ADAM10 mediates the cell invasion and metastasis of human esophageal squamous cell carcinoma via regulation of E-cadherin activity

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Abstract. A disintegrin and metalloprotease 10 (ADAM10) is involved in the tumorigenesis, invasion and metastasis of several types of solid tumors. However, the potential role of ADAM10 in human esophageal squamous cell carcinoma (ESCC) is not yet well understood. The present study showed that ADAM10 was overexpressed in human ESCC tissues *in vivo*, and positively associated with depth of tumor invasion, lymph node metastasis and TNM stage, contributing to tumor carcinogenesis, invasion and metastasis. Additionally, ADAM10 was overexpressed in 3 types of ESCC cell lines *in vitro*, as compared to that in normal esophageal epithelial cells (NEECs); and moreover, ESCC cells with high ADAM10 expression obtained enhanced invasion and migration ability. Subsequently, ADAM10 silencing by small interfering (si) RNA in ESCC cell line, EC-1, reduced cell invasion, migration and proliferation *in vitro*. Finally, ADAM10 negatively regulated E-cadherin in ESCC *in vivo* and *in vitro*. In conclusion, active ADAM10 promotes the carcinogenesis, invasion, metastasis and proliferation of ESCC and controls invasion and metastasis at least in part through the shedding of E-cadherin activity, which makes it a potential biomarker and a useful therapeutic target for ESCC.

Introduction

Esophageal carcinoma is one of the most common malignancies and has been ranked as the sixth leading cause of cancer-related death worldwide (1-4). Esophageal squamous cell carcinoma (ESCC), accounting for approximately 90% of all esophageal carcinoma cases, is more predominant in Asian countries (5). In the past decades, although tremendous strides have been made in therapeutic strategies including surgical resection, radiotherapy and chemotherapy, tumor invasion and metastasis still invariably heralds a poor prognosis and a low 5-year survival rate in ESCC patients (6-8). Therefore, a better understanding of the molecular mechanisms involved in tumor invasion and metastasis and discovery of a more effective target for anticancer therapy may help to prolong the survival and improve the life quality of patients with ESCC.

The a disintegrin and metalloproteases (ADAMs), a family of zinc-dependent transmembrane proteins, contain a metalloprotease and a disintegrin-like domain (9-11) and are closely associated with the degradation of the basement membrane, cell migration, cell fusion and signal transduction (12,13), thus playing important roles in the tumorigenesis, proliferation, differentiation, adhesion, invasion and metastasis of cancers (14-16). To date, approximately 40 ADAM members have been identified in this family, however only 21 are thought to function in humans (10). ADAM10 is an important proteolytically active member of this family (17). Emerging evidence suggests that ADAM10 is overexpressed in a variety of cancers and is associated with the tumor progression of breast cancer (18), non-small cell lung cancer (NSCLC) (19), nasopharyngeal carcinoma (20), oral squamous cell carcinoma (21,22) tongue squamous cell carcinoma (23), hepatocellular carcinoma (24,25), gastric cancer (26), colon cancer (27), pancreatic carcinoma (28), bladder cancer (29) and renal cell carcinoma (30). Proteolytically active ADAM10 acts as an ectodomain-sheddase, which can release the extracellular domain of membrane-bound proteins, such as adhesion molecules, growth factors, cytokines, chemokines and receptors; through these actions they are able to disrupt the tumor microenvironment and modulate key processes involved in tumor cell proliferation, migration and angiogenesis (31,32). E-cadherin is a transmembrane molecule that functions as an adhesion molecule, and is reported as an ADAM10 substrate (33,34). ADAM10 activity could lead to elevated shedding of E-cadherin and loss of cell-cell contact (35), further enhancing migratory and metastatic behavior (36).

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However, to date there has been no related study concerning the role of ADAM10 in the tumorigenesis, invasion and metastasis of ESCC. Moreover, whether the potential mechanism by which ADAM10 promotes tumor progression is associated with E-cadherin shedding is still unclear.

Herein, in the present study, we first detected expression of ADAM10 protein in ESCC tissues *in vivo* and compared it to E-cadherin expression and clinicopathological parameters associated with tumor invasion and metastasis, including tumor differentiation, depth of invasion, lymph node metastasis and TNM stage. In addition, ADAM10 expression in ESCC cell lines was examined *in vitro* and compared to cell invasion and migration ability. Furthermore, we inhibited the expression of ADAM10 in ESCC cell lines by using ADAM10 siRNA and analyzed the effect of the downregulation of ADAM10 on cell proliferation, invasion and migration and the expression levels of E-cadherin *in vitro*.

Materials and methods

Statement of ethics. This study was performed according to ethical protocol approved by the First Affiliated Hospital of Zhengzhou University, Henan, China. All of the clinical tissue samples used in this study were collected after obtaining written informed consents. All efforts were made to minimize suffering.

Tissue specimens. A total of 122 ESCC tissue specimens and 60 adjacent normal esophageal mucosa were obatined from patients (73 male and 49 female) who underwent surgical resection at The First Affiliated Hospital of Zhengzhou University, Henan, China. None of the patients had previously received neoadjuvant radiotherapy, chemotherapy or immunotherapy. Tissue sections were cut at a thickness of 4 μm for *in situ* hybridization (ISH) and immunohistochemistry (IHC). Clinical features of all the samples are listed in Table I. The diagnosis of ESCC was reconfirmed by two board-certified pathologists.

In situ hybridization (ISH) and evaluation of ISH staining. Paraffin-embedded slides were deparaffinized, rehydrated, and immersed in 3% hydrogen peroxide. Then the slides were digested in pepsin solution, and incubated with pre-hybridization solution and hybridization solution sequentially. After visualization of ISH using BCIP/NBT, the slides were counterstained with Nuclear Fast Red. The digoxin-labeled probe sequences used in this study were as follows: ADAM10 forward primer, 5-GAGGAGTGTACGTGTGCCAGTT-3 and reverse primer, 5-GACCACTGAAGTGCCTACTCCA-3; E-cadherin forward primer, 5-AAAGGCCCATTTCCTAAAAACCT-3 and reverse primer, 5-TGCGTTCTCTATCCAGAGGCT-3. ADAM10- and E-cadherin-positive staining was viewed as kyano-purple granule-like material that was located in the cytoplasm. The staining index was calculated by adding the scores for the percentage of positively stained cells (1, 0-10%; 2, >10-30%; 3, >30-70%; and 4, >70%) and the staining intensity (1, weak; 2, moderate; and 3, strong). A score of 0-2 was designated as a negative score (-), and ≥3 as a positive score (+). All of the staining analyses were independently performed by two pathologists.

Immunohistochemistry (IHC) and evaluation of IHC staining. Paraffin-embedded slides were deparaffinized, rehydrated, and subjected to antigen retrieval by using a microwave oven. Then the slides were incubated with primary antibodies anti-ADAM10 (1:200, Abcam, USA) or anti-E-cadherin (1:100, Santa Cruz, CA, USA) respectively, and subsequently incubated with biotinylated anti-rabbit IgG secondary antibody. After visualization of the immunoreactivity using DAB, the slides were counterstained with hematoxylin. ADAM10- and E-cadherin-positive staining was viewed as brown-yellow granule-like material that was located in the membrane and/or cytoplasm. The staining index was calculated by adding the scores for the percentage of positively stained cells (1, 0-10%; 2, >10-50%; and 3, >50%) and the staining intensity (1, weak; 2, moderate; and 3, strong). A score of 0-2 was designated as a negative score (-), and ≥3 as a positive score (+). All of the staining analyses were independently performed by two pathologists.

Cell lines and culture. Human ESCC cell line EC-1 was supplied by Professor Shihua Cao (Hong Kong University). Eca109 and TE-1 cells were maintained in the Key Laboratory of Tumor Pathology of Zhengzhou University. Normal esophageal epithelial cells (NEECs) of primary culture were obtained from fresh biopsies of esophageal mucosal tissues, during our previous experiment. All cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin, in a humidified incubator containing 5% CO_2 at 37˚C. Additionally, 1.0 ng/ml of epidermal growth factor (EGF) was added to the medium for the NEECs.

ADAM10 siRNA transfection of EC-1 cells. The sequences of ADAM10 siRNA and the control nonspecific siRNA were as follows: sense strand, AAAGGAUUCCCAUACUGAC and antisense strand, GUCAGUAUGGGAAUCCUUU for ADAM10; sense strand, AUCUUGAUCUUCAUUGUGC and antisense strand, GCACAAUGAAGAUCAAGAU for the nonspecific control. EC-1 cells grown until 80-90% confluency were transfected with 100 nM ADAM10 siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. EC-1 cells untreated or transfected with the nonspecific siRNA were used as control. The cells were cultured for an additional 48 h in a humidified incubator containing 5% CO_2 at 37˚C before being harvested for further assays.

RNA extraction, reverse transcription (RT) and real-time PCR. Total RNA was extracted from the cultured cells using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Two micrograms of total RNA was used for cDNA synthesis using the HiScript II RT kit (Vazyme, Nanjing, China). To evaluate ADAM10 and E-cadherin mRNA expression, real-time PCR was performed using a PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). β-actin was selected as an internal control. The primer sequences were as follows: forward, 5-CACGAGAAGCTGTGATTGCC-3 and reverse, 5-ATTCCGGAGAAGTCTGTGGTC-3 for ADAM10; forward, 5-TGATTCTGCTGCTCTTGCTG-3 and reverse,

Table I. Protein and mRNA expression of ADAM10 and E-cadherin in ESCC tissues and corresponding normal esophageal mucosa.

		ADAM10				E-cadherin			
	n	-	+	χ^2	P-value	-	+	χ^2	P-value
Protein									
Normal mucosa	60	49	11	37.164	0.000	7	53	51.120	0.000
ESCC	122	41	81			83	39		
mRNA									
Normal mucosa	60	51	9	31.550	0.000	0	60	59.946	0.000
ESCC	122	50	72			73	49		

5-CAAAGTCCTGGTCCTCTTCTCC-3 for E-cadherin; forward, 5-TTCGAGCAAGAGTGGCCA-3 and reverse, 5-AGGTAGTTTCGTGGATGCCA-3 for β-actin. ADAM10 and E-cadherin expression levels were normalized to β-actin and analyzed by using the $2^{-\Delta\Delta Ct}$ method (37). All experiments were carried out in triplicate.

Protein extraction and western blot analysis. Protein was extracted from the cultured cells using RIPA lysis buffer (Well, Shanghai, China) according to the manufacturer's protocol. The protein concentration was determined using the BCA kit (Bioss, Beijing, China). For western blot analysis, 50 μg of protein extract from each cell line was separated by SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% non-fat milk in PBST for 1 h at room temperature, the membrane was incubated with the primary antibody (ADAM10, 1:500, 84 kDa; E-cadherin, 1:200, 120 kDa) at 4˚C overnight. β-actin (1:5,000, 43 kDa) was selected as a loading control. Protein bands were detected with the ECL system (Well, Shanghai, China) and exposed to X-ray film. Quantification was performed by using ImageJ analysis tool. All experiments were carried out in triplicate.

Matrigel invasion assay. Tumor cell invasion ability was analyzed using an invasion chamber (Corning®, Corning, NY, USA). Briefly, a polycarbonate membrane with 8-μm pores was coated with Matrigel and dried at room temperature for 1 h in a 24-well plate. Cells in serum-free medium were seeded into the upper chamber of the wells and RPMI-1640 with 10% FBS was added into the lower chamber as a chemoattractant. Following incubation at 37˚C in 5% CO_2 for 24 h, cells on the upper surface of the membrane were scrubbed away by a cotton swab. Cells invading through the membrane and adhering to the lower surface of the membrane were fixed with 95% alcohol, stained with 0.05% crystal violet for 1 h, and quantitated by measuring the number of stained cells in five individual fields at a x400 magnification under a light microscope. All experiments were carries out in triplicate.

Scratch wound healing assay. Cell migration ability was measured by a scratch wounding healing assay. Cells were seeded into 12-well plates and incubated in RPMI-1640 with 10% FBS medium. Following incubation for 24 h, a scratch would was made across the center of the monolayer cells by using a sterile 200-μl pipette tip. Subsequently, the cells were washed with PBS to remove the detached cells and incubated with serum-free medium for an additional 48 h. Images of the cells that had migrated into the cell-free scratch wound area were acquired at a x200 magnification under a reversed light microscope. Cell migration ability was quantitated by measuring the width of the wounds in at least five representative fields and is expressed as 1 minus the average percent of the wound closure compared with the initial wound area measured. All experiments were carried out in triplicate.

Cell proliferation assay. Cell proliferation ability was measured by Cell Counting Kit-8 (CCK-8) assay. Briefly, the cells were seeded into 96-well plates at a density of $5x10^3$ cells/well and incubated for 0, 24, 48 and 72 h, respectively. At different time intervals, 10 μl of CCK-8 solution (Sangon, Shanghai, China) was added to the corresponding wells and incubated for an additional 4 h. The staining intensity in the medium was measured by determining the absorbance (OD values) at 450 nm to obtain cell growth curves. All experiments were carried out in triplicate.

Statistical analysis. All the data were analyzed using SPSS version 17.0 statistical package. The difference in counted data was examined by the χ^2 test. Meanwhile, quantitative data are expressed as mean ± SD, and differences in quantitative data were examined by the t-test. A P-value <0.05 was defined as indicating a statistically significant result.

Results

ADAM10 expression is elevated and E-cadherin expression is reduced in ESCC tissues. Emerging evidence suggests that ADAM10 is overexpressed in a variety of cancers and participates in tumor progression (18-30). Thus, IHC was initially performed to determine the protein expression level of ADAM10 in ESCC tissues and corresponding normal esophageal mucosal tissues. ADAM10-positive signals showed brown-yellow granules in the membrane and/or cytoplasm (Fig. 1A and B). Cytoplasmic expression of ADAM10 protein was strong in the ESCC tissues (Fig. 1A), but weak in the normal esophageal mucosa (Fig. 1B). The positive rate of ADAM10 was 66.4% (81/122) in the ESCC tissues, significantly higher than that in the corresponding normal esophageal mucosa [18.3% (11/60); P=0.000) (Table I).

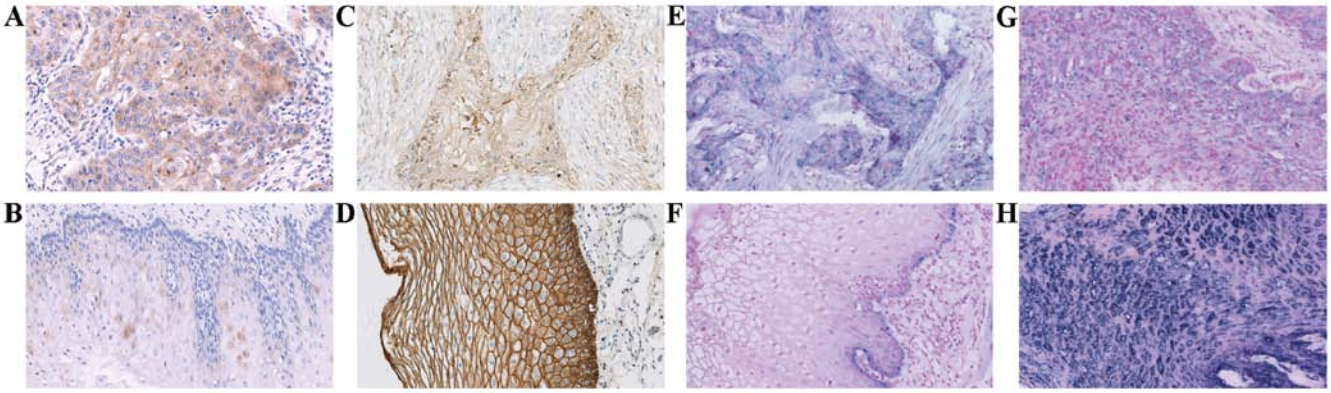


Figure 1. Immunohistochemistry (IHC) and *in situ* hybridization (ISH) images of ADAM10 and E-cadherin in representative samples from esophageal squamous cell carcinoma (ESCC) tissues and corresponding normal esophageal mucosa. Cytoplasmic expression of ADAM10 protein was strong in the ESCC tissues (A), but weak in the normal esophageal mucosa (B). Membranal/cytoplasmic expression of E-cadherin protein was moderate in the ESCC tissues (C), but strong in the normal esophageal mucosa (D). Cytoplasmic expression of ADAM10 mRNA was strong in the ESCC tissues (E), but was noted only in the basement of normal esophageal mucosa (F). Cytoplasmic expression of E-cadherin mRNA was weak in the ESCC tissues (G), but strong in the normal esophageal mucosa (H). Original magnification, x200.

Table II. Correlation of ADAM10 and E-cadherin proteins with clinicopathological parameters in the ESCC tissues.

		ADAM10				E-cadherin			
	n	-	+	χ^2	P-value	-	+	χ^2	P-value
Gender									
Male	73	44	29	0.015	0.904	48	25	0.434	0.510
Female	49	29	20			35	14		
Age (years)									
≥60	77	46	31	0.001	0.977	52	25	0.024	0.877
<60	45	27	18			31	14		
Histological grade									
I	33	16	17	4.510	0.105	16	17	7.981	0.018
II	51	14	37			38	13		
III	38	11	27			29	9		
Depth of invasion									
Superficial muscularis	19	11	8	6.014	0.049	8	11	7.360	0.025
Deep muscularis	46	14	32			32	14		
Fibrous membrane	57	16	41			43	14		
Lymph node metastasis									
No	74	34	40	12.835	0.000	44	30	6.357	0.012
Yes	48	7	41			39	9		
TNM stage									
I	17	11	6	8.920	0.012	7	10	6.790	0.034
II	44	14	30			33	11		
III	61	16	45			43	18		

Since ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion (33), we next detected the expression level of E-cadherin in the ESCC tissues and the corresponding normal esophageal mucosa tissues. E-cadherin-positive signals were presented as brown-yellow granules in the membrane and/or cytoplasm (Fig. 1C and D). Membranal and/or cytoplasmic expression of E-cadherin protein was negative or moderate (Fig. 1C) in the ESCC tissues, but strong in the normal esophageal mucosa (Fig. 1D). The positive rate of E-cadherin was 32.0% (39/122) in the ESCC tissues, significantly lower than that in the corresponding normal esophageal mucosa [88.3% (53/60); P=0.000) (Table I).

Furthermore, *in situ* hybridization (ISH) was used to detect the mRNA expression levels of ADAM10 and E-cadherin in the ESCC tissues and corresponding normal esophageal mucosa tissues. ADAM10-and E-cadherin-positive signals

Table III. Correlation of ADAM10 with E-cadherin protein in the ESCC tissues.

	ADAM10			
E-cadherin	+	-	r	P-value
+	11	28	-0.554	0.000
-	70	13		

both showed kyano-purple granule in cytoplasm (Fig. 1E-H). Expression of ADAM10 mRNA was strong in the ESCC tissues (Fig. 1E), but was present only in the basement of the normal esophageal mucosa (Fig. 1F). Expression of E-cadherin mRNA was negative or weak (Fig. 1G) in the ESCC tissues, but strong in the normal esophageal mucosa (Fig. 1H). The positive rates of ADAM10 and E-cadherin were 59.0 (72/122) and 40.2% (49/122) in the ESCC tissues, and 15.0 (9/60) and 100.0% (60/60) in the corresponding normal mucosa, respectively. Moreover, the expression of ADAM10 mRNA in the ESCC tissues was significantly higher than that in the corresponding normal esophageal mucosa (P=0.000), whereas contrary results were observed in the expression of E-cadherin mRNA (P=0.000) (Table I).

Taken together, the above data demonstrated that overexpression of ADAM10 contributes to carcinogenesis of ESCC and this action may be attributable to ADAM10-mediated E-cadherin shedding.

Elevated ADAM10 and reduced E-cadherin protein levels are associated with tumor progression in ESCC tissues. To investigate the clinical significance of ADAM10 and E-cadherin in ESCC tissues, we analyzed the correlation of their protein expression levels with clinicopathological features including histological grade, depth of invasion, lymph node metastasis and TNM stage. As shown in Table II, ADAM10 expression was positively correlated with depth of invasion, lymph node metastasis and TNM stage (P<0.05, respectively), but not correlated with histological grade (P>0.05). On the contrary, E-cadherin expression was negatively correlated with histological grade, depth of invasion, lymph node metastasis and TNM stage (P<0.05, respectively). These data demonstrated that in the ESCC tissues elevated ADAM10 and reduced E-cadherin levels play a pivotal role in tumor progression, such as invasion and metastasis.

Negative correlation of ADAM10 and E-cadherin protein levels in ESCC tissues. To explore the role of active ADAM10 involving E-cadherin in ESCC progression, we further studied the correlation of ADAM10 protein expression with E-cadherin in the ESCC tissues. As shown in Table III, ADAM10 protein expression was significantly negatively correlated with E-cadherin (r=-0.554, P=0.000), implying ADAM10 may control the invasion and metastasis of ESCC through E-cadherin shedding.

ADAM10 is overexpressed in ESCC cell lines and high expression of ADAM10 is correlated with increased cell invasion and migration in vitro. To further investigate the potential biological roles of ADAM10 in ESCC, cell experiments were carried out. We first used quantitative real-time PCR and western blot analysis to detect the expression levels of ADAM mRNA and protein in ESCC Eca109, EC-1 and TE-1 cells. Compared to the NEECs, Eca109, EC-1 and TE-1 cells showed significant overexpression of ADAM10 at both the mRNA and protein levels (P<0.01) (Fig. 2). Moreover, this increased expression was more apparent in the EC-1 cells than that in the Eca109 and TE-1 cells at the mRNA level (P<0.01), and than that in the TE-1 cells at the protein level (P<0.05) (Fig. 2).

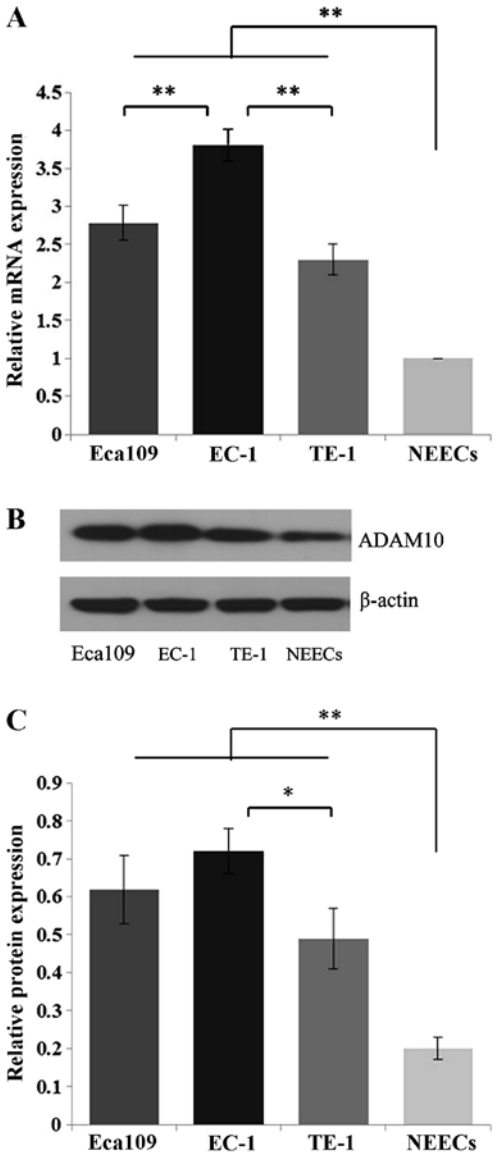


Figure 2. Quantitative real-time PCR and western blot analysis were used to detect mRNA and protein levels of ADAM10 in 3 types of ESCC cells. (A-C) Expression of ADAM10 mRNA and protein in ESCC cell lines (Eca109, EC-1, TE-1) and NEECs. ADAM10 expression at both the mRNA and protein level was significantly higher in the ESCC cells than levels in the NEECs. Moreover, this increase was more apparent in the EC-1 cells. β-actin was used as internal control. *P<0.05 and **P<0.01, compared to NEECs.

Based on the above clinical correlation analysis in the ESCC tissues, it is reasonable to hypothesize that ADAM10 plays an important role in promoting the invasion and migra-

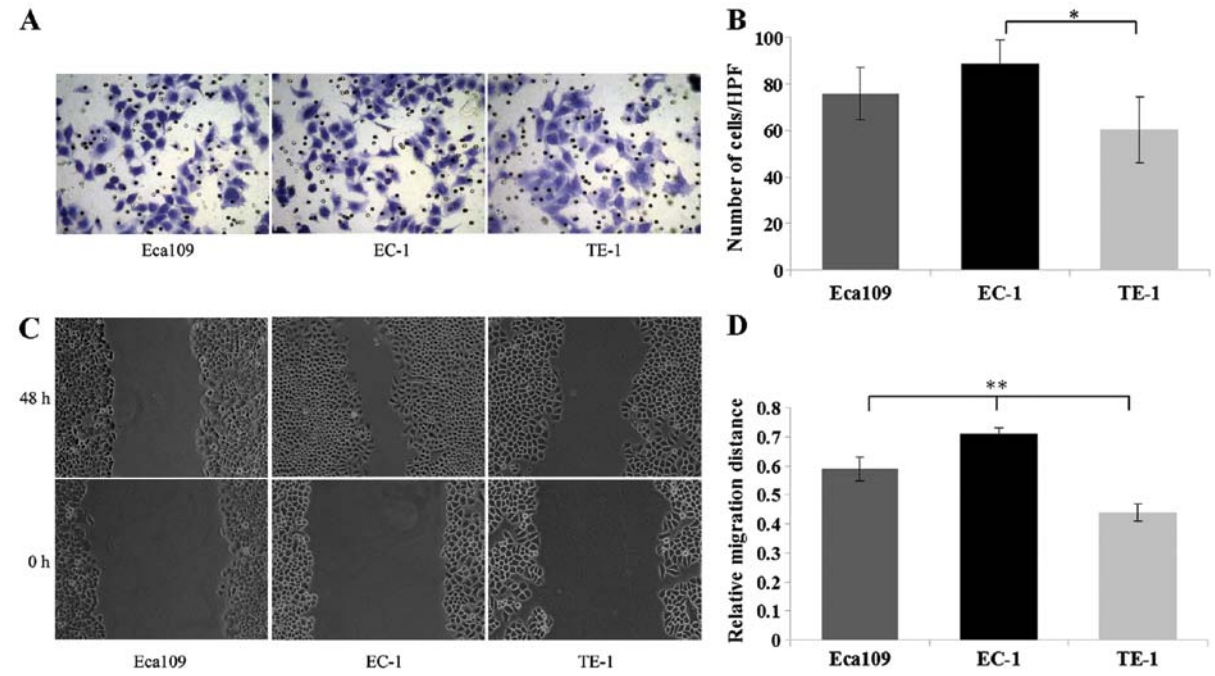
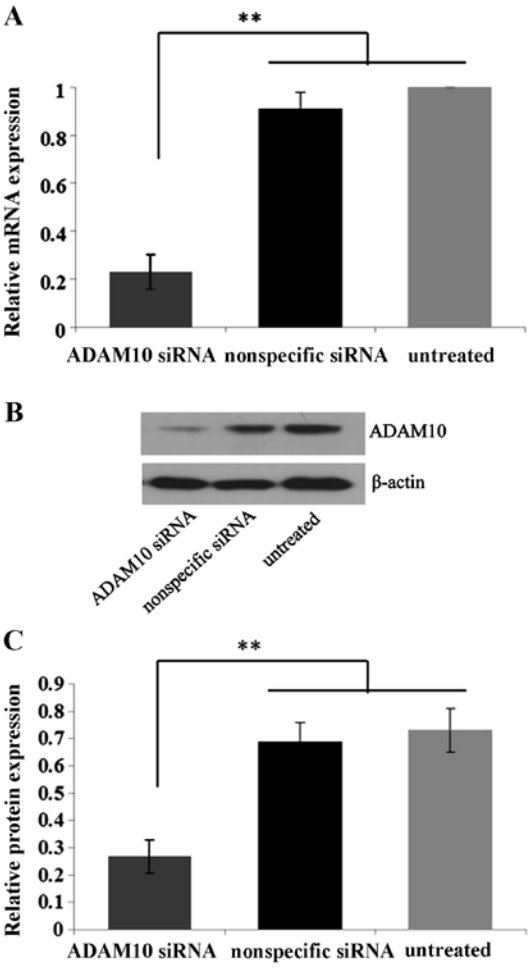


Figure 3. Matrigel invasion and scratch wound healing assays were used to detect the invasion and migration ability of ESCC cells. (A) For the Matrigel invasion assay, 3 ESCC cell lines (Eca109, EC-1, TE-1) in serum-free medium were seeded into the upper chamber and incubated for 24 h, respectively; subsequently cells invading through the Matrigel were fixed, stained and photographed under a light microscope at a x400 magnification. (B) EC-1 cells exhibited a significant increase in the number of traversed cells as compared with the TE-1 cells. (C) For the scratch wound healing assay, 3 ESCC cell lines (Eca109, EC-1, TE-1) were seeded into 12-well plates and incubated in medium containing 10% FBS for 24 h, respectively. Subsequently the wounds were made, and the cells were incubated in serum-free medium for an additional 48 h and photographed under a reversed light microscope at a x200 magnification. (D) The bar graph shows that the EC-1 cell migration was more rapid than the rate noted in the TE-1 cells. *P<0.05, **P<0.01.



tion of ESCC cells. Therefore, we next detected the invasion and migration ability of the ESCC cells using Matrigel invasion and scratch wound healing assays, respectively. The invasion assay demonstrated that the EC-1 cells exhibited a significant increase in the number of cells that traversed the membrane toward a chemoattractant as compared with the TE-1 cells (P<0.05) (Fig. 3A and B). Additionally, the scratch assay demonstrated that EC-1 cells showed a significant increase in the tumor cell migration distance as compared with the Eca109 and TE-1 cells (P<0.01) (Fig. 3C and D).

In line with the aberrant ADAM10 in these 3 types of ESCC cell lines, EC-1 cells with high ADAM10 expression demonstrated higher invasion and migration ability, whereas TE-1 cells with low ADAM10 expression demonstrated poor invasion and migration ability, suggesting that high ADAM10 expression is consistent with increased cell invasion and migration ability.

Specific blockage of ADAM10 by ADAM10 siRNA in EC-1 cells in vitro. In order to detect the knockdown efficiency

Figure 4. Quantitative real-time PCR and western blot analysis were used to detect the specificity and efficiency of ADAM10 knockdown by ADAM10 siRNA in the EC-1 cells. (A-C) Expression of ADAM10 mRNA and protein in EC-1 cells transfected with ADAM10 siRNA, EC-1 cells transfected with nonspecific siRNA and untreated EC-1 cells. The bar graphs show that the mRNA and protein levels of ADAM10 in the EC-1 cells transfected with ADAM10 siRNA were significantly lower than those in the control nonspecific siRNA-transfected and untreated cells, indicating that the knockdown of ADAM10 by ADAM10 siRNA was specific and efficient. **P<0.01. β-actin was used as an internal control.

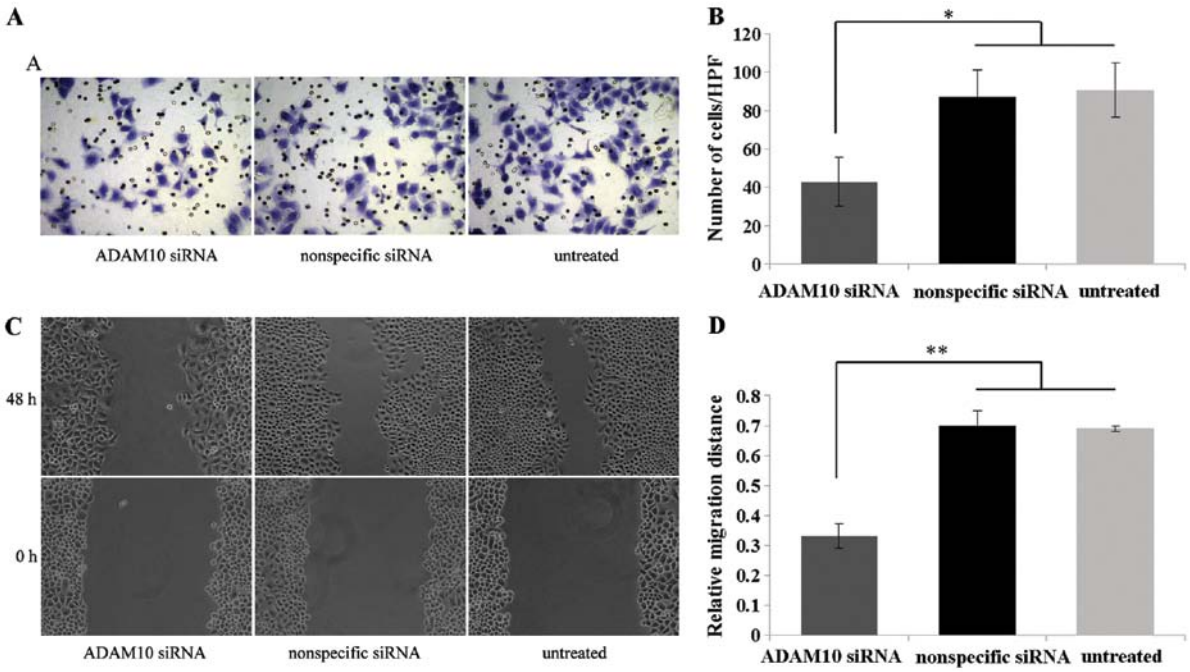


Figure 5. Matrigel invasion and scratch wound healing assays were used to detect the invasion and migration ability of the EC-1 cells transfected with ADAM10 siRNA. (A and C) Representative images of the Matrigel invasion assay (x400) and scratch wound healing assay (x200) for EC-1 cells transfected with ADAM10 siRNA, control nonspecific siRNA-transfected cells and untreated cells under a reversed light microscope, respectively. (B and D) EC-1 cells transfected with ADAM10 siRNA exhibited a significant decrease in the number of traversed cells and the cell migration distance in comparison to the control nonspecific siRNA-transfected and untreated cells, implying that ADAM10 knockdown inhibited the invasion and migration of the EC-1 cells *in vitro*. *P<0.05, **P<0.01.

of ADAM10 by ADAM10 siRNA, EC-1 cells were selected due to their elevated ADAM10 expression and enhanced invasion and migration ability compared to the Eca109 and TE-1 cell lines. Quantitative real-time PCR and western blot analysis showed that the expression levels of ADAM10 mRNA and protein in the ADAM10 siRNA-transfected cells were suppressed by ~75 and 60%, significantly lower than those in the control nonspecific siRNA-transfected and untreated cells (P<0.01) (Fig. 4), confirming the specific and efficient blockage of ADAM10 by ADAM10 siRNA in the EC-1 cells *in vitro*.

Knockdown of ADAM10 inhibits the invasion and migration of EC-1 cells in vitro. We examined the effect of ADAM10 knockdown on EC-1 cell invasion and migration using a Matrigel invasion and scratch wound healing assays. Matrigel invasion assay demonstrated that EC-1 cells transfected with the ADAM10 siRNA exhibited a significant decrease in the number of cells that traversed the membrane toward a chemoattractant as compared with the control nonspecific siRNA-transfected and untreated cells (P<0.05) (Fig. 5A and B). Furthermore, the scratch wound healing assay demonstrated that the EC-1 cells transfected with the ADAM10 siRNA resulted in a significant decrease in the tumor cell migration distance in comparison to the control nonspecific siRNA-transfected and untreated cells (P<0.01) (Fig. 5C and D). These data indicated that down-regulation of ADAM10 inhibited the invasion and migration of the EC-1 cells *in vitro*.

Knockdown of ADAM10 inhibits proliferation of EC-1 cells in vitro. To evaluate the effect of ADAM10 knockdown on EC-1 cell proliferation, CCK-8 assay was carried out. Compared with the control nonspecific siRNA-transfected and untreated cells, the EC-1 cells transfected with the ADAM10 siRNA showed significantly decreased OD values after incubation for 48 (P<0.05) and 72 h (P<0.01) (Fig. 6),

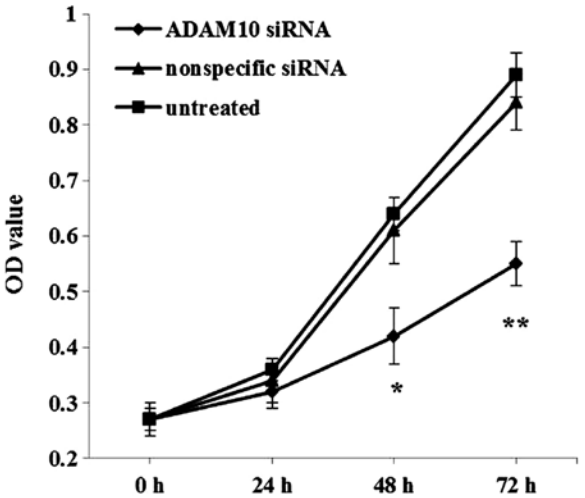


Figure 6. CCK-8 assay was used to detect the proliferation ability of the EC-1 cells transfected with ADAM10 siRNA. The EC-1 cells transfected with ADAM10 siRNA, control nonspecific siRNA-transfected cells and untreated cells were seeded into 96-well plates and incubated for 0, 24, 48 and 72 h, respectively. At different time intervals, CCK-8 reagent was added to each well and incubated for an additional 4 h. The staining intensity in the medium was measured by determining the absorbance (OD values) at 450 nm to obtain cell growth curves. Compared with the control nonspecific siRNA-transfected and untreated cells, EC-1 cells transfected with ADAM10 siRNA showed significantly decreased OD values at 48 and 72 h. *P<0.05, **P<0.01.

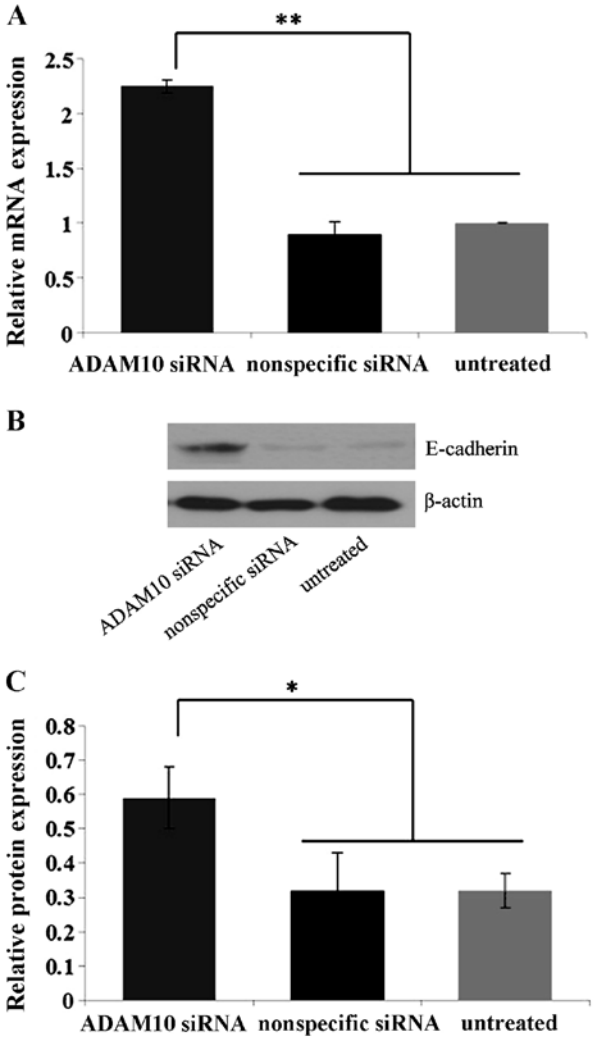


Figure 7. Quantitative real-time PCR and western blot analysis were used to detect the effect of ADAM10 knockdown by ADAM10 siRNA on E-cadherin expression in the EC-1 cells. (A-C) Expression of E-cadherin mRNA and protein in the EC-1 cells transfected with ADAM10 siRNA, EC-1 cells transfected with nonspecific siRNA and untreated EC-1 cells. The bar graphs show that the mRNA and protein levels of E-cadherin in the EC-1 cells transfected with ADAM10 siRNA were significantly higher than those in the control nonspecific siRNA-transfected and untreated cells, indicating that ADAM10 is essentially involved in E-cadherin cleavage in EC-1 cells *in vitro*. *P<0.05 and **P<0.01. β-actin was used as an internal control.

suggesting that downregulation of ADAM10 inhibited EC-1 cell growth *in vitro*.

Knockdown of ADAM10 suppresses E-cadherin expression in EC-1 cells in vitro. As described previously, expression levels of ADAM10 and E-cadherin were inversely correlated in the ESCC tissues. To further characterize the intrinsic relationship between ADAM10 and E-cadherin expression and explore the possible impact that ADAM10 exerts on the invasion and metastasis of ESCC, the expression changes in E-cadherin mRNA and protein in the EC-1 cells transfected with ADAM10 siRNA were determined by quantitative real-time PCR and western blot analysis assays. As shown in Fig. 7, downregulation of ADAM10 by ADAM10 siRNA dramatically increased E-cadherin mRNA and protein in the ADAM10 siRNA-transfected EC-1 cells as compared with the control nonspecific siRNA-transfected and untreated cells (P<0.05). These results demonstrated that ADAM10 is essentially involved in E-cadherin cleavage in EC-1 cells *in vitro*.

Discussion

The role of ADAM10 has been clarified in a variety of important biological processes, such as cell adhesion (33), cell migration (38), immunoreaction regulation (39,40) and apoptosis control (41). However recently, it has emerged as a major and key player in human diseases. ADAM10 is considered to contribute to Alzheimer's disease (42) and chronic inflammatory diseases (43) such as lung inflammation (44) and eczematous dermatitis (45); most importantly, it is overexpressed in many cancers and participates in tumor progression (18-30). Guo *et al* (19) demonstrated that ADAM10 protein expression is significantly increased in human NSCLC tissues, particularly in metastatic tissues; furthermore, downregulation of ADAM10 by ADAM10 shRNA reduced the invasion and migration of NSCLC cells. Consistent with this result, Gaida *et al* (28) also found that ADAM10 mRNA and protein were overexpressed in pancreatic carcinoma compared to normal pancreatic tissues, and ADAM10 silencing markedly reduced the invasiveness and migration of the tumor cells. However, the role of ADAM10 in ESCC is not yet well understood.

In the present study, we first examined the expression levels of ADAM10 mRNA and protein in human ESCC tissues and cells. As expected, ADAM10 mRNA and protein were both dramatically elevated in the ESCC tissues and cells in comparison to these levels in the normal esophageal epithelial mucosa tissues and cells, suggesting that aberrant activation of ADAM10 plays a fundamental role in the carcinogenesis of ESCC. Subsequently, the correlation of ADAM10 with tumor invasion and metastasis was analyzed. In ESCC tissues, ADAM10 expression was positively correlated with depth of invasion, lymph node metastasis and high TNM stage of the cancers; in addition, in ESCC cells, EC-1 with high ADAM10 expression exhibited higher invasion and migration ability, whereas TE-1 with low ADAM10 expression exhibited poor invasion and migration ability. Since EC-1 cells possessed a higher level of ADAM10 expression and markedly enhanced invasion and migration ability compared to the Eca109 and TE-1 cells, EC-1 cells were chosen to perform follow-up experiments to ascertain the effect of ADAM10 silencing on cell invasion and migration. After the knockdown of ADAM10 using ADAM10 siRNA, the EC-1 cells exhibited a significant decrease in the number of invasive cells, cell migration distance and cell proliferation in comparison to the control nonspecific siRNA-transfected and untreated cells. All these above results demonstrated that aberrant ADAM10 is associated with tumor cell invasion, migration, metastasis and proliferation in ESCC *in vitro* and *in vivo*.

A large body of evidence has shown that tumor invasion, metastasis and proliferation are associated with the shedding activity of ADAM10. Nevertheless, the precise mechanism ADAM10 exerts in the course of ESCC progression is still poorly understood. ADAM10 promotes tumor progression through the cleavage of its substrates, such as adhesion molecules (22). E-cadherin, an adhesion molecule, has been known

as an ADAM10 substrate (33). ADAM10 has been implicated in the shedding of E-cadherin in response to *Helicobacter (H.) pylori* infection in gastric cancer cell lines (46). *H. pylori* infection was found to correlate with increased ADAM10 expression in gastric cancer patient samples and to induce ADAM10 expression in gastric cancer cell lines (47). Indeed, chemical inhibition or shRNA-mediated knockdown of ADAM10 resulted in decreased soluble E-cadherin generation in response to *H. pylori* infection, suggesting that induction of ADAM10 by *H. pylori* in gastric cancer promotes E-cadherin cleavage (46). In the present study, we analyzed the correlation of ADAM10 with E-cadherin in ESCC tissues and found a significantly negative correlation between them, suggesting that ADAM10 promotes the progression of ESCC via E-cadherin shedding. Accordingly, we next detected the effect of ADAM10 silencing on the shedding of E-cadherin in EC-1 cells *in vitro*. The data showed that the knockdown of ADAM10 dramatically promoted E-cadherin mRNA and protein in the ADAM10 siRNA-transfected cells as compared with the control nonspecific siRNA-transfected and untreated cells, indicating that ADAM10 is critically involved in the proteolytic processing of E-cadherin in EC-1 cells *in vitro* and ADAM10-mediated cleavage of E-cadherin represents a potent mechanism for regulating invasion, migration, and proliferation of EC-1 cells. ADAM10 could shed the extracellular domain of E-cadherin, and allow β-catenin to translocate to the nucleus (33). Nuclear translocation of β-catenin further contributes to the activation and transcription of genes involved in cell proliferation such as c-myc and cyclin D1 (48). In addition to enhanced cell proliferation, E-cadherin shedding was found to simultaneously weaken cadherin-mediated cell-cell adhesion and promote dissociation of potentially invasive and metastatic cells located in primary cancers; moreover, such dissociation could directly place malignant cells on their pathway to metastasis (10).

In conclusion, the present study suggests that ADAM10 is a key regulator in the invasion and metastasis of ESCC, and may control invasion and metastasis at least in part through the regulation of E-cadherin activity. Disruption of ADAM10 expression may provide a possible route for therapeutic intervention for ESCC.

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