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Original Paper

Berberine Suppresses Cell Motility Through Downregulation of TGF-β1 in Triple Negative Breast Cancer Cells

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Key Words
Berberine • TGF-β1 • MMP-2 • MMP-9 • Metastasis

Abstract
Background/Aims: Transforming growth factor-beta proteins (TGF-βs) are multifunctional growth factors and powerful modulators of the epithelial-mesenchymal transition (EMT) in a variety of cancer types including breast and lung cancer cells. Here, we demonstrated the inhibitory effect of berberine (BBR) on tumor growth and metastasis of triple negative breast cancer (TNBC) cells via suppression of TGF-β1 expression. ***Methods:*** The levels of mRNA expression were analyzed by real-time PCR. The levels of MMP-2, MMP-9 and TGF-β1 protein expression were analyzed by zymography and confocal microscopy, respectively. Cell migration was analyzed by wound healing assay. Tumorigenicity of TNBC cells such as tumor growth and metastasis was analyzed using xenograft models. ***Results:*** In a clinical data set, aberrant TGF-β1 expression was associated with poor prognosis of breast cancer patients. Our *in vitro* results using TNBC cells showed that the expression levels of matrix metalloproteinase (MMP)-2 and MMP-9 and the capacity for cell migration were increased by TGF-β1 treatment. In contrast, basal levels of MMP-2 and MMP-9 were suppressed by a specific TGF-β receptor I inhibitor, SB431542. In addition, TGF-β1–induced MMP-2 and MMP-9 expression and cell migration were decreased by SB431542. Interestingly, we showed for the first time that BBR decreased the level of TGF-β1, but not TGF-β2, in TNBC cells. Furthermore, BBR significantly decreased the level of MMP-2 expression as well as the capacity for cell migration in TNBC cells. Finally, we examined the effect of BBR on *in vivo* tumor growth and lung metastasis in MDA-MB231 and 4T1 breast cancer xenograft models and showed that both were significantly decreased following BBR treatment. ***Conclusion:*** BBR suppresses tumorigenicity of TNBC cells through inhibition of TGF-β1 expression. Therefore, we demonstrate that BBR could be a promising drug for treatment of TNBC.

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Introduction

Triple negative breast cancers (TNBC) represent approximately 10 to 20% of all breast cancers and are defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [1]. TNBC is a heterogeneous disease, and 75% of cases are a basal-like molecular subtype [2]. TNBC generally occurs in younger women aged less than 50 years and is characterized by high proliferation rates, frequent metastases, and poor prognostic factors for disease-free survival and overall survival [3, 4]. In addition, there is no effective specific targeted therapy for TNBC, and therapeutic options for TNBC are currently limited to conventional chemotherapy. Thus, many researchers have been trying to find an effective targeted therapy for TNBC.

Transforming growth factor-β (TGF-β) proteins are multifunctional cytokines that control various processes in cancer development such as cell growth, invasion, migration, epithelial-mesenchymal transition (EMT), and apoptosis [5]. In a previous study, we reported that the levels of TGF-β1 and TGF-β2 are higher in TNBC cells than in non-TNBC cells [6]. The TGF-β signaling pathway also plays a critical role in breast cancer progression and metastasis [7]. Aberrant TGF-β2 expression is associated with poor prognosis in patients with basal type breast cancer [8]. As a result of the wide variety of effects of TGF-β on tumorigenesis, blockers of TGF-β and its signaling pathways such as ligand traps, antisense oligonucleotides, and small-molecule receptor kinase inhibitors have provided multiple therapeutic opportunities for the treatment of various diseases including cancer [9-11].

Berberine (BBR), an isoquinoline quaternary alkaloid, is a plant-derived compound used in traditional Chinese medicine that can be isolated from many kinds of medicinal plants such as *Hydrastis canadensis*, *Berberis aristata*, and *Coptis chinensis* [12]. BBR has multiple pharmacological properties including antioxidant, anti-apoptotic, and anticancer effects and activity against obesity and Alzheimer's disease [13-16]. BBR affects cancer cell proliferation with consequent impairment of cell division through downregulation of cyclin B1 and upregulation of p21 in a variety of cancer types, including lung and breast cancer cells [17-19]. In addition, BBR triggers pro-apoptotic effects through suppression of the HER2/PI3K/AKT signaling pathway [16]. We also reported that inhibition of AP-1 transcriptional activity by BBR treatment suppresses invasion of breast cancer cells [20].

Here, we explore the inhibitory effect of BBR on TGF-β1 expression and cell motility in TNBC models. We show that BBR significantly decreases the level of TGF-β1 expression, but not TGF-β2 expression, in TNBC cells. The reduction of TGF-β1 expression by BBR treatment triggers suppression of cell migration through downregulation of MMP-2. In addition, BBR also suppresses the phosphorylation level of smad3 in TNBC cells. Therefore, we suggest that BBR could have possible applications as a therapeutic drug for treatment of TNBC patients.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo Scientific (Hemel Hempstead, UK). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Berberine was purchased from Sigma-Aldrich (St. Louis, MO, USA). SB431542 was purchased from Tocris (Ellisville, MO, USA). Rabbit polyclonal anti–TGF-β1 and mouse monoclonal anti–β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti–phospho-smad3 (p-smad3) was from AbCam (Cambridge, United Kingdom). ECL Western Blotting Detection Reagents (West-Q Chemiluminescent Substrate Plus kit) were obtained from Genedepot (Barker, TX, USA).

Analysis of public database

Expression data were downloaded from a public database [Kaplan–Meier plotter database (http://kmplot.com/breast)]. The clinical value of TGF-β1 mRNA expression in patients with basal-type breast cancer was analyzed by Kaplan–Meier survival plots. The hazard ratio with 95% confidence interval and log-rank p-values were calculated.

Cell culture

MDA-MB231 human breast cancer cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. HCC1806 human breast cancer cells were grown in RPMI1640 conditioned media. Each cell line was maintained in culture medium without FBS for 24 h.

Western blotting

Cell lysates were prepared to detect p-smad3 and β-actin expression. Equal amounts of proteins (50 μg) were boiled for 5 min in Laemmli sample buffer and then electrophoresed in 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and the membranes were blocked with 10% skim milk in Tris-buffered saline (TBS) containing 0.01% Tween-20 (TBS/T) for 15 min. Blots were washed three times in TBS/T and then incubated with antibodies against p-smad3 and β-actin in TBS/T buffer at 4°C overnight. Blots were washed three times in TBS/T and subsequently incubated with secondary HRP-conjugated antibodies (1:2, 000 dilution) in TBS/T buffer. After 1-h incubation at room temperature (RT), the blots were washed three times in TBS/T. Positive immunoreactive proteins were detected using the West-Q Chemiluminescent Substrate Plus kit.

Real-Time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Isolated RNA samples were then used for RT-PCR. Samples (1 μg total RNA) were reverse-transcribed into cDNA in 20-μl reaction volumes using a first-strand RT-PCR cDNA synthesis kit according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA).

Gene expression was quantified by real-time PCR using a SensiMix SYBR Kit (Bioline Ltd., London, UK) and 100 ng cDNA per reaction. The specific primer sets used to detect gene mRNA expression are shown in Table 1. An annealing temperature of 60°C was used for all primers. PCR was performed in a standard 384-well plate format with an ABI 7900HT real-time PCR detection system. The raw threshold cycle (C_T) value was normalized to the housekeeping gene for each sample to obtain the ΔC_T. The normalized ΔC_T value was calibrated to the control cell samples to obtain the $\Delta\Delta C_T$ value.

Wound healing assay

HCC1806 and MDA-MB231 TNBC cells were seeded in six-well plates and cultured for 24 h. Cells were maintained in culture medium without FBS for 16-24 h. The cell monolayer was scratched with a 200-μl pipette tip to create a wound, which was washed twice with PBS to remove the suspended cells. The cells were maintained with or without 10 μM SB431542 or 50 μM BBR for 24 h in serum-containing medium, and cells migrating from the leading edge were photographed at 0 and 24 h using a CK40 inverted microscope (Olympus, Tokyo, Japan).

Zymography

Zymography was performed on 10% polyacrylamide gels that had been cast in the presence of gelatin, as described previously [21]. Briefly, samples (100 μl) were resuspended in loading buffer and separated on a 10% SDS-PAGE gel containing 0.5 mg/ml gelatin without prior denaturation. After electrophoresis, the gels were washed to remove SDS and incubated for 30 min at room temperature in a renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, and 1% Triton X-100). The gels were incubated for 48 h at 37°C in a developing buffer (50 mM Tris-HCl [pH 7.8], 5 mM $CaCl_2$, 0.15 M NaCl, and 1% Triton X-100) and then stained with Coomassie Brilliant Blue G-250, destained in 30% methanol, and flooded with 10% acetic acid to detect gelatinase secretion.

Table 1. Specific primer sequences for analysis of TGF-β1, TGF-β2, MMP-2, MMP-9, and GAPDH mRNA expression

Gene-Name	Forward	Reverse
TGF-β1	TGA ACC GGC CTT TCC TGC TTC TCA TG	GCG GAA GTC AAT GTA CAG CTG CCG C
TGF-β2	TAC TAC GCC AAG GAG GTT TAC AAA	TTG TTC AGG CAC TCT GGC TTT
MMP-2	GGC CTC GTA TAC CGC ATC AAT C	GGC CTC TCC TGA CAT TGA CCT T
MMP-9	CCC GGA CCA AGG ATA CAG	GGC TTT CTC TCG GTA CTG
GAPDH	ATT GTT GCC ATC AAT GAC CC	AGT AGA GGC AGG GAT GAT GT

Confocal microscopy

Human breast cancer BT474 and Hs578T cells grown on four-well Lab-Tek chamber slides were allowed to adhere overnight, fixed for 20 min in 4% paraformaldehyde, and incubated at 4°C overnight with anti–TGF-β1 antibody. Cells were washed three times in 1x phosphate buffered saline (PBS) and incubated with AlexaFluor 488-conjugated goat anti-mouse secondary antibody (1:50 dilution) for 60 min at RT. The slides were washed and mounted in Vectashield H-1200/DAPI mounting media (Vector Laboratories, Burlingame, CA, USA). Confocal images were analyzed using an LSM780 confocal laser-scanning microscope (Carl Zeiss, Zena, Germany).

Flow cytometry analysis (FACS)

Cells were trypsinized and harvested by centrifugation at 1, 500 rpm for 5 min. The cell pellets were then resuspended in 1 mL of PBS and fixed in 70% ethanol for 20 min at RT. The fixed cells were centrifuged and washed twice with PBS to wash out any apoptotic cells. The cells were resuspended in 1 mL of PBS with 100 μg/mL of DNase-free RNase A (Biopure, Canada) and then incubated for 30 min in a 37°C water bath. After centrifugation at 1, 500 rpm, the cell pellets were washed twice with PBS, resuspended in PBS containing 50 μg/mL propidium iodide (Sigma), and then analyzed using FACS-vantage (Becton–Dickinson, San Diego, CA, USA).

Soft agar colony formation assay

MDA-MB231 breast cancer cells were seeded at a density of 5 x 10^4 cells/well in 6-well plates in growth medium containing 0.7% agar (1.5 ml/well) on top of a layer of growth medium containing 1.4% agar (2 ml/well). Growth medium (500 μl) containing 20% FBS and top agar was mixed in equal volumes. In addition, 50 μM BBR was added on top of the agar in some plates. The cell suspension was plated and cultured in a 37°C incubator. After 2 weeks, viable colonies were stained with 0.01% crystal violet and observed using a CK40 inverted microscope (Olympus, Tokyo, Japan).

In vivo tumor growth inhibition by BBR in xenografts

We used 6- to 8-week-old female Balb/c nude mice (weight, 18-22 g; Orient Bio, Seoul, Korea) to establish a nude mice xenograft model. The mice were kept in pathogen-free animal housing in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were used according to protocols approved by the appropriate Institutional Review Board of Samsung Medical Center (Seoul, Korea).

Firefly luciferase 2 (Luc2) and td Tomato (L2T) fusion constructs were gifts of Dr. Sanjiv Sam Gambhir (Stanford University, CA, USA) [22]. Stable MDA-MB231-Luc cells were cultured and resuspended in matrigel (BD Biosciences, Bedford, MA, USA) to a final concentration of 5 × 10^6 cells/100 μL, which was injected directly into the right secondary mammary fat. The mice were randomly divided into two groups (n = 5/group), which were treated with water only (vehicle) or BBR in drinking water (0.1%) starting on the day of inoculation and continuing until the end of the experiment. The tumor size of mice in both groups was measured using digital calipers at set time points, and the volume was determined using the formula V=1/2 length × $(\text{width})^2$. Growth curves were calculated using average relative tumor volume within each group (vehicle- or BBR-treated) at the set time points. Bioluminescent imaging analysis was performed by a Xenogen IVIS® Spectrum (PerkinElmer). Mice were injected intraperitoneally with 100 μl of D-luciferin (10 mg/ml) in PBS and imaged 10 min later under anesthesia with 2.5% isofluorane. Luminescence images were captured as photons/sec/ROI (region of interest) minus background luminescence of a similarly sized region.

Inhibition of tumor metastasis by BBR in xenografts

TNBC 4T1 cells were cultured and resuspended in Matrigel (BD Biosciences, Bedford, MA, USA) to a final concentration of 1 × 10^5 cells/100 μL and were injected directly into the right secondary mammary gland fat pad. The mice were randomly divided into two groups (n = 5/group), which were treated with water only (vehicle) or BBR in drinking water (0.1%) starting on the day of inoculation and continuing until the end of the experiment. Lungs were removed, and histological features were analyzed using hematoxylin and eosin (H&E) staining. The number of tumor nodules was counted.

Statistical analysis

Statistical significance was determined using Student's t-test. Results are presented as mean ± SEM. All quoted p-values were two-tailed, and differences were considered statistically significant when the p-value was < 0.05. Statistical analyses were performed using Microsoft Excel.

Results

Aberrant TGF-β1 expression is associated with poor prognosis in breast cancer patients

Recently, we reported that aberrant expression of TGF-β1 and TGF-β2 triggers the EMT process in TNBC cells [6]. In a clinical data set, we investigated the relationship between TGF-β1 expression and clinical outcomes and the prognostic value of TGF-β1 in breast cancer patients using a Kaplan–Meier plotter database (http://kmplot.com/breast). Our results showed that induction of TGF-β1 was associated with poor prognosis in breast cancer patients (Fig. 1A). Patients with high TGF-β1 level showed poorer relapse-free survival (Fig. 1A, left, P = 0.004) and distant metastasis-free survival (Fig. 1A, right, P = 0.0098) than patients with low TGF-β1 level.

We next investigated the effect of TGF-β1 on cell motility in HCC1806 TNBC cells. As shown in Fig. 1B, the levels of MMP-2 and MMP-9 mRNA expression were increased by TGF-β1 treatment in HCC1806 TNBC cells. The level of MMP-2 mRNA expression was increased 4.0 ± 0.7-fold by treatment with 10 ng/ml TGF-β1 relative to the non-treated level (Fig. 1B). In addition, the level of MMP-9 mRNA expression was increased by 20.8 ± 4.3-fold relative to the control (Fig. 1B). We examined the levels of MMP-2 and MMP-9 protein expression under the same conditions. As expected, the levels of MMP-2 and MMP-9 protein expression, as well as the level of smad3 phosphorylation, were increased by TGF-β1 (Fig. 1C). Furthermore, the rate of cell migration of HCC1806 TNBC cells was significantly increased by TGF-β1 treatment (Fig. 1D). These results demonstrated that the level of TGF-β1 expression is directly associated with poor prognosis, and that TGF-β1 augments cell migration through the induction of MMP-2 and MMP-9 in TNBC cells.

The rate of cell migration is decreased by a specific TGF-β receptor I inhibitor, SB431542, in TNBC cells

To verify the effect of a specific TGF-β receptor I inhibitor, SB43154, on cells, we treated TNBC cells with 10 μM SB431542 for 24 h. As shown in Fig. 2A, basal MMP-2 and MMP-9 mRNA expression was significantly decreased by SB43154 treatment. The level of MMP-2 mRNA expression was decreased by 0.07 ± 0.01-fold by SB431542 compared with the non-treated level (Fig. 2A). In addition, the level of MMP-9 mRNA expression was also decreased by 0.17 ± 0.07-fold relative to the non-treated level (Fig. 2A). Under the same condition, basal levels of MMP-2 and MMP-9 protein and the phosphorylation level of smad3 were decreased by SB431542 (Fig. 2B). As expected, the rates of cell migration were significantly suppressed by SB43154 treatment in both HCC1806 and MDA-MB231 TNBC cells (Fig. 2C).

Next, we investigated the effect of SB43154 on TGF-β1–induced cell migration. After pretreatment with SB431542 for 30 min, HCC1806 TNBC cells were treated with 10 ng/ml TGF-β1 for 24 h. As shown in Fig. 3A, the levels of MMP-2 and MMP-9 mRNA expression were increased by TGF-β1, whereas TGF-β1–induced MMP-2 and MMP-9 expression was significantly decreased by SB431542. Under the same conditions, TGF-β1–induced MMP-2 and MMP-9 protein expression was also decreased by SB431542 (Fig. 3B). In addition, the enhancement of cell migration by TGF-β1 was significantly suppressed by SB43154 treatment in HCC1806 TNBC cells (Fig. 3C). These results indicate that a TGF-β1–dependent signaling pathway is directly associated with cell migration through the induction of MMP-2 and MMP-9 in TNBC cells.

BBR suppresses expression of TGF-β1, but not TGF-β2, in TNBC cells

In a previous study, we reported that BBR suppresses the invasiveness of breast cancer cells through inhibition of PKC-α [23]. Here, we investigated the effect of BBR on

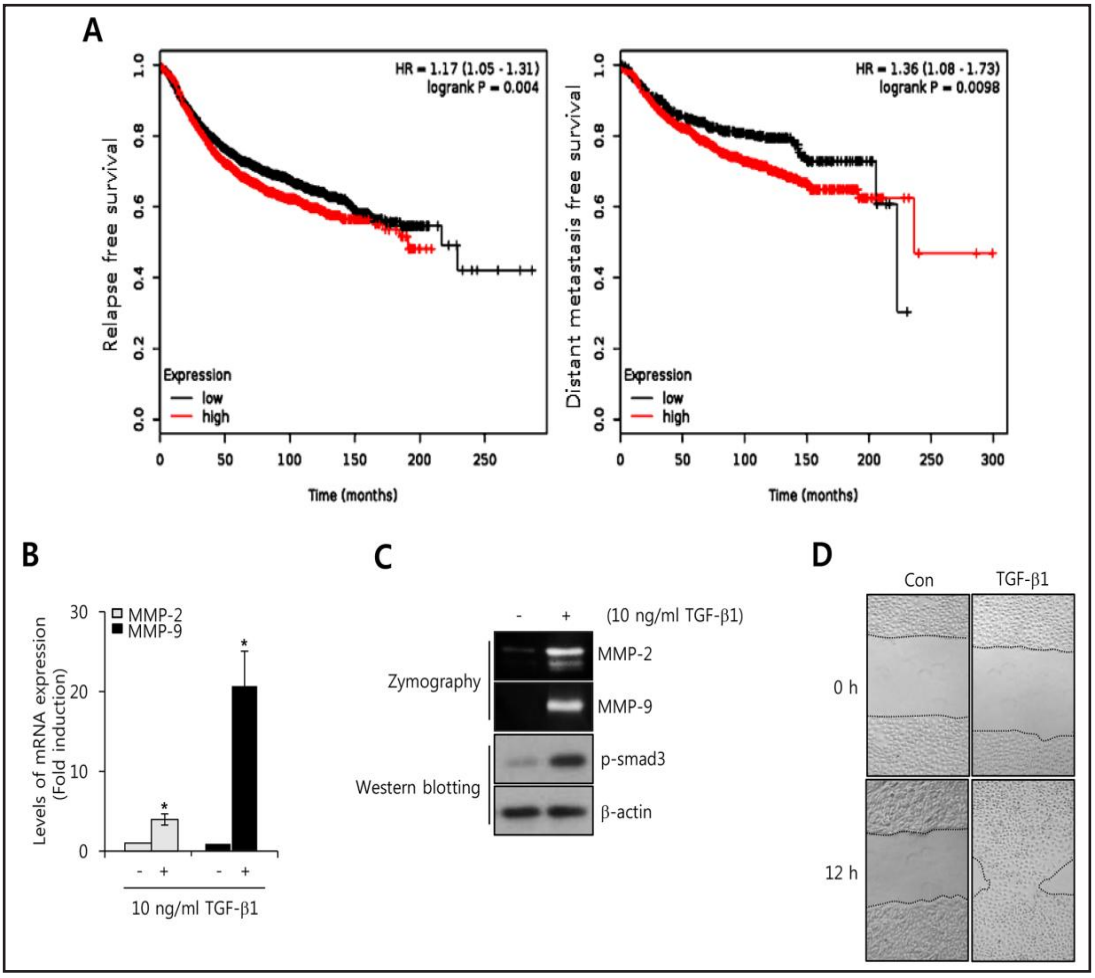

Fig. 1. Aberrant TGF-β1 expression is associated with poor prognosis in breast cancer patients. (A) Clinical values of TGF-β1 mRNA expression obtained from a public database [Kaplan–Meier plotter database (http://kmplot.com/breast)]. (B, C) After serum-starvation for 24 h, HCC1806 cells were treated with 10 ng/ml TGF-β1 for 24 h. (B) The levels of MMP-2 and MMP-9 mRNA expression were analyzed by real-time PCR. (C) The levels of MMP-2 and MMP-9 protein expression were analyzed by zymography, and the levels of smad3 and β-actin protein expression were analyzed by western blotting. (D) The migrating ability of HCC1806 cells was analyzed using the wound healing assay. The results are representative of three independent experiments. Values shown are mean ± SEM. * P<0.05 vs. control. Con: control.

the expression of TGF-β proteins, which are powerful modulators of the EMT in a variety of cancer cells including breast cancer cells. Interestingly, our results showed that BBR treatment decreased the level of TGF-β1 mRNA expression, but not TGF-β2 mRNA expression (Fig. 4B). The level of TGF-β1 mRNA expression was decreased 0.60 ± 0.04-fold (HCC1806 cells) and 0.67 ± 0.13-fold (MDA-MB231 cells) relative to the non-treated level by treatment with 50 μM BBR (Fig. 4B). Using confocal microscopy, we showed that the level of TGF-β1 protein expression was decreased by 50 μM BBR in both HCC1806 and MDA-MB231 TNBC cells (Fig. 4C).

To examine the inhibitory effect of BBR on cell migration in TNBC cells, we treated the cells with 50 μM BBR for 24 h. As shown in Fig. 5A, the basal level of MMP-2 mRNA expression was decreased by BBR. The level of MMP-2 mRNA expression in HCC1806 TNBC cells was decreased by 0.20 ± 0.06-fold compared to that of non-treated cells by 50 μM BBR (Fig. 5A). Under the same conditions, the level of MMP-2 protein expression was also decreased by BBR treatment (Fig. 5B). In addition, BBR completely suppressed smad3 phosphorylation

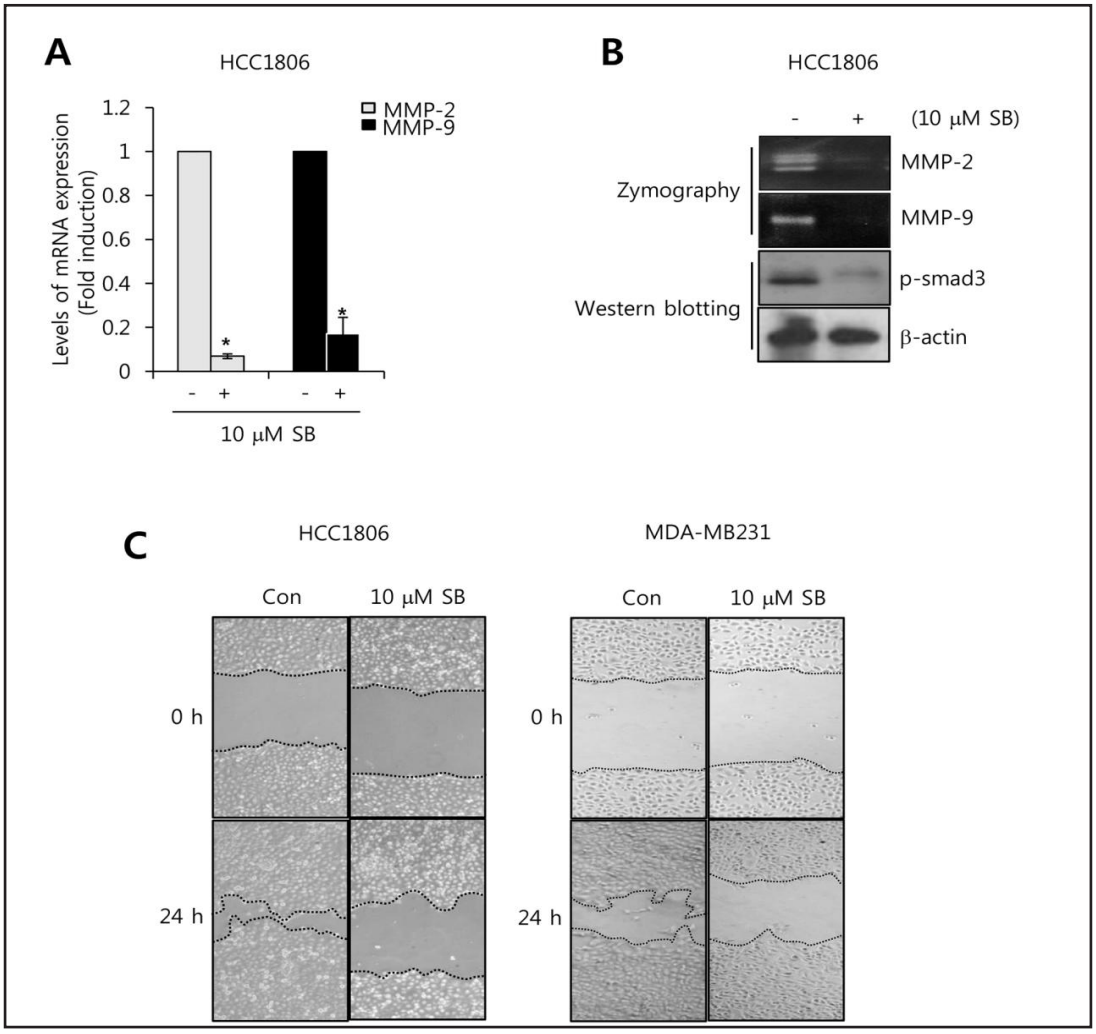

Fig. 2. Rate of cell migration in TNBC cells is decreased by the specific TGF-β receptor I inhibitor SB43154. (A, B) After serum starvation for 24 h, HCC1806 cells were treated with 10 μM SB431542 for 24 h. (A) Levels of MMP-2 and MMP-9 mRNA expression were analyzed by real-time PCR. (B) Levels of MMP-2 and MMP-9 protein expression were analyzed by zymography, and levels of smad3 and β-actin protein expression were analyzed by western blotting. (C) The migrating ability of TNBC cells was analyzed using the wound healing assay. The results are representative of three independent experiments. Values shown are mean ± SEM. * P<0.05 vs. control. Con: control.

in HCC1806 TNBC cells (Fig. 5B). As expected, the migration capacities of TNBC cells were significantly decreased by BBR treatment (Fig. 5C). We also investigated the effect of smad3 overexpression on MMP-2 expression in HCC1806 TNBC cells. As shown in Fig. 5D, the level of MMP-2 protein expression was increased by smad3 overexpression. These results demonstrated that BBR inhibits the TGF-β1/smad3 pathway and cell migration through downregulation of MMP-2 in TNBC cells.

BBR suppresses tumor growth and lung metastasis of TNBC cells in a xenograft model

Next, to verify the effect of BBR on TNBC cell growth, we treated MDA-MB231 cells with 50 μM BBR for 24 h. After 24 h, the cell cycle distribution was analyzed by FACS. As shown in Fig. 6A, our result showed that BBR triggered arrest in the G0/G1 phase (61.0%) compared with vehicle-treated cells (46.7%). Furthermore, we also investigated the effect of BBR on the invasion and anchorage-independent growth of TNBC cells. As expected, the invasiveness

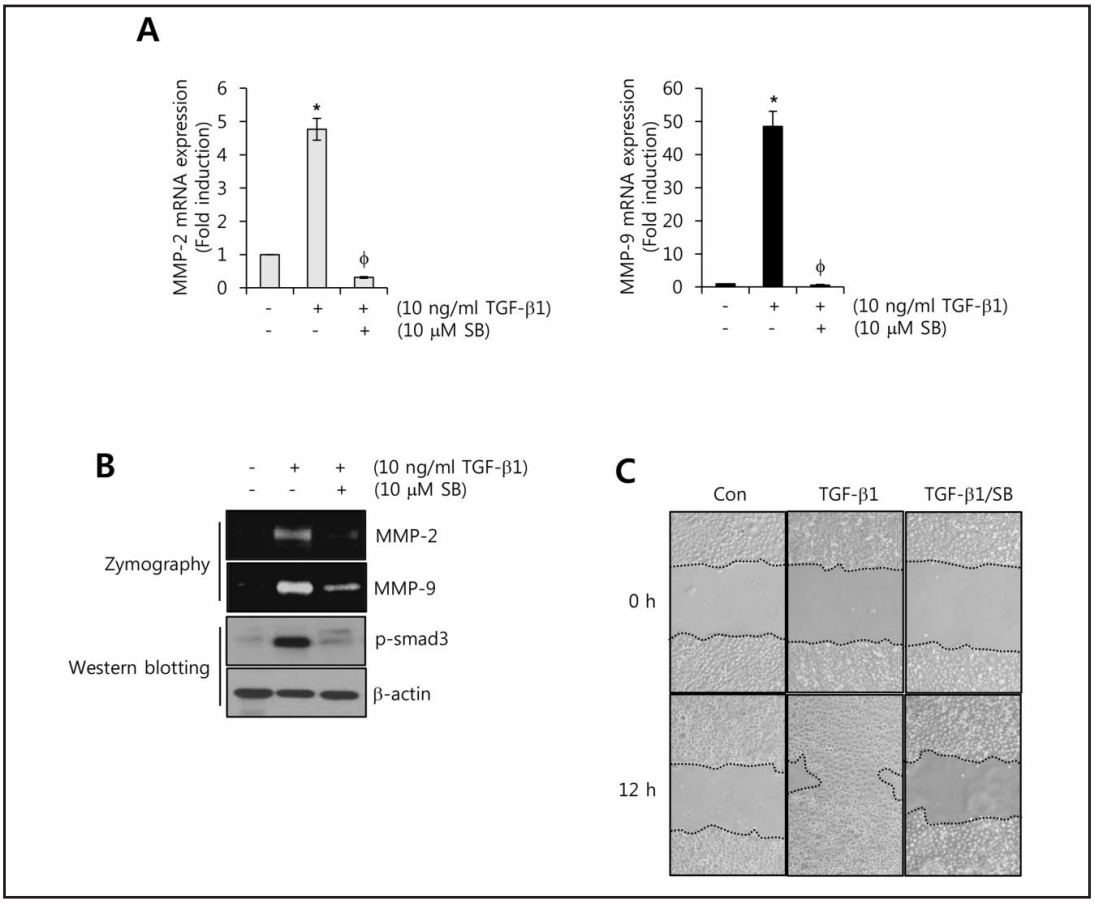

Fig. 3. TGF-β1–induced cell migration is decreased by SB431542 in HCC1806 TNBC cells. (A, B) After serum starvation for 24 h, HCC1806 cells were pretreated with 10 μM SB431542 for 30 min and then treated with 10 ng/ml TGF-β1 for 24 h. (A) Levels of MMP-2 and MMP-9 mRNA expression were analyzed by real-time PCR. (B) Levels of MMP-2 and MMP-9 protein expression were analyzed by zymography, and levels of the smad3 and β-actin protein expression were analyzed by western blotting. (C) The migrating ability of TNBC cells was analyzed using the wound healing assay. The results are representative of three independent experiments. Values shown are mean ± SEM. * P<0.01 vs. control, ϕ P<0.01 vs. TGF-β1-treated cells. Con: control.

and colony forming capacities of MDA-MB231 cells were significantly decreased by BBR treatment (Fig. 6B).

We next investigated the effects of BBR on *in vivo* tumor growth and lung metastasis of TNBC cells. To investigate the antitumor potential of BBR in an orthotopic xenograft model, we injected MDA-231-Luc cells (5 x 10^6 cell/100 μL) into the right secondary mammary fat pads of mice. In the established orthotopic model, tumor volume was reduced by BBR treatment compared with vehicle-treated mice (Fig. 6C). After 46 days, mice treated with 0.1% BRR developed significantly smaller tumors than the mice treated with vehicle (3.5 ± 0.9 × 10^9 photons/sec versus 6.9 ± 0.4 × 10^9 photons/sec), as measured by *in vivo* luciferase activity (Fig. 6C). In addition, tumor volume was also decreased by BBR treatment (Fig. 6D). Finally, we evaluated the effect of BBR on the metastasis of breast cancer cells using 4T1 mammary carcinoma cells, which are a highly tumorigenic and invasive model. As shown in Fig. 6E, BBR completely suppressed the metastatic potential of 4T1 xenograft tumors. These results demonstrated that BBR significantly suppresses the tumor growth and metastatic potential of TNBC cells.

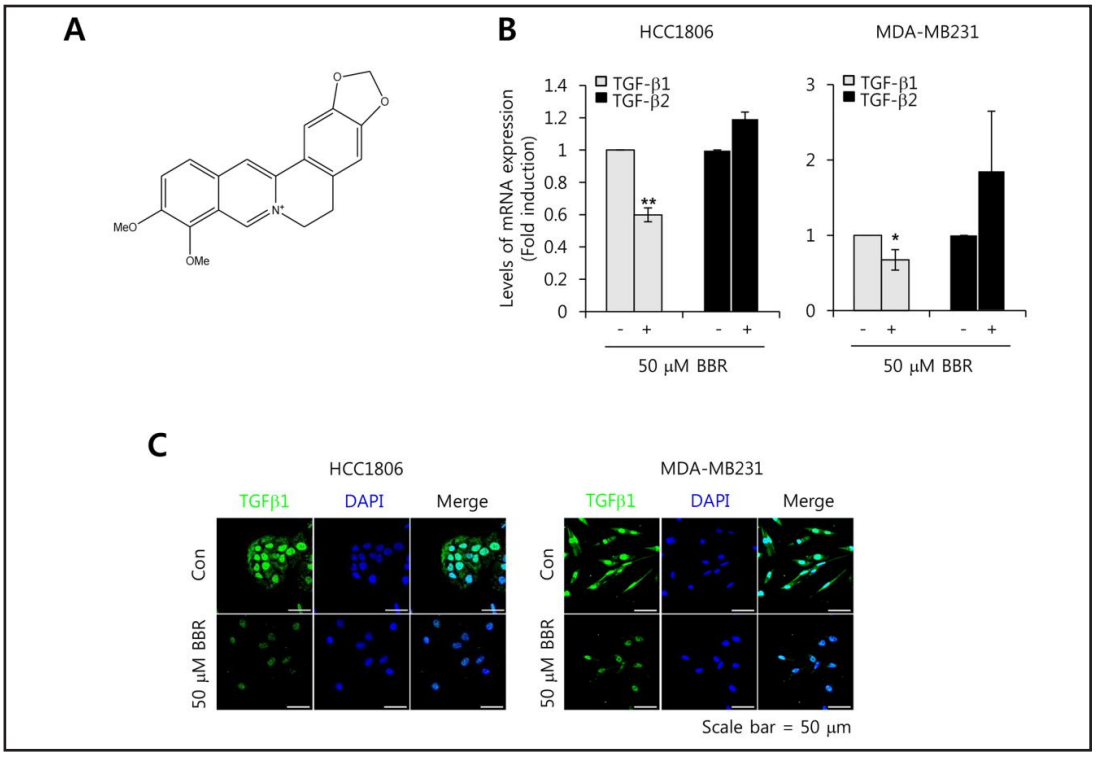

Fig. 4. BBR suppresses expression of TGF-β1, but not TGF-2, in TNBC cells. (A) The chemical structure of BBR. (B) After serum starvation for 24 h, TNBC cells were treated with 50 μM BBR for 24 h. Levels of TGF-β1 and TGF-β2 mRNA expression were analyzed by real-time PCR. (C) TGF-β1 protein expression level was analyzed by confocal microscopy. The results are representative of three independent experiments. Values shown are mean ± SEM. * P<0.05, ** P<0.01 vs. vs. control. Con: control, Scale bar = 50 μm.

Discussion

TGF-β is a ubiquitously expressed, multifunctional cytokine that controls various cancer biological events such as cell proliferation, invasion, apoptosis, and modification of the microenvironment [24-26]. Aberrant TGF-β1 induction is associated with poor prognoses in a variety of cancer cells including prostate and hepatocellular carcinoma [27, 28]. In this study, we investigated the role of TGF-β1 in breast cancer. Our results show that the level of TGF-β1 expression is associated with relapse-free and overall survival of breast cancer patients. The expression of genes related to cell invasion and migration, including MMP-2 and MMP-9, is significantly increased by TGF-β1 treatment in TNBC cells. In addition, the migration of TNBC cells is also enhanced by TGF-β1. Together, these results demonstrate that TGF-β1 expression is directly or indirectly associated with recurrence of breast cancer.

Because of the wide variety of effects of TGF-β on tumorigenesis, many TGF-β signaling antagonist agents to suppress TGF-β and its signaling pathway are under development at both the pre-clinical and clinical stages [29]. In breast cancer models with bone metastases, combined treatment with TGF-β antagonists and doxorubicin results in increased therapeutic efficacy and reduced dosage of chemotherapeutics [30]. Recently, we reported that expression of both TGF-β1 and TGF-β2 was significantly increased in TNBC cells, and the TGF-β receptor I/II inhibitor LY2109761 completely suppresses EMT-related gene expression (i.e., vimentin and fibronectin) and cell motility [6]. Consistent with these reports, we now show that SB431542, a TGF-β receptor kinase inhibitor, inhibits basal- and TGF-β1–induced MMP-2 and MMP-9 expression as well as the migration of TNBC cells. Therefore, we suggest that TGF-β and its signaling pathway could be therapeutic targets, and their blockade provides another therapeutic opportunity for treatment of TNBC patients.

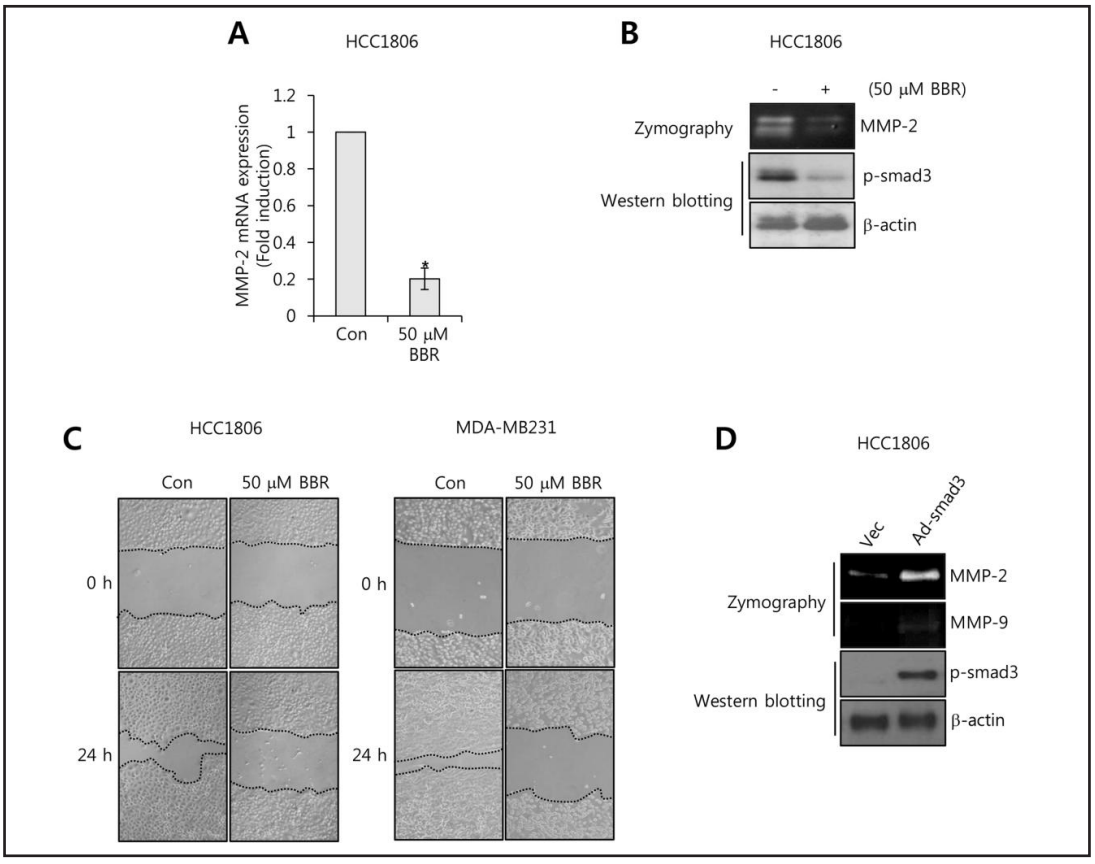

Fig. 5. BBR suppresses TNBC cell migration through downregulation of MMP-2 and MMP-9. (A, B) After serum starvation for 24 h, HCC1806 cells were treated with 50 μM BBR for 24 h. (A) Levels of MMP-2 and MMP-9 mRNA expression were analyzed by real-time PCR. (B) Levels of MMP-2 and MMP-9 protein expression were analyzed by zymography. (C) The migrating ability of TNBC cells was analyzed using the wound healing assay. The results are representative of three independent experiments. Values shown are mean ± SEM. * P<0.01 vs. control. Con: control.

BBR suppresses tumor progression by suppressing abnormal cell proliferation and arresting the cell cycle [31]. In particular, low-dose treatment (≤ 20 μM) with BBR induces G1/S arrest, while high-dose treatment (50 μM) with BBR inhibits the G2/M checkpoint in RM-1 prostate cancer cells [32]. BBR also inhibits tumor cell invasion and angiogenesis, which in turn suppresses cancer metastasis, by suppression of MMP-2, MMP-9, and VEGF [23, 33, 34]. Consistent with previous studies, we show that BBR induces arrest in the G0/G1 phase and inhibits anchorage-independent growth in MDA-MB231 breast cancer cells. Interestingly, we show for the first time that BBR decreases the basal level of TGF-β1 expression in TNBC cells, but does not affect TGF-β2 expression. Furthermore, BBR also decreases the level of smad3 phosphorylation in TNBC cells. Although the antitumor effect of BBR should be further proven in animal and clinical studies, we propose BBR as a novel anti-metastatic agent for treatment of TNBC through suppression of TGF-β1 expression, which is a trigger of EMT processes in a variety of cancer cells.

Conclusion

This study focused on the effect of BBR on TGF-β1 expression, cell growth, and metastasis in TNBC models. Breast cancer patients with a high level of TGF-β1 show a poor clinical

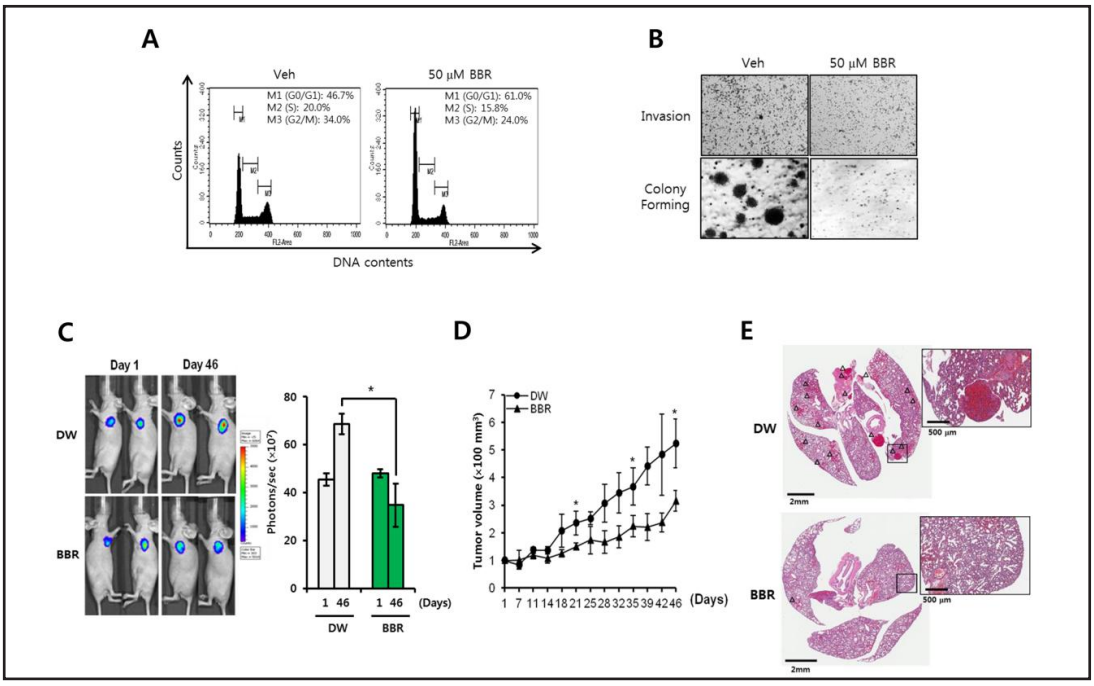

Fig. 6. BBR suppresses tumor growth and lung metastasis of TNBC cells in xenograft models. (A) After serum starvation for 24 h, MDA-MB231 cells were treated with 50 μM BBR for 24 h, and the cell cycle distribution was analyzed by FACS. (B) Cell invasion and colony formation were analyzed as described in Materials and Methods. (C, D) The secondary fat pads of mice were injected with MDA-MB231-Luc TNBC cells. (C) Bioluminescent imaging analysis was analyzed in a Xenogen IVIS 200 biophotonic imager as described in Materials and Methods. (D) Tumor sizes for each group (n = 5) were analyzed for 46 days. Values are mean ± standard errors. * P<0.05 vs. vehicle. (E) Secondary mammary fat pads of mice were injected with 4T1 TNBC cells (1×10^5 cells/100 μL). The mice were randomly divided into two groups (n = 5/group), which were treated with vehicle (dH_2O) or 0.1% BBR in the drinking water. After 14 days, lung tissue was collected from Veh and BBR-treated groups (n=5) and stained with H&E for analysis of metastatic nodules (arrow heads). Veh: vehicle, BBR: berberine.

outcome. The TGF-β receptor kinase inhibitor SB431542 decreases MMP-2 and MMP-9 expression and cell migration in TNBC cells. We show for the first time that BBR significantly decreases the level of TGF-β1 expression in TNBC cells. Basal levels of smad3 phosphorylation and MMP-2 expression are also decreased by BBR treatment in HCC1806 TNBC cells. In contrast, MMP-2 expression is significantly increased by smad3 overexpression. Finally, BBR suppresses tumor growth and metastasis of TNBC cells in an orthotopic mouse model. Therefore, our findings demonstrate that BBR could be a therapeutic drug for treatment of TNBC patients through inhibition of TGF-β1 as well as TGF-β–dependent signaling pathways.

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Disclosure Statement

The authors declare no conflict of interest.

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