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Original Articles

USP26 promotes esophageal squamous cell carcinoma metastasis through stabilizing Snail

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ABSTRACT

Snail is an important transcription factor of epithelial-mesenchymal transition (EMT) and related to poor prognosis and distant metastasis of tumor patients. Snail is a liable protein and degraded by ubiquitin-proteasome system. There are various E3 ligases mediating its degradation, but the deubiquitinating enzyme reversed Snail degradation is not fully understood. In this study, we screened a DUB library and found USP26 is a potent deubiquitinase mediating Snail stabilization. We also identified that USP26 is a booster of esophageal squamous cell carcinoma (ESCC) cell migration and invasion, and it is highly expressed in ESCC samples. Our observation demonstrates that USP26 is a novel deubiquitinating enzyme of Snail and it provides a potential target for the therapy of esophageal cancer metastasis.

1. Introduction

Esophageal carcinoma is a major health problem, being the sixth most common cause of cancer death worldwide [1]. Esophageal squamous cell carcinoma, which is the most common histological subtype of esophageal cancer, is high-incidence in eastern Asia, eastern and southern Africa [1–3]. The five-year survival rate of ESCC patients is between 15% and 25%, and a large proportion of patients have previously metastasized before diagnosis [4,5].

Metastasis is the primary cause of death in cancer patients [6]. Epithelial-mesenchymal transition (EMT) is an essential program in the process of metastasis. EMT is a process that epithelioid cells lose their differentiated epithelial-like properties and gain a mesenchymal-like phenotype [7], and losing the expression of E-cadherin is a hallmark of EMT [8]. EMT-activating transcription factors (EMT-TFs) are vital executors of the EMT process, such as ZEB, TWIST and Snail families which have been reported to induce EMT by down-regulating E-cadherin [9].

Snail is a transcription factor containing C2H2 zinc-finger domain and is first characterized in Drosophila [10,11]. As an EMT-TF, the well-studied function of Snail is inducing EMT progress, and repression of E-cadherin transcription is an important way to activate EMT [12,13]. In many types of human cancers, Snail is found overexpressed and associated with poor prognosis and distant metastasis of cancer patients [14,15]. Snail expression also correlated with metastasis in ESCC patients [16,17]. Thus, Snail protein level must be tightly regulated, and it is important to expound the mechanism of Snail regulation.

Snail is a liable protein and degraded by ubiquitin-proteasome system. There are various E3 ligases mediating its degradation, such as SPSB3, FBXO11, FBXL14 and β-TrCP [18–21]. For example, FBXO11 ubiquitinates Snail which is phosphorylated by PKD1, and β-TrCP or SPSB3 mediated Snail degradation in response to it phosphorylation by GSK3β [18,19,21]. It is well known that the ubiquitination process could be reversed by deubiquitinating enzymes. There are about 100 deubiquitinases, and they are classified in five families including UCHs, USPs, OTUs, Josephins and JAMMs [22]. But the mechanism of deubiquitinases stabilizing Snail is unclear. DUB3 is a deubiquitinating enzyme that has been reported to deubiquitinates and stabilizes Snail, and it is one of cytokine induced DUBs [23,24]. DUB3 is a target of CDK4/6, and phosphorylation by CDK4/6 is essential for DUB3 deubiquitinating and stabilizing Snail [23]. In recent studies, we identified OTUB1 and PSMD14 as deubiquitinases of Snail [25,26]. As an important transcription factor, Snail might be regulated by multi-deubiquitinating enzymes. Other deubiquitinases might participate in the

Abbreviations: USP26, ubiquitin specific peptidase 26; Snail, snail family transcriptional repressor 1; DUB, deubiquitinating enzyme; ESCC, esophageal squamous cell carcinoma; CHX, cycloheximide; EMT, epithelial mesenchymal transition

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post-translational network of Snail.

In this study, we screened a DUB expression library and found USP26 is a potent deubiquitinase responsible for Snail deubiquitination and stabilization. We also identified that USP26 is a booster of ESCC cell migration and invasion, and it is highly expressed in ESCC samples. Overall, our study demonstrates USP26 is a novel deubiquitinating enzyme of Snail and provides a possible target for the therapy of esophageal cancer metastasis.

2. Materials and methods

2.1. Cell culture

The human ESCC cell lines KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE410 and KYSE510 were munificently given by Dr. Shimada Y. and grown in RPMI/1640 medium (Hyclone, USA) plus with 10% fetal bovine serum (Hyclone, USA). HEK293T cell line was purchased from American Type Culture Collection (ATCC) and grown in DMEM medium (Hyclone, USA) plus with 10% fetal bovine serum.

2.2. Plasmids and antibodies

The USP26, USP26 mutant (C304S) and Snail were cloned into pLVX-IRES vector. The USP26 and Snail truncated mutants were constructed into pcDNA3 vector. The USP26 shRNA sequences (5′-TATCC CACTTTGTGTAACC-3′and 5′-CCGGCTAAGTGATAATATTCAA-3′) and Snail shRNA sequences (5′-CCACTCAGATGTCAAGAAGTA-3′) were cloned into pSIH-H1 vector.

Antibodies include: anti-Snail (#3879, Cell Signaling Technology, USA;), anti-USP26 (ab188239, ab101650, Abcam, USA), anti-N-cadherin (#13116, Cell Signaling Technology, USA), anti-E-cadherin (#3195, Cell Signaling Technology, USA), anti-Vimentin (#5741, Cell Signaling Technology, USA), anti-Flag (#8146, Cell Signaling Technology, USA).

2.3. Screening of DUB library

The DUB plasmid library includes 66 deubiquitinase plasmids were gifts from Wade Harper (Addgene) [27]. The vectors in the library were pDEST-TetON-PGK-puro and pDEST-LTR-IRES-puro with Flag-tag and HA-tag. HEK293T cells were plated in 24-well plates and transfected with individual DUB plasmid and Snail. After 24 h, the cells were treated with CHX for 2 h [21]. Then, the cells were harvested and subjected to immunoblotting.

2.4. Co-immunoprecipitation and ubiquitination assay

Indicated plasmids were transfected into HEK293T cells. After twenty-four hours, the transfected cells were treated with 20 μM MG132 for six hours [21]. The cells were harvested, and the total protein were extracted with lysis buffer. The cell lysates were incubated with anti-Flag M2 affinity gel (A2220, Sigma Aldrich, USA) at 4 °C overnight. The beads were washed with lysis buffer three times and then the immunoprecipitates were subjected to immunoblotting.

For endogenous immunoprecipitation or ubiquitination, the indicated cells were treated with 20 μM MG132 for six hours before being harvested and lysed. The cell lysates were incubated with anti-Snail antibody or IgG at 4 °C overnight, and then incubated with protein A/G agarose for four hours. The beads were washed with lysis buffer three times and then the immunoprecipitates were subjected to immunoblotting.

2.5. Transwell migration and invasion assay

Cells which suspended with serum-free medium were plated onto Boyden chambers (3422, Corning Incorporated, USA) with or without coated matrigel (Corning Incorporated, USA). The bottom chambers were filled with 10% fetal bovine serum medium. After 24 h, the bottom cells were fixed and stained with crystal violet, and cells in three randomly selected views were photographed and counted.

2.6. Immunohistochemical staining

The tissue microarrays of human ESCC and paired adjacent tissues were obtained from Nanjing First Hospital [26]. The tissues were stained with anti-USP26 and anti-Snail antibodies respectively. Each sample was scored according to the intensity, and the value of negative staining was 0, weak positive was 1, positive was 2, and strong positive was 3. The study was approved by the ethical committee of the Cancer Hospital Chinese Academy of Medical Sciences, and all patients were informed consent.

2.7. Immunofluorescence assay

Cells were plated into the μ-slide VI (ibidi, German), fixed with 4% paraformaldehyde and permeabilized by 0.1% Triton X-100. Then the cells were blocked with 5% BSA and incubated with primary antibodies overnight at 4 °C. The protein signals were detected by anti-rabbit IgG Fab2 conjugated with Alexa Fluor 488 (Cell Signaling Technology, USA) and anti-mouse IgG Fab2 conjugated with Alexa Fluor 555 (Cell Signaling Technology, USA). Finally, the cells were incubated with 4,6-diamidino-2-pheylindole for 15 min and visualized by a laser confocal microscope.

2.8. Animal experiments

1×10^6 cells were injected into the tail vein of 6 weeks old mail SCID/beige or NOD/SCID mice, from Vital River (Beijing, China). Each group includes at least ten mice, and the lungs were harvested 12 weeks after injection. The experimental procedures were approved by Institutional Animal Care and Use Committee of Chinese Academy of Medical Sciences Cancer Hospital.

2.9. Statistical analysis

The difference of protein or mRNA level between the two groups was analyzed by unpaired *t*-test. The correlation of USP26 and Snail in tissue specimens was analyzed using Pearson's test with SPSS software version 22.0. Other statistical analyses were all performed using GraphPad Prism 6.0. A p value < 0.05 was considered significant.

3. Results

3.1. USP26 increases stability of Snail

To find potential deubiquitinating enzymes that increased Snail stability, we screened a DUB cDNA expression library. We co-transfected various deubiquitinases and Snail into the HEK293T cells. In order to enlarge the differences and eliminate the fake positive results, the cells were treated with CHX for two hours before harvest. We found USP26 significantly increased Snail stability (Fig. 1A and Fig. S1). DUB3 also enhanced the stabilization of Snail (Fig. 1A). To further validate USP26 upregulated protein level of Snail, we co-expressed Snail and USP26 in HEK293T cells. Ectopic expression of USP26 significantly increased Snail protein level (Fig. 1B). In addition, the protein level of Snail was increased by overexpressing USP26 in a dose-dependent manner (Fig. 1C). Next, we tested the protein level of USP26 and Snail in ESCC cell lines and found that there was a positive correlation between them (Fig. 1D). Then we stably overexpressed USP26 in KYSE30 cells and knocked down USP26 in KYSE150 cells. The results of immunoblotting assay revealed that overexpression USP26 resulted in remarkable increase of Snail protein in KYSE30 cells (Fig. 1E), and

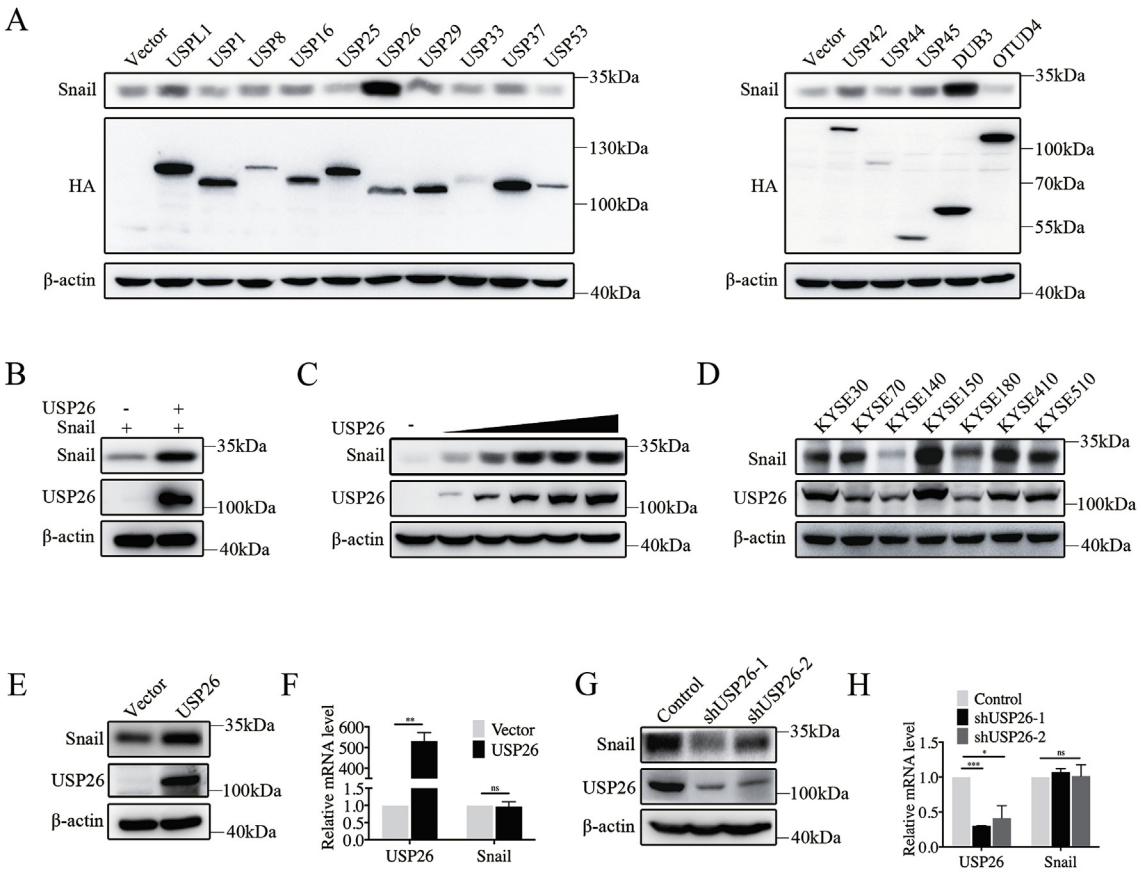

Fig. 1. The deubiquitinating enzyme USP26 stabilizes Snail.
(A) The DUBs and Snail were co-transfected into HEK293T cells. After 24 h, cells were treated with CHX (50 μg/ml) for 2 h and the Snail protein level were analyzed by Western blot. (B) USP26 and Snail were co-transfected into HEK293T cells, the Snail expression was detected by Western blot. (C) Increasing quantities of USP26 were transfected to HEK293T cells, USP26 and Snail expression were analyzed by Western blot. (D) The protein level of USP26 and Snail in ESCC cell lines were detected. (E and F) The protein (E) and mRNA level (F) of USP26 and Snail in the KYSE30 cells stably expressing USP26 were detected. (G and H) The protein (G) and mRNA level (H) of USP26 and Snail in USP26-knockdown KYSE150 cells were analyzed. Data are presented as the mean ± SEM, analyzed by unpaired *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

knockdown of USP26 was accompanied with decreased Snail protein expression in KYSE150 cells (Fig. 1G). Meanwhile, the mRNA level of Snail was not affected by the change of USP26 in KYSE30 (Fig. 1F) and KYSE150 cells (Fig. 1H). These results indicate that USP26 enhances protein level of Snail by post-translational modification and it may be a deubiquitinase of Snail.

3.2. USP26 interacts with Snail

In order to investigate the mechanism of USP26 mediated Snail protein upregulation, we co-transfected Flag-USP26 and Snail to HEK293T cells and performed a co-IP assay. The results showed that Snail was co-immunoprecipitated by USP26 (Fig. 2A). Moreover, a reciprocal co-IP experiment was performed with HEK293T cells co-transfected withUSP26 and Flag-Snail, which further demonstrated the interaction between USP26 and Snail (Fig. 2B). To further identify the region of USP26 responsible for mediating Snail interaction, we generated three truncated mutants of USP26. And the co-IP experiments revealed that USP26 mutants which included USP domain were able to interact with Snail (Fig. 2C), indicating that the USP domain of USP26 was in charge of its interaction with Snail. We also generated three truncated mutants of Snail to find the region of Snail which interacts with USP26. We found that the C-terminal of Snail which includes zinc finger domain mediated the interaction with USP26 (Fig. 2D). Furthermore, endogenous USP26 and Snail from lysates of KYSE150 cells were also co-immunoprecipitated, which confirmed the interaction of Snail and USP26 in the physiological condition (Fig. 2E). In addition, immunofluorescence assay demonstrated endogenous USP26 and Snail co-localized in the nucleus of KYSE30 and KYSE150 cells (Fig. 2F and Fig. S2). Taken together, our results indicate that USP26 physically interacts with Snail in the nuclear and the interaction is mediated by the UPS domain of USP26 and zinc finger region of Snail.

3.3. USP26 stabilizes Snail through deubiquitination

Since USP26 is a deubiquitinating enzyme, we tried to identify whether USP26 regulates Snail protein level by its deubiquitinase activity. We generated a USP26 mutant by replacing the catalytic cysteine with a serine residue (C304S). The result of co-IP assay revealed that USP26 mutant still interact with Snail (Fig. S3A). However, USP26 mutant failed to upregulate Snail protein level (Fig. S3B). Then, we performed CHX-pulse-chase assay by co-transfecting Snail with USP26 or USP26mutant to HEK293T cells. After treatment with cycloheximide to inhibit protein synthesis, Snail protein level decreased rapidly, and overexpression of USP26 but not USP26 mutant blocked the degradation of Snail (Fig. 3A). We further tested the effect of USP26 on Snail degradation in ESCC cell lines. We observed that overexpression of USP26 delayed Snail degradation in KYSE30 cells (Fig. 3B) and knockdown of USP26 in KYSE150 cells (Fig. 3C) shortened the half-life of Snail. Next, in order to determine whether USP26 stabilizes Snail protein is mediated by deubiquitinating, we tested the effect of USP26 on Snail ubiquitination. We co-transfected USP26 or USP26 mutant

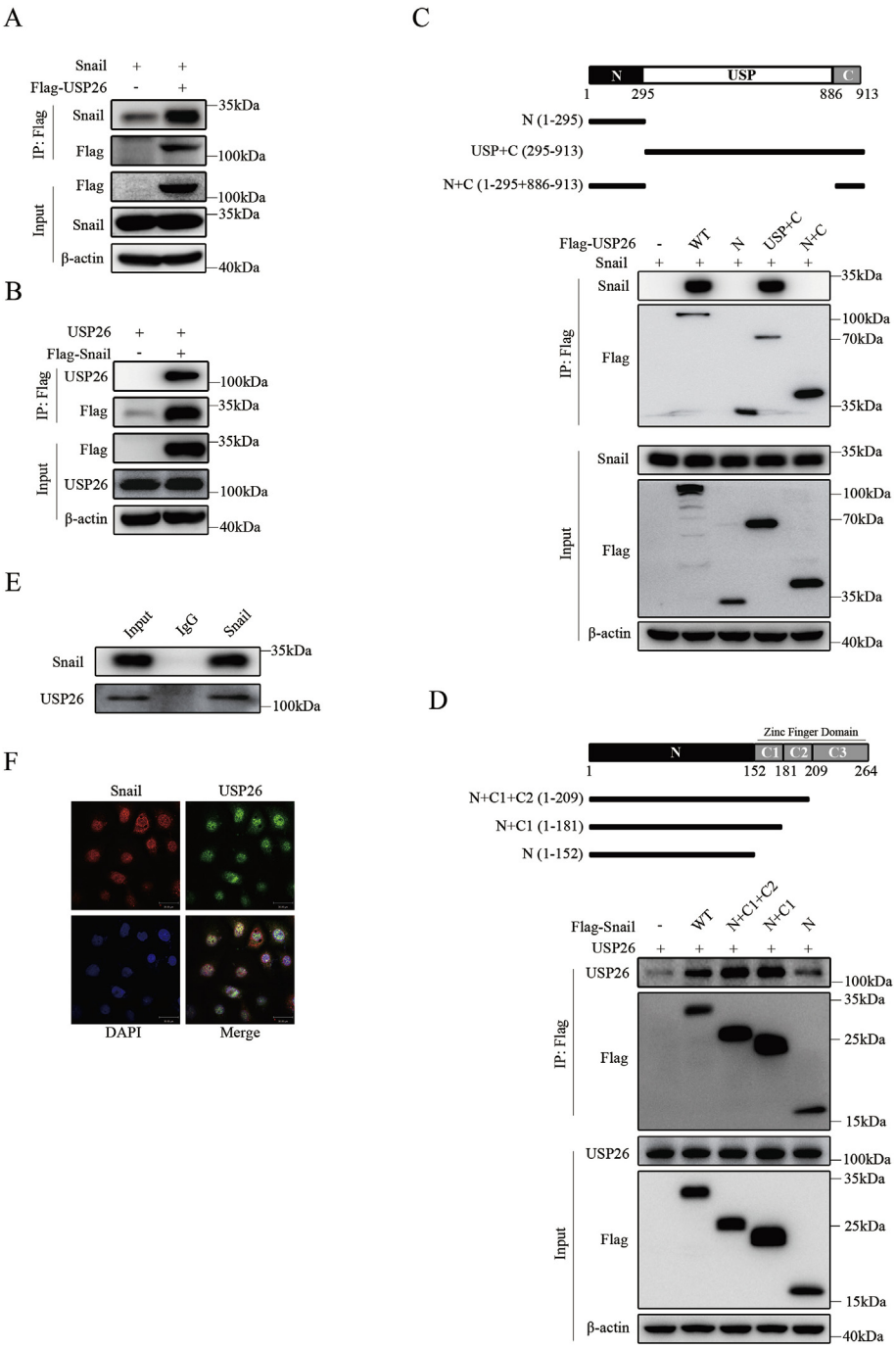

Fig. 2. USP26 interacts with Snail.
(A) Snail were co-transfected with Flag-USP26 or vector into HEK293T cells. After 24 h, the cells were treated with 20 μM MG132 for 6 h. Flag-USP26 were immunoprecipitated with anti-FLAG M2 affinity gel, and the associated Snail was detected by Western blot. (B) USP26 were co-transfected with Flag-Snail or vector into HEK293T cells. After 24 h, the cells were treated with 20 μM MG132 for 6 h. Flag-Snail were immunoprecipitated with anti-FLAG M2 affinity gel, and the associated USP26 was detected by Western blot. (C) A schematic diagram shows the structure of USP26 and UPS26 truncations. Flag-WT or truncated USP26 were co-transfected with Snail into HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel, and the associated Snail was detected by Western blot. (D) The structure of Snail and Snail truncations was showed in a schematic diagram. Flag-WT or truncated Snail were co-expressed with USP26 in HEK293T cells. USP26 was pulled down by IP assay, and analyzed by immunoblotting. (E) Endogenous USP26 co-immunoprecipitated with endogenous Snail. Endogenous USP26 and Snail were detected by Western blot. (F) Endogenous USP26 and Snail in KYSE30 cells were examined by immunofluorescence. Scale bars, 30 μm.

with Flag-Snail and HA-ubiquitin to HEK293T cells. After treatment with MG132, Flag-Snail were immunoprecipitated and the ubiquitinated Snail were detected with HA antibody. We found that ectopic expression of USP26 notably reduced its ubiquitination level but there was no such effect of USP26 mutant (Fig. 3D). Furthermore, ectopic expression of USP26 also decreased the ubiquitination of endogenous Snail in KYSE30 cells (Fig. 3E) and knockdown of USP26 increased Snail ubiquitination level in KYSE150 cells (Fig. 3F). We also performed ubiquitination assay with a series of mutant ubiquitin. The result showed USP26 could only remove the K48-linked ubiquitin chain from Snail protein (Fig. 3G). Collectively, these results indicated that USP26 maintained the stability of Snail by decreasing its ubiquitination.

3.4. USP26 promotes ESCC metastasis through inducing EMT

Since Snail is a transcription factor that inhibits E-cadherin expression to induce EMT progress, we detected several EMT markers such as E-cadherin, N-cadherin, and Vimentin in ESCC cells with overexpression or knockdown of USP26. Our results showed that the epithelial marker like E-cadherin was decreased, and mesenchymal markers such as N-cadherin and Vimentin were increased in KYSE30 cells overexpressing USP26 (Fig. 4A and Fig. S4A). Conversely, knockdown of USP26 increased E-cadherin and downregulated N-cadherin and Vimentin in KYSE150 cells (Fig. 4B and Fig. S4B).

EMT is an essential event in the metastatic of many solid tumors, and Snail is a vital regulator of EMT. Therefore, we tested whether USP26 promote metastasis in ESCC cells. In vitro, the migration and invasion ability of KYSE30 cells was enhanced by USP26 overexpression

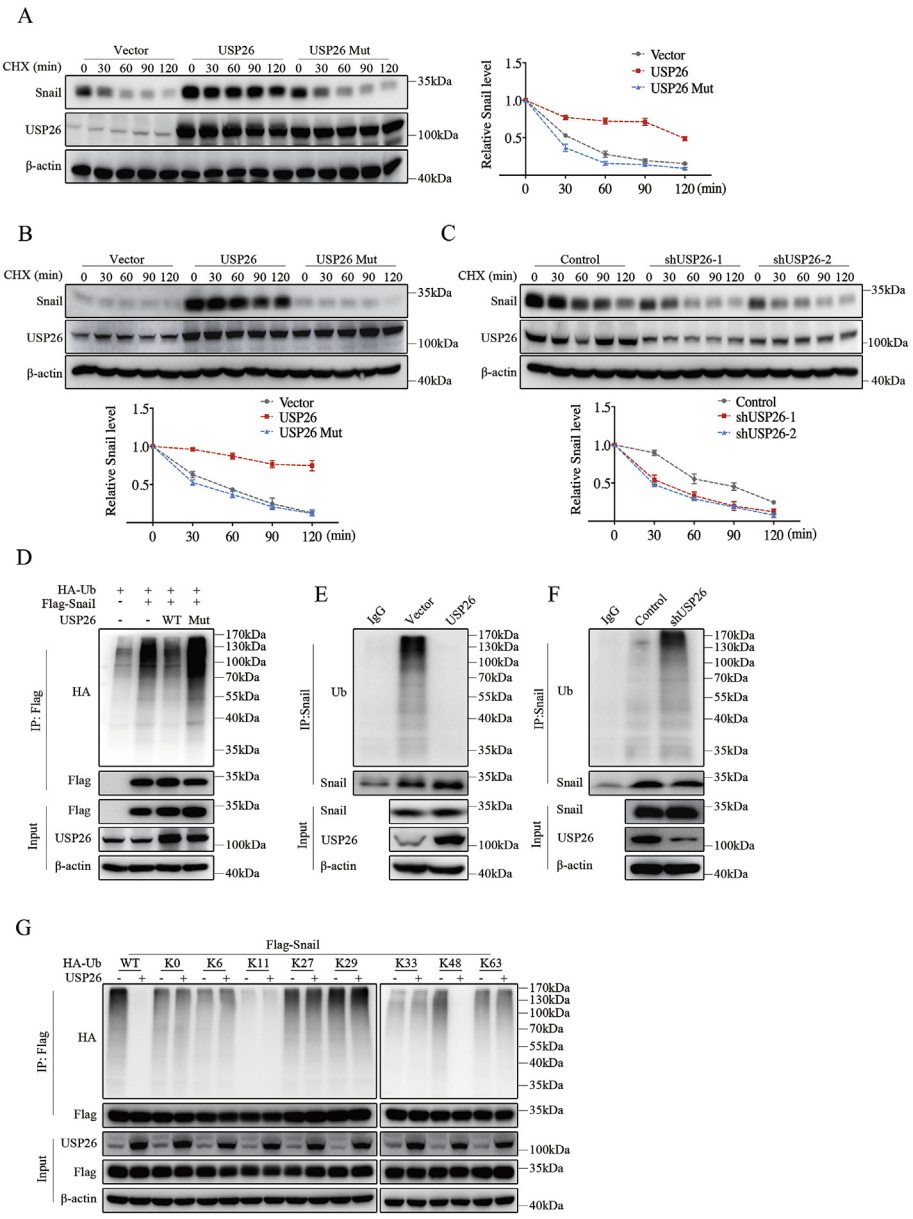

Fig. 3. USP26 stabilizes and deubiquitinates Snail. (A) Snail were co-transfected with USP26 or USP26 Mut into HEK293T cells. After treatment with CHX (50 μg/ml) for the indicated time, USP26 and Snail were detected by Western blot. Snail expression was quantified by ImageJ software. (B) KYSE30 cells stably expressing USP26 or USP26 Mut were treated with CHX (50 μg/ml) as indicated. Endogenous Snail was analyzed by Western blot. Snail expression was quantified by Image J software. (C) The USP26-knockdown KYSE150 cells were treated with CHX (50 μg/ml) as indicated. Endogenous Snail was analyzed by western blot. Snail expression was quantified by Image J software. (D) Flag-Snail and HA-Ub were co-expressed with USP26 or USP26 Mut in HEK293T cells. After treatment with 20 μM MG132 for 6 h, cell lysates were subjected to ubiquitination assay and the ubiquitination level of Snail was detected by Western blot with HA antibody. (E) The USP26 or vector stable overexpressing KYSE30 cells were treated with 20 μM MG132 for 6 h. Cell lysates were subjected to ubiquitination assay with Snail antibody, and the ubiquitination of endogenous Snail were detected by Western blot using ubiquitin antibody. (F) The USP26-knockdown KYSE150 cells were treated with 20 μM MG132 for 6 h. Cell lysates were subjected to ubiquitination assay. (G) HA-WT, K0, K6, K11, K27, K29, K33, K48 or K63 Ub were co-transfected with Flag-Snail and USP26 into HEK293T cells. After treatment with 20 μM MG132 for 6 h, cell lysates were subjected to ubiquitination assay and the ubiquitination level of Snail detected by HA antibody.

but not the USP26 mutant (Fig. 4C). Conversely, knockdown of USP26 inhibited the migration and invasion of KYSE150 cells (Fig. 4D). Next, we used a tail vein injection mouse model to detect the effect of USP26 on ESCC metastasis in vivo. The number of lung nodules was significantly upregulated by USP26 overexpression in KYSE30 cells (Fig. 4E). But the USP26 mutant lost the ability of promoting lung metastasis. And knockdown of USP26 inhibited the lung metastasis of KYSE150 cells (Fig. 4F).

To further identify USP26 promoted ESCC migration and invasion by increasing Snail stabilization, we knocked down Snail in USP26 overexpressing KYSE30 cells or ectopic expressed Snail in USP26 knockdown KYSE150 cells. Transwell assay showed the function of USP26 promoting migration and invasion of KYSE30 cells was largely reversed by knockdown of Snail (Fig. 4G). And overexpression of Snail could largely increase the ability of migration and invasion on USP26 knockdown cells (Fig. 4H). Taken together, these results reveal that USP26 promotes ESCC metastasis through increasing Snail stability and inducing the progress of EMT.

3.5. USP26 and Snail are uniformly overexpressed in ESCC samples

To further study the relationship of USP26 and Snail in ESCC, we examined the expression of USP26 and Snail in ESCC tissues and paired adjacent normal tissues. Immunohistochemistry staining showed that the USP26 protein level was much higher in the ESCC samples than the normal tissues (Fig. 5A), indicating an oncogenic role of USP26 in ESCC. In addition, there was a significant positive correlation between USP26 and Snail protein level in both ESCC and adjacent normal tissues (Fig. 5B, C and D). This positive correlation further proved the regulating relationship between USP26 and Snail.

4. Discussion

Despite the rapid development of antitumor therapy, metastasis is still a great threat to the cancer patients and the understanding of its biological mechanisms remains limited [5]. Metastasis is a complex multistep process that begins with cell detachment from the original sites and finally generated a micro metastatic lesions in the distant organs [28]. Before transferred and colonized to other sites, most

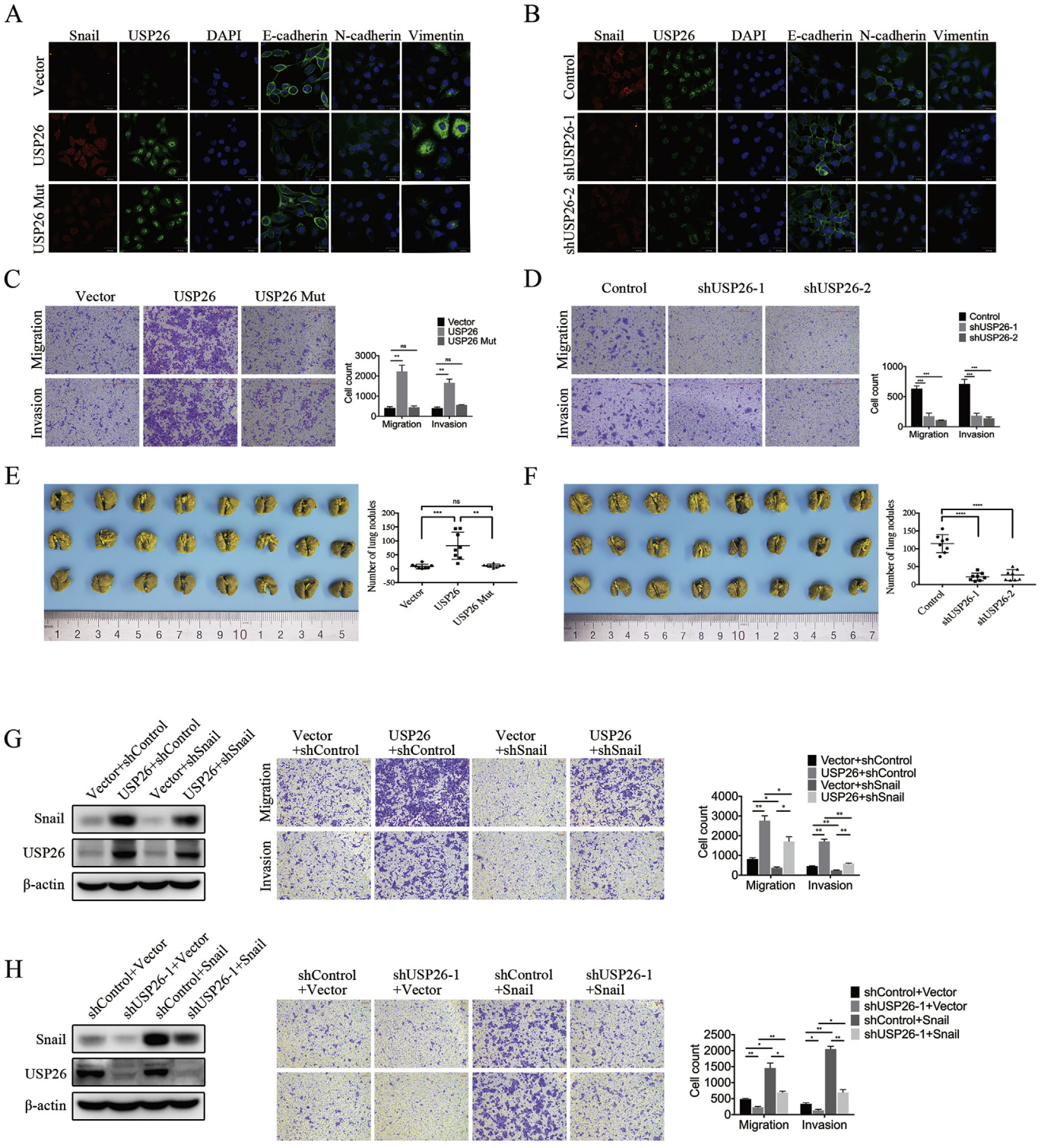

Fig. 4. USP26 promotes ESCC cell migration and invasion.
(A and B) Expression of USP26, Snail, E-cadherin, N-cadherin and Vimentin were detected by immunofluorescence assay in USP26 or USP26 Mut stably expression KYSE30 cells (A) and USP26-knockdown KYSE150 cells (B), Scale bars, 30 μm. (C) The migration and invasion of USP26 or USP26 Mut overexpressing KYSE30 cells and corresponding control cells were examined by transwell assay. (D) Migration and invasion assays were performed using USP26 knockdown KYSE150 cells and corresponding control cells. (E) USP26 or USP26 Mut stably overexpression KYSE30 cells were injected into the tail vein of SCID/Beige mice. After 12 weeks, the lungs were harvested, and the nodules were counted. (F) USP26-knockdown KYSE150 cells were injected into NOD/SCID mice. After 4 weeks, harvested the lungs and the nodules were counted. (G) Snail was simultaneously knocked down in USP26 stably overexpressing KYSE30 cells. The expression level of Snail and USP26 was detected by immunoblotting (left panel). The migration and invasion assays were performed with indicated cells. (H) Snail was simultaneously overexpressed in USP26 knockdown KYSE150 cells. The expression level of Snail and USP26 was detected by immunoblotting (left panel). The migration and invasion assays were performed with indicated cells. Data are presented as the mean ± SEM, analyzed by unpaired *t*-test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

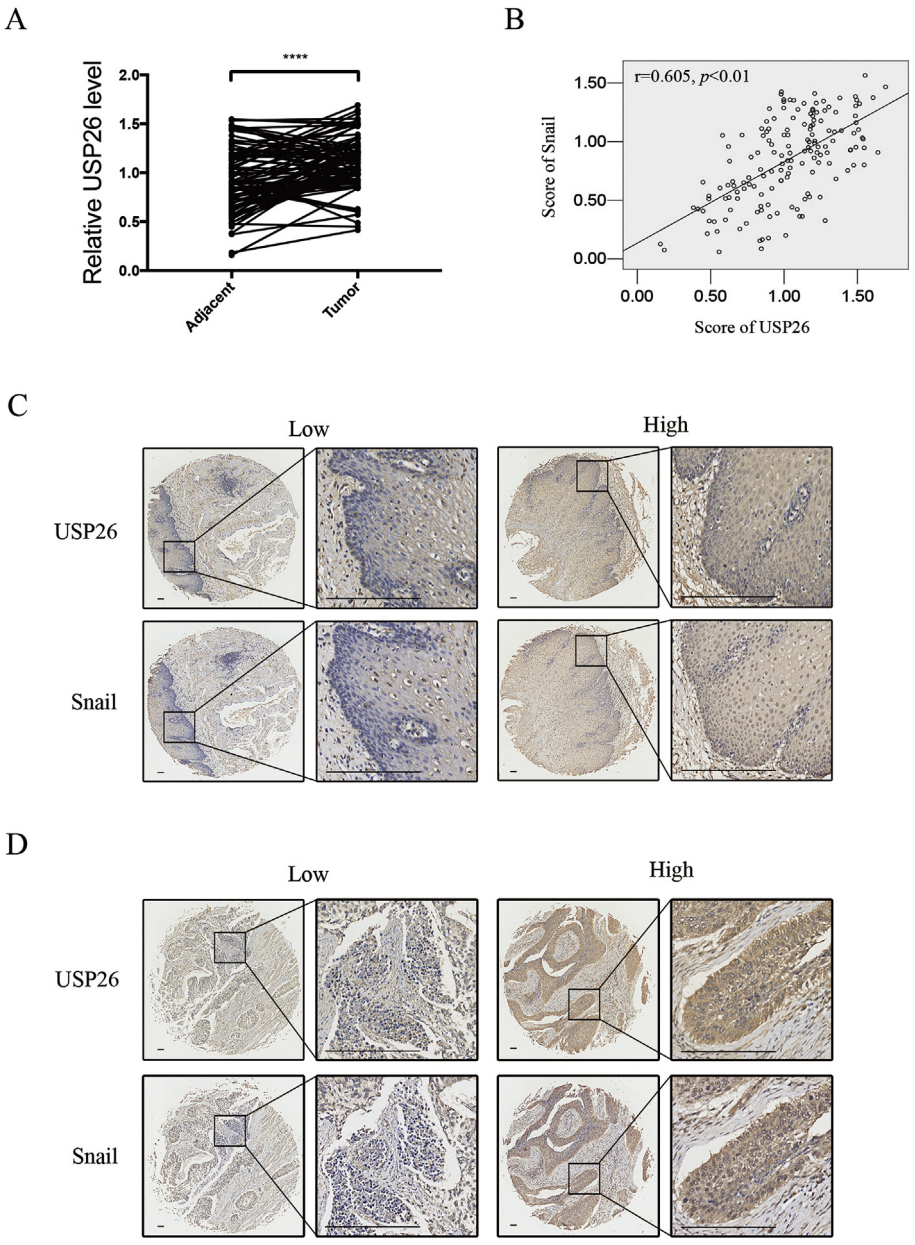

Fig. 5. Expression of Snail is positively correlated with USP26in ESCC.
(A) The expression of USP26 in ESCC and paired adjacent normal tissues was detected by immunohistochemistry, and the relative expression of USP26 was analyzed (unpaired *t*-test, ****$p < 0.0001$). (B) The expression of USP26 is positively correlated with Snail in ESCC samples (Pearson's test, $p < 0.01$). (C and D) The typical staining of USP26 and Snail in normal esophageal epithelial (C) and ESCC tissues (D). Scale bars, 100 μm.

epithelial cells detached from epithelial tissues went through a crucial step of changing their cell phenotypes to mesenchymal-like. The transition is known as EMT which plays a key role in the tumor metastasis [7]. EMT involves many key events, such as degradation of the cell junctions, loss of basal polarity, changing of the cell shape and architecture, downregulation of epithelial phenotype markers and activation of mesenchymal genes, increasing of cell motility and degraded extracellular matrix proteins [29,30]. Executers of EMT are EMT-TFs, consisted of ZEB, TWIST and Snail families, which performed an important function in tumor initiation, growth, invasion, chemoresistance and metastasis [9]. Snail as a vital EMT-TFs, its most studied role is initiating EMT progress. The momentous mechanism of Snail induced EMT is suppression of E-cadherin transcription, a key component of adherens junctions in epithelial cells [12,13]. In addition, Snail plays a critical role in the evolvement of metastatic, drug resistance, cancer stem cell (CSC)-like properties and tumor recurrence [31,32].

As Snail is a crucial factor of EMT and distant metastasis, we focused on the inherent mechanism of Snail regulation in ESCC metastasis. Firstly, it has been demonstrated that expression of Snail is post-translationally regulated through ubiquitin proteasome pathway. And many E3 ligases regulating Snail ubiquitination and degradation has been reported [18–21]. However, stabilization of Snail through deubiquitination is unclear. Although some deubiquitinases of Snail, such as DUB3, PSMD14 and OTUB1, had been demonstrated to regulate its ubiquitination and stabilization [23–26], it is common that multiple deubiquitinating enzymes targeted the same substrate to regulate its stability under different circumstances. Such as, the crucial oncogene Slug is deubiquitinated and stabilized by DUB3 and USP10 [33,34]. Ubiquitination of p53 is downregulated by deubiquitinating enzymes USP7, USP10 and USP11 [35–37]. In addition, β-catenin is deubiquitinated by USP15 and USP4 [38,39]. Therefore, we suppose Snail stability and ubiquitination also regulated by multiple deubiquitinating

enzymes. In this study, we found USP26 is a specific deubiquitinase of Snail which could significantly increase Snail stability, and USP26 specifically interacts with Snail. USP26 binds to the Snail zinc finger domain, a region which is necessary for Snail stability and nuclear localization [40]. The Snail regulators, NOTCH1 and PARP1, are both bound to zinc finger region [41–43]. USP26 interacted with Snail via its USP domain which is a conservative domain of USPs and where the catalytic sites located. USP26 was reported to remove K48-linked Ub chains of CBX4 and CBX6 in previously published studies [44]. In this study, we demonstrated that USP26 removed K48 ubiquitin chains from Snail which are signals for recognition and initiation of degradation [45].

We also identified that USP26 co-located with Snail and induced accumulation of Snail protein in ESCC cells. USP26-overexpressing ESCC cells showed decreased epithelial marker E-cadherin and increased mesenchymal labels N-cadherin and Vimentin protein level, and had a stronger ability of migration and invasion. The USP26 mutant showed no such effect. Conversely, knockdown of USP26 significantly repressed the transition of epithelial-like cells to mesenchymal phenotype and metastasis of ESCC cells. In addition, knockdown of Snail reversed the EMT process and the effect of USP26 overexpression on promoting metastasis of ESCC cells. Overexpression of Snail also rescued the USP26-knockdown cells' migration and invasion. These observations further proved that USP26 enhanced metastasis of ESCC cells through stabilization of Snail. As USP26 has been proved to be an oncogene in vivo and in vitro, we examined the expression of USP26 and Snail in clinical ESCC and adjacent normal esophageal epithelial tissues. We observed a higher expression of USP26 in ESCC tissues than the normal tissues, and there was a strong positive correlation between USP26 and Snail in ESCC samples. Thus, our results suggested that USP26 performed oncogenic role in ESCC and might serve as a potential therapeutic target in the treatment of ESCC metastasis. A specific small molecule inhibitor for USP26 might be a promising therapeutic agent for metastatic ESCC patients. This is also the direction of our further research.

In summary, our study demonstrates a vital role for USP26 in ESCC metastasis by mediating Snail stability. And the accumulated Snail enhances ESCC metastasis through inducing EMT. In addition. Our study provides a rationale and potential target for the treatment of human ESCC.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.007.

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