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Original Article

Inhibition of notch signaling reverses epithelial-mesenchymal transition in esophageal cancer

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Abstract: Notch signaling can promote Epithelial-mesenchymal transition (EMT) and enhance the invasion and metastasis of tumor cells, but the role of Notch induced EMT in esophageal cancer (EC) remains unclear. This study aimed to reveal the role of Notch signaling in EMT of EC cells. Eca-109 esophageal adenocarcinoma cells were treated with TGF-β1 or/and DAPT. The expression of E-cadherin and vimentin was evaluated by Western blot analysis and Immunofluorescence staining. Cell viability, proliferation and migration were examined by CCK-8, colony formation and wound healing assay. The status of Notch signaling was evaluated based on the detection of NICD and Hes1 levels. We found that TGF-β1 treatment led to decreased expression of E-cadherin and increased expression of vimentin in Eca-109 cells, accompanied by increased cell viability, proliferation and migration, as well as upregulated levels of NICD and Hes1. However, these TGF-β1 induced effects were inhibited after the cells were additionally treated with DAPT. In conclusion, the inhibition of Notch signaling reverses EMT and malignant phenotypes of esophageal cancer and is a potential approach for the treatment of EC.

Keywords: Notch, epithelial-mesenchymal transition, Hes1, esophageal cancer, TGF-β1

Introduction

Esophageal cancer (EC) is one of the most common malignant tumors in the world, the 5-year survival rate of patients with advanced EC after surgical treatment is less than 15% [1]. The high probability of invasion and metastasis is the main reason of treatment failure, therefore, how to reduce EC invasion and is an important challenge [2].

Epithelial-mesenchymal transition (EMT) is a fundamental biological process during which epithelial cells lose apical-basal polarity and acquire a mesenchymal phenotype accompanied by decreased epithelial marker E-cadherin and increased mesenchymal marker vimentin [3]. EMT is regarded as a hallmark for invasiveness and metastasis in many types of cancer [4-6]. Therefore, the reversal of EMT may be an effective way to inhibit the invasion and metastasis of EC.

Notch signaling is an important communication platform between adjacent cells. Following contact with its ligand Jagged/Delta, Notch is subjected to two cleavage and intracellular domain (NICD) is released into the nucleus where it binds transcription factor RBP-Jκ to initiate the transcription of target genes such as Hes and HRT, which regulate cell fate decision, differentiation, proliferation, apoptosis, and adhesion [7]. Emerging evidences have confirmed that Notch signaling is involved in regulating EMT by inhibiting E-cadherin expression and inducing vimentin expression. Consequently, Notch promotes the transformation of epithelial cell morphology and function into mesenchymal cell, leading to the invasion and metastasis of tumor cells [8]. However, the effects of Notch on EMT of EC cells remain unclear. This study aimed to reveal the role of Notch signaling in EMT of EC cells. We used TGF-β1 to induce EMT in human esophageal carcinoma Eca-109 cells and found that Notch signaling inhibition could reduce malignant phenotypes of EC cells via the reversal of EMT.

Methods

Reagents and antibodies

RPMI-1640 was purchased from Gibco BRL (USA), Fetal bovine serum (FBS) was from TransGen (China), N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-Sphenylglycine t-butyl ester (DAPT)

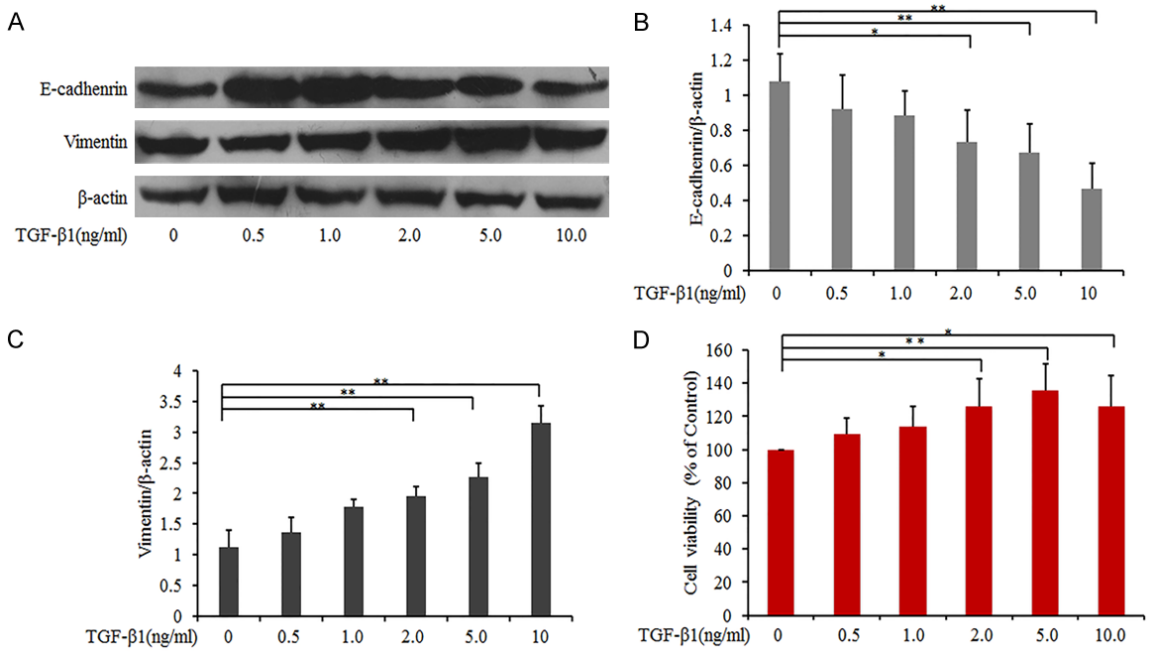

Figure 1. EMT induced by TGF-β1 enhanced EC cell viability. A. Western blot analysis of E-cadherin and Vimentin expression in Eca-109 cells treated with different concentrations of TGF-β1. β-actin was loading control. B. Densitometry analysis of E-cadherin expression level. C. Densitometry analysis of Vimentin level. D. CCK-8 assay of the viability of Eca-109 cells treated with different concentrations of TGF-β1. Data were presented as mean ± SD, n=3, **P*<0.05, ***P*<0.01.

was purchased from Sigma (USA), recombinant TGF-β1 was purchased from Peprotech (USA), Cell counting kit-8 (CCK-8) was from BestBio Technologies (China). Anti-Notch1 antibody-Cleaved-Val1744 and Anti-Hes1 antibody were from Abcam (USA), E-cadhenrin rabbit mAb and Vimentin rabbit mAb were from Cell Signaling Technology (USA), β-Actin antibody was from Anbo Biotechnology Company (USA), and enhanced chemiluminescence was from Thermo Scientific (USA).

Cell culture and treatment

Human esophageal carcinoma Eca-109 cells (ATCC Rockville, USA) were cultured in RPMI-1640 supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO_2. TGF-β1 and DAPT were dissolved in DMSO and RPMI-1640, respectively, and added to the medium before cell treatment.

Cell viability assay

Eca-109 cells were treated with 10 μl CCK-8 for 1 h, the absorbance was measured at 450 nm using a Microplate reader (Thermo, USA). The percentage of viable cells was calculated compared to control cells (100%).

Western blot analysis

Protein samples were separated by 8-10% SDS-PAGE, then transferred to nitrocellulose membranes (Millipore) and blocked in 10% nonfat milk in TBST (150 mM NaCl, 50 mM Tris pH 7.5, 0.1% Tween-20). Membranes were incubated with primary antibodies overnight at 4°C, washed thrice with TBST, and then incubated with secondary antibodies at 37°C for 1 h. After the membranes were washed with TBST, the signals were detected using enhanced chemiluminescence by ImageQuant LAS4000 (GE, USA). Relative protein levels were normalized to that of β-actin.

Immunofluorescence

The cells were fixed in 4% paraformaldehyde in PBS for 15 min and washed three times with PBS. After blocking (0.2% Triton X-100, 0.05% Tween 20, 1% FCS, 0.02% BSA) for 1 h at 37°C, the cells were incubated with primary antibodies overnight at 4°C followed by three washings with PBS. Afterwards they were incubated with the fluorochrome-conjugated antibody for 2 h at 37°C. The nuclei was stained with DAPI. The immunofluorescence signals were visualized and recorded with fluorescence microscope.

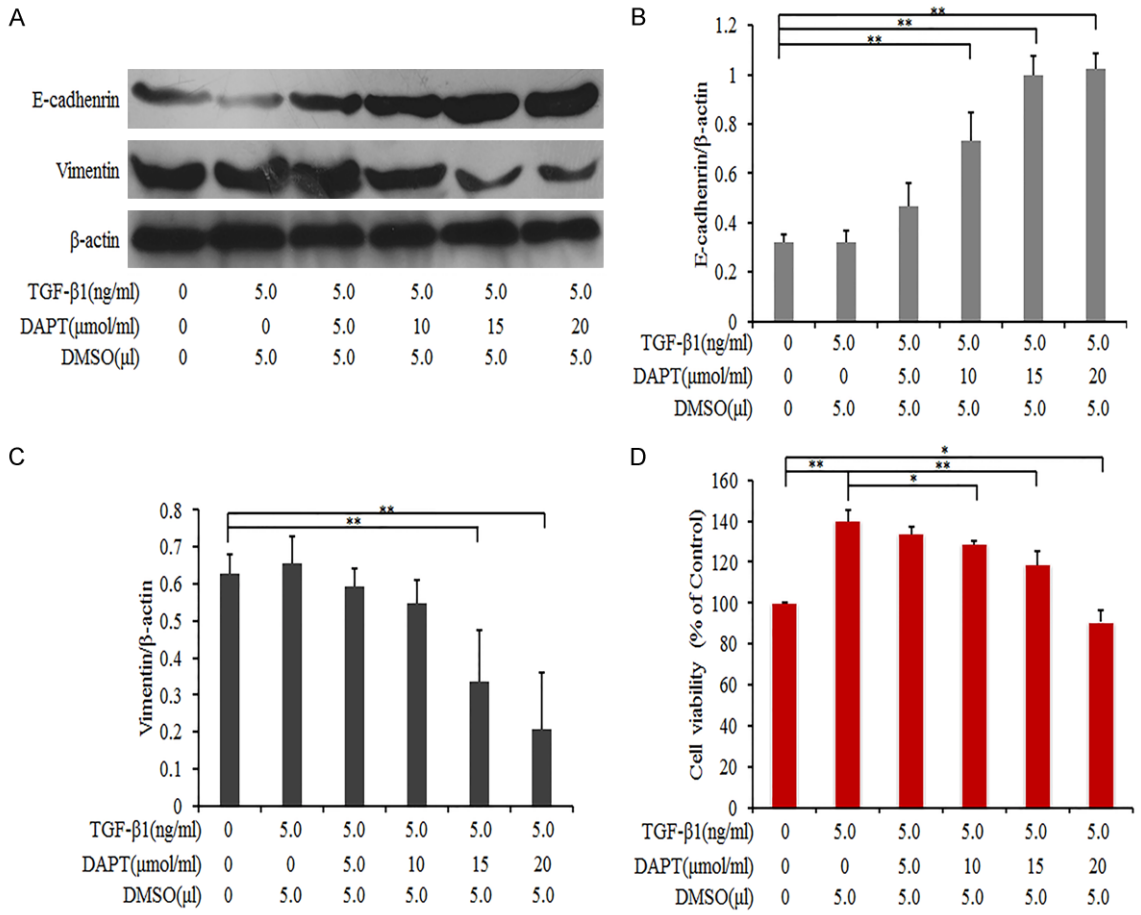

Figure 2. DAPT reversed EMT in EC cells. A. Western blot analysis of E-cadherin and Vimentin expression in Eca-109 cells treated with TGF-β1 or/and DAPT. β-actin was loading control. B. Densitometry analysis of E-cadherin expression level. C. Densitometry analysis of Vimentin level. D. CCK-8 assay of the viability of Eca-109 cells treated with TGF-β1 or/and DAPT. Data were presented as mean ± SD, n=3, **P*<0.05, ***P*<0.01.

Colony formation assay

100 cells were seeded in dish and cultured for macroscopic clone. The cells were washed by PBS and fixed by 4% paraformaldehyde for 15 min. Then the cells were stained by crystal violet for 10 min and washed by water. Finally the clones were taken pictures and counted. The colony formation rate = (number of clones)/(number of seeded cells) ×100%.

Wound healing assay

Cells were planted in 6-well plates and cultured to 80% confluence. Subsequently, the artificial wounds were created on the confluent cell monolayer using 200 μl pipette tips, and the detached cells were washed twice with FBS free culture medium. Then the cells were grown in FBS free RPMI-1640, the distance migrated by the cell monolayer to close the wounded area during this time period was measured. Results were expressed as a migration index: the distance migrated after treatment compared to control.

Statistical analysis

Data were analyzed using the SPSS version 12 statistical analysis package (SPSS Inc., Chicago, IL, USA). Examined data were assessed using ANOVA followed by Duncan's post-hoc test. In each test, the data were expressed as the mean ± SD, and $P<0.05$ was accepted as statistically significant.

Results

EMT induced by TGF-β1 enhances EC cell viability

To understand the role of EMT in esophageal cancer, we used TGF-β1 to induce EMT in Eca-

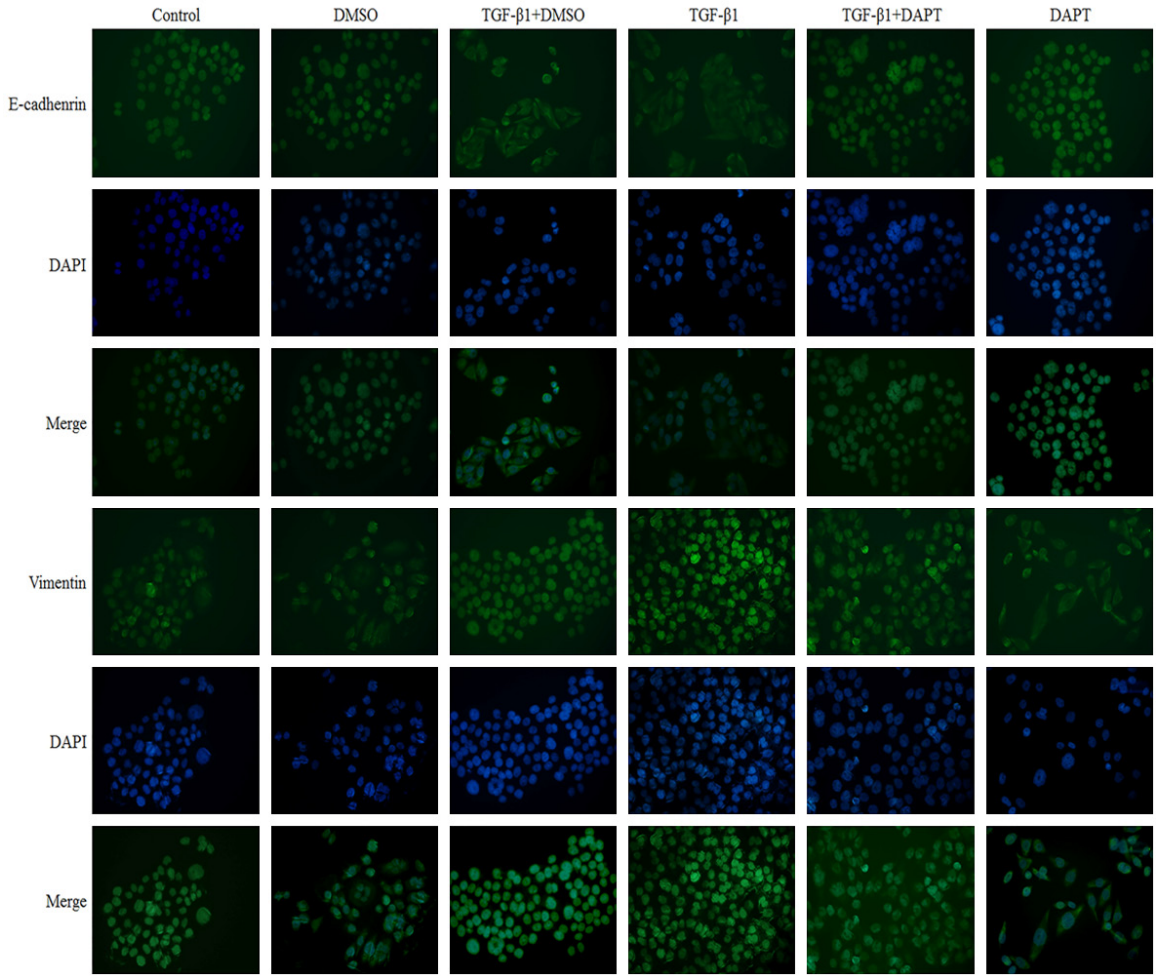

Figure 3. Immunofluorescence analysis of E-cadhenrin and Vimentin expression in Eca-109 cells treated with TGF-β1 or/and DAPT. The nuclei were stained as blue by DAPI.

109 esophageal adenocarcinoma cells. Western blot analysis showed that the expression of E-cadhenrin decreased gradually and the expression of Vimentin increased gradually when the concentration of TGF-β1 was above 2 ng/ml, indicating that Eca-109 cells underwent EMT (**Figure 1A-C**). In addition, we found that the cell viability was significantly increased when the cells were treated with TGF-β1 at the concentration above 2 ng/ml (**Figure 1D**). These data suggest that EMT can significantly enhance the viability of esophageal cancer.

Inhibition of notch signaling reverses EMT in EC cells

To explore the role of Notch signaling in EMT of esophageal cancer, we used DAPT to inhibit Notch1 signaling and found that E-cadhenrin expression was significantly increased while Vimentin expression was decreased when the concentration of DAPT was 15 μmol/ml, indicating that DAPT inhibited EMT of Eca-109 cells (**Figure 2A-C**). In addition, the viability of Eca-109 cells was significantly decreased after treatment with DAPT at the concentration above 10 μmol/ml (**Figure 2D**). Therefore, we consider that inhibition of Notch signaling can reverse EMT and decrease the viability of EC cells.

Notch signal inhibition reduces the proliferation and migration of EC cells

To examine the impact of inhibiting Notch signal on the proliferation and migration of esophageal cancer, we used 5 ng/ml TGF-β1 to induce EMT and used 15 μmol/ml DAPT to reverse EMT in Eca-109 cells. First we confirmed that upon the stimulation of TGF-β1 the

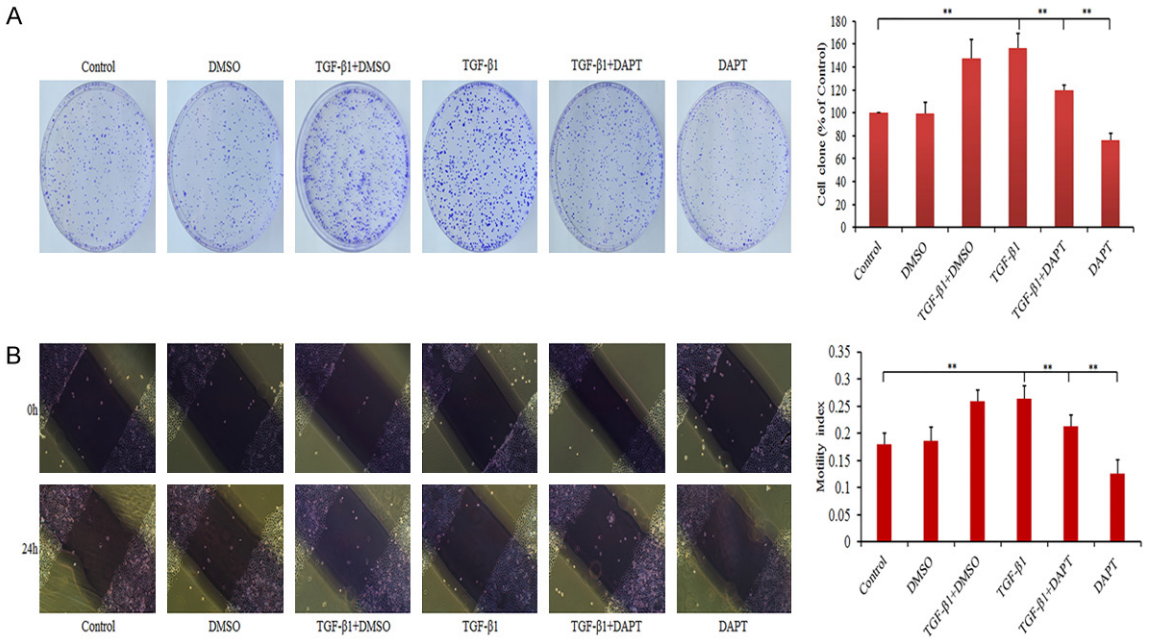

Figure 4. DAPT reduced the colony formation and migration of EC cells. A. Colony formation assay of Eca-109 cells treated with TGF-β1 or/and DAPT. B. Would healing assay of Eca-109 cells treated with TGF-β1 or/and DAPT. Data were presented as mean ± SD, n=3, $*P<0.05$, $**P<0.01$.

fluorescence intensity of E-cadhenrin was decreased while the fluorescence intensity of Vimentin was enhanced, but these changes were reversed by DAPT (**Figure 3**).

Next we evaluated the capacity of proliferation and migration of Eca-109 cells undergoing EMT induced by TGF-β1 or inhibited by DAPT. The results showed that the proliferation and migration of Eca-109 cells increased after treatment with TGF-β1 but these effects could be inhibited by DAPT (**Figure 4**). These data suggest that the reversal of EMT by the inhibition of Notch signaling contributes to the inhibition of EC cell proliferation and migration.

TGF-β1 induced activation of notch signaling is blocked by DAPT

To explore the possible role of Notch signaling in TGF-β1 induced EMT, we detected the expression of N1ICD and Hes1 in Eca-109 cells. Western blot analysis showed that the levels of N1ICD and Hes1 were significantly increased after TGF-β1 treatment, indicating the activation of Notch signaling. Furthermore, the levels of N1ICD and Hes1 in Eca-109 cells treated with TGF-β1 and DAPT were significantly lower than in cells treated with TGF-β1 alone (**Figure 5**). These data suggest that TGF-β1 induced activation of Notch signaling is blocked by DAPT.

Discussion

EC is a common cause of cancer-related death worldwide. Although the overall resection rate of EC has greatly improved, 5-year survival rate is still low due to the strong invasion and metastasis of EC [9]. EMT is an important physiological and pathological process in which epithelial cells lose their polarity and obtain high invasion and migration ability under certain conditions, such as embryonic development [10]. Recent study showed that inhibition of EMT by miR-204 could suppress EC invasion and metastasis [11]. In this study, we found that EMT gradually developed in Eca-109 esophageal adenocarcinoma cells when the cells were treated with increased concentration of TGF-β1. Accordingly, the cell viability, proliferation and migration were increased after the treatment with increased concentration of TGF-β1. These data indicate that EMT contributes to the growth and metastasis of EC.

A large number of studies have shown that Notch signaling pathway can induce EMT in a variety of tumor cells and promote the invasion

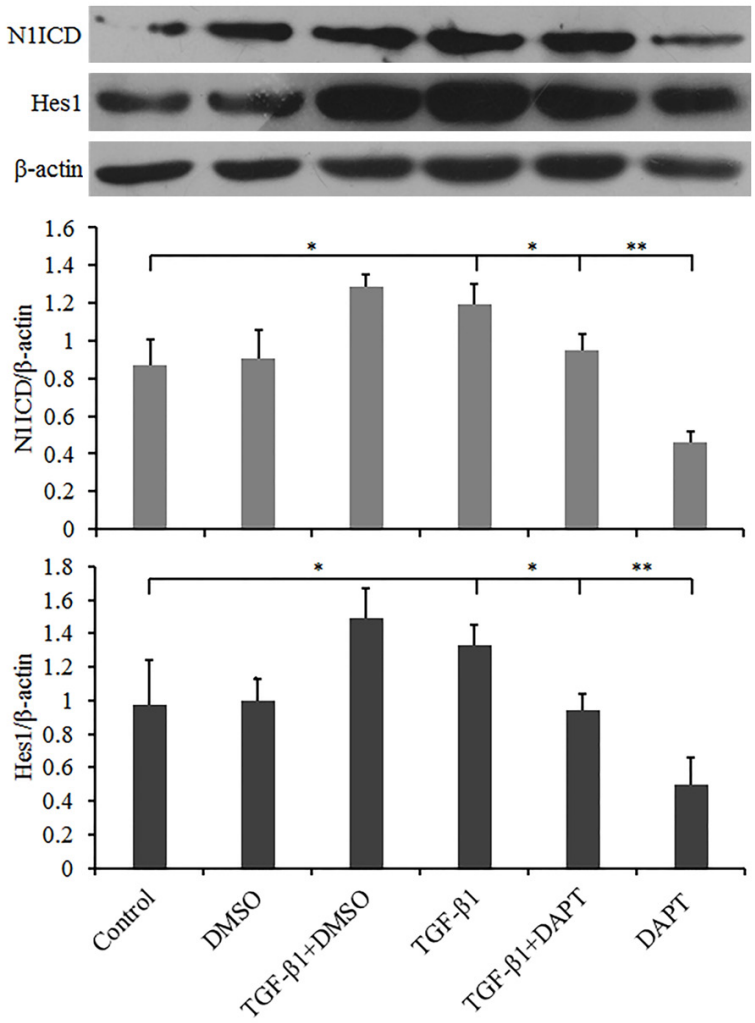

Figure 5. DAPT inhibited TGF-β1 induced increases of N1ICD and Hes1 levels. Western blot and densitometry analysis of N1ICD and Hes1 levels in Eca-109 cells treated with TGF-β1 or/and DAPT. β-actin was loading control. Data were presented as mean ± SD, n=3, **P*<0.05, ***P*<0.01.

and metastasis of tumor, which closely related to the survival rate, prognosis and drug resistance of cancer patients [12]. In particular, inhibiting Notch signal has been shown to reduce the invasion and metastasis of EC [13]. In this study, we employed γ-secretase inhibitor DAPT to block Notch signaling in Eca-109 cells. We found that DAPT reserved TGF-β1 induced EMT in Eca-109 cells. In detail, TGF-β1 induced changes in E-cadhenrin and Vimentin expression were abrogated by DAPT. Consistently, TGF-β1 stimulated increases in cell viability, proliferation and invasion were inhibited by DAPT. Collectively, these data suggest that Notch signaling promotes EMT in EC cells to initiate their malignant phenotypes.

E-cadherin is considered as the key molecule of EMT, whose expression and function decline during EMT, leading to loose connection and adhesion between tumor cells and strong invasion and migration of a variety of malignant tumors [14-16]. High expression of vimentin has been found in epithelial origin tumors such as liver cancer, cervical cancer, uterine endometrial cancer, prostate cancer, gastric cancer, lung cancer, esophageal cancer, oral squamous cell cancer and thyroid cancer, and is significantly associated with tumor, development, invasion and metastasis [17-20]. Using immunofluorescence we found that after TGF-β1 treatment, E-cadherin expression was weak while vimentin expression was strong in Eca-109 cells, the situation was completely reversed when Notch signaling was blocked by DAPT. Activation of Notch signaling is mechanistically linked with EMT of pancreatic cancer. RNAi mediated inhibition of Notch signaling partially reversed EMT of pancreatic cancer, and significantly reduced the invasion and migration of tumor cells [21]. In this study we performed CCK8, colony formation and wound healing assay to show that TGF-β1 induced EMT was associated with increased viability, proliferation and migration of Eca-109 cells. In contrast, the reversal of EMT by DAPT was associated with decreased viability, proliferation and migration of Eca-109 cells.

Curcumin mediated inhibition of Notch1 signaling could induce cell death in EC cells [22]. Therefore, the activation of Notch signaling plays an important role in EC tumorigenesis. Indeed, a recent study showed that Hes1, a downstream target of Notch signaling, was an independent prognostic marker in EC [23]. In this study, our results showed Hes1 expression

level was significantly increased when EMT was induced by TGF-β1 in Eca-109 cells, but Hes1 expression level decreased sharply when TGF-β1 induced EMT was reversed by DAPT. Therefore, we believe that the reversal of EMT by Notch1 inhibition may be closely related to the down-regulation of Hes1. Further studies are needed to elucidate the molecular mechanisms by which Notch signaling regulates EMT of EC cells.

In conclusion, our data show that the inhibition of Notch signaling reverses EMT and malignant phenotypes of esophageal cancer. Therefore, targeting Notch signaling is a potential approach for the treatment of EC.

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Disclosure of conflict of interest

None.

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