BMP9 mediates the anticancer activity of evodiamine through HIF-1α/p53 in human colon cancer cells

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Abstract. Colon cancer is one of the most common malignancies. Although there has been great development in treatment regimens over the last few decades, its prognosis remains poor. There is still a clinical need to find new drugs for colon cancer. Evodiamine (Evo) is a quinolone alkaloid extracted from the traditional herbal medicine plant *Evodia rutaecarpa*. In the present study, CCK-8, flow cytometry, reverse transcription quantitative polymerase chain reaction, western blot analysis and a xenograft tumor model were used to evaluate the anti-cancer activity of Evo in human colon cancer cells and determine the possible mechanism underlying this process. It was revealed that Evo exhibited prominent anti-proliferation and apoptosis-inducing effects in HCT116 cells. Bone morphogenetic protein 9 (BMP9) was notably upregulated by Evo in HCT116 cells. Exogenous BMP9 potentiated the anti-cancer activity of Evo, and BMP9 silencing reduced this effect. In addition, HIF-1α was also upregulated by Evo. The anticancer activity of Evo was enhanced by HIF-1α, but was reduced by HIF-1α silencing. BMP9 potentiated the effect of Evo on the upregulation of HIF-1α, and enhanced the antitumor effect of Evo in colon cancer, which was clearly reduced by HIF-1α silencing. In HCT116 cells, Evo increased the phosphorylation of p53, which was enhanced by BMP9 but reduced by BMP9 silencing. Furthermore, the effect of Evo on p53 was potentiated by HIF-1α and reduced by HIF-1α silencing. The present findings therefore strongly indicated that the anticancer activity of Evo may be partly mediated by BMP9 upregulation, which can activate p53 through upregulation of HIF-1α, at least in human colon cancer.

Introduction

Colon cancer is one of the most common gastrointestinal malignancies (1). Despite marked developments in the diagnosis and treatment of colon cancer in the past few decades, its prognosis remains poor. To date, the treatment of colon cancer is faced with major challenges, including the serious side effects caused by chemotherapy agents, drug resistance and metastasis. Thus, there is still a clinical need for the development of new treatment regimens for colon cancer.

Although chemotherapy drugs possess serious adverse effects, chemotherapy remains one of the major treatments for colon cancer (2). Natural products and their active derivates, including semi-synthetic and synthetic analogs, comprise one of the most important sources of chemotherapy drugs. Several plant-derived compounds, such as paclitaxel, vincristine, camptothecin and etoposide, have already been used against cancer for several decades (3-6). Evodiamine (Evo), a quinolone alkaloid extracted from traditional herbal medicine *Evodia rutaecarpa* (7), has multiple pharmacological actions and could be used for obesity, inflammation, infectious and cardiovascular diseases (8). A growing amount of evidence has indicated that Evo exhibits anticancer activity against various types of cancer, such as tongue, colon and breast cancer (9-12). According to studies, this activity of Evo may be mediated by NF-κB (9), transforming growth factor β1 (TGF-β1) (11) and/or the p53/p21/Rb pathway (12). However, the explicit mechanism underlying this activity requires further exploration.

Although the global pathogenesis of colon cancer remains unclear, the aberrant function of several important signals and genes, such as p53, APC, PIK3CA and Smad4 mutations, has been identified (13-16). TGF-β is a cytokine that plays an important role in deciding the fate of cells by regulating proliferation and differentiation. Bone morphogenetic proteins (BMPs) are another sub-group of TGF-β superfamily, which were reported by Urist in 1965 as osteogenic factor (17). In addition to the development of skeletal system, BMPs also play an important role in the development of gastrointestinal track

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by regulating the stromal microenvironment, protecting from polyposis initiation of the colonic mesenchyme and terminal differentiation of intestinal secretory progenitor cells (18,19). Thus, the aberrant signal transduction of BMP may be another major cause of colon cancer (20). Our previous study demonstrated that BMP9 partly mediated the anticancer activity of several natural products, such as resveratrol (21). Although Evo exhibited effective anticancer activity in colon cancer, it remains unknown whether BMP9 is involved in this process.

In the present study, it was determined whether BMP9 could mediate the anticancer activity of Evo in colon cancer, and the possible mechanism underlying this biological process was revealed.

Materials and methods

Chemicals and cell culture. Evo was purchased from Xi'an Hao-Xuan Bio-tech Co., Ltd. and dissolved with dimethyl sulfoxide (DMSO) for *in vitro* testing, or suspended with 0.4% carboxymethylcellulose sodium for *in vivo* testing. Human colon epithelial cell line (FHC) and human colon cancer cell lines (including HCT116, LoVo, SW620 and SW480) were obtained from the American Type Culture Collection (ATCC). Primary antibodies for PCNA (cat. no. sc-56; mouse, monoclonal; 1:1,000), GAPDH (cat. no. sc-32233; mouse, monoclonal; 1:1,000), Bad (cat. no. sc-8044; mouse, monoclonal; 1:1,000), Bcl-2 (cat. no. sc-7382; mouse, mono-clonal; 1:1,000), BMP9 (cat. no. sc-514211; rabbit, polyclonal; 1:1,000), HIF-1α (cat. no. sc-10790; rabbit, polyclonal; 1:1,000), Smad1/5/8 (cat. no. sc-6031-R; rabbit, polyclonal; 1:1,000), p53 (cat. no. sc-55476; mouse, monoclonal; 1:1,000) and p-p53 (cat. no. sc-13580; mouse, monoclonal; 1:1,000) were purchased from Santa Cruz Biotechnology Inc. Phosphorylated (p)-Smad1/5/9 (cat. no. 13820S; goat, mono-clonal; 1:1,000) was ordered from Cell signaling Technology. Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin (100 U/ml) and strep-tomycin (100 μg/ml) at 37˚C in 5% CO_2.

Cell viability assay. Cell viability was measured using CCK-8 kits (cat. no. C008-2; Seven Sea Biotechnology, Shanghai China). Briefly, subconfluent cells were placed in 96-well plates with 200 μl medium (2,000 cells/well), and treated with different concentrations of Evo (0.5, 1, 1.5, 2 and 2.5 μM) for 24, 48 and 72 h, according to the experimental design. CCK-8 (10 μl/well) was added and then the cells were incubated for another 2 h at 37˚C. The optical density of each well was measured at 450 nm using a microplate reader (ELx800; BioTek Instruments, Inc.). Each assay was repeated at least three times.

Crystal violet staining and colony formation assay. Crystal violet staining was performed as previously reported (22). In brief, subconfluent HCT116 cells were treated with Evo (0.5, 1 or 2 μM) for 24 h. Cells were then re-plated in 12-well cell culture plates without Evo at 100 or 200 cells/well. Colonies were subjected to crystal violet staining after treatment for 14 days. They were then carefully washed with cold (4˚C) phosphate-buffered saline (PBS) and stained with PBS-buffered 0.5% crystal violet formalin solution at room temperature for 20 min. Next, the plates were washed with tap water and air-dried for imaging under inverted microscope (magnification, x40, Ti Nikon; Nikon) or scanning. There are over 450 cell colonies in each well at least. Each assay was repeated at least three times.

Construction of recombinant adenovirus. Recombinant adenoviruses for the present study were constructed using the AdEasy system (23,24). In brief, the coding sequence of human BMP9 and HIF-1α was amplified and sub-cloned into a shuttle vector (pAdTrace-TO4); Oligo cassettes for BMP9 or HIF-1α silencing were cloned into a pSES1 shuttle vector. The shuttle vectors were then recombined with pAdEasy1 in BJ5183 cells. Finally, the correct recombinant vectors were transfected into 293 cells (obtained from ATCC) for packaging adenoviruses, which were designated as AdBMP9, AdHIF-1α, AdsiBMP9 and AdsiHIF-1α. All recombinant adenoviruses were tagged with green fluorescent protein (GFP) and AdGFP was used as the vector control.

Flow cytometry for cell cycle and apoptosis. Subconfluent cells were placed into 6-well culture plates and treated according to the experimental design for 48 h. For cell cycle analysis, cells were harvested and washed carefully with PBS (4˚C), fixed with cold (4˚C) 70% ethanol, washed with 50% ethanol, 30% ethanol, and PBS. Finally, the cells were stained with propidium iodide (PI) PBS solution (20 mg/ml, containing RNase 1 mg/ml) for 30 min, followed by flow cytometric analysis (BD FACSVantage SE; Kaluza Analysis ver 2.0). For apoptosis analysis, cells were collected and washed with PBS (4˚C), followed by incubation with Annexin V-EGFP and PI, following the manufacturer's instructions (cat. no. KGA104; Nanjing KeyGen Biotech Co., Ltd.). Finally, the cells were subjected to fluorescence-activated cell sorting. Each assay was repeated at least three times.

Protein harvest and western blot analysis. Subconfluent HCT116 cells were seeded in 6-well culture plates and treated with different concentrations of Evo (0.5, 1 or 2 μM) or DMSO. At each scheduled time-point, the cells were washed with PBS (4˚C) and lysed with 300 μl lysis buffer (cat. no. R0020; Solarbio Science and Technology Co., Ltd.). The protein level was assessed with BCA, and lysates were boiled for 10 min. The protein mass for each loading was 45 μg per lane, and proteins were subjected to 10% SDS-PAGE gel separation. Then, the proteins were transferred onto polyvinylidene fluo-ride membranes and blocked with 5% bovine serum albumin (BSA) (cat. no. SW3015; Solarbio Science and Technology Co., Ltd.) for 1 h. Finally, the membranes were incubated with corresponding primary antibodies (GAPDH, PCNA, Bad, Bcl-2, BMP9, HIF-1α, Smad1/5/8, p-Smad1/5/8, p53 and p-p53) for 2 h and horseradish peroxidase-conjugated secondary antibodies for anti-rabbit, anti-mouse or anti-goat (cat. nos. A0208, A0216 and A0181; Beyotime Institute of Biotechnology) for 1 h sequently at room temperature. Target proteins were visualized using SuperSignal West Pico Substrate (cat. no. 34096; Thermo Fisher Scientific, Inc.), images were captured with chemiluminescence imager (ChemiDoc XRS; Bio-Rad Laboratories Co., Ltd.) and quantified using Image Lab Software (version 4.1; Bio-Rad

Laboratories Co., Ltd.). Each assay was repeated at least three times.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Subconfluent HCT116 cells were seeded in a T25 culture flask and treated according to the experimental design. At 24 and/or 48 h after treatment, total RNA was extracted using TRIzol reagent (cat. no. 15596-026; Thermo Fisher Scientific, Inc.). The RNA was used to generate cDNA with RT kit (cat. no. R037A; Takara Biotechnology). Next, the cDNA products were used as templates for the following PCR assay. SYBR-Green kit (cat. no. B21202) was purchased from Bimake (Shanghai, China). The qPCR assays were performed with CFX Connect Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). The thermocycling conditions consisted of an initial denaturation, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. Analysis was conducted with CFX Connect system's software (Bio-Rad Laboratories, Inc.). GAPDH was used as internal control for mRNA expression levels and the relative mRNA levels were calculated with the $2^{-\Delta\Delta Cq}$ method (25). The primers used for this study are as follows: Bad forward, 5'-CGGAGGATGAGTGACGAGTT-3' and reverse, 5'-CGGAGGATGAGTGACGAGTT-3'; Bcl-2 forward, 5'-GGATGCCTTTGTGGAACTGT-3' and reverse, 5'-AGCCTGCAGCTTTGTTTCAT-3'; GAPDH forward, 5'-CAACGAATTTGGCTACAGCA-3' and reverse, 5'-AGG GGAGATTCAGTGTGGTG-3'. Each assay was repeated at least three times.

Xenograft tumor model of human colon cancer. The animal experiment was approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China). Twenty athymic nude mice (female, 4-6 weeks old, 18-22 g, 5/group) were purchased from the Animal Center of Chongqing Medical University (Chongqing, China). HCT116 cells were pretreated with AdGFP, AdBMP9, or AdBMP9 plus AdsiHIF-1α, and then collected and resuspended in PBS (4˚C). The final density was 2×10^7 cells/ml. Cells in 50 µl PBS (4˚C) were injected into the flanks of athymic mice. At 3 days following injection, animals were treated with Evo (10 mg/kg) or the same volume of solvent intragastrically once a day. Four weeks after injection, mice were euthanized by intraperitoneal injection of pentobarbital sodium (180 mg/kg body weight). Animals were sacrificed and the tumor masses were retrieved for histological evaluation when no autonomous breathing was produced for 2-3 min and no blink reflexes appeared.

Histological evaluation. Retrieved tumor masses were fixed in 10% formalin and embedded with paraffin. Sections were stained with hematoxylin and eosin (H&E) after being deparaffinized and rehydrated.

Statistical analysis. All quantitative experiments were performed in triplicate. Data are expressed as the mean ± SD. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, Inc.). A two-tailed t-test was used to compare differences between two groups, and one-way analysis of variance with Tukey's post hoc test was used to compare differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of Evo on HCT116 cell proliferation. CCK-8 assay results revealed that Evo decreased the viability of HCT116 cells in a concentration- and time-dependent manner (Fig. 1A). Crystal violet staining results revealed that Evo decreased the HCT116 cell colony formation ability when the concentration of cells was >1 µM (Fig. 1B). Western blot analysis results revealed that Evo significantly increased the protein expression of PCNA in HCT116 cells compared to the control (Fig. 1C and D). Flow cytometric results revealed that Evo markedly arrested the cell-cycle at the S phase in HCT116 cells compared to the control (Fig. 1E). These results indicated that Evo inhibited the proliferation of human colon cancer cells.

Effects of Evo on HCT116 cell apoptosis. RT-qPCR assay results revealed that Evo increased the mRNA expression of Bad, but significantly decreased that of Bcl-2 (Fig. 2A and B). In addition, western blot analysis results revealed that Evo (at 1 and 2 µM) increased the expression of Bad, and significantly decreased that of Bcl-2, which was more pronounced at 48 h compared to the control (Fig. 2C-E). Flow cytometric results revealed that Evo also slightly increased the percentage of apoptotic HCT116 cells, even at a concentration of 0.5 µM (Fig. 2F). These findings indicated that Evo induced apoptosis in HCT116 cells.

Effects of BMP9 on the anticancer activity of Evo in HCT116 cells. Western blot analysis results revealed that Evo significantly increased the level of BMP9 in HCT116 cells compared to the control (Fig. 3A and B), and that endogenous BMP9 could be detected in the FCH cells and several other colon cancer cell lines (Fig. 3C and D). RT-qPCR results revealed that BMP9 could slightly increase the mRNA expression of Bad and significantly decrease that of Bcl-2 compared to the control, and BMP9 promoted the increasing effect of Evo on the mRNA expression of Bad, and the reducing effect of Evo on Bcl-2 in HCT116 cells compared to the group treated with Evo only (Fig. 3E and F). Conversely, BMP9 silencing slightly decreased the mRNA expression of Bad and increased that of Bcl-2 compared to the control. It was also revealed that BMP9 silencing decreased the increasing effect of Evo on the mRNA expression of Bad, and reversed the decreasing effect of Evo on the mRNA expression of Bcl-2 in HCT116 cells compare to the group treated with Evo only (Fig. 3G and H). These results indicated that BMP9 may mediate the anticancer effect of Evo in colon cancer cells.

Effects of HIF-1α on the anticancer activity of Evo in HCT116 cells. HIF-1α has been revealed to be upregulated by BMP9 in progenitor cells. HIF-1α has also been revealed to be involved in tumorigenesis. Therefore, it was next determined whether HIF-1α could mediate the effect of BMP9 on the anticancer activity of Evo in HCT116 cells. RT-qPCR results revealed that Evo significantly increased the mRNA expression of HIF-1α in HCT116 cells compared to the control (Fig. 4A). Western blot analysis results also revealed that Evo increased HIF-1α in HCT116 cells compared to the control (Fig. 4B and C). Furthermore, AdHIF-1α-mediated exog-

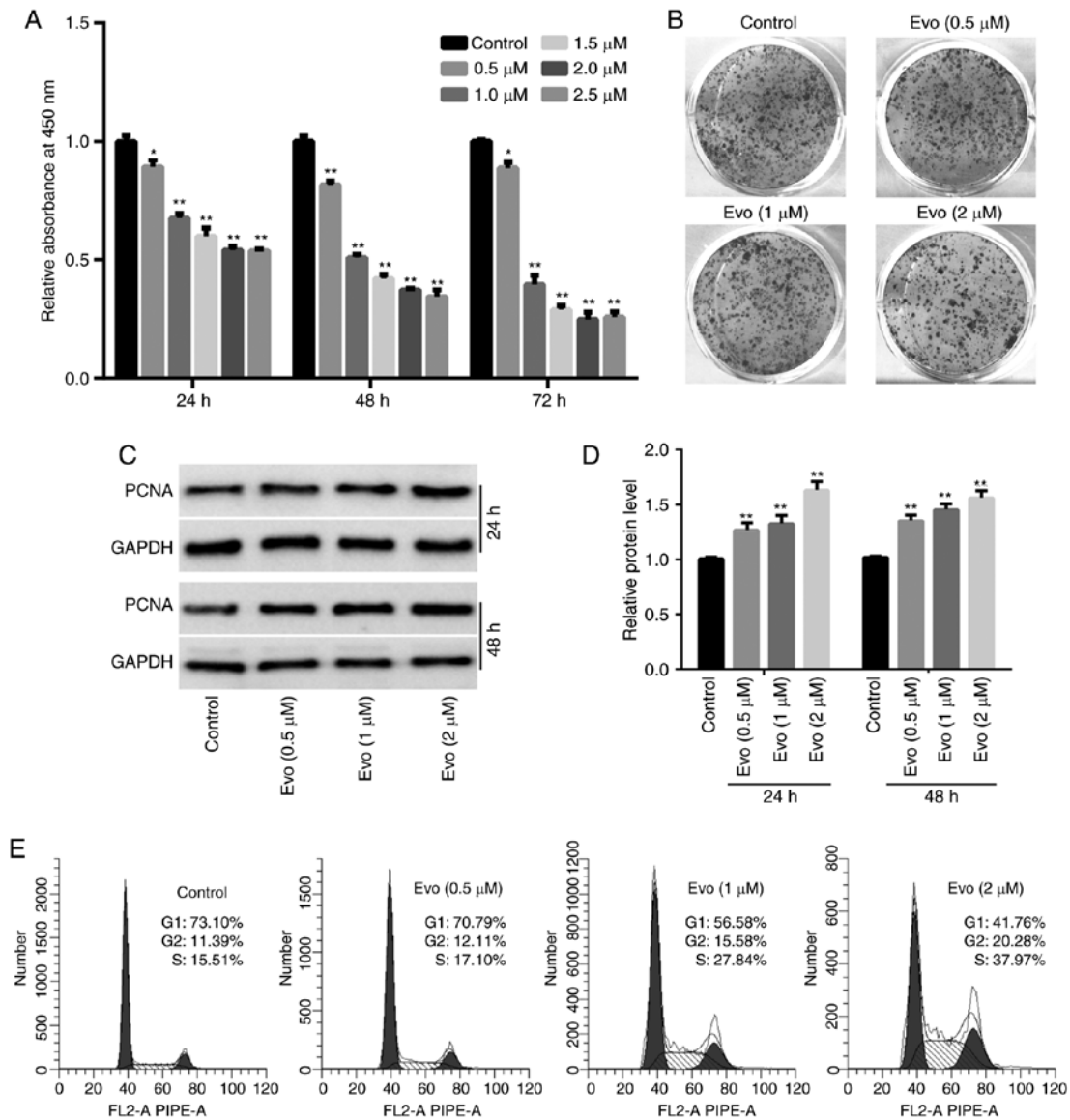Figure 1. Effects of Evo on HCT116 cell proliferation. (A) CCK-8 assay results revealed the anti-proliferative effect of Evo in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group). (B) Colony formation results revealed the anti-proliferative effect of Evo in HCT116 cells. (C) Western blot analysis results revealed the effect of Evo on the protein expression of PCNA in HCT116 cells; GAPDH was used as a loading control. (D) Quantification of the western blot analysis results revealed the effect of Evo on the protein expression of PCNA in HCT116 cells (**P<0.01 vs. the control group). (E) Flow cytometric results revealing the effect of Evo on cell cycle arrest in HCT116 cells. Evo, evodiamine.

enous HIF-1α increased the protein level of Bad and reduced the level of Bcl-2 compared to the control, and promoted the increasing effect of Evo on Bad and the decreasing effect of Evo on Bcl-2 compared to the group treated with Evo only (Fig. 4D-F). Conversely, HIF-1α silencing slightly reduced the protein level of Bad compared to the control, but attenuated the increasing effect of Evo on Bad and the decreasing effect of Evo on Bcl-2 in HCT116 cells compared to the group treated with Evo only (Fig. 4G-I). In addition, our previous study also indicated that the anticancer activity of Evo was associated with the downregulation of HIF-1α in human colon cancer cells (LoVo) (10). These results indicated that HIF-1α may also participate in mediating the anticancer activity of Evo in HCT116 cells.

Effects of BMP9 and/or HIF-1α on the anticancer activity of Evo in human colon cancer. BMP9 and HIF-1α both affected

the anticancer activity of Evo. Thus, whether HIF-1α could mediate the effect of BMP9 on Evo in colon cancer cells was next determined. Western blot analysis results revealed that Evo exhibited no substantial effect on the protein expression of Smad1/5/8 and p-Smad1/5/8 in HCT116 cells (Fig. 5A). The quantification of western blot analysis revealed that Evo exerted no obvious effect on the ratio of p-Smad1/5/8 to Smad1/5/8 in HCT116 cells (Fig. 5B). Further western blot analysis results revealed that BMP9 slightly increased HIF-1α compared to the control, but significantly promoted the increasing effect of Evo on HIF-1α in HCT116 cells compared to the group treated with Evo only (Fig. 5C and D). BMP9 silencing also exerted no substantial effect on HIF-1α compared to the control, but significantly reduced the increasing effect of Evo on HIF-1α in HCT116 cells compared to the group treated with Evo only (Fig. 5E and F). Xenograft tumor assay results revealed that Evo markedly inhibited tumor growth compared to the control, and
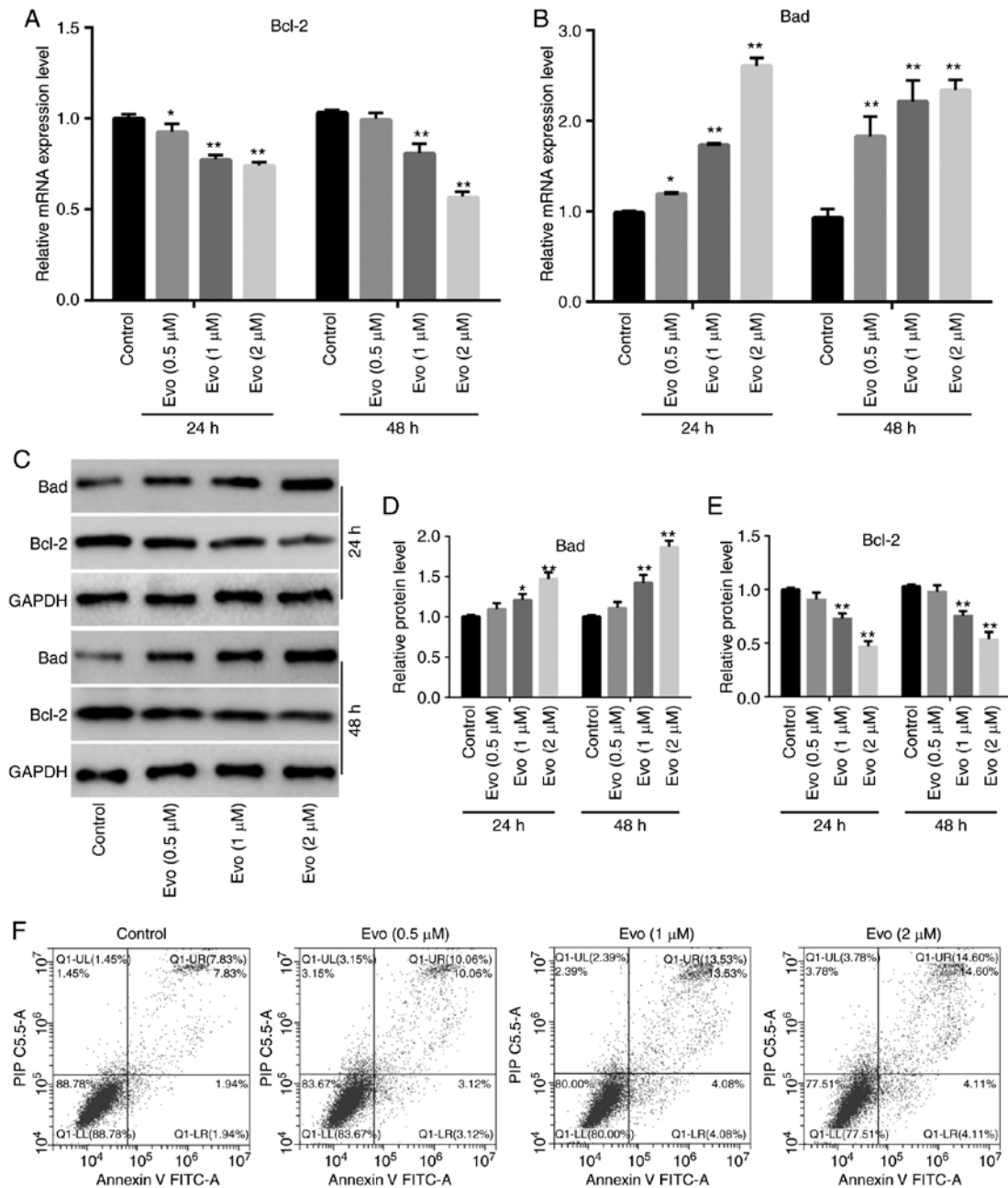
Figure 2. Effects of Evo on HCT116 cell apoptosis. (A) RT-qPCR results revealed the effect of Evo on the mRNA expression of Bcl-2 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group). (B) RT-qPCR results revealed the effect of Evo on the mRNA expression of Bad in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group). (C) Western blot analysis results revealed the effect of Evo on the protein expression of Bad and Bcl-2 in HCT116 cells. (D) Quantification of the western blot analysis results revealed the effect of Evo on the protein expression of Bad in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group). (E) Quantification of the western blot analysis results revealed the effect of Evo on the protein expression of Bcl-2 in HCT116 cells (**P<0.01 vs. the control group). (F) Flow cytometric results revealing g the effect of Evo on HCT116 cell apoptosis. Evo, evodiamine.

BMP9 enhanced the antitumor growth effect of Evo (the largest tumor mass appeared in the control group, and the maximum diameter of the tumor masses was 1.1 cm), which could almost be reversed by HIF-1α silencing (Fig. 5G). Histochemical staining (H&E) results revealed that Evo markedly increased karyopyknosis compared to the control, and BMP9 potentiated that effect, which was clearly attenuated by HIF-1α silencing (Fig. 5H). These results indicated that HIF-1α may mediate the effect of BMP9 on the anticancer activity of Evo in colon cancer.

Effects of Evo, BMP9 and/or HIF-1α on the activity of p53 in HCT116 cells. Finally, it was investigated how HIF-1α

mediates the effect of BMP9 on the anticancer activity of Evo in HCT116 cells. Western blot analysis results revealed that Evo (1 and 2 μM) significantly increased the protein level of p53 and p-p53 in HCT116 cells compared to the control (Fig. 6A and B). Evo increased the level of p53 and p-p53 in HCT116 cells compared to the control, and BMP9 potentiated the increasing effect of Evo on the level of p53 and p-p53 compared to the group treated with Evo only (Fig. 6C and D). Conversely, BMP9 silencing slightly decreased the level of p53 and p-p53 compared to the control, and significantly reduced the increasing effect of Evo on p53 and p-p53 in HCT116 cells compared to the group treated with Evo only (Fig. 6E and F).
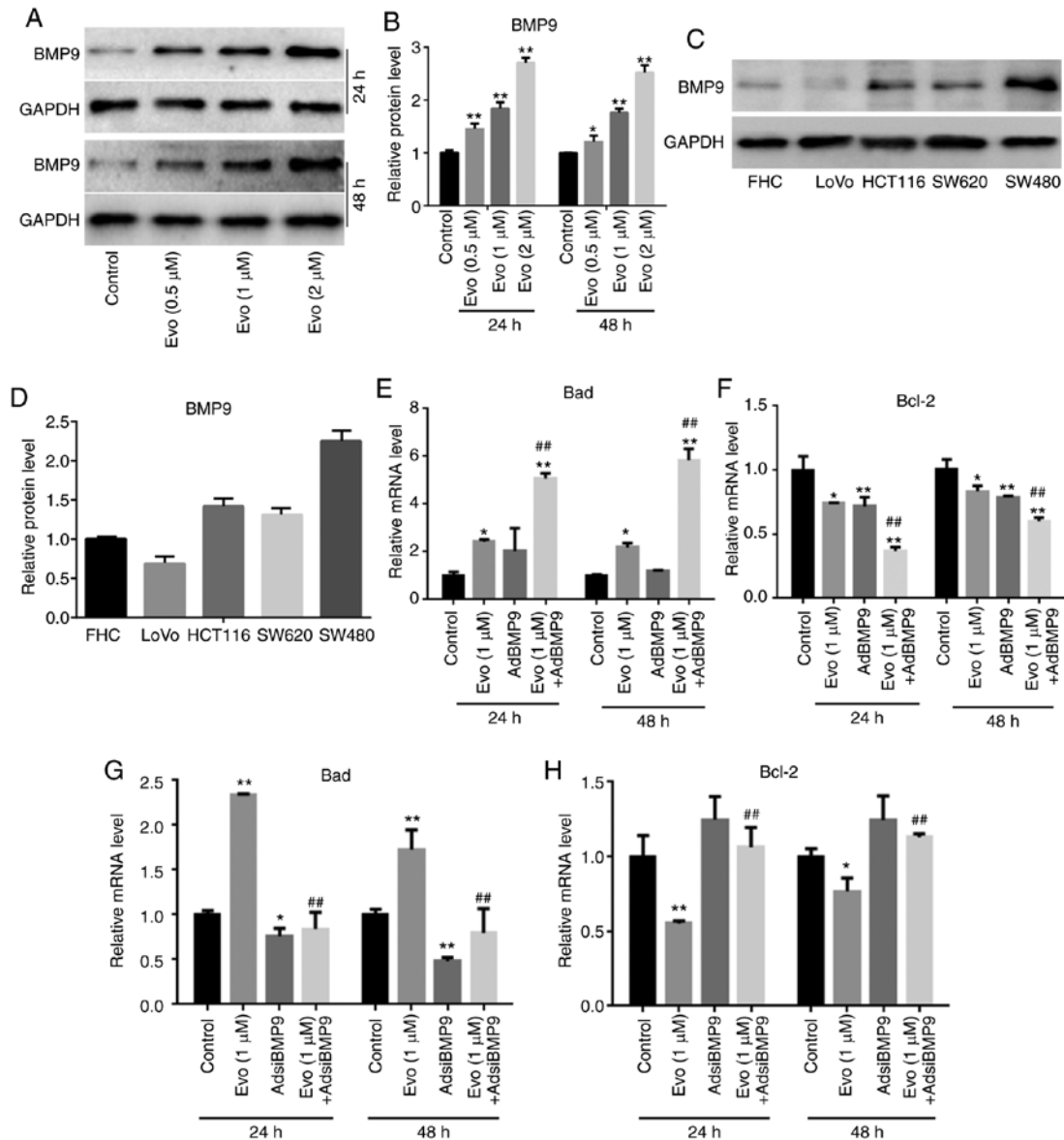
Figure 3. Effects of BMP9 on the anticancer activity of Evo in HCT116 cells. (A) Western blot analysis results revealed the effect of Evo on BMP9 in HCT116 cells. GAPDH was used as the loading control. (B) Quantification of the western blot analysis results revealed the effect of Evo on the protein expression of BMP9 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group). (C) Western blot analysis results revealed the expression of BMP9 in FHC and other available colon cancer cell lines; GAPDH was used as the loading control. (D) Quantification of the western blot analysis results revealed the protein expression of BMP9 in FHC and several colon cancer cell lines. (E) RT-qPCR results revealed the effect of Evo and/or BMP9 on the mRNA expression of Bad in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the group treated with Evo only). (F) RT-qPCR results revealed the effect of Evo and/or BMP9 on the mRNA expression of Bcl-2 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the group treated with Evo only). (G) RT-qPCR results revealed the effect of Evo and/or BMP9 silencing on the mRNA expression of Bad in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the group treated with Evo only). (H) RT-qPCR results revealed the effect of Evo and/or BMP9 silencing on the mRNA expression of Bcl-2 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the group treated with Evo only). Evo, evodiamine; BMP9, bone morphogenetic protein 9.

Furthermore, western blot analysis results revealed that HIF-1α slightly increased the level of p53 and p-p53 in HCT116 cells compared to the control, but significantly promoted the increasing effect of Evo on p53 and p-p53 in HCT116 cells compared to the group treated with Evo only (Fig. 6G and H). HIF-1α silencing also exerted no obvious effect on p53 and p-p53 compared to the control, but significantly decreased the increasing effect of Evo on p53 and p-p53 in HCT116 cells compared to the group treated with Evo only (Fig. 6I and J). These data indicated that HIF-1α may mediate the effect of BMP9 on the anticancer activity of Evo by partly enhancing the activity of p53 in colon cancer cells.

Discussion

Colon cancer remains one of the most common malignancies. In the past decades, there have been substantial developments in diagnostic and clinical treatment regimens for colon cancer. However, its prognosis remains poorer than expected. Therefore, there is still a need for the development of new and effective drugs or strategies for colon cancer treatment. In the present study, the effective anticancer activity of Evo, which may be mediated by BMP9 via the upregulation of HIF-1α to partly increase the activity of p53, was demonstrated in human colon cancer.
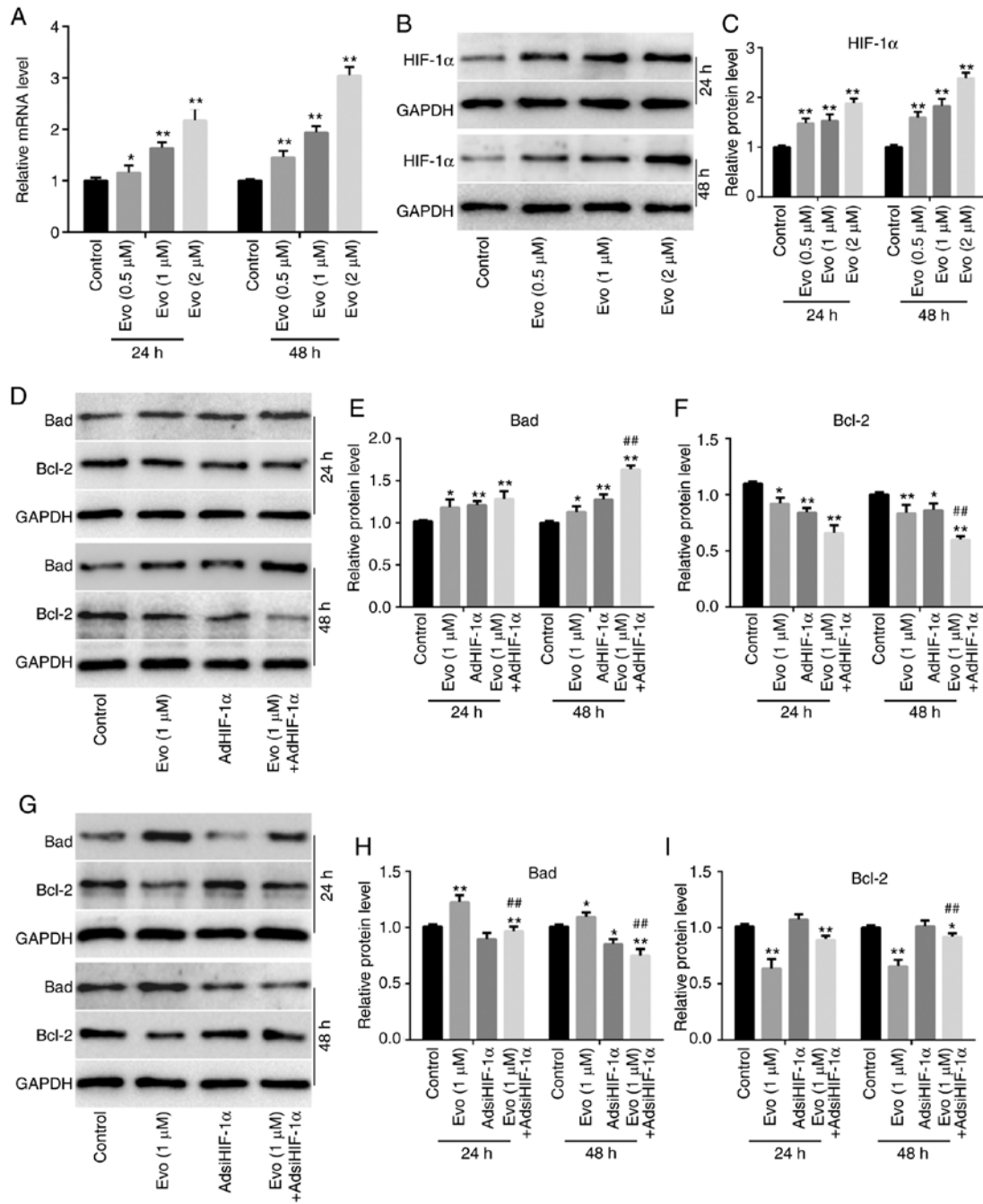
Figure 4. Effects of HIF-1α on the anticancer activity of Evo in HCT116 cells. (A) RT-qPCR revealed the effect of Evo on HIF-1α in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group). (B) Western blot analysis results revealed the effect of Evo on HIF-1α in HCT116 cells; GAPDH was used as the loading control. (C) Quantification of the western blot analysis results revealed the effect of Evo on the protein expression of HIF-1α in HCT116 cells (**P<0.01 vs. the control group). (D) Western blot analysis results revealed the effect of Evo and/or HIF-1α on Bad and Bcl-2 in HCT116 cells; GAPDH was used as the loading control. (E) Quantification of western blot analysis results revealed the effect of Evo and/or HIF-1α on the protein expression of Bad in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; ##P<0.01 vs. the Evo-treated group). (F) Quantification of the western blot analysis results revealed the effect of Evo and/or HIF-1α on the protein expression of Bcl-2 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; ##P<0.01 vs. the Evo-treated group). (G) Western blot analysis results revealed the effect of Evo and/or HIF-1α silencing on Bad and Bcl-2 in HCT116 cells; GAPDH was used as the loading control. (H) Quantification of the western blot analysis results revealed the effect of Evo and/or HIF-1α silencing on the protein expression of Bad in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; ##P<0.01 vs. the Evo-treated group). (I) Quantification of the western blot analysis results revealed the effect of Evo and/or HIF-1α silencing on the protein expression of Bcl-2 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; ##P<0.01 vs. the Evo-treated group). Evo, evodiamine.

It has been reported that Evo exhibits great anti-cancer activities in many types of cancer, such as tongue, breast, colon, prostate and lung cancer (9-12,26,27). As far as colon cancer is concerned, the anticancer activity of Evo may be mediated by inactivating the NF-κB (9) or TGF-β1 (11), or activating the p53/p21/Rb pathway (12). Our previous study indicated that the anti-cancer activity of Evo was associated with the downregulation of HIF-1α, at least in colon cancer (10). However, the explicit molecular mechanism underlying this activity of Evo needs to be further clarified.
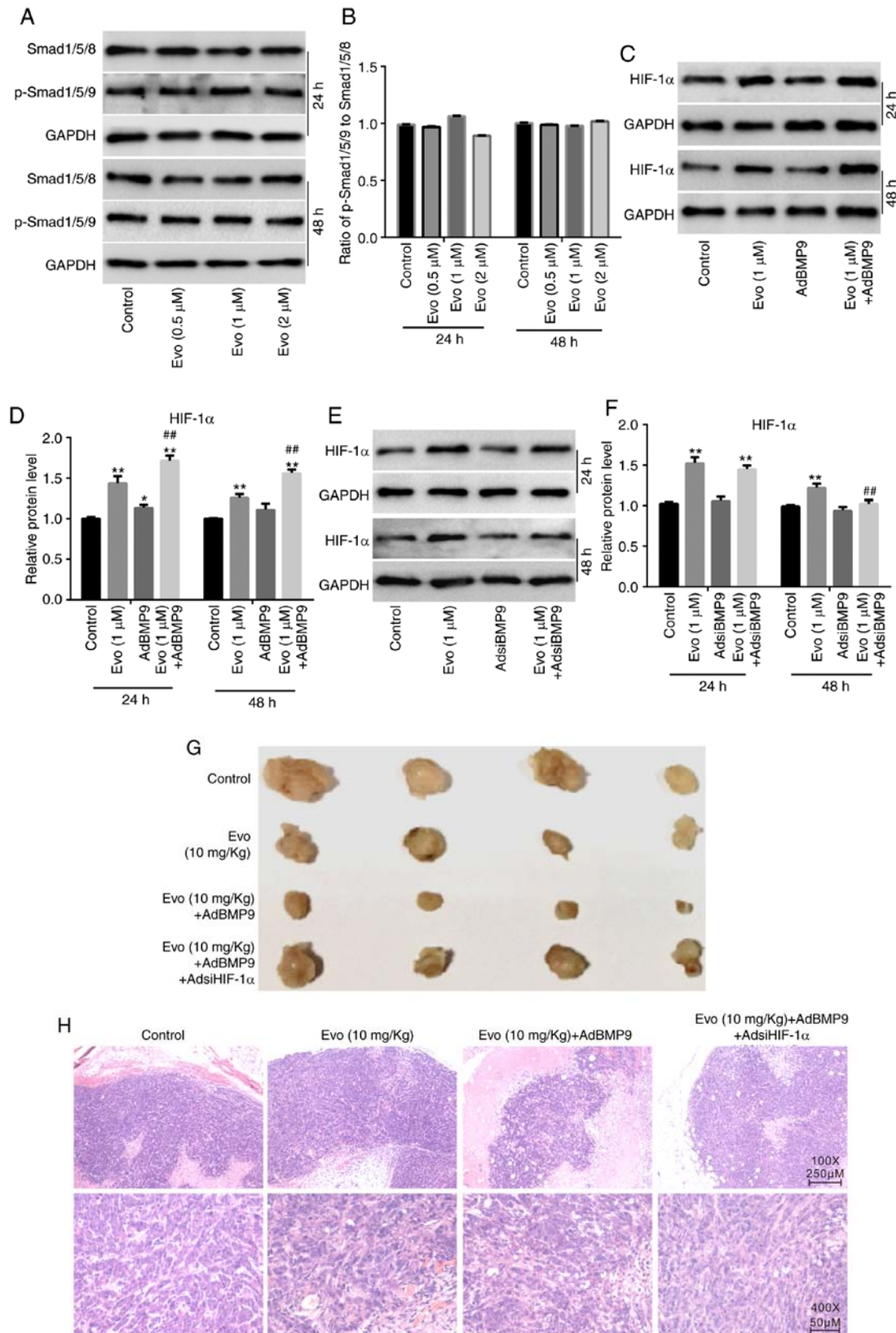
Figure 5. Effects of BMP9 and/or HIF-1α on the anticancer activity of Evo in human colon cancer. (A) Western blot analysis results revealed the effect of Evo on the expression of Smad1/5/8 and p-Smad1/5/8 in HCT116 cells; GAPDH was used as the loading control. (B) Quantification results of the western blot analysis revealed the effect of Evo on the ratio of p-Smad1/5/8 to p-Smad1/5/8 in HCT116 cells. (C) Western blot analysis results revealed the effect of Evo and/or BMP9 on the expression of HIF-1α in HCT116 cells; GAPDH was used as the loading control. (D) Quantification of the western blot analysis results revealed the effect of Evo and/or BMP9 on the protein expression of HIF-1α in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; $^{##}$P<0.01 vs. the Evo-treated group). (E) Western blot analysis results revealed the effect of Evo and/or BMP9 silencing on the expression of HIF-1α in HCT116 cells, GAPDH was used as the loading control. (F) Quantification of the western blot analysis results revealed the effect of Evo and/or BMP9 silencing on the protein expression of HIF-1α in HCT116 cells (**P<0.01 vs. the control group; $^{##}$P<0.01 vs. the Evo-treated group). (G) Representative tumor masses revealed the effect of BMP9 and/or HIF-1α on the effect of Evo on colon cancer tumor growth (Evo, 10 mg/kg). (H) H&E staining results revealed the anticancer effect of Evo in colon cancer (Evo, 10 mg/kg). Evo, evodiamine; BMP9, bone morphogenetic protein 9.
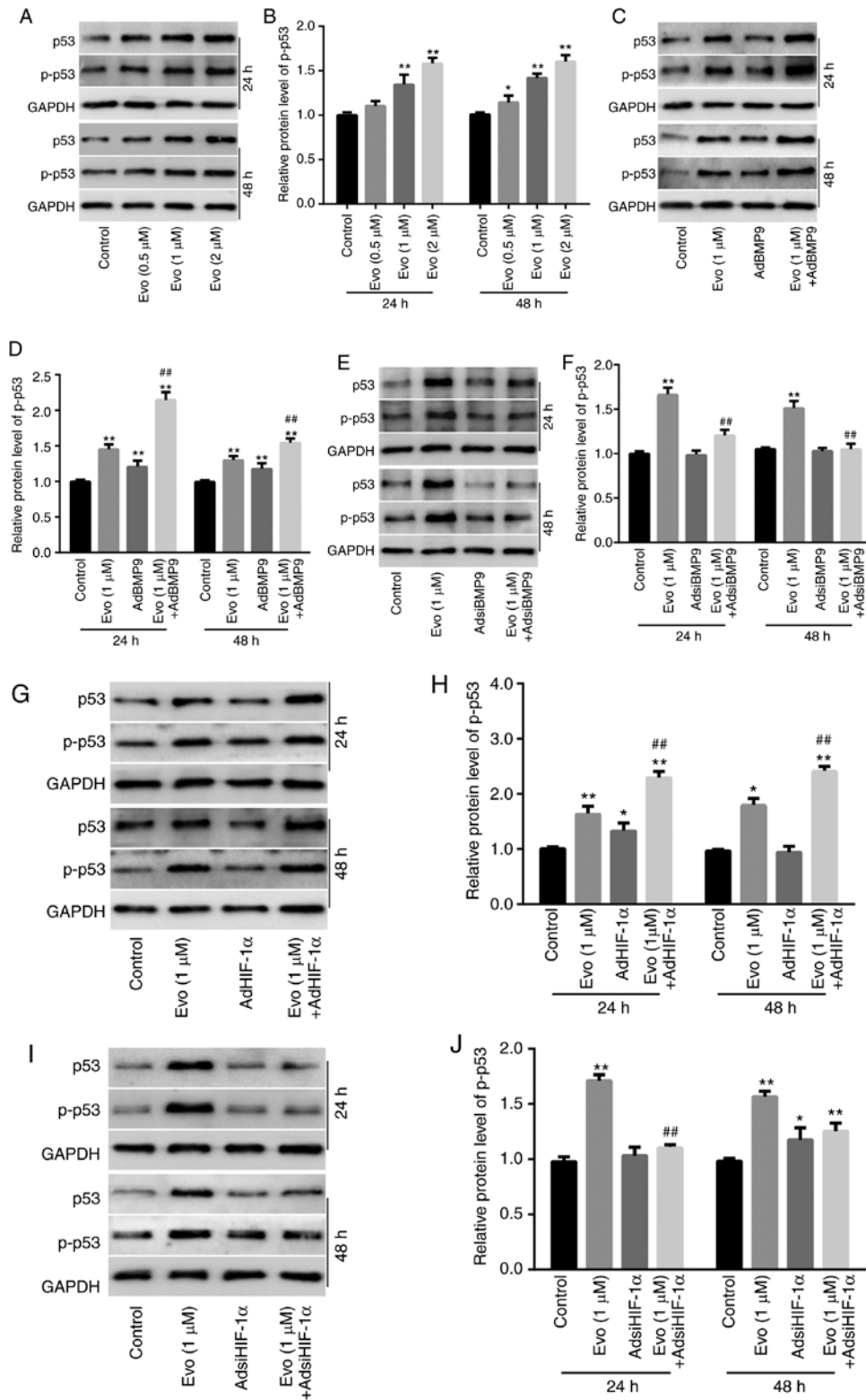
Figure 6. Effects of Evo, BMP9 and/or HIF-1α on the activity of p53 in HCT116 cells. (A) Western blot analysis results revealed the effect of Evo on p53 and p-p53 in HCT116 cells; GAPDH was used as the loading control. (B) Quantification of the western blot analysis results revealed the effect of Evo on p53 and p-p53 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group). (C) Western blot analysis results revealed the effect of Evo and/or BMP9 on p53 and p-p53 in HCT116 cells; GAPDH was used as the loading control. (D) Quantification of the western blot analysis results revealed the effect of Evo and/or BMP9 on the protein expression of p53 and p-p53 in HCT116 cells (**P<0.01 vs. the control group; $^{##}$P<0.01 vs. the Evo treated group). (E) Western blot analysis results revealed the effect of Evo and/or BMP9 silencing on p53 and p-p53 in HCT116 cells; GAPDH was used as the loading control. (F) Quantification of the western blot analysis results revealed the effect of Evo and/or BMP9 silencing on the protein expression of p53 and p-p53 in HCT116 cells (**P<0.01 vs. the control group; $^{##}$P<0.01 vs. the Evo treated group). (G) Western blot analysis results revealed the effect of Evo and/or HIF-1α on p53 and p-p53 in HCT116 cells; GAPDH was used as the loading control. (H) Quantification of the western blot analysis results revealed the effect of Evo and/or HIF-1α on the protein expression of p53 and p-p53 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; $^{##}$P<0.01 vs. the Evo treated group). (I) Western blot analysis results revealed the effect of Evo and/or HIF-1α silencing on p53 and p-p53 in HCT116 cells; GAPDH was used as the loading control. (J) Quantification of the western blot analysis results revealed the effect of Evo and/or HIF-1α silencing on the protein expression of p53 and p-p53 in HCT116 cells (*P<0.05 and **P<0.01 vs. the control group; $^{##}$P<0.01 vs. the Evo treated group). Evo, evodiamine; BMP9, bone morphogenetic protein 9.

TGF-β is a super-family that includes various cytokines, which have been revealed to regulate several essential cell physiological processes, such as cell proliferation and differentiation (28). BMPs belong to the TGF-β super-family, and it was reported that normal BMP signaling is necessary for the development of colon microenvironment and terminal differentiation of intestinal progenitor cells (18,19). The loss or inactivation of BMP signaling may lead to the development of gastric neoplasm, colorectal epithelial overgrowth and polyp formation (29-31). Therefore, the aberrant BMP/Smad signaling may be one of major causes of colon cancer, and a potential target for its treatment. Voorneveld *et al* reported that the administration of statins following diagnosis can significantly reduce the risk of colon cancer-associated mortality, if BMP signaling remains intact (32). Our previous studies have indicated that oridion and honokiol both exhibited effective anticancer activity in human colon cancer by upregulating BMP7 to increase p53 activity (33,34). BMP9 is another member of the BMP family, which has been less studied than any of the other BMPs. It was reported that BMP9 exhibited a potential to commit mesenchymal stem cell to osteoblastic lineage, which is much stronger than that of BMP2 or BMP7 (35). However, our previous results demonstrated that BMP9 may also mediate the anticancer effect of resveratrol in colon cancer in a p38 MAPK-dependent manner (21). We therefore speculated that BMP9 may also be associated with the anticancer activity of Evo in colon cancer. In the present study, it was demonstrated that Evo significantly increased the expression of BMP9. However, the endogenous BMP9 level in HCT116 cells (Fig. 3C) was slightly higher than that of the control group (Fig. 3A). This difference may due to the different treatments, namely cells were treated with the same volume of DMSO as the Evo-treated groups for the data in Fig. 3A, but no treatment was introduced for the data in Fig. 3C. Exogenous BMP9 enhanced the anti-cancer activity of Evo in HCT116 cells, and BMP9 silencing showed the reverse effect. Therefore, BMP9 may also contribute to the anticancer activity of Evo in colon cancer cells.

BMP9, also named growth and differentiation factor 2, is capable of regulating cell proliferation and differentiation. In general, BMP9 exerts its physiological function through the BMP/Smad pathway. However, it can also realize its function through the non-canonical BMP/Smad pathway, such as PI3K/Akt or MAPKs (36,37). A previous study revealed that the aberrant BMP/Smad signaling transduction is one of the pathogenic causes of colon cancer, along with Smad4 mutation or function loss (38). In the present study, it was revealed that Evo exhibited no substantial effect on increasing the activity of BMP/Smad signaling. The effect of BMP9 on the anticancer activity of Evo may therefore not be mediated through the BMP/Smad signaling pathway, at least in HCT116 cells. Our previous study demonstrated that Evo could downregulate hypoxia-inducible factor 1α (HIF-1α) in a human colon cancer cell line (LoVo), and HIF-1α silencing potentiated the anticancer effect of Evo (10). François *et al* reported that BMP2 can induce HIF-1α in chronic wounds (39). We therefore speculated that the effect of BMP9 on the anticancer activity of Evo may also be associated with HIF-1α. HIFs, including HIF-1, HIF-2 and HIF-3, are special transcriptional factors that respond to the decreased oxygen level in cellular context. HIF-1 consists of HIF-1α and HIF-1β subunits, which form heterodimers to regulate downstream targets. Although HIF-1α and HIF-1β are both constitutively expressed, the half-life of HIF-1α is so short, that the transcriptional activity of HIF-1 is mostly determined by HIF-1α (40). HIF-1α is critical for angiogenesis and new vascular formation, which is very important for tumor growth, metastasis and relapse (41). Therefore, HIF-1α may promote tumor progression and has been reported as a promising anticancer therapeutic target (42). Our previous studies also indicated that the downregulation of HIF-1α may mediate the anticancer activity of Evo in LoVo colon cancer cells (10). However, in the present study, it was revealed that Evo upregulated HIF-1α in HCT116 cells, BMP9 potentiated the upregulating effect of Evo on HIF-1α, and BMP9 silencing attenuated the upregulating effect of Evo on HIF-1α. Furthermore, BMP9 enhanced the antitumor growth effect of Evo in colon cancer, which could be partly reduced by silencing of HIF-1α. Therefore, the present data indicated that BMP9 may mediate the anticancer activity of Evo by upregulating HIF-1α in colon cancer cells. These controversial effects of Evo and/or HIF-1α in colon cancer cells may be dependent on the cell type or cellular context, and mechanism that needs to be thoroughly elucidated. In addition, the way in which HIF-1α mediated the anticancer activity of Evo and/or BMP9 in HCT116 cells remains unclear.

Usually, cancer cells are characteristic of mutations or the inactivation of tumor suppressors, such as p53 and PTEN (43,44). As one most famous tumor suppressor, p53 is considered as a potential target for several drugs. Further analysis indicated that Evo increased the activity of p53, which was potentiated by BMP9 and reduced by BMP9 silencing. Furthermore, HIF-1α potentiated the increasing effect of Evo on the activity of p53, and HIF-1α silencing had the reverse effect in HCT116 cells. These findings indicated that p53 may also be activated by BMP9 in cancer cells, which may be the result of the upregulation of HIF-1α by BMP9 in HCT116 cells. MDM2 is a specific p53 ubiquitin ligase and transcriptional inhibitor, which negatively regulates p53 activity (45). Therefore, the effect of BMP9 on p53 may be mediated by HIF-1α through regulation of the expression of MDM2. The results from further analysis also revealed that Evo decreased the expression of MDM2, which could be enhanced by HIF-1α and reduced by HIF-1α silencing in HCT116 cells (data not shown).

Collectively, the present study indicated that Evo exhibits effective anticancer activity in human colon cancer, and BMP9 may partly mediate this activity by upregulating HIF-1α to enhance the activity of p53, at least in HCT116 cells. BMP9 was revealed to suppress cancer cell proliferation in a p53-dependent manner in colon cancer. However, the exact mechanism underlying this process needs to be further studied.

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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

BCH and DZS designed the study. FSL, JH, MZC and JRZ conducted the experiments. PPL, LL, YD and YH analyzed the data. FSL and BCH wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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