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Original Article

Antimetastatic effects of norcantharidin on hepatocellular carcinoma cells by up-regulating FAM46C expression

Xu-Ying Wan[1*], Xiao-Feng Zhai[2*], Yi-Ping Jiang[3], Ting Han[3], Qiao-Yan Zhang[3], Hai-Liang Xin[3]

[1]The Integrated TCM & Western Medicine Department, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200433, P. R. China; [2]Department of Traditional Chinese Medicine, Changhai Hospital, Second Military Medical University, Shanghai 200433, P. R. China; [3]Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai 200433, P. R. China. [*]Co-first authors.

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Abstract: Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide. Norcantharidin (NCTD), a demethylated analog of cantharidin, possesses antimetastatic effects on HCC cells. The aim of this study was to identify target proteins of NCTD. In this study, we confirmed the antimetastatic effects of NCTD on SMMC-7721 and MHCC-97H cells. Through RNA sequencing, we found a non-canonical poly (A) polymerase, Family-with-sequence-similarity-46C (FAM46C) was up-regulated in response to NCTD exposure. Gene set enrichment analysis on The Cancer Genome Atlas liver HCC (LIHC) dataset revealed that metastasis down pathway was strongly associated with FAM46C expression. Overexpression of FAM46C in HCC cells suppressed cell migration and invasion via suppressing transforming growth factor-β (TGF-β)/Smad signaling and epithelial-mesenchymal transition (EMT) process. Additionally, the antimetastatic effects of NCTD on HCC cells were partially rescued by FAM46C knockdown. Collectively, our results suggested that FAM46C, up-regulated by NCTD treatment, played a critical role in promoting the migration and invasion of HCC cells via TGF-β/Smad signaling. We identified a new therapeutic target of NCTD.

Keywords: Antimetatasis, norcantharidin, hepatocellular carcinoma, FAM46C, TGF-β/Smad

Introduction

Hepatocellular carcinoma (HCC) is most common type of liver cancer and the third leading cause of cancer-related mortality in the world [1, 2]. Chronic Hepatitis B infection is the predominant cause of HCC in countries where Hepatitis B is endemic, such as China [3], whereas chronic Hepatitis C infection is a major risk factor in countries where Hepatitis B is rare [2]. HCC causes 662,000 deaths globally per year, about half of which is in China [1]. The 5-year survival rate for HCC patients remains less than 20% because of tumor recurrence and metastasis [1]. Understanding the molecular mechanisms of metastasis and development of antimetastatic therapy is continuously needed.

Cantharidin, the active constituent of a Chinese traditional medicine blister beetle (*Mylabris phalerata* Pallas), has antitumor properties [4]. Norcantharidin (NCTD), the demethylated form of cantharidin, is easier to synthesize and less toxic. NCTD possesses anticancer activity [5-19] and inhibits renal interstitial fibrosis [20]. NCTD can inhibit cell growth by inducing cell apoptosis of various cancer types, including HCC [5, 6, 8, 13, 14, 16-18]. NCTD also shows antimetastatic effect on several cancer cells. Luan et al. reported that NCTD could reduce the migration of A549 lung cancer cell [21]. Chen et al. showed that NCTD reduces the invasion of CT26 colon cancer cells via decreasing the activity of matrix metalloproteinase (MMP)-2 and -9 and expression of several cadherin-catenin adhesion molecules [19, 22]. A recent study has reported the inhibitory effects of NCTD on the invasion and migration of Huh7 HCC cell [11]. Epithelial-mesenchymal transition (EMT) is thought to contribute to the complex pathogenesis of tumors and fibrosis [23,

24]. NCTD could antagonizes EMT process in renal proximal tubule cells [25] and colon cancer cells [26].

In the current study, we confirmed the antimetastatic effects of NCTD on SMMC-7721 and MHCC-97H cells. RNA samples from SMMC-7721 cells treated with DMSO or NCTD were then subjected to next-generation RNA Sequencing. Family-with-sequence-similarity-46C (FAM46C), a member of FAM46 family [27], was found significantly increased in response to NCTD treatment. Little is known about the function of FAM46 proteins. Here, we found that FAM46C reduced cell migration and invasion by *in vitro* overexpression experiments. Further experiments indicated the critical role of FAM46C in the antimetastatic effects of NCTD on HCC cells via regulating EMT.

Materials and methods

Cell culture

Human HCC cell lines, MHCC-97H, HepG2, Bel-7404, SMMC-7721, MHCC-97L and SK-Hep-1 were obtained from Chinese Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and penicillin (Life Technologies) at 37°C in a humidified atmosphere with 5% CO_2.

Migration and invasion assay

Migration and invasion assays were performed in uncoated or Matrigel (BD Biosciences, Bedford, MA, USA)-coated 24-well Transwell chamber (8-μm pore size, Corning Life Sciences, Corning, NY, USA). Briefly, SMMC-7721 and MHCC-97H cells (3.0×10^4 cells/well) in 200 μl serum-free medium with various concentrations of NCTD (original dissolved in DMSO; final concentrations: 5 and 10 μg/mL; Sigma, St. Louis, MO, USA) or DMSO (Sigma) were seeded into the upper chamber, while 600 μl medium with 10% FBS were added into the lower chamber. After 24 h of incubation at 37°C and 5% CO_2, cells remaining in the upper chamber were completely removed using a cotton swab. Cells attached to the bottom of the membranes were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. The degree of migration/invasion was expressed as the average number of cells counted in five randomly selected filed. Experiments were performed in triplicate.

RNA extraction and sequencing

SMMC-7721 cells were treated with 10 μg/mL NCTD or DMSO. After 24 h, cells were collected and RNA was isolated by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA integrity was verified by electrophoresis in denaturing formaldehyde gel. Libraries for RNA-sequencing were prepared with TruSeq Sample Preparation Kit (Illumina, San Diego, CA, USA). Next-generation sequencing was performed with the Illumina Genome Analyzer (Illumina, San Diego, CA). The original sequence data have been deposited in NCBI's Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra, AC: SRP075227).

Lentivirus and RNA interference

The control vector lentivirus and FAM46C overexpression lentivirus were constructed by Shanghai Genechem Co., Ltd. (Shanghai, China).

To knock down FAM46C expression, specific siRNA fragments targeting FAM46C (siFAM-46C) and control siRNA (siNC) were designed and synthesized by Genepharma Co., Ltd (Shanghai, China).

Real-time PCR

Total RNA was isolated with the TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) and then reverse-transcribed to first strand cDNA using the cDNA Synthesis kit (Fermentas). Then, the cDNA was subjected to real-time PCR with specific primers for FAM46C (sense, 5'-TGTGCTC-CAGGTTCTTCATC-3' and antisense, 5'-GAGGTA-GTCGTACTTGCTTCTC-3'); and GAPDH (sense, 5'-CACCCACTCCTCCACCTTTG-3' and antisense, 5'-CCACCACCCTGTTGCTGTAG-3') by using SYBR Green PCR kit (Thermo Fisher Scientific) protocol on ABI7300 (Applied Biosystem) thermal cycler. All samples were performed in triplicate, and results were calculated by the 2-ΔΔCt method.

Gene set enrichment analysis (GSEA)

Gene expression data were downloaded from The Cancer Genome Atlas website (TCGA,

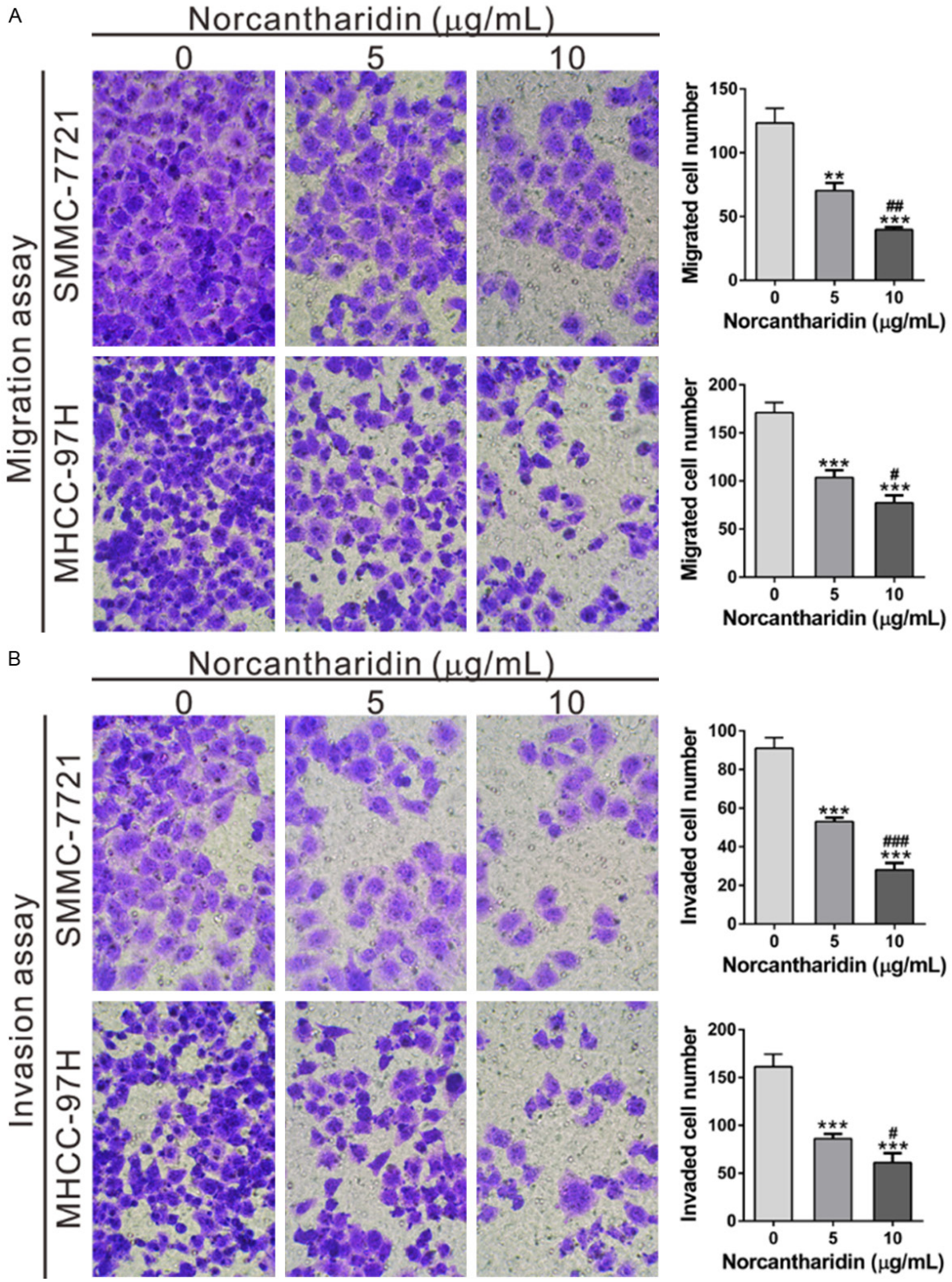
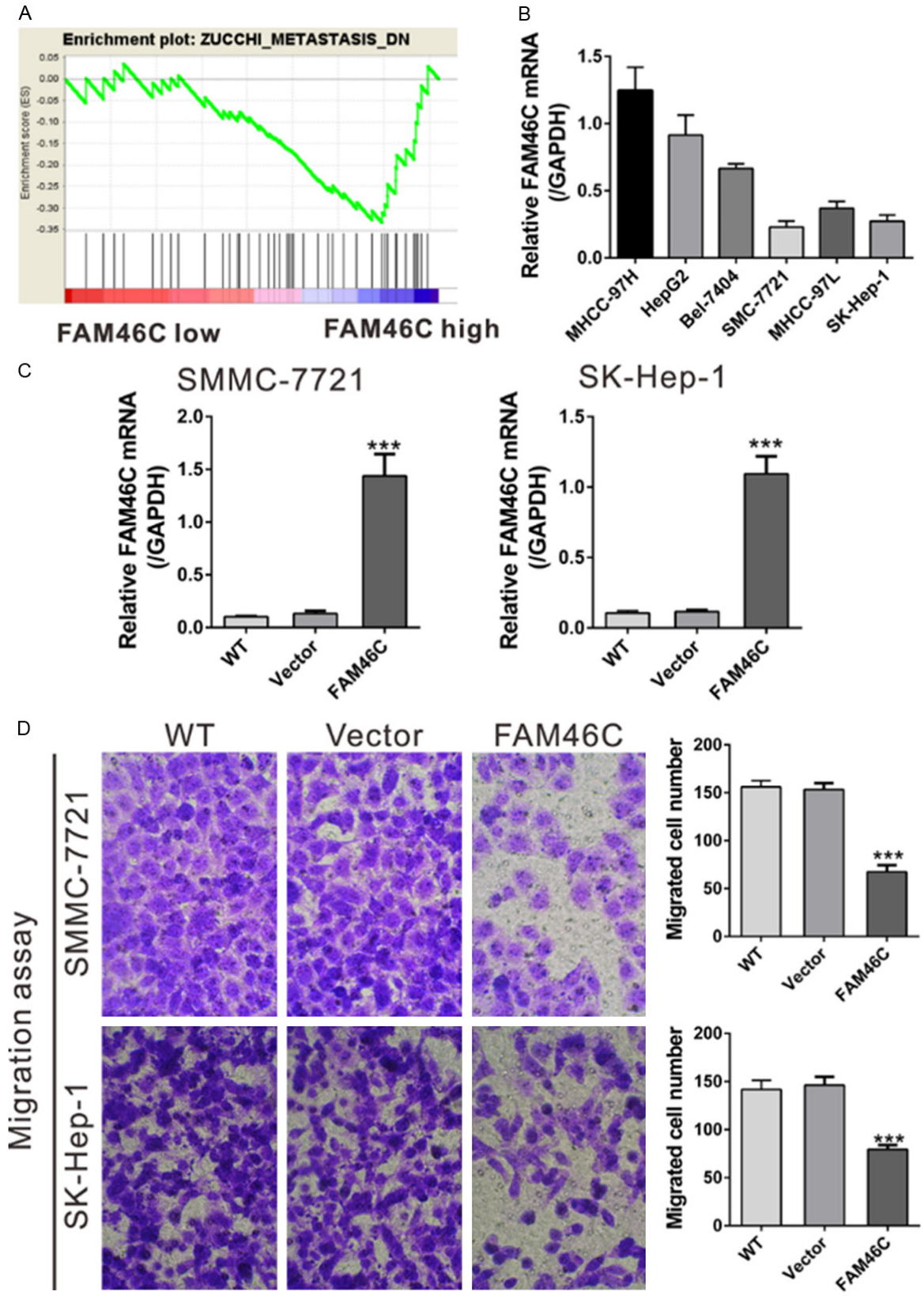


Figure 1. Effect of NCTD on cell migration and invasion in HCC cells. Migration and invasion assays were performed in uncoated or Matrigel-coated chambers. SMCC-7721 and MHCC-97H cells (3.0×10^4 cells/well) in 200 μl serum-free medium with various concentrations of NCTD (5 and 10 μg/mL) or DMSO were seeded into the upper chamber, while 600 μl medium with 10% FBS were added into the lower chamber. After 24 h of incubation, the migration (A) and invasion (B) abilities of SMCC-7721 and MHCC-97H cells were quantified as described in the Materials and Methods section. $^{**}P<0.01$, $^{***}P<0.001$ as compared with DMSO group; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ as compared with 5 μg/mL NCTD-treated group.


A
Enrichment plot: ZUCCHI_METASTASIS_DN
Enrichment score (ES)
FAM46C low
FAM46C high
B
Relative FAM46C mRNA (/GAPDH)
MHCC-97H
HepG2
Bel-7404
SMC-7721
MHCC-97L
SK-Hep-1
C
SMMC-7721
SK-Hep-1
Relative FAM46C mRNA (/GAPDH)
***
WT
Vector
FAM46C
D
WT
Vector
FAM46C
Migration assay
SMMC-7721
SK-Hep-1
Migrated cell number
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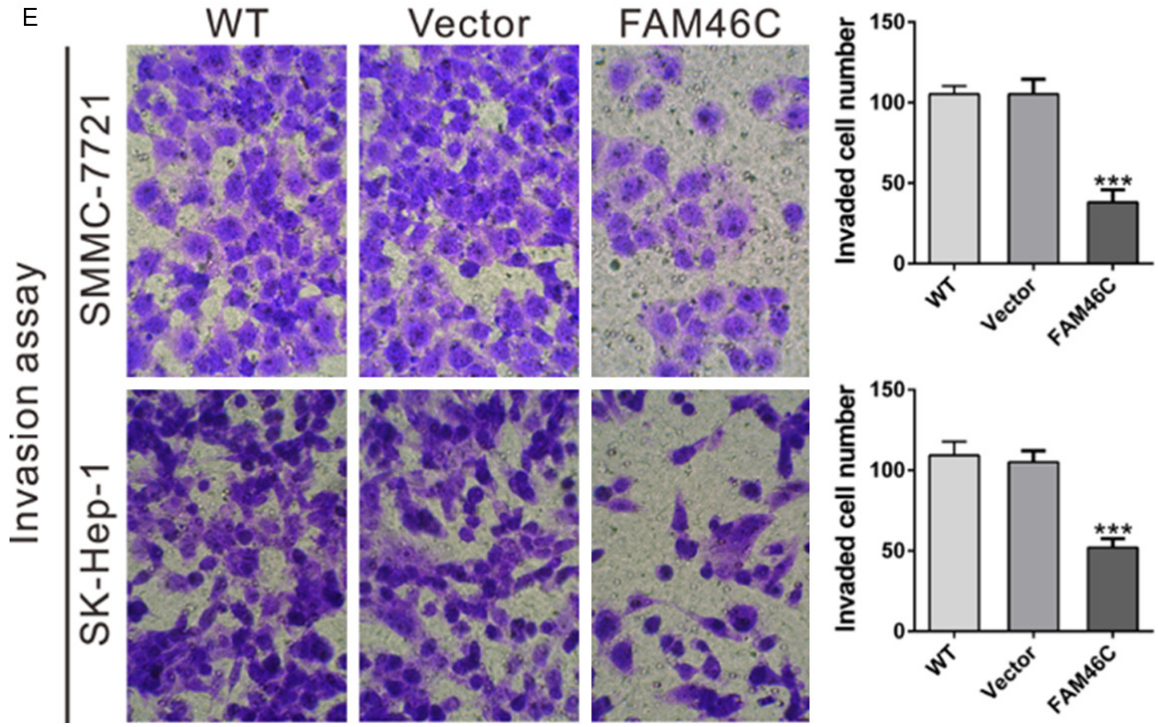

Figure 2. Effect of FAM46C overexpression on cell migration and invasion in HCC cells. (A) GSEA analysis in HCC patients with lower FAM46C expression versus higher FAM46C expression based on TCGA IHC datasets showed that metastasis down pathway was strongly associated with FAM46C expression. (B) mRNA levels of FAM46C were checked in 6 human HCC cell lines using real-time PCR. (C) Expression of FAM46C in SMCC-7721 and SK-Hep-1 cells was analyzed by real-time PCR. Migration (D) and invasion (E) of SMCC-7721 and SK-Hep-1cells infected with FAM46C virus or Vector virus. ***P<0.001 as compared with Vector virus-infected cells.

https://tcga-data.nci.nih.gov/tcga/) for the LIHC (liver hepatocellular carcinoma) project. To investigate biological pathways involved in NCTD treatment or HCC pathogenesis through FAM46C, Gene set enrichment analysis (GSEA) was carried out based on our sequencing data and TCGA LIHC dataset as previously described [28, 29].

Antibodies and Western blotting

Antibodies against MMP-2 (ab92536), Twist (ab175430), CXCL1 (ab86436) and CXCL2 (ab91511) were purchased from Abcam (Cambridge, MA, USA). Anti-MMP-9 (sc-393859) was from Santa Cruz Biotech. (Santa Cruz, CA, USA). Antibodies against RhoA (#2117s), E-cadherin (#14472), p-Smad2/3 (#8828), Smad2/3 (#8685) and GAPDH (#5174) were purchased from Cell Signaling (Danvers, MA, USA).

Cells were washed with PBS and then lysed with ice-cold radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). Protein concentrations were measured by BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% non-fat milk in TBST buffer at room temperature, the membranes were incubated with indicated primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 h. Membranes were visualized using the enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, USA) following the instructions of the manufacturer. The expression of target proteins was quantified by the densitometry with Image J software (NIH, Bethesda, MD, USA) with GAPDH as loading control.

Statistical analysis

Data from three independently experiments were analyzed by using GraphPad Prism software (version 6.0, San Diego, CA, USA) and shown as means ± SD. Statistical significances of differences were analyzed by One-way analysis of variance (ANOVA) test followed Sidak's multiple comparison post-test. P<0.05 was considered as significantly different.

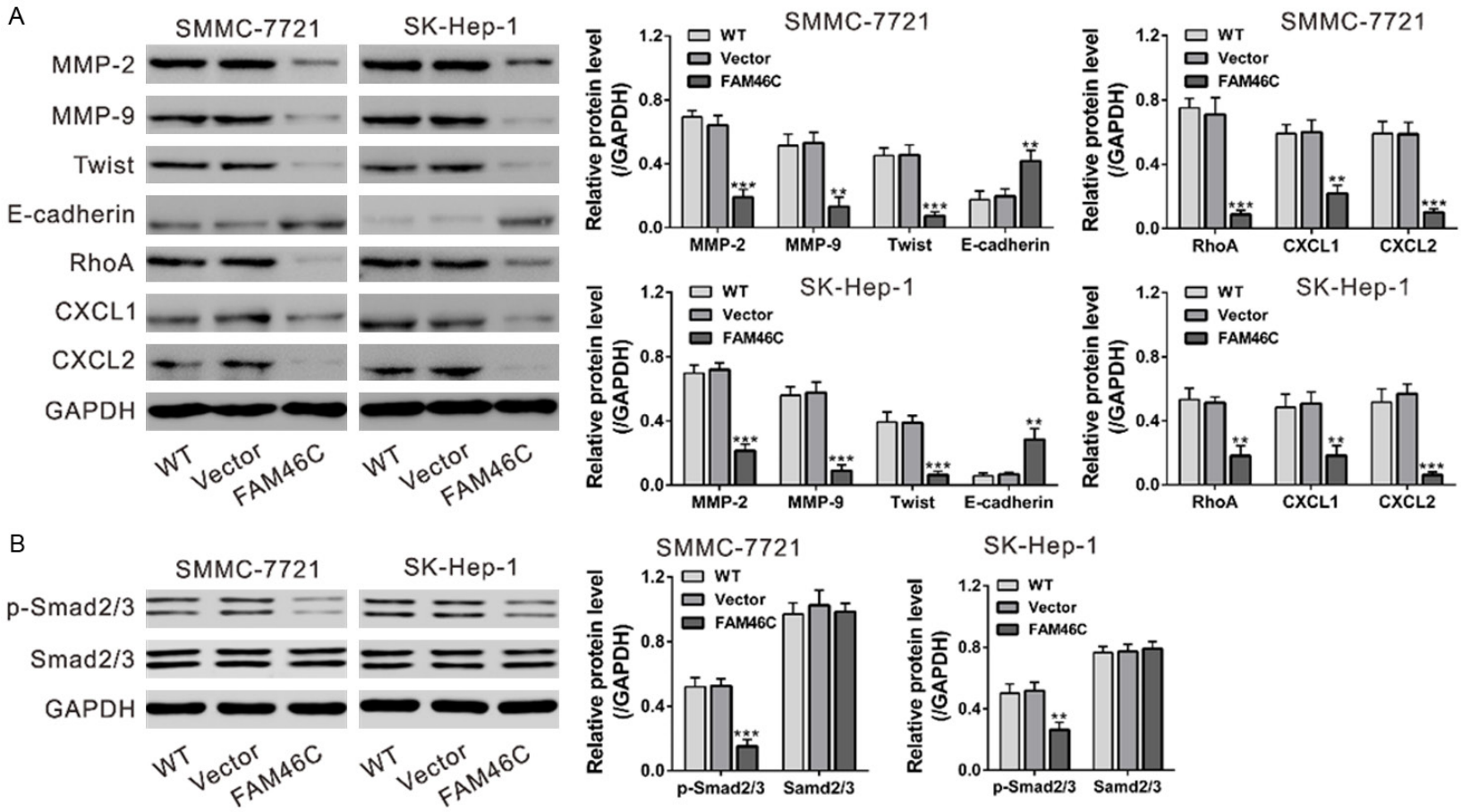

Figure 3. Mechanisms of FAM46C exerts its function. A: Protein levels of cell EMT-related factors (MMP-2, MMP-9, Twist and E-cadherin) and metastasis down pathway-related proteins (RhoA, CXCL1 and CXCL2) were evaluated by Western blotting. B: p-Smad2/3 and Smad2/3 were evaluated. ***P*<0.01 and ****P*<0.001 as compared with Vector virus-infected cells.

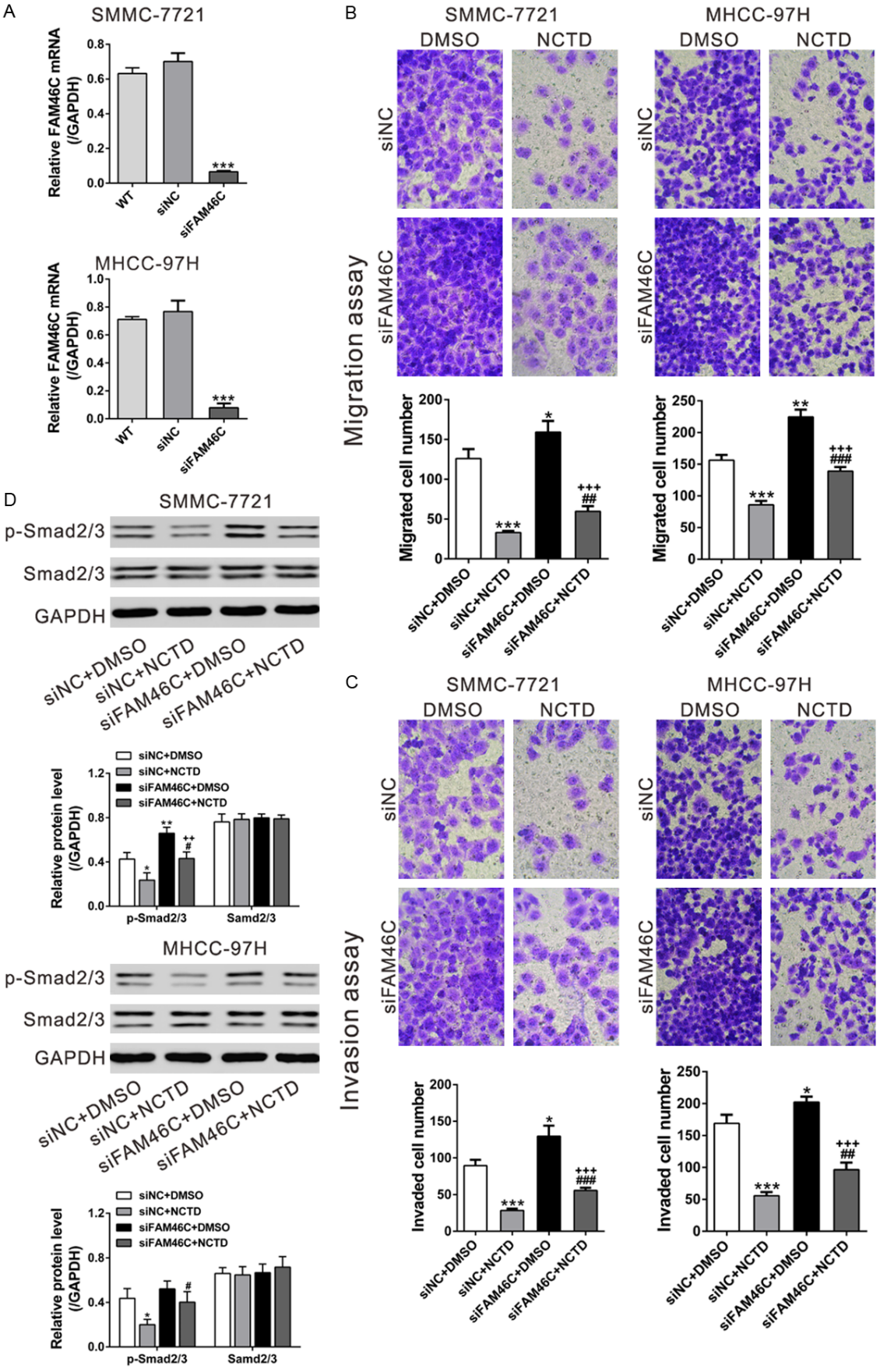
A
SMMC-7721
Relative FAM46C mRNA (/GAPDH)
WT
siNC
siFAM46C
MHCC-97H
B
SMMC-7721
MHCC-97H
DMSO
NCTD
siNC
siFAM46C
Migration assay
Migrated cell number
siNC+DMSO
siNC+NCTD
siFAM46C+DMSO
siFAM46C+NCTD
C
Invasion assay
Invaded cell number
D
p-Smad2/3
Smad2/3
GAPDH
Relative protein level (/GAPDH)
Samd2/3

Figure 4. FAM46C is critical for the antimetastatic effects of NCTD on HCC cells. (A) SMMC-7721 and MHCC-97H cells were transfected with siNC or siFAM46C. At 48 h after transfection, FAM46C mRNA expression was detected by real-time PCR. (B-D) SMMC-7721 and MHCC-97H cells were divided into 4 groups: siNC+DMSO, siNC+NCTD (10 μg/mL), siFAM46C+DMSO and siFAM46C+ NCTD (10 μg/mL). At 24 h after treatment, the migrated (B) and invaded cells (C) were counted. (D) Western blot analysis of Smad2/3 phosphorylation at 48 h after treatment. **P*<0.05, ***P*<0.01 and ****P*<0.001 as compared with siNC+DMSO; #*P*<0.05, ##*P*<0.01 and ###*P*<0.001 as compared with siNC+NCTD; ++*P*<0.01 and +++*P*<0.001 as compared with siFAM46C+DMSO.

Results

NCTD treatment suppresses HCC cell motility and invasiveness

Cell migration was determined using Transwell chambers in the present of NCTD (5 and 10 μg/mL) or DMSO. Cells that migrated into the lower chamber were fixed and then stained with crystal violet. NCTD treatment dose-dependently decreased cell motility, showing less migrating cells than DMSO-treated cells (**Figure 1A**). Invasive activity was also tested using Matrigel-coated chambers, which showed that NCTD treatment impaired invasive ability in a dose-dependent manner (**Figure 1B**).

FAM46C regulates cell migration and invasion of HCC cell lines

To explore how NCTD exerted antimetastatic effects on HCC cells, RNA samples were extracted from DMSO or NCTD (10 μg/mL)-treated SMCC-7721 cells (n=3) and then subjected to RNA sequencing. FAM46C mRNA level was found increased more than 3 folds when treated with 10 μg/mL NCTD for 24 h.

To probe the FAM46C-associated pathways on an unbiased basis, we performed GSEA based on TCGA LIHC dataset, which showed that metastasis down pathway was strongly associated with FAM46C expression (**Figure 2A**). These data suggested that FAM46C may play a key role in the metastasis of HCC.

To investigate the function of FAM46C in the metastasis of HCC cells, FAM46C lentivirus and vector control lentivirus were produced. The mRNA level of FAM46C in 6 HCC cells, MHCC-97H, HepG2, Bel-7404, SMCC-7721, MHCC-97L and SK-Hep-1, was evaluated by real-time PCR. Two cell lines, SMCC-7721 and SK-Hep-1, had lower mRNA expression and chosen for overexpression experiments (**Figure 2B**). The ectopic expression of FAM46C in both cells was confirmed by real-time PCR (**Figure 2C**). Infection of FAM46C lentivirus into SMCC-7721 and SK-Hep-1 cells significantly impaired the migration (**Figure 2D**) and invasion (**Figure 2E**) ability compared with Vector lentivirus infected cells and wild-type cells. These data suggested that FAM46C inhibited the metastasis of HCC cells.

FAM46C regulates the expression of EMT pathway and metastasis down pathway-related proteins

The expression of EMT pathway proteins, which are closely related to metastasis of tumor cells, were analyzed by Western blot. FAM46C overexpression significantly reduced the expression levels of MMP-2, MMP-9 and Twist, while increased the main factor of EMT, E-cadherin [30] (**Figure 3A**).

As mentioned above, GSEA revealed that metastasis down pathway was strongly associated with FAM46C expression (**Figure 2A**). To validate the GSEA results, we then detected the protein expression of metastasis down pathway-related proteins (RhoA, CXCL1 and CXCL2) in HCC cells transiently overexpressed FAM46C. The expression of detected protein was significantly reduced in both SMCC-7721 and SK-Hep-1 cells after the overexpression of FAM46C (**Figure 3A**).

FAM46C suppresses the activation of TGF-β pathway in HCC cells

The phosphorylation levels of Smad2/3, downstream of TGF-β signaling [30], were also analyzed by Western blot. As shown in **Figure 3B**, FAM46C overexpression significantly decreased the relative phosphorylation levels of p-Smad2/3. These data suggested FAM46C may promote cell invasion by activating TGF-β pathway.

FAM46C is critical for the antimetastatic effects of NCTD on HCC cells

Further, to further discover the relationship between FAM46C and NCTD, FAM46C expres-

sion was knocked by siRNA transfection in HCC cells. As shown in **Figure 4A**, FAM46 siRNA (siFAM46C) significantly reduced its mRNA expression in SMCC-7721 and MHCC-97H cells compared with control siRNA (siNC) and wild-type cells (WT).

SMCC-7721 and MHCC-97H cells were then divided into 4 groups and subjected to Transwell assays: Group 1, cells treated with siNC and DMSO; Group 2, cells treated with siNC and 10 μg/mL NCTD; Group 3, cells treated with siFAM46C and DMSO; and Group 4, cells treated with siFAM46C and 10 μg/mL NCTD. Silencing of FAM46C could partially counteract the antimetastatic effects of NCTD (**Figure 4B** and **4C**).

We then detected phosphorylation status of Smad2/3 (**Figure 4D**). The activity of Smad2/3 was significantly decreased by NCTD treatment, and partially rescued by FAM46C knockdown. These data indicated that the antimetastatic effect of NCTD on HCC was dependent on FAM46C and TGF-β signaling.

Discussion

The antimetastatic effects of norcantharidin (NCTD) on HCC cells have been studied [11]. Here, we found that FAM46C was the target of NCTD and might play a role during HCC metastasis via regulating EMT.

Firstly, we demonstrated that NCTD treatment dose-dependently decreased the migration and invasion ability of SMCC-7721 and MHCC-97H cells (**Figure 1**), which was in line with the previous study on Huh7 cells [11]. We then found that FAM46C mRNA expression was significantly increased in response to NCTD treatment by RNA sequencing analysis, suggesting that FAM46C was a potential target for NCTD.

Little is known about the function of FAM46C except its involvement in viral replication [31, 32] and malignancies [27, 33-36]. FAM46C is a potential tumor suppressor for multiple myeloma [33-37]. Point mutations of FAM46C have been observed in various tumors including HCC [27], strongly suggesting the involvement of FAM46C in cancer pathogenesis. Here, GSEA revealed that metastasis down pathway was strongly associated with FAM46C expression (**Figure 2A**). We then examined the functions of FAM46C in the metastasis of HCC cells. Ectopic expression of FAM46C in HCC cells with lower expression of FAM46C could impaired cell migration and invasion (**Figure 2**). On the contrary, FAM46C knockdown in HCC cells resulted in inverse effects (**Figure 4**). Our data indicated the antimetastatic effects of FAM46C on cancer cells.

Furthermore, we tried to explore the molecular mechanism by which FAM46C exerted antimetastasis functions. CXCL1 [38], CXCL2 [39] and RhoA [40], three factors in metastasis down pathway, are strongly correlated with the ability of tumor cells to invade and successfully establish metastases. EMT process is proposed to be required for cancer cell metastasis by increasing cell motility and invasiveness [30]. Here, FAM46C overexpression stimulated the expression of the main factor of EMT (E-cadherin), but decreased the expression of an inducer of EMT (Twist) and metastasis down pathway-related factors (CXCL1, CXCL2 and RhoA) (**Figure 3A**). Moreover, TGF-β signaling pathway is the best-studied pathway involved in EMT [30]. We found that FAM46C overexpression led to a remarkable decrease in Smad2/3 phosphorylation (**Figure 3B**). These findings suggested that FAM46C may suppress TGF-β signaling and EMT process, thus leading to the inhibition of HCC cell invasion.

To further investigate the association between NCTD and FAM46C, FAM46C expression was knockdown in HCC cells. The antimetastatic effects of NCTD on HCC cells were significantly attenuated by FAM46C knockdown (**Figure 4**), indicating that FAM46C was a target of NCTD. Moreover, NCTD can antagonize EMT by inhibiting the TGF-β/Smad pathway in HK-2 renal proximal tubule cells [25]. We found that the phosphorylation of smad2/3 was reduced in response to NCTD treatment, and partially rescued by FAM46C knockdown. These findings clearly indicated that NCTD treatment up-regulated FAM46C, suppressed TGF-β/Smad pathway, thus inhibiting cell metastasis.

In our study, the antimetastatic role of NCTD was confirmed in HCC cells. FAM46C, a potential target of NCTD, which could inhibited HCC cell migration and invasion via inhibiting TGF-β/Smad pathway and EMT process. This study identified a new therapeutic target of NCTD for inhibiting EMT.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Hai-Liang Xin, Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, P. R. China. Tel: (+86) 21-81871300; Fax: (+86) 21-81871300; E-mail: hailiangxin@163.com

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