

Inhibitor of β -catenin and TCF (ICAT) promotes cervical cancer growth and metastasis by disrupting E-cadherin/ β -catenin complex

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Abstract. The inhibitor of β -catenin and TCF (ICAT) blocks the binding of TCF to β -catenin and has been demonstrated as a suppressor of the Wnt/ β -catenin signaling pathway. It has been reported to exert a different function around a wide variety of cancers. However, its function and underlying mechanisms in human cervical cancer remains unknown. In the present study, the expression of ICAT in 41 human cervical cancer tissues and 30 normal cervical tissues was evaluated by immunohistochemical analysis. ICAT was found highly expressed in cancer tissues. ICAT overexpression significantly promoted SiHa cell proliferation *in vitro* by causing G1 arrest, and enhanced cell migration and invasion whereas, ICAT knockdown induced opposite effects in Caski cells which have higher expression of ICAT. Downregulation or overexpression of ICAT resulted in an altered expression of the epithelial-mesenchymal transition (EMT). Furthermore, immunoprecipitation assays revealed that ICAT promoted cervical cancer EMT by competing in E-cadherin binding to β -catenin. Overexpression of ICAT in SiHa cells promoted tumor growth and EMT was also demonstrated by the xenograft mouse experiment. These results demonstrate that ICAT contributed to the progression of cervical cancer and may play a role in the regulation of EMT by disrupting the E-cadherin/ β -catenin complex. It may be a novel potential therapeutic target for therapy in human cervical cancer.

Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth in mortality among females worldwide (1,2). The incidence is lower in developed countries as a consequence of screening by cervical cytology and human papillomavirus (HPV) (3). However, the prognosis of patients with advanced-stage cervical cancer remains unsatisfactory, especially for those in developing countries where widespread screening is unavailable (4). What is more, cervical cytology and HPV screening also have some shortcomings (5). Metastasis of cervical cancer is responsible for ~90% of all poor prognosis (3). Therefore, it is urgent for cervical cancer to identify causes and potential markers of metastasis in order to find novel therapeutic strategies.

Inhibitor of β -catenin and T-cell factor (ICAT) is a small protein of 81 amino acids that was first reported in 2000 (6). ICAT was demonstrated as an inhibitor of the Wnt/ β -catenin signaling pathway at first (7). Subsequently, crystallographic analyses of ICAT have proved its β -catenin binding sites which was a β -sheet-like conformation and located in carboxy-terminal domain (8-10). As research continued, ICAT was also found to inhibit the intercellular adhesion function (11).

Epithelial-to-mesenchymal transition (EMT) has been recognized as an important process in embryogenesis and carcinogenesis (12,13). During EMT, epithelial cells convert to mesenchymal cells, which involves profound phenotypic changes including loss of cell-cell adhesion and cell polarity (14). Increasing evidence implicates that EMT is a key process for metastasis in cervical cancer (15,16). However, whether ICAT is associated with the process of EMT in cervical cancer is still unknown.

In the present study, we demonstrated that ICAT was highly expressed in cervical cancer tissues and had a role in control of EMT in cervical cancer cells. Overexpression of ICAT promoted cell proliferation, migration, invasion and resulted in EMT by disrupting the stability of E-cadherin/ β -catenin

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complex, whereas the opposite effect was observed in down-regulation of ICAT in Caski cells. We thus, identified a novel role and regulatory mechanism of ICAT in EMT in cervical cancer.

Materials and methods

Human tissue samples. Cervical cancer and normal cervical tissues were collected from patients who undergone hysterectomy at the First Affiliated Hospital, Chongqing Medical University. None of the patients received any preoperative chemotherapy, immunotherapy, or radiotherapy. Written informed consent was obtained from each patient at the time of surgery, and all collections were approved by the Clinical Ethics Committee of the hospital.

Cell culture. The human cervical cancer cell lines SiHa, HeLa and Caski were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 1% penicillin and 1% streptomycin. All cells were cultured at 37°C in a 5% CO₂ incubator under humidified atmosphere.

Immunohistochemistry (IHC). Cervical cancer tissues, normal cervical tissue and paraffin-embedded nude mouse xenograft cervical tumors were sliced into 4- μ m consecutive sections. Sections were then dewaxed in xylene, rehydrated in graded ethanol and heat-treated for antigen retrieval with citric acid buffer. Sections were cooled and incubated in a 0.3% hydrogen peroxide solution for 20 min, blocked with normal goat serum for 30 min and then incubate with corresponding antibody at 4°C overnight. Subsequently, the sections were performed using an immunohistochemistry SP-9000 kit (Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing, China). Staining procedures were performed under standardized conditions. Subsequently, hematoxylin were used for counterstain. All slides were scored by a pathologist in a blinded manner. The evaluation of ICAT immunoreactivity was performed using the immunoreactivity scores. The score was determined by multiplying the staining intensity by the staining extent. The staining intensity for ICAT was scored as 0, negative; 1, weak; 2, moderate; and 3 strong. The staining extent was scored as 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100% according to the percentage of positively stained cells. The immunoreactivity was divided to two grades on the basis of the final score: negative immunoreactivity was defined as a total score of 0-4 and positive was defined as a total score >4.

Western blot analysis. The cells (2x10⁶) were washed three times with cold phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Cell lysis solution was centrifuged at 13,000 x g for 15 min at 4°C and protein solution were collected. The BCA protein assay kit (Beyotime Institute of Biotechnology) was used to measure the protein concentration. Equivalent amounts of protein were loaded in SDS-PAGE polyacrylamide gels and then transferred onto PVDF membranes. The PVDF membranes were blocked

with 5% bovine serum albumin (BSA; Beijing Solarbio Science and Technology, Co., Ltd., Beijing, China) in TBST for 2 h at 37°C. Subsequently, the membranes were incubated with the primary antibodies overnight at 4°C. The following primary antibodies were used in the present study: monoclonal rabbit anti-ICAT, anti-MMP9 (1:5,000; Abcam, Cambridge, MA, USA); monoclonal rabbit anti- β -catenin (1:1,000, Abcam), polyclonal rabbit anti-E-cadherin, anti-vimentin and anti-Snail (1:1,000; Bioworld Technology, Inc., Nanjing, China), monoclonal mouse anti- β -actin (1:1,000; Beijing Zhongshan Golden Bridge Biotechnology); polyclonal rabbit anti-PCNA, anti-cyclin D1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed with TBST 3 times and incubation with a secondary antibody (1:5,000; Beijing Zhongshan Golden Bridge Biotechnology) for 1 h at 37°C. The SuperSignal West Pico Chemiluminescent substrate kit was used to quantify protein levels.

RNA isolation and quantitative real-time reverse transcription-PCR (qRT-PCR). Cells were treated with AdICAT in FBS-free DMEM for 48 h. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the RNA extraction protocol. Total RNA (1.5 μ g) was used for cDNA synthesis by reverse transcriptase PCR. The cDNA was amplified by a real-time polymerase chain reaction (qPCR) system (Bio-Rad Laboratories) using SYBR-Green PCR Master Mix. β -actin was used as the endogenous control. Reaction conditions were as follows: denaturation at 94°C for 10 sec, annealing at 57°C for 20 sec and extension at 72°C for 10 sec. The specific primers for ICAT were 5'-ATGAACCG CGAGGGAGCTCC-3' (forward) and 5'-CTACTGCCTCCG GTCTTCCG-3' (reverse). Those for β -actin were 5'-GATGA CCCAGATCATGTTTGAG-3' (forward) 5'-AGGGCATACC CCTCGTAGAT-3' (reverse).

Adenoviral transfections and small interfering RNA. Recombinant adenovirus AdICAT and negative control AdRFP were kindly donated by Dr Tongchuan He (University of Chicago Medical Center, Chicago, IL, USA). AdICAT or AdRFP was transfected into the SiHa cells with polybrene (Sigma-Aldrich). After 8 h of cultivation, the medium was replaced with a fresh medium without FBS. The fluorescence was then observed 36 h later.

Three ICAT-siRNAs and negative control (NC) were purchased from Shanghai GenePharma, Co., Ltd. (Shanghai, China). The sequences are shown in Table I. Cells were transfected using a Lipofectamine RNAiMAX kit (Invitrogen) according to the manufacturer's instructions.

MTT assay. Cell viability was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A total of 4x10³ cells were seeded into each well of 96-well plates in quintuplicate and cultured for 24, 48, 72 and 96 h. At the indicated time, 10 μ l of MTT (5 mg/ml; Sigma-Aldrich) was added into each well and then incubated for 4 h at 37°C. Next, 150 μ l of dimethyl sulfoxide (DMSO) was added to the 96-well plates to dissolve the formazan product. Finally, the absorbance was measured at 492 nm on a microplate reader. the overall experiments were repeated at least three times.

Table I. The sequences of the siRNAs and negative control.

siRNAs	Sense	Antisense
siICAT	#1:GGUGCUUUAGUUAGGUCAUTT #2:GGUCCACAGCUGGAAAGUUTT #3:GGCUCCAUAUUUCAACUAATT	AUGACCUAACUAAAGCACCTT AACUUUCCAGCUGUGGACCTT UUAGUUGAAAUAUGGAGCCTT
siNC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Colony-forming assay. Log-phase cells were collected and ~500 cells were plated into each well of a 6-well plate. The cells were cultured in 10% FBS medium and the medium was replaced every four days. When clones were observed, the cells were fixed with 4% paraformaldehyde, washed twice with PBS and stained with 0.1% crystal violet. The visible colonies were counted. Each experiment was repeated thrice.

Flow cytometric analysis. Cells (1×10^6) from each group were harvested and washed thrice with cold PBS, followed by fixation with 70% cold ethanol at 4°C overnight. After being washed in PBS, the cells were incubated with propidium iodide (PI; Sigma-Aldrich) and RNaseA for 30 min at room temperature. The cell cycle distribution was measured by a FACSVantage SE flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Migration and invasion assay. Transwell chambers (24-well Transwell chambers, 8- μ m pore size; Corning, Inc., Corning, NY, USA) were utilized for migration and invasion assays. For the migration assay, $\sim 4 \times 10^5/400 \mu$ l cells in serum-free media were seeded into upper chambers after infection for 48 h. The lower chamber contained medium with 20% FBS. Following 24-h incubation, the cells which invaded to the lower surface of the chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet, and counted from five random fields by bright field microscopy. The Transwell invasion assay course was similar to the migration assay except that the Transwell membrane was coated with 1:4 diluted Matrigel beforehand. Each experiment was repeated thrice.

Immunofluorescence staining. Cells cultured on chamber coverslips were fixed with 4% paraformaldehyde for 15 min and blocked with 10% goat serum (0.2% Triton X-100 in PBS) for 1 h at 37°C. Then, the sections were incubated with anti-E-cadherin rabbit, anti-vimentin rabbit (1:100; Bioworld Technology) and anti- β -catenin (1:100; Abcam) rabbit at 4°C overnight. Thereafter, cells were incubated with DyLight 488 AffiniPure goat anti-rabbit IgG (H+L) (1:800; EarthOx, LLC, San Francisco, CA, USA) for 1 h at 37°C. Images were captured under a fluorescence microscope (Eclipse Ti-S; Nikon, Tokyo, Japan).

Immunoprecipitation. Whole cells were washed three times with cold PBS and resuspended in RIPA lysis buffer at 4°C for 30 min. Anti- β -catenin antibody (5 μ g; Santa Cruz Biotechnology) was incubated with Protein A/G Magnetic Beads (Biotools Co., Ltd., Shanghai, China) at room temperature for 20 min. Cell lysis solution was centrifuged at 14,000 x g for 10 min and protein solution was added into the Protein

A/G Magnetic Beads and incubated overnight at 4°C. Then, the beads were washed three times with wash buffer. Proteins were eluted by boiling with 20 μ l 1X SDS-PAGE loading buffer. Finally, the expression of the target protein was evaluated by western blot analysis.

Xenograft mouse experiment. The *in vivo* experiments were approved by the guidelines established by the Animal Care and Use Committee of Chongqing Medical University Laboratory Animal Research. The 4-6-week old female nude mice were randomly divided into 3 groups (n=5/group). Untreated SiHa cells (2×10^7 /each nude mouse), AdRFP-infected SiHa cells (2×10^7 /each nude mouse) and AdICAT-infected SiHa cells (2×10^7 /each nude mouse) were injected subcutaneously into the posterior flank position of the nude mice. Untreated SiHa cells and SiHa/AdRFP served as control groups, whereas SiHa/AdICAT served as the treatment group. Tumor dimensions were recorded every week with vernier calipers, and the volumes were calculated using the following formula: $\pi/6 \times (\text{length} \times \text{width}^2)$. The mice were sacrificed by cervical vertebra dislocation after 5 weeks, and tumor tissues were collected, embedded in paraffin for H&E and immunohistochemical analysis.

Results

ICAT is upregulated in human cervical cancer tissues, and verification of recombinant SiHa/ICAT. To investigate the role of ICAT in human cervical carcinogenesis, we first detected the endogenous expression of ICAT in human normal cervix and cervical cancer by immunohistochemistry (IHC). The representative ICAT staining is shown in Fig. 1A. Samples were scored based on the immunoreactivity scores: negative (1-4) and positive (5-12) (17). The average scores of IHC for ICAT were 5.000 ± 0.6215 in normal cervix samples and 7.366 ± 0.3916 in cervical cancer (Fig. 1B). The positive ICAT expression rates were 40.0% (12/30) in normal cervix and 87.8% (36/41) in cervical cancer (Fig. 1C; $P < 0.01$). To further confirm the role of ICAT in human cervical cancer, we detected the expression of ICAT by qRT-PCR and western blot analysis in three human cervical cancer cell lines (HeLa, SiHa and Caski) (Fig. 1C and D). The results showed that ICAT mRNA and protein were detected in all three cervical cancer cell lines and Caski cells showed higher expression of ICAT; however, SiHa showed lower expression. These data suggested that ICAT was upregulated in cervical cancer and it may be involved in carcinogenesis. Thus, we used SiHa and Caski cells as a model to investigate the function of ICAT on cell proliferation, migration and invasion. SiHa cells were

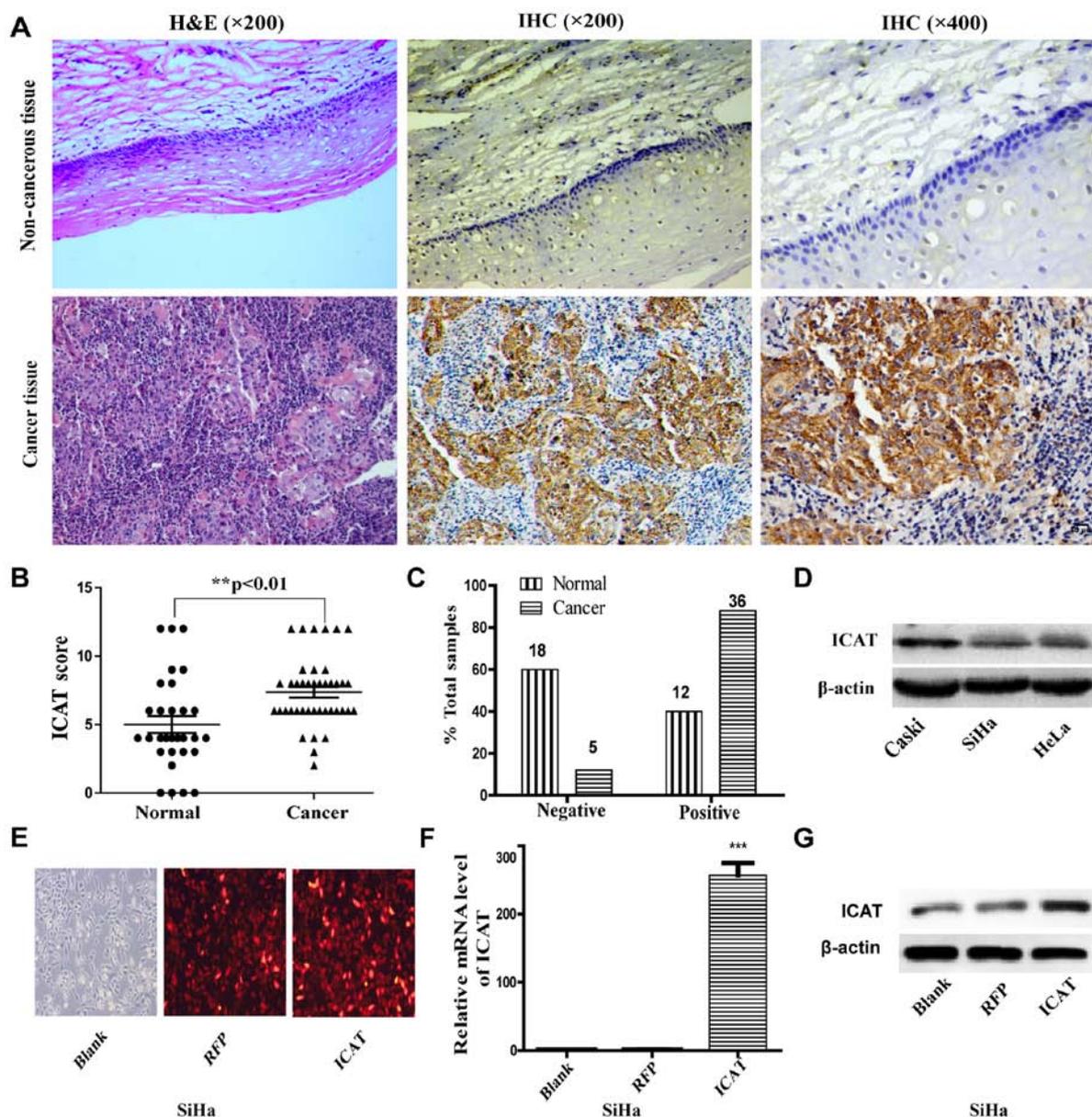


Figure 1. Expression of ICAT in samples, cervical cancer cells and verification of recombinant SiHa/ICAT. (A) Representative H&E staining and immunohistochemical staining of ICAT in normal cervix ($n=30$) and cervical cancer tissue ($n=41$) paraffin sections. Scale bar, $50 \mu\text{m}$; (B) the immunoreactivity scores of ICAT staining in normal cervix and cervical cancer tissue; (C) percentage of ICAT negative and positive staining scores in normal cervix and cervical cancer tissue; (D) the expression of ICAT in Caski, SiHa and HeLa cells was measured by western blot analysis; (E) infection efficiency of the SiHa cells infected with AdRFP and AdICAT for 36 h observed by a fluorescence microscope; (F) expression of ICAT in SiHa cells was analyzed by qRT-PCR; (G) expression of ICAT in SiHa cells was analyzed by western blot analysis. Data are shown as mean \pm SD of 3 individual measurements ($^*P < 0.05$, $^{**}P < 0.01$, as compared with Blank and RFP).

transfected with ICAT-expressing adenoviruses (AdICAT) to generate recombinant SiHa/ICAT. The transfection efficiency of SiHa cells at 36 h was observed under a fluorescence microscope (Fig. 1E). qRT-PCR and western blot assay showed that recombinant SiHa/ICAT cells were successfully established and were appropriately prepared for the subsequent experiments (Fig. 1F and G).

ICAT overexpression promotes proliferation, migration and invasion in SiHa cells. After AdICAT-mediated enforced ICAT expression in SiHa cells, cell proliferation ability was assessed by MTT assay and colony formation assay. The MTT assay showed that the proliferation of the SiHa/ICAT cells

was increased significantly compared with the Blank and RFP groups (Fig. 2A). Colony formation assay results showed that the number of colonies of SiHa/ICAT were approximately double that of the controls (Fig. 2B). Flow cytometry revealed that ICAT overexpression led to an increase in the percentage of cells in the S phase and a decrease in the number of cells in the G1 phase compared with the controls (Fig. 2C). Cell migration and invasion abilities were detected by Transwell migration assays and Transwell invasion assays, respectively. As shown in Fig. 2D and E, ICAT introduction caused a remarkable increase of SiHa cells that invaded to the lower surface of the chamber. Furthermore, we detected the protein levels of migration-related factor 9 (MMP9), DNA replication

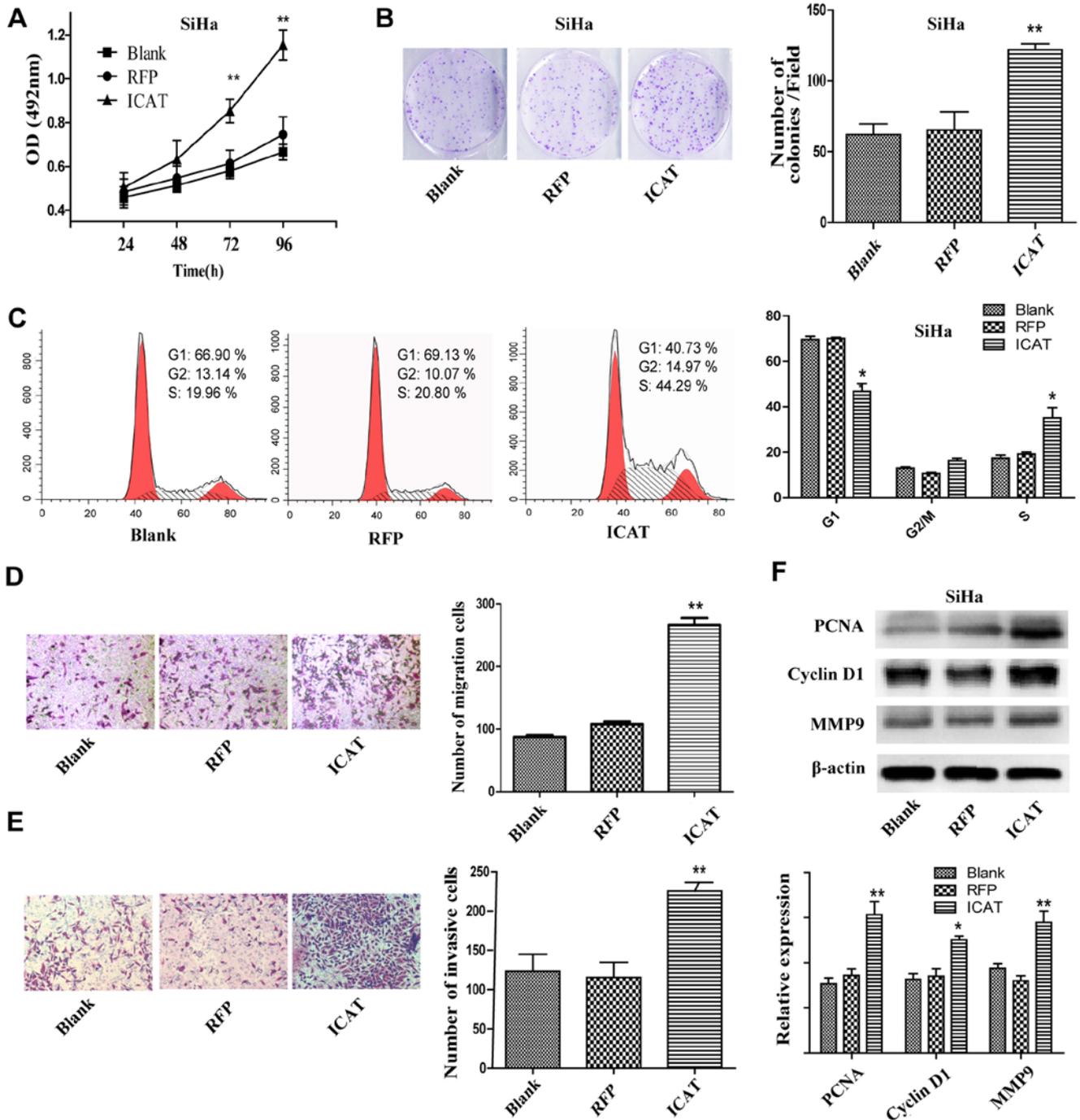


Figure 2. ICAT overexpression promotes proliferation, migration and invasion in SiHa cells. (A and B) MTT and colony formation analysis of the effect of ICAT overexpression on SiHa cell proliferation. (C) Flow cytometric analysis of the effect of ICAT overexpression on SiHa cell cycle; (D and E) cell migration and invasion were measured by Transwell assay (x100 magnification); (F) the expression levels of migration-related factor (MMP9), DNA replication factor (PCNA) and cyclin D1 were detected by western blot analysis after infection with AdICAT. Data are shown as mean \pm SD of 3 individual measurements (* P <0.05, ** P <0.01, as compared with Blank and RFP).

factor (PCNA) and cyclin D1 by western blotting. The results showed that PCNA, cyclin D1 and MMP9 were upregulated after ICAT overexpression in the SiHa cells (Fig. 2F).

Downregulation of ICAT inhibits the proliferation, migration and invasion in Caski cells. To further examine whether ICAT is involved in cervical cancer progression, we synthesized three siRNAs specifically targeting ICAT. The interference efficiency of the three siRNAs targeting ICAT was verified

by qRT-PCR and western blotting (Fig. 3A and B). As shown in Fig. 3A and B, ICAT-siRNA-3 had a significant inhibitory effect on ICAT and were used in the following assays. MTT assay and colony formation assay results showed that knockdown of ICAT by RNAi significantly decreased cells proliferation of Caski cells (Fig. 3C and D). Furthermore, we compared the cell cycle profiles of ICAT knockdown cells by flow cytometry. Suppression of ICAT led to a decrease in the number of cells in the S-phase and an increase in the

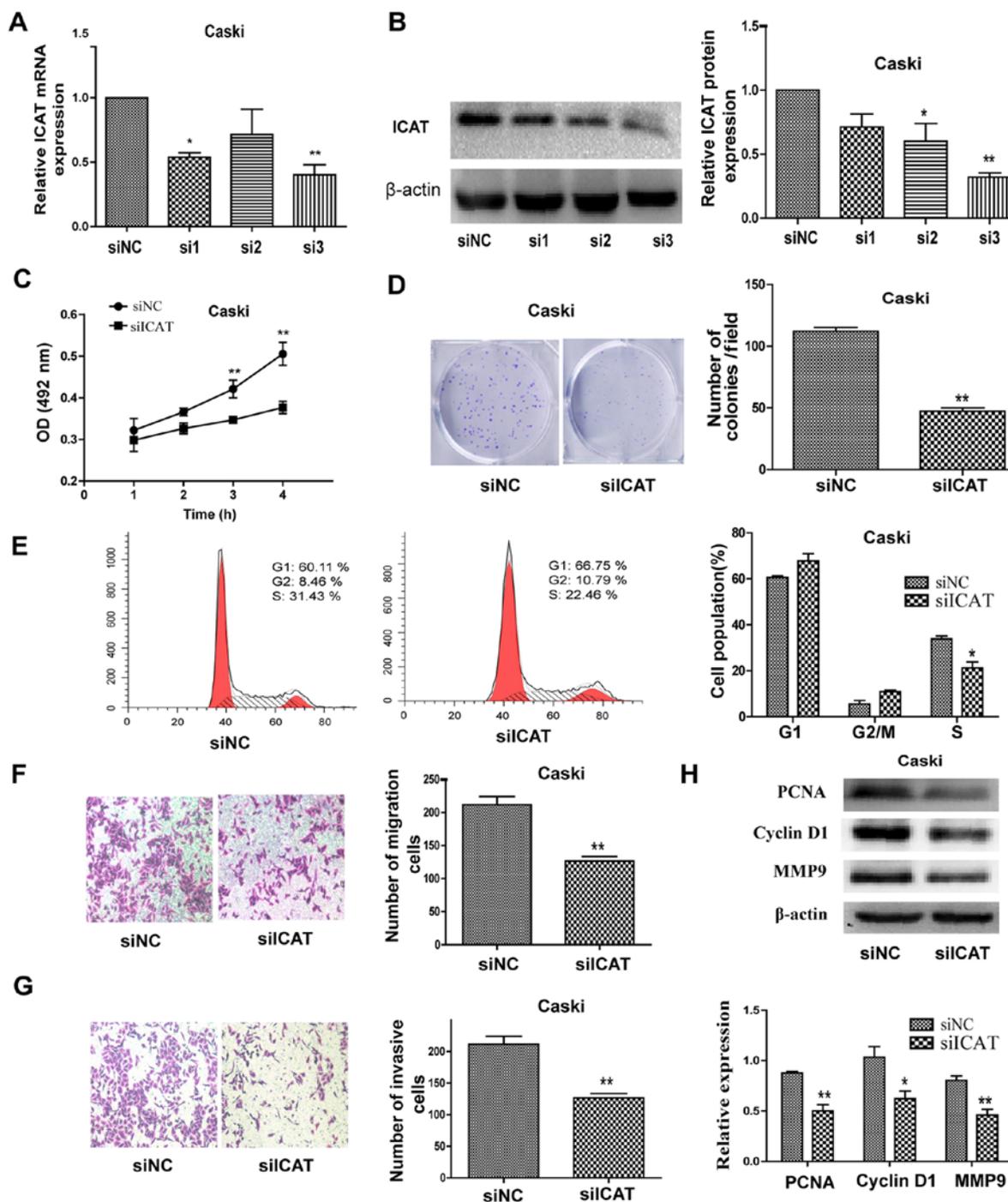


Figure 3. Downregulation of ICAT inhibits the proliferation, migration and invasion of cervical cancer Caski cells. (A and B) The inhibitory effect of siRNA on ICAT in the Caski cells were analyzed by qRT-PCR and western blot analysis; (C and D) MTT and colony formation assay analysis of the effect of ICAT knockdown on Caski cell proliferation; (E) flow cytometric analysis of the effect of ICAT knockdown on Caski cell cycle; (F and G) cell migration and invasion were measured by Transwell assay (x100 magnification); (H) the expression levels of MMP9, PCNA and cyclin D1 were detected by western blot analysis following ICAT siRNA treatment. Data are shown as mean \pm SD of 3 individual measurements (* P <0.05, ** P <0.01 as compared with siNC).

percentage of cells in the G1 phase (Fig. 3E). The migration and invasion effects in Caski cells by inhibiting ICAT were also investigated. Following transfection with siICAT for 48 h, both the migratory and invasive effects of Caski cells were significantly decreased compared with the control groups (Fig. 3F and G). Quantification of invading cells revealed a significant decrease in the number of migrating and invading cells for Caski cells after ICAT knockdown. In addition, western blotting showed that PCNA, cyclin D1 and

MMP9 were downregulated after ICAT knockdown in the Caski cells (Fig. 3H).

ICAT promotes EMT of cervical cancer cells and disrupts E-cadherin/ β -catenin complex. A significant morphological alteration was observed by microscopy after ICAT-overexpressing in the course of experiments. As seen in Fig. 4A, AdICAT-mediated enforced ICAT expression in SiHa cells exhibited more mesenchymal morphology with

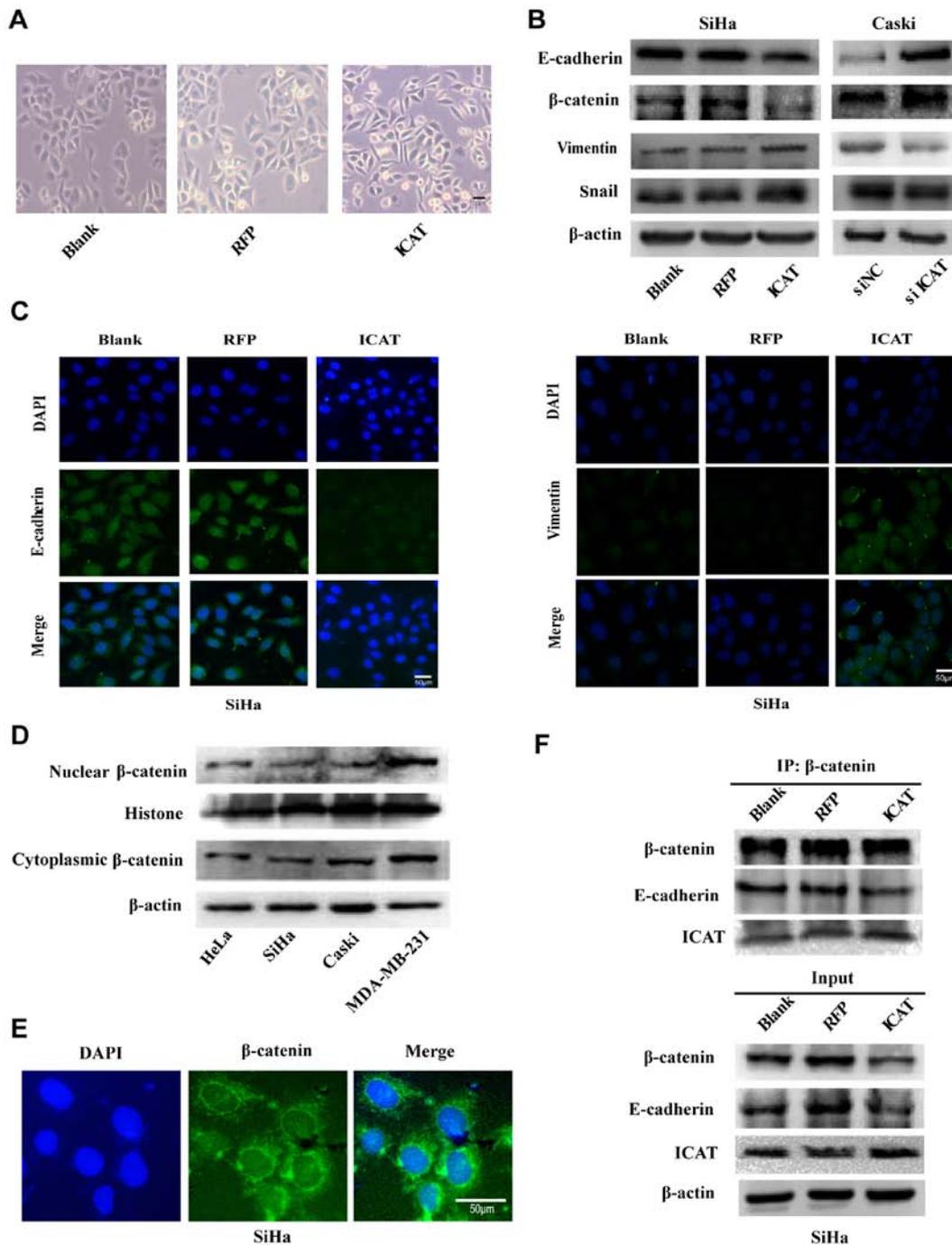


Figure 4. ICAT regulates epithelial-mesenchymal transition of cervical cancer cells and disrupts E-cadherin/ β -catenin complex. (A) The images of cell morphological change caused by ICAT overexpression observed by microscopy; (B) western blot analysis of the expression levels of EMT markers (E-cadherin, β -catenin, vimentin and snail) in SiHa, SiHa/RFP, SiHa/ICAT, Caski/NC and Caski/siICAT cells; (C) immunofluorescence assay for E-cadherin and vimentin expression, proteins were stained green, and the nuclei were stained with DAPI (blue). The images are magnified x400. Scale bar, 50 μ m; (D) endogenous expression of nuclear β -catenin and cytoplasm β -catenin in SiHa, Caski, HeLa and MDA-MB-231; (E) immunofluorescence assay for cellular distribution of β -catenin proteins in SiHa. Proteins were stained green, and the nuclei were stained with DAPI (blue). Scale bar, 50 μ m; (F) immunoprecipitation (IP) of E-cadherin and ICAT proteins with β -catenin in SiHa cells.

spindle-like shape, while control cells displayed more cobblestone-shaped morphology (18,19). Thus, we investigated whether ICAT have a regulatory effect on EMT program in cervical cancer cells. The expression of EMT-related markers were detected by western blot analysis in SiHa and Caski cells after transfection with AdICAT or siICAT. As expected, the overexpression of ICAT induced marked upregulation of mesenchymal marker vimentin and snail but downregulation

of epithelial marker E-cadherin and β -catenin in SiHa cells. Furthermore, ICAT knockdown remarkably elevated the expression of E-cadherin and β -catenin and at the same time significantly suppressed the expression of snail and vimentin in Caski cells (Fig. 4B). In order to better demonstrate the ICAT regulated EMT in cervical cancer cells, we tested the EMT-related marker changes by immunofluorescence analysis. The results showed that the vimentin staining was

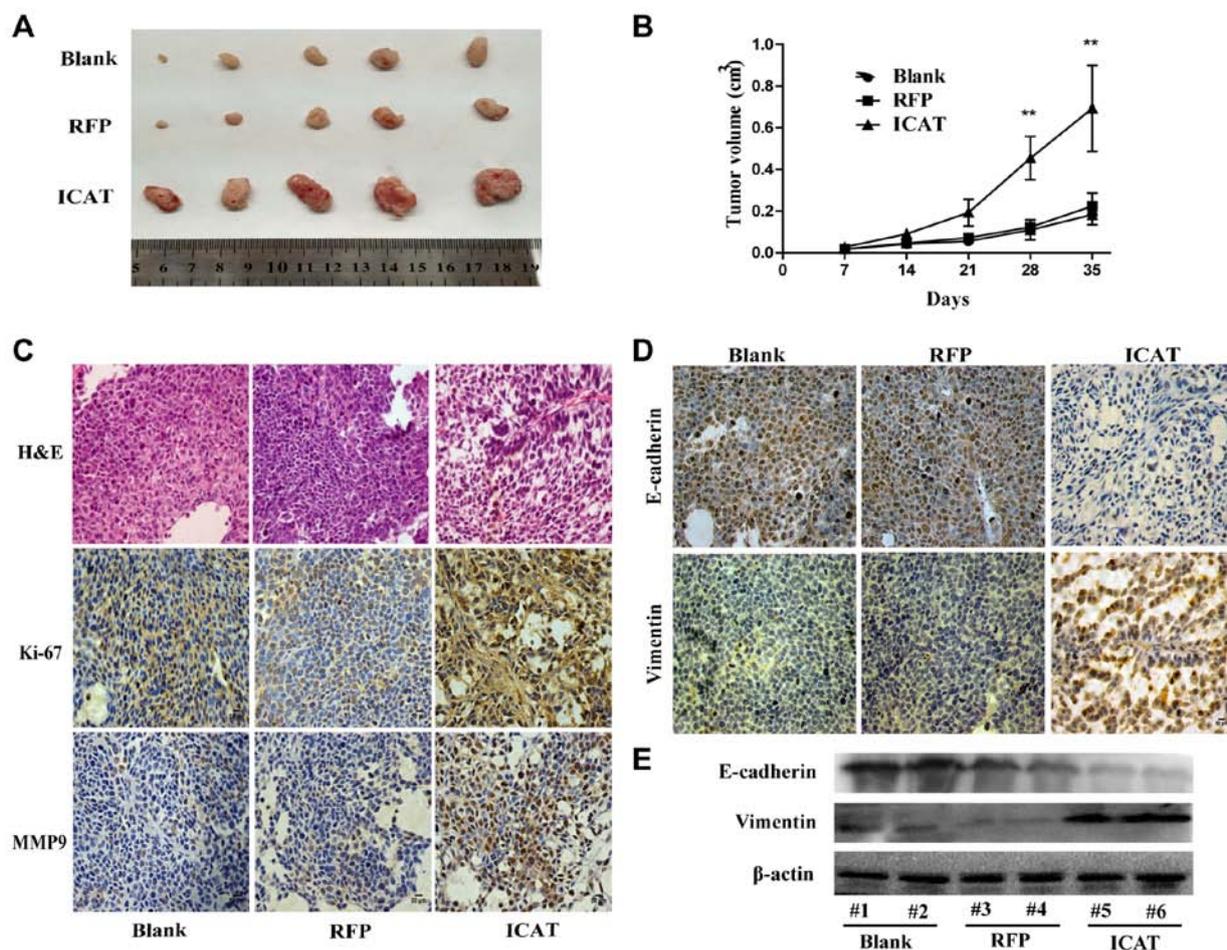


Figure 5. Overexpression of ICAT in SiHa cells promotes tumor growth *in vivo*. (A) The tumor sizes in the SiHa, SiHa/RFP, SiHa/ICAT groups; (B) the tumor growth curves of the SiHa, SiHa/RFP, SiHa/ICAT groups. (n=5 in each group); (C) H&E staining of tumor tissues (x200 magnification), Ki-67 and MMP9 staining of the SiHa, SiHa/RFP, SiHa/ICAT groups by immunohistochemical staining (x200 magnification). Scale bar, 50 μ m; (D) immunohistochemical staining of EMT markers (E-cadherin and vimentin) in various groups; (E) western blot analysis of the expression levels of EMT markers in various groups. Data are shown as mean \pm SD of three individual measurements ($P < 0.05$, $^{**}P < 0.01$, as compared with Blank or RFP).

enhanced while E-cadherin staining was reduced in ICAT groups compared with the negative controls (Fig. 4C). Having identified ICAT as a promoter of EMT, we were interested in identifying the mechanism behind these effects. It has been reported that ICAT plays a role in β -catenin-dependent nuclear signaling and E-cadherin/ β -catenin complex of the cell adhesion (11), thus, we turned our attention on β -catenin in cervical cancer. Koay *et al* (20) found that β -catenin has aberrant localization in the cytoplasm and no expression was found in the nucleus in 126 invasive carcinomas of different histological types. We also illustrated the endogenous expression of nuclear β -catenin and cytoplasm β -catenin in SiHa, Caski, HeLa and MDA-MB-231 which is aberrantly activated in Wnt/ β -catenin signaling (22), and used as positive control in western blot analysis. As seen in Fig. 4D, β -catenin protein was observed in the cytoplasm mainly and the expression of nuclear β -catenin was very faint. Immunofluorescence staining analysis of the cellular distribution of β -catenin proteins in SiHa (Fig. 4E) also proved β -catenin was mainly localized in the cytoplasm. Thus, we hypothesized that ICAT influence the E-cadherin/ β -catenin complex and EMT mainly in cervical cancer. As confirmation, we further verified that ICAT competes with β -catenin

binding to E-cadherin when overexpressed in SiHa by immunoprecipitation (Fig. 4F).

Overexpression of ICAT in SiHa cells promotes tumor growth and EMT in vivo. To investigate the effects of ICAT on the tumor growth and EMT of cervical cancer cells *in vivo*, SiHa, SiHa/RFP and SiHa/ICAT cells were subcutaneously implanted into nude mice. The tumors were monitored weekly and dissected after xenografting for 5 weeks. As shown in Fig. 5A the tumor volume of the SiHa/ICAT cells was significantly increased relative to the controls after 3 weeks (Fig. 5B). Hence, ICAT expression increased the proliferation of cervical cancer cells relative to the control in nude mice and significantly increased tumor burden over time. These results were consistent with those *in vitro*. The immunohistochemical analysis of Ki-67 and MMP9 expression revealed that compared with the SiHa, SiHa/RFP groups Ki-67 and MMP9 positive cell rates were increased in the SiHa/ICAT group (Fig. 5C). The expression of EMT-related markers also showed corresponding changes, the vimentin staining was enhanced while E-cadherin staining was reduced in the SiHa/ICAT group (Fig. 5D). Furthermore, western blot results also showed that the protein expression level of vimentin was

increased in SiHa/ICAT group, while the protein expression level of E-cadherin was significantly decreased (Fig. 5E).

Discussion

ICAT, a β -catenin interacting protein and WNT/ β -catenin pathway inhibitor, that was originally identified to disrupts the formation of β -catenin and TCF complexes and inhibit β -catenin-TCF-4-mediated transactivation. Dysregulation of WNT signaling is associated with various human cancer including colon (21), breast (22), prostate cancer (23) and glioma (24). As a negative regulator of WNT signaling, ICAT was at first presumed to be a tumor suppressor gene and its inactivation may result in carcinogenesis. Sekiya *et al* (25) found that overexpression of ICAT induces G2 arrest and cell death in colorectal cancer cells carrying an APC or β -catenin mutation and hepatocellular tumor cells with an Axin mutation (9). Other studies have demonstrated that ICAT inhibited glioma cell proliferation, invasion and induced apoptosis by suppressing Wnt/ β -catenin activity *in vitro* and *in vivo* (26,27). In addition, in accordance with two other WNT/ β -catenin pathway inhibitors, niclosamide and XAV939, ICAT inhibited WNT/ β -catenin pathway activation and exerted antitumor effects in primary cultures of human leiomyoma cells (28). All of these results seem to indicate that ICAT may serve as a tumor suppressor gene. However, some research showed different results. Ectopic overexpression of ICAT increased melanoma cells motility and matrigel invasion which was associated with conversion of an elongated/mesenchymal phenotype to a round/amoeboid phenotype (29,30). Zhang *et al* (31) found that miR-424-5p could block EMT process of anchorage-independent hepatocellular carcinoma cells by directly targeting ICAT, and suppressed hepatocellular carcinoma progression. Therefore, the effects of ICAT is different in various types of cancers. However, its role in cervical cancer was unknown, and we investigated the effects and its mechanism of ICAT on cervical cancer.

β -catenin as the major biochemical target of ICAT is not only a downstream transcriptional activator of the Wnt signaling pathway but also has a significant role in cell-cell adhesion. It binds the cytoplasmic domain of E-cadherin and links the actin cytoskeleton. This complex constitutes a key element in intracellular adhesion (32).

β -catenin is a critical epithelial marker, the lack of its expression could promote EMT of epithelial cells and the cell morphology changed from epithelial morphology to a mesenchymal phenotype characterized (33). This function has been recognized as an important process in the invasion and metastasis of cancer.

In this study, we first detected the expression of ICAT by immunohistochemistry in 41 cervical cancer samples and 30 normal cervical tissues. The results indicated that ICAT was upregulated in cervical cancer and it may play an important role in cervical carcinogenesis and progression. Then, we enforced ICAT expression by recombinant adenovirus to investigate the effects of ICAT on SiHa cells. We used MTT, colony formation assay and flow cytometry assays to analyze cells proliferation. The results showed that ICAT overexpression promoted SiHa cells proliferation and the percentage of cells in the S phase. As is known, metastasis of cervical cancer is

responsible for the vast majority of all poor prognosis. Whether ICAT is involved in cervical cancer invasion and metastasis has been proved. Transwell migration and Matrigel invasion assay revealed that the migration and invasion of SiHa cells were significantly promoted by ICAT, whereas ICAT silencing by siRNAs targeting ICAT induced opposite effects in Caski cells. The above suggested that ICAT promoted metastasis of cervical cancer.

We then investigated how ICAT affects cell migration and invasion of cervical cancer cells. Interestingly, a major cell morphological change from a cobblestone-like morphology to a more mesenchymal morphology with spindle-like shape was observed. The findings indicated that ICAT may be involved in the cervical cancer tumorigenesis and metastases through the regulation of EMT. To further investigate the role of ICAT in the EMT of cervical cancer, the expression of EMT related markers were detected by western blot analysis in SiHa and Caski cells after ICAT overexpression or downregulation. We proved that overexpression of ICAT induced marked upregulation of mesenchymal marker but a downregulation of epithelial marker in SiHa cells and ICAT knockdown induced opposite effects in Caski cells. Immunofluorescence analysis also proved that overexpression of ICAT promotes EMT in SiHa cells. Furthermore, we illuminated that ICAT could disrupt the E-cadherin/ β -catenin complex and reduce the expression of E-cadherin in SiHa cells by immunoprecipitation. The *in vivo* function of ICAT on cervical cancer was also demonstrated in our studies with a nude mouse xenograft model and were consistent with previous studies. ICAT was found more than a decade ago, but most of the studies only focus on its role in β -catenin-dependent nuclear signaling while ignoring its role in EMT. We found ICAT could disrupt the stability of E-cadherin/ β -catenin complex and result in EMT in cervical cancer. However, the precise mechanisms underlying the feature of ICAT and its relation to EMT remain to be defined in cervical cancer.

In summary, we revealed that ICAT expression was upregulated in cervical cancer and participated in the EMT of cervical cancer by inhibiting β -catenin binding to E-cadherin. The *in vivo* function of ICAT on cervical cancer was also demonstrated and was consistent with previous studies. These results support the notion that inhibiting ICAT expression and function may be an effective strategy for cervical cancer therapy.

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