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Original Article

TBX3 promotes the epithelial mesenchymal transition of cervical cancer by upregulating ID1

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Abstract: In this study, we aim to investigate the role and mechanism of T-box transcription factor 3 (TBX3) in cervical cancer. The mRNA and protein expression of TBX3, inhibitor of DNA binding 1 (ID1), and epithelial mesenchymal transition (EMT) markers (E-Cadherin, N-Cadherin, and vimentin) were measured using qRT-PCR and Western blot. shTBX3 and shID1 were transfected into SiHa cells to knockdown TBX3 and ID1. The metastasis and invasion abilities of cervical cancer cells were determined using a wound healing assay and an invasive assay. The shTBX3- and shID1-transfected SiHa cells were injected into nude mice using a xenograft tumor growth model. We found that TBX3 and ID1 were highly expressed in cervical cancer cells. Importantly, silencing TBX3 and ID1 significantly reduced the migration and metastasis of cervical cancer cells. In addition, silencing TBX3 and ID1 significantly inhibited the EMT, evidenced by the increased E-cadherin, and decreased N-cadherin and vimentin. The size and weight of the xenograft tumor were significantly reduced by shTBX3 and shID1. We demonstrate that TBX3 or ID1 knockdown can effectively inhibit cervical cancer cells migration and invasion. These findings indicate that TBX3 and ID1 can act as potential therapeutic targets for the prevention and treatment of cervical cancer.

Keywords: Cervical cancer, TBX3, ID1, EMT

Introduction

Cervical cancer is one type of common tumor among women worldwide, negatively influencing the quality of life in women [1-3]. Persistent high-risk human papillomavirus (HPV) infection is the main cause of cervical cancer. However, the prevention and treatment for cervical cancer are still an issue. Currently, surgical treatment of patients with early cervical cancer is ideal, which can preserve nerves and even fertility. However, for patients with advanced cervical cancer, surgical treatment has a higher risk of complications post-surgery. Although the survival period of patients is effectively prolonged [4], pelvic organ resection may negatively influence the quality of life in women in the long-term post-surgery. Moreover, radiotherapy has been used to kill tumor cells with radiation. The radiotherapy technology is relatively mature, the positioning is more accurate, and the application is relatively wide [5]. However, the long-term adverse reactions caused by radiotherapy remain a problem. Currently, there are no effective pharmacological agents that are sensitive to cervical cancer.

T-box transcription factor 3 (TBX3) is a transcription factor that has been shown to be closely related to various types of cancers including cervical cancer. It was found that overexpression of TBX3 is associated with the progression and migration of several types of tumor cells, e.g., hepatocellular carcinoma [6], breast cancer tumors [7], and melanoma [8]. Studies have found that over-expression of TBX3 in HeLa cells leads to the reduction of mRNA and protein levels of endogenous phosphatase and tensin homolog (PTEN) [9]. In addition, transcriptional activity analysis showed

that TBX3 inhibits PTEN activity, which affects the progression and metastasis of cancer cells [10]. These findings suggest that the overexpression of TBX3 suppresses cell apoptosis and promotes cell proliferation and migration [11]. TBX3 could be a potential target for the treatment and prevention of cervical cancer.

A large number of research results show that the inhibitor of DNA binding (ID) gene is a key regulator of tumor cells [12, 13]. It has been found that ID is highly expressed in human tumor specimens and cultured tumor cells *in vitro* [14, 15]. The role of ID1 in tumorigenesis and development has been widely recognized in a variety of tumors including pancreatic cancer, breast cancer, cervical cancer, melanoma, prostate cancer, liver cancer [14-18]. Studies have shown that in melanoma, TBX3 promotes epithelial mesenchymal transition (EMT) and tumor progression by up-regulating the expression of ID1. However, the role and mechanism of TBX3 and ID1 on the invasion of cervical cancer are not clear [19]. In this study, we aim to investigate whether TBX3-ID1 signaling pathway promotes the EMT and migration in SiHa cervical cancer cells and promotes the progress of xenograft tumors using *in vitro* and animal xenograft tumor models.

Methods

Clinical specimens

Cervical tissue samples were collected from cervical cancer patients and normal subjects. The mRNA was extracted using RNA extraction kit (Qiagen, Valencia, CA, USA). The total protein samples were extracted from cervical tissues using radioimmunoprecipitation assay buffer (RIPA) lysate containing protease inhibitors. The study was approved by the ethics committee of Bethune International Peace Hospital, and written consent was obtained from all participants.

Cell culture

Human cervical cancer cell line (SiHa cells) was purchased from the Cell Resource Center of the Institute of Basic Medicine (Chinese Academy of Medical Sciences, Beijing, China). The cells were cultured in Iscove's modified Dulbecco medium supplemented with 10% fetal bovine serum (FBS, Gibco).

Xenograft transplant

Animal studies were approved by the ethics committee of the 980th Hospital of the Joint Logistic Support Force of PLA (Bethune International Peace Hospital). BALB/C nude mice were purchased from GemPharmatech (Nanjing, China). Eight- to ten-week-old of female mice were randomly divided into two groups, control or SiHa cells treated with shTBX3 or shID1. SiHa cells (1×10^7 cells/mouse) were injected subcutaneously into the right groin. The tumor size was measured every 3 days for 28 days. The following equation was used to calculate the tumor volume:

Volume = [length (in millimeters) × width^2 [in square millimeters])/2.

Plasmid transfection

SiHa cells were transfected with an *ID1*-overpressing plasmid, sh*TBX3* plasmid (sh*TBX3*-1 sequence: CGT GGT TTA TAT GTC CGG GAT, sh*TBX3*-2 sequence: GCT GCT GAT GAC TGT CGT TAT), sh*ID1* plasmid (sh*ID1*-1 sequence: CCT ACT AGT CAC CAG AGA CTT, sh*ID1*-2 sequence: ACT CGG AAT CCG AAG TTG GAA) or a controlled scrambled plasmid (Genechem, Shanghai, China). The transfection was carried out using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Cells were harvested for mRNA and protein analysis after being transfected for 48 hours or for wound healing, invasion assay and tumor plantation on mice after being transfected for 24 hours.

RNA isolation and real-time qPCR

Total RNA was extracted from cervical tissue samples or cultured cells using an RNA extraction kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using a cDNA Synthesis Kit (TaKaRa, DaLian, China). The primers for the genes were listed below: *TBX3*: forward: 5'-CCCGGTTCCACATTGTAAG-AG-3'; reverse: 5'-GTATGCAGTCACAGCGATGA-AT-3'. *ID1*: forward: 5'-CTGCTCTACGACATGAAC-GG-3'; reverse: 5'-GAAGGTCCCTGATGTAGTCG-AT-3'. *GAPDH*: forward: 5'-GTCCACCACCCTGT-TGCTGTA-3'; reverse: 5'-CTTCAACAGCGACACC-CACTC-3'. *E-Cadherin*: forward: 5'-CGAGAGC-TACACGTTCACGG-3'; reverse: 5'-GGGTGTCGA-GGGAAAAATAGG-3'. *N-Cadherin*: forward: 5'-

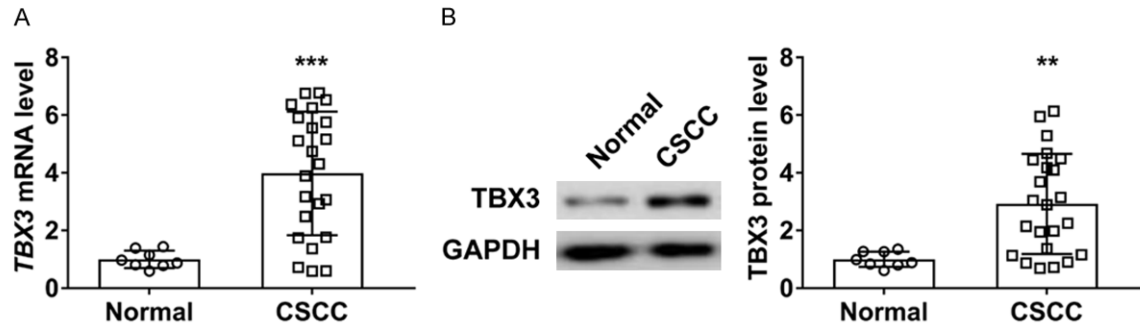

Figure 1. TBX3 was upregulated in cervical squamous cell carcinoma specimens. A. The mRNA level of *TBX3* in normal and CSCC specimens was determined by RT-qPCR. B. The protein level of TBX3 in normal and CSCC specimens was determined by western blot. 8 normal cervix specimens, 23 CSCC specimens. $**P < 0.01$, $***P < 0.001$.

TCAGGCGTCTGTAGAGGCTT-3'; reverse: 5'-ATGCACATCCTTCGATAAGACTG-3'. *Vimentin*: forward: 5'-GACGCCATCAACACCGAGTT-3'; reverse: 5'-CTTTGTCGTTGGTTAGCTGGT-3'. The relative expression was normalized to *GAPDH*, and analyzed by the comparative threshold cycle ($2^{-\Delta\Delta ct}$) method.

Western blot

The total protein samples were extracted from cervical tissues or cultured cells using RIPA lysate containing protease inhibitors. The total protein concentrations were measured using a BCA Protein Assay Kit (Beyotime). The same number of proteins (50 μg) were loaded and separated by a 10% SDS-PAGE gel. Subsequently, the proteins were transferred to polyvinylidene difluoride membranes. The blots were blocked with 5% bovine serum albumin or non-fat milk and probed overnight with primary antibodies against E-cadherin (1:500, Cell Signaling Technology, Cat #3195), N-cadherin (1:500, Cell Signaling Technology, Cat #13116), Vimentin (1:1000, Cell Signaling Technology, Cat #5741), TBX3 (1:1000, R&D, Cat #AF4509), ID1 (1:500, Abcam, Cat #ab168256) and GAPDH (1:5000, Cell Signaling Technology, Cat #2118). Secondary antibodies were applied for 1 hour. Finally, the target bands were detected using enhanced chemiluminescence (ECL) reagents (ThermoFisher, MA, USA).

Wound healing and invasion

SiHa cells were seeded at a density of 1×10^7 cells/well and transfected with shRNA oligonucleotides or plasmid for 24 hours. Then a scratch wound was created using a sterile pipette tip. The wound healing and invasion were imaged using an inverted microscope at 48 hours post scratch. The relative wound closure was measured as a ratio of the occupied area to the total area using an Olympus IX71 imager.

Statistical analysis

The software GraphPad Prism version 7 (San Diego, CA) was used for data analysis. The measurement data were tested for normality and expressed as mean ± standard deviation (SD). Comparisons between the two groups were performed with a Student's t-test. Comparisons between multiple groups were performed with a two-way ANOVA test or a one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

TBX3 was upregulated in cervical squamous cell carcinoma specimens

To determine the mRNA and protein expression levels of the TBX3, we collected cervical specimens from patients with cervical cancer and healthy subjects and analyzed the TBX3 mRNA and protein expression using qRT-PCR and Western blot. We found that compared with normal tissues, TBX3 mRNA and protein levels were significantly higher in cervical cancer tissues in patients with cervical cancer (**Figure 1A** and **1B**, $P < 0.001$, $P < 0.01$, respectively). These findings suggest that TBX3 could be a potential target for the treatment of cervical cancer.

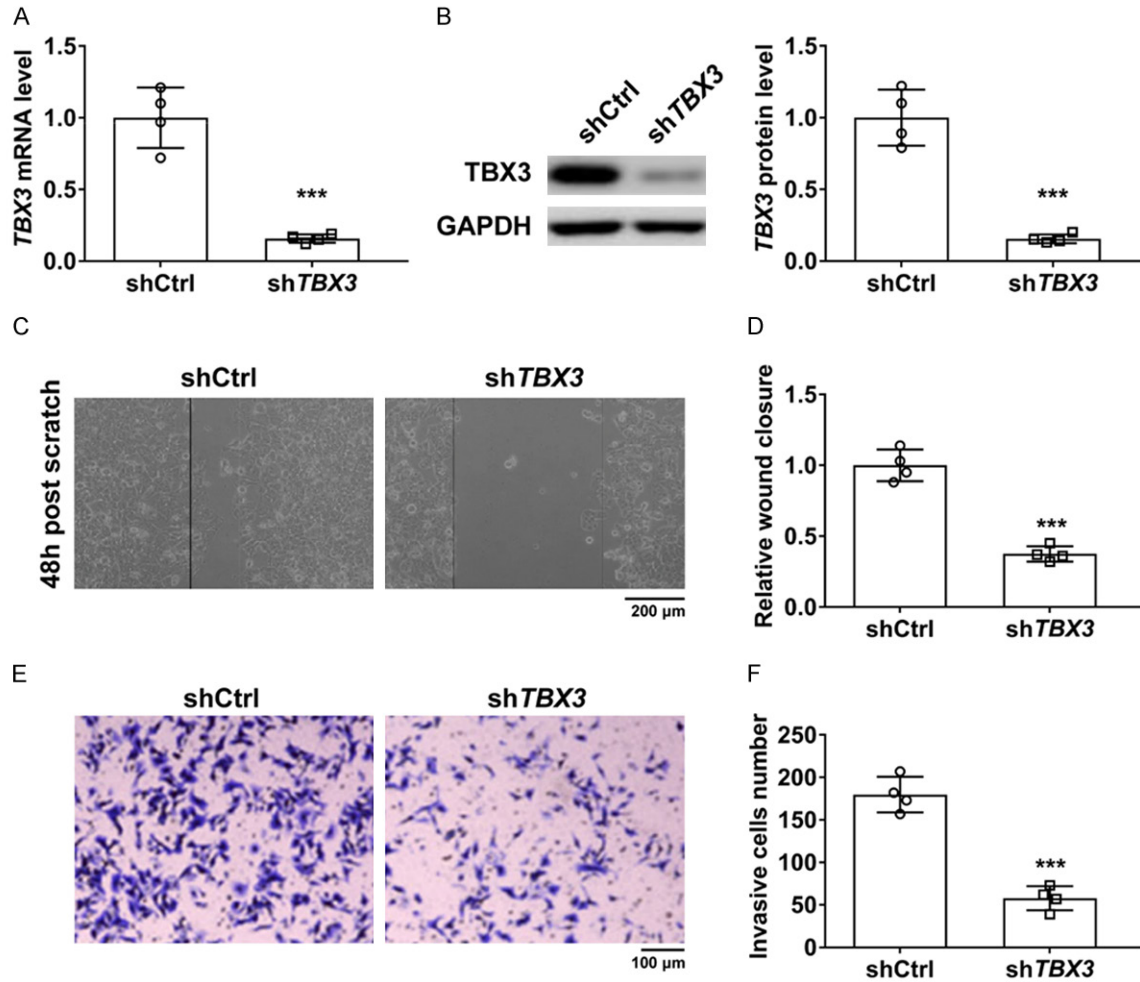

Figure 2. TBX3 promoted the migration and invasion of cervical cancer cells. (A and B) The mRNA (A) and protein (B) level of TBX3 in shCtrl- (shControl) and sh*TBX3*-1-treated SiHa cells. (C and D) The relative wound closure in wound healing assay. (E and F) Invasive cells in trans-well invasion assay. 4 wells per group. ****P* < 0.001.

TBX3 promoted the migration and invasion of cervical cancer cells

Next, to investigate whether TBX3 can serve as a target to treat and manage cervical cancer, we transfected the SiHa cells with shTBX3 to knockdown TBX3 using shRNA. To confirm TBX3 knockdown, we measured the mRNA and protein expressions of TBX3. The mRNA and protein levels of TBX3 in shTBX3 cells were significantly reduced compared with shCtrl cells ($P < 0.001$, **Figures 2A**, **2B**, S1A, S1C, S1D). The results showed that knocking down TBX3 remarkably inhibited the ability of wound closure of cervical cancer cells relative to the shCtrl cells (**Figures 2C**, **2D**, S1I). In parallel, the invasive cell number was significantly reduced in the shTBX3 cells when compared with shCtrl cells (**Figures 2E**, **2F**, S1J).

TBX3 mediated the expression of EMT related genes in cervical cancer cells

To investigate the role of TBX3 in regulating the ability of tumors to migrate, we evaluated the proteins related to epithelial-mesenchymal transition (EMT), including E-Cadherin, N-Cadherin and Vimentin. We observed that the protein level of E-Cadherin was significantly augmented when silencing TBX3 compared to shCtrl (**Figures 3A**, **3B**, S1K, S1L). In contrast, our results showed that the protein levels of N-Cadherin and Vimentin were significantly reduced in the shTBX3 cells when compared to

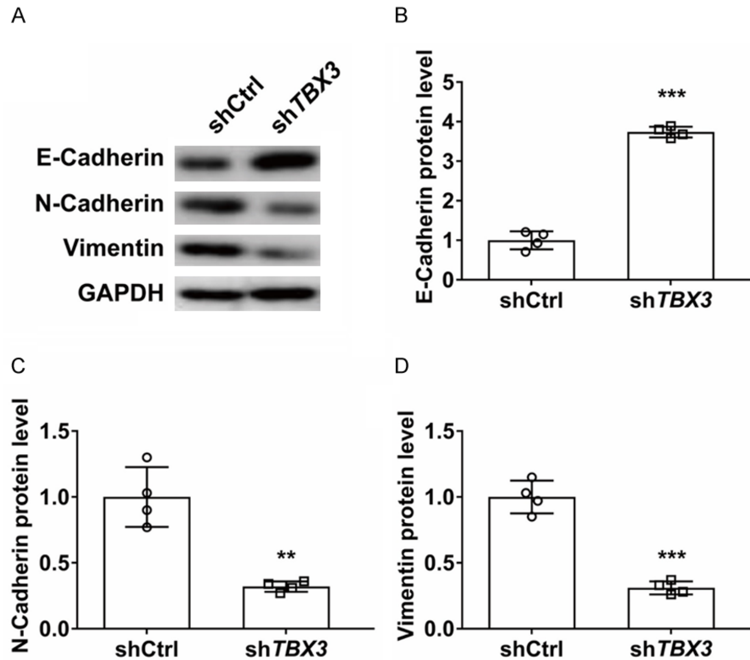

Figure 3. TBX3 mediated the expression of EMT related genes in cervical cancer cells. The protein level of E-Cadherin (A and B), N-Cadherin (A and C) and Vimentin (A and D) in shCtrl- and sh*TBX3*-1-treated SiHa cells. 4 wells per group. $**P < 0.01$, $***P < 0.001$.

the shCtrl cells (**Figures 3A**, **3C**, **3D**, S1K, S1M, S1N). These results suggest that silencing TBX3 effectively inhibits EMT in cervical cancer cells.

TBX3 mediated the expression of ID1 in cervical cancer cells

Furthermore, to determine whether silencing TBX3 has an impact on ID1 expression, we measured the mRNA and protein levels of ID1. We observed that both mRNA and protein expression of ID1 were significantly reduced in shTBX3 cells relative to shCtrl (**Figures 4A**, **4B**, S1B, S1C, S1E).

ID1 promoted the migration and invasion of cervical cancer cells

Therefore, to investigate the role of ID1 in promoting migration and invasion of cervical cancer cells, we used sh*ID1*-treated SiHa cells to knockdown ID1. ID1 gene knockdown was confirmed using qRT-PCR and western blot (**Figures 5A**, **5B**, S1F-H). The wound healing assay showed that silencing ID1 significantly inhibited cell wound closure (**Figures 5C**, **5D**, S1I). In line with the wound healing results, we observed that the invasion ability in shID1 cells was significantly reduced when compared to shCtrl cells (**Figures 5E**, **5F**, S1J). These results suggest that ID1 plays an essential role in promoting the migration and invasion of cervical cancer cells.

Similarly, we observed that shID1 significantly increased E-Cadherin protein expression compared to shCtrl cells, whereas the protein levels of N-Cadherin and Vimentin were significantly reduced in shID1 cells vs. shCtrl cells (**Figures 6A-D**, S1K-N).

We treated ID1-overexpressing SiHa cells with shTBX3 and analyzed their migration and invasion and the mRNA and protein expression levels of EMT-related genes. The data revealed that ID1 overexpression promoted migration and invasion (Figure S2A and S2B), and increased mRNA and protein expression levels of EMT-related genes (Figure S2C-I), which could not be altered by shTBX3 treatment (Figure S2). In addition, we examined the expression of ID1 and EMT-related genes in cervical cancer tissues. An upregulation of ID1, N-Cadherin and Vimentin and a downregulation of E-Cadherin were observed in cervical cancer tissues compared to normal tissues (Figure S3A-E).

TBX3-ID1 axis mediated the progression of cervical cancer cells in xenograft tumor model

To further investigate the role of TBX3-ID1 in regulating tumors *in vivo*, we used a xenograft tumor model by injecting cervical cancer cells in nude mice. Tumor volume and tumor weight in shTBX3 and shID1 were significantly reduced after 28 days when compared with shCtrl. In agreement with the *in vitro* results, the protein level of E-cadherin was remarkably increased in shTBX3 and shID1 treatment groups relative to the shCtrl group (**Figure 7A**, **7B**). The protein levels of ID1, N-Cadherin and Vimentin were significantly lower in shTBX3 and shID1 groups

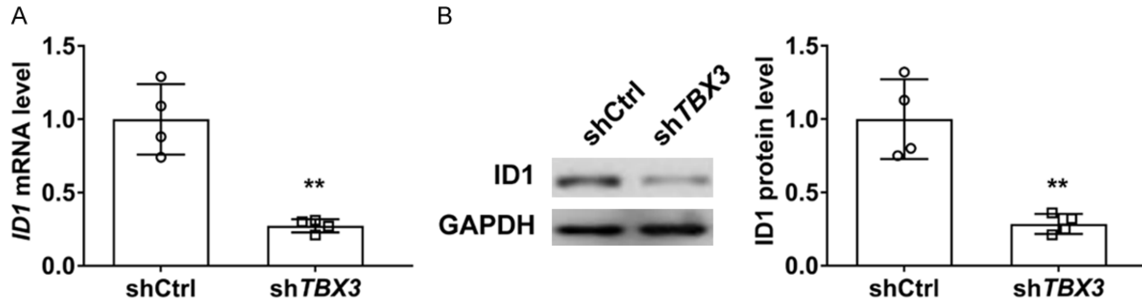

Figure 4. TBX3 mediated the expression of ID1 in cervical cancer cells. The mRNA (A) and protein (B) level of ID1 in shCtrl- and sh*TBX3*-1-treated SiHa cells. 4 wells per group. $^{**}P < 0.01$.

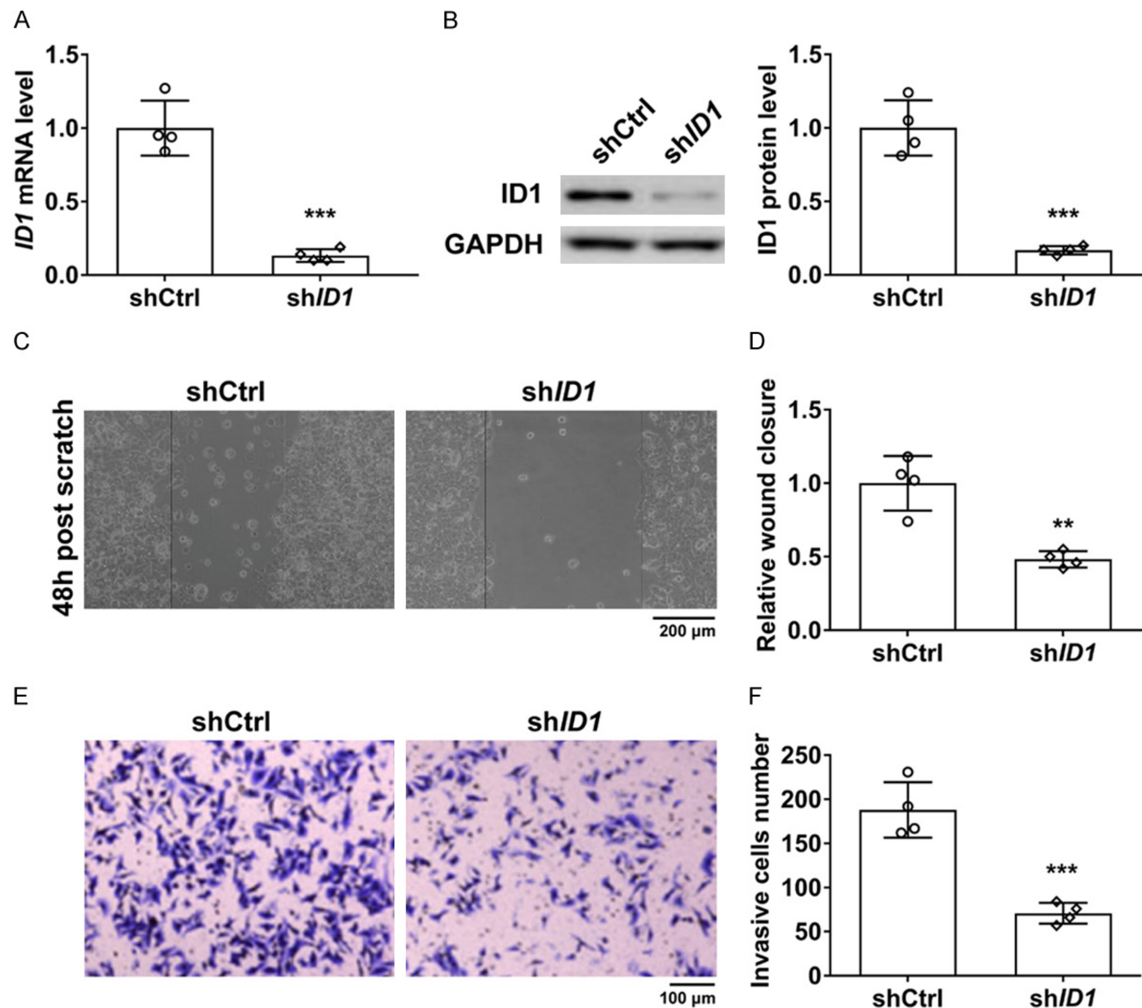

Figure 5. ID1 promoted the migration and invasion of cervical cancer cells. (A and B) The mRNA (A) and protein (B) level of ID1 in shCtrl- and shID1-1-treated SiHa cells. (C and D) The relative wound closure in wound healing assay. (E and F) Invasive cells in trans-well invasion assay. 4 wells per group. $^{**}P < 0.01$, $^{***}P < 0.001$.

than the shCtrl group (**Figure 7C-G**). These findings suggest that silencing TBX3 or ID1 can effectively inhibit cervical tumor progression and migration.

Discussion

Data from GLOBOCAN show that in 2020, there were 604,000 new cases of cervical cancer

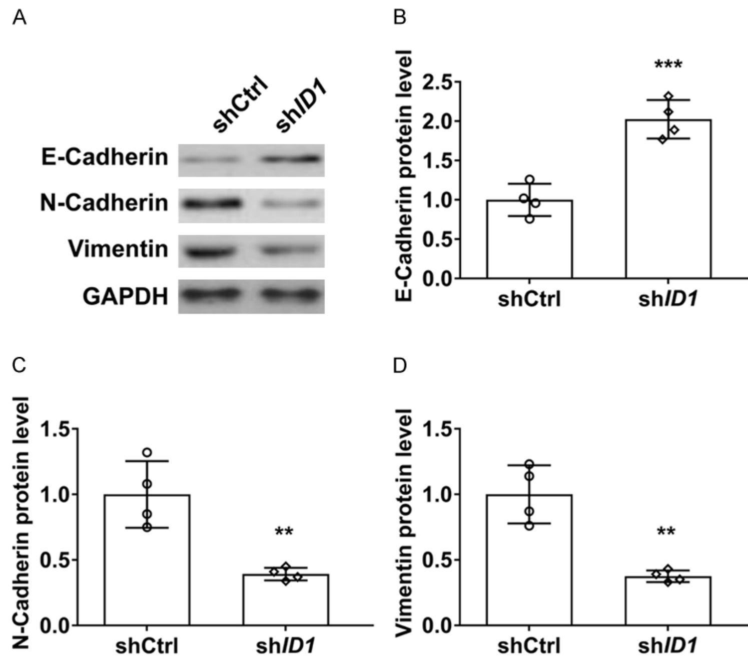

Figure 6. ID1 mediated the expression of EMT related genes in cervical cancer cells. The protein level of E-Cadherin (A and B), N-Cadherin (A and C) and Vimentin (A and D) in shCtrl- and sh*ID1*-1-treated SiHa cells. 4 wells per group. $^{**}P < 0.01$, $^{***}P < 0.001$.

worldwide, associated with high mortality and morbidity rate [20]. A number of studies have shown that persistent high-risk HPV infection is the main cause of cervical lesions and cervical cancer [21]. Currently, surgical treatment of patients with early cervical cancer is more effective and can preserve nerves and even fertility. However, for patients with advanced cervical cancer, surgical treatment is relatively risky due to severe complications during and post-surgery. Irradiation increases the probability of normal tissue being affected, causing complications such as bone marrow suppression, radiation enteritis, and radiation cystitis [22]. Excessive radiation exposure may cause patients to relapse and worsen their condition. Cisplatin is the most effective drug in the treatment of such patients [23]. The choice of chemotherapy drugs is generally based on platinum and combined with other chemotherapy drugs. However, developing and optimizing the most effective combination of chemotherapeutical agents is still under debate.

There is emerging evidence that the TBX3 gene is involved in several important signaling pathways [24]. Studies have shown that the TBX3 gene can negatively regulate the expression of P19ARF (human P14ARF) and inhibit the process of cell apoptosis [25]. The overexpression of the TBX3 gene in malignant tumors can effectively suppress the P19ARF/p53 pathway, thereby strongly limiting cell senescence and death [26]. The abnormal expression and excessive accumulation of TBX3 has been demonstrated to promote cell malignant transformation. Correlation analysis showed that the expression of TBX3 is negatively correlated with E-cadherin and positively correlated with N-cadherin in colorectal cancer [27, 28]. In our study, we found that silencing TBX3 can effectively increase E-cadherin and inhibit N-cadherin protein levels in cervical cancer cells, suggesting the inhibitory role of silencing TBX3 in migration and invasion. Moreover, Kristic et al. strongly showed that TBX3 is closely related to enhancing the invasiveness and the transition of breast cancer at early stage via the SLUG signaling pathway [19]. The overexpression of TBX3 can be used as a potential indicator of the prognosis of patients with colorectal cancer. As a potential indicator, TBX3 may assist doctors in clinical decision-making and follow-up arrangements.

ID is one of the members of the helix-loop-helix (HLH) transcription factor family [29]. ID plays an important role in negatively regulating the activity of basic HLH (bHLH) transcription factors [30]. Mammalian cells contain four ID factors, namely ID1-ID44, participating in the cell cycle regulation process, including cell growth, differentiation, death, and embryonic development [31]. A large number of research results have demonstrated that the ID genes are highly expressed in human cancer cells. In particular, overexpression of ID1 is associated with the occurrence and development of a variety of tumors, such as neuroblastoma, pancreatic cancer, cervical cancer, melanoma, hepatocellular carcinoma, etc. The antisense of ID1 can be used to inhibit ID1 expression in tumor cells, thereby limiting cell proliferation [32-34]. Highly

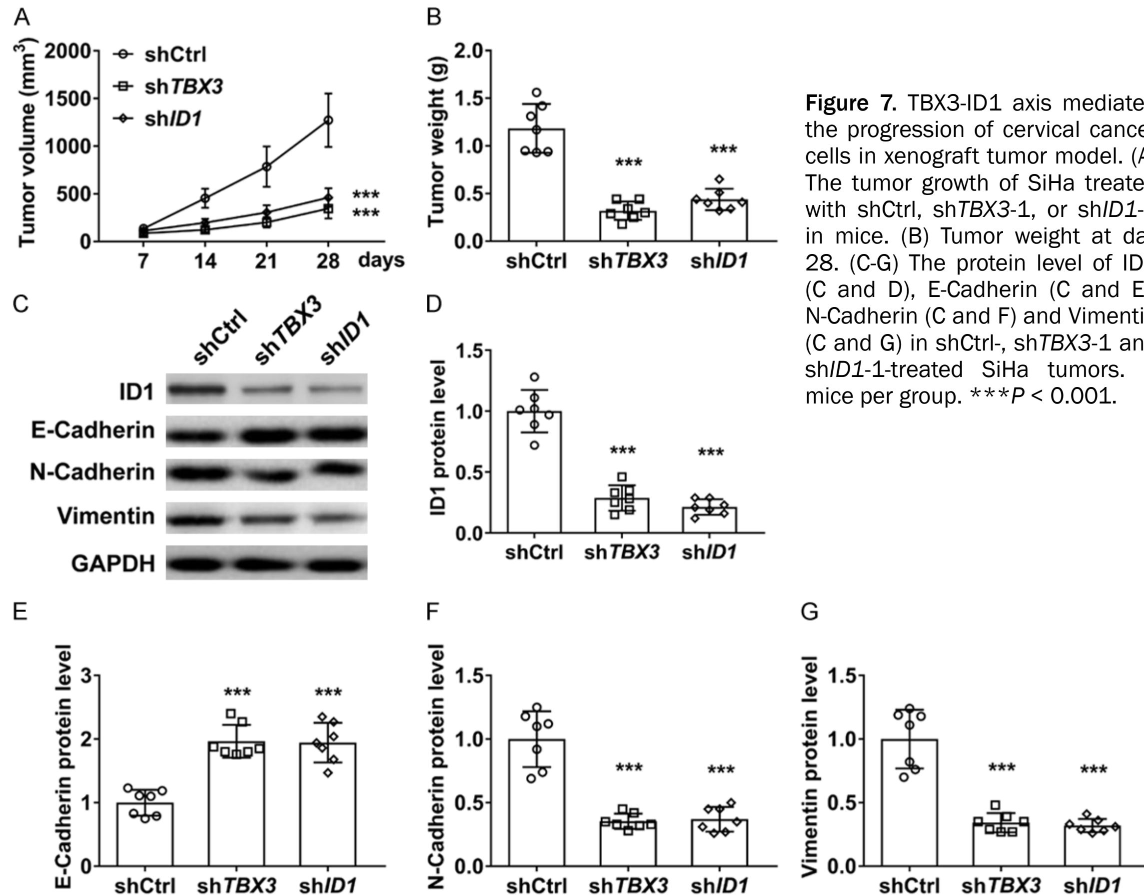

Figure 7. TBX3-ID1 axis mediated the progression of cervical cancer cells in xenograft tumor model. (A) The tumor growth of SiHa treated with shCtrl, sh*TBX3*-1, or sh*ID1*-1 in mice. (B) Tumor weight at day 28. (C-G) The protein level of ID1 (C and D), E-Cadherin (C and E), N-Cadherin (C and F) and Vimentin (C and G) in shCtrl-, sh*TBX3*-1 and sh*ID1*-1-treated SiHa tumors. 7 mice per group. ***$P < 0.001$.

expressed ID1 has been found in prostate tumor and cervical tumor [17, 35]. In agreement with these findings, we found that silencing ID1 significantly suppressed cervical cancer cells migration and invasion. To confirm that silencing TBX3 and ID1 can reduce cervical cancer cells progression and metastasis, we used tumor xenograft model. Of note, silencing TBX3 or ID1 significantly inhibited tumor growth as well as the ability of migration and invasion. These findings suggest that TBX3 and ID1 can potentially serve as a target to treat cervical cancer.

However, there are a few limitations in this study. First, although our findings indicate that ID1 might be a downstream mediator of TBX3, there are no direct data to demonstrate whether TBX3 directly regulates ID1. A study to determine the signaling pathway between TBX3 and ID1 will be included in the future. Secondly, we showed that pretreatment with shTBX3 or ID1 can inhibit tumor growth. However, whether shTBX3 or ID1 can reduce tumor proliferation, migration and invasion in advanced cervical tumor remain unclear.

Conclusion

In conclusion, we demonstrated that TBX3 and ID1 expression promote cervical cancer cells migration and invasion. Silencing TBX3 and ID1 effectively inhibited cervical cancer cells metastasis and invasion *in vitro* and *in vivo*. These data provide evidence to support that targeting TBX3 and ID1 can serve as potential therapeutic approach to prevent and treat cervical cancer.

Disclosure of conflict of interest

None.

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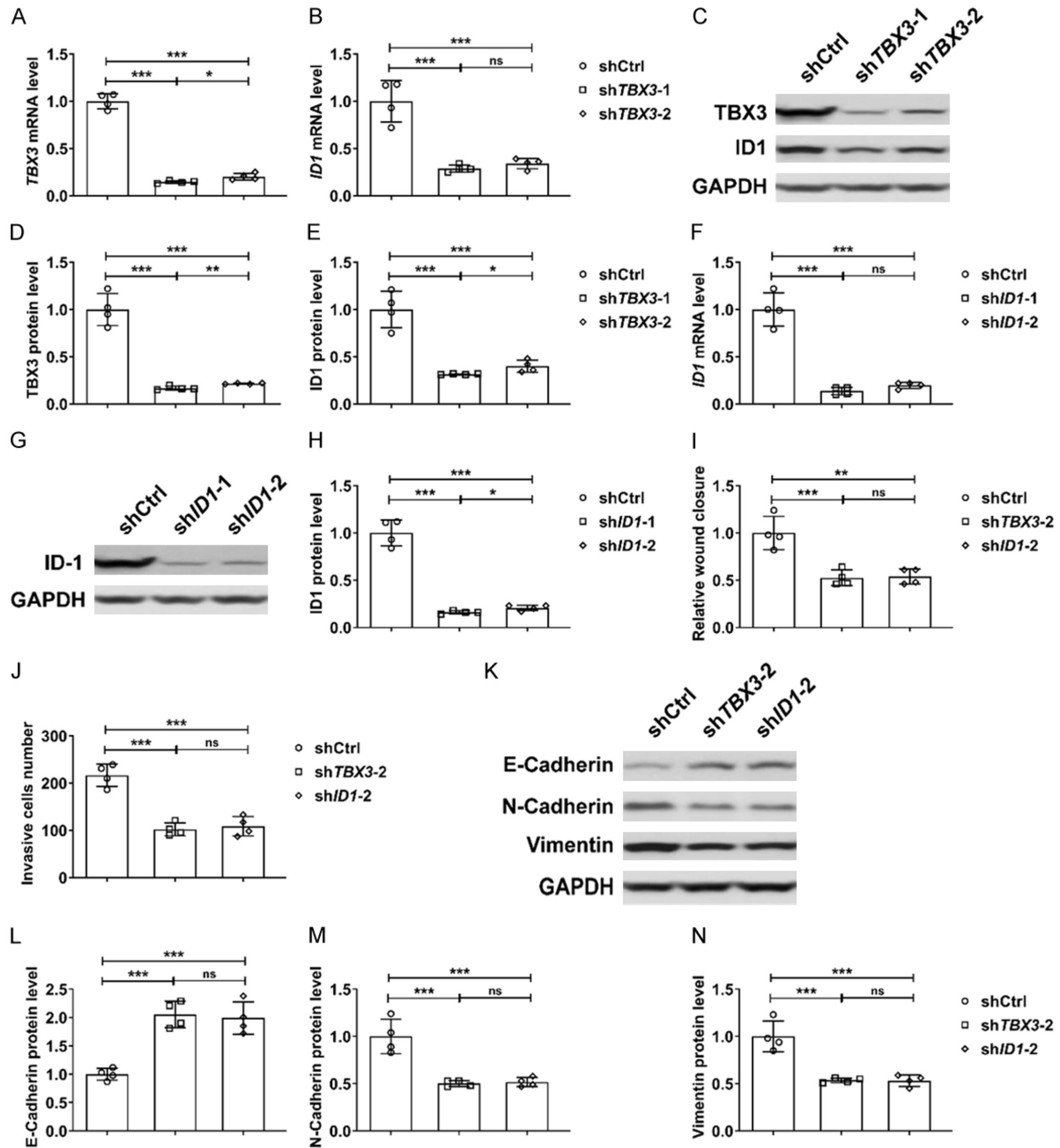

Figure S1. TBX3-ID1 axis mediated the migration, invasion and EMT related genes in cervical cancer *in vitro*. (A-E) The mRNA (A and B) and protein (C-E) level of TBX3 (A, C, D) and ID1 (B, C, E) in shCtrl-, sh*TBX3*-1 and sh*TBX3*-2-treated SiHa cells. (F-H) The mRNA (F) and protein (G and H) level of ID1 in shCtrl-, sh*ID1*-1 and sh*ID1*-2-treated SiHa cells. (I) The relative wound closure in wound healing assay of shCtrl-, sh*TBX3*-2 and sh*ID1*-2-treated SiHa cells. (J) Invasive cells in trans-well invasion assay of shCtrl-, sh*TBX3*-2 and sh*ID1*-2-treated SiHa cells. (K-N) The protein level of E-Cadherin (K and L), N-Cadherin (K and M), and Vimentin (K and N) in shCtrl-, sh*TBX3*-2 and sh*ID1*-2-treated SiHa cells was determined by western blot. 4 wells per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant.

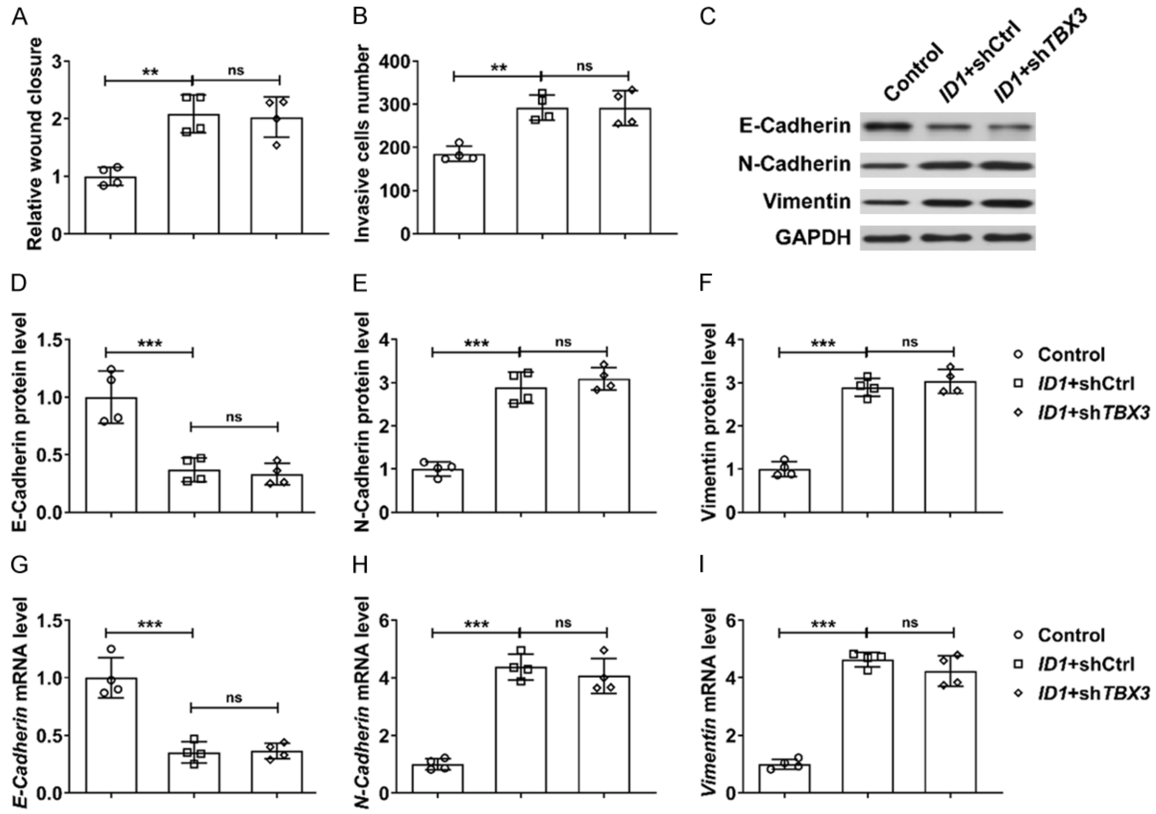

Figure S2. Knockdown of TBX3 did not reverse the phenotype under overexpression of ID1. (A) The relative wound closure in wound healing assay. (B) Invasive cells in trans-well invasion assay. (C-F) The protein level of E-Cadherin (C and D), N-Cadherin (C and E), and Vimentin (C and F) in control or *ID1*-overexpressing SiHa cells treated with shCtrl- and sh*TBX3* was determined by western blot. (G-I) The mRNA level of *E-Cadherin* (G), *N-Cadherin* (H), and *Vimentin* (I) in control or *ID1*-overexpressing SiHa cells treated with shCtrl- and sh*TBX3* was determined by RT-qPCR. 4 wells per group. ***P* < 0.01, ****P* < 0.001, ns, not significant.

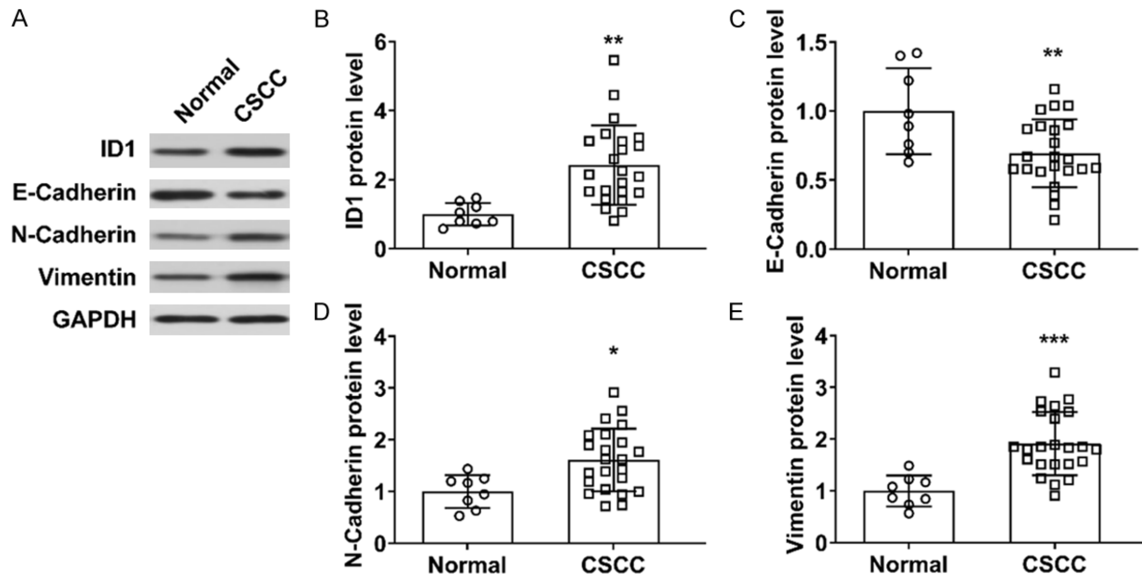

Figure S3. Expression level of ID1 and EMT related genes in cervical squamous cell carcinoma specimens. The protein level of ID1 (A and B), E-Cadherin (A and C), N-Cadherin (A and D), and Vimentin (A and E) in normal and CSCC specimens was determined by western blot. 8 normal cervix specimens, 23 CSCC specimens. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.