Molecular Cancer Therapeutics

Embelin Reduces Colitis-Associated Tumorigenesis through Limiting IL-6/STAT3 Signaling

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Abstract

The interleukin-6 (IL-6)/STAT3 signaling regulates survival and proliferation of intestinal epithelial cells and plays an important role in the pathogenesis of inflammatory bowel disease and colorectal cancer. Embelin is a small molecule inhibitor of X-linked inhibitor of apoptosis protein (XIAP), with antioxidant, anti-inflammatory, and antitumor activities. We previously showed that embelin inhibits the growth of colon cancer cells *in vitro*, and effectively suppresses 1,2-dimethylhydrazine dihydrochloride-induced colon carcinogenesis in mice. Here, we explored the antitumor effects and mechanisms of embelin on colitis-associated cancer (CAC) using the azoxymethane/dextran sulfate sodium (AOM/DSS) model, with a particular focus on whether embelin exerts its effect through the IL-6/STAT3 pathway. We found that embelin significantly reduced incidence and tumor size in CAC-bearing mice. In addition to inhibiting proliferation of tumor epithelial cells, embelin suppressed colonic IL-6 expression and secretion, and subsequently STAT3 activation *in vivo*. Importantly, *in vitro* studies have revealed that in colon cancer cells, embelin diminished both the constitutive and IL-6–induced STAT3 activation by stimulating Src homology domain 2-containing protein tyrosine phosphatase (SHP2) activity. Moreover, embelin protected mice from AOM/DSS-induced colitis before tumor development. Embelin decreased IL-1β, IL-17a, and IL-23a expression as well as the number of $CD4^+$ T cells and macrophages infiltrating the colonic tissues. Thus, our findings demonstrated that embelin suppresses CAC tumorigenesis, and its antitumor effect is partly mediated by limiting IL-6/STAT3 activation and Th17 immune response. Embelin may be a potential agent in the prevention and treatment of CAC. *Mol Cancer Ther; 1206–16. ©2014 AACR.*

Introduction

Colorectal cancer is the third most common malignancy in males and second most common cancer in females worldwide (1). Epidemiologic and experimental studies have shown that patients with inflammatory bowel disease (IBD) are at a greater risk of developing colorectal cancer than the general population, and colitis-associated cancer (CAC) is the major cause of death in patients with IBD (2).

There is growing evidence that tumors are sustained and promoted by inflammatory signals from the surrounding microenvironment (3). Numerous cytokines and growth factors produced by inflammatory cells or immune cells can affect the regulation of genes mediating proliferation and preventing apoptosis, thereby promoting carcinogenesis. NF-κB plays a central role in mediating the link between inflammation and cancer development. NF-κB exerts a procarcinogenic effect principally on immune (myeloid) cells and epithelial cells (4). Interleukin-6 (IL-6) is one of the cytokines produced upon NF-κB activation in myeloid cells. IL-6 plays important roles in immune response, cell proliferation, and apoptosis. In fact, the correlation between IL-6 levels and the clinical activity of IBD and colorectal cancer has been demonstrated (5). Furthermore, animal studies have addressed the tumor-promoting role of IL-6 during CAC development (6). The protumorigenic effect of IL-6 is largely mediated by the transcription factor STAT3, and the IL-6/STAT3 cascade is an important regulator of proliferation in tumor cells (7, 8).

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is a potent, nonpeptidic, cell-permeable small molecule inhibitor of X-linked inhibitor of apoptosis protein (XIAP). The structure of embelin has been previously published (9). Embelin possesses a variety of biologic activities such as antioxidant, anti-inflammatory, and antitumor effects (10, 11). However, the molecular mechanisms involved in

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these effects remain largely unknown. We have previously found that embelin inhibits the growth of colon cancer cells by reducing cell proliferation and inducing apoptosis. Moreover, embelin effectively suppresses 1,2-dimethylhydrazine dihydrochloride–induced colon carcinogenesis in mice. The antitumor effects of embelin could be partly attributed to its inhibition of NF-κB activity (12). Recently, embelin was found to inhibit constitutive STAT3 activation in human cancer cell lines (13). Other studies have shown that embelin could ameliorate pro-inflammatory agent-induced acute colitis in mice (14, 15). These studies suggested that embelin may have potential therapeutic benefit for human IBD. However, none of these earlier studies examined the role of embelin in an inflammatory model of *de novo* tumorigenesis.

In this study, we aimed to determine the long-term effects of embelin on CAC carcinogenesis in a mouse model, with a particular emphasis on whether embelin exerts its antitumor effects through interfering with the IL-6/STAT3 pathway.

Materials and Methods

Induction of colitis-associated colon cancer

The CAC model was induced as described previously (16, 17) using azoxymethane (AOM; Sigma-Aldrich) and dextran sulfate sodium (DSS; Affymetrix). Male C57BL/6 mice (6–8 weeks) were randomly divided into 4 groups: control, embelin alone (embelin), CAC challenge (AOM/DSS), and CAC challenge and embelin treatment (AOM/DSS+embelin). AOM (10 mg/kg) was injected intraperitoneally on day 0. Seven days after the AOM injection, mice were given 2% DSS in the drinking water for 7 days. Mice were then maintained on regular water for 7 days before receiving a second intraperitoneal injection of AOM (5 mg/kg). Seven days after the second AOM injection, mice were subjected to 2 more cycles of 2% DSS treatment (7 days/cycle), each separated by 14 days of regular water. Embelin (50 mg/d/kg body weight; Advance Scientific & Chemical, Inc.) or vehicle (dimethyl sulfoxide) was added to the diet and given to mice 10 days before the CAC challenge, and then continued until harvest. To address the effect of embelin on colitis during the CAC development, mice were sacrificed 5 days after the first cycle of DSS treatment, which correspond to day 19 of CAC regimen. The analysis of tumor development was performed on day 85. Macroscopic tumors were counted and measured with a caliper. One half of the colon tissue was snap frozen in liquid nitrogen. The other half was fixed in 10% phosphate-buffered formalin for subsequent paraffin embedding and histologic analysis. Animal experimentation was approved by the Animal Studies Committee of Peking University First Hospital.

Histopathologic analysis

Histologic changes in the colonic tissues were examined by standard hematoxylin and eosin (H&E) staining. Colitis was evaluated in nondysplastic areas, determining by a blinded pathologist and the scoring was based on the morphologic criteria described previously (18). The criteria are as follows: loss of mucosal architecture (0, absent; 1–3, mild-serve), inflammatory cell infiltration (0, absent; 1–3, mild-extensive), muscle thickening (0, absent; 1–3, mild-extensive), goblet cell depletion (0, absent; 1, present), and crypt abscess formation (0, absent; 1, present). The score of each variable was added to give a total microscopic damage score. Colonic neoplasms were diagnosed as previously described (12).

Cell-proliferation assay *in vivo*

Three hours before sacrifice, mice were injected intraperitoneally with 100 mg/kg 5-bromo-2-deoxyuridine (BrdUrd; Sigma). To determine BrdUrd incorporation, sections were stained using a BrdUrd *In Situ* Detection Kit (BD Pharmingen) according to the manufacturer's recommendations. Cell proliferation was assessed as described previously (19).

Immunohistochemical analysis

The expression of p-STAT3 (Tyr705 phospho-STAT3), IL-6, CD68, and CD4 was detected respectively using monoclonal rabbit anti-p-STAT3 (Cell Signaling Technology), polyclonal rabbit anti-IL-6, polyclonal rabbit anti-CD68, and monoclonal mouse anti-CD4 antibodies (Abcam). After retrieving of antigens, primary antibody or control isotype immunoglobulin G (IgG; Santa Cruz) was applied overnight at 4°C followed by incubation with horseradish peroxidase–labeled secondary antibody (Dako). The slides were developed with diaminobenzidine substrate (BD Pharmingen) and the results were evaluated by a pathologist.

Determination of cytokine levels in mouse serum and colonic mucosa

The protein levels of cytokines were measured by ELISA using commercial kits (R&D Systems and eBioscience) according to the manufacturer's instructions. The serum level of IL-6 was expressed in pg/mL. IL-1β, IL-17a, and IL-23 levels in the colonic mucosa homogenate were expressed as picogram per milligram of protein (pg/mg pro).

Cell culture and siRNA transfection

Human colon cancer cell line HCT116 was purchased from the American Type Culture Collection and passaged in our laboratory for fewer than 6 months after resuscitation. Mycoplasma contamination was tested by PCR during culture, and no addition authentication was done as cells came from national repositories. Cells were cultured in McCoy's 5A medium supplemented with 10% FBS and 1% Pen-Strep (all from Invitrogen) at 37°C with 5% CO_2. Human recombinant IL-6 was purchased from R&D System.

HCT 116 cells were grown to 50% confluence and SHP2 (which is encoded by *PTPN11*) siRNA (5′-GGAAAGAAGCAGAGAAAUUUU-3′) was transfected into cells using Lipofectamin 2000 (Invitrogen). The

effect of gene knockdown was assessed by Western blot analysis 48 hours later.

Western blot analysis and qPCR assays

Western blot analysis and qPCR were performed as we described (12, 20), and the following primary antibodies were used: anti-glyceraldehyde-3-phosphatedehydrogenase (GAPDH, Abcam), anti-p-STAT3, anti-STAT3, anti-Tyr580 phoshho-SHP2 (p-SHP2), anti-SHP2 (Cell Signaling Technology), anti-IL-6Rα and anti-gp130 (Santa Cruz). All secondary antibodies were from Dako. The sequences of primers used in qPCR are as follows: *IL-6* forward: 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and reverse: 5′-TTGGTCCTTAGCCACTCCTTC-3′; *IL-1*β forward: 5′-GCAACTGTTCCTGAACTCAACT-3′ and reverse: 5′-ATCTTTTGGGGTCCGTCAACT-3′; *IL-17a* forward: 5′-TCAGCGTGTCCAAACACTGAG-3′ and reverse: 5′-CGCCAAGGGAGTTAAAGACTT-3′; *IL-23a* forward: 5′-ATGCTGGATTGCAGAGCAGTA-3′ and reverse: 5′-ACGGGGCACATTATTTTTAGTCT-3′; *GAPDH* forward: 5′-AGGTCGGTGTGAACGGATTTG-3′ and reverse: 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

Nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared with a commercial Kit (Pierce Biotechnology) following the manufacturer's instructions. STAT3-specific DNA binding activity was determined using a DIG Gel Shift Kit (Roche). Briefly, a double-stranded DNA probe (5′-CGCGTAGCTTAGGTTTCCGGGAAAGCACG-3′) containing ICAM-pIRE consensus sequence and mutant probe (5′-CGCGTAGCTTAGGTTTCCGGGCCCGCACG-3′) were 3′ end labeled with digoxigenin (DIG)-ddUTP by terminal transferase. Fifteen micrograms of nuclear extract was incubated with DIG-labeled probe and poly[d(I-C)] for 15 minutes at room temperature. The mixtures were electrophoresed on 6% nondenaturing polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. The membrane was blocked and incubated with anti-digoxigenin-AP. After detection with alkaline phosphatase buffer, the shifted bands corresponding to the STAT3–DNA complexes were visualized by exposure of X-ray films.

Statistical analysis

Data are expressed as mean ± SD. Differences were analyzed by Student *t* test or one-way ANOVA. A *P* value of less than 0.05 was considered significant.

Results

Embelin inhibited CAC tumorigenesis in mice

The CAC mouse model was induced by injection with procarcinogen AOM followed by 3 cycles of DSS exposure to elicit colitis. As expected, multiple colonic tumors, either flat or polypoid, were seen in all mice receiving AOM/DSS, and the tumors were confined to the middle and distal colon (Fig. 1A). Notably, embelin treatment significantly reduced multiplicity of frequency and size of lesions (Fig. 1A and B). Most tumors were of smaller size (<2 mm) in embelin-treated mice (Fig. 1C). Correspondingly, average tumor load, as indicated by the sum of the diameters of all tumors in a given mouse was lower in embelin-treated mice (Fig. 1D). Histopathologically, the tumors were adenomas with high-grade dysplasia or adenocarcinomas (Fig. 1E). No mice in the embelin alone or untreated control group developed any colonic tumors.

Embelin inhibited cell proliferation in tumor epithelia of CAC mice

To assess the effect of embelin on cell proliferation, we determined BrdUrd incorporation in colonic crypt cells and tumor epithelia. In agreement with the effect on tumor size, treatment of AOM/DSS exposed mice with embelin led to a 10.8% decrease in the BrdUrd incorporation in dysplastic areas (Fig. 1F, a, b, and e). However, no significant difference in the proliferation of crypt cells in normal mucosa was observed (Fig. 1F, c, d, and e). Thus, embelin does not seem to affect the growth of normal colonic crypt cells.

Embelin protected mice from AOM/DSS-induced colitis during early tumor promotion

Colitis was evaluated after completion of the CAC challenge. There was no significant difference in total microscopic inflammation score between the mice treated with or without embelin at day 85 (data not shown). Thus, we focused our analysis on the role of embelin in inflammation at the beginning of CAC regimen. On DSS administration, embelin untreated mice lost more body weight than embelin-treated mice (Fig. 2A). Moreover, without embelin treatment, mice exhibited a delayed weight recovery after removal of DSS. This was in line with histopathologic analysis of mice at day 19 of the CAC protocol, 5 days after the first cycle of DSS administration. As shown in Fig. 2B, a more severe form of acute colitis was found in embelin untreated mice, with extensive epithelial denudation and prominent inflammatory cells infiltration. Importantly, crypt structures were well preserved and inflammatory reactions were significantly milder in colons from mice treated with embelin. There was a significant decrease in the microscopic inflammatory score in embelin-treated mice at day 19 (Fig. 2C). Embelin treatment also attenuated the colon shorting effect elicited by AOM/DSS exposure at this time point (Fig. 2D). These results suggested that embelin ameliorates AOM/DSS-induced colitis during the early stages of tumor initiation.

Embelin limited IL-6/STAT3 signaling in CAC

IL-6 is a critical tumor promoter during CAC tumorigenesis, and its effects are largely mediated by STAT3. Previous study demonstrated that IL-$6^{-/-}$ mice exhibited reduced tumor formation in CAC model (7). We therefore sought to investigate whether embelin exerts the antitumor effects through interfering with IL-6/STAT3

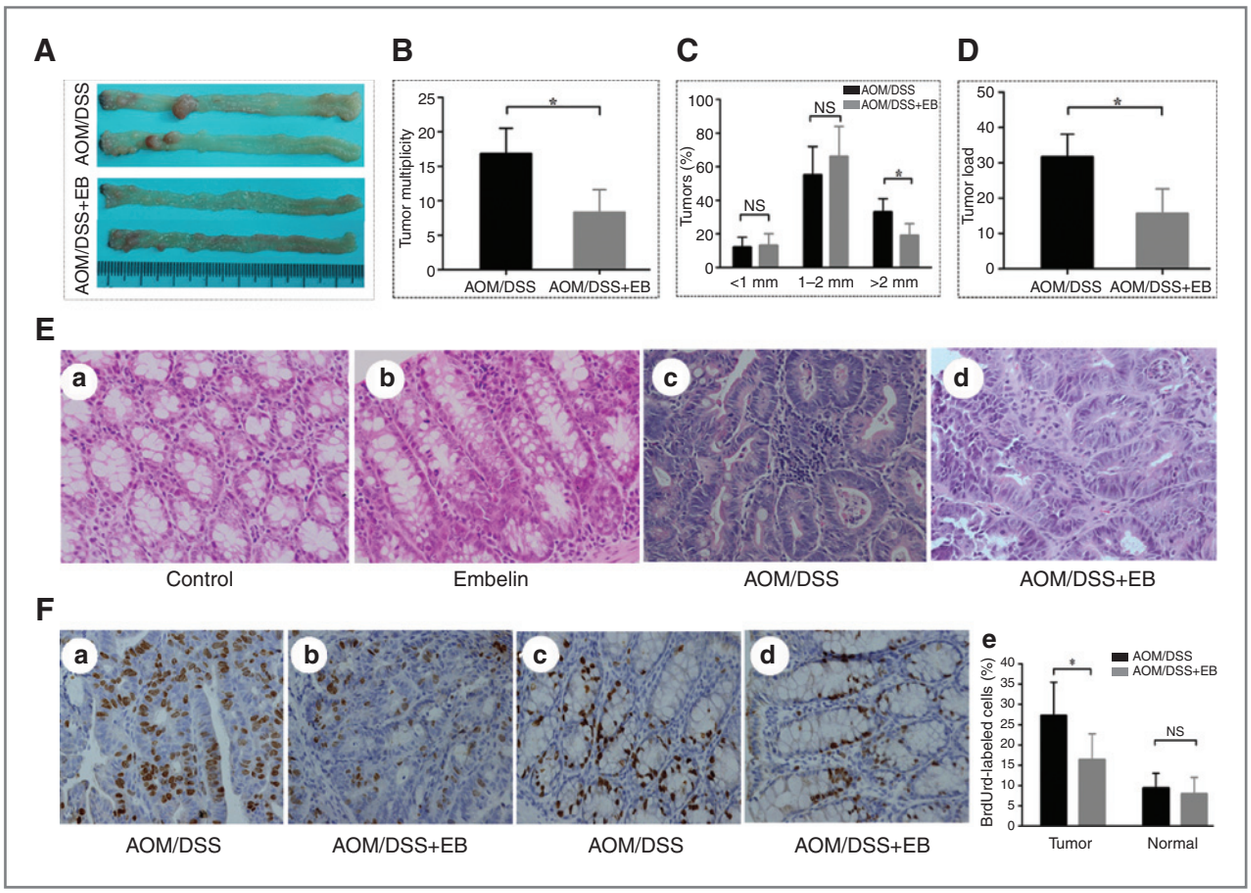

Figure 1. Embelin (EB) suppressed CAC tumorigenesis in mice. Embelin reduced colonic tumor formation (A and B). Tumors were confined to the middle and distal colon (A). Tumor multiplicity (B), size distribution (C), and average tumor load (D) were determined at day 85. Results, mean ± SD ($n = 10$; *, $P < 0.05$). E, H&E-stained sections of colons from control (a), embelin- (b), AOM/DSS- (c), and AOM/DSS+embelin-treated (d) mice. F, the colonic epithelial cell proliferation was determined by BrdUrd labeling and immunostaining. Dysplastic (a, b) and nondysplastic (c, d) areas of CAC mice treated with (b, d) or without (a, c) embelin are shown. Original magnification, ×400. The immunostaining data are better illustrated by quantitative analysis (e). Data, mean ± SD ($n = 10$; *, $P < 0.05$). NS, not significant.

signaling. As shown in Fig. 3A, AOM/DSS exposure increased IL-6 mRNA expression in colonic mucosa (>3.7-fold), and embelin significantly reduced colonic IL-6 expression by 53.8% in CAC model. Immunohistochemical staining showed that IL-6 expression was strongest in the stroma of dysplastic and peripheric nondysplastic areas, whereas much weaker staining was seen in the epithelia (Fig. 3B, c and d). Importantly, in dysplastic and adjacent non-dysplastic colonic mucosa of CAC mice, embelin treatment markedly diminished stromal IL-6 expression (Fig. 3B, e and f). Embelin also significantly decreased the serum level of IL-6 in CAC mice (>2.2-fold; Fig. 3C). Thus, embelin may block IL-6 secretion from myeloid cells.

Immunohistochemical analysis revealed a strong expression of activated form of STAT3 (p-STAT3) in the epithelial and stromal cells in dysplastic areas (Fig. 3D, c and d) and undetectable expression in normal colonic mucosa (Fig. 3D, a and b). In comparison, treatment of CAC-bearing mice with embelin promoted a significant reduction in the expression of p-STAT3 in the epithelial and stromal compartments of the colons (Fig. 3D, e and f). Taken together, these data indicated that embelin inhibits IL-6 expression and secretion, and subsequent STAT3 activation, which may be responsible for its antitumor effects in CAC development.

Embelin regulated cytokine expression, and $CD4^+$ T-cell and macrophage infiltration in CAC

To confirm the role of embelin in inflammatory reaction and tumorigenesis, we determined the levels of cytokines in colonic mucosa of CAC-bearing mice. Significantly decreased expression of mRNAs encoding IL-1β, IL-17a, and IL-23a were observed in embelin-treated mice (Fig. 4A). Similarly, the protein levels of IL-1β, IL-17a, and IL-23 were significantly decreased by embelin administration (Fig. 4B). No difference was observed in the mRNAs expression of IFN-γ, IL-10, and TNF-α between mice treated with and without embelin (data not shown).

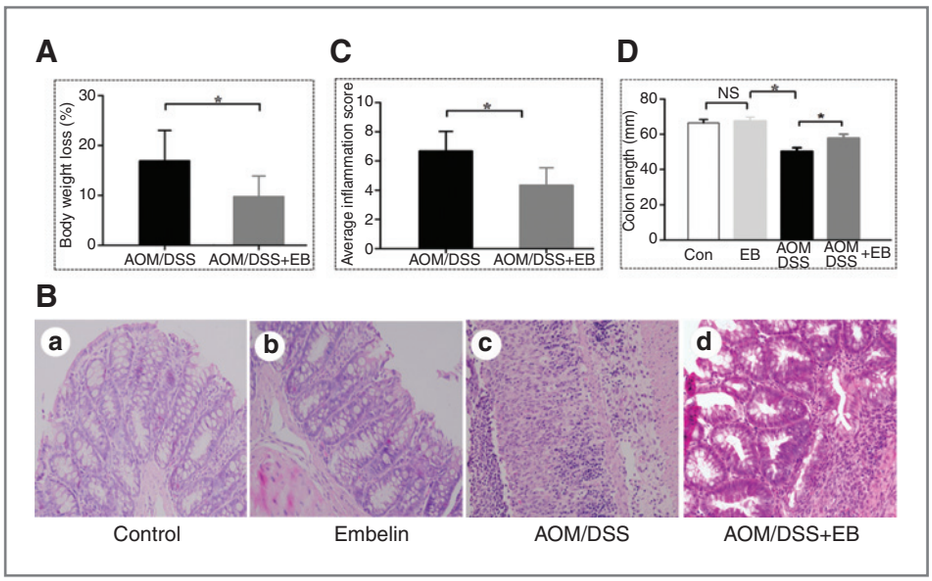

Figure 2. Embelin protected mice from AOM/DSS-induced colitis during early tumor promotion. A, extent of body weight loss in mice treated with or without embelin at day 19 of the CAC regimen. B, H&E-stained sections of colons from control (a), embelin- (b), AOM/DSS- (c), and AOM/DSS+embelin-treated (d) mice. Original magnification, ×200. Microscopic inflammation score (C) and colon length (D) in mice were analyzed. Results, mean ± SD ($n = 8$; *, $P < 0.05$).

To determine the composition of the inflammatory infiltration, we analyzed colon tissues of CAC-bearing mice by immunohistochemistry. There was decreased $CD4^+$ T-cell infiltration in dysplastic and nondysplastic colonic areas of embelin-treated mice (Fig. 5A). Staining with anti-CD8 demonstrated very few positive cells for either group (data not shown). Lamina propria and tumor-infiltrating macrophages and dendritic cells are the major IL-6 producers during the colitis phase and developed CAC (7). CD68 is a specific immunomarker for macrophage (21). There were a large number of $CD68^+$ macrophages infiltrated the colonic tumor stroma, and less was detected in the peripheral nondysplastic areas (Fig. 5B, c and d). A marked reduction of macrophage infiltration was observed following embelin treatment, in both the tumor stroma and peripheral nondysplastic tissues (Fig. 5B, e and f). These indicated that most of the infiltrating T cells are $CD4^+$, and reduced IL-6 level may be the result of decreased macrophage infiltration after embelin treatment.

Embelin suppressed both the constitutive and IL-6–induced STAT3 activation in HCT116 cells

To further elucidate the molecular mechanisms mediating the effects of embelin on CAC development, we tested the effects of embelin on IL-6/STAT3 signaling *in vitro*. Constitutively active STAT3 was observed in HCT116 cells, which could be suppressed by embelin in a time-dependent manner, as shown in Fig. 6A, where 20 μmol/L of embelin could completely inhibit STAT3 activation at 6 hours, and total STAT3 protein expression was unaffected. Treatment of HCT116 cells with IL-6 led to a significant increase in STAT3 activation, with the highest level at 4 hours (Fig. 6B). Moreover, pretreatment with embelin abolished IL-6–induced STAT3 phosphorylation in HCT116 cells (Fig. 6B). Furthermore, electrophoretic mobility shift assay (EMSA) analysis showed that embelin abrogated the DNA binding ability of STAT3 (Fig. 6C).

Because IL-6 *trans*-signaling in epithelial cells plays a crucial role in the development of CAC (6, 22), we analyzed cell lysates of HCT116 cells treated with or without embelin for the expression of effectors of this pathway. As shown in Fig. 6A, embelin did not affect the expression of IL-6Rα and gp130. Moreover, embelin did not change the mRNA levels of IL-6Rα, gp130, and TNF-α–converting enzyme (TACE) in the colonic mucosa of CAC-bearing mice (data not shown).

Inhibition of STAT3 activation by embelin was mediated by SHP2

The Src homology domain 2-containing protein tyrosine phosphatase (SHP2) is a negative regulator of STAT3 activation through the dephosphorylation of phosphotyrosine (23). We examined whether embelin can modulate the function of SHP2 in HCT116 cells. Embelin treatment resulted in an increase in the activated form of SHP2, Tyr^{580} phospho-SHP2 (p-SHP2), with the highest level after 2 hours, without changing total SHP2 expression (Fig. 6D). To confirm that the effect of embelin on the suppression of STAT3 activation was SHP2-dependent, the silencing of SHP2 was performed using siRNA. Western blotting showed that the knockdown of SHP2 abrogated embelin-induced p-STAT3 dephosphorylation (Fig. 6E), suggesting that embelin inhibits constitutive STAT3 activation by stimulating SHP2 activity.

Discussion

In this study, we showed that embelin intensely inhibited tumorigenesis in the AOM/DSS model of inflammation-induced colon cancer. Our novel finding is that the antitumor effect of embelin is mediated, in part, by limiting IL-6/STAT3 signaling. Embelin significantly reduced colonic IL-6 expression and secretion,

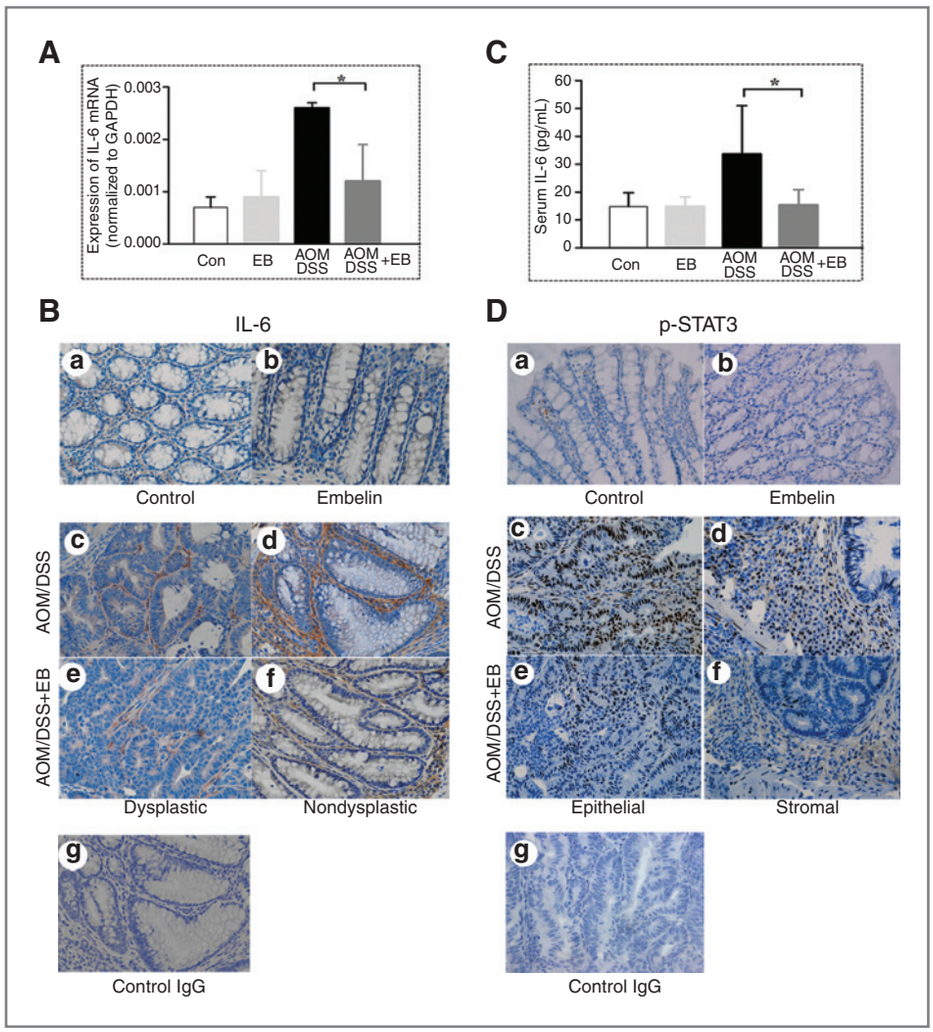

Figure 3. Embelin decreased IL-6 level and inhibited STAT3 activation in CAC. A, relative expression level of IL-6 mRNA in colonic mucosa was analyzed by qPCR. Data are mean ± SD ($n = 8$; *, $P < 0.05$). B, immunohistochemical analysis of IL-6 expression in colonic tissues from control (a), embelin-treated (b), and CAC-bearing (c–f) mice. Dysplastic (c, e) and peripheral nondysplastic (d, f) areas of CAC mice treated with (e, f) or without (c, d) embelin are shown. g, control isotype IgG. C, serum level of IL-6 was measured by ELISA. Data are mean ± SD ($n = 8$; *, $P < 0.05$). D, immunohistochemical analysis of p-STAT3 expression in the colonic tissues from control (a), embelin-treated (b), and CAC-bearing (c–f) mice. Epithelia (c, e) and stromal (d, f) compartments of CAC-bearing mice treated with (e, f) or without (c, d) embelin are shown. g, control isotype IgG. Original magnification, ×400.

and subsequent STAT3 activation. In addition, embelin suppressed both the constitutive and IL-6–induced STAT3 activation *in vitro*. The inhibitory effect of embelin on STAT3 activation was mediated by SHP2. Embelin protected mice from AOM/DSS-induced colitis before tumor initiation. The potent anti-inflammatory effect of embelin was further demonstrated by the findings that embelin significantly decreased the expression of IL-1β, IL-17a, and IL-23a, and the infiltration of $CD4^+$ T cells and macrophages into colonic tissues during CAC development.

IL-6 is a cytokine with multiple functions (24, 25). It exerts its biologic actions by binding to 2 membrane receptors, IL-6Rα and gp130. IL-6Rα is expressed by specific cells, such as neutrophils, macrophages, and certain lymphocytes, whereas gp130 is widely expressed by various cell types (24). Classic signaling of IL-6 involves the binding of IL-6 to IL-6Rα on the target cells and association with gp130, thereby inducing downstream signal transduction. Alternatively, IL-6 can activate cells lacking the membrane bound IL-6R through IL-6 *trans*-signaling. In this process, the matrix metalloproteinase TACE (also known as ADAM17, a disintegrin and metalloproteinase 17) releases soluble IL-6Rα (sIL-6Rα) by cleaving membranous IL-6Rα. sIL-6Rα can also bind IL-6, to form the IL-6/sIL-6Rα complex that interact with membrane gp130 to induce signal transduction. The importance of IL-6 *trans*-signaling in chronic colitis and CAC development has been well demonstrated (6, 22). TACE plays a major role in the inflammatory processes by promoting the shedding of the extracellular domain of several transmembrane proteins such as receptors and adhesion molecules (26, 27). In the CAC model, tumor epithelial cells express high level of TACE, which control IL-6R shedding and thus IL-6 *trans*-signaling (6, 22). The specific inhibition of this signaling by the antibody against IL-6Rα or the blockade of sIL-6Rα using gp130-Fc prevented CAC tumorigenesis (6). In this study, we analyzed the effect of embelin on the effectors of IL-6 *trans*-signaling. The expression of IL-6Rα, gp130, and TACE was not altered by embelin both *in vitro* and *in vivo*, implicating that the antitumor effect of embelin may not be mediated

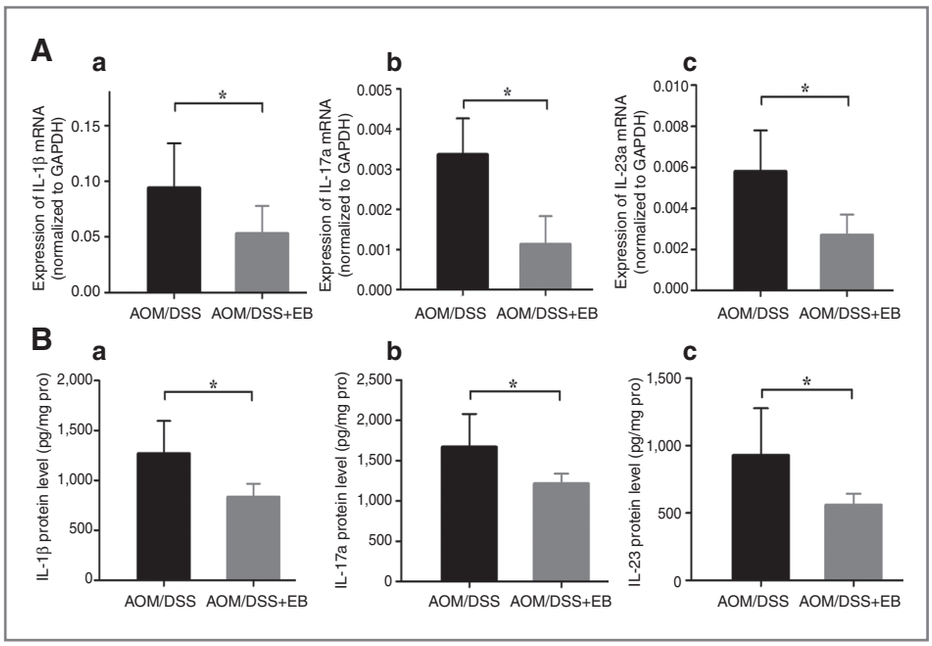

Figure 4. Effect of embelin on cytokine production in CAC. A, relative expression levels of IL-1β (a), IL-17a (b), and IL-23a (c) mRNA in colonic mucosa were analyzed by qPCR. B, protein levels of L-1β (a), IL-17a (b), and IL-23 (c) were measured by ELISA. Data are mean ± SD ($n = 8$; *, $P < 0.05$).

by the suppression of IL-6 *trans*-signaling. However, the activity of TACE can be regulated at the posttranscriptional level (27), thus the regulation of embelin on TACE activation may still need to be further investigated.

Accumulating studies have suggested a potential role of IL-6 in colon cancer. IL-6 effectively promotes the growth and invasion of colon cancer cells *in vitro* (28). In patients suffering from colon cancer, serum levels of IL-6 are increased and correlated with the tumor load (29). Furthermore, it has been recently documented that IL-6 and sIL-6Rα regulate colitis and CAC development *in vivo* (7, 22, 24). In our study, we have shown that embelin powerfully inhibited tumorigenesis in the CAC model, and the antitumor effect was associated with diminished IL-6 expression and secretion. We also found that embelin treatment reduced macrophage infiltration in colonic tumor stroma, where IL-6 expression was correspondingly decreased. Our data are in line with the previous studies (7) that macrophage may be a major source of IL-6 during CAC development.

IL-6 is not only a driving factor for tumor initiation, but also an important player in tumor progression (30). It was reported that the interference of tumor initiation could result in changes in tumor number, whereas differences in tumor size and tumor load provided evidence for factors involved in tumor progression (31). Previous studies have confirmed that continuous treatment with recombinant IL-6 during early or late stages of CAC resulted in an increase in tumor size. Moreover, when IL-6 was administered during early CAC induction, they enhanced tumor multiplicity (7). Here, our data showed that embelin significantly reduced the size and multiplicity of colon tumors, indicating embelin has an impact on both tumor formation and growth in CAC development. Furthermore, mice treated with embelin plus recombinant IL-6 during early CAC induction tend to develop larger tumors than CAC-bearing mice receiving embelin alone, whereas a marginal increase in tumor multiplicity was seen after excess IL-6 exposure (data not shown). Thus, implying the antitumor action of embelin is partly mediated by limiting IL-6 signaling, and the effect may be more pronounced on tumor initiation stage.

STAT3 is a critical protumorigenic effector for IL-6 signaling. Specific STAT3 ablation in intestinal epithelial cells interferes with tumor formation and growth in CAC (7, 8). The possible role of STAT3 in the development of inflammation related colon cancer has been suggested by the findings that the activation of STAT3 signaling is persistently present in patients with IBD and colorectal cancer (32, 33). Thus, the intervention of IL-6/STAT3 signaling holds a preventive and therapeutic potential for CAC.

IL-6 is not the sole STAT3 activator, our data demonstrated that embelin can directly suppress STAT3 activity and IL-6–induced STAT3 activation in colon cancer cells. However, p-STAT3 expression was also downregulated in the colons from embelin-treated mice, confirming that embelin inhibited STAT3 activation during CAC. STAT3 induces the expression of genes involved in proliferation (cyclinD1, c-Myc, and PCNA) and anti-apoptosis (Bcl-XL, Bcl-2, and survivin; ref. 6). Here, we found that embelin inhibited cell proliferation in the tumor epithelia of CAC mice, which was consistent with our previous studies showing that embelin can downregulate survivin, cyclin D1, and c-Myc expression both *in vitro* and *in vivo* (12). Therefore, embelin seems to exert antitumor effects by suppression of cell proliferation, and this is in part through STAT3 inhibition.

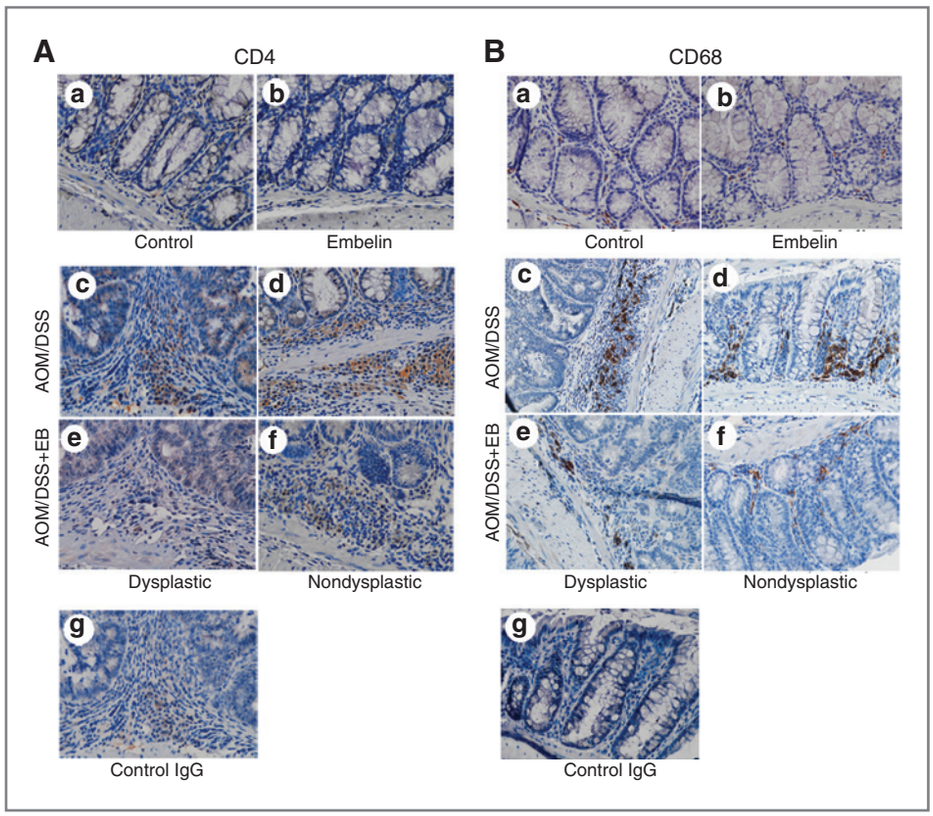

Figure 5. Embelin reduced $CD4^+$ T-cell and macrophage infiltration in colon tissues of CAC. Immunohistochemical analysis of $CD4^+$ T-cell (A) and $CD68^+$ macrophage (B) infiltration in colonic tissues from control (a), embelin-treated (b), and CAC-bearing (c–f) mice. Dysplastic (c, e) and peripheral nondysplastic (d, f) areas of CAC mice treated with (e, f) or without (c, d) embelin are shown. g, control isotype IgG. Original magnification, ×400.

We also found that the embelin-induced inhibition of STAT3 activation involves the protein tyrosine phosphatases (PTP). PTPs have been considered potential tumor suppressors because of their antagonistic effects on oncogenic protein tyrosine kinases signaling (34). Inactivating mutations of PTPs are frequent events in colorectal cancer (35). SHP2 is an intracellular PTP that negatively regulates IL-6 signaling (36). Moreover, Tyr^{705} phospho-STAT3 is a substrate of SHP2, and SHP2 seems to directly inhibit the activation of STAT3 (23). SHP2 acts as a tumor suppressor in hepatocellular carcinogenesis. The specific deletion of SHP2 promotes inflammatory signaling through the STAT3 pathway, resulting in tumor development (37). Our current observations indicated that siRNA targeted knockdown of SHP2 resulted in the abrogation of embelin-mediated effects on p-STAT3 dephosphorylation. The activation of SHP2 by embelin was verified by the phosphorylation of Tyr^{580}. Thus, embelin-induced inhibition of STAT3 activation in colon cancer cells is to some degree mediated by SHP2. The regulation of SHP2 by embelin occurs at the posttranscriptional level. A recent study reported that embelin induced the expression of PTEN, another member of the PTPs family, in human multiple myeloma cells, and this correlated with the downregulation of constitutive STAT3 phosphorylation (13). Therefore, the modulating effects on PTPs activity are a possible mechanism for embelin-induced inhibition of STAT3 activation.

The tumor microenvironment influences the physiology of cancer cells. Immune cells in the tumor produce cytokines and other factors that promote tumor growth and survival (3, 38, 39). In this study, we identified that embelin reduced the expression of the pro-inflammatory cytokines (IL-1β, IL-17a, and IL-23a) and the infiltration of $CD4^+$ T cells and macrophages in colonic tissues, suggesting that the effects of this agent on CAC may be because of its impact on immune cells. IL-17–producing effector T helper (Th17) cells are crucial for inflammation and may have a potential role in carcinogenesis (40, 41). The roles of IL-6 and IL-23 in the maturation of Th17 cells have been identified (42). STAT3 is critical for IL-6–driven differentiation and IL-23–mediated expansion of Th17 cells (43). Conversely, Th17 cells may produce IL-6, which in turn activates STAT3. Thus, the Th17 response can promote tumor growth in part via the IL-6/STAT3 pathway (41). Recently, we found that embelin inhibited inflammation by decreasing Th17 and IL-6–producing Th cells both in the tumor-infiltrating lymphocytes and splenocytes in a mouse pancreatic cancer model (unpublished observations). Indeed, in work presented here, other effector cytokines that are subsequently produced by Th1 and Th2 cells (i.e., IFN-γ, IL-10, and TNF-α) were not altered by embelin treatment. This suggests that the antitumor action of embelin is to some extent mediated by suppressing the Th17 response, possibly as a result of the downregulation of IL-6/STAT3 signaling.

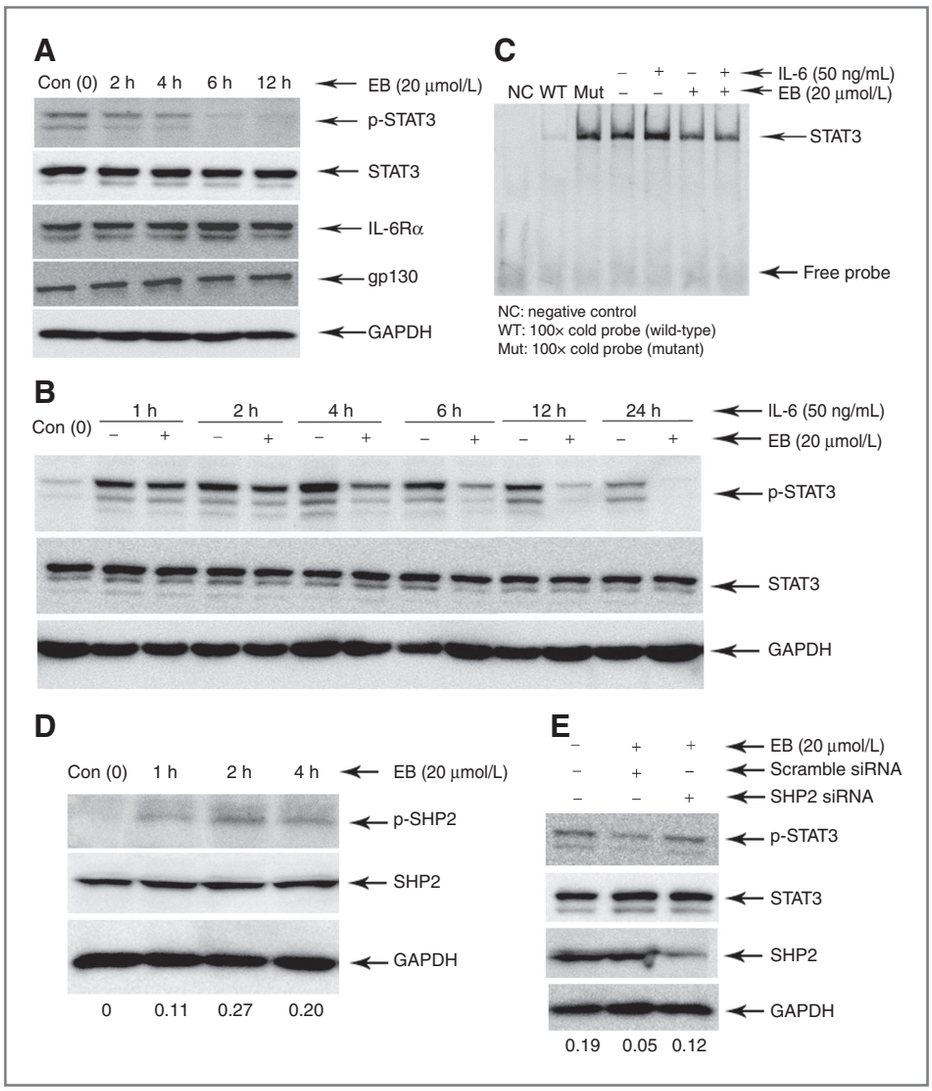

Figure 6. Embelin suppressed STAT3 activation in HCT116 cells, and the effect was mediated by SHP2. A, treatment of HCT116 cells with embelin for indicated durations followed by immunoblot analysis. HCT116 cells were incubated with embelin for 1 hour before treatment with IL-6. p-STAT expression and STAT3-DNA binding ability was assessed by Western blot analysis (B) and EMSA (C). D, HCT116 cells were treated with embelin and then the expression of p-SHP2 and total SHP2 were detected. E, HCT116 cells were transfected with scrambled or SHP2 siRNA followed by treatment with embelin for 4 hours. Expression of p-STAT3, STAT3, and SHP2 were determined. The relative differences in p-SHP2 (D) and p-STAT3 (E) were quantified by densitometric analysis. All results shown are representative of 3 independent experiments.

To understand whether the embelin-mediated inhibition of CAC carcinogenesis relies on the negative effect of embelin on the ongoing colitis, a time-course study was performed. Indeed, the protective effect of embelin was observed on AOM/DSS-induced colitis before tumor development. Embelin treatment resolved inflammation and promoted mucosal healing at the beginning of CAC regimen. It should be noted that IL-6 and STAT3 are both required for survival of intestinal epithelial cells and maintenance of mucosal integrity (7, 8). Excessive interference with systemic STAT3 activation could potentially cause gastrointestinal damage (44). This context revealed that embelin inhibited the proliferation of neoplastic but not normal colonic crypt cells, and did not affect normal mucosal regeneration processes *in vivo*. Thus, it is tempting to speculate that embelin interferes exclusively with excessive IL-6/STAT3 activation that sustains colon cancer cells growth.

In conclusion, embelin effectively suppressed CAC tumorigenesis in mice and the antitumor effects may in part be because of its inhibition on IL-6/STAT3 activation and Th17 immune response. Acting as an IL-6 blocker and STAT3 inhibitor, embelin may be a potential agent in the prevention and treatment of CAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Dai, W. Wang, L. Qiao
Development of methodology: H. Jiao, W. Wang, L. Qiao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Dai, H. Jiao, W. Wang, Y. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Dai, L. Hebbard
Writing, review, and/or revision of the manuscript: Y. Dai, R. Zhang, L. Hebbard, J. George, L. Qiao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Teng, W. Wang
Study supervision: Y. Dai, W. Wang, L. Qiao

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