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Up-regulation of Bone Morphogenetic Protein 7 by 2-Hydroxycinnamaldehyde Attenuates HNSCC Cell Invasion

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Abstract. *Background/Aim: Few studies have examined the effect of 2'-hydroxycinnamaldehyde (HCA) on head and neck squamous cell carcinoma (HNSCC) cell invasion. This study examined the role of BMP7 on the anti-migration and anti-invasion activity of HCA using HNSCC cells. Materials and Methods: Matrigel invasion and wound healing assays were conducted to investigate cell migration or invasion. BMP7 overexpression vector or siRNA mixture was used for transient regulation of gene expression. Results: HCA attenuated HNSCC cell migration and spheroids Matrigel invasion without cytotoxicity. mRNA and protein expression of BMP7 increased with HCA treatment. Exogenous BMP7 overexpression without HCA treatment attenuated Matrigel invasion of cells. Furthermore, suppression of BMP7 by siRNA alleviated the inhibitory effect of HCA on the invasion of Matrigel by the cell, indicating that BMP7 is responsible for the anti-migration effect of HCA in HNSCC cells. Conclusion: HCA treatment led to a remarkable up-regulation of BMP7, which resulted in the attenuation of HNSCC cell invasion.*

A World Health Organization report states that head and neck squamous cell carcinoma (HNSCC) is among the most prevalent cancers worldwide, ranking consistently in the top 12 most common cancers. Despite many advances in cancer treatment, the 5-year survival rate of patients with HNSCC has only marginally improved. Cancer metastasis, rather than primary tumors, is responsible for most cancer deaths (1). Understanding the molecular mechanisms mediating HNSCC invasion and metastasis may enable the prevention of HNSCC recurrence.

Cinnamaldehyde is commonly used as a flavoring and ingredient in food, beverages, medical products, cosmetics, and perfumes. 2'-Hydroxycinnamaldehyde (HCA) is a natural compound isolated from the bark of *Cinnamomum cassia* Blume (2). CB-PIC ((E)-4-((2-(3-oxoprop-1-enyl)phenoxy) methyl)pyridinium malonic acid), a cinnamaldehyde derivative, has higher water solubility than other derivatives. These compounds have been characterized as antitumor agents in diverse cancer cell lines (3, 4). HCA has shown therapeutic selectivity in K-ras-transformed cells by down-regulating thiol antioxidants (5), a promising result for antitumor drug development. Although these cinnamaldehydes exert cytotoxic effects at micromolar concentrations and have a terminal half-life of approximately 2 h (3), their systemic administration significantly suppressed tumor formation *in vivo* (6), suggesting that these compounds suppress tumorigenesis at physiological levels. Our previous study showed that 100 nM HCA inhibits *in vitro* cancer invasion, and HCA exerts an inhibitory effect on breast cancer metastasis *in vivo* (7). BCA, an HCA derivative, decreased epithelial-mesenchymal transition (EMT) and invasion through early growth response protein 1-dependent phosphatase and tensin homolog activation in colon cancer (8). However, the molecular mechanism of action of these compounds has not been clearly identified in other cancers. Our previous study showed that HCA has antitumor activity in HNSCC cells by inducing cell-cycle arrest and apoptosis. More importantly, HCA effectively arrested tumor growth by inhibiting cell proliferation and inducing apoptosis in a rat oral tumor model (9). These results suggest the potential value of HCA as a candidate for oral cancer therapies. However, no studies have examined the effect of HCA on the migration or invasion of HNSCC cells.

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The transforming growth factor β (TGFβ) superfamily signaling regulates cell growth, differentiation, and various physiological and pathological events (10-12). TGFβ1 and bone morphogenetic protein 7 (BMP7) are two key members in the TGFβ superfamily, and they play important, but diverse roles in chronic diseases. BMP7 is known to regulate cancer cell proliferation. Honokiol inhibited colon cancer cell growth *via* up-regulation of p53 in a BMP7-dependent manner (13). Resveratrol attenuated colon cancer cell growth by inactivating PI3K/Akt signaling through up-regulating BMP7 (14). Interestingly, BMP7 also antagonized TGFβ1 in tumorigenesis-associated EMT in breast cancer (15). Furthermore, BMP7 induced breast cancer cell aging by repressing the *hTERT* gene (16). In contrast, overexpression of BMP7 promoted gene amplification and mutation consequence in cell proliferation and survival (17). In addition, BMP7 is associated with the metastasis and poor prognosis of cancers (18-20). The function of BMP7 in HNSCC progression is not well-known. A recent study showed that inhibition of BMP signaling overcomes acquired resistance to cetuximab in oral cancer (21). Lappin *et al*. showed that the expression of BMP receptors was significantly high in HNSCC cells compared to that in oral keratinocytes. They also showed that recombinant human BMP7 has significant inhibitory effects on oral cancer cells, but not on the viability of oral keratinocytes. However, there was no significant effect on Matrigel invasion in HNSCC cells (22).

Three-dimensional (3-D) *in vitro* models have been used in cancer research as intermediate models between *in vitro* cancer cell line cultures and *in vivo* tumors. Smalley *et al*. suggested that the 3-D spheroid model resembles human tumors *in vivo* by establishing a gradient of oxygen and nutrients (23). The 3-D invasion assay of Matrigel-embedded spheroids performed in a 96-well plate with one spheroid per well is a highly reproducible and standardized method (24, 25). Therefore, this method was used to evaluate the effect of HCA on HNSCC cell invasion. d. Additionally, the role of BMP7 expression on the inhibitory effect of HCA was investigated.

Materials and Methods

Chemicals and reagents. MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) was purchased from Sigma (St. Louis, MO, USA). DMEM, RPMI, fetal bovine serum, and penicillin/streptomycin antibiotics were obtained from Gibco (Grand Island, NY, USA). Qiazol was acquired from Qiagen (Hilden, Germany), and 2X SYBR green PCR master mix was obtained from Takara Biotechnology (Shiga, Japan). Rabbit polyclonal anti-BMP7 and rabbit monoclonal anti-E-cadherin were acquired from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated mouse monoclonal IgG anti-β-actin antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase-conjugated secondary antibodies were acquired from Pierce (Rockford, IL, USA). All other reagents were obtained from standard commercial sources.

Cell culture and cell viability analysis. FaDU cells (ATCC HTB-43, pharynx) were obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin solution. UMSCC1 (SCC070, mouth floor) was obtained from Merck KGaA (Darmstadt, Germany). YD-10B (KCLB 60503, tongue) was purchased from the Korea Cell Line Bank (Seoul, Korea). UMSCC1 and YD-10B cells were maintained in RPMI medium containing 10% fetal bovine serum and 1% penicillin/streptomycin solution. Mycoplasma testing for the cell lines was performed by PCR method (26). Cells were grown at 37˚C in a 5% CO_2 humidified atmosphere. To evaluate cell viability, cells were seeded into 96-well plates at 5,000 cells/well. On the following day, the cells were treated with HCA or CB-PIC in fresh media and incubated for another 48 h. Dimethyl sulfoxide (DMSO) in 0.1% (v/v) was used as a control. Cell viability was then assessed by MTT assay and absorbance was read at 540 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). All experiments were repeated three times.

Wound healing and Transwell Matrigel invasion assay. The effects of HCA or CB-PIC on cell migration were assessed by a wound healing assay. Briefly, cells were seeded in 60-mm culture plates and then incubated for 16-24 h. When the cell confluency reached approximately 80-90%, wounds were created by scratching with pipette tips. The medium was then changed to that containing either DMSO or compounds. The plates were photographed by phase contrast microscopy immediately after changing the media to provide a zero-time control and wound healing was assessed after 48 h of incubation.

In vitro cell invasion was investigated using Matrigel-coated 8.0-μm filter invasion chambers (BD Biosciences, San Jose, CA. USA) (27). Briefly, 100 μl of a Matrigel solution was placed on a Transwell insert (Costar, Cambridge, MA, USA), allowed to harden and form a gel at 37˚C for 2 h, and dried on a clean bench. The cells were suspended in medium (contained with DMSO control or compounds) and 300 μl aliquots were added to each Matrigel-coated Transwell insert. The lower chamber of the Transwell was filled with 400 μl of medium (contained with DMSO or compounds), followed by incubation for 48 h. The inserts were washed twice with PBS and stained with crystal violet (0.2% in 10% ethanol). The cells on the upper side of the insert membrane were removed with a cotton swab, and the number of cells that had migrated to the lower side of the membrane was counted. For each membrane, six random fields were selected and the number of cells was counted on an inverted microscope (100×). The invasion index was calculated as the fold-change of invaded cells in compounds-treated groups over the DMSO control group. All experiments were repeated three times.

3-D Matrigel invasion assay. The effect of HCA on the Matrigel invasion of FaDU cell's 3-D spheroids was evaluated. Briefly, FaDU cells were seeded into a 96-well U-bottom ultra-low attachment plate (6000 cells per well) (Corning, Inc., Corning, NY, USA) and cultured for 4 days to form spheroids with uniform size in each well (>700 μm in diameter). Spheroid size was determined

using a Cell^3 iMager scanner CC-5000 (SCREEN Holdings Co., Ltd. Kyoto, Japan). The spheroids were centrifuged, and 50 μl Matrigel matrix was added directly to each well containing 100 μl medium to provide a semi-solid matrix into which tumor cells could invade from the spheroid body. HCA or CB-PIC was added at final concentrations of 100, 250, and 500 nM in each well, and DMSO was used as a control. Matrigel invasion was monitored over a period of 14 days by phase-contrast microscopy (5× magnification) and quantified by measuring the number and average length of tube-like structures from the surface of each spheroid. All experiments were repeated two times.

Quantitative RT-PCR. Total RNA was extracted using Qiazol (Qiagen) according to the manufacturer's instructions. Five micrograms of extracted RNA were reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA), and the resulting cDNA was diluted by 10-fold and stored at –20˚C until used. The following primers were used for qRT-PCR: E-cadherin forward, 5'-CGACCCAACCCAAGAATCTA-3', reverse, 5'-CTCCAAGAATCCCCAGAATG-3'; BMP7 forward, 5'-TGGAAAGATCAAACCGGAACTC-3', reverse, 5'-TCGTGGAA CATGACAAGGAATT-3'; MMP2 forward, 5'-AGCTGCAACCTG TTTGTGCTG-3', reverse, 5'-CGCATGGTCTCGATGGTATTCT-3'; MMP9 forward, 5'-ACGACGTCTTCCAGTACCGAGA-3', reverse, 5'-TAGGTCACGTAGCCCACTTGGT-3'; GAPDH forward, 5'-AGATCATCAGCAATGCCTCCTG-3', reverse, 5'-CTGGGCAGG GCTTATTCCTTTTCT-3'. Gene expression was normalized using GAPDH as a housekeeping gene. qRT-PCR was carried out using an ABI Prism 7500 sequence detection system (Applied Biosystems). Calculations were made based on the values of the Δcycle threshold (ΔCt), which was determined by normalizing the average Ct value of each treatment to that of the endogenous GAPDH control and then calculating $2^{-\Delta\Delta Ct}$ for each treatment. Statistical analyses were performed as previously described (28). All experiments were repeated three times.

Western blot analysis. Cells were washed twice with cold PBS, after which 200 μl of PRO-PREP protein extraction solution (Intron, Daejon, South Korea) was added. The cell lysates were centrifuged and protein concentrations were estimated using Coomassie protein assay reagent (Thermo Scientific, Waltham, MA, USA). Next, 30-40 μg aliquots of the protein samples were electrophoresed on 8-15% SDS-PAGE gels. The proteins were then transferred to nitrocellulose membranes blocked in 5% skim milk in TBS (25 mM Tris base and 150 mM NaCl) for 2 h at room temperature, followed by incubation with primary antibody overnight at 4˚C. Horseradish peroxidase-conjugated secondary antibody was then applied at 1:5000 dilution for 1 h at room temperature, after which the samples were washed three times in TBST (TBS and 0.1% Tween 20). β-Actin was used as a loading control. The target proteins were detected using electrochemiluminescence detection reagents and the relative intensities of the bands were analyzed using ImageJ software (NIH, Bethesda, MD, USA). All experiments were repeated 2-3 times.

siRNA or overexpression vector transfection. Cells were transiently transfected with a BMP7 siRNA mixture (Santa Cruz Biotechnology). Briefly, 1×10^5 cells were seeded into 60-mm plates and the medium was replaced on the following day with serum-free medium immediately prior to transfection. Cells were then transfected with each siRNA at a final concentration of 10 nM using Lipofectaminetm 2000 (Invitrogen, Carlsbad, CA, USA) as a transfecting agent in Opti-MEM (Invitrogen). After 8 h, the medium containing the transfection mixture was replaced with fresh serum-containing medium. Exogenous BMP7 overexpression vector (pCMV-BMP7) was obtained from Origene Technologies (Rockville, MD, USA).

Statistical analysis. Significant variation analysis was used to carry out parametric two-tailed non-paired *t*-test. All analyses were performed using Origin 8.0 (OriginLab, Northampton, MA, USA), and p-values ≤ 0.05 were considered statistically significant.

Results

Effects of HCA and CB-PIC on HNSCC cell cytotoxicity. The cytotoxic effects of HCA and CB-PIC on HNSCC cells such as FaDU and UMSCC1 were analyzed by the MTT assay. Figure 1A shows the structures of HCA and CB-PIC. As shown in Figure 1B, there was no significant effect of these compounds on cell viability when applied at concentrations between 50 and 1000 nM.

Effect of HCA and CB-PIC on in vitro migration and Matrigel invasion of HNSCC cells. As shown in Figure 2A, HCA at 250 or 500 nM showed anti-migratory effects on FaDU cells. There was no remarkable effect on cell migration following CB-PIC treatment. Next, the effects of these compounds on Transwell Matrigel invasion was examined at the same concentrations. HCA at 250 or 500 nM showed higher anti-invasion effect in FaDU and UMSCC1 cells as compared to CB-PIC (Figure 2B).

Application of the 3-D spheroids' Matrigel invasion assay indicated that treatment of FaDU cells with HCA at 250 or 500 nM resulted in remarkable inhibition of cell invasion (Figure 3A). The mRNA expression of MMP2 and MMP9, which are important for HNSCC invasion, decreased remarkably following HCA treatment of spheroids for 14 days (Figure 3B). CB-PIC treatment had no effect on FaDU spheroid Matrigel invasion under the same conditions (data not shown).

Effect of HCA on E-cadherin expression. HCA, applied at a 100-500 nM concentration, remarkably increased the mRNA expression of E-cadherin in HNSCC cell lines (Figure 4A). CB-PIC did not affect E-cadherin mRNA expression under the same conditions (data not shown). Regarding the effect of HCA and CB-PIC on HNSCC cell migration, Matrigel invasion, and E-cadherin expression, 250 nM HCA were found to be the most appropriate concentration, and subsequent experiments were performed at this concentration. The time-dependent expression of E-cadherin mRNA and protein was evaluated following treatment of cells with 250 nM HCA for 24 h. As shown in Figure 4B, E-cadherin expression in HNSCC cells increased after treatment with HCA for 6-12 h.

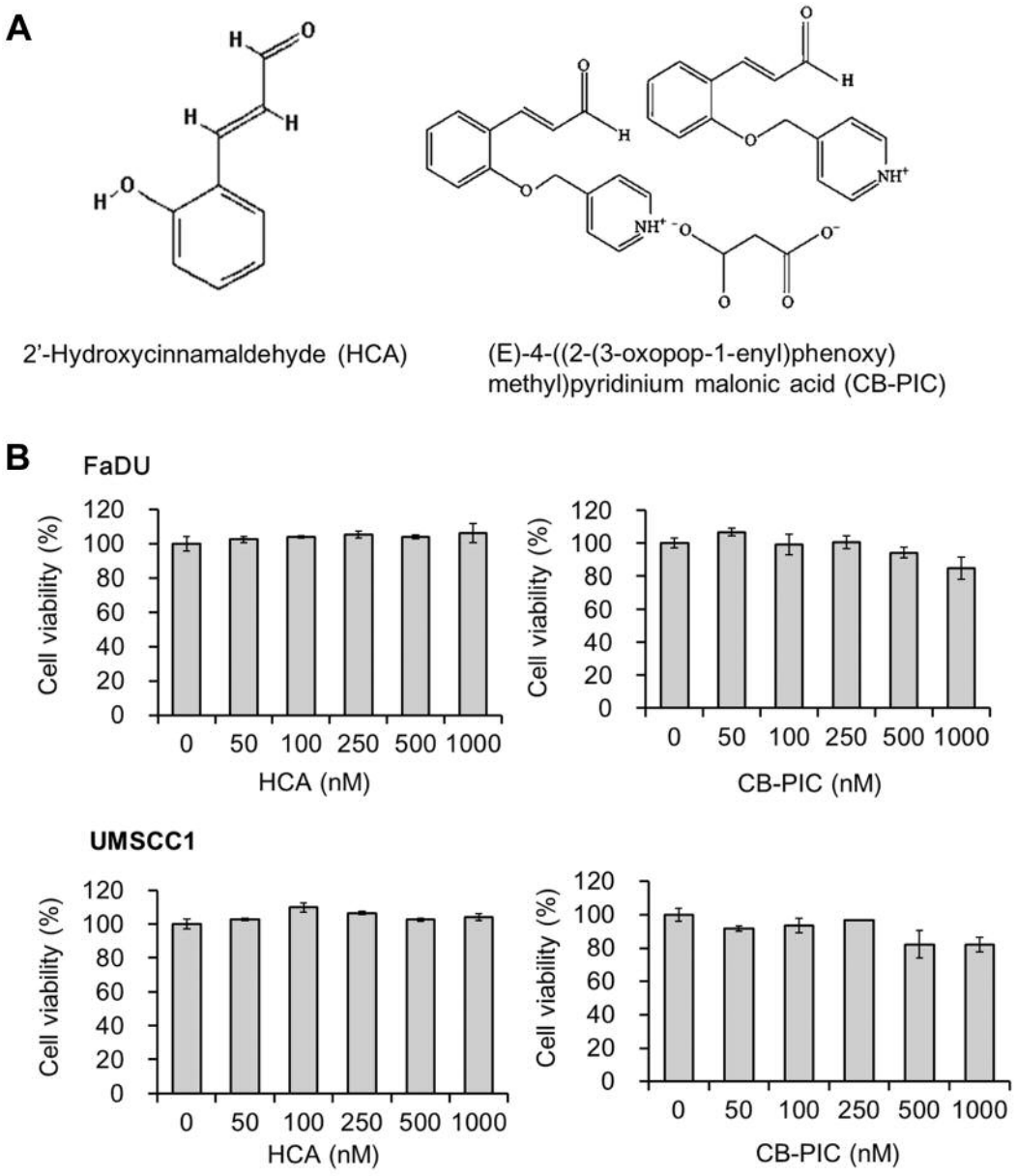

Figure 1. *Effects of HCA and CB-PIC on HNSCC cell viability. (A) Chemical structures of HCA and CB-PIC. (B) Cells were incubated with the indicated doses of HCA or CB-PIC for 48 h, after which cell viability was assessed using the MTT assay. The results, from three independent experiments, are expressed as percentages of the control±standard deviation.*

Effect of HCA on the expression of BMP7 mRNA and protein. Treatment with 250 nM HCA increased BMP7 mRNA levels by 6 h. The levels of BMP7 mRNA, peake at 12 h and continued to be increased at 24 h. BMP7 protein expression was continuously increased for 24 h under the same conditions (Figure 5A). Next, the effect of exogenous BMP7 overexpression without HCA treatment on Matrigel invasion of HSNCC cells was evaluated. As shown in Figure 5B, BMP7 overexpression was confirmed at the mRNA and protein expression level. BMP7 overexpression resulted in increased mRNA and protein expression levels of E-cadherin at 24 h following transfection (Figure 5C). Furthermore, Transwell Matrigel invasion was decreased significantly under the same conditions (Figure 5D) (*p<0.01).

Effect of BMP7 on chemosensitivity to HCA. To determine whether cell invasion by HCA was attenuated by BMP7

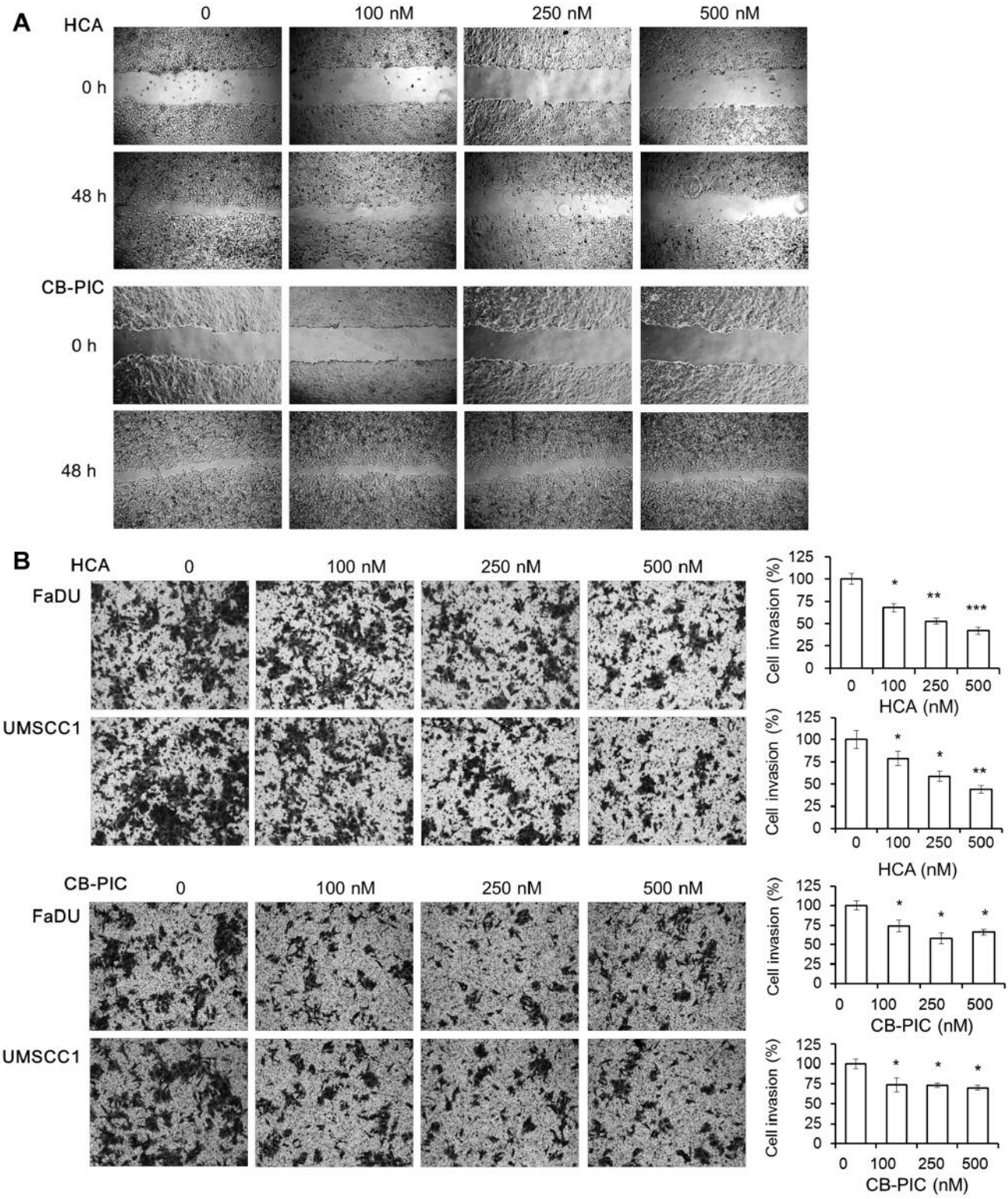

Figure 2. *Effects of HCA and CB-PIC on HNSCC cell migration and Transwell Matrigel invasion. (A) A wound healing assay was performed to evaluate the effect of HCA or CB-PIC for 48 h on FaDU cell migration. (B) A Transwell Matrigel invasion assay was performed under the same conditions. Cell invasion was analyzed microscopically (×100). The results, from three independent experiments, are expressed as percentages of the control±standard deviation. (*p<0.05, **p<0.01, ***p<0.005).*

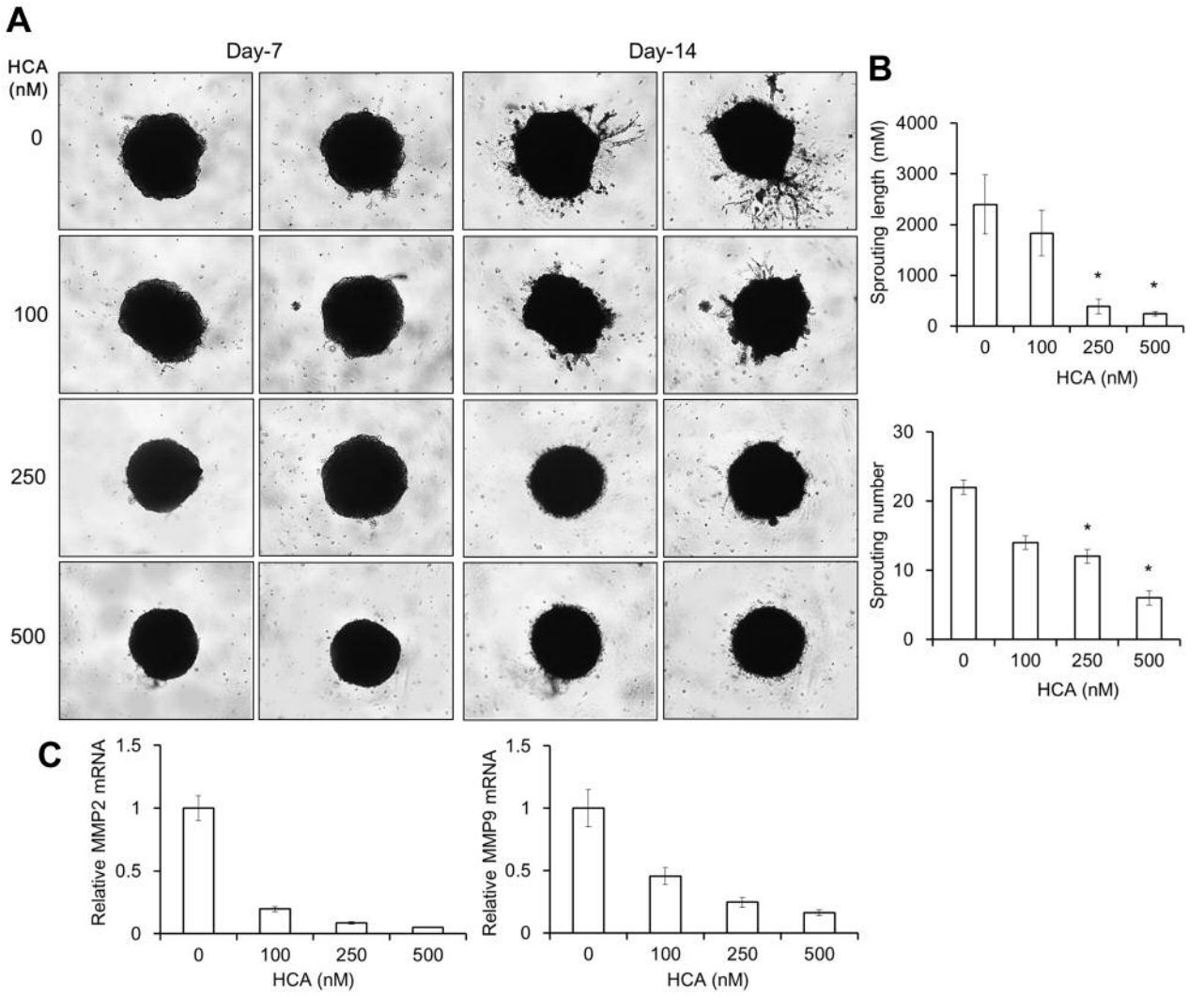

Figure 3. *Effect of HCA on FaDU 3-D spheroid invasion. (A) FaDU cells were seeded in a 96-well ultralow attachment U-bottom plate (6×10^3 cells per well) and cultured for 4 days to form one spheroid per well (>700 μm in diameter). After removing the media, 150 μl of new media containing 50 μl Matrigel solution were added to each well to provide a semi-solid matrix into which tumor cells could migrate from the spheroid body. The final HCA concentration was 0, 100, 250, or 500 nM in each well. Matrigel invasion was monitored over a period of 14 days by phase-contrast microscopy (5× magnification). (B) After 14 days, Matrigel invasion was quantified as the length or number of tube-like structures from the surface of each spheroid (*$p<0.05$). (C) HCA-treated FaDU spheroids were analyzed for mRNA expression of MMP2 and MMP9 by qPCR after 14 days. The results, from two independent experiments, are expressed as percentages of the control±standard deviation.*

down-regulation, cell invasion and E-cadherin expression was investigated in siBMP7-pretreated cells. To evaluate the efficiency of the siBMP7 mixture, the mRNA and protein expression of BMP7 was analyzed after 48 h of transfection (Figure 6A). Down-regulation of both BMP7 and E-cadherin mRNA was observed in cells transfected with siBMP7 (Figure 6B).

The effect of BMP7 knockdown on FaDU invasion was investigated by comparing the invasion index of siBMP7-transfected cells with that of control siRNA-transfected cells. BMP7 knockdown significantly increased Matrigel invasion (*$p<0.05$) (Figure 6C). To evaluate the effect of BMP7 on HCA-induced inhibition of invasion, the invasion index of siBMP7-treated cells with or without HCA treatment was compared. A decreased invasion was observed in cells treated with HCA under siBMP7 transfection, but the difference was not statistically significant (Figure 6C). These data suggest that BMP7 plays an important role in the HCA-dependent inhibition of cancer cell invasion.

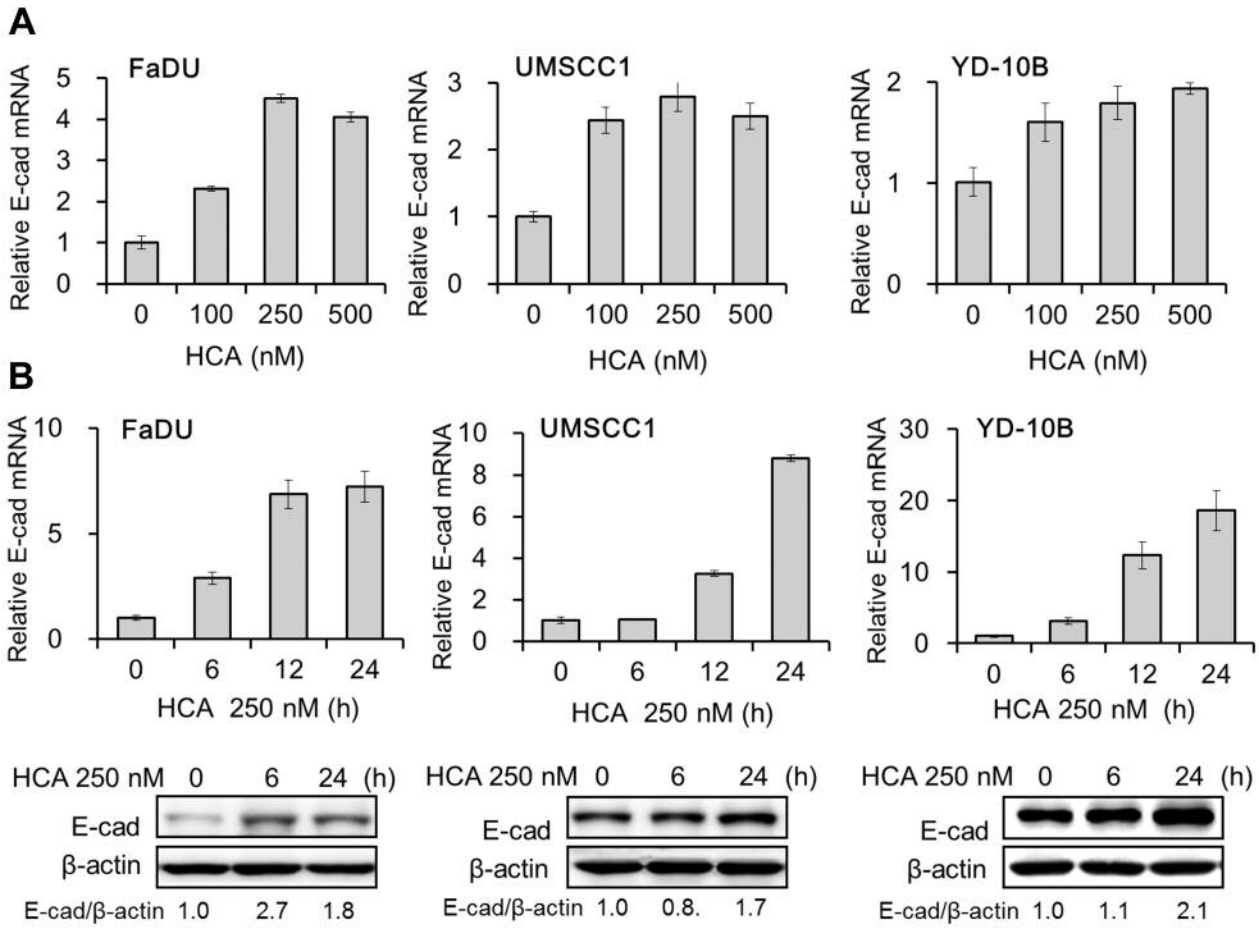

Figure 4. *Effect of HCA on E-cadherin expression in HNSCC cells. (A) HNSCC cells were treated with 0-500 nM HCA for 24 h, and E-cadherin mRNA expression was analyzed by qRT-PCR. (B) Time-dependent E-cadherin expression was analyzed upon treatment with 250 nM HCA for 24 h by qRT-PCR and western blotting. The results, from two or three independent experiments, are expressed as the control±standard deviation.*

Discussion

BMPs and their receptors are abnormally expressed in various carcinomas, such as prostate, breast, and ovarian cancers (29-31). Studies have indicated that malfunctions in BMP signaling pathways are responsible for cancer progression, by inducing proliferation, invasion, and metastasis and protecting cells from death. However, the complexity and diversity of BMPs and their signaling pathways vary widely and show conflicting effects on tumor properties. BMP7 has been shown to promote EMT in melanoma and to be related to metastasis in breast cancer (32, 33). BMP7 has also been associated with the nodal invasion of colon cancer (34) and the promotion of metastasis of colorectal cancer cells (35). However, BMP7 exhibits contradictory roles by inhibiting TGF-β-induced EMT in esophageal adenocarcinoma (36) and breast cancer cells (15), resulting in a significant reduction in TGFβ1-triggered cell growth and metastasis. Interestingly, recombinant human BMP7 significantly reduced the viability of HNSCC cells, but not the viability of oral keratinocytes (22). No studies have examined the effect of BMP7 on the cell invasion of HNSCC cells. In the present study, the inhibitory effects of HCA on HNSCC cell migration and invasion have been found by up-regulation of BMP7. CB-PIC induced apoptosis of SW620 colorectal cancer cells by activating AMP-dependent protein kinase and ERK (37). Furthermore, CB-PIC decreased MDR1 mRNA expression by suppressing the STAT3 and AKT signaling pathways, thus overcoming drug resistance in breast cancer cells (38). There has been no data showing the effect of CB-PIC on cell migration or invasion.

3-D *in vitro* models have been widely used in cancer research as an intermediate model between *in vitro* cell

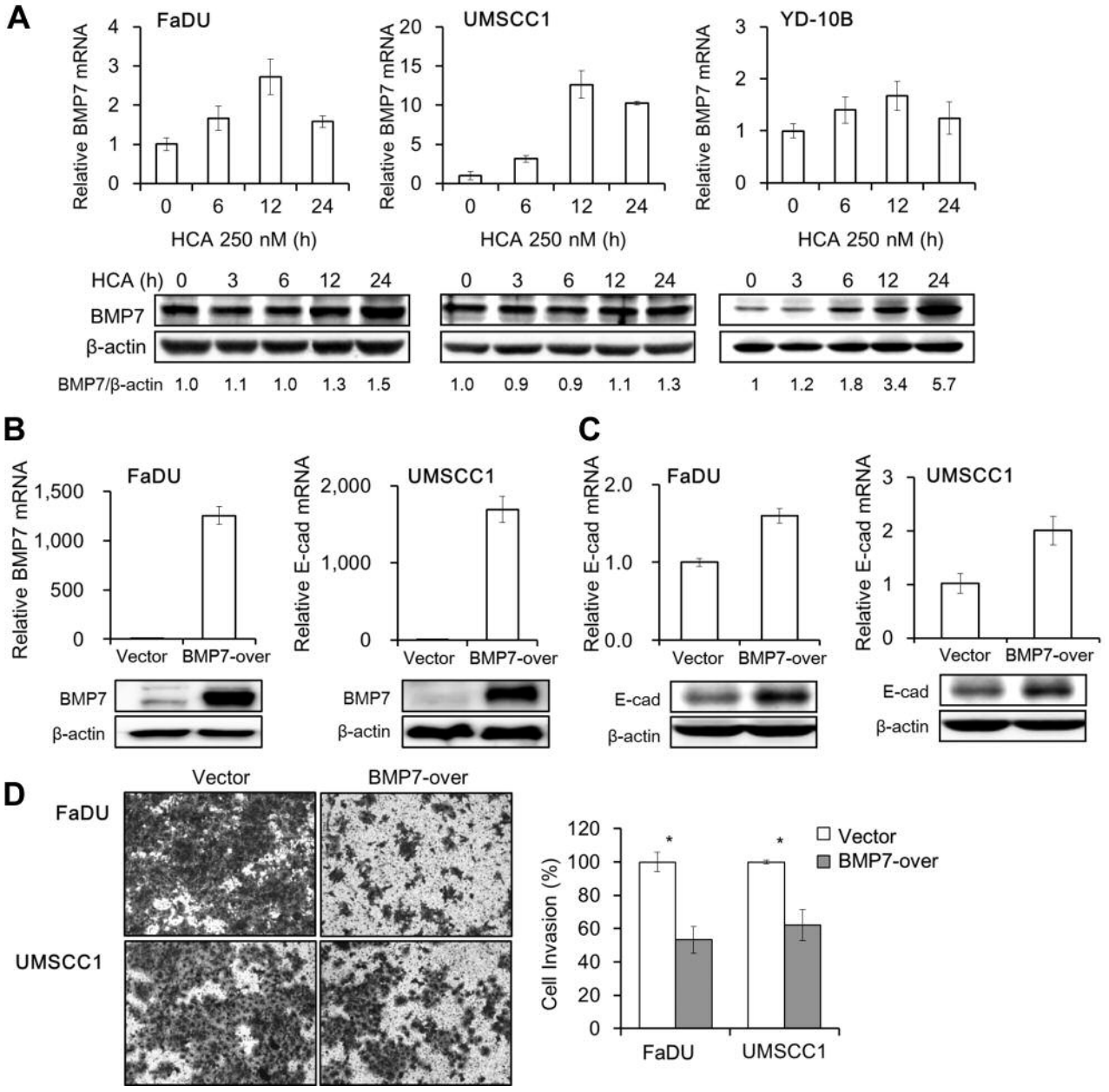

Figure 5. *Effect of BMP7 up-regulation on Transwell Matrigel invasion of HNSCC cells. (A) Time-dependent expression of BMP7 mRNA and protein upon treatment with 250 nM HCA for 24 h was analyzed by qRT-PCR and western blotting. (B) BMP7-overexpression vector was transfected using Lipofectamine™ 2000 for 48 h, followed by analysis of mRNA and protein expression of BMP7. pCMV vector was used as a control vector. (C) mRNA and protein expression of E-cadherin was analyzed under the same conditions. (D) A Transwell Matrigel invasion assay was performed to analyze the effect of exogenous BMP7 overexpression on cell invasion. Cell invasion index was calculated as a percentage of invaded cells and comparisons were made between cells transfected with BMP7 and cells transfected with control pCMV vector (*p<0.05). The results, from two or three independent experiments, are expressed as percentages of the control±standard deviation.*

culture and *in vivo* tumor. Particularly, screening assay for chemotherapy with tumor cell spheroids growing in a 3-D model has been developed. In the present study, a 3-D invasion assay of Matrigel-embedded spheroids was performed to evaluate the effect of HCA on FaDU spheroid invasion. Interestingly, HCA significantly decreased Matrigel invasion of FaDU spheroids for 14 days. mRNA expression of MMP2 and MMP9 was remarkably decreased under the

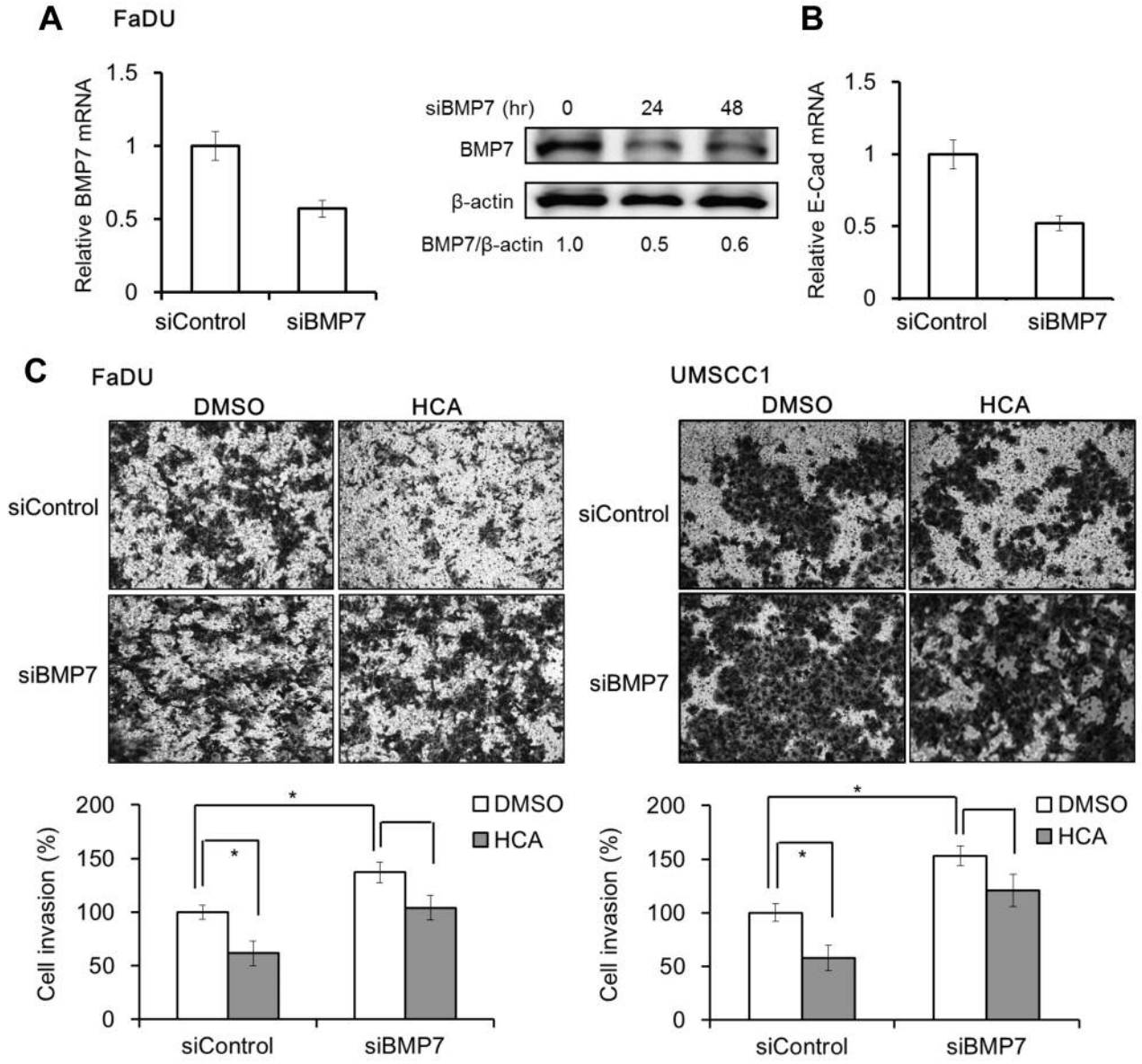

Figure 6. *Effects of BMP7 knockdown on cell invasion and chemosensitivity of HNSCC cells. (A) Cells were transfected with siBMP7 for 48 h, after which BMP7 mRNA and protein expression was investigated by qPCR and western blot analyses, respectively. (B) E-Cadherin mRNA expression was investigated under the same conditions. (C) Cells were transfected with control siRNA or siBMP7 for 24 h, and then subjected to DMSO or HCA treatment for another 24 h, after which Transwell Matrigel invasion was analyzed. The effect of BMP7 knockdown on FaDU invasion was investigated by comparing the invasion index of siBMP7-transfected cells with that of control siRNA-transfected cells ($p<0.05$). The effect of BMP7 on HCA-induced anti-invasion was evaluated by comparing the invasion index of HCA-treated cells with that of DMSO-treated cells under siBMP7 transfection ($p>0.05$). The results, from two or three independent experiments, are expressed as percentages of the control ± the standard deviation.*

same conditions. The optimal concentration of HCA to inhibit Matrigel invasion of FaDU spheroids was the same under both 2-D and 3-D conditions. However, CB-PIC showed inhibitory activity towards HNSCC cells only in the 2-D Matrigel invasion assay, but not in the 3-D spheroids. Previous study showed that hypoxic or necrotic areas are observed in spheroids of approximately 500 μm in diameter (39), which is an important factor known to contribute to resistance to chemotherapeutics (40). The spheroids' size used in this study for Matrigel invasion was above 700 μm in diameter. Interestingly, a previous study showed that CB-PIC had better anti-tumorigenic activity under hypoxia than under

normoxia conditions. Based on these results, CB-PIC does not seem to inhibit HNSCC cancer cell invasion. It would be more effective to develop CB-PIC as an anti-proliferating agent by highlighting the characteristics of its improved solubility. On the contrary, HCA showed significant inhibition of FaDU spheroids' Matrigel invasion at the same condition, suggesting that HCA would be effective for the inhibition of cancer cell invasion even at hypoxic condition.

E-Cadherin-mediated cell-cell adhesion is well-known to inhibit cell invasion. Previous data showed that BMP7 completely abolished TGFβ1-induced suppression of E-cadherin in breast cancer cells (15). Additionally, BMP7 up-regulated E-cadherin in an *in vivo* rat silicosis model by inhibiting the p38 MAPK/Snail pathway, which resulted in the inhibition of EMT (41). In the present study, HCA-dependent BMP7 up-regulation or HCA-independent exogenous BMP7 overexpression increased E-cadherin levels, consistent with this previous study.

The molecular mechanisms underlying the anticancer effects of HCA are diverse, suggesting that HCA is a multi-targeting compound. However, few studies have shown that HCA can regulate cancer cell's invasion. As shown in this study, the anti-invasion effect of non-cytotoxic amounts of HCA would be beneficial for cancer treatment, specifically for combination therapies with commonly used anticancer drugs. However, more research is required to clarify the *in vivo* anti-invasion efficacy of HCA and its derivatives on various cancer types, as well as the molecular mechanisms through which HCA inhibits cell invasion.

In conclusion, our data suggest that sub-toxic doses of HCA have anti-invasion effects on oral cancer cells by up-regulating BMP7. Our study showed that BMP7 would be an effective target molecule for inhibiting HNSCC metastasis. Further studies are needed to evaluate these effects *in vivo*. This study also suggests that HCA can be used in prophylactic chemotherapy to inhibit cancer metastasis.

Conflicts of Interest

No competing financial interests exist.

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