Hypermethylation-induced silencing of ITGA4 promotes oral squamous cell carcinoma progression through SNX5 upregulation

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Abstract. Epigenetic modifications, especially DNA methylation, play an increasingly important role in oral cancer. However, their specific contributions to the progression of oral squamous cell carcinoma (OSCC) remain unclear. The present study used the Shiny Methylation Analysis Resource Tool (SMART) database (https://smart.embl.de/smart/change_mode.cgi) to identify methylation-driven genes associated with OSCC. Among the identified candidates, integrin subunit α4 (ITGA4) exhibited significantly elevated methylation levels in head and neck cancers. A methylation-specific PCR assay showed that ITGA4 is highly methylated in OSCC cells compared with normal immortalized human normal oral keratinocyte (iNOK) cells. Additionally, the mRNA expression levels of ITGA4 were significantly lower in OSCC cell lines compared with normal iNOK cells. ITGA4 overexpression markedly inhibited the cell proliferation, migratory ability and capacity of colony formation and induced apoptosis in FaDu and YD-15 cells. In proteomic analysis, ITGA4 suppressed the expression of Sorting Nexin 5 (SNX5), a protein linked to cancer progression. siRNA-mediated knockdown of SNX5 importantly inhibited cell proliferation, migration, and colony formation in FaDu and YD-15 cells. Moreover, in a chick chorioallantoic membrane xenograft model, overexpression of ITGA4 or small interfering SNX5 significantly inhibited OSCC tumor growth and angiogenesis *in vivo*. Collectively, these findings demonstrated that ITGA4 acts as a tumor suppressor in OSCC by downregulating SNX5 and suggested that ITGA4 may serve as a valuable prognostic biomarker and potential therapeutic target for OSCC.

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Introduction

Recent evidence highlights the pivotal role of epigenetic dysregulation in cancer development and progression. Among epigenetics, DNA methylation is one of the most well-characterized epigenetic mechanisms (1-3). Aberrant DNA methylation, particularly hypermethylation of CpG islands in promoter regions, can lead to transcriptional silencing of tumor suppressor genes. Conversely, global hypomethylation can activate oncogenes and promote genomic instability (4-6). These methylation abnormalities are frequently implicated in oral squamous cell carcinoma (OSCC), contributing to carcinogenesis through deregulation of critical pathways such as Wnt and MAPK signaling (7). In OSCC, promoter hypermethylation has been identified as a key epigenetic mechanism leading to the silencing of over 40 tumor suppressor genes. These genes are involved in crucial cellular processes such as cell cycle control, apoptosis, DNA repair and cell adhesion. The cumulative effect of such epigenetic silencing, often working in tandem with genetic alterations, contributes significantly to the malignant transformation of oral epithelial cells (7,8). The present study used a DNA methylation database to screen for genes that are epigenetically repressed in OSCC. Among several candidates, integrin subunit α 4 (ITGA4) was selected for further investigation, based on its promoter methylation profile and its involvement in cancer. It showed one of the most OSCC-specific hypermethylation patterns in the Shiny Methylation Analysis Resource Tool (SMART) database (https://smart.embl.de/smart/change_mode.cgi). screening, being unmethylated in normal immortalized human normal oral keratinocyte (iNOK) cells but strongly hypermethylated and transcriptionally repressed in OSCC lines. ITGA4 encodes a member of the integrin family, which mediates cell-extracellular matrix interactions and regulates adhesion, migration, proliferation and survival through intracellular signaling cascades (9,10). Additionally, ITGA4 forms a heterodimer with the β1 or β7 integrin subunit and activates downstream pathways such as PI3K/AKT, MAPK/ERK, and NF-κB, which are critically involved in promoting cell survival, epithelial-mesenchymal transition (EMT) and resistance to apoptosis (10). These pathways also contribute to cytoskeletal remodeling and enhanced motility, facilitating tumor invasion and metastasis (11,12). Conversely, under certain conditions, ITGA4 expression has been associated with reduced proliferation and increased

cell-cell adhesion, indicating a possible tumor-suppressive function (13). However, its specific role in OSCC remains poorly understood. The present study aimed to investigate the epigenetic regulation and molecular function of ITGA4 in OSCC cells, providing insight into whether it acts as an oncogene or tumor suppressor, and whether it may serve as a potential biomarker or therapeutic target.

The present study aimed to determine the epigenetic status and biological function of ITGA4 in OSCC cells. It demonstrated that ITGA4 was silenced by promoter hypermethylation in OSCC cells and that overexpression of ITGA4 suppressed tumor cell proliferation, migration and colony formation and promoted apoptosis. Furthermore, it suggested that ITGA4 acted as a tumor suppressor gene, at least in part, by downregulating Sorting Nexin 5 (SNX5), a potential oncogenic effector in OSCC. These findings were further validated *in vivo* using a chick chorioallantoic membrane (CAM) xenograft model, establishing the therapeutic relevance of the ITGA4/SNX5 axis in OSCC progression.

Materials and methods

Cell culture and reagents. Human oral squamous cell carcinoma cell lines (FaDu, YD-8, YD-10B and YD-15) were purchased from the Korea Cell Line Bank in July, 2015. Immortalized normal oral keratinocytes (iNOK) were provided by Dr E.C. Kim (Wonkwang University, Korea) in March, 2010 (14). Cell lines were subjected to identity authentication using a short tandem repeat profiling method. Last tested in May 20, 2021. FaDu cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.); YD-8, YD-10B, and YD-15 in RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc.); and iNOK cells in defined keratinocyte serum-free medium (K-SFM, Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37˚C in 5% CO_2. The DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine was obtained from MilliporeSigma.

Bioinformatics analysis. The SMART database was used to investigate CpG-methylated gene expression in tumors and adjacent normal tissues. The β value was used in the DNA methylation analysis. The bioinformatics analysis utilized publicly available datasets derived from the SMART database (https://smart.embl.de/smart/change_mode.cgi). The distributions of the methylation expression levels were displayed in box plots. Plots based on Kaplan-Meier analyses were generated to compare the overall survival rates between the high- and low-expression groups (*P<0.05, **P<0.01, and ***P<0.001).

DNA extraction and methylation-specific PCR (MSP). Genomic DNA was purified using the Wizard Genomic DNA Purification Kit (Promega Corporation) and eluted in 100 μl of ddH_2O from $2x10^6$ cells. Bisulfite treatment was performed using the EpiJET bisulfite conversion kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For denaturation and DNA bisulfite conversion, a PCR tube was placed in a thermal cycler under Protocol A conditions: 98˚C for 10 min and 60˚C for 150 min. In the initial step, MSP was conducted in a reaction volume of 10 μl. This reaction volume consisted of 2 μl of bisulfite DNA serving as the template, 300 nmol/l of both forward and reverse primers, 7 μl of deionized water, and AccuPower® ProFi Taq PCR PreMix (Bioneer Corporation). PCR was performed under the following conditions: Denaturation at 94˚C for 5 min; 35 cycles of 90˚C for 30 sec, annealing at 50˚C for 30 sec, and extension at 72˚C for 45 sec; and a final extension at 72˚C for 5 min. Subsequently, the MSP products were visualized via 1.5% agarose gel electrophoresis and HiQ Blue Mango Dye (Bio-D Co. Ltd.) staining. The specific sequences of the primers used for PCR amplification are listed in Table I.

Construction of the pcDNA4-ITGA4-Myc-HisA plasmid. The pcDNA4-Myc-HisA vector served as the backbone for constructing pcDNA4-ITGA4-Myc-HisA using an In-Fusion® Cloning Kit (Takara Bio USA, Inc.). Briefly, the pcDNA4-Myc-HisA vector was linearized with *Kpn*I and *Xho*I restriction enzymes and purified. The ITGA4 coding sequence was PCR-amplified from the pITGA4-GFP plasmid (Addgene, Inc.) using GoTaq® Long PCR Master Mix (Promega Corporation) and the designed primers. The vector and the insert were both purified from agarose gels using a DNA-spin™ Plasmid DNA Purification Kit (Intron Biotechnology, Inc.) according to the manufacturer's instructions. The In-Fusion cloning reaction was then established according to the recommended protocol. The In-Fusion mixture was transformed into DH5α competent cells, and the resulting plasmids were isolated and confirmed by DNA sequencing.

Western blot analysis. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (1 μg/ml) and 1X Xpert phosphatase inhibitor cocktail (GenDEPOT). The protein concentration was determined using a Bradford assay. Protein lysates (30 μg) were separated on 10% SDS-PAGE gels and then transferred to PVDF membranes (MilliporeSigma). The membranes were then blocked with 5% skimmed milk for 2 h at room temperature before incubation with primary antibodies (1:1,000 dilution) against ITGA4 (cat. no. Sc-365569), Pro-Caspase 7 (cat. no. Sc-28295), Pro-Caspase 9 (cat. no. Sc-8355), Slug (cat. no. Sc-166476), Vimentin (cat. no. Sc-6260), N-cadherin (cat. no. Sc-8424) and β-actin (cat. no. Sc-47778), all purchased from Santa Cruz Biotechnology, Inc. The membranes were washed three times with TBST (Tris-buffered saline with 0.1% Tween 20) and incubated with an anti-rabbit IgG conjugated with horseradish peroxidase (W4018) or an anti-mouse IgG conjugated with horseradish peroxidase (cat. no. W4028; 1:2,500 dilution) from Promega Corporation for 1 h at room temperature. Protein signals were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.) and detected via an Amersham ImageQuant 800 western blot imaging system (Cytiva).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from the samples. Total RNA was extracted from approximately $1x10^6$ cells per sample. The RNA quality and concentration were determined via a Nanodrop Denovix Ds-11 Series (DeNovix Inc.), where the aim was to obtain an OD 230/260 greater than 1.8 before

Table I. DNA sequences of the primers.

Target	Sequences	Purpose
GAPDH	F: 5'-CACTGCCACCCAGAAGACT-3'	RT-qPCR
	R: 5'-GGACACGGAAGGCCATGC-3'	
ITGA4	F: 5'-ACACTTTCCAGACAGCCAGG-3'	RT-qPCR
	R: 5'-GGCCCCCATCACAATTAAATCC-3'	
ZFP82	F: 5'-CTCACTGGCAGGATGCTTCA-3'	RT-qPCR
	R: 5'-AGACCCTTAAGGCCATGGTT-3'	
ITGA4-Met	F: 5'-TATTTTAGGTCGGTTCGAACGT-3'	MSP
	R: 5'-AACACAACAACAACATCACCG-3'	
ITGA4-UnMet	F: 5'-GGTATTTTAGGTTGGTTTGAATGT-3'	MSP
	R: 5'-AACACAACAACAACATCACCATC-3'	
ZFP82-Met	F: 5'-CTGGGTCTGGAAGTAGAAGTA-3'	MSP
	R: 5'-TCTAAGCCACAGAAGGAGAT-3'	
ZFP82-UnMet	F: 5'-TGGCTCTGCAACACAACAGT-3'	MSP
	R: 5'-GGCGAAAAGCTCCCAGGTAA-3'	

RT-qPCR, reverse transcription-quantitative PCR; MSP, methylation-specific PCR; ITGA4, integrin subunit α 4; ZFP82, zinc finger protein 82.

downstream experiments. For reverse transcription quantitative polymerase chain reaction, both cDNA synthesis and qPCR were conducted within a single reaction tube, Reverse transcription and quantitative PCR were performed using the GoTaq® Probe 1-Step RT-qPCR System (Promega Corporation) according to the manufacturer's instructions, with reverse transcription at 37˚C for 15 min, initial denaturation at 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. The primers used for RT-qPCR are listed in Table I. Relative quantification was performed according to the $2^{-\Delta\Delta Cq}$ method, as previously described (15). All experiments were performed in triplicate and data are presented as the mean ± standard deviation.

Transfection. All oral cancer cell lines were transiently transfected with the ITGA4 expression plasmid or the pcDNA4 empty vector using the Neon Electroporation System (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Electroporation was performed under the following conditions: 1,100 V, one pulse, and a pulse width of 30 msec. For the FaDu cell line, transfection was carried out in DMEM, whereas RPMI-1640 medium without antibiotics was used for the YD cell lines. After electroporation, cells were incubated for 24 h, transferred to fresh complete culture medium, and incubated for an additional 24 h before being subjected to subsequent analyses and experiments.

Cell proliferation assay. An MTT assay was used to evaluate cell proliferation. Cells were seeded at a density of $2x10^5$ cells/well in a 12-well plate 12 h prior to transfection with pcDNA4-ITGA4. Then, the cells were rinsed with PBS, and the media were replaced with fresh media containing a 0.5 mg/ml MTT solution. The cells were incubated for an additional 3 h for formazan crystal formation. Next, the insoluble formazan was dissolved in an acidified isopropanol solution containing 40 mM HCl. Finally, the absorbance of the dissolved solution was evaluated at 540 nm using a DTX 880 multimode detector (Beckman Coulter, Inc.).

Transwell chamber assay. Transwell chamber assays were performed to investigate the effects of ITGA4 on the migration and invasiveness of oral cancer cell lines. FaDu and YD-15 cells ($2.5x10^6$/ml) were transfected with either the ITGA4 plasmid or the empty vector control for 24 h. Each chamber was prepared by adding 200 μl of serum-free medium, followed by the addition of a suspension of $2.5x10^5$ control and transfected cells in another 200 μl of serum-free medium. Then, 600 μl of culture medium was added to the lower compartment. The chambers were placed in the wells containing culture medium and incubated at 37˚C for an additional 24 h. Following this incubation, the chambers were washed twice with PBS to remove nonadherent cells. The cells were subsequently fixed, washed, and stained with crystal violet at room temperature for 15 min. Nonadherent cells were removed, and the remaining stained cells were visualized under an inverted microscope (Olympus IX2-SLP; Olympus Corporation) at 40x magnification. Finally, the stain was dissolved in 10% acetic acid, and the absorbance of the solution was measured to quantify the number of migrated cells.

Wound-healing assay. FaDu, YD-15 and YD-8 oral cancer cell lines were seeded in a 6-well plate and transfected with either the ITGA4 plasmid or the empty vector control for 24 h in culture medium without antibiotics and containing fetal bovine serum (FBS). The next day, the medium was replaced with fresh complete culture medium, and the monolayer of adherent cells was scratched with a pipette tip to generate an approximately 2 mm wide scratch. Wound closure was monitored by capturing images under an Olympus IX2-SLP inverted microscope (Olympus) at 40x magnification. The wound-healing assay was evaluated qualitatively based on representative images.

Cell cycle and cell apoptosis analysis. A Guava Muse® Cell Analyzer (Luminex Corporation) was used to examine the cell cycle distribution and cell apoptosis according to the manufacturer's instructions. Briefly, cells were seeded in a 6-well plate and transfected with either an empty vector control or an ITGA4 plasmid. The cells were harvested and diluted to a concentration of $1x10^6$ cells/ml and then fixed in 70% cold EtOH overnight at -20˚C. For cell cycle analysis, cells were treated with Muse® Cell Cycle Reagent (Luminex Corporation) at room temperature for 30 min and analyzed via the Guava Muse® Cell Analyzer (Luminex Corporation). For the apoptosis analysis, a cell suspension (10^6 cells/ml) was incubated with 100 μl of Muse Annexin V and Dead Cell Reagent (Luminex Corporation). The cells were then incubated for 20 min at room temperature in the dark before analysis with a Guava Muse Cell Analyzer (Luminex Corporation). Data acquisition and analysis were performed using Muse Cell Analyzer Software (Luminex Corporation). The apoptotic rate was calculated as the percentage of early and late apoptotic cells combined, as defined by Annexin V-positive staining.

Clonogenic assay. The cells were seeded in a 6-well plate and transfected with either the ITGA4 plasmid or the empty vector. The cells were subsequently harvested and seeded in 6-well plates at a density of 2,000 cells per well. The plates were incubated for 14 days for colony formation. Next, the colonies were fixed in 10% formalin at room temperature for 20 min and stained with 0.05% crystal violet at room temperature for 30 min. Subsequently, the cell colonies were imaged and counted under an IX2-SLP inverted microscope (Olympus Corporation).

Proteomic analysis. For the protein network analysis, cells were transfected with/without the pcDNA4-ITGA4 plasmid for 48 h. The proteins were identified by proteomic analysis. Total proteins were extracted by filter-aided sample preparation digestion, which was performed according to the protein concentration measured in a BCA assay according to the manufacturer's instructions. The proteomic profile was determined via LC-MS/MS analysis according to the manufacturer's instructions. Briefly, a proteomic analysis was conducted on protein samples (150 μg) from each group using UPLC Exactive Equipment (Agilent 1290; Agilent Technologies, Inc.). LC-MS/MS data were analyzed using Proteome Discoverer. Raw MS data were analyzed using Proteome Discoverer software and searched against the UniProt Homo sapiens database. Carbamidomethylation of cysteine was set as a fixed modification, while oxidation of methionine and acetylation were considered variable modifications. The proteomics data have been uploaded to the PRIDE database and are now fully accessible via the provided accession number (project accession: PXD057268, ID: IlgbsEDjjjFO).

siRNA transfection. Predesigned siRNAs targeting human SNX5 (ID: 27131, NM_001282454.2) were purchased from Bioneer Corporation and transfected into cultured oral cancer cells using Lipofectamine® RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.). siRNA (1 μl) and 6 μl of Lipofectamine® RNAiMAX were diluted in 100 μl of OptiMEM (Invitrogen; Thermo Fisher Scientific, Inc.) separately; the mixture was mixed together and incubated at room temperature for 5 min. The final complexes were added to the cell culture medium. Cells were cultured with the siRNA complex for 48 h prior to downstream analysis. The specific primer pairs were as follows: siSNX5 sense (5'-GUGAAGGGUCUAUGACCA A-3') and anti-sense (5'-UUGGUCAUAGACCCUUCA-3'); siRNA control sense (5'-GCAGCGAGAGAATGAATTA-3') and anti-sense (5'-CAGTCGCGTTTGCGACTGG-3').

CAM. For the CAM assay, fertilized chicken eggs (1 day) were sterilized with 70% ethanol and incubated at 37˚C for 3 days. After 3 days incubation, a window approximately 2 cm in diameter was carefully generated in the eggshell using nippers to form an air pocket without damaging the CAM. The CAM was then visualized by meticulously separating a section of the eggshell membrane. This exposed area was then injected with control cells ($1x10^7$ cells) or ITGA4-transfected cells. After placement in a 60-mm culture plate sealed with parafilm, the fertilized eggs were incubated at 37˚C in suitable humidity for 5 days additionally. After 5 days incubation, blood vessel formation within the injected area was visually assessed and documented with images. Angiogenesis was quantified by counting the number of blood vessel branching points. At the end of the CAM assay, chicken embryos were humanely euthanized by rapid cooling on ice, and death was confirmed prior to tissue collection, in accordance with established ethical guidelines for embryos (16).

Statistical analysis. Data were obtained from three independent replicates in each experiment. The results are presented as the means ± standard deviations for variables with a normal distribution. Statistical analysis was performed via two-way ANOVA or one-way ANOVA followed by Tukey's post hoc test for multigroup comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Methylation status in human head and neck squamous cell carcinoma. According to the SMART database, several genes, including ITGA4 and zinc finger protein 82 (ZFP82), are significantly hypermethylated in head and neck squamous cell carcinoma (HNSSC) tissues compared with normal tissues (Table SI). The present study further examined the correlation between these hypermethylated genes and OSCC patient survival using Kaplan-Meier analysis. Low expression of ITGA4 and ZFP82 was significantly associated with reduced survival in OSCC patients ($P\leq0.05$), while high expression correlated with prolonged survival, indicating an inverse relationship between gene hypermethylation and prognosis (Fig. S1A-D). MSP was performed to assess ITGA4 and ZFP82 methylation in OSCC cell lines (FaDu, YD-8, YD-10B and YD-15). The two genes were highly methylated in cancer cells. Notably, ZFP82 was also methylated in normal iNOK cells, whereas ITGA4 was completely unmethylated in iNOK cells (Fig. 1A). Additional candidate genes were likewise hypermethylated in OSCC cells (Fig. S2). The present study next examined whether methylation affected gene expression. ITGA4 and ZFP82 mRNA levels were significantly lower in OSCC cells than in iNOK cells (Fig. 1B-D), supporting the

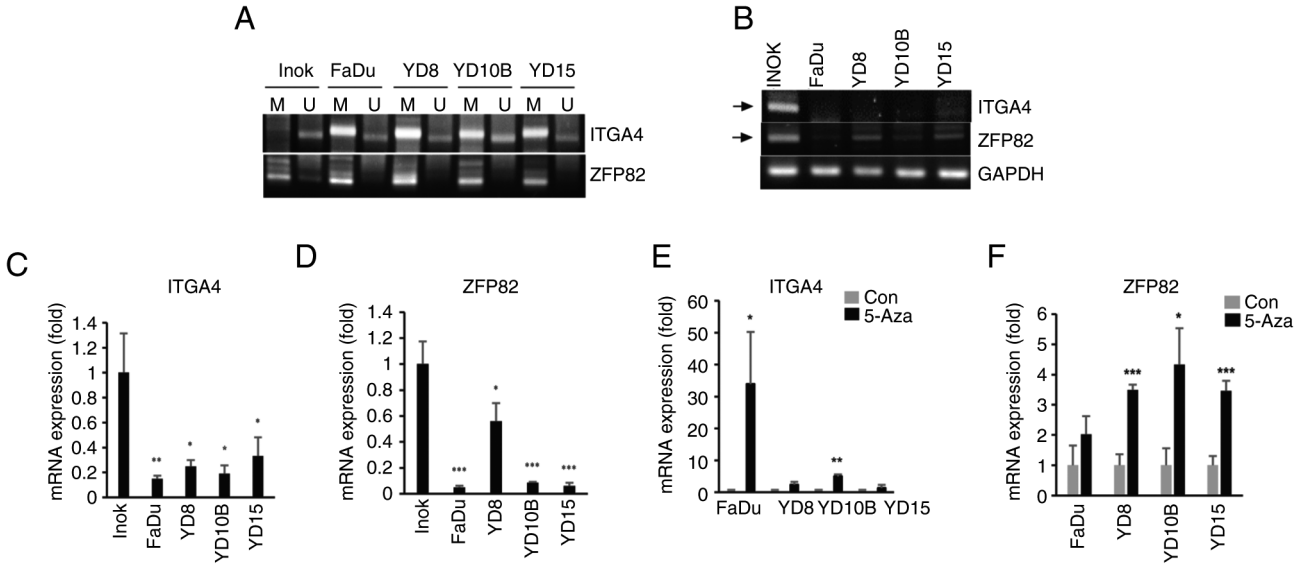

Figure 1. Methylation levels of ITGA4 and ZFP82 in different types of human cancers compared with normal tissues in the SMART database. (A) Representative results of the MSP. Verification of ITGA4 and ZFP82 gene promoter methylation in oral cancer cell lines (FaDu, YD-8, YD-10B and YD-15) and iNOKs via MSP. PCR products of unmethylated (U) and methylated (M) ITGA4 and ZFP82 from sodium bisulfite-treated genomic DNA from cell lines were visualized by ethidium bromide staining. (B-D) The expression of ITGA4 and ZFP82 was determined by RT-PCR and RT-qPCR. ITGA4 and ZFP82 mRNA expression. The quantities of the ITGA4 and ZFP82 mRNAs were determined in each sample using RT-qPCR. The data are presented as the mean ± standard deviation., n=3. Statistical significance of the differences between oral cancer cells (FaDu, YD-8, YD-10B, and YD-15) and iNOKs: *P<0.05, **P<0.01 and ***P<0.001 (E and F) Effect of 5-aza-2'-deoxycytidine (5-aza) treatment on oral cancer cells. RT-qPCR analysis of ITGA4 and ZFP82 expression in oral cancer cells treated with or without 10 μM 5-Aza for 48 h. The columns represent the mean ± standard deviation of triplicate qPCR experiments. **P<0.01 and ***P<0.001. ITGA4, integrin subunit α 4; ZFP82, zinc finger protein 82; SMART, Shiny Methylation Analysis Resource Tool; iNOKs, immortalized human normal oral keratinocytes; MSP, methylation-specific PCR; RT-PCR, reverse transcription PCR; RT-qPCR, reverse transcription-quantitative PCR.

association between hypermethylation and transcriptional repression. To assess methylation-dependent suppression, we treated OSCC cells with 10 μM 5-aza-2'-deoxycytidine (5-aza), a DNA demethylating agent. After 48 h, RT-qPCR revealed significant upregulation of ITGA4 and ZFP82 in treated cells compared with controls (Fig. 1E and F).

ITGA4 inhibits cell proliferation and induces cell cycle arrest and apoptosis in oral cancer cells. The present study overexpressed ITGA4 in FaDu and YD-15 cells to evaluate its role in oral cancer progression. The pcDNA4-ITGA4 plasmid and empty vector were transfected via electroporation. As shown in Fig. 2A and B, ITGA4 expression significantly increased at both mRNA and protein levels following transfection. Cell proliferation was significantly reduced in ITGA4-overexpressing FaDu and YD-15 cells at 48 and 72 h (Fig. 2C). To investigate the underlying mechanism, cell cycle distribution and apoptosis were analyzed using flow cytometry. ITGA4 overexpression increased the G_1-phase population and decreased the S/G2-phase population, indicating G_1-phase arrest (Fig. 2D). Moreover, apoptotic cell percentages increased in both cell lines: viability dropped from ~90% in controls to 77% in FaDu and 59% in YD-15 cells (Fig. 2E). Western blotting confirmed increased cleavage of procaspase-7 and -9 in ITGA4-overexpressing FaDu and YD-15 cells (Fig. 2F). Supporting these results, MTT and apoptosis assays in YD-8 and YD-10B cells showed ~43 and 46% proliferation inhibition at 48 h, respectively (Fig. S3A). Similarly, apoptotic cells (late apoptosis) increased to 38% in YD-8 and 35% in YD-10B cells, respectively, compared with controls (Fig. S3B).

ITGA4 inhibits the motility and colony formation ability of oral cancer cells. Next, the effect of ITGA4 on cell migration was detected. For this experiment, a wound healing assay and a Transwell migration assay were performed. ITGA4-transfected cells were incubated for 24 h to create a monolayer prior to generating a scratch ~2 mm wide, after which a wound healing assay was performed to evaluate cell motility at 24 and 48 h. ITGA4 overexpression reduced the wound closure rate of both FaDu and YD-15 cells (Fig. 3A), while ITGA4 overexpression inhibited wound closure in YD-8 cells (Fig. S4). Next, the effects of ITGA4 overexpression on the migration of FaDu and YD-15 cells was observed via Transwell migration assays. Cell migration was markedly reduced in ITGA4-overexpressing cells compared with control cells (Fig. 3B and C). These results indicated that ITGA4 inhibited the migration and invasiveness of oral cancer cells. EMT is a process by which epithelial cells lose their cell polarity and cell adhesion ability and gain migratory and invasive properties to become mesenchymal cells. EMT is broadly recognized for its involvement in cell migration and invasion. Therefore, the expression of mesenchymal markers in ITGA4-overexpressing cells was detected. Notably, the present study showed that ITGA4 overexpression inhibited the expression of mesenchymal proteins (slug, vimentin and N-cadherin) in FaDu and YD-15 cells (Fig. 3D and E). The long-term proliferative activity of ITGA4 was further evaluated via colony formation assays, which suggested that the colony-forming ability of FaDu and YD-15 cells was markedly reduced at 14 days after transfection with ITGA4 (Fig. 3F and G). Compared with control cells, FaDu and YD-15 cells transfected with ITGA4 presented colony formation rates of 0 and 11.4%, respectively. The ability of

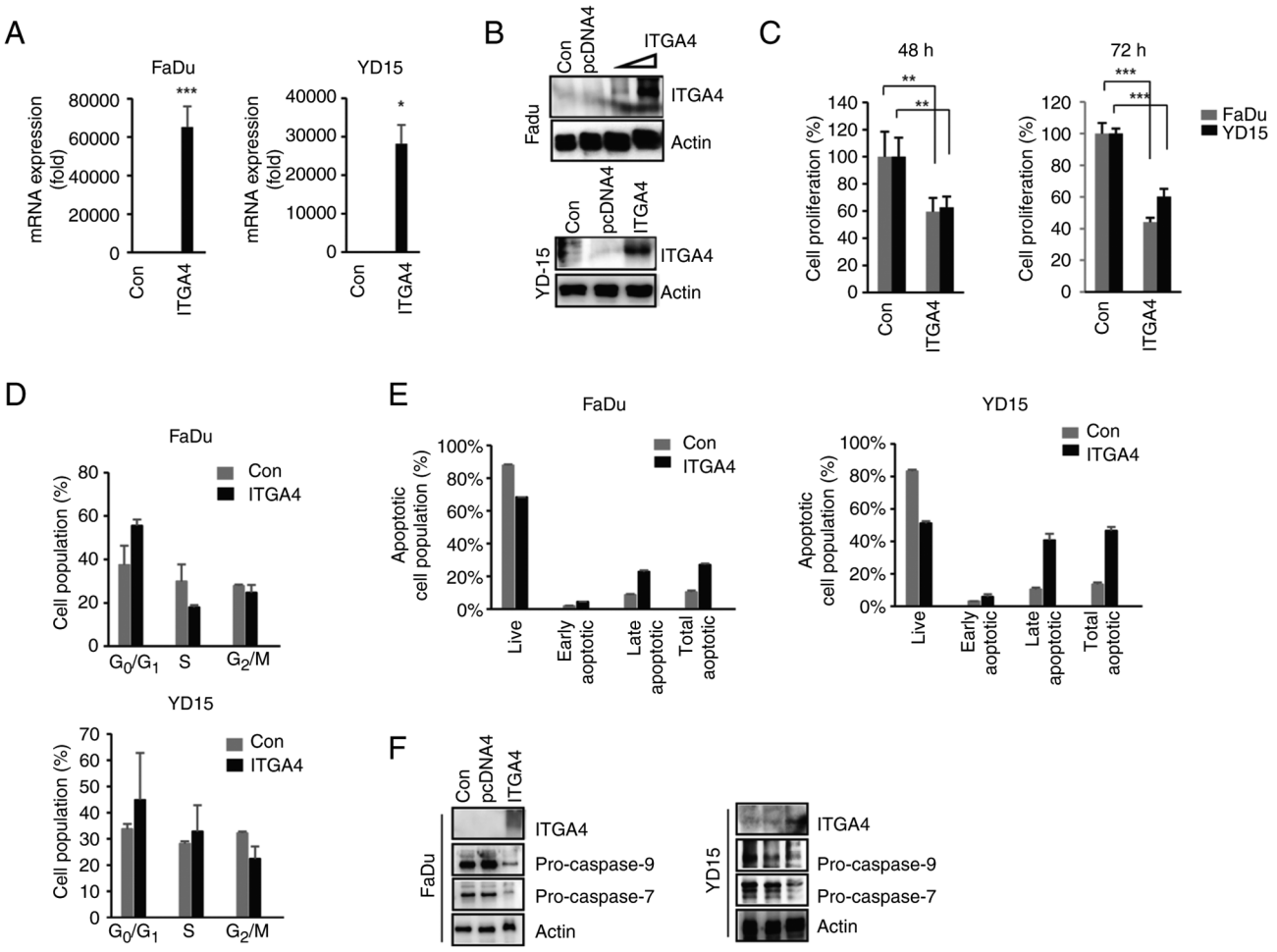

Figure 2. ITGA4 inhibits cell proliferation and induces cell cycle arrest and death in oral cancer cells. FaDu and YD-15 cells were transfected with the vector or the pcDNA4-ITGA4 plasmid for 48 h. (A) RT-qPCR analysis of ITGA4 mRNA levels in FaDu and YD-15 cells transfected with the pcDNA4-ITGA4 plasmid. β-Actin served as an internal control for RT-qPCR and western blot analyses. *P<0.05 vs. the control. (B) Western blot analysis of ITGA4 expression levels in ITGA4-overexpressing FaDu and YD-15 cells. (C) Cell proliferation was examined by MTT assays at the indicated time points after transfection with the pcDNA4-ITGA4 plasmid. The data are shown as the mean ± standard deviation of three separate experiments. **P<0.01 and ***P<0.001 vs. the control. (D) FaDu and YD-15 cells were transfected with pcDNA4-ITGA4 for 48 h, and then the cell cycle distribution was assessed by flow cytometry analysis after staining with PI. (E) Apoptotic cells were evaluated by dual staining with Annexin V/PI and counted via flow cytometry. The graph shows the quantification of the relative numbers of apoptotic cells among the ITGA4-overexpressing cells. (F) Procaspase-9 and procaspase-7 expression in FaDu and YD-15 cells 48 h after ITGA4 transfection, as measured by western blotting. ITGA4, integrin subunit α 4; RT-qPCR, reverse transcription-quantitative PCR.

ITGA4 to affect long-term colony formation may be partially due to both the inhibition of cell proliferation and/or the induction of cell death.

ITGA4 inhibits OSCC progression by inhibiting SNX5 expression. Proteins differentially expressed after ITGA4 overexpression in two oral cancer cell lines, FaDu and YD-15, were identified via LC-MS/MS analysis to examine the protein network through which ITGA4 may exert its anticancer effects on oral cancer cells. The present study identified 2,813 proteins, of which 2,597 could be quantified. The heatmap and volcano plots of the proteomic data revealed the differentially expressed proteins in both FaDu and YD-15 cells following ITGA4 overexpression. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to determine the pathways associated with the differentially expressed proteins. The KEGG-based enrichment analysis revealed that the differentially expressed proteins were mostly related to cell migration, cell differentiation, apoptosis, the extracellular matrix and secretion. Proteins that were upregulated >2.0-fold or downregulated <2.0-fold were considered significantly differentially expressed. According to this standard, compared with those in control cells, the levels of 68 and 295 proteins were increased and the levels of 117 and 173 proteins were decreased in FaDu and YD-15 cells, respectively, as a result of ITGA4 expression. The Venn diagram also revealed that 34 proteins were significantly upregulated, whereas 16 proteins were downregulated in both FaDu and YD-15 cells (Fig. S5). The top 50 upregulated and downregulated proteins according to the ITGA4 expression/control ratio are listed in Tables SII and SIII. The proteomic results were confirmed by quantifying 16 proteins downregulated by ITGA4 in both cell lines (data not shown). Many of these proteins have oncogenic potential. Among them, SNX5 was consistently downregulated in both ITGA4-overexpressing lines and selected for further study. Using The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus datasets, it was found that SNX5 expression was significantly upregulated in OSCC tissues

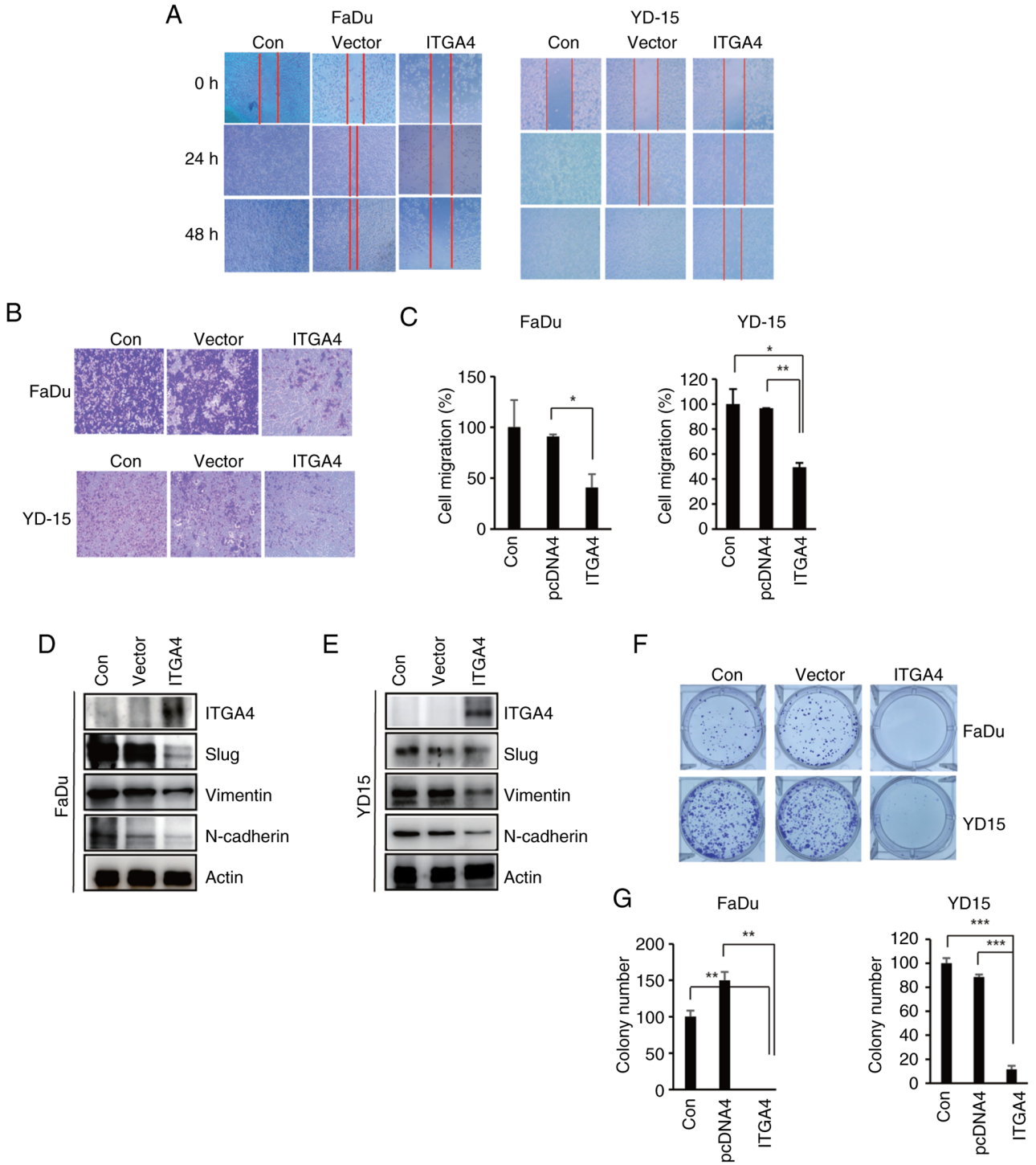

Figure 3. ITGA4 inhibits the migration and invasiveness of oral cancer cells. FaDu and YD-15 cells were transfected with the ITGA4 plasmid or the control empty vector. (A) Wound healing assays were used to assess the effect of ITGA4 overexpression on cell migration at the indicated times. Original magnification, x10. (B and C) Transwell migration assays. The graph summarizes the data from three independent experiments. The bars indicate the mean ± standard deviation (n=3). *P<0.05 and **P<0.01. Effects of ITGA4 on the expression of EMT markers. (D) FaDu and (E) YD-15 cells were transfected with the ITGA4 plasmid or the control empty vector for 48 h. The expression levels of mesenchymal markers in the indicated cells were analyzed by western blotting. (F) Colony formation assay in ITGA4-overexpressing cells. Vector- or ITGA4-transfected cells were plated in 12-well plates and cultured for 14 days. The colonies were stained with crystal violet for quantification. (G) Results of the quantification of Fig. 3F. The data are presented as the mean ± standard deviation. from three independent experiments. **P<0.01 and ***P<0.001. ITGA4, integrin subunit α 4.

compared with normal tissues (Fig. 4A), and high SNX5 levels were significantly associated with poor survival in OSCC patients (P=0.042; Fig. 4B). SNX5 mRNA and protein expression in oral cancer cells were analyzed via RT-qPCR and western blotting. The two were elevated in cancer cells compared with normal iNOK cells (Fig. 4C and D). Western blotting showed that SNX5 expression was markedly decreased in FaDu and YD-15 cells overexpressing ITGA4 (Fig. 4E). To validate this, SNX5 was knocked down using siRNA in FaDu and YD-15 cells. RT-qPCR and western

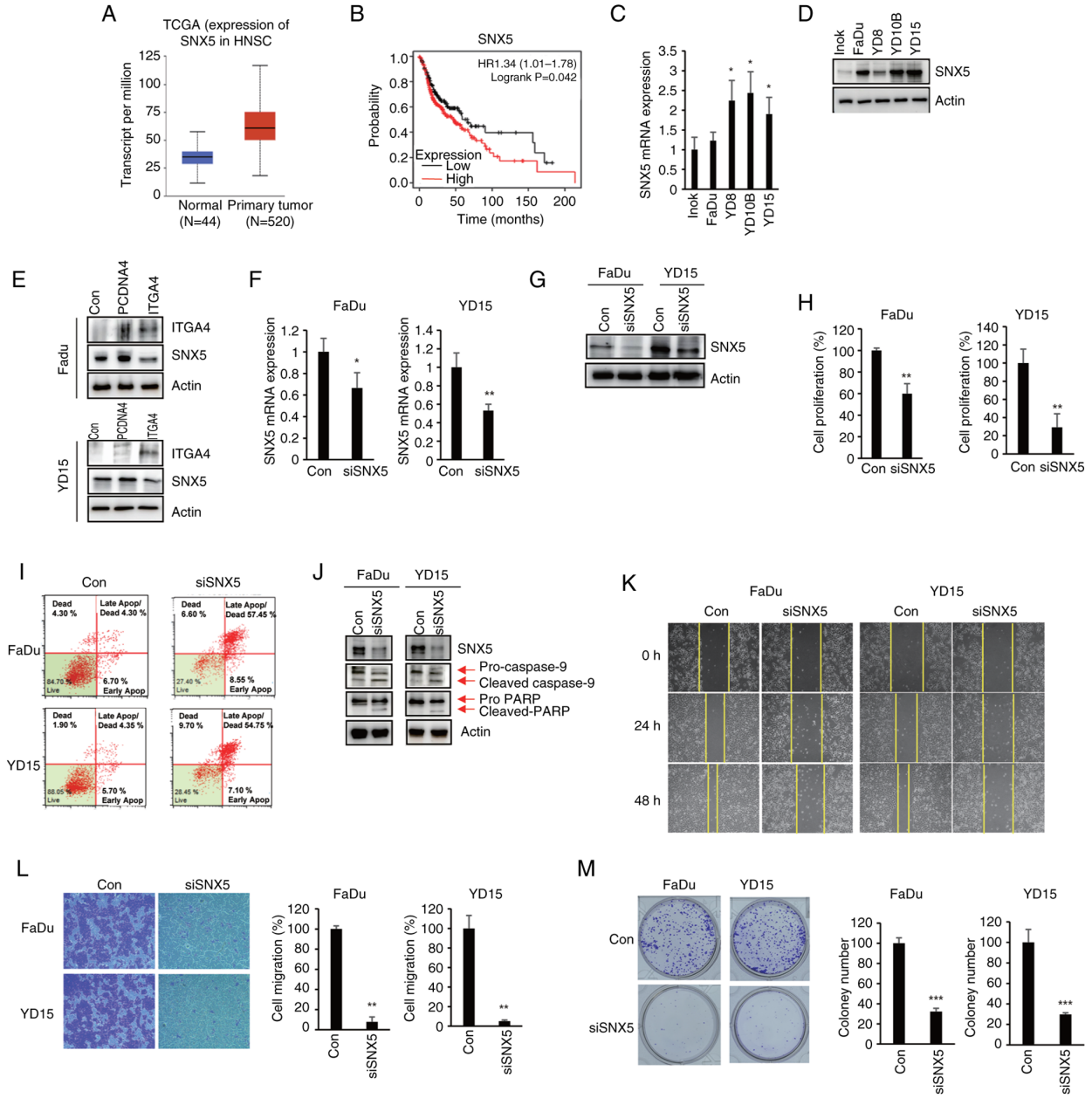

Figure 4. SNX5 is upregulated and associated with a poor prognosis of OSCC patients. (A) The expression of SNX5 in HNSCC tissues (red) was compared with that in corresponding noncancerous normal tissues (blue) in TCGA datasets (n=520). (B) Kaplan-Meier analysis of the overall survival of OSCC patients stratified by the SNX5 expression level. The black plot indicates the survival of patients in the low-risk group, and the red plot represents the survival of patients in the high-risk group. The survival curves were statistically significant (P<0.05). The expression of SNX5 mRNA in oral cancer cells was compared with that in corresponding noncancerous iNOK cells using (C) RT-qPCR and (D) western blotting. *P<0.05 vs. control iNOK cells. (E) ITGA4 reduced SNX5 expression in FaDu and YD-15 cells. The cells were transfected with the ITGA4 plasmid or the control empty vector for 48 h. The expression levels of SNX5 in the indicated cells were analyzed by western blotting. SNX5 protein expression in siSNX5-induced FaDu and YD-15 cells was confirmed by (F) RT-qPCR analysis and (G) western blotting. Actin served as a loading control. (H) SNX5 knockout attenuated cell growth and induced cell death in FaDu and YD-15 cells. The growth of control or SNX5-knockout FaDu and YD-15 cells was detected by an MTT assay. The bars indicate the mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01. (I) Apoptotic cells were evaluated by dual staining with Annexin V/PI and counted using flow cytometry. The percentage represents the relative number of apoptotic siSNX5-treated cells. (J) Caspase-9 and PARP expression/activation in FaDu and YD-15 cells 48 h after the transfection with siSNX5, as measured by western blotting. (K) SNX5 silencing inhibits oral cancer cell migration. FaDu and YD-15 cells were transfected with 100 nM siRNA for 48 h. Cell migration was determined via a wound healing assay at the indicated times after siRNA transfection. (L) Transwell migration assay in siSNX5-overexpressing cells. siSNX5-transfected cells were plated on soft agar and incubated for 48 h. (M) A clonogenic assay was performed, and the colonies were stained with crystal violet for quantification. Images of 12-well plates with colonies were captured on day 14, and a bar graph was generated by calculating the percentages of colonies from each cell line relative to those of the controls. The data are representative of three independent experiments. **P<0.01, ***P<0.001. SNX5, Sorting Nexin 5; OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; TCGA, The Cancer Genome Atlas; iNOK, immortalized human normal oral keratinocyte; RT-qPCR, reverse transcription-quantitative PCR; si, short interfering.

blotting confirmed effective silencing of SNX5 mRNA and protein (Fig. 4F and G). MTT assays showed significantly reduced proliferation in SNX5-knockdown cells (Fig. 4H). Flow cytometry revealed a marked increase in apoptosis: late apoptotic cell percentages rose to 57.95% (FaDu) and 54.75% (YD-15) vs. controls (Fig. 4I). Western blotting showed

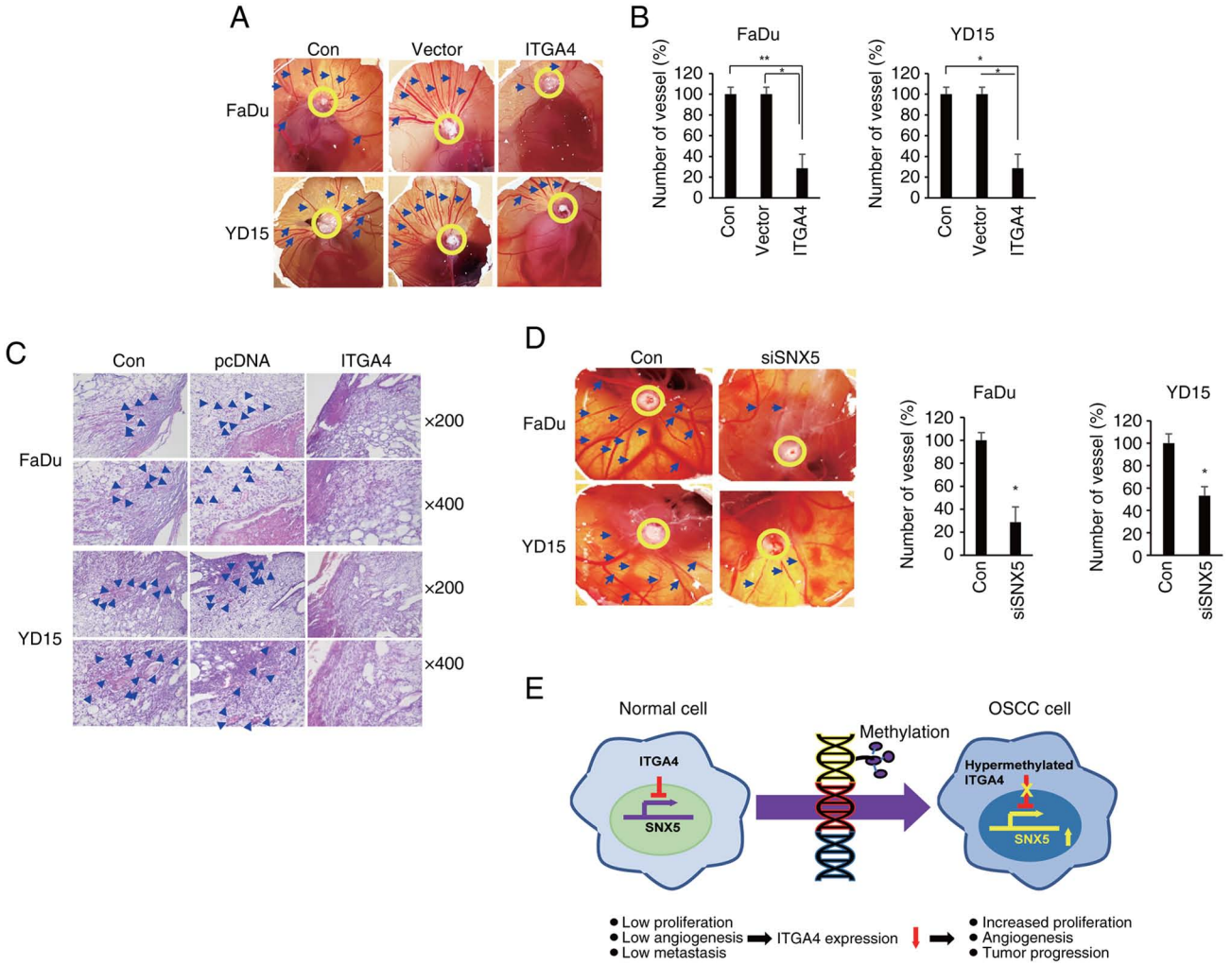

Figure 5. ITGA4 inhibits angiogenesis. A CAM assay was used to analyze the effects of ITGA4 and siSNX5 on angiogenesis *in vivo*. (A) Fertilized eggs were implanted with ITGA4- or siSNX5-transfected FaDu and YD-15 cells ($1x10^7$/ml) and maintained in a humidified incubator at 37˚C for an additional 5 days (n=5). (B) Graphs showing the results of the quantitative evaluation of angiogenesis. The values are presented as the means ± S.D $^*P<0.05$ and $^{**}P<0.01$. (C) H&E staining of tumors (blue arrow head). (D) Representative images of oral cancer cell xenografts from the CAM assay. Fertilized eggs were inoculated with siSNX5-transfected FaDu and YD-15 cells and incubated for an additional 5 days (n=5). The graphs show the quantitative evaluation of angiogenesis. $^*P<0.05$. (E) Images of summarized the main findings. ITGA4, integrin subunit α 4; CAM, chick chorioallantoic membrane; si, short interfering; SNX5, Sorting Nexin 5; OSCC, oral squamous cell carcinoma.

cleavage of procaspase-9 and PARP-1 after SNX5 knockdown (Fig. 4J), consistent with results from ITGA4 overexpression. The present study then assessed the effect of SNX5 depletion on migration using scratch and Transwell assays. Knockdown significantly impaired migration in FaDu and YD-15 cells (Fig. 4K and L). Finally, colony formation was significantly reduced after 14 days of SNX5 knockdown, with FaDu and YD-15 cells showing >68 and 71% reductions, respectively, compared with controls (Fig. 4M).

ITGA4 prevents angiogenesis in xenografts derived from FaDu and YD-15 cells in the CAM model. Angiogenesis and invasion are crucial processes in tissue expansion, which, when enhanced, lead to the formation of malignant tumors. After ITGA4-overexpressing cells were implanted in fertilized eggs using a microsyringe, they were allowed to grow for 5 days to further clarify the role of the ITGA4/SNX5 axis in OSCC angiogenesis and invasion *in vivo*. The findings revealed a significant reduction of ~70% in angiogenesis surrounding ITGA4-overexpressing tumors compared with the control groups for both FaDu and YD-15 cells (Fig. 5A and B). Histology of the tumor tissues revealed that the number of invading tumor cells was markedly increased in the control group. Conversely, fertilized eggs bearing ITGA4-overexpressing tumors had lower numbers of invading tumor cells than the control eggs (Fig. 5C). SNX5 siRNA-transfected FaDu and YD-15 cells were seeded on chicken embryo CAMs and incubated for 3 days. The results revealed that the number of blood vessels on the surface of the tumors in the siSNX5-transfected group was greater than that in the control group (Fig. 5D), and the microvessel density was markedly suppressed by siSNX5. Fig. 5E summarized the main findings of the results.

Discussion

The present study performed an integrated epigenetic and functional analysis to investigate the role of DNA methylation in oral squamous cell carcinoma. Through a meta-analysis

of methylation datasets from head and neck squamous cell carcinoma, it identified seven hypermethylated genes (ITGA4, ITGA8, ZFP82, PCDH17, PHYHIPL, USP44 and ZFN132) frequently altered in tumor tissues (Table SI) (17-27). Among these candidates, ITGA4 were consistently hypermethylated in OSCC cell lines but unmethylated in normal oral keratinocytes, suggesting their potential roles as tumor suppressor genes epigenetically silenced during OSCC development. These findings were further supported by TCGA gene expression data and Kaplan-Meier survival analyses, which showed a negative correlation between the methylation of these genes and patient prognosis.

Focusing on ITGA4, the present study explored its tumor-suppressive function and regulatory mechanisms in OSCC. The results supported the following key conclusions: i) In MSP, ITGA4 is a DNA hypermethylation-driven gene in OSCC cell lines; ii) ITGA4 expression is significantly lower in OSCC cells than in normal cells, which suggests that epigenetic silencing of ITGA4 may contribute to OSCC development; iii) ITGA4 overexpression inhibits OSCC cell proliferation, migration and colony formation and induces apoptosis, which indicates its regulatory role as a tumor suppressor gene; and iv) ITGA4 inhibits the expression of key mesenchymal markers involved in EMT, further supporting its regulatory role in OSCC progression. In addition, MSP analysis confirmed that ITGA4 was methylated in various OSCC-derived cell lines, and treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine restored ITGA4 expression, indicating that promoter hypermethylation is a primary mechanism of its transcriptional silencing. However, these results, while strongly suggestive, do not fully establish methylation as the direct causal mechanism. To conclusively demonstrate causality, additional mechanistic approaches such as targeted promoter demethylation using CRISPR/dCas9-TET1 will be used to investigate the methylation-specific luciferase reporter assays, or bisulfite sequencing before and after epigenetic editing. In addition, DNA methylation often cooperates with repressive histone marks such as H3K9me3 or H3K27me3 and analyzing these additional layers would provide a more complete understanding of the epigenetic mechanisms underlying ITGA4 inactivation in further study.

ITGA4 is a heterodimeric transmembrane protein that promotes cell survival, proliferation, migration, invasion and tumor invasion and metastasis (10). However, the role of ITGA4 in tumorigenesis remains controversial. Previous studies have suggested that ITGA4 expression has been implicated in tumor progression and poor prognosis across multiple cancer types, including gastric, OSCC, chronic lymphocytic leukemia and HER2-positive breast cancers (9-12,28-31). Furthermore, elevated ITGA4 levels contribute to the pathogenesis of gastrointestinal stromal tumors and MYCN-low neuroblastoma, correlating with reduced overall survival (30). By contrast, a study focusing on colorectal cancer and rectal adenocarcinoma patients found that low ITGA4 expression was associated with poorer overall survival, indicating a possible protective role of ITGA4 in this cancer type (10,11). This association highlights the potential of ITGA4 not only as a diagnostic marker but also as a therapeutic target in the management of OSCC. Despite the evidence supporting the potential significance of ITGA4 in OSCC, additional studies are needed to elucidate the mechanisms by which ITGA4 methylation influences various stages of tumor formation and to explore its potential clinical applications.

In addition to ITGA4, another candidate, ZFP82 (also known as ZNF545), a KRAB-ZFP family tumor suppressor, is frequently hypermethylated in various cancers, leading to its downregulation (32-35). ZFP82 has been shown to inhibit tumorigenesis in esophageal cancer by suppressing proliferation and invasion while promoting apoptosis (36). In the present study, Kaplan-Meier and SMART analyses indicated that ZFP82 hypermethylation was associated with poorer survival in OSCC patients. Although ZFP82 also appeared as a hypermethylated candidate in the initial screening, several factors led the present study to prioritize ITGA4 for functional investigation. Unlike ITGA4, ZFP82 showed partial methylation even in iNOK cells and displayed considerable variability in mRNA expression among OSCC cell lines, suggesting that its epigenetic alteration is less OSCC-specific. In addition, 5-aza treatment produced only modest restoration of ZFP82 expression, and no consistent evidence linked ZFP82 to tumor-suppressive pathways.

By analyzing the proteomic profile, the present study demonstrated that ITGA4 overexpression significantly decreased SNX5 levels in FaDu cells. SNX5 is a member of the sorting nexin family involved in endosomal trafficking, which has recently been implicated in tumorigenesis. Beyond its role in endosomal trafficking, SNX5 has been reported to regulate receptor recycling, intracellular signaling, and apoptotic pathways, including modulation of caspase-2 activity (37). SNX5-mediated control of growth factor receptor turnover can influence proliferative and invasive behavior and dysregulated SNX5 expression has been associated with aggressive phenotypes in papillary thyroid carcinoma and head and neck cancers (38,39). These findings indicate that SNX5 functions as a critical regulator linking endosomal homeostasis to cancer-related signaling, supporting its relevance in OSCC biology. The present study observed consistent downregulation of SNX5 at both the mRNA and protein levels upon ITGA4 overexpression. Moreover, siRNA-mediated knockdown of SNX5 was similar to the above observed anti-proliferative and pro-apoptotic effects of ITGA4, providing functional validation of this regulatory axis. However, the mechanism that regulates SNX5 in carcinogenesis has not been clearly elucidated. The finding of the present study that ITGA4-mediated suppression of SNX5 expression is a key mechanism underlying the antitumor effects of ITGA4 on OSCC is a novel and important contribution to understanding OSCC pathogenesis. While the results consistently showed that ITGA4 overexpression suppressed SNX5 and that SNX5 depletion reproduced the effects of ITGA4, the direct signaling mechanism linking these molecules remains undefined. The observed reduction in EMT markers and cytoskeletal remodeling suggested that ITGA4-dependent modulation of FAK/Src/Rho pathways may indirectly affect SNX5, but further studies are needed.

Experiments using a CAM xenograft model indicated that ITGA4 overexpression or siSNX5 significantly inhibited OSCC cells driven angiogenesis. This finding is clinically relevant, as angiogenesis is a critical process in the progression and metastasis of OSCC. It also enhances our understanding of not only the anticancer role of ITGA4 in inhibiting tumor

progression but also its potential therapeutic value through its ability to suppress angiogenesis. Although the CAM assays demonstrated tumor-suppressive effects of ITGA4, these findings cannot fully establish its role in OSCC progression. Future, more comprehensive *in vivo* studies will investigate the effect of ITGA4 on tumor growth, invasion and metastasis. Despite these findings, studies on epigenetic regulation and implications of ITGA4 in OSCC are still limited in identifying precancerous tissues, prognosis and treatment-related biomarkers for clinical applications and in developing therapeutics. In particular, further research is needed to evaluate the methylation status of ITGA4 in precancerous lesions and to determine its prognostic and predictive value in clinical settings. Additionally, the therapeutic potential of epigenetic drugs (epidrugs) to restore ITGA4 function warrants further investigation. The roles of other hypermethylated genes, such as ZFP82, also deserve exploration as possible diagnostic or therapeutic targets. In the present study, iNOK cells were used as a non-malignant comparator to assess OSCC-specific epigenetic alterations. Multiple independent studies have shown that iNOK cells exhibit low expression of oncogenic markers such as PLK1, HSF1, HDAC8 and HOXC6/Bcl-2 and display biophysical properties distinct from OSCC cell lines, supporting their use as a reliable non-tumorigenic epithelial reference (40-43). Although the detailed immortalization procedure for iNOK has been described for related HPV16 E6/E7-immortalized oral keratinocyte models within the same research network, additional molecular characterization, including baseline methylation and transcriptomic profiling, would further strengthen their validation as a normal comparator in future studies.

In conclusion, the present study demonstrated that ITGA4 is a key epigenetically silenced tumor suppressor gene in OSCC and that its antitumor effects are at least partially mediated by the downregulation of SNX5. These findings not only improve novel mechanistic insights into OSCC pathogenesis but also suggest that ITGA4 could serve as a potential prognostic biomarker and a therapeutic target for OSCC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author or public database (http://www.ebi.ac.uk/pride; accession numbers: PXD057268).

Authors' contributions

NT and HC were responsible for conception, methodology, data validation, writing the original draft, writing, reviewing and editing. SA was responsible for conception, resources, funding acquisition, writing, reviewing and editing and supervision. NT. and SA confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

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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