Norcantharidin inhibits IL-6-induced epithelial-mesenchymal transition via the JAK2/STAT3/TWIST signaling pathway in hepatocellular carcinoma cells

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Abstract. Epithelial-mesenchymal transition (EMT), plays a vital role in hepatocellular carcinoma (HCC) development and metastasis. Norcantharidin (NCTD; 7-oxabicyclo (2.2.1) heptane-2,3-dicarboxylic anhydride) plays anticancer roles in the regulation of tumor cell proliferation, apoptosis and migration. However, the molecular mechanism of HCC EMT and the effects of NCTD in the HCC EMT process have been either poorly elucidated or not studied. In this study, HCC EMT was induced by the treatment of IL-6 and various concentrations of NCTD (0, 30, 60 and 120 μM) were treated with HCC cell lines, HCCLM3 and SMMC-7721. We investigated the effect of NCTD on the invasion of HCC cells by using Transwell assay. Immunofluorescence staining, western blot analysis and quantitative RT-PCR were performed to evaluate the protein and mRNA expression levels of HCC cells. Here, using cell line models, our data demonstrated that interleukin 6 (IL-6) induced EMT through the JAK/STAT3/TWIST pathway in HCC. Moreover, our studies revealed that NCTD markedly inhibited IL-6-induced EMT and cell invasiveness. Signaling studies revealed that NCTD sufficiently suppressed JAK/STAT3/TWIST signaling to reverse the IL-6-promoting effects. Collectively, these data provide evidence for the use of NCTD as a potential anticancer drug in HCC metastatic patients.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed cancer and second most frequent cause of cancer-related deaths for males worldwide. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer-related deaths (1). HCC is associated with a poor prognosis due to late diagnosis and a lack of effective treatment options. Although great advances in surgical techniques and medical care have been achieved over the last several decades, the 5-year survival rate worldwide of HCC is still less than 5%, mainly due to the high rate of recurrence and metastasis (2). In fact, increasing evidence suggests that HCC metastasis is a multistep process. During progression to metastasis, cancer cells are thought to acquire a mesenchymal phenotype, which allows them to leave the site of the primary tumor, invade surrounding tissues, and migrate to distant organs. After seeding, these cells switch back to an epithelial phenotype and proliferate to form metastases (3,4). The process by which cells switch from epithelial-mesenchymal (EMT) phenotypes is known as EMT transition (5). EMT is a critical prognostic factor in HCC and is involved in early recurrence or metastases after surgery (6). Blocking EMT is currently considered as a promising strategy to inhibit cancer metastasis and improve patient survival.

Growing evidence suggests that inflammation promotes EMT (7,8). Interleukin 6 (IL-6) a multifunctional cytokine in the tumor microenvironment, has been regarded as the main factor involved in EMT, contributing to tumor invasion and metastasis (9-11). Previous studies revealed that IL-6 leads to the development of HCC (12,13), as an independent predictor of HCC tumor recurrence, poor survival, and tumor metastasis (14). Furthermore, IL-6 appears to contribute as a potent factor to the initiation of EMT via activation of Janus kinase (JAK) family members (JAK1, JAK2, and TYK2), leading to the activation of transcription factors of the signal transducer and activator of transcription 3 (STAT3) signaling (15-17). Emerging evidence suggests that STAT3 regulated the expression of Twist by directly binding to the transcriptional starting site in the Twist promoter (18). Moreover, blocking STAT3

markedly suppressed the expression of Twist, confirming the regulation of Twist by STAT3 (17). However, little is known about the mechanisms of IL-6-induced EMT in HCC.

Increasing evidence has indicated that traditional Chinese medicines contain anticancer ingredients. Norcantharidin (NCTD; 7-oxabicyclo (2.2.1) heptane-2,3-dicarboxylic anhydride) is a demethylated and low-cytotoxic analog of cantharidin, an active ingredient of the Chinese blister beetle *Mylabris*, which has been used in China to treat tumors, inflammation and many other conditions for a long time (19,20). Previous studies have reported that NCTD could modulate the expression of Bcl-2 and Mcl-1 to effectively inhibit proliferation and induce apoptosis in a variety of human tumor cells, as well as producing fewer side effects and leukocytosis (21-27). Several studies have suggested that NCTD inhibits cell migration and invasion through the c-Jun N-terminal kinase (JNK) and mitogen-activated protein (MAP) kinase signaling pathways in human lung cancer cells and CRC cells (28-30). However, the effects of NCTD on HCC metastasis have not been elucidated thus far.

In the present study, we investigated the anti-metastatic effects of NCTD using IL-6-treated HCC cells. We found that NCTD inhibited IL-6-induced EMT and invasiveness through the suppression of the JAK2 and STAT3 pathways to regulate TWIST expression in HCC cell lines.

Materials and methods

Compound preparations and reagents. NCTD (molecular formular: $C_8H_8O_4$, molecular weight: 168.1467, HPLC ≥98%) was purchased from the National Standard Network (Beijing, China). NCTD was dissolved in dimethyl sulphoxide (DMSO) to a concentration of 40 mg/ml as stock solution, stored at room temperature and protected from the light. Serial concentrations of NCTD were diluted in culture medium before use (DMSO <1%, 0-120 μM). Cucurbitacin I (JSI-124, #C4493-1MG; Sigma-Aldrich, St. Louis, MO, USA) a novel inhibitor of the JAK2/STAT3 signaling pathway (molecular weight: 514.65, HPLC ≥95%) (31), was dissolved in DMSO to a concentration of 5 mg/ml as stock solution, stored at -20˚C and protected from the light. Then, cucurbitacin I was diluted to a concentration of 0.5 μM in culture medium before use (DMSO <1%). Human recombinant IL-6 (#I1395-50UG; Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) to a concentration of 100 μg/ml as stock solution and stored at -20˚C. The final concentration of IL-6 was diluted to 100 ng/ml in culture medium before use.

Cell culture. HCC cell lines, HCCLM3 and SMMC-7721, were purchased from the China Infrastructure of Cell Line Resources (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah, USA) and 1% penicillin and streptomycin (Hyclone) in a 5% CO_2 humidified incubator at 37˚C.

Cell invasion assays. Transwell Matrigel invasion chambers (#3422; Corning Costar Corporation, USA) with 8-μm membrane pores coated with 100 μl of 1:6 diluted Matrigel in serum-free DMEM (#356234; BD Biosciences, CA, USA) were used for the cell invasion assay. Cells were pretreated with 100 ng/ml of IL-6 for 48 h. Cells ($1x10^4$) were plated in 100 μl of serum-free DMEM containing NCTD (0, 30, 60 and 120 μM) and JSI-124 (0.5 μM) as the positive control group into the upper chamber. The lower chamber was filled with 600 μl of DMEM and 10% FBS medium. After incubation at 37˚C for 24 h in a 5% CO_2 atmosphere, the non-invaded cells in the inserts were removed with cotton swabs. The invaded cells on the underside were treated with a fixative/staining solution (0.1% crystal violet, 4% paraformaldehyde) for visualization. The number of invading cells on the filters was counted in 5 random fields per filter at an x100 magnification in triplicate wells for each group.

Immunofluorescence staining. Cells were treated with 100 ng/ml of IL-6 and grown in 96-well plates. The cells were fixed for 15 min with 4% paraformaldehyde, and permeabilized for 10 min in PBS containing 0.1% Triton X-100 at room temperature. The cells were rinsed in PBS (pH 7.4) three times for 5 min. After blocking in Immunol staining blocking buffer (Beyotime Biotechnology, Shanghai, China) for 1 h at room temperature, the cells were probed with a primary antibody at 4˚C overnight. After rinsing in PBS, the cells were incubated with secondary antibodies [anti-rabbit IgG-Alexa Fluor 555-conjugated (red) or anti-rabbit IgG-Alexa Fluor 488-conjugated (green)] for 1 h at room temperature and the nuclei were stained with DAPI (Beyotime Biotechnology) for 10 min. All of the images were semi-quantitatively analyzed, and the 96-well plates were mounted and viewed using ImageXpress Micro (high content analysis; Molecular Devices, CA, USA). For analysis of E-cadherin, N-cadherin and vimentin expression, fluorescence intensity was quantified by assessing the intensity in the cells using Image-Pro Plus software, version 6.0 (Media Cybernetics, Bethesda, MD, USA).

Western blot analysis. HCCLM3 cells were pretreated with 100 ng/ml of IL-6 for 48 h and then treated with NCTD (0, 30, 60, and 120 μM) for 24 h. The positive control group was subjected to JSI-124 treatment (JAK2/STAT3 inhibitor, 0.5 μM) for 24 h. The cells were lysed in a RIPA buffer with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (both from Beyotime Biotechnology), and then clarified by centrifugation. Total cell lysates were resuspended in SDS sample buffer and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA), and then blocked with 5% non-fat milk for 1 h at room temperature. The membranes were then incubated with primary antibodies [E-cadherin (#3195), N-cadherin (#13116), vimentin (#5741), JAK2 (#3230), pJAK2 (Tyr1007/1008, #3776), STAT3 (#12640) and pSTAT3 (Tyr705, #9145), (all from Cell Signaling Technology, Inc., Danvers, MA, USA), and Twist (H-81, #sc-15393; Santa Cruz Biotechnology, Dallas, Texas, USA)], overnight at 4˚C before incubation with the corresponding HRP-conjugated secondary antibodies (1:10,000) diluted in TBST. Then, the membranes were washed extensively with TBST and visualized using an enhanced chemiluminescence detection system, followed by quantification using the Image Quant LAS 4000 (GE Healthcare, Bucks, UK).

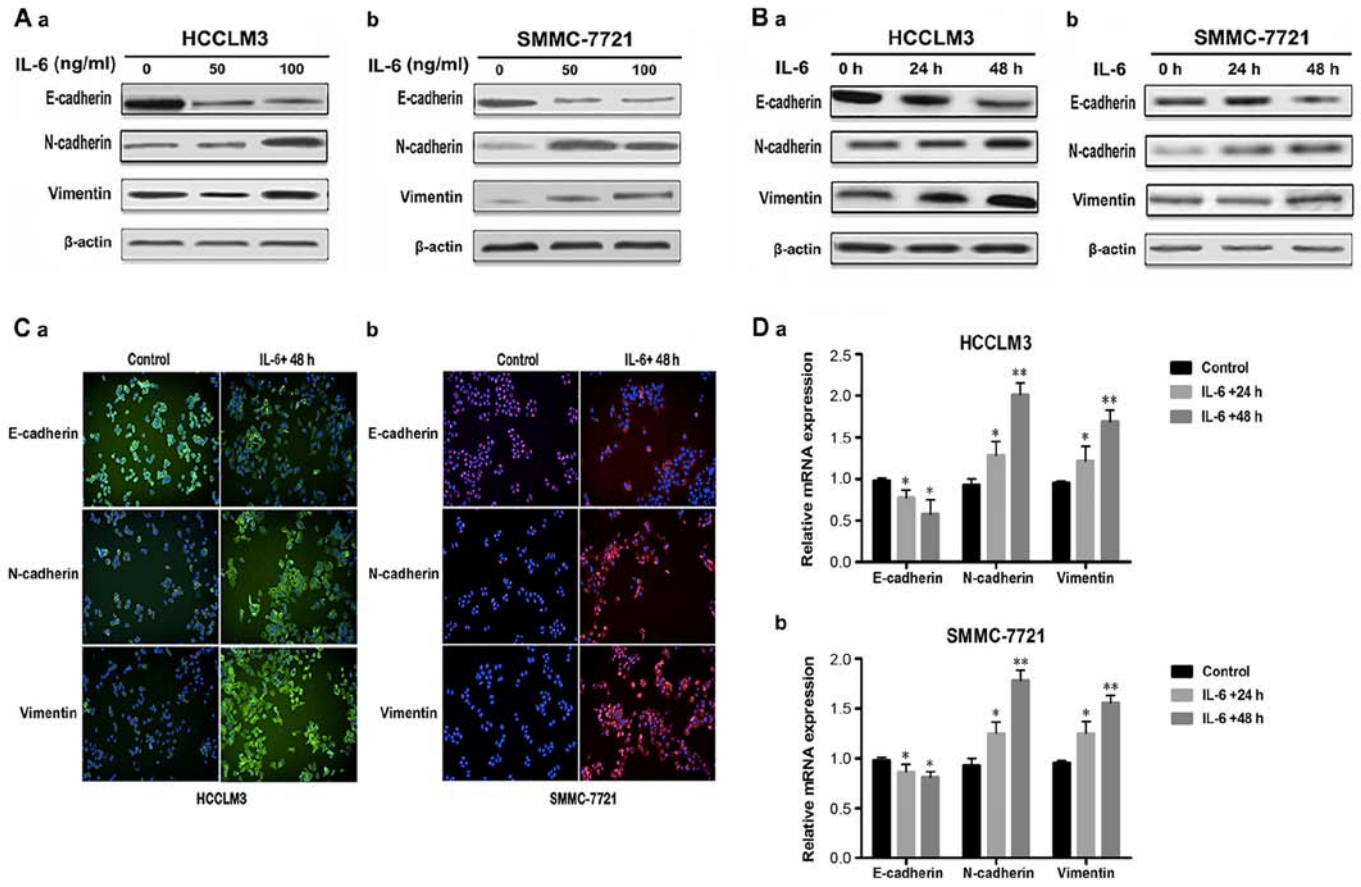

Figure 1. IL-6 treatment induces EMT in HCC cell lines. (A-a and b) Cells were treated with IL-6 (0, 50 and 100 ng/ml) for 48 h.The protein levels of EMT markers were assessed by western blot analysis in HCCLM3 and SMMC-7721 cell lines. (B-a and b) Cells were treated with IL-6 (100 ng/ml) for 0, 24 and 48 h. The protein levels of EMT markers were assessed by western blot analysis in HCCLM3 and SMMC-7721 cell lines. (C-a) HCCLM3 cells were treated without or with IL-6 (100 ng/ml) for 48 h. Cells were stained with primary antibodies against E-cadherin, N-cadherin and vimentin, followed by Alexa Fluor 488-conjugated (green) secondary antibodies. Nuclei were stained with DAPI (blue) (original magnification, x400). (C-b) SMMC-7721 cells were treated without or with IL-6 (100 ng/ml) for 48 h. Cells were stained with primary antibodies against E-cadherin, N-cadherin and vimentin, followed by Alexa Fluor 555-conjugated (red) secondary antibodies. Nuclei were stained with DAPI (blue) (original magnification, x400). (D-a and b) Cells were exposed to 100 ng/ml of IL-6 for 24 and 48 h. The mRNA expression levels of E-cadherin, N-cadherin and vimentin were determined by quantitative RT-PCR (mean values ± SD, n=3, are provided. *P<0.05, **P<0.01). IL-6, interleukin 6; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma.

Table I. The sequences of primers.

Target gene	Primers sequences
E-cadherin	F: 5'-CCCATCAGCTGCCCAGAAAATGAA-3'
	R: 5'-CTGTCACCTTCAGCCATCCTGTTT-3'
N-cadherin	F: 5'-AAGAACGCCAGGCCAAACAAC-3'
	R: 5'-CTGGCTCAAGTCATAGTCCTGGTCT-3'
Vimentin	F: 5'-GACAATGCGTCTCTGGCACGTCTT-3'
	R: 5'-TCCTCCGCCTCCTGCAGGTTCTT-3'
JAK2	F:5'-TGGACAGAGAGAGAATTTTCTGAACT-3'
	R: 5'-TTCATTGCTTTCCTTTTTCACA-3'
STAT3	F: 5'-TTGCCAGTTGTGGTGATC-3'
	R: 5'-AGAACCCAGAAGGAGAAGC-3'
TWIST	F: 5'-GTCCGCAGTCTTACGAGGAG-3'
	R: 5'-GCTTGAGGGTCTGAATCTTGCT-3'
GAPDH	F: 5'-TGCACCACCAACTGCTTAGC-3'
	R: 5'-GGCATGGACTGTGGTCATGAG-3'

F, forward; R, reverse; JAK, Janus tyrosine kinase; STAT3, signal transducer and activator of transcription 3.

Quantitative RT-PCR. Total RNA was extracted from individual groups of cells using TRIzol reagent and reversely transcribed to cDNA using the First Strand cDNA Synthesis kit (Invitrogen). cDNA was synthesized using 1 μg of total RNA in a 20-μl final volume by reverse transcription utilizing SuperScript II Reverse Transcriptase with oligo-dT(18)-primers (both from Invitrogen). The relative levels of target gene mRNA transcripts were determined by quantitative RT-PCR using the SYBR-Green Master Mix kit. RNA was amplified using ABI Prism 7000 Sequence Detection System (Applied Biosystems). The sequences of the primers are shown in Table I. The PCR amplification was performed in triplicate at 95˚C for 10 min and was subjected to 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. The relative levels of each gene to GAPDH mRNA transcripts were calculated.

Statistical analysis. Data were expressed as the mean ± standard deviation (SD) and statistical differences were determined by two-way repeated ANOVA or by one-way ANOVA, followed by a Newman-Keuls test using SPSS 13.0 statistical software. A p-value of <0.05 was considered significant.

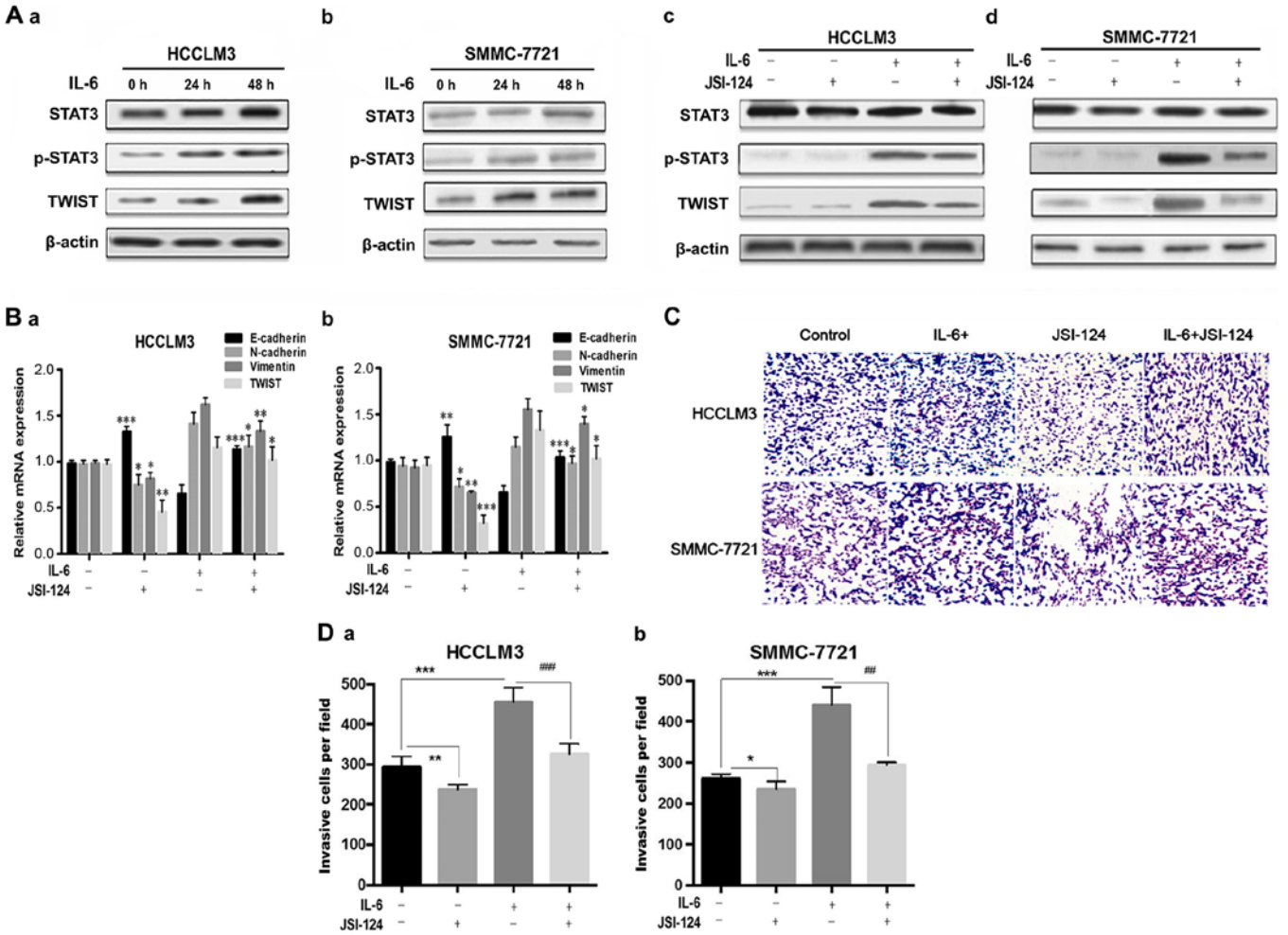

Figure 2. JAK2/STAT3/TWIST signaling is required for IL-6-induced EMT in HCC cell lines.(A-a and b) Western blot analysis of p-STAT3-Y705, STAT3 and TWIST in HCCLM3 and SMMC-7721 cells after IL-6 treatment for the indicated periods (0, 24 and 48 h). (A-c and d) JSI-124 inhibited IL-6-induced STAT3 phosphorylation and TWIST expression in HCCLM3 and SMMC-7721 cell lines. (B-a and b) Cells were pretreated with 100 ng/ml of IL-6 for 48 h and then exposed to 0.5 μM of JAK2/STAT3 inhibitor (JSI-124) for 24 h. The mRNA levels of E-cadherin, N-cadherin, vimentin and Twist were determined by RT-PCR in HCCLM3 and SMMC-7721 cell lines. JSI-124 significantly reversed IL-6-mediated downregulation of E-cadherin, upregulation of N-cadherin and vimentin, and decreased mRNA levels of Twist. (C and D-a and b) JSI-124 inhibited IL-6-induced cell invasion in HCCLM3 and SMMC-7721 cells. Cells were treated with 100 ng/ml of IL-6 and/or 0.5 μM of JSI-124 for 24 h, and allowed to pass through 8-μm membrane pores coated with 100 μl of Matrigel in Transwells. Invasive cells through the pores were stained with 1% crystal violet (magnification, x200). The mean values ± SD, n=3 are provided. *P<0.05, **P<0.01, ***P<0.001 vs. the control group, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the IL-6+ and JSI-124-). JAK, Janus tyrosine kinase; STAT3, signal transducer and activator of transcription 3; IL-6, interleukin 6; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma.

Results

IL-6 induces EMT in HCC cells. To investigate whether recombinant IL-6 could induce EMT in HCC cells, we treated the human HCC cell lines, HCCLM3 and SMMC-7721, which exhibit an epithelial phenotype, with various concentrations (0, 50, and 100 ng/ml) of recombinant IL-6 for 48 h or with 100 ng/ml of IL-6 for the indicated durations (0, 24, and 48 h). We assessed the expression of epithelial marker, E-cadherin, and the mesenchymal markers, N-cadherin and vimentin. The results demonstrated that E-cadherin expression was significantly suppressed, although N-cadherin and vimentin expression was increased with IL-6 treatment in HCCLM3 and SMMC-7721 cells as compared to the control group (absence of IL-6) in a dose-dependent manner (Fig. 1A). When the HCCLM3 and SMMC-7721 cells were treated with 100 ng/ml of IL-6 for 0, 24, and 48 h, the data revealed that EMT had occurred in a time-dependent manner (Fig. 1B). In addition, we examined the expression of EMT markers in hepatocellular cells by immunofluorescence staining. After the HCCLM3 and SMMC-7721 cells were treated with IL-6 (100 ng/ml) for 48 h, similar effects of E-cadherin, N-cadherin and vimentin were observed (Fig. 1C). We next determined the mRNA expression levels of E-cadherin, N-cadherin and vimentin in response to IL-6 (100 ng/ml) treatment for 0, 24 and 48 h by quantitative RT-PCR. The mRNA levels of E-cadherin were significantly decreased in HCCLM3 and SMMC-7721 cells treated with IL-6 as compared to the control cells without IL-6 treatment. In contrast, the mRNA levels of N-cadherin and vimentin were markedly increased in HCCLM3 and SMMC-7721 cells treated with IL-6 as compared to the control cells without IL-6 treatment (Fig. 1D). In summary, these results indicated that HCCLM3 and SMMC-7721 cells undergo EMT in response to IL-6 exposure, via downregulation of epithelial marker E-cadherin, and upregulation of mesenchymal markers N-cadherin and vimentin.

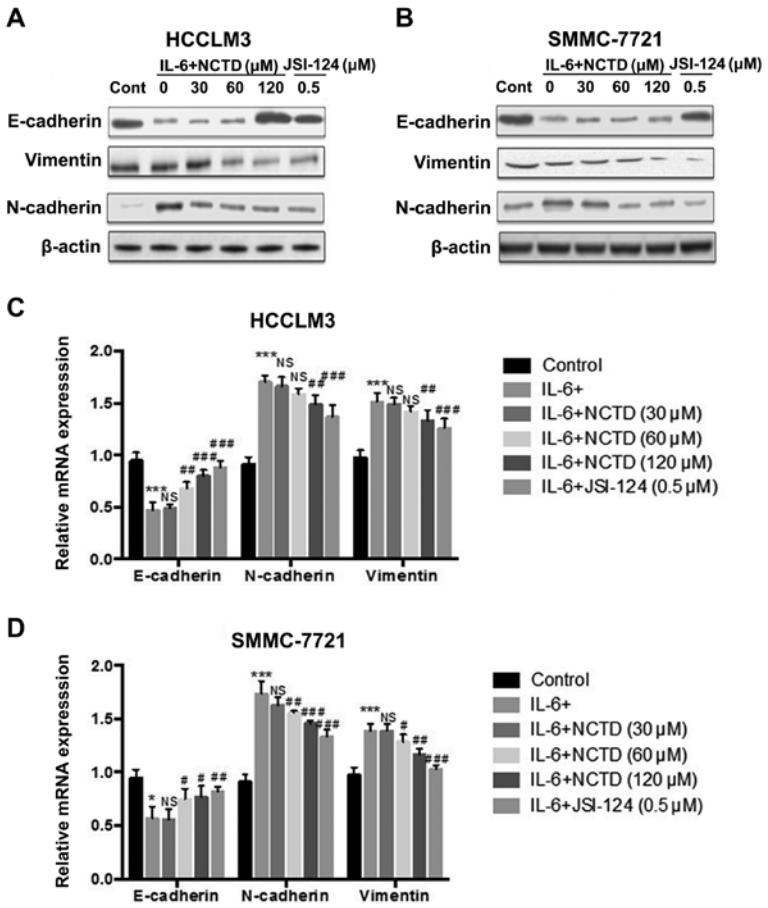

Figure 3. NCTD effectively blocks the IL-6 induced EMT in HCC cell lines. (A and B) After pretreatment with IL-6 (100 ng/ml) for 48 h, western blot analysis of E-cadherin, N-cadherin and vimentin protein expression was performed in HCCLM3 and SMMC-7721 cells after exposure to various concentrations of NCTD (30, 60 and 120 μM) or JSI-124 (0.5 μM) for 24 h. (C and D) Cells were pretreated with IL-6 (100 ng/ml) for 48 h and the levels of E-cadherin, N-cadherin and vimentin mRNA were determined by RT-PCR after exposure to various concentrations of NCTD (30, 60 and 120 μM) or JSI-124 (0.5 μM) for 24 h in HCCLM3 and SMMC-7721 cell lines. E-cadherin was upregulated, whereas N-cadherin and vimentin were downregulated following incubation with NCTD for 24 h as compared to the IL-6-treated group (0 μM of NCTD). The mean values ± SD, n=3 are provided. *P<0.05, **P<0.01, ***P<0.001 vs. the control group, NS P>0.05, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the IL-6 group). NCTD, norcantharidin; IL-6, interleukin 6; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma.

IL-6-induced EMT is mediated by the activation of the JAK2/STAT3/TWIST pathway in HCC cells. JAK2/STAT3 signaling regulates different cellular functions, including the EMT process, in multiple cancer types. Herein, western blot results revealed that STAT3 was phosphorylated and activated to upregulate EMT transcription factor, TWIST, after IL-6 (100 ng/ml) treatment for the indicated durations (0, 24 and 48 h) in HCC cell lines HCCLM3 and SMMC-7721 (Fig. 2A-a and b). We hypothesized that JAK2/STAT3 signaling is required for IL-6-mediated EMT in HCC. To test this hypothesis, we used a JAK2/STAT3 specific inhibitor cucurbitacin I (JSI-124) to suppress the activation of STAT3. JSI-124 decreased STAT3 phosphorylation and TWIST expression induced by IL-6, respectively, in HCCLM3 and SMMC-7721 (Fig. 2A-c and d). We further examined the role of JSI-124 in IL-6-mediated EMT in HCCLM3 and SMMC-7721 cells. As observed, JSI-124 significantly reversed IL-6-mediated downregulation of E-cadherin, upregulation of N-cadherin and vimentin, and decreased the mRNA levels of Twist (Fig. 2B).

We used Transwell assays to further investigate the invasive capacities of HCCLM3 and SMMC-7721 cells. IL-6 treatment with 100 ng/ml for 24 h enhanced cell invasiveness, whereas incubation with JSI-124 markedly suppressed the IL-6-induced increase of invaded cells. The results revealed that IL-6 enhanced the invasion abilities of HCCLM3 and SMMC-7721 cell lines, and that the JAK2/STAT3 inhibitor (JSI-124) suppressed IL-6-induced cell invasion (Fig. 2C and D). These findings demonstrated that upregulation of TWIST by STAT3 was required for IL-6-induced EMT and invasion in the HCCLM3 and SMMC-7721 cell lines.

Norcantharidin suppresses IL-6-induced EMT in HCC cells. To investigate the effect of NCTD treatment on IL-6-induced EMT in the HCCLM3 and SMMC-7721 cell lines, the cells were treated with 100 ng/ml of IL-6 for 48 h. Western blot analysis revealed that the expression of epithelial marker, E-cadherin, was significantly increased, while mesenchymal markers, N-cadherin and vimentin, were downregulated following incubation with various NCTD concentrations (0, 30, 60 and 120 μM) for 24 h when compared to the IL-6-treated group (0 μM of NCTD) (Fig. 3A and B). Furthermore, the relative mRNA levels of EMT markers from the NCTD groups after treatment with 100 ng/ml of IL-6 for 48 h, were analyzed

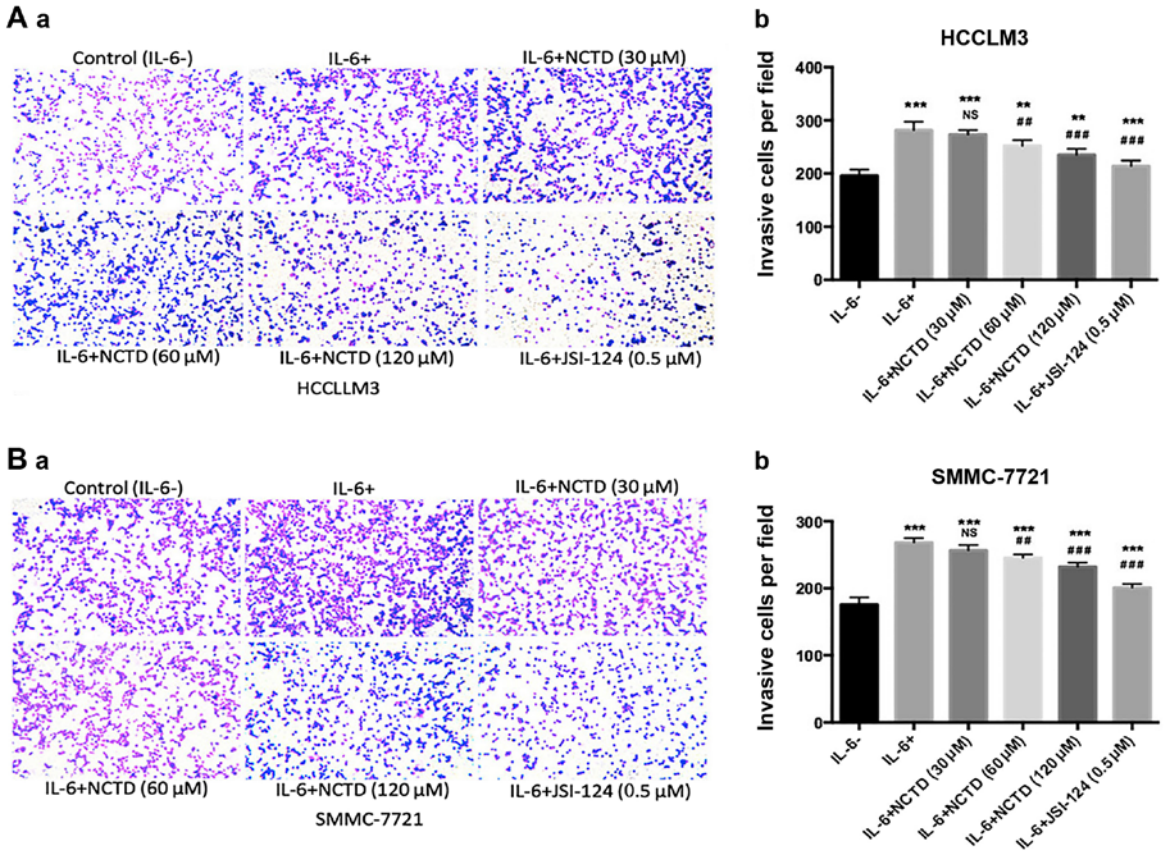

Figure 4. NCTD significantly suppresses the IL-6-induced invasiveness in HCC cell lines. (A-a and b and B-a and b) Cell invasive abilities were assessed by Transwell assay with different treatments in HCCLM3 and SMMC-7721 cells (magnification, x200). IL-6 significantly enhanced the invasive abilities after pretreatment (100 ng/ml of IL-6) for 48 h. However, NCTD and JSI-124 decreased cell invasion after exposure to various concentrations of NCTD (30, 60 and 120 μM) and JSI-124 (0.5 μM), for 24 h. The mean values ± SD, n=3 are provided. *P<0.05, **P<0.01, ***P<0.001 vs. the control group, NS P>0.05, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the IL-6 group). NCTD, norcantharidin; IL-6, interleukin 6; HCC, hepatocellular carcinoma.

by quantitative RT-PCR. E-cadherin mRNA was suppressed after IL-6 treatment. However, this suppression was reversed by treatment with different NCTD concentrations (60 and 120 μM). In contrast, the relative levels of N-cadherin and vimentin mRNA transcripts were increased after IL-6 treatment. However, this induction was prevented in the NCTD-treated (120 μM) group (Fig. 3C and D). Collectively, the data demonstrated that NCTD treatment reversed the EMT process induced by IL-6 in the HCCLM3 and SMMC-7721 cell lines.

NCTD inhibits IL-6-induced cell invasion in HCC cells. The effects of NCTD on IL-6-induced cell invasion in the HCCLM3 and SMMC-7721 cell lines was further investigated by Transwell assays. The results revealed that IL-6 significantly enhanced the invasion ability of the two cell lines (P<0.001), whereas concomitant incubation with NCTD or JSI-124 suppressed the IL-6-induced invasion ability of the cells (Fig. 4). Thus, these findings demonstrated that NCTD suppressed IL-6-induced cellular invasiveness in a dose-dependent manner.

NCTD inhibits IL-6-induced EMT through the JAK/STAT3/TWIST signaling pathway in HCC cells. To explore the underlying mechanisms of the inhibitory activities of NCTD on IL-6-induced EMT, we determined its effect on the activation of the JAK/STAT3/TWIST pathway. The expression of JAK2, phosphorylated JAK2 (p-JAK2), STAT3, phosphorylated STAT3 (p-STAT3) and TWIST after IL-6 treatment in the different concentration NCTD-treated groups was analyzed by western blot analysis. Notably, the expression of p-JAK2 and p-STAT3 increased after exposure of HCCLM3 and SMMC-7721 cells to IL-6. Furthermore, the expression of p-JAK2 and p-STAT3 decreased after treatment with NCTD in a dose-dependent manner (Fig. 5A-a and b). Notably, the JAK2/STAT3 inhibitor JSI-124 significantly suppressed the p-JAK2 and p-STAT3 levels. Treatment with NCTD and JSI-124 also markedly decreased JAK2 and STAT3 phosphorylation induced by IL-6 (Fig. 5B).

Finally quantitative RT-PCR analysis revealed that the levels of JAK2 and STAT3 increased after IL-6 treatment, whereas after treatment with different concentrations of NCTD, the relative levels of JAK2, STAT3 and TWIST decreased in a dose-dependent manner in HCCLM3 and SMMC-7721 cells. Notably, the decrease in the expression of JAK2, STAT3 and TWIST with the NCTD (30 μM) treatment was not in a statistically significant manner in the HCCLM3 cell lines (Fig. 5C). Moreover, NCTD (120 μM) treatment resulted in a significant decrease of IL-6-mediated TWIST expression. Likewise the expression of TWIST in the two cell lines was compared to the suppression of JAK2/STAT3, suggesting that NCTD directly counteracted the IL-6-mediated induction of

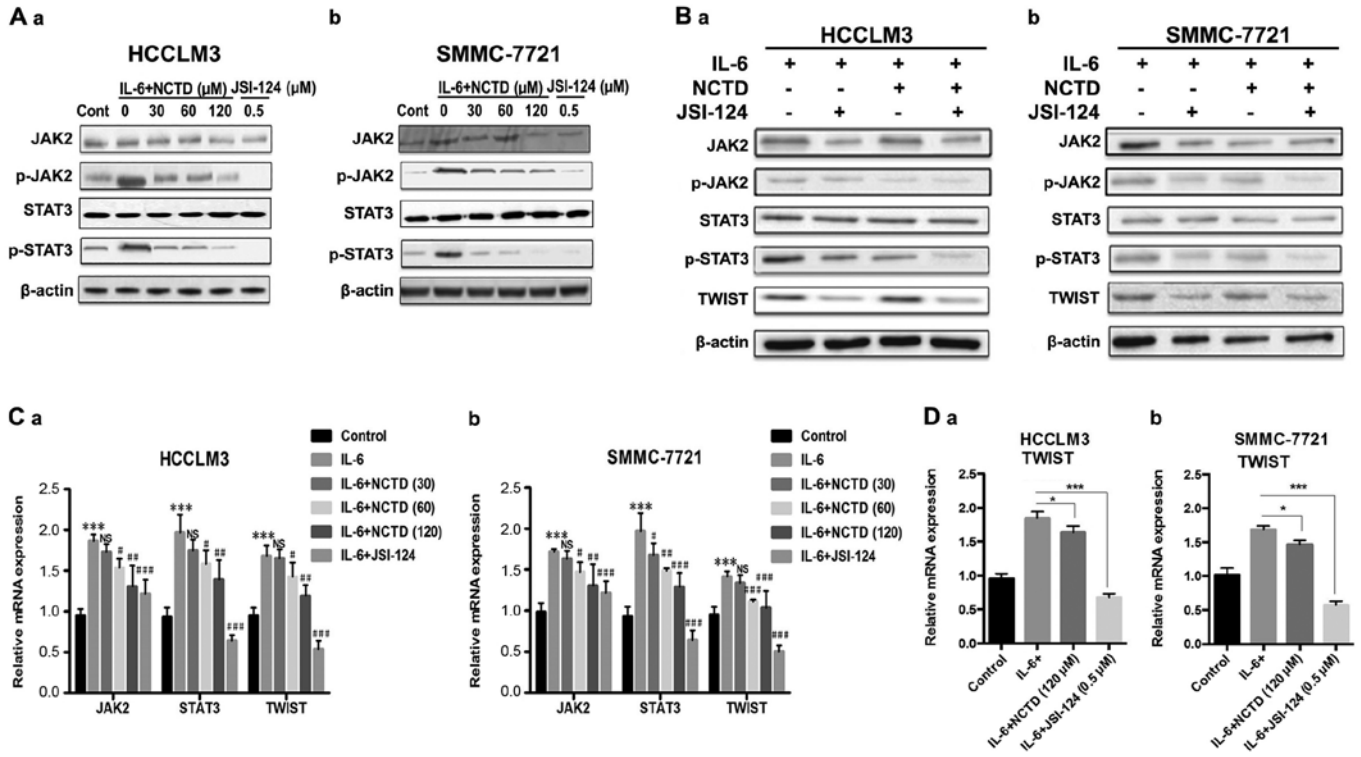

Figure 5. NCTD inhibits JAK2/STAT3/TWIST signaling in HCC cell lines. (A-a and b) After pretreatment with IL-6 (100 ng/ml) for 48 h, western blot analysis of the protein expression of JAK2, phosphorylated JAK2 (p-JAK2), STAT3, and phosphorylated STAT3 (p-STAT3) was performed in HCCLM3 and SMMC-7721 cells after exposure to various concentrations of NCTD (30, 60 and 120 μM) or JSI-124 (0.5 μM) for 24 h. (B-a and b) Cells were pretreated with IL-6 (100 ng/ml) for 48 h, and the protein levels of JAK2, p-JAK2, STAT3, p-STAT3 and TWIST were determined by western blot analysis after exposure to NCTD (120 μM) or JSI-124 (0.5 μM) for 24 h in HCCLM3 and SMMC-7721 cell lines. (C-a and b) Cells were pretreated with IL-6 (100 ng/ml) for 48 h, and the levels of JAK2, STAT3, TWIST mRNA were determined by RT-PCR after exposure to various concentrations of NCTD or JSI-124 (0.5 μM) for 24 h in HCCLM3 and SMMC-7721 cell lines. (D-a and b) Cells were pretreated with IL-6 (100 ng/ml) for 48 h, the mRNA levels of TWIST were determined by RT-PCR after exposure to NCTD (120 μM) or JSI-124 (0.5 μM) for 24 h in HCCLM3 and SMMC-7721 cell lines. (Mean values ± SD, n=3, are provided. *P<0.05, **P<0.01, ***P<0.001 vs. the control group, NS P>0.05, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the IL-6 group). NCTD, norcantharidin; JAK, Janus tyrosine kinase; STAT3, signal transducer and activator of transcription 3; IL-6, interleukin 6; HCC, hepatocellular carcinoma.

TWIST (Fig. 5D). In summary, these results demonstrated that NCTD inhibited IL-6-induced EMT via the downregulation of the TWIST transcription factor through JAK2/STAT3 signaling.

Discussion

EMT has been recognized as one of the universal mechanisms by which cancer cells acquire migratory and invasive capacity (5,32-34). Invasion is a key step to progression toward a malignant phenotype, and occurs when tumor cells translocate from the relatively constrained initial neoplastic mass into neighboring host tissues. During the process of EMT, epithelial cells acquire a fibroblastoid appearance due to downregulation of epithelial markers and upregulation of mesenchymal markers, thus generating a migratory phenotype (35,36). EMT which has been actively investigated in HCC (37-39), can be induced by numerous cytokines and growth factors, including IL-6 (40). Previous studies revealed that IL-6 induces a complex reciprocally regulated cytokine network in tumor cells which leads to the development of malignant and invasive tumors (40-42). IL-6 was shown to promote EMT changes in mesenchymal tumors from HCC patients as well as mesenchymal HCC cell lines (6). HCCLM3 and SMMC-7721 cell lines have highly invasive properties and were used in metastasis studies (43,44). In this study, we established the IL-6-induced EMT model in HCC cell lines (HCCLM3 and SMMC-7721), and demonstrated that IL-6 stimulated EMT in a time- and dose-dependent manner accompanied by downregulation of E-cadherin and upregulation of N-cadherin and vimentin in HCCLM3 and SMMC-7722 cells. In addition, administration of IL-6 significantly enhanced the invasion potential of HCC cell lines as a result of EMT. Previous studies have suggested that IL-6 can induce EMT changes by activating its downstream protein STAT3 within the tumor microenvironment, and plays an important role in the pathogenesis of human cervical carcinoma and breast cancer (41,45). Whether STAT3 plays a role in EMT induction in human HCC still remains to be determined. JSI-124 is a selective inhibitor of JAK2/STAT3 and has been demonstrated to exert anti-proliferative and antitumor effects both *in vitro* and *in vivo* (46). In the present study, we found that the JAK2/STAT3 inhibitor (JSI-124) reversed the IL-6-induced EMT process and STAT3 phosphorylation in HCC cell lines, suggesting that JAK2/STAT3 may be involved in IL-6-induced EMT change and may be one of the mechanisms responsible for this change. Twist has been reported as a major transcriptional suppressor downregulating E-cadherin expression and thus resulting in initial EMT and ultimate tumor metastasis (47,48). Emerging evidence suggests that STAT3 activation upregulates Twist expression (17,41).

Our data revealed that Twist, as an EMT activator, was significantly increased during EMT induction by IL-6. However, blocking STAT3 activation markedly suppressed the expression of Twist, confirming the regulation of Twist by STAT3.

In addition to EMT change, both HCCLM3 and SMMC-7721 cells after treatment with IL-6 gained invasive capacities. Using the cell line model, we investigated the effects of NCTD on IL-6-induced EMT and cell invasion. The results revealed that NCTD hindered the upregulation of N-cadherin and vimentin, and the downregulation of E-cadherin induced by IL-6 in HCCLM3 and SMMC-7721 cells, as well as the increased cell invasiveness induced by IL-6. IL-6-mediated activation of JAK2/STAT3 implicated in EMT and metastasis, and inhibition of JAK2 or blockade of activated STAT3 significantly suppressed the EMT process, cell migration and invasion in colon and pancreatic cancer (49,50). During the investigation process into the effects of NCTD on the levels of p-JAK2 and p-STAT3 in liver cancer cells after the treatment of IL-6, we found that NCTD significantly inhibited the activation of JAK2/ STAT3 induced by IL-6, although there was a slight variation in the levels of JAK2 in the presence of NCTD or JSI-124. The variation could be caused by different cell activity states. Moreover, we observed that the EMT-related transcription factor TWIST was downregulated after NCTD treatment, both at the mRNA and protein levels. All of these results clearly demonstrated that NCTD could block IL-6-induced EMT via the JAK2/STAT3/TWIST signaling pathway, which is an important mechanism underlying HCC development and metastasis.

To conclude, IL-6 promotes the EMT process during HCC development. NCTD can effectively target cell EMT by suppressing JAK2/STAT3/TWIST signaling under IL-6 stimulation. These findings warrant further assessment of NCTD, as an anticancer drug, in clinically relevant cancer models to explore its potential role in the treatment of HCC patients.

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