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CD82/KAI1 inhibits invasion and metastasis of esophageal squamous cell carcinoma via TGF-β1

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Abstract. – OBJECTIVE: Previous studies have found that CD82/KAI1 is a tumor suppressor gene. However, the role of CD82/KAI1 in esophageal squamous cell carcinoma (ESCC) has not been reported. This study aims to investigate the specific role of CD82/KAI1 in ESCC, so as to further explore the relationship between CD82/KAI1 expression and clinical characteristics of ESCC.

PATIENTS AND METHODS: The expression of CD82/KAI1 in 96 pairs of ESCC tissues and adjacent normal tissues was detected by Real-time quantitative polymerase chain reaction (qRT-PCR). The relationship between CD82/KAI1 expression and the pathological indicators of ESCC patients was analyzed by Kaplan-Meier method. QRT-PCR further validated the expression level of CD82/KAI1 in ESCC cells. In addition, the CD82/KAI1 knockdown expression model was constructed using small interfering RNA in ESCC cell lines TE-1 and EC-109 cells. Cell counting kit-8 (CCK-8) and transwell assay were performed to detect cell viability, invasion and migration. Finally, the potential mechanism of CD82/KAI1 in regulating ESCC was explored using Western blot.

RESULTS: QRT-PCR results showed that the expression level of CD82/KAI1 in ESCC was significantly lower than that of normal tissues, and the difference was statistically significant. Higher rates of lymph node metastasis and distant metastasis, as well as shorter overall survival were observed in ESCC patients with lower expression of CD82/KAI1 compared with those with higher expression. CD82/KAI1 overexpression decreased cell proliferation, invasion and metastasis in ESCC cells. Western blot results showed that the expressions of TGF-β1, Smad2/3, MMP-2 and MMP-9 were regulated by CD82/KAI1. In addition, rescue experiments demonstrated an interaction between CD82/KAI1 and TGF-β1, indicating that CD82/KAI1 inhibits the malignant progression of ESCC via regulating TGF-β1.

CONCLUSIONS: Lowly expressed CD82/KAI1 in ESCC was significantly associated with the pathological stage, distant metastasis and poor prognosis of ESCC patients. CD82/KAI1 may inhibit the malignant progression of ESCC by interacting with TGF-β1.

Key Words:
CD82/KAI1, TGF-β1, Esophageal squamous cell carcinoma, Invasion and metastasis.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies in the world, with a high mortality rate of more than 40,000 deaths per year[1,2]. In China, although the incidence of ESCC has a declining trend, its mortality is still relatively high. ESCC has become one of the most severe tumors that seriously affects Chinese patients[3,4]. Current treatment methods include surgical treatment, radiation therapy, chemotherapy and targeted therapy[5-7]. With the rapid development of molecular biology, genetic diagnosis technology and other disciplines, ESCC is considered as the long-term interaction of genetic and environmental factors. These changes eventually lead to physiological dysfunctions, including cell proliferation, apoptosis, and differentiation[8,9]. Although great achievements have been made, whether there are other epigenetic regulations involved in the occurrence and development of ESCC still need further discussion. Diagnostic and therapeutic difficulties resulting from the unclear pathogenesis of ESCC are one of the important reasons for its high morbidity and mortality[9,10]. Because of insidious symptoms in early stage of ESCC, most patients are already in late stage when first diagnosed. 40-60% of ESCC patients lost the surgical opportunity because of the late stage or high surgical risk[11].

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Exploration on differentially expressed genes in ESCC and their specific mechanism contribute to develop new strategies for ESCC treatment[10,12]. Tumorigenesis and metastasis are the result of multiple genes and multiple factors[13,14]. It is reported that the CD82/KAI1 gene is a novel tumor metastasis suppressor gene, which is located on chromosome 11p11.2. CD2/KAI1 is a member of transmembrane protein 4 superfamily (TM4SF), which is widely expressed in many tissues[15,16]. Its expression is closely related to the evolution and prognosis of various tumors and the implantation of embryos[17,18]. A large number[18-20] of experiments have shown that the differentially expressed CD82/KAI1 is closely related to malignant tumors, which can be served as a biomarker in tumors. Recent studies have found that CD82/KAI1 is differentially expressed in many tumors with certain tissue specificity[20]. It can inhibit the proliferation, invasion and metastasis of tumor cells, thus regulating the occurrence and development of tumors[21]. CD82/KAI1 is closely related to the occurrence and development of various tumors, such as bladder cancer, gastrointestinal tumor, and endometrial cancer[22-24]. However, the specific mechanism of CD82/KAI1 in tumors is not yet fully understood. In general, CD82/KAI1 may involve in chromosome recombination, gene imprinting, epigenetic regulation, nucleoplasm transport, mRNA splicing and translation. It also participates in the biological processes of tumor cell proliferation, cycle, apoptosis, differentiation and metastasis[25,26]. The role of CD82/KAI1 in ESCC is poorly understood, which requires for further investigation. In this study, we analyzed the expression of CD82/KAI1 in 96 pairs of ESCC tissues and adjacent tissues. The effect of CD82/KAI1 on the biological function of ESCC cells was also explored. Previous studies have pointed out that CD82/KAI1 can inhibit tumor cell division, metastasis and other processes, thereby regulating tumor development. This study aims to investigate the role of CD82/KAI1 in pathological indicators and prognosis of ESCC.

Patients and Methods

Patients and ESCC Samples

We collected 96 ESCC tumors and paracancerous tissue specimens from ESCC patients. ESCC was diagnosed based on the 8th UICC/AJCC TNM staging criteria for esophageal squamous cell carcinoma. Enrolled patients did not receive preoperative radiotherapy or chemotherapy. This study was approved by the Ethics Supervision Committee of our Hospital. Patients and their family members have been fully informed and signed relevant informed consent.

Cell Lines and Reagents

Four human ESCC cell lines (OE19, OE33, TE-1 and EC-109 cells) and a human normal esophageal epithelial cell (HEEC cells) were purchased from ATCC (American Type Culture Collection) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (American Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Transfection

Negative controls (NC) and RNA containing CD82/KAI1 overexpression sequences (CD82/KAI1) were purchased from GenePharma (Shanghai, China). Cells were plated in 6-well plates and transfection was performed after cell confluence was up to 70%. Cell transfection procedures were based on the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Cells transfected for 48 h were collected and plated in 96-well plates with 2000 cells per well. After cell culture for 6 h, 24 h, 48 h, and 72 h, 10 μL of CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added in each well. After incubation for 2 h, the OD value of each well was measured at the wavelength of 490 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Transfected cells were collected and 200 cells were seeded in each well of a 6-well plate. After cell culture for 2 weeks, cells were washed with phosphate buffered saline (PBS) and fixed in 2 ml of methanol for 20 min. After PBS wash for three times, colonies were stained with 0.1% crystal violet for 20 min. Colonies were captured in a light microscope (Olympus, Tokyo, Japan).

Transwell Assay

After 48 hours of transfection, cells were digested and resuspended in serum-free medium. Cell density was adjusted to 2.0×105/mL. Transwell chambers containing Matrigel were placed in 24-well plates. 200 μL of cell suspension and

500 μL of medium containing 10% fetal bovine serum (FBS) were added in the upper and lower chamber, respectively. After cell culture for 48 h, cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. Inner cells were carefully cleaned. Penetrating cells were captured in 5 randomly selected fields of each sample.

Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from ESCC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The qRT-PCR reaction was performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan), and StepOne Plus Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The following primers were used for the qRT-PCR reaction: CD82/KAI1: forward: 5'-GCTCATTCGAGACTACAACAGC-3', reverse: 5'-GTGACCTCAGGGCGATTCA-3'; TGF-β1: forward: 5'-AGGACCTCGGCTGGAAGTGGAT-3', reverse: 5'-AGGACCTTGCTGTACTGCGTGT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. Data were analyzed using ABI Step One software and relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Transfected cells were lysed using a cell lysis buffer, shaken on ice for 30 min, and centrifuged at 4°C, 14,000 ×g for 15 min. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The extracted proteins were separated on a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. The primary antibodies were TGF-β1, Smad2/3, MMP-2, MMP-9, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The secondary antibodies were anti-mouse and anti-rabbit.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used to data analyses. Data were expressed as mean ± standard deviation. Continuous variables and categorical variables were analyzed the t-test and χ2-test, respectively. Kaplan-Meier method was used to evaluate the survival time of patients, followed by Log-rank test for comparing the differences between different curves. $p<0.05$ was considered statistically significant.

Results

CD82/KAI1 was Lowly Expressed in ESCC Tissues and Cell Lines

We detected the expression of CD82/KAI1 in 96 pairs of ESCC tissues and paracancerous tissues by qRT-PCR. CD82/KAI1 was lowly expressed in ESCC tissues compared with that of paracancerous tissues (Figure 1A and 1B). We also detected CD82/KAI1 expression in ESCC cell lines and normal esophageal epithelial cell line. CD82/KAI1 was downregulated in ESCC cells than that of HEEC cells (Figure 1C) TE-1 and EC-109 cells expressed the lowest level of CD82/KAI1, which were selected for the following experiments.

CD82/KAI1 Expression was Correlated with Clinical Stage, Lymph Node Metastasis, Distance Metastasis and Overall Survival in ESCC Patients

According to the qRT-PCR results of CD82/KAI1 expressions in 57 pairs of ESCC tissues and paracancerous tissues, ESCC patients were divided into high expression group and low expression group. The relationship between CD82/KAI1 expression and age, sex, clinical stage, lymph node metastasis and distant metastasis of ESCC patients was analyzed by x2-test. CD82/KAI1 expression was correlated to clinical stage, lymph node metastasis, and distant metastasis, whereas not correlated to age and sex of ESCC patients (Table I). Follow-up data of ESCC patients were collected for analyzing the prognosis. The Kaplan-Meier survival curve showed that the high expression of CD82/KAI1 was significantly associated with poor prognosis of ESCC ($p<0.05$, Figure 1D). The above results suggested that CD82/KAI1 may be a new biological indicator for predicting the prognosis of ESCC.

Overexpression of CD82/KAI1 Inhibited Cell Proliferation

In order to explore the effect of CD82/KAI1 on proliferation of ESCC cells, we first successfully constructed a CD82/KAI1 overexpression expression

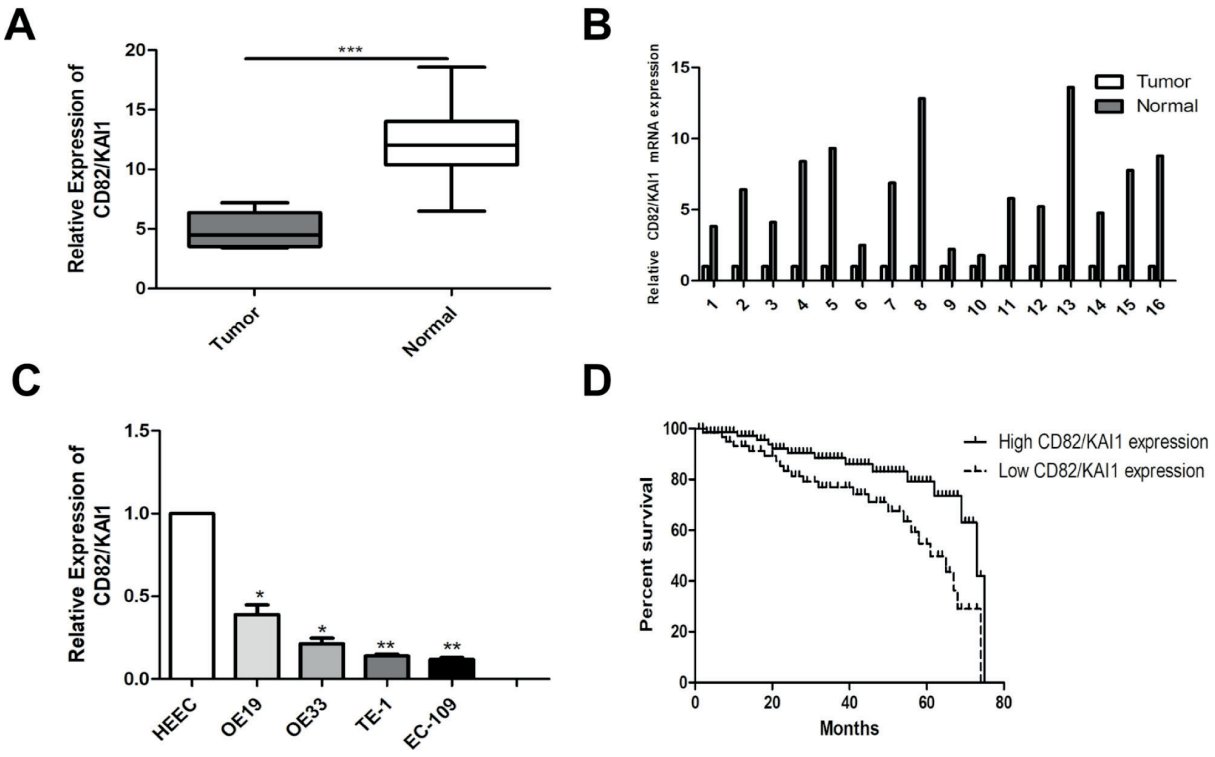

Figure 1. ***A-B***, The expression of CD82/KAI1 in 96 ESCC tissues was significantly decreased. ***C,*** Expression levels of CD82/KAI1 in 4 ESCC cell lines (OE19, OE33, TE-1 and EC-109) and normal esophageal epithelium cell line (HEEC). ***D,*** Kaplan-Meier survival curves of ESCC patients based on CD82/KAI1 expression. Patients in the low expression group had a significantly more unfavorable prognosis than those in low expression group. A representative data set was displayed as mean ± SD values (**p*<0.05, ***p*<0.01).

model (Figure 2A and 2B). CD82/KAI1 overexpression remarkably decreased proliferation of ESCC cells (Figure 2C and 2D). Similar results were obtained in colony formation assay (Figure 2E and 2F).

Overexpression of CD82/KAI1 Inhibited Cell Migration and Invasion

The effect of CD82/KAI1 on migration and invasion of ESCC cells was detected by tran-

Table I. Association of CD82/KAI1 expression with clinicopathologic characteristics of esophageal squamous cell carcinoma.

		CD82/KAI1 expression		
Parameters	**Number of cases**	**High (%)**	**Low (%)**	***p*-value**
Age (years)				0.569
< 60	42	25	17	
≥ 60	54	29	25	
Gender				0.143
Male	47	30	17	
Female	49	24	25	
T stage				0.035
T1-T2	55	36	19	
T3-T4	41	18	23	
Lymph node metastasis				0.032
No	53	35	18	
Yes	43	19	24	
Distance metastasis	0.023			
No	70	45	25	
Yes	26	10	16	

swell assay. Compared with that of NC group, the number of penetrating cells was remarkably reduced after CD82/KAI1 overexpression, suggesting that the migration ability was inhibited (Figure 3A and 3B). Invasive experiment obtained the similar results (Figure 3C and 3D).

Overexpression of CD82/KAI1 Changed the Expression of TGF-β1/Smad Signaling Pathway

To analyze the potential mechanism of CD82/KAI1 in inhibiting cell proliferation, invasion and migration, we detected the expressions of

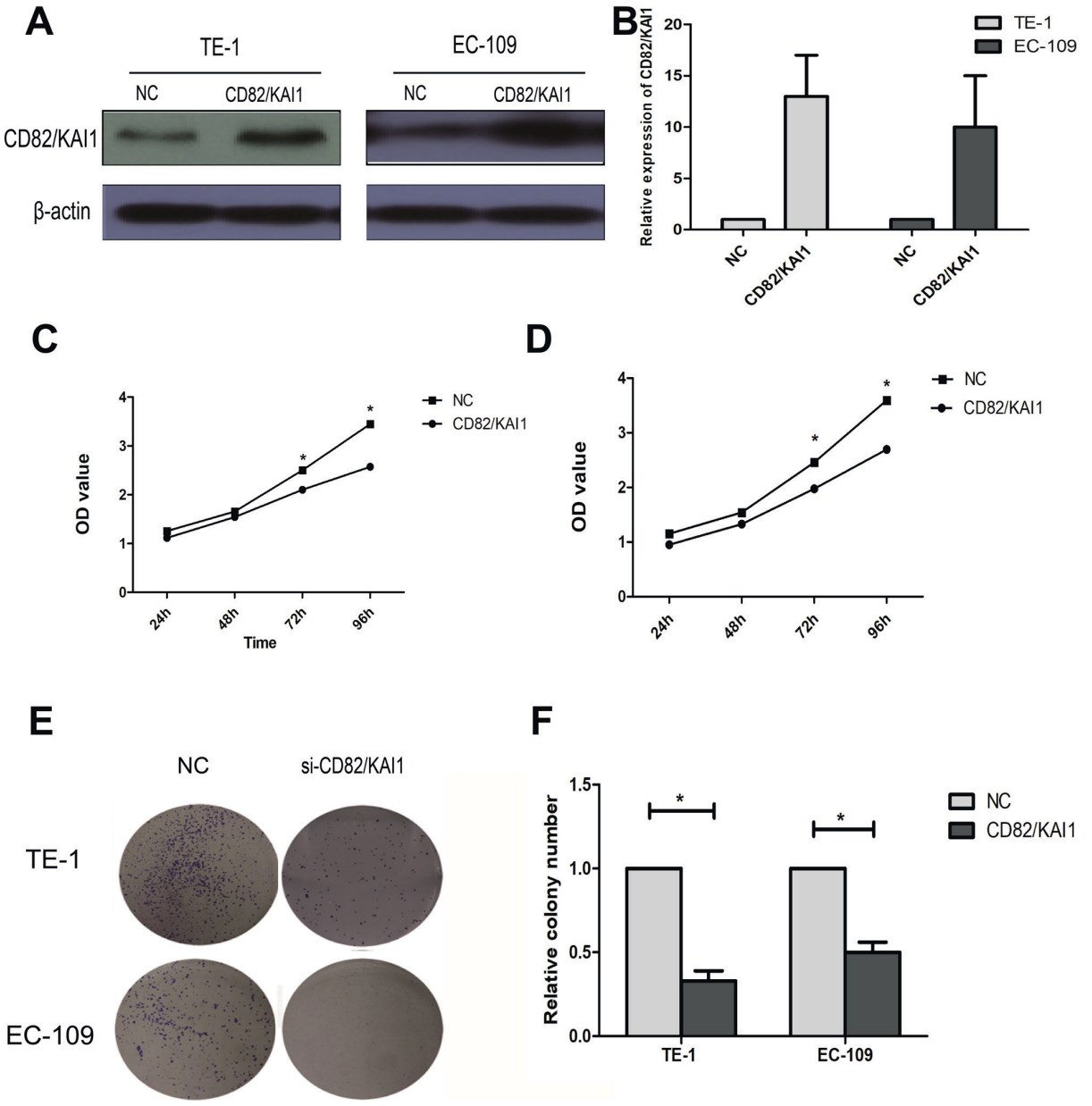

Figure 2. ***A-B***, Western Blot and qRT-PCR were used to verify the efficiency of CD82/KAI1 overexpression in TE-1 and EC-109 cell lines. ***C-D***, Growth curve analysis showed the cell growth of TE-1 and EC-109 cells with CD82/KAI1 overexpression. ***E-F***, The efficiencies of cell colony formation in TE-1 and EC-109 cells with CD82/KAI1 overexpression. A representative data set was displayed as mean ± SD values (*p<0.05, **p<0.01).

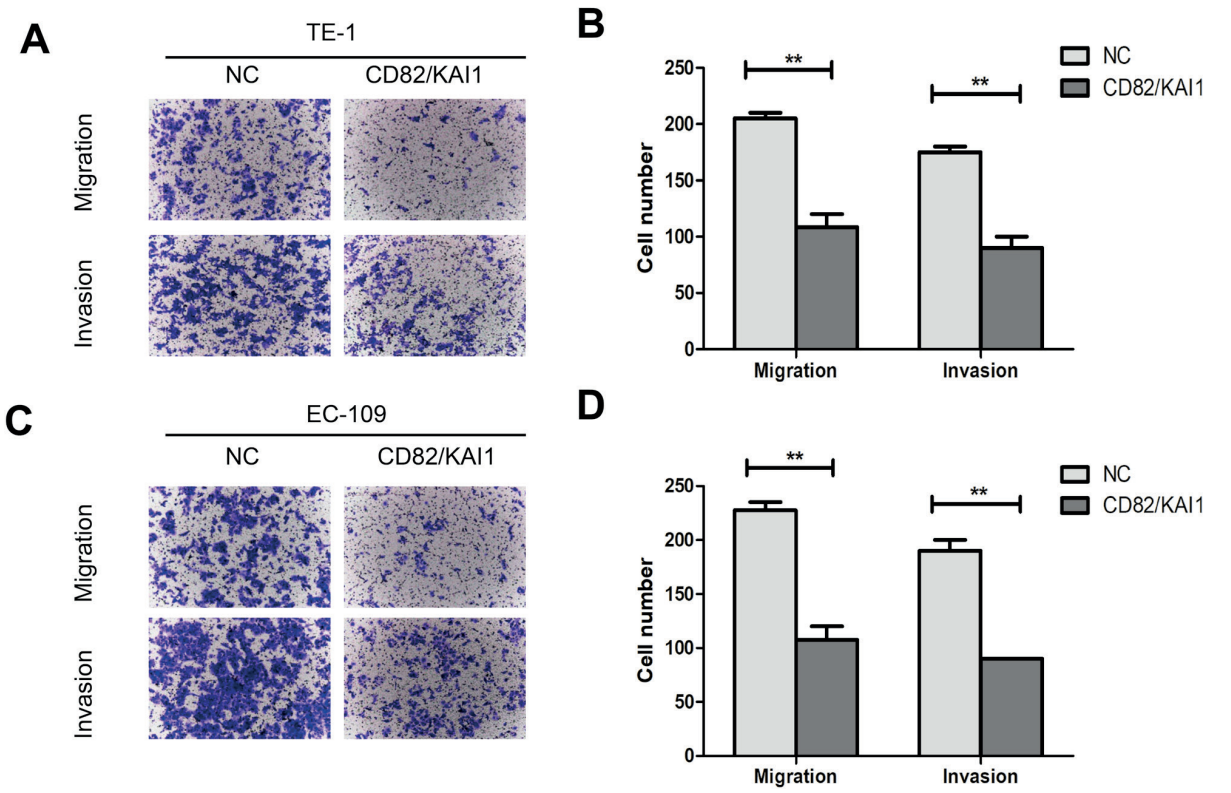

Figure 3. ***A-B,*** TE-1 cells transfected with CD82/KAI1 displayed significantly lower migration and invasion capacity. ***C-D***, EC-109 cells transfected with CD82/KAI1 displayed significantly lower migration and invasion capacity. A representative data set was displayed as mean ± SD values (**p*<0.05, ***p*<0.01).

key genes in TGF-β1/Smad pathway by Western blot. Our data showed that protein expressions of TGF-β, Smad2/3, MMP-2 and MMP-9 were remarkably downregulated after CD82/KAI1 overexpression (Figure 4).

TGF-β1 Modulated CD82/KAI1 Expression in Human ESCC Cells

To further explore how CD82/KAI1 inhibits the malignant progression of ESCC, we found that TGF-β1 may be related to CD82/KAI1 through bioinformatics analysis. Our results indicated that TGF-β1 was overexpressed in ESCC tissues than that of paracancerous tissues (Figure 5A and 5B). Besides, TGF-β1 was also overexpressed in ESCC cells compared with that of HEEC cells (Figure 5C).

Subsequently, we investigated the interaction between TGF-β1 and CD82/KAI1 in ESCC cells. TGF-β1 knockdown in ESCC cells upregulated CD82/KAI1 expression. Further experiments demonstrated that both mRNA and protein levels of CD82/KAI1 were negatively regulated by TGF-β1 (Figure 5D). Small interference sequence of TGF-β1 was constructed. Rescue experiments indicated that inhibited proliferation and migration of ESCC cells induced by CD82/KAI1 overexpression were reversed after TGF-β1 knockdown (Figure 6).

Discussion

ESCC is one of the common malignant tumors of the upper gastrointestinal tract. It is of great significance to study of the occurrence and development mechanism of ESCC for improving the diagnosis and prognosis of ESCC patients[2,3]. Molecular genetic changes in ESCC cells, such as changes in gene copy number and disruption of coding sequences, have important effects on tumor phenotype[9,10]. In recent years, the incidence and mortality of ESCC in China have gradually increased, and the early diagnosis rate of ESCC patients in China is extremely low[7-9]. ESCC has a high degree of malignancy and is prone to recur-

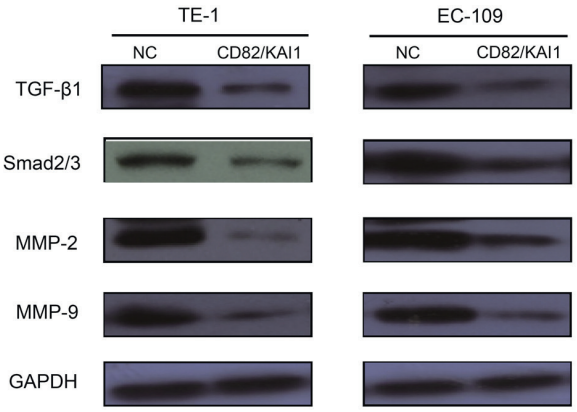

Figure 4. Overexpression of CD82/KAI1 expression significantly decreased the expression of TGF-β1/Smad signal pathway, including TGF-β1, Smad2/3, MMP-2 and MMP-9.

rence and metastasis, thus leading to a very poor prognosis. Early diagnosis and accurate prognosis contribute to improve the clinical outcomes. Genetics, diet, unhealthy lifestyle, and precancerous lesions are all closely related to ESCC occurrence. Clinically, more than half of ESCC patients experience micrometastases before radical surgery. It is a direct cause of metastasis and recurrence after ESCC. Researches on early diagnosis of metastasis and recurrence after advanced ESCC have been well recognized[27]. Recent studies have found that CD82/KAI1 exerts a vital role in many diseases, including tumors[20,22,26]. The differentially expressed CD82/KAI1 may affect the diagnosis, treatment and prognosis of ESCC.

In recent years, a variety of molecular targets regulated by CD82/KAI1 have been continuously revealed. Accumulating evidence has shown that CD82/KAI1 regulates the biological behaviors of ESCC, which provides a new direction in diagnosing and treating ESCC[15-18]. CD82/KAI1 is a tumor metastasis suppressor gene that is located

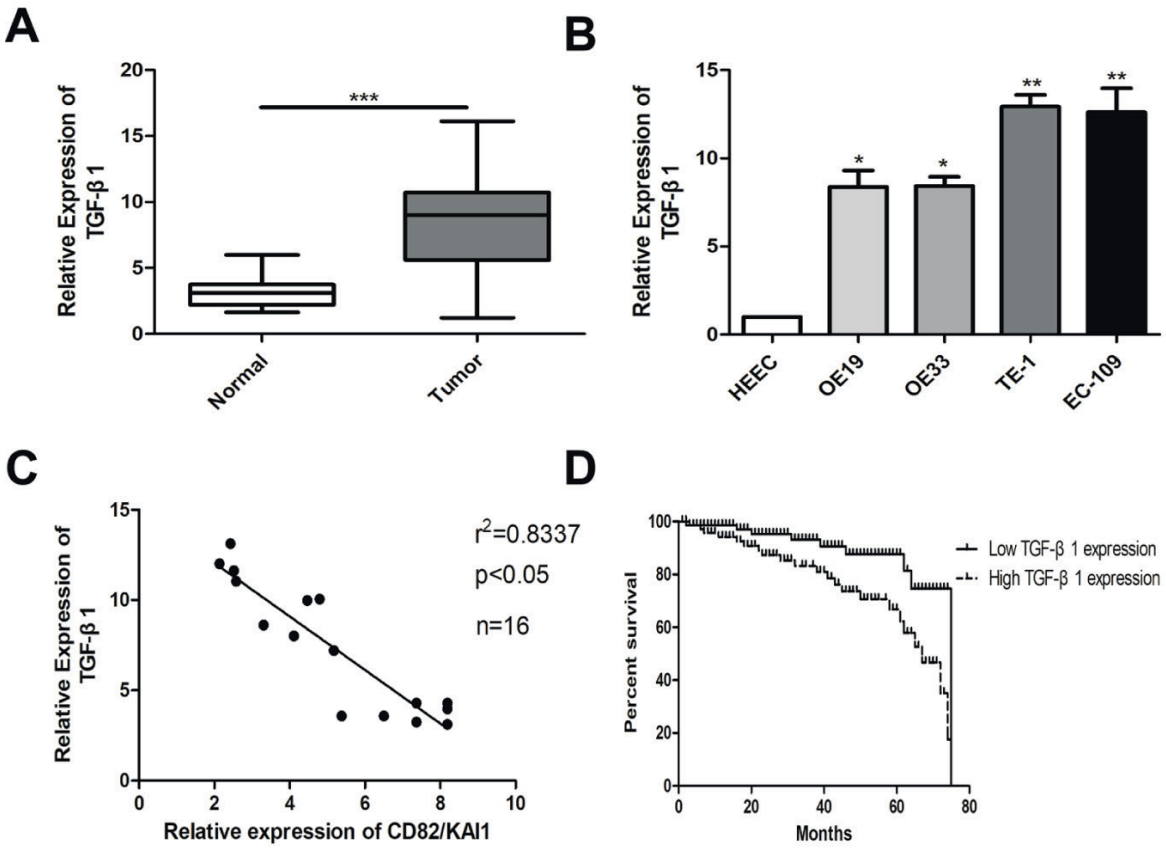

Figure 5. ***A-B,*** The mRNA expression of TGF-β1 relative to GAPDH in human ESCC tissues, corresponding adjacent tissues, and ESCC cell lines were detected using qRT-PCR. ***C,*** A negative correlation was found between CD82/KAI1 and TGF-β1 in tumor samples. ***D,*** Kaplan-Meier survival curves of ESCC patients based on TGF-β1 expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in low expression group. A representative data set was displayed as mean ± SD values (*p<0.05, **p<0.01).

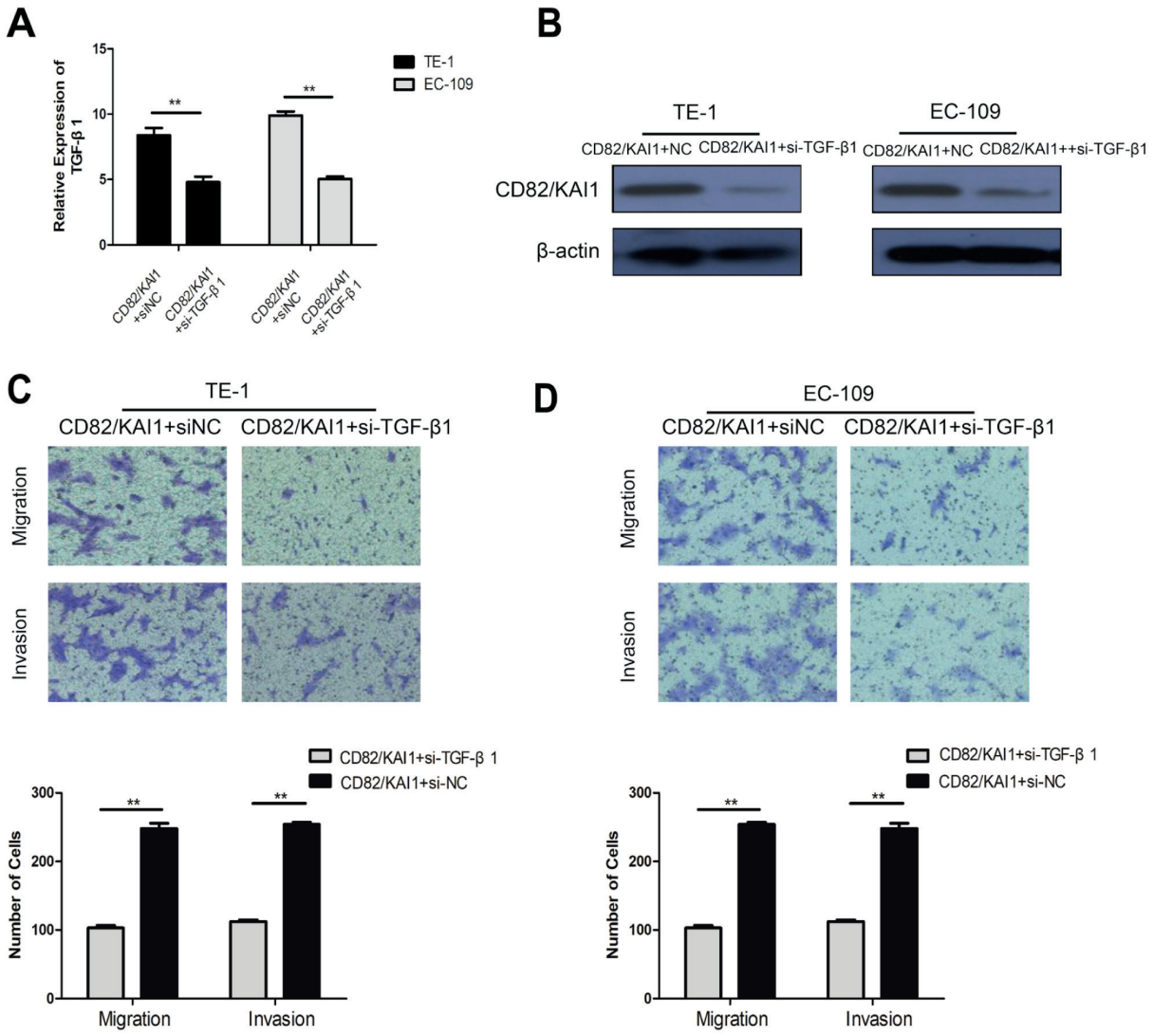

Figure 6. ***A,*** The expression of TGF-β1 was verified by qRT-PCR in co-transfected cell lines. ***B,*** Western blot was used to verify the expression of TGF-β1. ***C-D***, The roles of CD82/KAI1 and TGF-β1 in the regulation of ESCC cell migration and invasion were examined by transwell assay. A representative data set was displayed as mean ± SD values (*p<0.05, **p<0.01).

on chromosome 11 p11.220. The CD82/KAI1 protein belongs to the 4 transmembrane superfamily (TM4SF) and is mainly present on the surface of leukocytes and other tissue cells, with short amino and carboxyl tails inside the membrane[23]. Functionally, CD82/KAI1 inhibits tumor metastasis by regulating cell-cell adhesion and inhibiting tumor cell detachment from the primary tumor[20]. CD82/KAI1 is expressed in most tissues of the body and is widely distributed in prostate, liver, lung, spleen, bone marrow, placenta and other organs[15,17,25]. Current studies have suggested that CD82/KAI1 is involved in the intercellular and extracellular matrix reactions, which further regulates tumor invasion and metastasis[20]. It is reported that CD82/KAI1 expression is correlated to metastasis and prognosis of pancreatic cancer, gastric cancer and prostate cancer[15-17]. Revealing the role and mechanism of CD82/KAI1 contributes to search for novel targets for ESCC. In the present study, CD82/KAI1 was found to be downregulated in ESCC, which was negatively correlated to tumor stage, lymph node metastasis, distant metastasis and prognosis of ESCC patients. Further *in vitro* experiments demonstrated that CD82/KAI1 inhibited proliferation and migration of ESCC cells.

TGF-β1/Smad pathway is an essential pathway involved in tumorigenesis[26,27]. As a classical pathway[28,29], TGF-β activates and phosphorylates its receptors containing serine/threonine kinase domains of type I and II with high affinity. After ligand induction, TGF-β activates TGF-β type I receptor (TβR1) via binding to TGF-β type II receptor (TβR2). Smads is the downstream factor of TGF-β, which is activated by TβR1, whereas inhibited by TGF-β type III receptor[30,31]. TGF-β can be divided into three subtypes, namely TGF-β1, TGF-β2 and TGF-β3, among which TGF-β1 plays a key role in the regulation of tumorigenesis and development[32]. Our data suggested that CD82/KAI1 overexpression downregulated key genes in TGF-β1/Smad pathway, indicating that CD82/KAI1 inhibits proliferation and migration of ESCC via TGF-β1/Smad pathway.

TGF-β exerts multiple effects in physiology (embryo development, differentiation, cell growth) and pathological processes (inflammation, fibrosis, angiogenesis, tumorigenesis and development)[33]. TGF-β1 is closely related to the occurrence and development of tumors[34].

TGF-β1 gene amplification is associated with increased cell differentiation, local and distant metastasis, migration, decreased apoptosis, accelerated angiogenesis and tumor invasion35. This study found that TGF-β1 expression was positively correlated to tumor differentiation, lymph node metastasis and clinical stage. Relative studies have confirmed that TGF-β1 could inhibit migration via upregulating adhesion factors36, which was in consistent with our study.

Conclusions

We found that lowly expressed CD82/KAI1 in ESCC was significantly associated with the pathological stage, distant metastasis and poor prognosis of ESCC patients. CD82/KAI1 may inhibit the malignant progression of ESCC by interacting with TGF-β1.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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