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Original Paper

CDC27 Facilitates Gastric Cancer Cell Proliferation, Invasion and Metastasis via Twist-Induced Epithelial-Mesenchymal Transition

Yongfan Xin[a] Shili Ning[b] Liang Zhang[c] Ming Cui[d]

[a]Department of Interventional Therapy, the First hospital of Shanxi Medical University, Taiyuan, [b]Department of Acute Abdominal Surgery, the Second Affiliated Hospital of Dalian Medical University, Dalian, [c]Department of Interventional Therapy, the Second Affiliated Hospital of Dalian Medical University, Dalian, [d]Department of Emergency, the Second Affiliated Hospital of Dalian Medical University, Dalian, China

Key Words
CDC27 • Gastric Cancer • Twist • EMT

Abstract
Background/Aims: Lymph node metastasis is the primary cause of cancer-related death among patients with gastric cancer (GC), and cell division cycle 27 (CDC27) promotes the metastasis and epithelial-mesenchymal transition in many cancers. Till now, the mechanisms underlying CDC27-induced the epithelial-mesenchymal transition (EMT) of GC are still unclear. ***Methods:*** We analyzed the expression levels of CDC27 and EMT-related biomarkers using immunohistochemistry and Western blot in 60 cases of GC tissues, and then GC cells with CDC27 shRNAs or plasmids were subjected to *in vitro* and *in vivo* assays, including CCK-8, wound healing and transwell assays. ***Results:*** The CDC27 expression was obviously increased in GC tissues, and significantly correlates with EMT-related biomarkers, lymph node metastasis and poor 5-year overall survival. Additionally, *in vitro* and *in vivo* assays demonstrated that silencing of CDC27 expression effectively inhibited GC cell proliferation, invasion and metastasis. Conversely, CDC27 overexpression led to the opposite results. Finally, we demonstrated that Twist shRNA inhibited CDC27-meditated invasion and EMT of GC cells. ***Conclusion:*** CDC27 facilitates gastric cancer cell proliferation, invasion and metastasis via Twist-induced EMT; thus, this study offered a new therapy method for GC patients.

Y.Xin and S. Ning contributed equally to this work.

Liang Zhang
and Ming Cui

Department of Interventional Therapy, the 2nd Affiliated Hospital of Dalian Med. Univ.
Dalian, 116027, Liaoning (China)
E-Mail zhangliangdlmu@163.com; cuimingdlmu@163.com

Introduction

Gastric cancer is the leading cause of cancer-related death among most common malignancy [1-3]. The prognosis in GC patients is unsatisfactory, especially those with advanced TNM stages. The main reason is the occurrence of local and systemic metastasis [4-6]. However, the molecular mechanisms underlying GC metastasis remain largely unknown. Investigating the mechanisms and regulatory networks of GC metastasis will contribute to the identification of novel biomarkers and therapeutic targets of GC.

EMT has a critical role in invasive and metastatic potential of different types of cancers [7-9]. During EMT, the expression of some epithelial cell markers, such as E-cadherin decreases, while the expression of mesenchymal cell markers increases, such as twist and vimentin [10-12]. Besides, increasing reports revealed that CDC27 expression was deregulated in varies of cancers, including breast cancer, and colorectal cancer [13-15]. Feng Z et al. reported that mir-218-2 promotes glioblastomas growth, invasion and drug resistance by targeting CDC27, and that CDC27 expression may be a significant indicator [16]. Accumulating evidence indicates CDC27 may be involved in the invasion and metastasis of gastric cancer cells. However, to our knowledge, the mechanisms of CDC27 in metastasis of gastric cancer remain unclear.

In the present study, we analyzed the expression levels of CDC27 and EMT-related biomarkers using immunohistochemistry and Western blot in 60 cases of GC tissues, and then GC cells with CDC27 silencing or over-expression were subjected to *in vitro* and *in vivo* assays.

Materials and Methods

Clinical specimens

Sixty cases of GC tissues and adjacent non-tumor tissues were collected from GC patients who received surgical treatments between 2012 and 2017 in the Second Affiliated Hospital of Dalian Medical University (Dalian, China). The age range of the patients with GC was 36–79 years old (mean age, 60 years old), with males, 44 and females, 16. None of the patients received chemotherapy prior to surgical treatment. The clinical samples were collected with informed consent from all patients. The specimens were stored in liquid nitrogen prior to being subjected to further experiments. The protocols were approved by the Institutional Research Ethics Committee of the Second Affiliated Hospital of Dalian Medical University (Dalian, China).

Cell culture

The human gastric cancer cell lines (AGS, SNU216, SGC7901, MKN45, MGC803 and KATO-III) and normal gastric epithelial GES-1 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml penicillin and 100 mg/ml streptomycin. All cell cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C.

Immunohistochemistry (IHC)

Paraffin sections (5 μm) of tissues were deparaffinized and dehydrated with graded ethanol. After blocking the endogenous peroxidase with 3.0% H_2O_2, slides were incubated with citric acid buffer to retrieve antigen by heating in 120°C for 15 min. The slides were then blocked with 5% BSA (Sigma, St. Louis, MO, USA) for 1 h and incubated with related primary rabbit monoclonal antibody (Santa Cruz Biotechnologies, Dallas, TX, USA) overnight at 4°C. After that, slides were washed with PBS and further incubated with biotinylated second antibody for 15 min at room temperature. Finally, the slides were stained using the VECTASTAIN Elite ABC HRP kit and DAB reagents. For negative control, PBS was used instead of primary antibody.

IHC evaluation

Briefly, the positivity percentage was scored as 0 (0–10%), 1 (11–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The staining intensity was scored as 0 (no immunoreactivity), 1 (weakly stained), 2 (moderate stained), and 3 (strong stained). The final IHC score (IHCS) was weighted by multiplying the intensity score with percentage score (range, 0-12). Patients were classified into a low expression group (IHCS $\geqslant$6) and a high expression group (IHCS <6) according to the IHCS.

Western blot analysis

RIPA lysis solution and phenylmethylsulfonyl fluoride solution were added to cells, which were then centrifuged at 12, 000 rpm for 15 min at 4°C. The extracted total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, which was subsequently immersed in 5% skim-milk blocking solution for 2 h. The membrane was then incubated with a rabbit anti-human monoclonal antibody and mouse anti-human GAPDH monoclonal antibody (1:1, 000; Santa Cruz Biotechnologies, Dallas, TX, USA) separately at 4°C overnight, before being exposed to a secondary antibody at 25°C for 1 h. Finally, chemiluminescence reagent was added. Relative expression was normalized to GAPDH levels, which were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Transfection

Cells were transfected with CDC27 shRNA using Lipofectamine RNAi reagent (Thermo Fisher Scientific, Rockford, IL, USA). CDC27 mRNA sequences were obtained from Genebank to design primers. Then PCR amplification was performed and the PCR product was subjected to 1% agarose gel (SeaKem GTG; FMC BioProducts, Rockland, Maine) electrophoresis. The recovered CDC27 fragment and vector PCDNA3.1 were digested with HindIII and XhoI (Takara Shuzo Co, Japan), and the gel was recovered by electrophoresis, and then ligated with T4 DNA ligase (New England Biolabs, Beverly, MA) overnight at 4°C. At last, the ligation product was transformed into DH5a. After the single clones were picked, the extracted plasmids were identified and sequenced by double digestion. Then cells were transfected with PCDNA3.1-CDC27 by Lipofectamine-2000 (Thermo Fisher Scientific, Rockford, IL, USA). The protein expression of CDC27 was assessed by Western blot analysis.

Wound healing and transwell assays

For the wound healing migration assay, cells were plated in 24-well plates and allowed to attach overnight. Confluent monolayer cells were scraped using 10 μl pipette tips. At the indicated time points (0 and 48 h), the wound areas were photographed under a microscope (Olympus, Tokyo, Japan). For the transwell invasion assay, 1×10^5 cells were added into the upper chamber of an insert precoated with matrigel (Costar, Cambridge, MA, USA). And 100 μl medium containing 20% FBS were added to the lower part of the chamber. After 24 h of incubation, the invaded cells were fixed with methanol and stained with eosin solution (Beyotime Company, Shanghai, China).

Cell proliferation assay

The transfected cells were seeded into 96-well plates, in DMEM medium supplemented with 10% FBS, at a density of 2, 000 cells/well, and cultured at 37° C. At the end of each experiment, a 10-μl Cell Counting kit-8 (CCK-8) solution was added to each well and subsequently incubated for 2 h at 37°C. The relative optical density (OD) level was evaluated at an absorbance of 450 nm and normalized to that of the controls using a standard microplate reader (Thermo Fisher Scientific, Inc.).

Animal experiment

Balb/c nude mice were used under the animal laboratory conditions according to the approved guidelines of the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Dalian Medical University (Dalian, China) and were performed on the basis of the institutional guidelines. Human tumour xenograft models were created by subcutaneous injection of SGC7901 cells (sh-NC/sh-CDC27) or AGS cells (Vector/CDC27), and then grown for 5 weeks. The size of the generated tumour was evaluated

using a tumour volume calculation formula of (W × W × L)/2. Lung metastatic models were developed by slow injection into the tail vein of mouse. Mice were sacrificed after 5 weeks, and tumours were removed for RNA and protein extraction, haematoxylin and eosin (H&E) staining or IHC staining.

Statistical analysis

Data were analyzed using SPSS software 19.0 version (IBM Corp., Armonk, NY, USA) or GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) and presented as the mean ± standard deviation. Comparisons between two groups were assessed using a non-paired Student's t-test. Comparison among three groups was performed by one-way analysis of variance, and differences between two groups were determined by Newman-Keuls multiple comparison unless the data were not normally distributed, in which case a Kruskal-Wallis test and Nemenyi test were applied. Correlation analysis was conducted using Pearson's or Spearman's correlation. The $\chi 2$ test was performed to analyze the association between protein expression and clinicopathological indicators. Survival curves were generated using the Kaplan-Meier estimator method, which were then compared using the log-rank test. Overall survival was defined as the time between the date of initial therapy and the final follow-up or the time of patient mortality due to any cause. P<0.05 was considered as a statistically significant difference.

Results

CDC27 expression and its association with EMT-related biomarkers in GC samples

Firstly, we detected the expression of CDC27 protein in 60 cases of GC tissues and adjacent normal tissues using Western blot analysis and IHC. We found that CDC27 protein has a significantly higher relative expression level compared with that in adjacent normal tissues (*P*<0.001, Fig. 1A). Furthermore, Twist and Vimentin also had higher relative expressions in GC tissues than those in adjacent normal tissues (both *P*<0.001, Table 1). However, E-cadherin expression was significantly relatively lower in GC tissues than that in adjacent normal tissues (*P*<0.001, Table 1). And then, the clinicopathological analysis

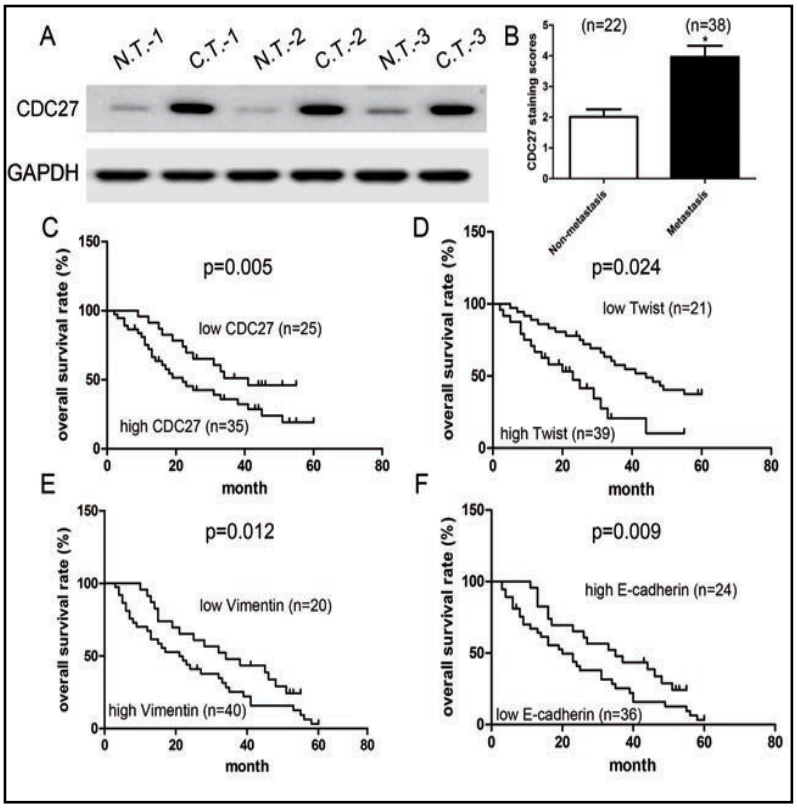

Fig. 1. Expression and significance of CDC27 and EMT biomarkers in gastric cancer tissues and normal gastric tissues. (A) Western blot assay was used to compare CDC27 expression in three representative gastric cancer tissues (C.T.) and normal tissues (N.T.) from different patients. GAPDH was used as internal control. (B) The average staining scores of CDC27 expression in GC patients with or without metastasis. (C-F) Survival curves of gastric cancer patients with CDC27, E-cadherin, Twist or Vimentin expression. *P<0.05 v.s. non-metastasis. Bars show the means ± SD of three independent experiments.

from 60 cases of GC tissues suggested that the relative expressions of CDC27, E-cadherin, Twist and Vimentin proteins obviously correlated with T stage, pTNM stage and lymph node metastasis, respectively (all $P<0.05$, Table 2). It should be noted that patients with lymph node metastasis had significantly higher relative CDC27 expression levels than those without lymph node metastasis ($P<0.001$, Fig. 1B), indicating that CDC27 may have an important role in GC metastasis. Besides, relative CDC27 expression was observed to markedly correlate with the relative expression of E-cadherin, Twist and Vimentin ($P<0.001$, $P<0.001$, $P=0.046$, respectively; Table 3). Finally, our survival analysis exhibited that positive expression of CDC27, Twist or Vimentin had a negative relationship with five-year overall survival of post-operative 60 cases of GC patients ($P=0.005$, $P=0.024$, $P=0.012$, respectively; Fig. 1C-E). However, positive expression of E-cadherin had a positive relationship with overall survival of post-operative GC patients ($P=0.009$, Fig. 1F).

CDC27 facilitates GC cell metastasis

To explore the role of CDC27 in the metastasis of gastric cancer, the expression level of CDC27 was analyzed in 6 kinds of GC cell lines, including AGS, SNU216, SGC7901, MKN45, MGC803 and KATO-III, and normal gastric epithelial GES-1 cells. We found that CDC27 showed the highest expression in SGC7901 and SNU216 cells, and had the lowest expression in AGS cells and GES-1 cells ($P<0.05$, Fig. 2A). Accordingly, SGC7901 and SNU216 cells were used for subsequent transfection

Table 1. Expressions of CDC27, E-cadherin, Twist and Vimentin in gastric cancer and normal tissues

Proteins	cancer tissues	normal tissues	P-value
CDC27			
Positive	35	19	0.003
Negative	25	41	
E-cadherin			
Positive	24	45	<0.001
Negative	36	15	
Twist			
Positive	39	18	<0.001
Negative	21	42	
Vimentin			
Positive	40	20	<0.001
Negative	20	40	

Table 2. Correlation between CDC27, E-cadherin, Twist and Vimentin expression and clinicopathological features in gastric cancer

Indicators	n	CDC27			E-cadherin			Twist			Vimentin		
		+	–	P-value	+	–	P-value	+	–	P-value	+	–	P-value
Age (year)													
≥60	41	23	18	0.606	15	26	0.428	25	16	0.337	25	16	0.169
<60	19	12	7		9	10		14	5		15	4	
Gender													
Male	44	26	18	0.694	16	28	0.340	27	17	0.327	29	15	0.837
Female	16	9	7		8	8		12	4		11	5	
Tumor size (cm)													
≥5	36	23	13	0.285	9	27	0.004	21	15	0.185	23	13	0.576
<5	24	12	12		15	9		18	6		17	7	
Lauren's classification													
Diffuse	16	9	7	0.843	6	10	0.812	9	7	0.392	8	8	0.099
Intestinal	44	26	18		18	26		30	14		32	12	
Lymphatic vessel invasion													
Yes	28	17	11	0.726	10	18	0.526	21	7	0.129	20	8	0.464
No	32	18	14		14	18		18	14		20	12	
T stage													
$T_1 + T_2$	29	21	8	0.032	6	23	0.003	24	5	0.005	23	6	0.045
$T_3 + T_4$	31	14	17		18	13		15	16		17	14	
pTNM stage													
I + II	33	23	10	0.048	9	24	0.026	26	7	0.013	27	6	0.006
III + IV	27	12	15		15	12		13	14		13	14	
Lymph node metastasis													
Yes	38	29	9	<0.001	10	28	0.005	29	9	0.016	29	9	0.037
No	22	6	16		14	8		10	12		11	11	

using CDC27 shRNA, and AGS cell lines were selected for CDC27-overexpression. Western blot analysis revealed the endogenous expression of CDC27 protein was obviously silenced by CDC27 shRNA (P<0.05, Fig. 2B and C), and obviously increased by CDC27 overexpression plasmids (P<0.05, Fig. 2 B and C). As shown in Fig. 3, both the migration and invasion ability of SGC7901 cells that were transfected with the shRNAs targeting CDC27 were significantly impaired, which was identical with those in SNU216 cells (P<0.05). Further, our data revealed CDC27 overexpression reversed the impaired migration and invasion capabilities of gastric cancer cells and increased the migration and invasion cell number in AGS cells (P<0.05). These data indicated that CDC27 facilitated the metastasis of GC cell lines.

CDC27 facilitates GC cell invasion via Twist-induced EMT

In order to figure out whether CDC27 enhanced the invasiveness of GC cells via EMT, the EMT biomarkers (E-cadherin, Snail, Twist, and Vimentin) were detected using Western blot analysis. Results showed that the expression of E-cadherin was obviously elevated in CDC27-silenced SGC7901 or SNU216 cells, accompanied by an obvious reduce in the expressions of Vimentin and Twist (P<0.05, Fig. 4A and B). However, AGS cells with CDC27 overexpression had opposite effects (P<0.05, Fig. 4C). Notably, the expression of Snail was not obviously altered in

Table 3. Correlation of CDC27 with E-cadherin, Twist and Vimentin in gastric cancer tissues

	CDC27		χ^2	P-value
	Positive	Negative		
E-cadherin				
Positive	6	18	18.29	<0.001
Negative	29	7		
Twist				
Positive	33	6	31.67	<0.001
Negative	2	19		
Vimentin				
Positive	32	18	3.96	0.046
Negative	3	7		

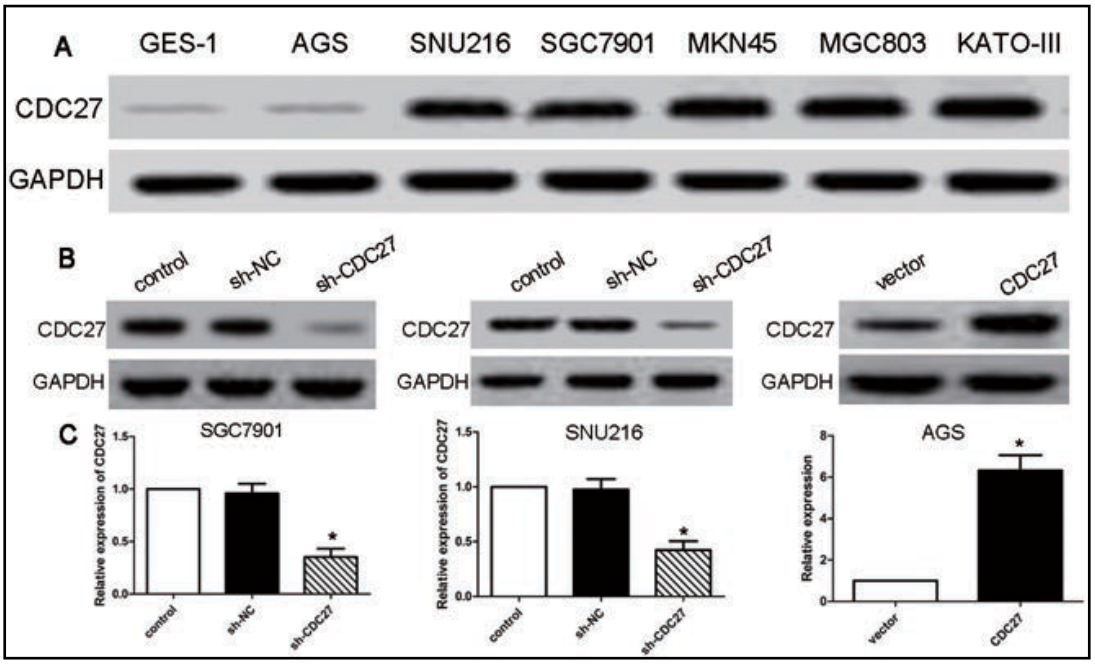

Fig. 2. Expression of CDC27 protein in GC cells and normal gastric cells. (A) Relative expression of CDC27 protein in gastric cancer cell lines (AGS, SNU216, SGC7901, MKN45, MGC803 and KATO-III) and normal gastric epithelial GES-1 cells were measured by Western blot. (B-C) Relative expression of CDC27 protein was detected by Western blot in CDC27-knockdown SGC7901 and SNU216 cells and CDC27 overexpressing AGS cells. GAPDH was used as internal control. *P<0.05 v.s. control or sh-NC. Bars show the means ± SD of three independent experiments.

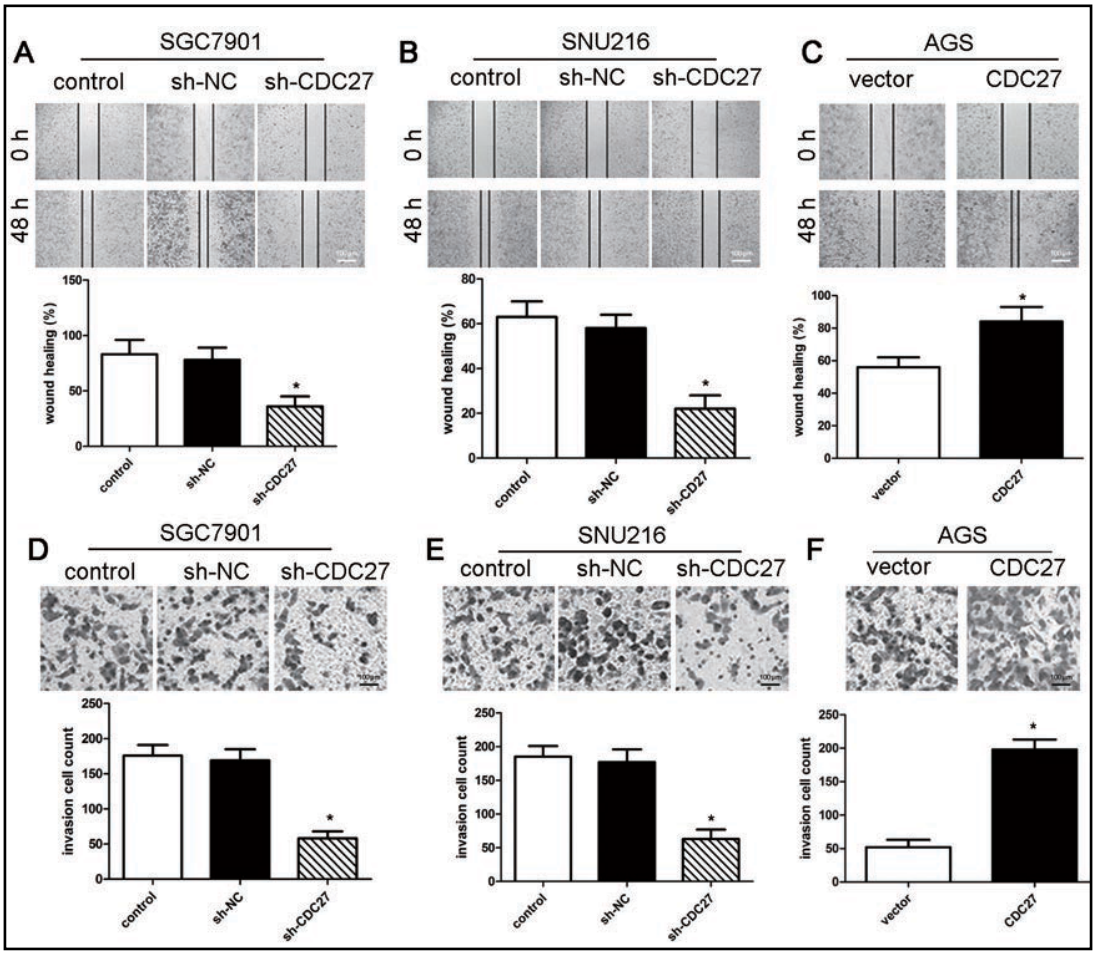

Fig. 3. Effects of CDC27 silencing and overexpression on GC cells migration and invasion. (A–C) Effects of silenced CDC27 expression on migratory ability of SGC7901 and SNU216 cells by wound-healing assay. (D–F) Effects of overexpressed CDC27 on invasive ability of AGS cells by transwell invasion assay. *P<0.05 v.s. control or sh-NC. Bars show the means ± SD of three independent experiments.

SGC7901, SNU216 and AGS cells (Fig. 4A-C). Next, we continued to explore the role of Twist in CDC27-mediated EMT, and found that Twist shRNA reversed CDC27-mediated EMT biomarkers in AGS cells (P<0.05, Fig. 4D). As expected, Twist shRNA significantly inhibited the CDC27-mediated invasion of AGS cells (P<0.05, Fig. 4E).

CDC27 facilitates GC cell metastasis in vivo

In this work, six-week-old male BALB/c nude mice were utilized for tumourigenicity. At 24 h after transfection, SGC7901 cells (sh-NC/sh-CDC27) or AGS cells (Vector/CDC27) were inoculated in nude mouse armpit to construct xenograft tumor model nude mice. Vital signs of nude mice were observed, and the mice were sacrificed in 5th week to extract tumor tissues. According to HE staining, we observed that nude mice containing SGC7901 cells with sh-CDC27 had significantly less lung metastasis nodules than the sh-NC group (P<0.05, Fig. 4F). Conversely, nude mice containing AGS cells with CDC27 overexpression had more lung metastasis nodules than the vector control (P<0.05, Fig. 4G). In addition, the immunohistochemical analysis revealed that the expression of CDC27 in tumor tissues containing SGC7901 cells with sh-CDC27 was lower compared with the sh-NC group. However, the expression of E-cadherin in tumor tissues containing SGC7901 cells with sh-CDC27 was higher compared with the sh-NC group. As expected, AGS cells with CDC27 overexpression had the inverse effects.

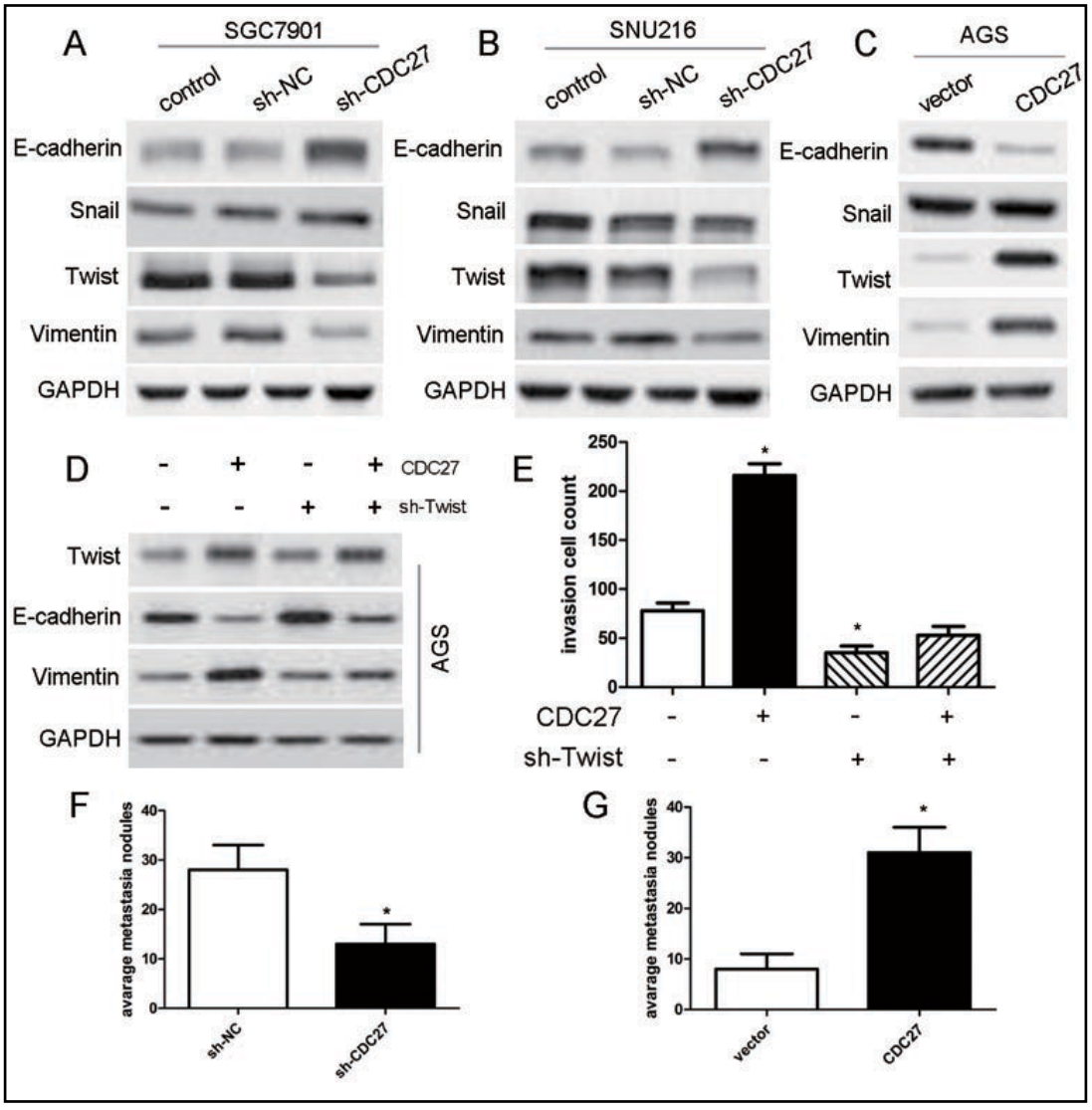

Fig. 4. CDC27 induced cell EMT and invasion via a Twist-dependent mechanism in vitro and in vivo. (A,B) Influences of CDC27-depletion on EMT-related biomarkers in SGC7901 and SNU216 cells. (C) Effects of CDC27 overexpressing on EMT-related markers in AGS cells. (D-E) Insight into a Twist-dependent mechanism of CDC27-meditated cell EMT and invasion, by Western blot and transwell assay. GAPDH was used as internal control. *P<0.05. (F-G) Lung metastatic nodules were observed in HE-stained samples. The number of average lung metastatic nodules was calculated. *P<0.05 v.s. vector or sh-NC. Bars show the means ± SD of three independent experiments.

CDC27 also promotes GC cell proliferation

In this work, we investigated the cell proliferation using in-vitro and in-vivo assay to investigate the potential effect of cell proliferation on CDC27-induced migration and invasion. CCK-8 assay revealed that proliferation rates of three GC cells showed significant differences (P<0.05, Fig. 5A). Based on in-vitro assay, we further determine the effect of CDC27 on tumor formation using *in vivo* assay, SGC7901 cells with sh-CDC27 or AGS cells with CDC27 plasmids were inoculated subcutaneously into nude mice respectively. The mice were sacrificed in 5th week to extract tumor tissues. The tumour volume was calculated based on the formula: volume (mm^3) = (short diameter)^2 × (long diameter)/2. As expected,

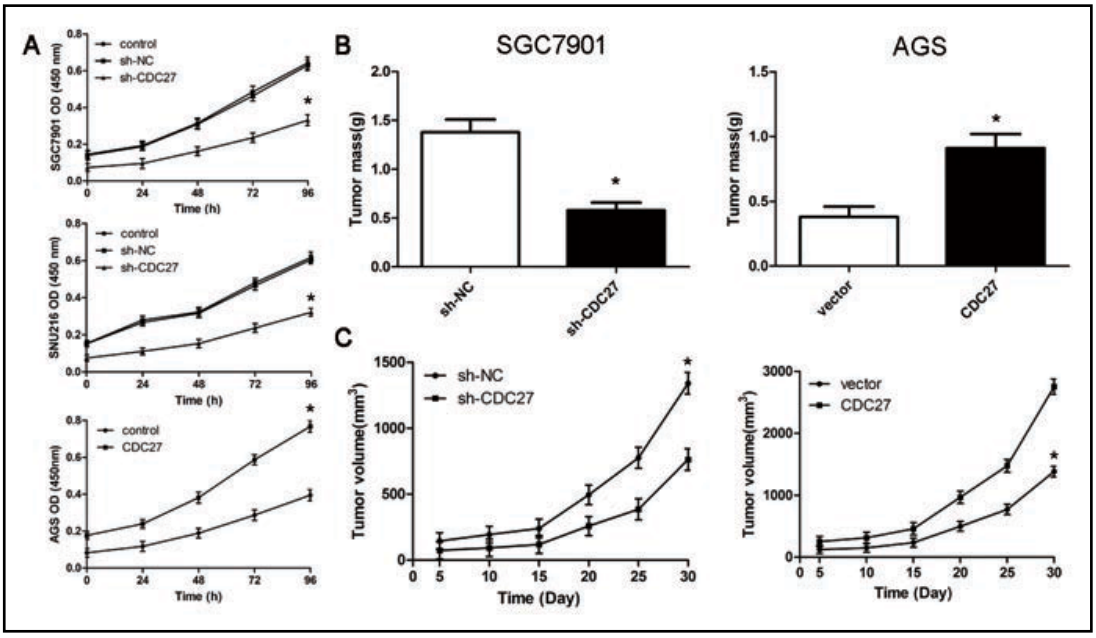

Fig. 5. Effects of CDC27 silencing and over-expression on GC cells proliferation. (A) Effects of CDC27 knockdown and over-expression on the proliferation of GC cells (SGC7901, SNU216 and AGS) in vitro by CCK-8 assay. (B-C) Influences of CDC27 knockdown and overexpression on the tumor formation of GC cells (SGC7901 and AGS) via the subcutaneous injection into nude mice, as well as the measurement of tumor mass and tumor volume. *P<0.05 v.s. vector or sh-NC. Bars show the means ± SD of three independent experiments.

both weight and the volume of implanted tumors also showed significant differences from their controls (P<0.05, Fig. 5B and C). These results indicated that CDC27 promotes GC cell proliferation, which further facilitated GC cell migration and invasion.

Discussion

CDC27 is a core subunit of anaphase-promoting complex/cyclosome (APC/C), which is required for binding of CDH1 and CDC20 to recognize and degrade target substrates. In recent years, CDC27 has been reported to be involved in cell proliferation, migration and invasion in many kinds of cancers [17, 18]. However, the effect of CDC27 expression on the invasion and metastasis of gastric cancer has not yet been clearly elucidated. In the present study, CDC27 was highly expressed in GC tissues than that in adjacent normal tissues, and obviously correlated with lymph node metastasis, EMT biomarkers and an unfavourable prognosis, suggesting that CDC27 may be involved into the metastasis and EMT of gastric cancer cells.

As known to all, EMT is closely linked to tumor metastasis and invasion. EMT process is featured by loss of cell-cell adhesion mediated by E-cadherin, mesenchymal marker expression and thus increased cell motility and invasiveness [19-21]. In view of the role of CDC27 in the migration and invasion of GC cells, we first examined the effects of CDC27 on the EMT process [20, 21]. Qiu L et al. reported that CDC27 induces metastasis and invasion in colorectal cancer via the promotion of epithelial-to-mesenchymal transition [15]. Feng Z et al. also demonstrated that Cdc27 is a target for glioblastomas growth, invasion and drug resistance [16]. Our findings showed that the expression levels of E-cadherin were dramatically increased and that the expression levels of Snail and Twist were correspondingly significantly decreased in the shCDC27 cells. Furthermore, we explored corresponding signalling pathways involved into CDC27-induced EMT. We found that CDC27 facilitated

cells invasion and metastasis via Twist pathway, leading to initiation of EMT. And silencing of Twist expression could reverse this process, suggesting that Twist played an important role in CDC27-induced EMT. Therefore, more signals involved in CDC27-induced EMT in the metastasis of gastric cancer need further identification.

Besides, we explored whether the role of CDC27 in GC cell migration and invasion was attributed to GC cell proliferation. We demonstrated that CDC27 indeed affected gastric cancer cell proliferation to potentially influence cell migration and invasion. On the other hand, there are still many issues that remain to be addressed. For example, current reports showed that CDC27 expressions are involved in different cancers. So, we need to investigate other downstream molecules of CDC27 in more future studies.

Conclusion

Taken together, our study validated that the high expression of CDC27 was obviously related with GC metastasis and poor prognosis, and CDC27 facilitates gastric cancer cell invasion and metastasis via Twist-induced EMT. Overall, our work may provide a new and useful therapy target for gastric cancer patients.

Disclosure Statement

The authors have declared that no conflict of interests exists.

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