Anti-proliferative effect of honokiol on SW620 cells through upregulating BMP7 expression via the TGF-β1/p53 signaling pathway

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Abstract. Honokiol (HNK), a natural pharmaceutically active component extracted from magnolia bark, has been used for clinical treatments and has anti-inflammatory, antiviral and antioxidative effects. In recent years, anticancer research has become a major hotspot. However, the underlying molecular mechanisms of how HNK inhibits colorectal cancer have remained elusive. The present study focused on elucidating the effects of HNK on the expression of bone morphogenetic protein (BMP)7 and its downstream interaction with transforming growth factor (TGF)-β1 and p53 in colon cancer. In vitro assays, cell viability, cell cycle distribution and apoptosis were examined using Cell Counting Kit-8, flow cytometry and reverse transcription-quantitative PCR, respectively. In addition, the expression of BMP7, TGF-β1 and relevant signaling proteins was determined by western blot analysis. In vivo, the anticancer effect of HNK was assessed in xenografts in nude mice. Furthermore, immunohistochemistry was performed to evaluate the association between BMP7 and TGF-β1 expression in colon cancer. The results indicated that HNK inhibited the proliferation of colon cancer cell lines, with SW620 cells being more sensitive than other colon cancer cell lines. Furthermore, HNK markedly promoted the expression of BMP7 at the mRNA and protein level. Exogenous BMP7 potentiated the effect of HNK on SW620 cells, while knocking down BMP7 inhibited it. As a downstream mechanism, HNK increased the expression of TGF-β1 and p53, which was enhanced by exogenous BMP7 in SW620 cells. In addition, immunohistochemical analysis indicated a positive association between BMP7 and TGF-β1 expression. Hence, the present results suggested that HNK is a promising agent for the treatment of colon cancer and enhanced the expression TGF-β1 and p53 through stimulating BMP7 activity via the non-canonical TGF-β signaling pathway.

Introduction

Colorectal cancer (CRC) was the third most commonly diagnosed malignancy in males and females and the fourth most common cause of cancer-associated mortality worldwide in 2012 (1,2). As the diagnosis of early-stage CRC is difficult, the mortality of patients has increased in developed regions, such as Australia, New Zealand, Europe and North America over the past decade. There was an estimated 1.4 million cases and 693,900 CRC-related deaths in 2012 worldwide, and the mortality rate was approximately 50% (2). Currently, the primary treatment strategy for CRC is surgery and subsequent adjuvant chemotherapy (3). However, surgical treatment may cause significant physical and psychological damage to patients, affecting their quality of life (4,5). Thus, there is still an urgent requirement to explore potential and effective agents for the clinical treatment of colon cancer with fewer adverse effects.

In recent years, an increasing number of studies have reported on natural products with potent antitumor properties, which has thus become a hotspot in the field of cancer research (6,7). Honokiol [HNK; 3',5-di-(2-propenyl)-1,1'-biphenyl-2,4'-diphenol] is a bioactive compound extracted from the bark and branches of the Traditional Chinese Medicinal plant magnolia (8). It has been reported that HNK exhibits multiple pharmacological activities, including antitumor, antioxidative, antiviral and anti-inflammatory effects (9). Previously, Wang et al (10) demonstrated that HNK possesses potential anti-inflammatory effects through inhibiting tumor necrosis factor-α-induced interleukin (IL)-1β and IL-8 expression in peripheral blood mononuclear cells from patients with rheumatoid arthritis. A study by Liu et al (11) indicated that HNK is a promising agent for several chronic diseases, and inhibits cell proliferation and induces apoptosis in several cancer cell lines, such as human leukemia, colon cancer and lung cancer cell lines.

The transforming growth factor-β (TGF-β) superfamily consists of >30 members, including TGF-βs (comprising...
the three highly homologous isoforms TGF-β1, TGF-β2 and TGF-β3, activins, inhibins, nodal factors, bone morphogenetic proteins (BMPs), anti-Müllerian hormone, and growth and differentiation factors (12). Previous studies have indicated that TGF-β signaling is a relatively conventional membrane receptor to the nuclear transcription activation pathway and participates in diverse biological events, including embryonic stem cell self-renewal and differentiation, the homeostasis of differentiated cells and suppression of cancer development (13,14). The TGF-β pathway has dichotomous roles during tumor progression. In premalignant cancer cells, TGF-β signaling inhibits cell proliferation and enhances cell-cycle arrest and apoptosis (15). Furthermore, activation of this pathway in late-stage cancer cells is able to stimulate epithelial-to-mesenchymal transition and promote invasiveness and metastasis (16,17). Therefore, the opposing roles of TGF-β signaling during tumor progression make it a challenging target for developing anticancer interventions. TGF-β1, a multifunctional cytokine, is the primary member of the TGF-β superfamily (18). An increasing number of studies have indicated that TGF-β1 also exerts critical roles in multiple processes, including cell proliferation, development, wound repair and immune responses (19). The present study primarily focused on investigating whether the anti-neoplastic effect of HNK in colon cancer involves the modulation of TGF-β1 signaling.

BMPs also belong to the TGF-β superfamily (12). It has been indicated that BMPs serve vital roles in numerous processes during embryonic development and adult homeostasis, exerting functions to regulate stem cell proliferation and differentiation, cell growth and apoptosis, as well as the progression of cancer (20). BMP2 has been indicated to inhibit the proliferation of colon cancer cells and inactivation of BMP3 is relevant for regulating the development of colon cancer (21,22). Furthermore, BMP9 has been indicated to mediate the inhibitory effect of resveratrol in colon cancer cells (23). BMP7, which may be isolated from bone extracts, is a broad-spectrum growth factor that has a role in the development of bone and cartilage (24). BMP7 has been recognized as a potent target to inhibit cell growth and induce apoptosis (25,26). In fact, studies have demonstrated that BMP7 is involved in the development of several cancer types, including breast cancer, prostate cancer and esophageal squamous cancer (26). Liu et al (27) reported that oridonin exhibits efficacious anticancer activity through upregulating BMP7 in colon cancer. Furthermore, Zeng et al (28) indicated that resveratrol exerts an anti-proliferative effect on colon cancer cells through upregulating BMP7 and inactivating PI3K/Akt signaling.

p53, a well-known tumor suppressor protein and an essential mediator of the cellular stress response, has been regarded as a valid therapeutic target (29). Functional loss or mutations in p53 have been considered a primary cause of cancer development (29). For instance, a recent study by Li et al (30) reported that aberrant protein phosphatase 2Cδ activity decreases p53 acetylation and its transcriptional activity, and suppresses doxorubicin-induced cell apoptosis in breast cancer. Furthermore, Nigro et al (31) demonstrated that p53 mutations have a role in the development of numerous common human malignancies, such as breast, lung and colon cancer.

In the present study, the role of HNK in regulating cell proliferation and apoptosis in human colon cancer was investigated and the underlying molecular mechanisms were explored. Western blot and immunohistochemical analyses were performed to evaluate the association between HNK and TGF-β1 expression. Furthermore, the role of HNK in BMP7-mediated regulation of TGF-β1 expression in SW620 cells was demonstrated in vivo and in vitro. Finally, the influence of HNK on the association between BMP7, TGF-β1 and p53 in colon cancer was preliminarily confirmed. Taken together, the present results demonstrated that HNK is a potential candidate regulating BMP7 activation to enhance p53 expression via TGF-β1/p53 signaling for the treatment of colon cancer.

Materials and methods

Cell culture and reagents. The SW620, HCT116, SW480 and LoVo colon cancer cell lines, the FHC normal colonic epithelial cell line, and 293 cells were obtained from the American Type Culture Collection. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were from HyClone (Cytiva). Cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO2. HNK was purchased from Hao-Xuan Bio-Tech Co., Ltd. and its purity was 98.7%. At our laboratory, HNK was dissolved to 10 mM in dimethyl sulfoxide (DMSO) and stored at -20°C (the final concentration of DMSO reached 0.25% following the addition of HNK stock solution to cell cultures). Inhibitor of TGF-β1 (LY364947) was purchased from Targetmol Co., Ltd. LY364947 was dissolved to 10 mM in dimethyl sulfoxide (DMSO) and stored at -20°C. SW620 cells were treated with 5 µM LY364947 at 37°C for 24 or 48 h. The primary antibodies used were as follows: GAPDH (cat. no. 10494-1-AP), Bad (cat. no. 10435-1-AP), Bcl-2 (cat. no. 60178-1-lg), proliferating cell nuclear antigen (PCNA; cat. no. 10205-2-AP), BMP7 (cat. no. 10221-1-AP) and TGF-β1 (cat. no. 21898-1-AP; all from Proteintech Technology, Inc.; Smad1/5/9 (cat. no. sc-6031-R), phosphorylated (p)-Smad1/5/9 (cat. no. sc-12335), Smad2/3 (cat. no. sc-8332) and p-Smad2/3 (cat. no. sc-11769; all from Santa Cruz Biotechnology, Inc.; p53 (cat. no. A11232) and p-p53 (cat. no. AP0083; both from Abclonal Technology, Inc.). Biotin-labeled goat anti-rabbit IgG (cat. no. A0277; 1:3,000), biotin-labeled goat anti-mouse IgG (cat. no. A0286; 1:3,000) and HRP-labeled goat anti-rabbit IgG (cat. no. A0208; 1:3,000) were obtained from Beyotime Institute of Biotechnology. Cell Counting Kit-8 (CCK-8) assay kit (cat. no. C008-2) was purchased from Shanghai Seven Sea Biotechnology Co., Ltd.

Cell viability assay. Cell viability was determined using CCK-8. In brief, cells were harvested and plated at a density of 2,000 cells in 200 µl fresh growth medium (DMEM) containing 10% FBS per well in 96-well plates. Subsequently, the cells were treated with different concentrations of HNK (SW620 cells: 15, 20, 25, 30 and 35 µM; HCT116 cells: 10, 15, 20, 25 and 30 µM; SW480 cells: 17.5, 22.5, 25, 27.5 and 30 µM; LoVo cells: 20, 25, 30, 35 and 40 µM) for different time periods (24, 48 or 72 h) at 37°C. At the indicated time-points, CCK-8 (10 µl per 100 µl medium) was added to each well of a 96-well plate, and the cells were incubated for 2 h at 37°C. The optical density was measured at 450 nm using a Multimode
Recombinant vectors were linearized and transfected into BJ5183/AdEasy cells, respectively. Recombinant adenoviruses combined with pAdEasy1 in BJ5183/AdEasy cells, respectively. PCR products or siRNA fragments for BMP7 were cloned into a shuttle vector (pAdTrace-TO4). Subsequently, the present study were constructed using an AdEasy system (33). In brief, a coding sequence of human BMP7 was amplified and sub-cloned into a shuttle vector (pAdTrace-TO4). Subsequently, PCR products or siRNA fragments for BMP7 were cloned into a pSSEI shuttle vector. The shuttle vectors were then recombined with pAdEasy1 in BJ5183/AdEasy cells, respectively. Recombinant vectors were linearized and transfected into 293 cells for packaging recombinant adenoviruses, which were designated as AdBMP7 and AdsiBMP7, respectively. Finally, recombinant adenoviruses were harvested 14-20 days later. Recombinant adenoviruses were tagged with green fluorescent protein (GFP) and red fluorescent protein (RFP) for tracking of the viruses, respectively. Recombinant adenoviruses that expressed GFP (AdGFP) or RFP (AdRFP) only were used as a vector control. All recombinant adenoviruses used in the present study were provided by Professor Tong-Chuan He (Medical Center of the University of Chicago, Chicago, IL, USA).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Cells (2X10^5 per well) were cultured in 6-well plates and exposed to 2 ml medium with increasing concentrations of HNK (final concentrations, 0, 20, 25 or 30 µM) for different time periods (24 or 48 h) at 37°C. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), followed by RT at 37°C for 15 min and 85°C for 5 sec to generate complementary (c)DNA using a PrimeScript™ RT Reagent kit (Takara Bio, Inc.). The cDNA products were used as templates for qPCR with 2X SYBR-Green qPCR Master mix (Bimake) to determine the expression levels of the target genes.

Flow cytometric analysis of the cell cycle. SW620 cells were trypsinized with trypsin (cat. no. AS-10; T&L Biological Technology, Inc.) to obtain single-cell suspensions and seeded into 6-well plates containing 2 ml growth medium and different concentrations (20, 25 and 30 µM) of HNK at 37°C, followed by culture for 48 h. For cell cycle analysis, cells were harvested and washed with cold PBS, fixed with cold 70% ethanol at 4°C or 30 min, and sequentially washed with 50% ethanol, 30% ethanol and PBS (4°C). Finally, the cells were stained with 1 ml propidium iodide (20 mg/ml) containing RNase (1 mg/ml) in PBS (4°C) for 30 min, after which the cell cycle was analyzed using a flow cytometer and Kaluza Analysis software (version 2.0; Beckman Coulter, Inc.); 20,000 cells were gauged for each sample.

Detection of apoptosis. Annexin V-enhanced green fluorescence protein (EGFP) staining was a method of detecting apoptosis (32). Cells (2X10^5 per well) were resuspended, cultured in 24-well plates and treated with different concentrations of HNK (20, 25 and 30 µM) for 24 h at 37°C. Cells were stained with an Annexin V-EGFP Apoptosis Detection kit (Nanjing Keygen Biotech Co., Ltd.) in accordance with the manufacturer's protocol. In brief, cells were washed with PBS (4°C) and treated with 200 µl binding buffer and 2 µl Annexin V-EGFP at room temperature for 10 min. Subsequently, working solution was added to each well and cells were incubated for 10 min. Finally, cells were extensively washed and images were captured using a fluorescence microscope (magnification, x40), and the fluorescence intensity was used for quantification with ImageJ software (version 1.5; National Institutes of Health). Each assay was performed in triplicate.

Construction of BMP7 and BMP7 small interfering (si)RNA recombinant adenovirus. Recombinant adenoviruses used in the present study were constructed using an AdEasy system (33). In brief, a coding sequence of human BMP7 was amplified and sub-cloned into a shuttle vector (pAdTrace-TO4). Subsequently, PCR products or siRNA fragments for BMP7 were cloned into a pSSEI shuttle vector. The shuttle vectors were then recombined with pAdEasy1 in BJ5183/AdEasy cells, respectively. Recombinant vectors were linearized and transfected into 293 cells for packaging recombinant adenoviruses, which were

Immunofluorescence assay. In brief, cells were seeded into 48-well plates in medium supplemented with 10% FBS on cover slides. After different treatments at 37°C for 24 h, cells were fixed with 4% pre-cooled paraformaldehyde at 4°C for 20 min, and then washed with cold PBS and permeabilized with 0.5% Triton X-100 for 8 min. Subsequently, the cells were blocked with 5% bovine serum albumin (HyClone; Cytiva) at 37°C for 1 h. Cells were sequentially incubated with primary antibodies (1:200) against TGF-β1 (cat. no. 21898-1-AP; ProteinTech Group, Inc.) and BMP7 (cat. no. 12221-1-AP; ProteinTech Group, Inc.) overnight at 4°C. Homologous IgG was used as a negative control, and samples were incubated with the corresponding secondary antibodies (goat anti-mouse IgG, cat. no. SA00001-1, 1:100; rabbit anti-goat IgG, cat. no. SA00004-4, 1:100; both Santa Cruz Biotechnology, Inc.) at room temperature in the dark for 1 h. Finally, cells were stained with DAPI (1:1,000) at room temperature for 6 min. Images were captured under an inverted fluorescence microscope (magnification, x400) (LV100ND; Nikon Corporation).

Western blot analysis. Cells (2X10^5 per well) were seeded in 6-well plates and treated with different concentrations of HNK and/or other reagents followed by culture for 24
or 48 h at 37°C. At the scheduled time-points, cells were washed twice with PBS (4°C) and lysed with 300 µl lysis buffer (cat. no. R0020; Solarbio Science and Technology Co., Ltd.). The protein concentration was assessed with BCA, and lysates were collected and denatured by boiling for 15 min. A total of 30 µg protein was loaded per lane. Samples were subjected to 10% SDS-PAGE, transferred onto nitrocellulose membranes and incubated with primary antibodies against (GAPDH, PCNA, Bad, Bcl-2, BMP7, TGF-GF, Smad1/5/9, p-Smad1/5/9, Smad2/3, p-Smad2/3, p53 and p-p53, 1:1,000) for 2 h at room temperature, followed by secondary antibodies biotin-labeled goat anti-rabbit Ig (cat. no. A0277; Beyotime Institute of Biotechnology; 1:3,000), biotin-labeled goat anti-mouse IgG (cat. no. A0286; Beyotime Institute of Biotechnology; 1:3,000) and HRP-labeled goat anti-rabbit IgG (cat. no. A0208; Beyotime Institute of Biotechnology; 1:3,000) for 30 min at room temperature. Subsequently, target proteins were visualized using an enhanced chemiluminescence kit (cat. no. 34095; Thermo Fisher Scientific, Inc.). Assays were performed in triplicate. The band densities were standardized to GAPDH and protein levels were quantified using ImageJ software (version 1.5; National Institutes of Health).

Animal experiment. A total of 20 female athymic nude mice (BALB/c nu/nu; age, 4-6 weeks; body weight, 18-20 g) were obtained from the animal center of Chongqing Medical University and maintained under pathogen-free conditions at room temperature, with free access to food and water and a light/dark cycle of 12/12 h. The animal experiment was approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (approval no. 2019-225). The mice were randomly divided into four groups with 5 mice in each group: i) Control group (injected with untransfected cells and treated with 100 µl 0.4% carboxy-methylcellulose sodium solution); ii) HNK group; iii) AdBMP7 + HNK group; and iv) AdsiBMP7 + HNK group. SW620 cells were transfected with AdBMP7 or AdsiBMP7 for 24 h, harvested and resuspended in cold PBS. Subsequently, transfected cells (2x10⁷ in 50 µl) were injected subcutaneously into the flanks of each nude mouse in AdBMP7 + HNK and AdsiBMP7 + HNK groups. After 2 weeks, animals were treated with HNK (50 mg/kg) by intragastric administration in 100 µl solution per mouse once a day for 2 weeks. At 4 weeks post-injection, the experimental mice were euthanized and all the tumor samples were retrieved for the following histological evaluation after the mice exhibited no autonomous breathing for ≥2-3 min and no blink reflex. The weights and diameters of the tumor samples were measured with digital calipers, and tumor volume was calculated using the following formula: Tumor volume (cm³)=1/2 (longer diameter x shorter diameter)³. The maximum tumor volume observed was 0.6 cm³. Tumors were excised for histological evaluation.

All animals were given free access to sterilized food and water and were habituated for a week before the experiments. Animal health and behavior were monitored once a day, and no animal death was observed during the experiment. Euthanasia was performed by administrating sodium pentobarbital intraperitoneally (180 mg/kg body weight) to minimize suffering and distress. All procedures were carried out in strict accordance with the recommendations established by Animal Care and Ethics Committee of Chongqing Medical University as well as the guidelines by U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals.

Histological evaluation. Retrieved tumor samples were fixed in 10% formalin at room temperature for 2 weeks, and embedded with paraffin. Sections (4-µm thick) were then stained with hematoxylin for 5 min and eosin for 2 min at room temperature, after deparaffinization and rehydration. Images were captured under a fluorescence microscope (magnifications, x100, x200 and x400).

Statistical analysis. Data are expressed as the mean ± standard deviation of at least three independent experiments. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, Inc.). One-way analysis of variance with Tukey's post hoc test was used to compare the difference among multiple groups. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

HNK suppresses the viability of colon cancer cells. In the present study, the cytotoxic effect of HNK in several colon cancer cell lines was first determined by CCK-8 assay. The results indicated that HNK significantly suppressed the viability of various cancer cell lines, including SW620, LoVo, SW480 and HCT116. As presented in Fig. 1A, HCT116 cells were most susceptible to HNK treatment among the four cell lines examined. However, western blot analysis was used to detect the endogenous levels of BMP7 among the cell lines, and the level of BMP7 was lower in SW620 cells compared with HCT116 cells (Fig. 1C). Therefore, SW620 cells were selected for the subsequent studies. The results of the flow cytometric analysis indicated that HNK may cause cell cycle arrest of SW620 cells at the G1 phase (Fig. 1B). The effect of HNK on colony formation of SW620 and FHC cells was also evaluated. The results suggested that HNK significantly inhibited the colony formation of SW620 cells to a greater extent than that of FHC cells (Fig. 1D). Furthermore, western blot analysis indicated that HNK significantly decreased the level of PCNA in SW620 cells (Fig. 1E). Taken together, these results suggested that HNK exerted a marked inhibitory effect on the viability and proliferation of colon cancer cells.

Effects of HNK on the apoptosis of SW620 cells. Apoptosis may be regarded as a measure of the efficacy of various anticancer treatments (35). The present study investigated the effects of HNK on the expression of Bcl-2 family genes Bcl-2 and Bad, which are involved in the growth and development of cancer (36,37). RT-qPCR and western blot analysis were used to determine the apoptosis-inducing effects of HNK in SW620 cells. As presented in Fig. 2A and C, HNK induced a dose- and time-dependent increase of Bad expression, while reducing the levels of Bcl-2 in SW620 cells (Fig. 2B and D). To further confirm the effects of HNK on the induction of cell apoptosis, an Annexin V-EGFP staining assay was used, which indicated that HNK significantly promoted apoptosis in SW620 cells (Fig. 2E). Thus, these results suggested that HNK induces apoptosis in colon cancer cells.
Effects of HNK on BMP7 in SW620 cells. A previous study demonstrated that exogenous BMP7 inhibits the growth of colon cancer cells (38). Thus, it was then assessed whether HNK is able to regulate the expression of BMP7 in SW620 cells. RT-qPCR demonstrated that HNK significantly increased the mRNA levels of BMP7 in SW620 cells in a dose-dependent manner (Fig. 3A), which was consistent with the results of the western blot analysis (Fig. 3B). Furthermore, immunofluorescence analysis was employed to evaluate whether HNK was able to promote the expression of BMP7 in SW620 cells. As presented in Fig. 3C, HNK was able to increase the expression of BMP7 in SW620 cells. To determine the roles of BMP7, the cells were transfected with BMP7 or BMP7 siRNA recombinant adenovirus, and western blot analysis of BMP7 was performed. The results revealed that AdBMP7 and AdsBMP7 recombinant adenovirus successfully increased or decreased BMP7 expression, respectively (Fig. 3D). These results suggested that the inhibitory effect of HNK on SW620 cells was mediated through regulating the activity of BMP7 signaling.

Effects of BMP7 on the anticancer activity of HNK in SW620 cells. The inhibitory effect of HNK on colon cancer cells is well documented (39). In the present study, the role of BMP7 in the anticancer activity of HNK in SW620 cells was determined. The effect of BMP7 on SW620 cell growth was evaluated by a colony formation assay (Fig. 4A). Exogenous BMP7 significantly enhanced the effect of HNK to inhibit the colony formation of SW620 cells. By contrast, BMP7-knockdown did not significantly reduce this effect. Similar results were obtained in the CCK-8 viability assay (Fig. 4C). In addition, western blot analysis suggested that exogenous BMP7 promoted the effect of HNK to decrease the protein levels of PCNA (Fig. 4B). In vivo, the results suggested that exogenous BMP7 improved the effect of HNK to reduce tumor volume and tumor weight when compared to HNK treatment alone (Fig. 4D). Subsequently, western blot analysis was used to examine the effect of BMP7 on the expression of the apoptosis-associated proteins Bad and Bcl-2. The results indicated that exogenous BMP7 enhanced the effect of HNK on Bad and Bcl-2 expression (Fig. 4E and F). By contrast, knocking down BMP7 attenuated this effect.
Figure 2. Effects of HNK on apoptosis in SW620 cells. SW620 cells were treated with HNK at the indicated concentrations for the appropriate durations. The effects of HNK on the mRNA levels of (A) Bad and (B) Bcl-2 in SW620 cells were determined by reverse transcription-quantitative PCR analysis. SW620 cells were exposed to increasing concentrations of HNK for 24 or 48 h and the levels of (C) Bad and (D) Bcl-2 were determined by western blot analysis, (E) The effect of HNK on pro-apoptosis was observed by Annexin V-EGFP staining analysis in SW620 cells. The control was treated with 6 µl DMSO (equivalent to HNK 30 µM). *P<0.05, **P<0.01 vs. control groups. HNK, honokiol; EGFP, enhanced green fluorescence protein.

Figure 3. Effects of HNK on BMP7 in SW620 cells. (A) Following HNK treatment for 24 or 48 h, the mRNA levels of BMP7 in SW620 cells were determined by reverse transcription-quantitative PCR analysis. (B) Western blot analysis was used to determine the effect of HNK on the expression levels of BMP7 protein in SW620 cells. (C) Immunofluorescence staining indicated that HNK treatment for 24 h promoted BMP7 expression in SW620 cells. Magnification, x400. (D) Western blot analysis was performed to confirm the effect of recombinant adenovirus on the protein levels of BMP7 in SW620 cells (upper panels show the results following transfection of recombinant adenoviruses). The control was treated with 6 µl DMSO (equivalent to HNK 30 µM). *P<0.05, **P<0.01 vs. control groups. HNK, honokiol; BMP, bone morphogenetic protein; AdBMP7, adenovirus overexpressing BMP7; AdsibMP7, adenovirus expressing small interfering RNA targeting BMP7.
These results indicated that BMP7 has a vital role in the inhibition of colon cancer cells.

*Effects of HNK on TGF-β1 and p53 in SW620 cells.* BMP7, as a member of the family of BMPs, exerts its function through BMPs/Smad signaling or the non-canonical BMPs/Smad signaling pathway, such as p38-MAPK and PI3K/Akt (40). As presented in Fig. 5A, HNK treatment did not significantly affect the levels of total Smad1/5/9 or p-Smad1/5/9 in SW620 cells, as indicated by western blot analysis and quantitative analysis, thereby indicating that BMP7 exerts its function through a non-canonical signaling pathway.
pathway. According to previous research results, the p53 status in SW620 cells is inactivated (27). Therefore, it was next evaluated whether HNK affects the protein levels of TGF-β1 and p53 (S15) by using western blot analysis. As presented in Fig. 5B and C, HNK promoted TGF-β1 expression. Similarly, the results revealed that HNK increased the levels of p-p53 (Fig. 5D and E). Taken together, these results demonstrated that the effect of HNK to regulate TGF-β1 and p-p53 expression was exerted through a non-typical signaling pathway.

Effects of BMP7 on the activation of TGF-β1 and p53 in SW620 cells. In the present study, it was demonstrated that HNK was able to increase the protein levels of TGF-β1 and
Next, the effects of BMP7 on the activation of TGF-β1 and p53 by HNK in SW620 cells were determined. As presented in Fig. 6A and B, western blot analysis indicated that exogenous BMP7 significantly promoted the effect of HNK to upregulate the ratio of p-p53/p53. By contrast, knocking down BMP7 attenuated the effect of HNK to decrease the ratio of p-p53/p53 in SW620 cells (Fig. 6C and D).

Next, the effect of BMP7 on TGF-β1 expression was examined. Western blot analysis suggested that exogenous BMP7 significantly enhanced the HNK-induced upregulation of the TGF-β1 protein level (Fig. 7A and B). By contrast, knocking down BMP7 attenuated the effect of HNK to decrease the ratio of p-p53/p53 in SW620 cells (Fig. 6C and D).

Lastly, SW620 cells transfected with AdBMP7 or AdsiBMP7 were injected into nude mice to establish a human tumor xenograft. Hematoxylin and eosin staining demonstrated that more necrotic cells were present in the AdBMP7 + HNK-treated group compared with the control group, while knocking down BMP7 partially attenuated this effect (Fig. 7G). As presented in Fig. 7H, the expression of TGF-β1 in transplanted tumors was determined by using an immunohistochemistry assay. It was indicated that the expression of TGF-β1 was positively associated with BMP7 expression. These results suggested that HNK promoted TGF-β1 expression, which was mediated by HNK-induced expression of BMP7 in vivo.

Effects of TGF-β1 to regulate p53 expression. In the present study, it was demonstrated that treatment with HNK led to upregulation of BMP7 and p53 activation in SW620 cells and activated TGF-β1, but did not trigger the BMPs/Smad signaling pathway in SW620 cells. Next, the proteins in the TGF-β1/Smad signaling pathway were assessed by western blot analysis. The results indicated that HNK did not markedly affect the levels of total Smad2/3 or p-Smad2/3 in SW620 cells (Fig. 8A). Thus, TGF-β1 may regulate p53 activation through the non-canonical BMPs/Smad signaling pathway. Next, it was evaluated whether HNK affected the levels of p53 by activating...
TGF-β1. For this, SW620 cells underwent different treatments and the protein levels of p53 and p-p53 (S15) were evaluated by western blot analysis. As presented in Fig. 8B and C, the TGF-β1-selective inhibitor LY364947 (5 µm) significantly reduced p-p53 levels and the ratio of p-p53/p53 compared with treatment with HNK alone. Furthermore, combined treatment of HNK and BMP7 attenuated the effect of LY364947 on the protein levels of p-p53 and the ratio of p-p53/p53, while its effects were reversed by knocking down BMP7. These results demonstrated that during HNK treatment, TGF-β1 regulated the expression of p53, which may be mediated by BMP7.

Discussion

Colon cancer is one of the most common malignancies of the digestive system (3). With the development of technology and medicine, early screening, diagnosis and treatment for colon cancer have been significantly improved in the past decade (41). However, due to the major difficulty of designing individualized treatments, improving the prognosis of patients with colon cancer currently poses a great challenge (42). Thus, there is a requirement to develop less toxic and more effective agents for the treatment of colon cancer. A growing number of studies have focused on natural products due to their beneficial properties, including low toxicity and a good safety profile for human health (43,44). Previous studies have reported on the use of compounds with anticancer activity that are natural products and/or their derivatives for colon cancer treatment in the clinic, including vincristine, paclitaxel and camptothecin (6,45,46). Therefore, natural products may be an abundant source for chemotherapeutic agents against several human cancers.

HNK, a biphenyldiol natural product, is isolated from the bark and branches of the magnolia tree (9). HNK possesses an expansive medicinal prospect and clinical need, and has been reported to have a beneficial effect in the treatment of several diseases (47). Of note, several studies have suggested that HNK exerts various biological activities, including antitumor, antioxidation, antiviral and anti-inflammatory effects (8,9). The antitumor effects of HNK have attracted increasing attention, including its activity against breast cancer, lung cancer, leukemia and colon cancer (11,48). Furthermore, it has been indicated that HNK is able to inhibit the proliferation and induce apoptosis in HCT116 cells, thereby supporting that HNK may be a potential anticancer drug (38). Mechanistically, the anticancer activities of HNK may be mediated through...
various signaling pathways and molecules, including STAT3, epidermal growth factor receptor, NF-κB, cell survival signaling and inflammatory mediators (47,48). However, to the best of our knowledge, whether any further signaling pathways are involved in the tumor-inhibitory effect of HNK remains to be elucidated.

With the increase in understanding of colon cancer, its etiological causes have become more extensively elucidated. Various molecules and signaling pathways have been implicated in this malignancy, including the Wnt and TGF-β signaling pathway, as well as MAPK signaling (40,49,50). The TGF-β super-family contains various members, mainly consisting of the TGF-βs, activins, inhibins, nodal factors and BMPs (49). Previous studies have reported that the TGF-β signaling pathway has a role in numerous biological processes and has pleiotropic functions in regulating cell growth, differentiation, apoptosis, motility, invasion, cancer progression and immune response (19,51). TGF-β1, a multifunctional cytokine, is the primary member of the TGF-β superfamily, which has become a breakthrough point for investigating the causes of cancer and preventive treatments (14). It has a crucial role in multiple events, including cell proliferation, differentiation and development, tissue repair and regeneration (13). However, certain other studies have indicated that abnormal function of TGF-β1 is involved in multiple human diseases, including fibrosis, autoimmune diseases and cancer, which pose a significant threat to human health (13,52,53). Based on the specific properties of TGF-β1 that have previously been demonstrated, the effects of HNK on the expression of TGF-β1 were mainly investigated in the present study. The results confirmed that HNK caused significant upregulation of TGF-β1 expression. To the best of our knowledge, the present study was the first to demonstrate that HNK augmented TGF-β1 expression in SW620 cells. The
detailed molecular mechanisms underlying this process were then further elucidated.

BMPs are among the primary members of the TGF-β superfamily. BMPs were firstly identified by Urist (54) in 1965 as osteoinductive factors. It has since been reported that BMPs have a vital role in a multitude of processes of embryonic development and adult homeostasis, regulating cell proliferation and apoptosis throughout the whole body, and are involved in cancer progression (55,56). Mutations in members of the BMP pathway or disorders thereof have been reported in juvenile polyposis and in inherited polyposis syndrome that predisposes to colorectal cancer (57). In addition, it has been indicated that BMP2 suppresses cell growth and enhances chemosensitivity of colon cancer cells and that exogenous expression of BMP3 in HCT116 cells inhibits cell growth, migration and invasion, and increases the rate of apoptosis. Furthermore, BMP9 may mediate the anticancer effect of resveratrol in colon cancer cells (22,23,58).

BMP7, as one of the members of the BMPs, is also known as osteogenic protein-1 (55). An increasing number of studies have also demonstrated that BMP7 is implicated in the development of several cancer types (38,59). Shen et al (59) indicated that recombinant human BMP7 significantly inhibits cell proliferation, motility and invasion in SBC-3 and SBC-5 cells. However, another study reported that the level of BMP7 in breast cancer cells is higher compared with that in normal cells (26). A previous study by our group demonstrated that oridonin exhibits efficacious anticancer activity through upregulating BMP7 in colon cancer (39). Thus, it was speculated that the anticancer activity of HNK in colon cancer may also be associated with BMP7. Unlike previous studies, the colony formation assay was used to evaluate the anti-proliferation effect of HNK on SW620 cells in the present study. The results demonstrated that HNK significantly inhibited the proliferation of SW620 cells, which is combined with exogenous BMP7. By contrast, BMP7-knockdown did not markedly reduce those effects. Similar results were obtained in the CCK-8 viability assay. Hence, HNK was observed to lead to the upregulation of BMP7 expression in SW620 cells and that exogenous BMP7 potentiated the effect of HNK to inhibit cell viability and induce apoptosis, while BMP7-knockdown did not significantly attenuate this effect. One of the primary reasons is that HNK may exert the anticancer effect on multiple molecular targets in colon cancer (8). Exogenous BMP7 could cooperate with HNK to enhance its antitumor activity, and the effect of knocking down BMP7 on reducing the anticancer activity of HNK may be mitigated by others mechanisms. Hence, the inhibitory effect of HNK may in part be mediated by the upregulation of BMP7 in colon cancer. Next, it was hypothesized that the anticancer effect of HNK was exerted through HNK-induced BMP7 augmenting the activity of TGF-β1. Thus, the effect of BMP7 on TGF-β1 expression was further assessed. The results demonstrated a positive association between the expression of TGF-β1 and BMP7 in colon cancer cells.

BMPs conventionally perform their biological functions through the BMPs/Smad signaling pathway, which is known as the canonical BMPs/Smad signaling pathway (55). In addition, BMPs may transmit their signal through the non-canonical BMPs/Smad signaling pathway, including MAPKs, TGF-β and PI3K/Akt (28). In the canonical BMPs/Smad signaling pathway, BMPs exert their function through binding to their receptors, which are composed of type I BMP receptor (BMPR1) and BMPRII (60). BMPRII recruits and phosphorylates BMPRI, which in turn initiates signal transduction mediated by the downstream Smad
proteins. Subsequently, Smad1/5/9 are phosphorylated and form a complex with Smad4, thereby allowing them to translocate into the nucleus. Smad4 acts as a transcriptional co-activator with Smad1/5/9 to facilitate this process (55). However, several studies have indicated that BMP7 may exert anticancer effects in a Smad4-independent manner (38,61). Furthermore, in the present study, western blot analysis demonstrated that HNK exerted no significant effect on the level of total and phosphorylated Smad1/5/9 in SW620 cells.

p53, a well-known tumor suppressor protein that exerts its functions as a critical mediator of the cellular response to exogenous and endogenous stresses, is considered a valid therapeutic target in various cancer types (62-64). Functional loss or mutation in p53 has been regarded as a primary cause of cancer. MAPK is another crucial cell-growth regulator in the pathogenesis of cancer. Abrerrant p38-MAPK signaling has been noted in solid tumors, including breast cancer and colon cancer (65). Results from previous studies have indicated that HNK affects the status of p53, and BMP7 regulates the activity of p53 in colon cancer cells (38,66). Zerbini et al (67) indicated the importance of Ser15 phosphorylation in regulating the oncogenic function of mutant p53 and apoptosis induction. For this reason, the present study investigated whether the anticancer effect of HNK on colon cancer cells was via other signaling pathways. The present results suggested that HNK led to the upregulation of TGF-β1 and increased the phosphorylation at the site S15 of p53 expression, which may be at least partially mediated by HNK-induced BMP7. Thus, it was hypothesized that inhibiting TGF-β1 activation may regulate p53 expression. The results of the present study indicated that the TGF-β1-selective inhibitor LY364947 reduced the protein levels of p-p53 and its function was significantly enhanced by knocking down BMP7. Conversely, HNK reversed the inhibitory effect of LY364947 on p-p53 expression through exogenous BMP7, as demonstrated by using western blot and quantitative analyses. Furthermore, HNK exerted no significant effect on the level of total and p-Smad2/3 in SW620 cells. Hence, TGF-β1 may activate p53 through the non-canonical signaling pathway.

The current study focused on whether the anticancer activity of HNK in colon cancer is associated with BMP7. Unlike previous studies, the most obvious difference a study by Liu et al (27) is that AdBMP7 or AdsiBMP7 (which is superior to the specific antibody of BMP7) exerts its function for a certain period of time (~4 or 5 weeks) after infecting cells. In addition, it was more convenient to conduct animal experiments. However, there were still some limitations in the present study. Firstly, regarding the effect of HNK on inducing cell apoptosis, only the apoptosis-related proteins Bad and Bcl-2 were assessed by RT-qPCR and western blot analysis. Flow cytometry and the pan-caspase inhibitor z-VAD-fmk were not used to detect honokiol-induced cell death in the present study. In addition, only the anticancer function of HNK on regulating TGF-β1 through BMP7 and the activation p53 was investigated, but its functions through other signaling pathways, including p38-MAPK and PI3K/Akt, were not explored.

In conclusion, in the present study, the anticancer activity of HNK was investigated in colon cancer cells and the underlying mechanisms were explored. The results indicated that HNK inhibited colon cancer cell growth and induced cell apoptosis through upregulating BMP7 to enhance TGF-β1 and p-p53 expression. Moreover, TGF-β1 may regulate p53 activation. These results were further confirmed in a human colon cancer xenograft nude mouse model. Of note, the present study demonstrated that HNK significantly increased the activity of TGF-β1 via upregulating the expression of BMP7.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

WJS, BCH and KW designed the study. QL, YM and XLL conducted the experiments. QL, YM and LM analyzed the data. QL and WJS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (approval no. 2019-255).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


