

Silencing of integrin subunit $\alpha 3$ inhibits the proliferation, invasion, migration and autophagy of esophageal squamous cell carcinoma cells

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Abstract. Esophageal squamous cell carcinoma (ESCC) is a deadly disease that seriously affects global public health. The aim of the present study was to explore the role of integrin subunit $\alpha 3$ (ITGA3) in ESCC and investigate its detailed molecular mechanisms. Using reverse transcription-quantitative PCR (RT-qPCR) and western blotting, the mRNA and protein expression of ITGA3 in cell lines was detected. In addition, a series of cellular biological experiments, including Cell Counting Kit-8, wound-healing, Transwell and TUNEL assays, were used to evaluate proliferation, migration, invasion and apoptosis, respectively. Furthermore, western blotting was used to measure the expression of corresponding proteins. ITGA3 was found to be upregulated in ESCC cell lines (ECA109 and TE1). It was also found that ITGA3 silencing inhibited the proliferation, migration, invasion and autophagy of ECA109 and TE1 cells but promoted their apoptosis. In addition, ITGA3 silencing was found to inhibit the FAK/PI3K/AKT signaling pathway. In conclusion, ITGA3 knockdown suppressed cell proliferation, invasion, migration and autophagy in ECA109 and TE1 cells, suggesting that ITGA3 may be a potential therapeutic target for the treatment of ESCC.

Introduction

A total of 572,000 new cases and 508,000 deaths from esophageal cancer are recorded annually. Esophageal cancer is mainly divided into esophageal adenocarcinoma (EAC)

and esophageal squamous cell carcinoma (ESCC) (1). As one of the most frequently diagnosed malignancies in the world, ESCC is associated with late diagnosis, metastasis, therapy resistance and frequent recurrence (2). It has been reported that ESCC, which accounts for >90% of all esophageal cancers, is prevalent in Asia, East Africa and South America (3). In addition, the five-year survival rate of ESCC is <10%, rendering it a cancer with a high mortality rate (4). The causes of ESCC include smoking, alcohol consumption, high intake of pickled vegetables, low intake of fresh fruit and vegetables and frequent exposure to polycyclic aromatic hydrocarbons (5). Despite the fact that tremendous progress has been made in the diagnosis and treatment of ESCC, its survival rate is still far from satisfactory and needs to be improved.

Integrins are heterodimeric integral membrane proteins that act as cell surface adhesion proteins (6,7). Integrin subunit $\alpha 3$ (ITGA3), also known as integrin $\alpha 3$, belongs to the integrin family (8). ITGA3 exists in abundance in normal organisms, while under the induction of oncogenes, there are changes in chromatin structure, extracellular matrix, growth factors and their receptors, and the transcription of integrin, eventually leading to the induction of cancer (9). Several studies have reported that ITGA3 is closely linked to the progression of several human cancers, such as pancreatic (10), colorectal (11), breast (12) and prostate cancer (13), and oral squamous cell carcinoma (14).

The aim of the present study was to investigate the role of ITGA3 in ESCC and identify a molecular biomarker and a potential therapeutic target for its diagnosis and treatment.

Materials and methods

Bioinformatic analysis. DepMap database (portals.broadinstitute.org/ccle/page?gene=ITGA3) was used to determine expression level of ITGA3 in various cancer cell lines.

Cell culture and transfection. The Het-1A immortalized human normal esophageal epithelium cell line was provided by the American Type Culture Collection, and ESCC cell lines (ECA109 and TE1) were obtained from Procell Life Science & Technology Co., Ltd. RPMI-1640 medium containing

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10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin were used to incubate the cells at 37°C with 5% CO₂.

In order to knock down ITGA3, a small interfering RNA (siRNA) targeting ITGA3 (siRNA-ITGA3; product ID: HSS179967), as well as its corresponding negative control (siRNA-NC; product ID: D-001810-10) were obtained from Invitrogen; Thermo Fisher Scientific, Inc. The transfection was carried out using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h strictly in line with the protocol of the manufacturer. At 48 h post transfection, the cells were harvested for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.) and PrimerScript reverse transcriptase (Takara Bio, Inc.), total RNA was extracted and then reverse-transcribed into complementary (cDNA). Next, SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform the RT-qPCR reaction on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Primers used in this study was as follows: ITGA3, forward, 5'-TCA ACCTGGATACCCGATTCC-3' and reverse, 5'-GCTCTG TCTGCCGATGGAG-3'; GAPDH, forward, 5'-CCATGG GGAAGGTGAAGGTC-3' and reverse, 5'-AGTGATGGC ATGGACTGTGG-3'. Finally, relative gene expression was calculated using the 2^{- $\Delta\Delta\text{C}_q$} method (15).

Western blotting. The proteins extracted using radioimmuno-precipitation lysis buffer (Beyotime Institute of Biotechnology) were subsequently quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology). The proteins were subjected to SDS/PAGE and then transferred onto PVDF membranes. Following blocking with 5% non-fat milk, the membranes were incubated with primary antibodies against ITGA3 (cat. no. ab131055; dilution, 1:500; Abcam), Ki67 (cat. no. ab92742; 1:5,000; Abcam), proliferating cell nuclear antigen (PCNA; cat. no. ab92552; dilution, 1:1,000; Abcam), matrix metalloproteinase 2 (MMP2; cat. no. ab92536; dilution, 1:1,000; Abcam), MMP9 (cat. no. ab76003; dilution, 1:1,000; Abcam), Bcl-2 (cat. no. ab32124; dilution, 1:1,000; Abcam), cleaved caspase-3 (cat. no. 9661; dilution, 1:1,000; Cell Signaling Technology), Bax (cat. no. ab32503; dilution, 1:1,000; Abcam), light chain 3 (LC3; cat. no. ab192890; dilution, 1:2,000; Abcam), beclin-1 (cat. no. ab207612; dilution, 1:2,000; Abcam), phosphorylated focal adhesion kinase (p-FAK; cat. no. ab81298; dilution, 1:1,000; Abcam), phosphorylated phosphoinositide 3-kinase (p-PI3K; cat. no. 17366; dilution, 1:1,000; CST), p-AKT (cat. no. ab38449; dilution, 1:500; Abcam), FAK (cat. no. ab40794; dilution, 1:2,000; Abcam), PI3K (cat. no. 17366; dilution, 1:1,000; CST) and AKT (cat. no. ab8805; dilution, 1:500; Abcam) at 4°C overnight. The next day, membranes were incubated with secondary antibodies for 2 h. Finally, the protein signals were captured using enhanced chemiluminescence reagents (Beyotime Institute of Biotechnology).

Cell Counting Kit-8 (CCK-8). ECA109 and TE1 cell proliferation was detected using CCK-8. The transfected or

un-transfected ECA109 and TE1 cells were seeded into 96-well plates and incubated for 24, 48, and 72 h, respectively. Next, 10 μl CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well and cells were cultured for a further 3 h. Finally, absorbance was detected at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

TUNEL assay. A TUNEL assay kit (Roche Biochemicals) was applied to determine cell apoptosis. In brief, 4% paraformaldehyde and 0.25% Triton-X 100 were used to fix and permeabilize ECA109 and TE1 cells. Cells were then labeled with TUNEL for 1 h and DAPI staining solution (1 $\mu\text{g}/\text{ml}$) was used to stain the nucleus for 5 min. Finally, images of positive apoptotic cells were captured using a fluorescence microscope (magnification, x200; Olympus BX53).

Wound-healing assay. ECA109 and TE1 cells were inoculated into 6-well plates and cultured until the cells reached 90-100% confluence. Sterile pipette tips were used to make a wound in the cell monolayer. Subsequently, cells were washed three times with PBS, and then incubated at 37°C with 5% CO₂. Finally, the images of the wound at 0 and 24 h were observed using a light microscope (magnification, x100; Olympus Corp.). The migration rate was calculated using the following formula: $(S_{0\text{h}} - S_{24\text{h}}) / S_{0\text{h}}$, where S represents the width of the wound.

Transwell assay. A Transwell assay was performed to detect cell invasion. Briefly, ECA109 and TE1 cells were resuspended in serum-free medium and inoculated in the upper Transwell chamber, which was precoated with Matrigel[®] (BD Biosciences), while the complete medium containing 10% FBS was added in the lower chamber. After 24 h, 4% paraformaldehyde and 0.1% crystal violet were used to fix and stain ECA109 and TE1 cells. Finally, the invaded cells were observed under a light microscope (Magnification x200; Olympus Corp.). The invasive rate was presented as the ratio of invasive cells in each group/invasive cells in the control group.

Statistical analysis. All data obtained from the experiments are presented as the mean \pm standard deviation, and were analyzed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). For comparisons among different groups, one-way ANOVA and Tukey's test were used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ITGA3 is upregulated in ESCC cell lines. Data from the DepMap database that ITGA3 is upregulated in various cancer cell lines, including ESCC (Fig. 1A). To confirm this finding, RT-qPCR and western blotting were used to measure the expression of ITGA3 in the Het-1A immortalized human normal esophageal epithelium cell line and ESCC cell lines (ECA109 and TE1). As shown in Fig. 1B-C, the mRNA and protein expression of ITGA3 was significantly increased in the ECA109 and TE1 cells, as compared with that in the Het-1A cells, which was consistent with the data obtained from the DepMap database.

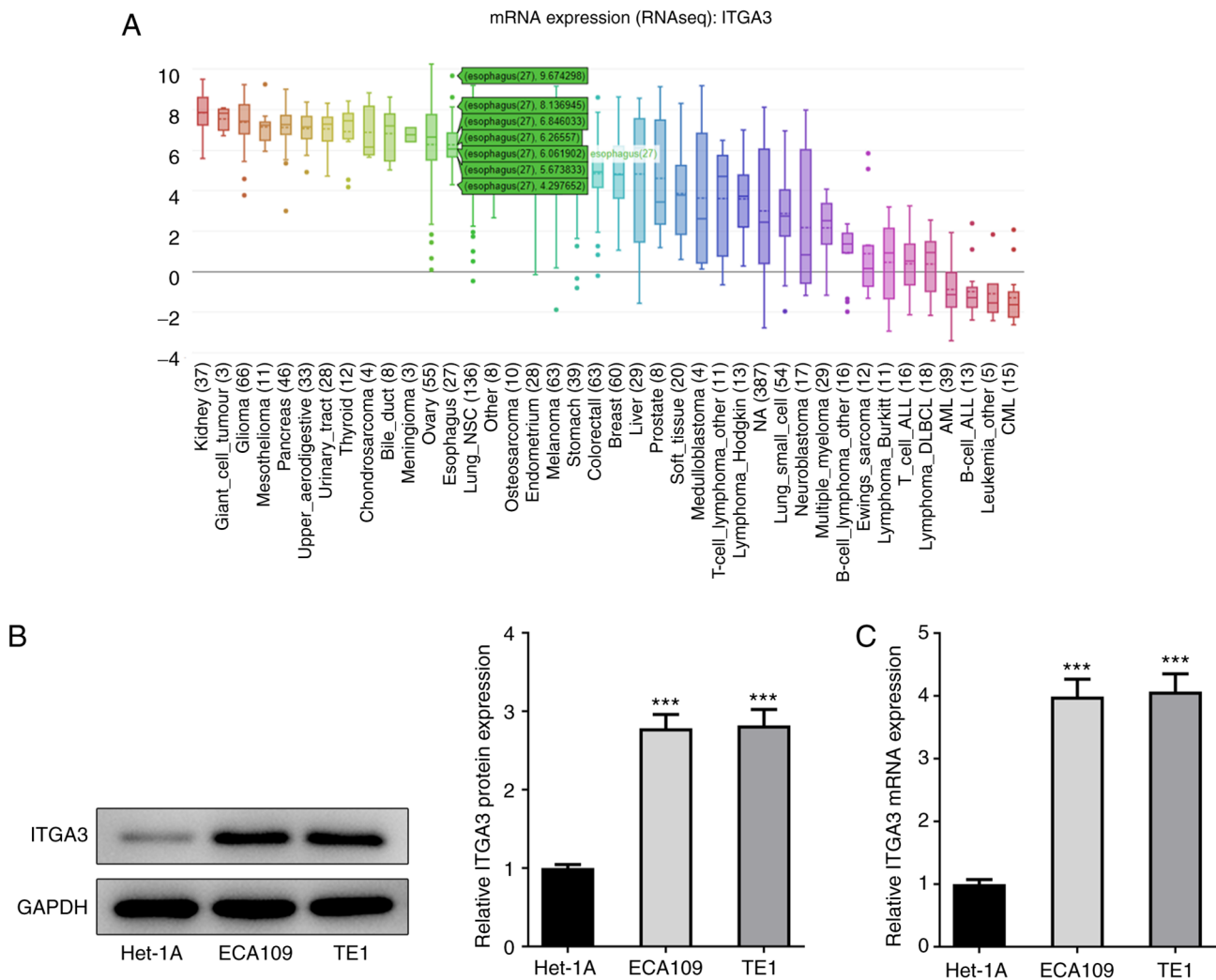


Figure 1. ITGA3 expression is upregulated in ESCC cell lines. (A) According to the DepMap database, the expression level of ITGA3 is upregulated in various types of cancer cells. (B-C) The protein and mRNA expression of ITGA3 in normal esophageal epithelial Het-1A and ESCC cell lines (ECA109 and TE1) was detected using western blotting and RT-qPCR. ***P<0.001 vs. Het-1A. ITGA3, integrin subunit $\alpha 3$; DepMap, Dependency Map; ESCC, esophageal squamous carcinoma cell.

ITGA3 silencing inhibits ESCC cell proliferation. With the aim of knocking down ITGA3 expression, ECA109 and TE1 cells were transfected with siRNA-ITGA3 for 48 h. To evaluate the transfection efficacy, RT-qPCR and western blot analysis were performed to measure the expression of ECA109 and TE1 cells. Compared with siRNA-NC, the expression of ITGA3 in ECA109 and TE1 cells was significantly downregulated following transfection with siRNA-ITGA3 (Fig. 2A-E). To detect the effects of ITGA3 silencing on the proliferation of ECA109 and TE1 cells, a CCK-8 assay was performed. As shown in Fig. 2F and G, cell proliferation was markedly decreased in the ITGA3-silenced ECA109 and TE1 cells, revealing the inhibitory effects of ITGA3 silencing on the proliferation of ECA109 and TE1 cells. Likewise, the expression of proliferation-related proteins, including Ki67 and PCNA, was also decreased by ITGA3 silencing, as compared with that in the siRNA-NC group (Fig. 2H-J).

ITGA3 silencing promotes ESCC cell apoptosis. The effects of ITGA3 silencing on the apoptosis of ECA109 and TE1

cells were assessed using TUNEL assay. The results shown in Fig. 2K-M suggested that ITGA3 silencing promoted relative cell apoptosis in the ECA109 and TE1 cells compared with that in the siRNA-NC group. The expression of apoptosis-related proteins was also measured using western blotting, and it was found that ITGA3 silencing significantly downregulated Bcl-2 but significantly upregulated cleaved caspase-3 and Bax, revealing that ITGA3 silencing promoted cell apoptosis in ESCC (Fig. 2N-Q).

ITGA3 silencing inhibits ESCC cell migration and invasion. Using wound-healing and Transwell assays, the effects of ITGA3 silencing on the migration and invasion of ECA109 and TE1 cells were evaluated. As shown in Fig. 3A and B, the relative cell migration rate of ECA109 and TE1 cells was inhibited following transfection with siRNA-ITGA3. Similarly, the relative invasion rate in the ECA109 and TE1 cells was also inhibited by ITGA3 silencing, compared with that in the siRNA-NC group (Fig. 3C and D). In addition, it was found that the expression of migration-related proteins, including MMP2 and MMP9, was significantly decreased following

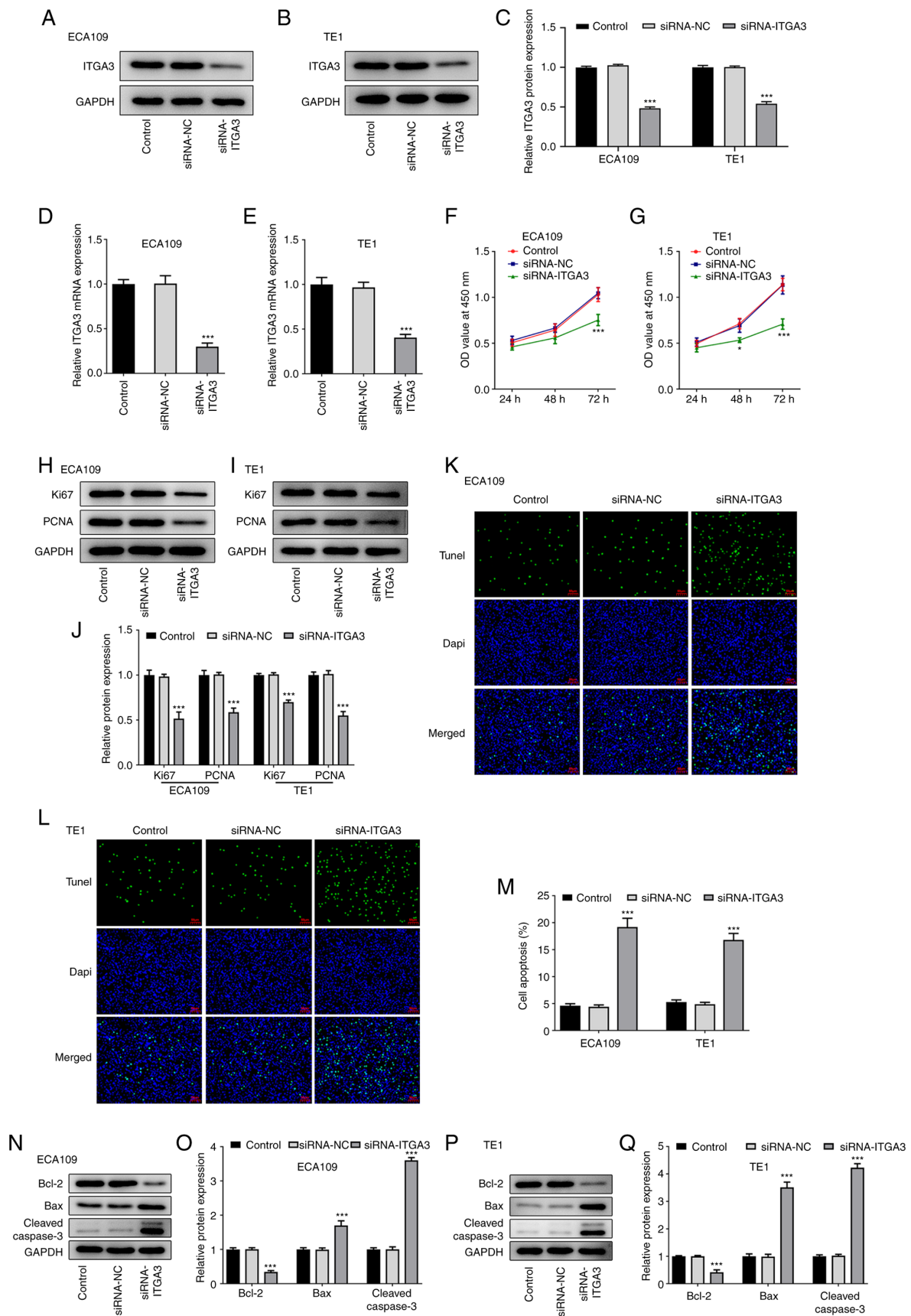


Figure 2. ITGA3 silencing inhibits the proliferation and promotes the apoptosis of ESCC cells. (A-C) The expression of ITGA3 in transfected ECA109 and TE1 cells was detected using western blot analysis. (D and E) Expression of ITGA3 in transfected ECA109 and TE1 cells was detected using RT-qPCR. (F and G) The proliferation of ECA109 and TE1 cells was detected using CCK-8 assay. (H-J) The expression of proliferation-related proteins was detected using western blotting. (K-M) The apoptosis of ECA109 and TE1 cells was detected using TUNEL assay. Magnification x200. (N-Q) The expression of apoptosis-related proteins in ECA109 and TE1 cells was detected using western blotting. * $P < 0.05$, *** $P < 0.001$ vs. siRNA-NC. ITGA3, integrin subunit $\alpha 3$; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; siRNA, small interfering RNA; NC, negative control; PCNA, proliferating cell nuclear antigen.

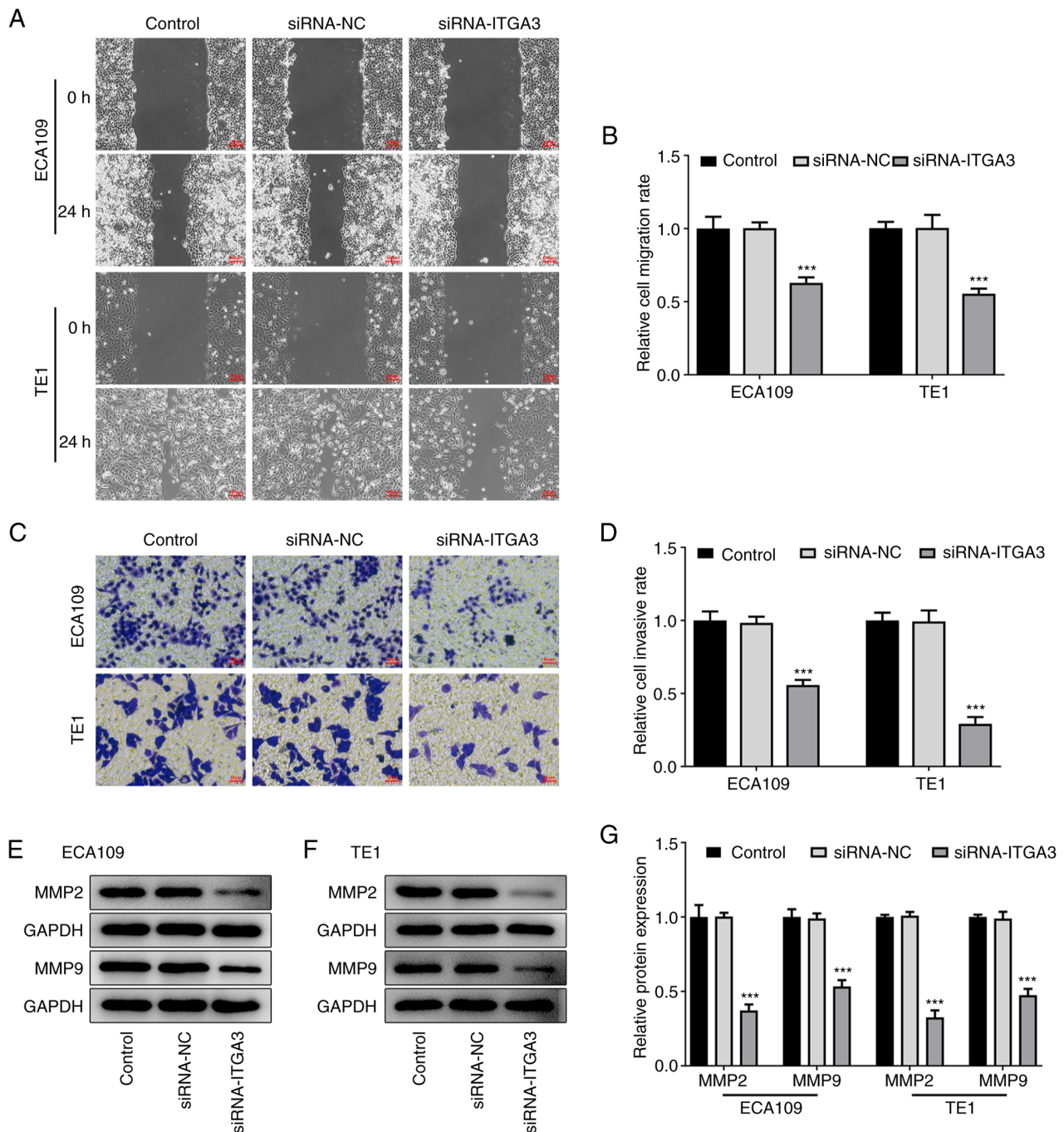


Figure 3. ITGA3 silencing inhibits the migration and invasion of ESCC cells. (A and B) The migration of ECA109 and TE1 cells was detected using wound-healing assay. Magnification x100. (C and D) The invasion of ECA109 and TE1 cells was detected using Transwell assay. Magnification x200. (E-G) The expression of MMP2 and MMP9 in ECA109 and TE1 cells was detected using western blotting. ***P<0.001 vs. siRNA-NC. ITGA3, integrin subunit α 3; siRNA, small interfering RNA; NC, negative control.

transfection with siRNA-ITGA3 (Fig. 3E-G). In conclusion, these results indicated that ITGA3 silencing suppressed ESCC cell migration and invasion.

ITGA3 silencing inhibits ESCC autophagy. As a conserved, self-degradation system, autophagy plays an indispensable role in maintaining cellular homeostasis under stress conditions (16). To determine the effects of ITGA3 silencing on the autophagy in ESCC, the expression of autophagy-related proteins, including LC3 II/I and beclin-1, was evaluated using western blotting. According to Fig. 4A-C, the expression of LC3

II/I and beclin-1 was significantly decreased in the ECA109 and TE1 cells following transfection with siRNA-ITGA3, as compared with that following transfection with siRNA-NC, suggesting that ITGA3 silencing suppressed the autophagy in ESCC.

ITGA3 silencing inhibits the FAK/PI3K/AKT signaling pathway in ESCC. According to the Kyoto Encyclopedia of Genes and Genomes database (<https://www.kegg.jp/>), ITGA3 regulates cell adhesion mainly through the FAK pathway. In view of this, the effects of ITGA3 silencing on

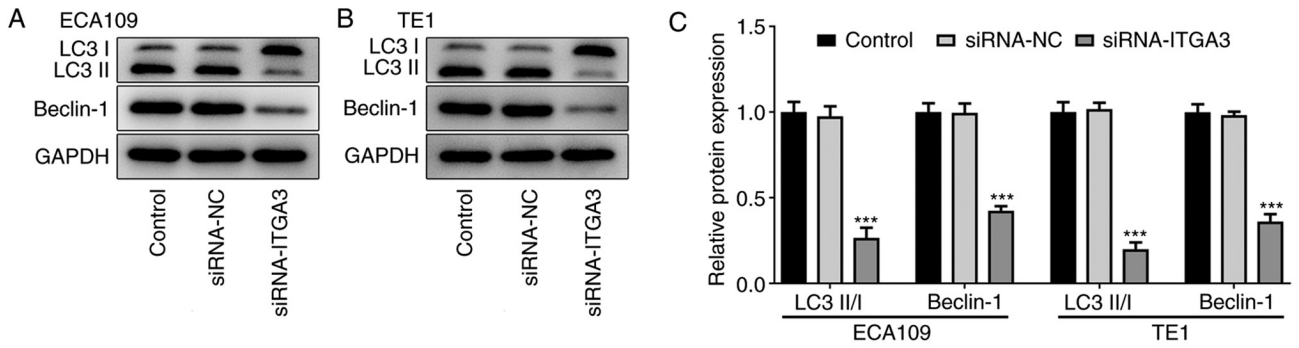


Figure 4. ITGA3 silencing inhibits the autophagy of ESCC cells. (A-C) The expression of LC3-II/I and beclin-1 in ECA109 and TE1 cells was detected using western blotting. *** $P < 0.001$ vs. siRNA-NC. ITGA3, integrin subunit $\alpha 3$; LC3-II, light chain 3-II; siRNA, small interfering RNA; NC, negative control.

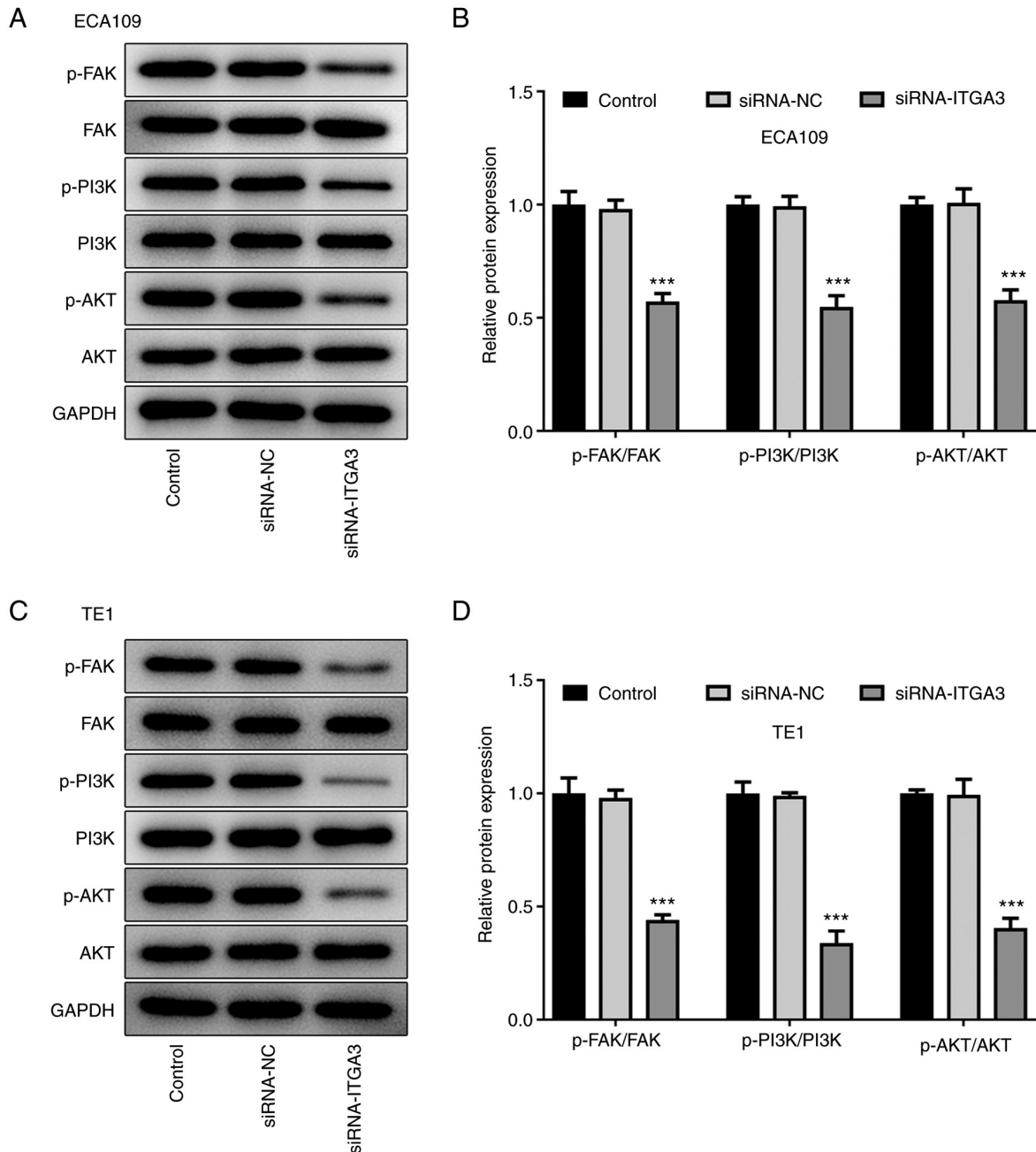


Figure 5. ITGA3 silencing inhibits the FAK/PI3K/AKT signaling pathway in ESCC cells. (A and B) The expression of p-FAK, p-PI3K, p-AKT, FAK, PI3K and AKT in ECA109 cells was detected using western blotting. (C and D) The expression of p-FAK, p-PI3K, p-AKT, FAK, PI3K and AKT in TE1 cells was detected using western blotting. *** $P < 0.001$ vs. siRNA-NC. ITGA3, integrin subunit $\alpha 3$; p-FAK, phosphorylated FAK; siRNA, small interfering RNA; NC, negative control.

the FAK/PI3K/AKT signaling pathway were investigated in both the ECA109 and TE1 cell lines. As shown in Fig. 5A-D, the expression of p-FAK, p-PI3K and p-AKT was markedly decreased in ITGA3-silenced ECA109 and TE1 cells, implying that ITGA3 silencing suppresses the activity of the FAK/PI3K/AKT signaling pathway.

Discussion

To the best of our knowledge, the present study was the first to investigate the role of integrin subunit $\alpha 3$ (ITGA3) in esophageal squamous cell carcinoma (ESCC), as well as its detailed molecular mechanism. It was found herein that ITGA3 was markedly upregulated in ECA109 and TE1 ESCC cell lines. Subsequently, to determine the role of ITGA3 in ESCC, a series of cellular biological experiments were conducted, and the results revealed that ITGA3 silencing inhibited the proliferation, migration, invasion and autophagy of ECA109 and TE1 cells but promoted their apoptosis. Considering that ITGA3 regulates cell adhesion mainly through the focal adhesion kinase (FAK) pathway, the expression of p-FAK, p-PI3K, p-AKT, FAK, PI3K and AKT was measured, and it was found that ITGA3 silencing suppressed the activity of the FAK/PI3K/AKT signaling pathway.

ITGA3, a member of the integrin family, was not only found to be abnormally expressed in several malignant human tumors, but also found to participate in the regulation of tumorigenesis (9,17,18). For example, ITGA3 downregulation has been shown to inhibit the invasion and migration of breast cancer cells but promote their apoptosis (19). Li *et al* (20) reported that ITGA3 was upregulated in human tongue squamous cell carcinoma cell lines and its knockdown could inhibit the invasion, migration and proliferation of tongue squamous cell carcinoma cells. In addition, ITGA3 was found to suppress the migration and invasion of head and neck squamous cell carcinoma cells by silencing its expression (21). In the present study, it was found that ITGA3 silencing inhibited cell proliferation, migration, invasion and promoted apoptosis in ESCC, which was consistent with the aforementioned results.

The abnormal proliferation and migration of tumor cells play an important role in the pathological processes of malignant tumors (22-25). It has been reported that the excessive proliferation and migration of tumor cells can induce metastasis (26); therefore, the search for an effective method that can inhibit the cell proliferation and migration of ESCC and improve prognosis is crucial. Herein, it was found that the proliferation and migration of ECA109 and TE1 cells were suppressed following transfection with siRNA-ITGA3. In addition, Ki-67 and proliferating cell nuclear antigen (PCNA) are considered to be classical markers of cell proliferation (27) and matrix metalloproteinases (MMPs), including MMP2 and MMP9, are vital prognostic markers for cancer invasion and metastasis (28). Considering this, western blotting was performed to assess the expression of proliferation- and migration-related proteins, and it was found that the expression of Ki67, PCNA, MMP2 and MMP9 was decreased in ITGA3-silenced ECA109 and TE1 cells, indicating the suppressive effects of ITGA3 silencing on the cell proliferation and migration of ESCC.

Apoptosis, a type of programmed cell death, is a normal physiological process that often occurs in multicellular organisms (29,30). The deregulation of apoptosis is closely associated with the advancement and progression of cancer (31). Bcl-2 family proteins exert a crucial role in regulating apoptosis pathway. The ratio of Bax/Bcl-2 is a critical determinant of apoptosis, and the cascade cleavage of caspase is an important execution process of cell apoptosis (32). The results of this study suggested that ITGA3 silencing downregulated Bcl-2 but upregulated cleaved caspase-3 and Bax, highlighting the promoting effects of ITGA3 silencing on the cell apoptosis of ESCC.

Autophagy inhibits benign tumor growth but promotes advanced cancer growth (16). Furthermore, evidence has suggested that autophagy plays an important role in ESCC (33). For example, it has been found that autophagy can promote the proliferation and invasion of tumor cells and is recognized as a tumor promoter in ESCC (34). Furthermore, Xie *et al* (35) testified that the induction of mitochondrial autophagy could inhibit the tumor growth of ESCC treated with mefloquine. Therefore, the expression of autophagy-related proteins was also measured herein. Of note, the expression of LC3 II/I and beclin-1 was decreased in ITGA3-silenced ECA109 and TE1 cells, suggesting that ITGA3 knockdown could inhibit autophagy in ESCC, which might partly explain the antitumor activity of ITGA3 in ESCC.

In conclusion, ITGA3 silencing inhibited cell proliferation, migration, invasion and autophagy but promoted the apoptosis in ESCC, indicating that ITGA3 may act as a therapeutic target for the improvement of ESCC.

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Availability of data and materials

All data in this study have been included in this article.

Authors' contributions

KS and JD conceived and designed the study. JD, YZ, DH, HL, LG and ZL performed the experiments and confirm the authenticity of all the raw data. JD, YZ, DH and HL analyzed and interpreted the data. KS and JD drafted and revised the manuscript. All authors have read and approved the final manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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