

scraped with a 200 μl pipette tip to create a wound. After washing with 1×PBS, cell cultures were re-fed with fresh medium. The cells were allowed to move to close the wound for 5 h. Photographs of the wound were taken at 0 and 5 h at the same position.

Statistical analysis. Following a normality check, *t*-test was employed for normally distributed data whilst non-normally distributed data was analysed using Mann-Whitney test. All experiments were repeated three times, and the results are expressed as the mean±SD, $p<0.05$ was considered as statistically significant.

Results

BMP8B expression was up-regulated in the gastric cancer tissues. In the TCGA database, the expression of BMP8B at the mRNA level was significantly up-regulated in the GC tissues (n=274) compared with adjacent normal tissues (n=33) (Figure 1A). As shown in Figure 1B, the up-regulation of BMP8B in GC tissues is more pronounced in our own database from Beijing Cancer Hospital.

To clarify the role of BMP8B in the progression of gastric cancer, we analysed the correlation between the expression of BMP8B and clinical pathological parameters in patients with GC in the TCGA database and found that poorly differentiated GC samples presented a significantly reduced BMP8B expression in relation to well differentiated and moderately differentiated GC ($p=0.007$) (Figure 1G). In GC tissues from the Beijing Cancer Hospital, although the difference of BMP8B expression between poorly differentiated adenocarcinoma and moderately differentiated adenocarcinoma did not reach statistical significance, a decreasing trend was also observed (0.00071±0.00035 *vs*. 0.00237±0.00107) (Table I). In addition, we found that the expression of BMP8B in signet ring cell carcinoma was significantly lower than that in adenocarcinoma (Table I).

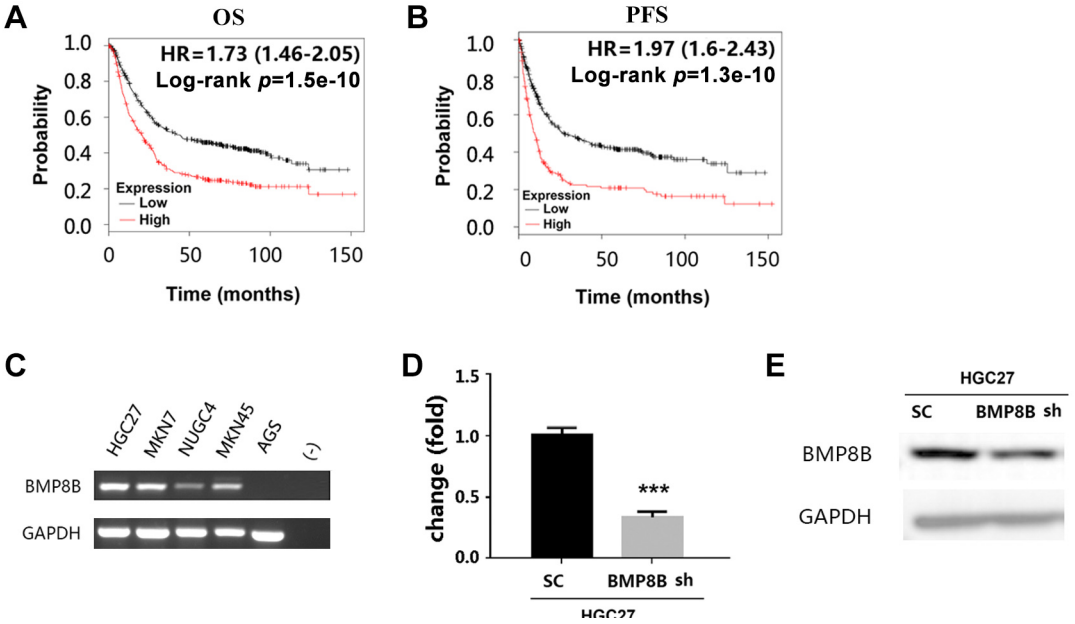

Figure 2. *Higher BMP8B expression correlates with poorer overall survival of patients with gastric cancer (GC). (A) Kaplan-Meier survival analyses show correlations between BMP8B expression and overall survival of patients with GC using the online platform. The cut off value used in the analysis was 47. (B) Correlation between BMP8B expression and progression-free survival (PFS) of GC was analysed, and the cut off value used in the analysis was 47. (C) Evaluation of BMP8B expression in GC cell lines using PCR. BMP8B knockdown in HGC27 was confirmed using Q-PCR (D) and western blot (E), respectively. ***p<0.001. HR: Hazard ratio.*

These results suggest that BMP8B is most likely to be involved in the differentiation of gastric cancer. However, BMP8B expression was not significantly correlated with T stage, N stage, and M stage of GC, in both TCGA database (Figure 1C-F) and our own cohort from Beijing Cancer Hospital (Table I).

KM plotter analysis (http://kmplot.com/) showed that patients with higher expression of BMP8B had shorter OS than those with low expression (n=876) (Figure 2A). In addition, higher expression of BMP8B was also related to poorer progression free survival (PFS) (n=641) (Figure 2B).

Evaluation of BMP8B expression in gastric cancer cell lines using PCR. We examined the expression of BMP8B in five GC cell lines (HGC27, MKN7, NUGC4, MKN45 and AGS) using PCR. Four cell lines had different degrees of expression (except AGS) and among them the HGC27 cell line had the strongest expression (Figure 2C).

BMP8B knockdown inhibits proliferation of GC cells in vitro. Knockdown of BMP8B was employed in HGC27 cells using shRNA, and the knockdown was confirmed using Q-PCR and western blot (Figure 2D and E). Then, we examined the effect of BMP8B on cell functions, including cell proliferation, adhesion, invasion, and migration. As shown in Figure 3A, a growth assay showed that BMP8B knockdown inhibited proliferation of HGC27 cells. The difference in proliferation between BMP8B knockdown HGC27 cell line and scramble control cells was observed on the third day and became more apparent on the fifth day.

To explore the molecular mechanism of BMP8B in the progression of GC, the correlation between BMP8B and cell proliferation indices MKI67 and PCNA was determined. We found a significant positive correlation between BMP8B and Ki67 (Figure 3B-D). More importantly, we found that BMP8B was also significantly positively related to most of the cell cycle-promoting molecules, especially CCNE1, CDK2, CCNB2, CDK1, CCNB3, CCNA2, CCNB1 and CCNC (Figure 3D and E). The correlation between BMP8B and cell cycle inhibitor molecules P21 and P27 did not reach statistical significance, but there was a trend of negative correlation (Figure 3D and E). These results suggest that the BMP8B's effect on GC cell proliferation is likely to be accomplished by regulating the cell cycle.

BMP8B knockdown promotes invasion and migration of GC cells in vitro. The adhesion assay showed that there was no significant difference in adhesion between the BMP8B knockdown HGC27 cell line and scramble control cells (Figure 4A). Invasion assay showed that BMP8B knockdown

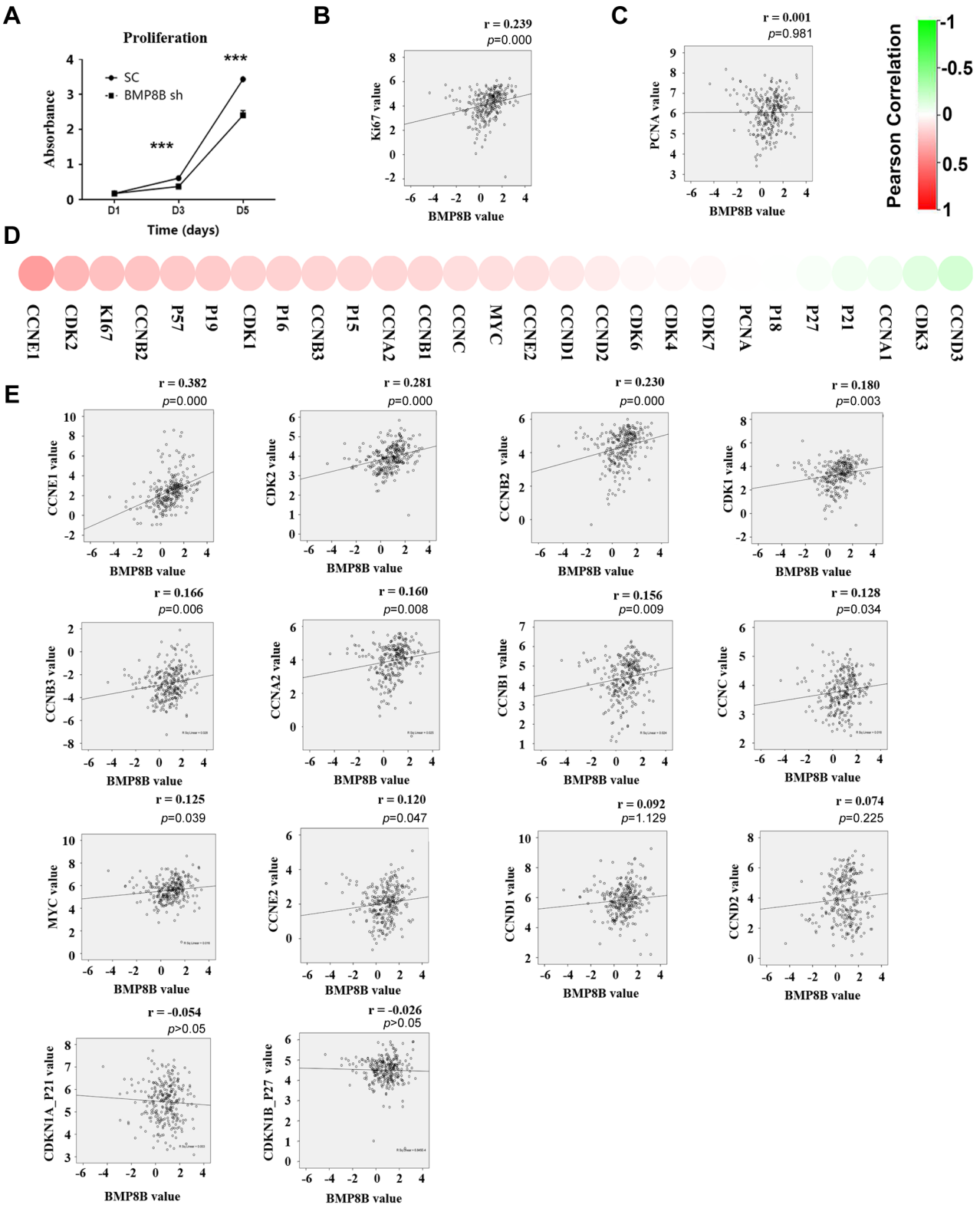

Figure 3. *BMP8B and tumor growth in gastric cancer (GC). (A) Cell proliferation assay was performed using HGC27$^{BMP8B\ sh}$ cells. Correlation between BMP8B mRNA expression and Ki67/PCNA was analysed using Spearman tests; results are shown as a heatmap (D) and scatter plots (B-C). Correlations between BMP8B mRNA expression and cell cycle regulators are shown as heatmap (D) and scatter plots (E). Three independent experiments were performed.*

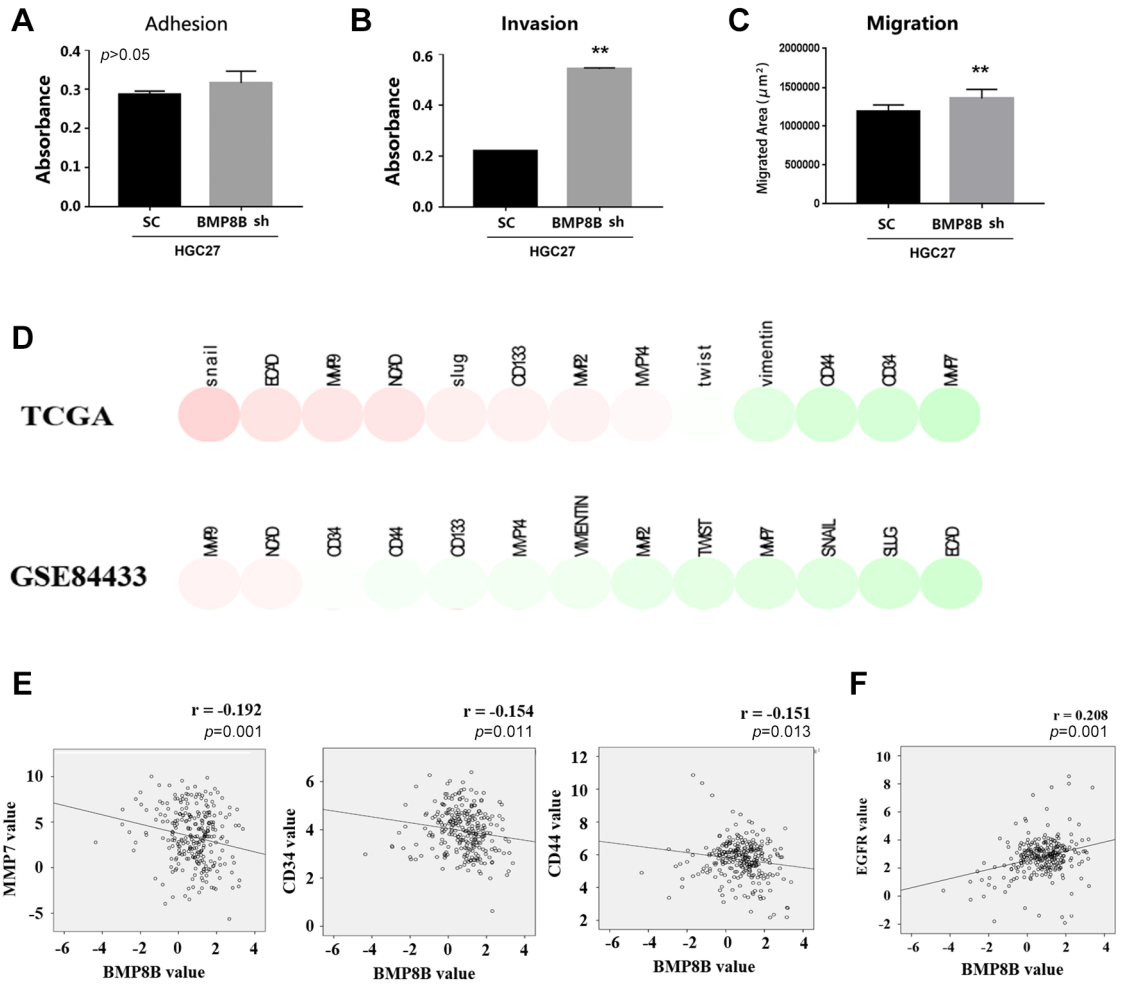

Figure 4. *BMP8B regulates the adhesion, invasion, and migration of gastric cancer (GC) cells. Cell adhesion assay (A) and transwell invasion assay (B) were performed to evaluate the impact of BMP8B on the adhesion and invasiveness of GC cell lines. Wound healing assays were performed using HGC27^{SC} and HGC27$^{BMP8B\ sh}$ and semi-quantification of migration area was performed using Image J (C). Aberrant expression of BMP8B correlates with the EMT, MMPs and stemness in GC. Correlations between BMP8B mRNA expression and EMT markers, MMPs and stem cell markers are shown as heatmap (D) and scatter plots (E). (F) Correlation between BMP8B and EGFR in TCGA database. Three independent experiments were performed. **p<0.01.*

promotes invasion of GC cells (Figure 4B). In addition, wound healing assays showed that BMP8B knockdown cells had increased migratory capacity (Figure 4C). These results show that BMP8B knockdown promotes cell invasion and migration, which is different from its effect on cell proliferation.

We analyzed the correlation between the expression of BMP8B and some important molecules including EMT-related molecules (SNAI1, SNAI2, and TWIST1), matrix metalloproteinases (MMP2, MMP7, MMP9 and MMP14) and stemness markers (CD34, CD44 and CD133) in the TCGA database and GSE84433 database and found that BMP8B was significantly negatively correlated with MMP7, CD34 and CD44 in both databases (Figure 4D and E).

In addition, we found that the expression of BMP8B and EGFR were significantly positively correlated in the TCGA database (Figure 4F), indicating that EGFR may also be involved in the effect of BMP8B on gastric cancer.

Correlation between BMP8B and other BMPs and BMP receptors (BMPRs). We analysed the correlation between BMP8B and other BMPs and BMPRs in TCGA database, GSE84433 database, and GSE36139 database, and the results were consistent (Figure 5B). As shown in the Figure 5A, BMP8B has a significant positive correlation with BMP7, ACVR2B, ACVR2A, ALK7 and ALK6, while BMP8B has a significant negative correlation with ALK2, BMP6 and TGFBR2.

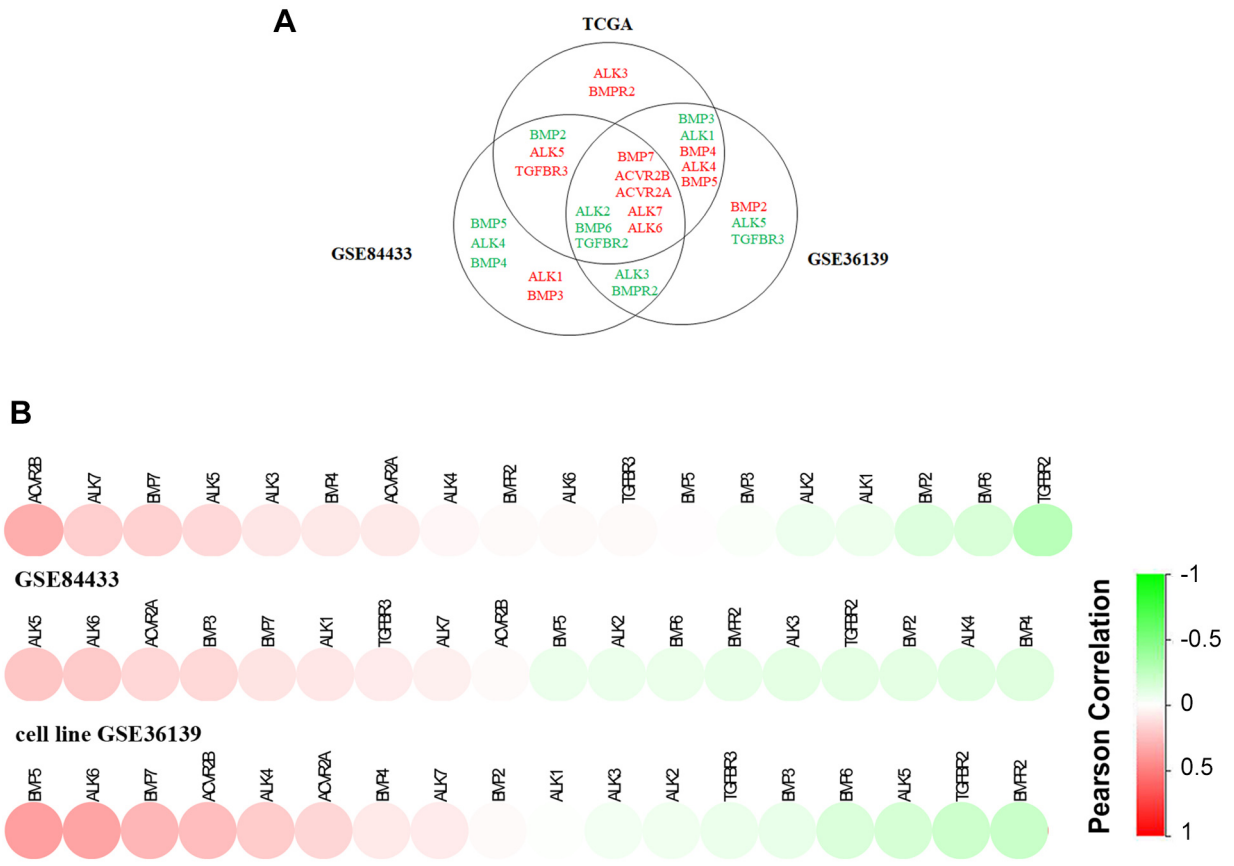

Figure 5. *Correlations between BMP8B and other bone morphogenetic proteins (BMPs) and bone morphogenetic protein receptors (BMPRs). (A) The overlapping BMPs/BMPRs that are more closely associated with BMP8B in TCGA, GSE84433, and GSE36139 database are shown. (C) Correlations between BMP8B mRNA expression and other BMPs/BMPRs. Red represents positive correlation, and green represents negative correlation.*

Discussion

Previous studies have shown that BMPs can regulate the homeostasis of the gastric epithelium (22, 23, 32), and also play an important role in the progression of GC through regulating the proliferation or invasion, migration of cancer cells (28, 29, 33-35). They may function as tumor-suppressors or tumor-promoters, depending on the different BMP ligands (29, 30, 36, 37). For example, BMP-2 and BMP-4 suppress the proliferation of GC cells *via* the induction of p21 (36). BMP-4 expression rate was inversely related to the prevalence of lymph node metastasis and tumor invasiveness (29). BMP-7 promoted metastasis of GC and correlated with poor prognosis (37).

However, the role of BMP8B in GC remains uncertain because there have been only two relevant studies. Mima *et al*. (30) reported that higher BMP8B in the GC was significantly associated with poorer survival, and Wisnieski (31) demonstrated that BMP8B expression was reduced in GC compared to nontumor samples, and it was associated with differentiation of tumor. This study systematically analyzed the expression of BMP8B in GC compared to normal tissues, and its correlation with clinicopathological factors. In addition, the study is the first to explore the effect of BMP8B on the function of GC cell lines by establishing a BMP8B knockdown model. Our results contribute to understanding the mechanism of BMP8B involved in the disease progression of GC.

As mentioned above, only one research has compared the difference of BMP8B expression between GC and normal tissues. Wisnieski (31) detected the expression of BMP8B in 42 matched pairs of GCs and corresponding adjacent nontumor tissues, showing that BMP8B mRNA expression was significantly reduced in GC tissues ($p<0.01$). However, in this study, we found that the expression of BMP8B at the mRNA level was significantly up-regulated in the GC tissues compared with adjacent normal tissues in both TCGA database and our own database from Beijing Cancer Hospital. The difference may be related to ethnic differences, and different experimental conditions such as

mRNA quantification methods may also have some impact on the results.

We found that the expression of BMP8B in GC is related to the prognosis of patients in the KM-plotter analysis. Higher expression of BMP8B correlated with both shorter OS and shorter PFS. This result is consistent with a previous report by Mima *et al*. (30), showing that high BMP8B mRNA expression was associated with a shorter survival of patients with GC following a curative resection. Furthermore, the multivariate analysis revealed that the prognostic power of BMP8B mRNA expression in the tumor was independent of other standard prognostic markers (HR=2.066; 95%CI=1.132-3.772; p=0.018) such as tumor size and the presence of the histological diffuse-type GC. However, we found that BMP8B expression was not significantly correlated with T stage, N stage and M stage of GC, in both TCGA database and our own cohort from Beijing Cancer Hospital, which was also consistent with a previous report by Mima *et al*. (30).

Pathologic grade classifies tumors into well, moderately, or poorly differentiated/anaplastic (38, 39). Previous studies have shown that the degree of differentiation of tumor cells correlates with the aggressiveness of the tumor (40-43). Poorly differentiated tumors are more invasive than well and moderately differentiated tumors. And some studies reported (44) that histology types (differentiated or undifferentiated) are strong indicators of poor prognosis in node negative patients with GC. In the present study, we found that poorly differentiated GC samples presented a significantly reduced BMP8B expression compared to well-differentiated and moderately differentiated GC, and the expression of BMP8B in signet ring cell carcinoma was also significantly lower than that in adenocarcinoma. In addition, we can find the same result in Wisnieski's research (31). Furthermore, a study has revealed that a BMP can modulate the differentiation of gastric cells by increasing pepsinogen II, a differentiation marker of the stomach (27). These results suggest that BMP8B is most likely to be involved in the differentiation of GC.

To explore the mechanism of BMP8B acting on GC cells, we established BMP8B knockdown model and performed the experiment *in vitro*, showing that BMP8B knockdown significantly inhibited proliferation of HGC27 cells. Cheng (45) has studied the effects of BMP8B on the proliferation of pancreatic cancer cell lines, and found that the over-expression of BMP8B inhibited cell growth and promoted activation of caspase-3 and -9, decreased the mitochondrial membrane potential, and inhibited PANC-1 cell apoptosis, while silencing the BMP8B gene expression with BMP8B shRNA exerted anti-apoptotic effects and boosted the growth of pancreatic cancer cells. It seemed that the effect of BMP8B on the proliferation of GC cell lines and pancreatic cancer cell lines may be reversed. In our research, we also found that BMP8B is significantly positively correlated with Ki67 and most cell cycle-promoting molecules. It is speculated that BMP8B's promotion of GC cell proliferation is likely to be achieved by regulating the cell cycle. However, the specific mechanism needs further confirmation in further research.

To the best of our knowledge, there are no studies on the effect of BMP8B on cancer cell invasion and migration. We are the first to report that BMP8B knockdown promotes invasion and migration of GC cells. Furthermore, we found that BMP8B was significantly negatively correlated with MMP7 and stem cell markers CD34 and CD44. MMPs play an important role in local invasion and distant metastasis of tumors, and our previous studies have also confirmed that many BMP receptors, including ACVRL1, ACVR1, TGFBR1, BMPR1B and TGFBR2 were also related to the expression of various MMP2, MMP7 and MMP14 (46). Tumor stem cells are a group of tumor cells with self-renewal ability and multi-directional differentiation potential. Some studies showed that cancer stem cells might form the basis of cancer invasion and metastasis (47-49). Taken together, it was shown that BMP8B was significant negatively associated with MMP7 and stem cell markers CD34 and CD44, which may contribute to the regulation of GC invasion by BMP8B. In addition, we found that the expression of BMP8B and EGFR were significantly positively correlated in the TCGA database, indicating that EGFR may also be involved in the effect of BMP8B on GC.

In our previous research, we systematically analysed the expression and clinical significance of BMPs (BMP2-BMP7) and BMP receptors (BMPR) in TCGA GC database and Gene Expression Omnibus (GEO) database and explored the possible mechanism of action (46). We found that most of the BMPs and BMPRs may inhibit proliferation of GC cells, and also, promote disease progression through a promotion of invasion, EMT and stemness. Among the BMPs and BMPRs, ALK1, ALK5, ALK6, TGFBR2, TGFBR3 and BMPR2 had the most statistically significant effect. The results are contrary to the effect of BMP8B on GC cells in this study, which showed that BMP8B knockdown inhibited proliferation of GC cells, while promoted invasion and migration of GC cancer cells. Thus, we hypothesize that BMP8B is a different from other BMPs and BMPRs. Then we analysed the correlation between BMP8B and other BMPs and BMPRs in TCGA database, GSE84433 database, and GSE36139 database, and found that BMP8B has a significant positive correlation with BMP7, ACVR2B, ACVR2A, ALK7 and ALK6, while BMP8B has a significant negative correlation with ALK2, BMP6 and TGFBR2 in these three databases. In addition, BMP8B was found to be mostly negatively correlated with the majority molecules that may play a crucial role in GC, such as TGFBR2, TGFBR3, BMPR2 and ALK5. It can be hypothesized that the

mechanism of BMP8B in GC is likely to be different from other BMPs and BMPRs, but the specific molecular mechanism needs to be further studied.

In conclusion, BMP8B expression was significantly up-regulated in GC tissues compared with adjacent normal tissues, and high BMP8B expression was associated with poor prognosis. BMP8B is most likely to be involved in the differentiation of gastric cancer. Poorly differentiated GC samples presented a significantly reduced BMP8B expression in relation to well-differentiated and moderately differentiated GC. BMP8B knockdown inhibited proliferation of GC cells, while promoted invasion and migration of cancer cells. These findings provide possible mechanisms of GC progression influenced by BMP8B, a potential therapeutic target for the treatment.

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Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

LY and WGJ designed the study. ZS, SC, XL, WGJ and LY did the experiments. ZS, SC, WGJ and LY contributed to data analyses. ZS, CS, WGJ and LY prepared the manuscript. ZS, SC, XL, WGJ and LY revised and proofread the article.

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