

Integrin alpha 6: A novel therapeutic target in esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC), the most common subtype of esophageal cancer in East Asian countries, is a devastating disease characterized by distinctly high incidence and mortality rates. Our previous expression profile analysis showed that integrin alpha 6 (ITGA6) is highly expressed in ESCC tissues. To validate cell surface expression of ITGA6 as a novel target in ESCC, we investigated ITGA6 expression in tumor tissue samples and cell lines of ESCC and found that ITGA6 was upregulated in these cells. *In vitro* knockdown of ITGA6 in ESCC cells resulted in inhibition of cell proliferation, invasion and colony formation. In addition, we demonstrated that ITGA6 associates with integrin beta 4 (ITGB4), and that this heterodimer complex is upregulated in both ESCC tissues and cell lines. Moreover, our biodistribution results in an ESCC xenograft model indicated that ITGA6 is a possible target for antibody-related diagnostic and therapeutic modalities in ESCC. Thus, our findings suggest that ITGA6 plays an important role in tumorigenesis in ESCC and represents a potential therapeutic target in the treatment of ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a devastating disease characterized by distinctly high incidence and mortality rates. Epidemiological evidence shows that ESCC is the 5th most common cause of death from cancer in men (1). Although great improvements have been made in the diagnostics, surgical treatment, chemotherapy, and radiotherapy for esophageal cancer, the overall survival rate of esophageal cancer remains

poor, with a 5-year survival rate of 15-34% (2-5). Therefore, it is necessary to investigate the molecular mechanisms related to ESCC development and progression. We sought to identify novel tumor-related genes and clarify their roles in order to elucidate the mechanisms of initiation and progression of ESCC that may ultimately lead to early diagnosis and increased patient survival.

Integrins are a family of transmembrane glycoprotein adhesion receptors that mediate cell-matrix and cell-cell adhesion (6). They are heterodimeric glycoproteins with 18 α subunits and 8 β subunits, which can associate to form 24 unique integrin heterodimers (7). Numerous studies have reported that integrins are involved in various intracellular pathways, including those involved in cell adhesion, migration, polarity, survival, growth, and death, suggesting their important role in cancer (8-10). Furthermore, integrins have been shown to be differentially regulated during tumor growth and progression, making them potential targets for cancer diagnostics and therapy. Although many integrins contribute to tumor progression, integrin alpha 6 (ITGA6), in particular, has been implicated in breast cancer progression in several studies (11-14). ITGA6 is synthesized as a 140-kDa precursor that is converted into 2 disulfide-linked polypeptides of 120 and 25 kDa by endoproteolytic cleavage of the C-terminal domain (15). The ITGA6 subunit can form heterodimers with either the $\beta 1$ or $\beta 4$ integrin subunit to form $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins, respectively. Both $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins function as receptors for the laminin receptor family of extracellular matrix proteins. A recent study showed that expression of certain integrins is highly upregulated in ESCC tissue and cell lines (16). However, neither ITGA6 regulation nor its function has been previously examined in ESCC.

In this study, we have shown that ITGA6 is highly expressed in ESCC and plays a role in the progression of cancer cells by regulating the proliferation and invasiveness of these cells. Furthermore, we have demonstrated the prognostic and therapeutic potential of ITGA6 by *in vitro* and *in vivo* analyses.

Materials and methods

Tissue samples. This study was approved by the institutional review board of the Korea Cancer Center Hospital. Ten ESCC

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tissue samples were obtained from patients undergoing surgery at the Korea Cancer Center Hospital, Seoul, Korea. All the specimens were histologically diagnosed as squamous cell carcinoma.

Total RNA preparation and RT-PCR. Total RNA was isolated from ESCC cell lines, frozen normal esophagus epithelial and tumor tissues from patients with ESCC by using the RNeasy Mini Kit (Qiagen, Cambridge, MA, USA) according to the manufacturer's instructions. A total of 2 μg RNA from each sample was subjected to reverse transcription to produce cDNAs using Oligo-dT primers and Superscript II (Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out with sets of synthesized primers specific to ITGA6; GAPDH was used as an internal control. PCR reactions were optimized for the number of cycles and annealing temperature to obtain an appropriately sized amplicon.

Cell culture. Human ESCC cell lines, TE-1, TE-2, TE-4, TE-5, TE-6, TE-8, TE-9, TE-10, TE-11, TE-14, and TE-15, and human primary esophageal epithelial cells, Het-1A, were purchased from RIKEN, Japan and ATCC, USA, respectively. All cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and antibiotics (100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) and were grown at 37°C under 5% CO₂ and 95% air.

siRNA treatment. Two siRNAs specific for human ITGA6 were purchased from Ambion. TE-8 cells were transfected with ITGA6 and control siRNA by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell proliferation assay. Cells (4x10³/well) were seeded in 96-well plates and incubated with 0.1 ml of RPMI-1640 supplemented with 10% FBS. At the indicated time points, cells were incubated with CellTiter 96 Aqueous (MTS) solution (Promega, Madison, WI, USA) in serum-free RPMI-1640. After 2 h of incubation with CellTiter 96 Aqueous solution, the colored MTS products in the supernatant were measured using a Microplate Reader at 490 nm absorbance.

Migration and invasion assay. Cells (5x10⁵/well) were seeded in a 24-well plate and incubated with fresh medium. After overnight incubation, a wound was introduced by scraping the monolayer with a 1-ml micropipette tip. The cells were washed twice with PBS to remove debris, incubated for 24 h in RPMI-1640 supplemented with 10% FBS, and evaluated by light microscopy. Invasion assays were performed using the 24-well Transwell system (8 μm pore size, BD Biosciences). Cells were starved in serum-free medium overnight, trypsinized, and washed 3 times with RPMI-1640 containing 1% FBS. Then, 2x10³ cells in 1% FBS-RPMI-1640 were seeded into the upper chamber, and 600 μl of RPMI-1640 containing 10% FBS was placed in the lower chamber. After 24-h incubation, the cells remaining in the upper chamber were removed. The cells on the lower surface of the membrane were fixed in 4% paraformaldehyde, stained with 0.5% crystal violet, and scored.

Colony formation assay. For colony formation assays, cells were plated into three 6-well cell culture plates and incubated

for 14 days. The plates were washed with PBS and stained with 0.2% crystal violet staining solution. For the colony formation assay in soft agar, cells (0.5x10³ cells/well) were suspended in RPMI containing 0.3% agarose and 10% FBS and layered on RPMI containing 0.8% agarose and 10% FBS in a 6-well plate. The colonies were analyzed morphologically using nitro tetrazolium blue chloride (NBT), and the number of colonies formed per well after 14 days of incubation was quantified.

Western blotting and antibodies. Protein extraction from frozen tissues and cell lines was performed using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitors (Roche, Basel, Switzerland). Lysates of the same amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Antibodies were purchased from Santa Cruz Biotechnology (ITGB4 and ITGB1) and Cell Signaling Technology (ITGA6). Membranes were developed with the ECL detection system (Thermo Fisher Scientific) after incubation with peroxidase-conjugated secondary antibodies.

Immunoprecipitation and FACS analysis. Cells were washed once with PBS and lysed for 30 min on ice in RIPA buffer. Cell debris was removed by centrifugation. Cell lysates were incubated at 4°C overnight with either anti-ITGB4 or rat IgG as a negative control. Immune complexes were precipitated with Sepharose beads and washed with washing buffer. Samples were resolved by electrophoresis on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with anti-ITGA6 followed by a peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized using the ECL detection system. To measure cell surface ITGA6 and ITGB4 expression, the suspended cells were incubated for 1 h on ice with 1 μg of primary antibodies per 3x10³ cells, washed and stained with FITC-conjugated secondary antibodies, and scanned using a FACSCalibur cytometer (Becton-Dickinson, San Jose, CA, USA).

Blocking antibody experiments. For the ITGA6 functional study, TE-8 cells were exposed to ITGA6 blocking antibody for 3 days; cell proliferation was measured with CellTiter 96 Aqueous solution and cell invasion by a Matrigel Transwell assay. For studies involving ITGA6 blocking antibody (GoH3, MAB1378, Millipore), 2 doses of ITGA6 (1 or 10 $\mu\text{g}/\text{ml}$) were used, and the results were compared with those obtained using a rat isotype control antibody (10 $\mu\text{g}/\text{ml}$, Invitrogen).

Radioiodination of anti-human ITGA6 antibody. The Iodogen-coated tube method (Pierce) was used for radiolabeling anti-human ITGA6 antibody (hITGA6 Ab, 3H1512, Santa Cruz Biotechnology) with ¹²⁵I (Perkin-Elmer). Radiolabeled antibody was purified by gel filtration on a PD-10 column (GE Healthcare Life Sciences) and sterilized by filtration (0.22 μm ; Millipore Co). The radiolabeling yield and radiochemical purity were determined with instant thin layer chromatography-silica gel (Gelman Scientific) in the stationary phase and acetone in the mobile phase.

Biodistribution study. Female athymic mice (BALB/c nu/nu; 5-6 weeks old; 17-23 g) were obtained from Japan SLC, Inc.

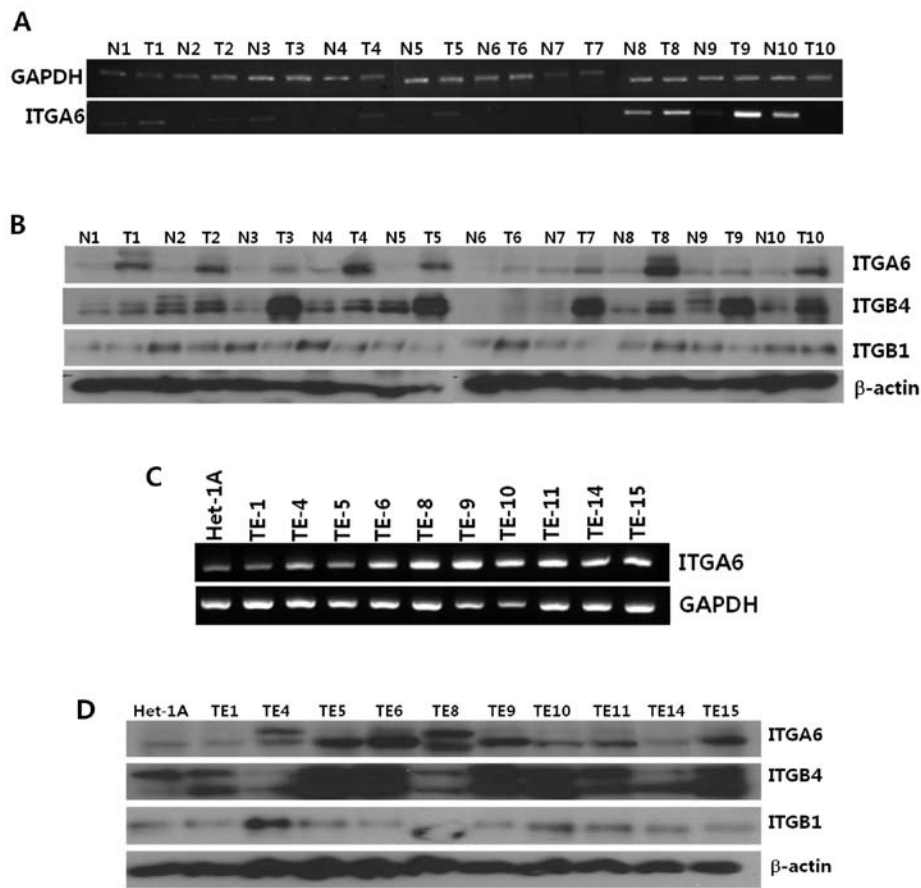


Figure 1. ITGA6 expression patterns in ESCC tissue and cell lines. (A), Relative levels of ITGA6 mRNA expression in ESCC patient tissues were determined by RT-PCR. GAPDH mRNA was used as an internal control. (B), Relative levels of ITGA6 protein expression in ESCC patient tissues were determined by western blot analysis. β -actin was used as a loading control. N, adjacent normal esophageal tissue. T, tumor tissue. (C), Relative levels of ITGA6 mRNA expression in ESCC cell lines were determined by RT-PCR. GAPDH mRNA was used as an internal control. (D), Relative levels of ITGA6 protein expression in ESCC cell lines were determined by western blot analysis. The β -actin protein level was used for normalization.

Tumors were grown after subcutaneous injection of 1×10^7 human esophageal squamous cell carcinoma cells (TE-8) in the right flank. After 28 days, ^{125}I -hITGA6 Ab (20 g/740 kBq) was injected into the tail vein of mice bearing a TE-8 tumor. For each time point, 4 mice were sacrificed to collect and weigh blood, tumors, and tissues. Radioactivity was measured with a scintillation counter. The percentage of injected radioactivity dose per gram of tissue (% ID/g) was calculated.

Statistical analysis. All data were obtained from at least 3 independent experiments and are expressed as the mean \pm standard deviation values. Statistical significance was analyzed by Student's t-test.

Results

ITGA6 is overexpressed in ESCC tissues and ESCC cell lines. In our previous study, we analyzed gene expression profiles for samples obtained from ESCC patients (17) by using specific criteria to identify genes associated with cell surface expression. Among the genes identified on comparison between normal and tumor tissues, we selected 7 upregulated and cell surface-expressed genes (ARL6IP, LAPTM5, ITGA6, SCARB2, MSN, CDH13, and T1A-2; $p < 8 \times 10^{-7}$). After validation using RT-PCR, we selected ITGA6 as a target molecule for ESCC.

Expression levels of ITGA6 mRNA and protein in tumor and normal tissue from 10 ESCC patients were determined by RT-PCR and western blot analyses, respectively. ITGA6 expression was significantly higher at both the mRNA and protein levels in ESCC tissue than in normal esophageal epithelial tissue (Fig. 1A and B). Next, we examined ITGA6 mRNA and protein expression in 10 esophageal squamous cancer cell lines by using RT-PCR and western blot analyses, respectively. ITGA6 expression was upregulated in most of the ESCC cell lines compared to the levels in the normal esophageal epithelial cell line, Het-1A (Fig. 1C and D).

Knockdown of ITGA6 decreases proliferation, invasion, and colony forming ability of ESCC cells. To understand the role of ITGA6 in ESCC cells, we transfected 2 different types of ITGA6 siRNAs in TE-8 cell lines. ITGA6 expression was confirmed in ITGA6 knockdown cells by RT-PCR and western blot analyses compared with that in control siRNA-transfected cells. As shown in Fig. 2A, the results demonstrated that both siRNAs effectively inhibited ITGA6 expression in TE-8 cell lines at both the mRNA and protein levels. The effect of ITGA6 knockdown on the proliferation of ESCC cells was evaluated by MTS assays. The results of the MTS assays showed that decrease in ITGA6 expression significantly reduced the viability of TE-8 cell lines ($p < 0.0001$, Fig. 2B). We next investigated the role of ITGA6 in wound

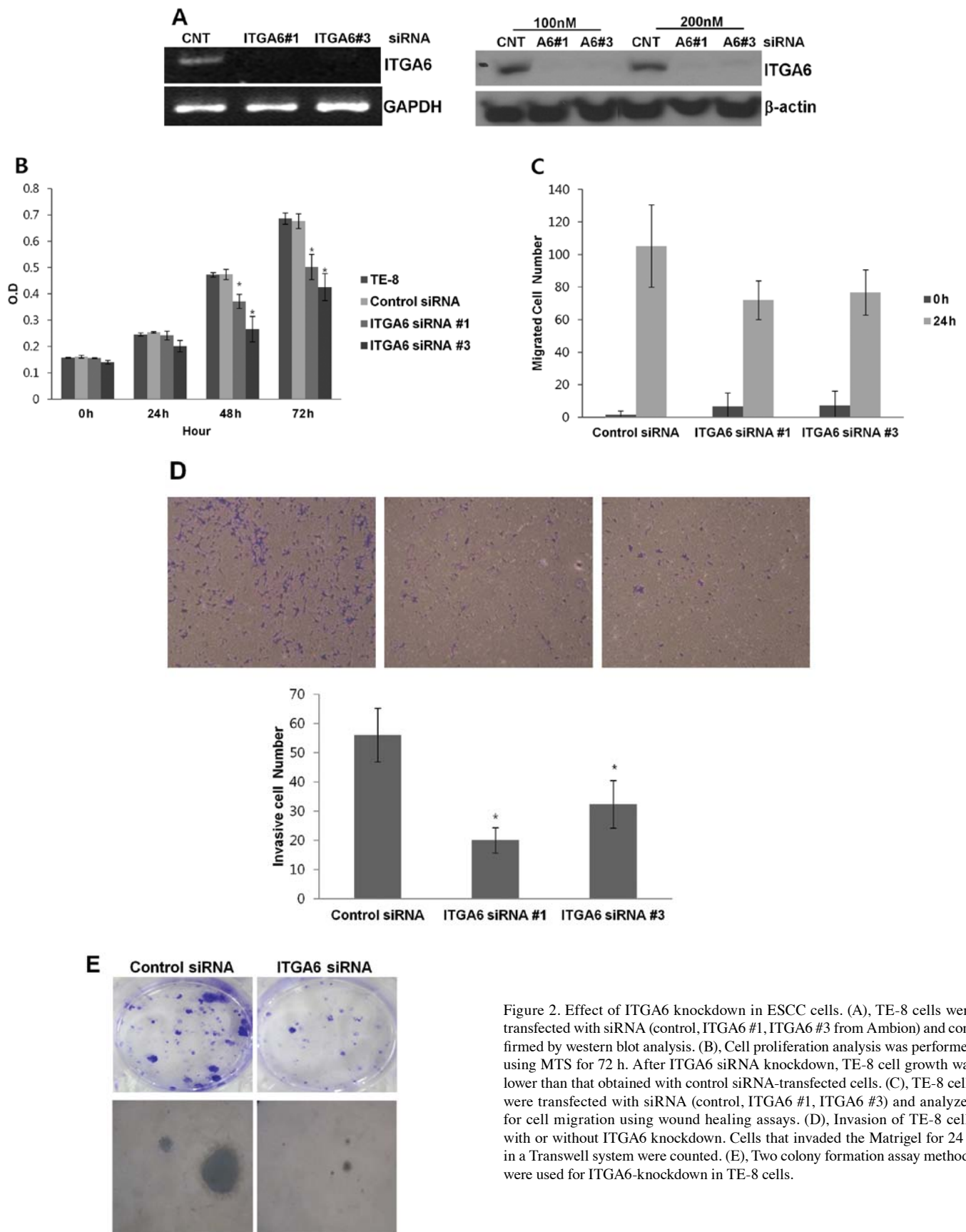


Figure 2. Effect of ITGA6 knockdown in ESCC cells. (A), TE-8 cells were transfected with siRNA (control, ITGA6 #1, ITGA6 #3 from Ambion) and confirmed by western blot analysis. (B), Cell proliferation analysis was performed using MTS for 72 h. After ITGA6 siRNA knockdown, TE-8 cell growth was lower than that obtained with control siRNA-transfected cells. (C), TE-8 cells were transfected with siRNA (control, ITGA6 #1, ITGA6 #3) and analyzed for cell migration using wound healing assays. (D), Invasion of TE-8 cells with or without ITGA6 knockdown. Cells that invaded the Matrigel for 24 h in a Transwell system were counted. (E), Two colony formation assay methods were used for ITGA6-knockdown in TE-8 cells.

healing and invasion in TE-8 cells. In the wound healing assay, ITGA6 siRNA-transfected cells showed a slight delay in wound repair compared to control siRNA-transfected cells (Fig. 2C). In Transwell invasion assays, in the absence of serum, the invasion

of control cells was similar to that of ITGA6-knockdown cells. However, when 10% serum was added as an attractant, invasion of ITGA6 knockdown cells was approximately 60% lower than that in control cells ($p < 0.05$, Fig. 2D).

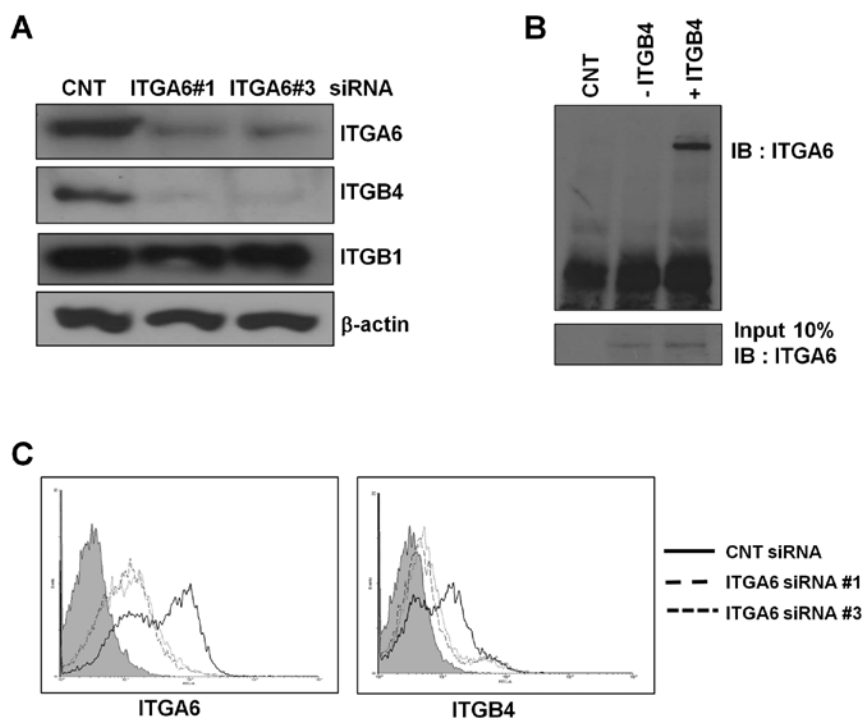


Figure 3. ITGA6 knockdown decreased ITGB4 expression but not ITGB1 expression. (A), ITGA6 knockdown decreased ITGB4 expression in TE-8 cells. ITGB1 did not show any difference after ITGA6 knockdown. (B), Immunoprecipitation assays showed that ITGA6 binds to ITGB4 in TE-8 cells. (C), FACS analysis showed results consistent with those of the immunoprecipitation assay. In ITGA6 knockdown TE-8 cells, cell surface expression of ITGB4 also decreased.

The inhibitory effect of ITGA6 knockdown on ESCC cell growth was also confirmed by 2 different types of colony formation assays: a monolayer assay and a soft agar assay (Fig. 2E).

Collectively, our results demonstrate that downregulation of ITGA6 suppresses ESCC cell proliferation, invasion, and colony formation *in vitro*.

ITGA6 knockdown decreases ITGB4 expression but does not affect ITGB1. Previous studies have reported that ITGA6 forms a complex with ITGB4 or ITGB1. To determine which integrin beta form is the dominant complex formed with ITGA6 in ESCC, we measured ITGB4 and ITGB1 expression levels in ESCC tissue samples and ESCC cell lines (Fig. 1A and B). ITGB4 was upregulated in ESCC tissue and ESCC cell lines along with ITGA6, whereas ITGB1 did not show much change. In ITGA6-knockdown cells, ITGB4 expression decreased but ITGB1 expression was unchanged compared to that for the control in TE-8 cell lines (Fig. 3A). These results were confirmed by FACS analysis. Following ITGA6 knockdown, ITGA6 cell surface expression significantly decreased in TE-8 cells. ITGB4 surface expression levels decreased in ITGA6-knockdown cells (Fig. 3C).

To confirm that ITGA6 and ITGB4 formed a complex, we performed immunoprecipitation assays. In the presence of an ITGB4 antibody, we detected an ITGA6 band by immunoblotting (Fig. 3B). Taken together, we found that the ITGA6/ITGB4 complex is the dominant form in ESCC and that ITGB4 expression is regulated by ITGA6 expression.

ITGA6-blocking antibody decreases proliferation and invasion in ESCC cells. Integrin function has been disrupted with a number of blocking antibodies and peptides, some of

which have entered clinical trials (18). To investigate potential targeting of ITGA6, we utilized an ITGA6-blocking antibody to evaluate ITGA6 function in ESCC cell lines. TE-8 cell treated with 10 μ g/ml of ITGA6-blocking antibody (GoH3, Millipore) showed lower cell proliferation ($p < 0.002$) and invasiveness than an isotype control antibody (Fig. 4).

125 I-labeled anti-ITGA6 targets ESCC tumors in a xenograft model. The tumor-targeting ability of the human ITGA6 antibody was studied in athymic mice bearing human esophageal squamous cell carcinoma xenografts. The 125 I-hITGA6 antibody was injected into the mouse model, and biodistribution was examined at 2, 24, 48, and 72 h post-injection. The percentages of the injected dose/g of tissue for tumor and normal tissues are shown in Fig. 5. The human ITGA6 antibody showed tumor localization, peaking at 24 h, with $7.30 \pm 1.32\%$ ID/g of tumor uptake. The uptake of radiolabeled anti-hITGA6 antibody in TE-8 tumors was higher than those in other tissues at 24, 48, and 72 h post-injection, except for the uptake in blood. The tumor-to-muscle (T/M) ratio was above 4.0 at all time points and showed a peak of 5.99 ± 0.94 at 24 h. The tumor-to-blood (T/B) ratio was below 1.0 at 2, 24, and 48 h; however, the T/B ratio peaked showed a peak of 1.16 ± 0.35 at 72 h. These uptake patterns suggest that the anti-hITGA6 antibody specifically targeted human integrin 6 in TE-8 tumors.

Discussion

ESCC is known to have the worst prognosis in the malignant tumors of the digestive tract. Despite existing therapies for ESCC such as surgery, chemotherapy, and radiotherapy, the clinical outcome for patients with ESCC remains poor, with an overall

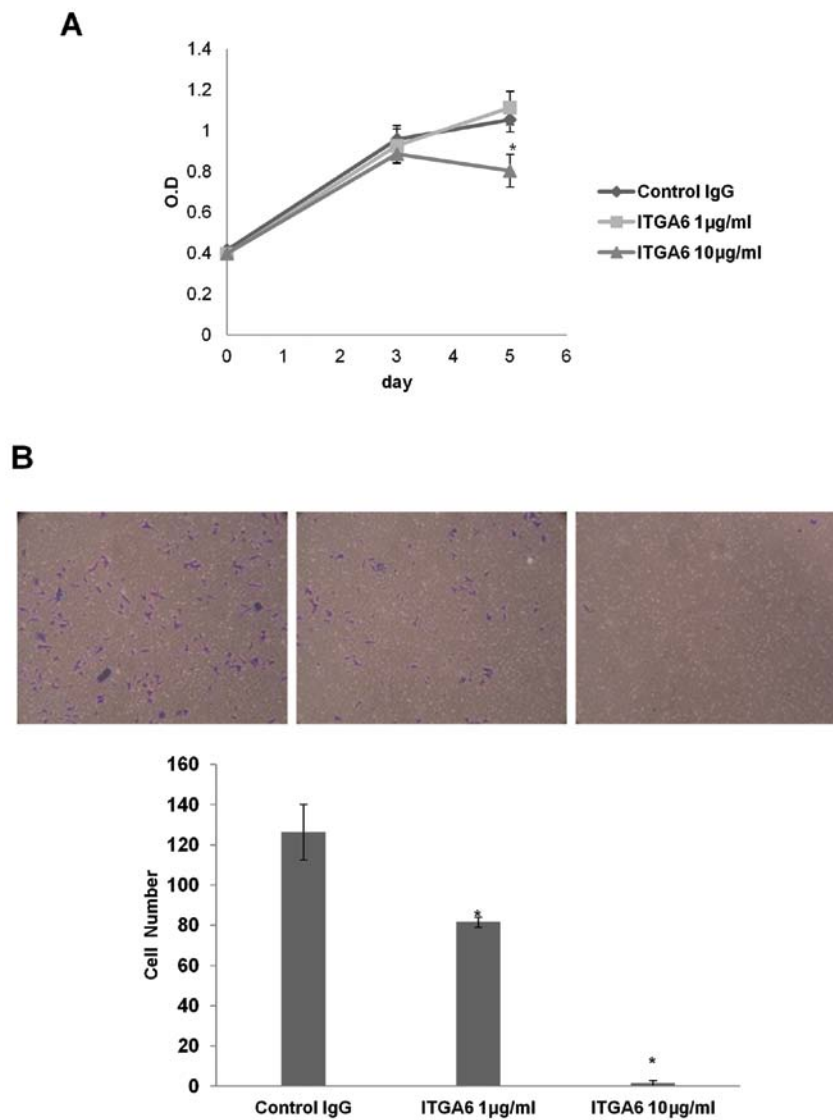


Figure 4. ITGA6-blocking antibody decreased proliferation and invasion in ESCC cells. (A), Decreased cell proliferation was observed with a blocking antibody to ITGA6 in a concentration-dependent manner as compared to the results for an isotype control antibody. (B), Invasion ability was also inhibited by the addition of the blocking antibody in TE-8 cells.

5-year survival rate of 15-34% (2-5). Hence, extensive research on more reliable and effective therapeutic strategies for ESCC is urgently required, particularly with respect to the aspects of efficacy, minimal toxicity, and predictive response to treatment.

Drug development for cancer has been transformed with the identification of and ability to direct treatment at specific molecular targets (19). Novel targeted treatments for esophageal cancer are in early developmental stages, and encouraging results have been reported with antibodies directed against the EGFR and VEGF ligands, as well as tyrosine-kinase inhibitors (20,21). To date, the HER2, EGFR, and VEGF pathways have been most extensively studied in esophageal cancers. However, despite a strong preclinical rationale and evidence of activity of these targets in other cancers, the efficacy of these drugs has been controversial when studied in the metastatic setting or in combination with chemoradiation.

In our previous microarray analysis, we observed that the level of ITGA6 expression increases greatly in ESCC (17). Furthermore, since ITGA6 is expressed on the cell surface, it is a

potential target for antibody therapy. Our present results showed that ITGA6 siRNA could effectively downregulate ITGA6 expression and that this downregulation of ITGA6 resulted in decreased cell proliferation, and invasion by colony formation in ESCC cell lines. Consistent with these findings, enhancement of cell proliferation and invasion by ITGA6 (integrin $\alpha 6\beta 1$ and $\alpha 6\beta 4$) has been shown in pancreatic carcinoma and breast cancer (22,23). Unlike ITGA5, which functions as both a tumor suppressor and oncogene, ITGA6 has been implicated only in promoting tumor progression (12,24). Recent studies have demonstrated that ITGA6 is necessary for the tumorigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line and that targeting ITGA6 in cancer stem cells inhibits self-renewal, proliferation, and tumor-formation capacity (18,25). These results suggest that ITGA6 plays an important role in tumorigenesis.

Changes in ITGA5 expression have been observed in several cancerous tissues (26). Furthermore, a previous study indicated that overexpression of ITGA5B1 inhibits the proliferation of

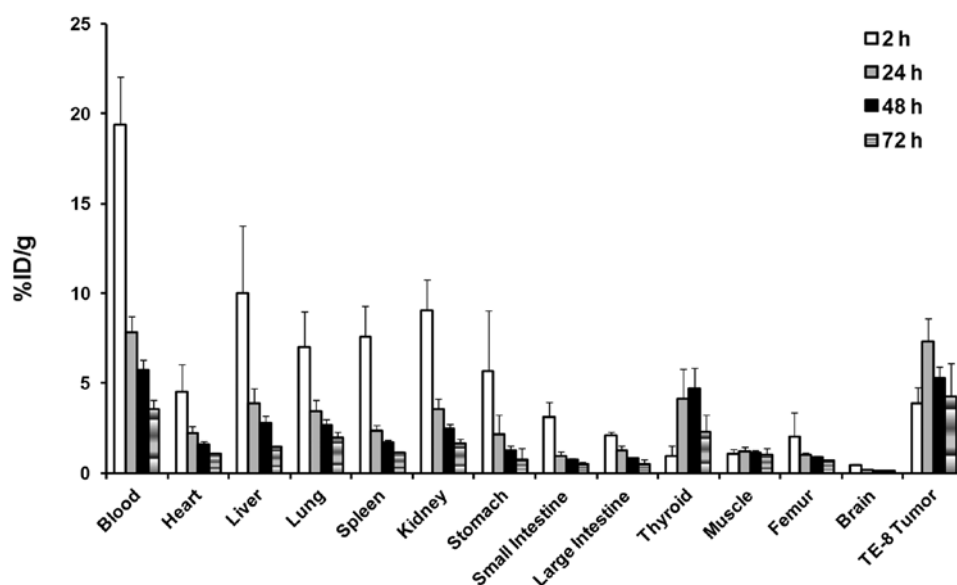


Figure 5. Biodistribution of ¹²⁵I-labeled anti-human ITGA6 antibody in athymic mice with TE-8 tumor xenografts. ¹²⁵I-anti-hITGA6 Ab (20 μg/740 kBq) was injected intravenously into mice. For each time point, 4 mice were sacrificed, and the percentage of the injected radioactivity dose/gram (% ID/g) was determined in various tissues.

human HT29 colon carcinoma cells *in vitro* and reduces the formation of lung colonies and cutaneous metastases *in vivo* (27). However, we did not find any differences in the ITGA5 expression patterns in ESCC tumor tissues compared with those in normal tissues (data not shown).

Additionally, we found that ITGB4 expression increases greatly in ESCC tissues and cell lines. Expression of the ITGA6/ITGB4 complex promoted tumor progression and metastasis of various cancer cells, including breast, colorectal, and thyroid carcinomas (22,28-30). Although some studies have reported that ITGA6B1 can facilitate survival of breast carcinoma cells, especially in response to environmental stress (24), we did not find any difference in the expression patterns of ITGB1 in ESCC tumor tissue and cell lines. In addition, we only detected decreased expression of ITGB4, but not of ITGB1, in ITGA6-knockdown cell lines using western blot and FACS analyses. These findings indicated that the ITGA6B4 complex might be a major form that controls cell survival in ESCC.

According to previous studies, integrins represent ideal pharmacological targets in tumors as they are cell surface receptors interacting with extracellular ligands and play important roles in tumor angiogenesis and tumorigenesis (19,31). In our present study, ITGA6 inhibitory antibody experiments demonstrated that blocking ITGA6 decreased cell proliferation and invasion in ESCC. The results for biodistribution analysis involving ¹²⁵I-labeled anti-ITGA6 showed that the ITGA6-targeting antibody could rapidly and specifically localize to esophageal tumors. These results showed that new combination strategies targeting integrins, especially ITGA6, with anti-angiogenesis or tyrosine kinase-inhibitor agents could be effective therapeutic tools for ESCC. Therefore, ITGA6 represents a potential therapeutic antibody target in ESCC, although highly effective humanized ITGA6 antibody development is required to maximize therapeutic efficiency.

In conclusion, our research demonstrated that the ECM receptor ITGA6 is overexpressed in ESCC tumor tissue and cell lines. Using ITGA6 knockdown, we found that ITGA6 plays an important role in the proliferation, and invasion by colony formation by ESCC cells. We found that ITGA6 was associated with ITGB4 and that this heterodimer complex was upregulated in both ESCC tissue and cell lines. We also found that the ITGA6 inhibitory antibody has the same effect as siRNA in ESCC cell lines. Moreover, the results for biodistribution analysis in an ESCC xenograft model suggested that ITGA6 could be used as a target of antibody-related therapy in ESCC. Taken together, our results indicate that ITGA6 plays an important role in tumorigenesis and has potential as a therapeutic target in ESCC.

Acknowledgements

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