

Mechanistic role of the ILK-S100A4 axis in modulating invasion and EMT in salivary adenoid cystic carcinoma

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Abstract. Salivary adenoid cystic carcinoma (SACC) is a prevalent malignant tumor of the salivary glands, characterized by invasive growth and perineural invasion, resulting in high rates of local recurrence, distant metastasis and poor long-term survival. Thus, elucidating the molecular mechanisms underlying SACC invasion and identifying effective therapeutic targets are of clinical importance. Functional cellular assays including proliferation, migration, and flow cytometry, and molecular experiments, such as western blot, were conducted *in vitro* to explore the effects of integrin-linked kinase (ILK) knockdown on the biological behavior of SACC cells and the expression of epithelial-mesenchymal transition (EMT) markers. Transcriptome sequencing identified S100 calcium-binding protein A4 (S100A4) as a key downstream effector regulated by ILK. Additionally, 52 clinical SACC specimens were analyzed to evaluate the correlation between S100A4 expression and clinicopathological features, as well as ILK expression. Rescue experiments validated the role of S100A4 in mediating the effects of ILK. ILK knockdown significantly suppressed the malignant behavior of SACC cells, including migration and invasion, and reversed EMT phenotypes. S100A4 expression was significantly associated with clinical features such as clinical stage and perineural invasion, along with ILK expression. Overexpression of S100A4 restored Snail expression, promoted the EMT process and rescued the impaired migration and invasion capabilities of SACC cells caused by ILK knockdown. ILK may facilitate EMT-mediated invasion in SACC cells via the Glycogen synthase kinase-3 β signaling pathway by modulating the transcription factor Snail. S100A4 may contribute to this

mechanism by modulating Snail protein. These findings imply that simultaneously targeting both ILK and S100A4 represents a novel and promising therapeutic strategy to suppress SACC progression.

Introduction

Salivary adenoid cystic carcinoma (SACC) is a relatively rare but aggressive malignancy of the salivary glands, accounting for ~25% of all salivary gland tumors (1,2). The tendency of SACC to invade locally and metastasize distantly makes it difficult to manage clinically. Despite advancements in multimodal therapies, including surgery in combination with radiotherapy or chemotherapy, >30% of patients experience local recurrence and face poor prognosis (3-5). Thus, elucidating the molecular mechanisms underlying SACC invasion and metastasis is key for improving patient outcomes.

Epithelial-mesenchymal transition (EMT), a key biological process during tumor progression, endows cancer cells with increased plasticity, promoting their migration and invasion capabilities (6). EMT is marked by the loss of epithelial characteristics, such as polarity and intercellular adhesion, and the acquisition of mesenchymal markers (7,8). Integrin-linked kinase (ILK) serves as a serine/threonine kinase and scaffolding protein involved in diverse signaling pathways, such as the PI3K/Akt and Wnt/ β -catenin pathways (9,10). Its N-terminal ankyrin repeat domain binds particularly interesting new cysteine-histidine-rich protein (PINCH), while the C-terminal kinase domain associates with Parvin to form the ILK-PINCH-Parvin complex. This complex broadly participates in the molecular regulation of malignant phenotypes, including cell cycle progression, migration, invasion and apoptosis resistance (10-14). ILK upregulation is observed in various types of cancer, such as prostate and colorectal cancer, and demonstrates significant associations with tumor progression and poor prognosis (15,16). A previous study demonstrated that ILK knockdown induces apoptosis of prostate cancer cells by decreasing AKT activity (15). Our previous research also revealed that overexpression of ILK is associated with perineural invasion, local recurrence and distant metastasis in SACC (17). Moreover, ILK levels are associated with the expression of EMT markers, suggesting its involvement in EMT regulation (16). However, the precise molecular mechanism remains unclear.

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S100 calcium-binding protein A4 (S100A4), a member of the S100 protein family, exerts its functions in a calcium-dependent manner and serves both intracellular and extracellular roles. Within cells, S100A4 expression is associated with regulation of cell migration, apoptosis and maintenance of stemness (18,19). In the extracellular space, S100A4 primarily stimulates pro-inflammatory signaling pathways and induces the expression of effector molecules, including growth factors, extracellular matrix components and MMPs, thereby activating diverse physiological processes (20). Recognized as a promoter of tumor progression, metastasis and poor clinical outcomes (21,22), S100A4 is frequently upregulated in tumor cells and the tumor microenvironment. It contributes to the pathogenesis and advancement of multiple malignancies, such as breast and lung cancer (23-25). Although both ILK and S100A4 have been independently linked to the regulation of EMT (26,27), the mechanistic connection between them remains poorly understood. The present study aimed to determine the contribution of ILK to the malignant phenotypes of SACC cells and the regulatory association between ILK and S100A4 in the context of EMT and tumor invasion. The present results could offer new perspectives on SACC pathogenesis and reveal promising targets for therapeutic intervention.

Materials and methods

Patients and specimen collection. A total of 52 SACC tissue samples were obtained following approval by the Ethics Committee of the Affiliated Stomatological Hospital of Southwest Medical University (approval no. 20211129004; Luzhou, China). The study enrolled 52 patients (21 male and 31 female), aged 23-84 years, who underwent radical resection between January 2020 and December 2024 and had not received any prior radiotherapy or chemotherapy. Patients who had previously undergone radiotherapy or chemotherapy, or those with ill-defined primary lesions accompanied by distant metastasis, were excluded. Written informed consent was obtained from each participant before tissue collection. Clinicopathological characteristics and follow-up data were obtained from the database of the Affiliated Stomatological Hospital of Southwest Medical University. According to the World Health Organization Classification of Head and Neck Tumors (28), the dominant histological pattern was determined based on the pattern occupying the largest proportion of the tumor, and those with a solid component >30% were classified as the solid subtype. Tumor staging was performed according to the 8th edition of the Union for International Cancer Control/American Joint Committee on Cancer (AJCC) TNM classification system (29). Normal parotid gland tissue samples (n=5) were collected from the disease-free surgical margins of patients undergoing parotidectomy for benign pleomorphic adenoma at the Affiliated Stomatological Hospital of Southwest Medical University between January 2024 and December 2024. Exclusion criteria included prior head and neck radiotherapy or chemotherapy. The control cohort consisted of 2 males and 3 females, aged 35 to 56 years. All tissues were reviewed and confirmed to be histologically normal by two independent pathologists.

Cell lines and culture. The human SACC cell lines SACC-83 and SACC-LM were obtained from the State Key Laboratory of Oral Diseases, West China Hospital of Stomatology. SACC-83 and SACC-LM cell lines are derived from ductal epithelial cells (30). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Vazyme) and 1% penicillin-streptomycin solution (Beyotime Institute of Biotechnology), and maintained at 37°C in a humidified incubator with 5% CO₂.

Cell transfection. Lentiviruses targeting ILK and S100A4 were constructed and synthesized by OBIO Technology. Short hairpin (sh)RNA sequences targeting ILK were inserted into the pSLenti-U6-shRNA-CMV-EGFP-F2A-Puro-WPRE silencing vector. S100A4 sequences were cloned into the pSLenti-EF1-EGFP-P2A-Puro-CMV-S100A4-3X FLAG-WPRE overexpression vector. The negative control lentivirus was obtained from OBIO Technology. Lentiviral particles were harvested at 48 and 72 h after transfecting the recombinant vectors into 293T cells (ATCC). SACC-83 and SACC-LM cells were seeded in 12-well plates at a density of 5x10⁴ cells per well and cultured at 37°C for 24 h until 70% confluence. Cells were then transduced with lentivirus at a multiplicity of infection of 60 in the presence of 5 µg/ml Polybrene Plus (OBIO Technology). Following 72 h transfection at 37°C, 2 µg/ml puromycin (Beijing Solarbio Science & Technology Co., Ltd.) was introduced for selection to establish stably transfected cell lines. Subsequent experiments utilized stable cells that were cultured for 5-7 days post-selection and maintained within five passages of transfection. The sequences of ILK-targeting shRNAs were as follows: shRNA-1, 5'-CCGTAGTGTAATGATTGATGA-3'; shRNA-2, 5'-CGACCCAAATTTGACATGATT-3' and shRNA-3, 5'-GCAATGACATTGTCGTGAAGG-3'. Untreated cells were defined as the control group (CON). Cells transfected with a scramble non-targeting shRNA lentivirus served as the negative control for the ILK knock-down experiment (NC-ILK), and cells transfected with an empty vector lentivirus served as the negative control for the S100A4 overexpression experiment (NC-S100A4).

Cell proliferation assay. SACC cells in the logarithmic growth phase were trypsinized and seeded into 96-well plates at a density of 3x10³ cells/well (n=5/group). After 24 h incubation at 37°C, Cell Counting Kit (CCK)-8 reagent (Dojindo Laboratories, Inc.) was administered at 0, 24, 48, 72, 96 and 120 h. Optical density at 450 nm following 1 h incubation at 37°C was measured to quantify cell proliferation.

Colony formation assay. SACC cells were seeded in 6-well plates at a density of 700 cells/well and cultured in complete RPMI-1640 medium at 37°C under 5% CO₂ for 14 days, with the medium (Gibco; Thermo Fisher Scientific, Inc.) replaced every 48 h. Colonies (defined as cell clusters containing ≥50 cells) were fixed with 4% paraformaldehyde for 20 min at room temperature, stained with 0.1% crystal violet for 15 min at room temperature, rinsed with PBS, and then imaged using a light microscope. Colony counting was performed automatically using ImageJ (v1.54, National Institutes of Health).

Cell cycle analysis. SACC cells (5×10^5) were fixed in 75% ethanol at -20°C overnight. Cells were treated with RNase A ($50 \mu\text{g}/\text{ml}$ at 37°C for 30 min) and stained with PI ($5 \mu\text{l}$; 4°C for 30 min in the dark). Cell cycle distribution was analyzed using a flow cytometer (BD FACSCalibur), and data were processed with FlowJo software (v10.8, FlowJo LLC).

Wound healing assay. SACC cells (2×10^6 cells/well) were cultured at 37°C for 24 h in 6-well plates until $>95\%$ confluency. The monolayers were scratched using a sterile pipette tip and incubated at 37°C in serum-free medium. Images were captured at 0, 12 and 24 h using an inverted light microscope (Olympus GX53). The wound area was quantified using ImageJ software (v1.54, National Institutes of Health) to evaluate migration.

Transwell invasion assay. SACC cells (5×10^4 /insert) suspended in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) were seeded into the upper chamber of Matrigel-coated Transwell inserts (Corning, Inc.). The lower chamber was filled with medium containing 10% FBS (Vazyme) as a chemoattractant. Following 48 h incubation at 37°C , non-invading cells were removed and invaded cells were fixed with 4% paraformaldehyde for 20 min at room temperature, stained with 0.1% crystal violet for 15 min at room temperature, and then imaged using a light microscope. A total of five randomly selected fields per membrane were analyzed using ImageJ software (v1.54, National Institutes of Health).

Western blotting. Total protein was extracted from SACC cells using RIPA buffer containing protease and phosphatase inhibitors (AbMole Bioscience). Protein concentration was determined using a BCA assay (Vazyme). Equal amounts of protein ($30 \mu\text{g}/\text{lane}$) were separated by 10% SDS-PAGE and transferred to PVDF membranes. Following blocking with 5% skimmed milk for 2 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies against ILK (1:1,000, Abcam; cat. no. ab76468), S100A4 (1:5,000, Proteintech Group, Inc.; cat. no. 16105-1-AP), E-cadherin (cat. no. ET1607-75), N-cadherin (cat. no. ET1607-37), Snail (cat. no. ER1706-22), GSK-3 β (cat. no. ET1607-71) and phosphorylated (p-)GSK-3 β (all 1:1,000; all HuaBio; cat. no. ET1607-60). Membranes were incubated with HRP-conjugated secondary antibodies (1:20,000; HuaBio; cat. no. HA1023) for 1 h at room temperature. Protein bands were visualized using ECL reagents (Biosharp Life Sciences) and analyzed with ImageJ, using GAPDH (1:5,000, HuaBio; cat. no. HA721136) as the internal control.

RNA sequencing. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and RNA integrity was confirmed using an Agilent 2100 Bioanalyzer. RNA libraries were prepared using the NEBNext Ultra RNA Library Prep kit (cat. no. #7530; New England Biolabs) and subjected to paired-end sequencing on an Illumina NovaSeq 6000 platform (Gene Denovo) with a 150 bp read length and a 20 pM loading concentration. To ensure high-quality data for assembly and analysis, raw reads were filtered using fastp v0.18.0 (31) to remove adapters and low-quality bases, yielding high-quality clean reads.

Bioinformatics analysis. Differentially expressed genes (DEGs) were analyzed using the Omicsmart platform (omicsmart.com), applying false discovery rate (FDR) <0.05 and absolute fold-change >2 . Gene Set Enrichment Analysis (GSEA) was performed to identify signaling pathways and molecular functions significantly enriched in the gene expression profile. EMT-associated gene sets were obtained from EMTome (emtome.org). Functional associations were explored by construction of protein-protein interaction (PPI) networks using the STRING database (string-db.org).

Immunohistochemistry and HE staining. Fresh tissue was fixed in 10% formalin for 24 h at room temperature and paraffin-embedded. Sections ($4 \mu\text{m}$ thickness) were deparaffinized in xylene for 20 min and rehydrated through a graded ethanol series. Heat-induced antigen retrieval was conducted in citrate buffer (pH 6.0) at $92-98^\circ\text{C}$ for 10 min. After endogenous peroxidase activity was blocked with 3% hydrogen peroxide, sections were incubated with 5% normal goat serum (ZSGB-BIO; cat. no. ZLI-9056) for 30 min at 37°C to block non-specific binding. Subsequently, the sections were incubated overnight at 4°C with primary antibodies against ILK (1:100, Abcam; cat. no. ab76468), S100A4 (1:100, Proteintech Group, Inc.; cat. no. 16105-1-AP), E-cadherin (1:100; cat. no. ET1607-75), N-cadherin (1:10,000; cat. no. ET1607-37) and Snail (1:100; cat. no. ER1706-22; all HuaBio). After washing with PBS, the sections were incubated with a ready-to-use, enhanced horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG polymer (ZSGB-BIO; cat. no. PV-9000) at 37°C for 40 min. Signal detection was carried out using DAB chromogen (ZSGB-BIO; cat. no. ZLI-9017), and nuclei were counterstained with hematoxylin for 3 min at room temperature. Slides were examined under a light microscope. Image analysis was performed using ImageJ software (v1.54, National Institutes of Health). For HE staining, serial sections were prepared from the same paraffin-embedded tissue blocks. Briefly, after standard deparaffinization and rehydration, sections were stained with hematoxylin for 5 min, followed by eosin for 2 min. The slides were then dehydrated, cleared in xylene for morphological evaluation. A total of two independent pathologists, blinded to the clinical data, performed the evaluation. A total of five randomly selected fields of view were examined under high-power magnification, and the pathologists recorded the percentage of cells at each intensity level (0, negative; 1, weak; 2, moderate and 3, strong) for both ILK and S100A4. The H-score was calculated as: $[0 \times (\% \text{ of cells with score } 0) + 1 \times (\% \text{ of cells with score } 1) + 2 \times (\% \text{ of cells with score } 2) + 3 \times (\% \text{ of cells with score } 3)] (32)$.

Statistical analysis. All statistical analyses were conducted using SPSS version 27.0 (IBM Corp.). Data are presented as the mean $\pm$ SD of at least three independent biological replicates. The normality of data distribution was assessed using the Shapiro-Wilk test. Associations between S100A4 expression and clinicopathological features were evaluated using the independent samples unpaired t-test or one-way ANOVA followed by Tukey's post hoc test. The correlation between ILK and S100A4 expression was validated using Spearman

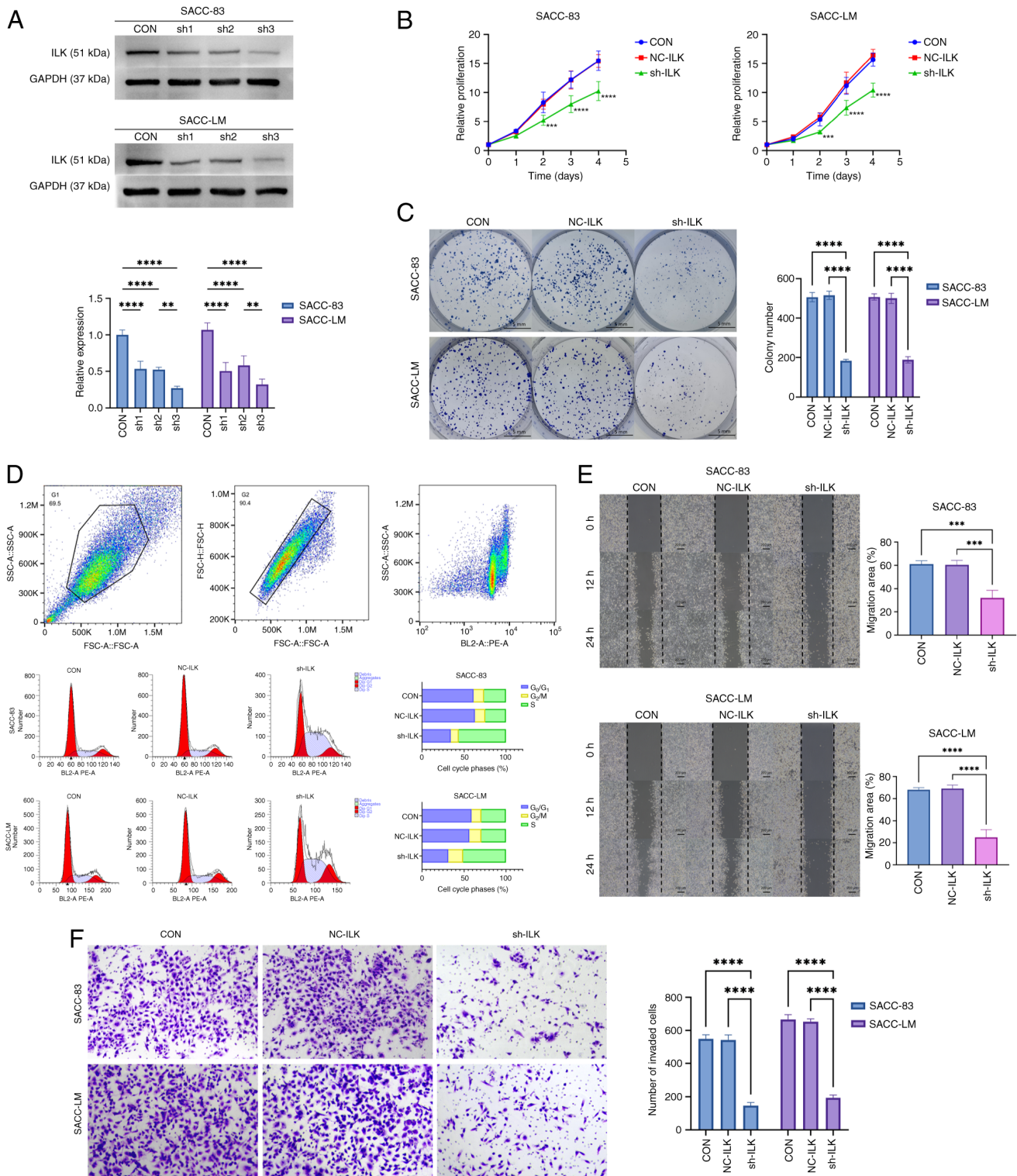


Figure 1. ILK contributes to the malignant biological behavior of SACC. (A) Western blot analysis confirmed effective ILK knockdown using shRNA. (B) Cell Counting Kit-8 assay to determine the inhibitory effect of ILK knockdown on SACC cell proliferation. (C) Colony formation assay demonstrated decreased colony-forming ability upon ILK knockdown. (D) Flow cytometry analysis showed ILK knockdown-induced cell cycle arrest in SACC cells. (E) Wound healing assay demonstrated reduced migratory capacity after ILK knockdown. (F) Transwell invasion assay revealed decreased invasive potential following ILK knockdown. Results are shown as the mean $\pm$ SD of three independent assays. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. control. ns, not significant. ILK, integrin-linked kinase; sh, short hairpin; SACC, salivary adenoid cystic carcinoma; CON, control; NC, negative control.

correlation analysis. Graphs and data visualizations were generated using GraphPad Prism version 10.0 (Dotmatics, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Knockdown ILK suppresses malignant phenotypes in SACC cells. To investigate the biological function of ILK

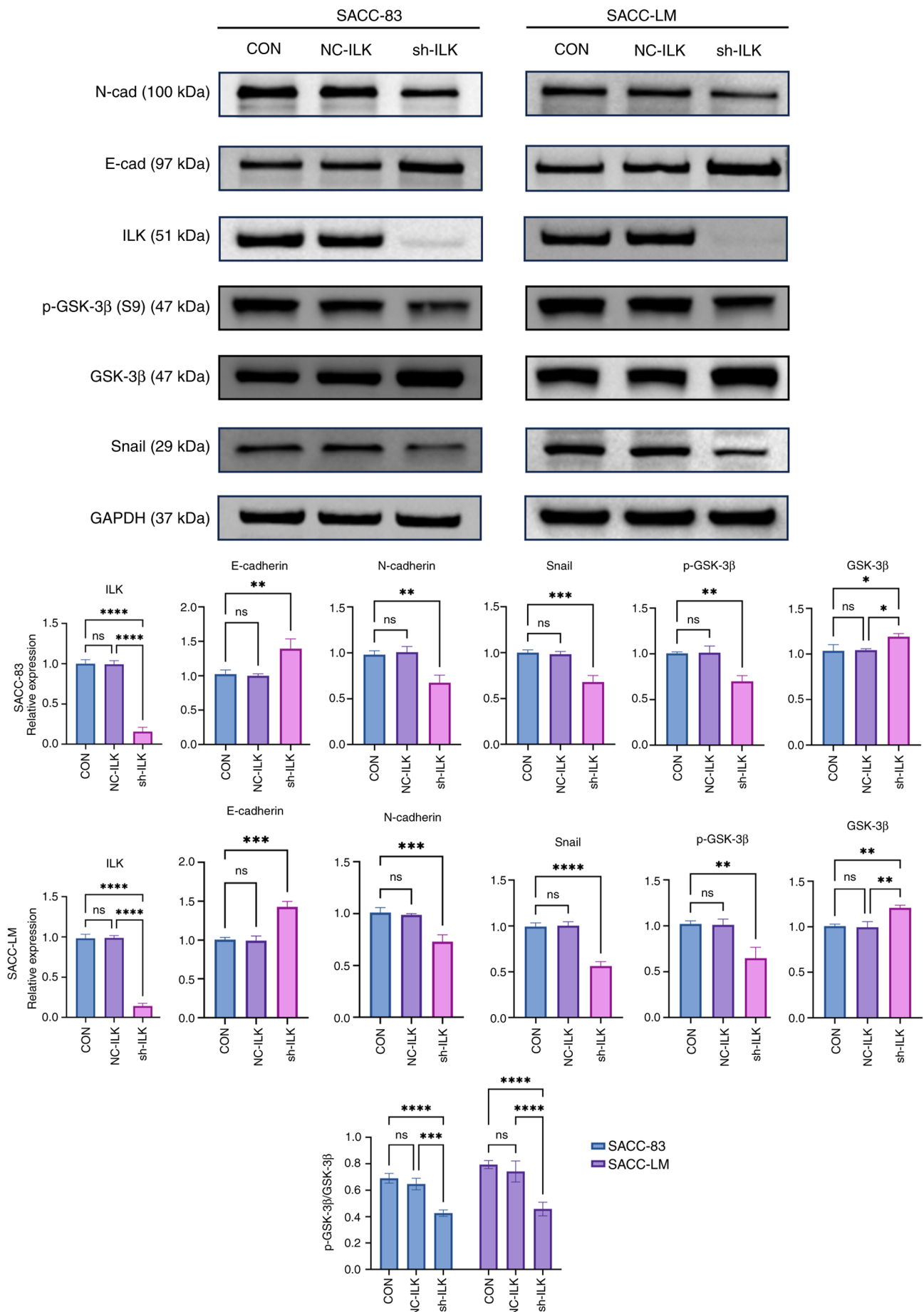


Figure 2. Western blot analysis of the effects of ILK knockdown on EMT markers and signaling. Results are shown as the mean $\pm$ SD of three independent assays. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. control group. ns, not significant.

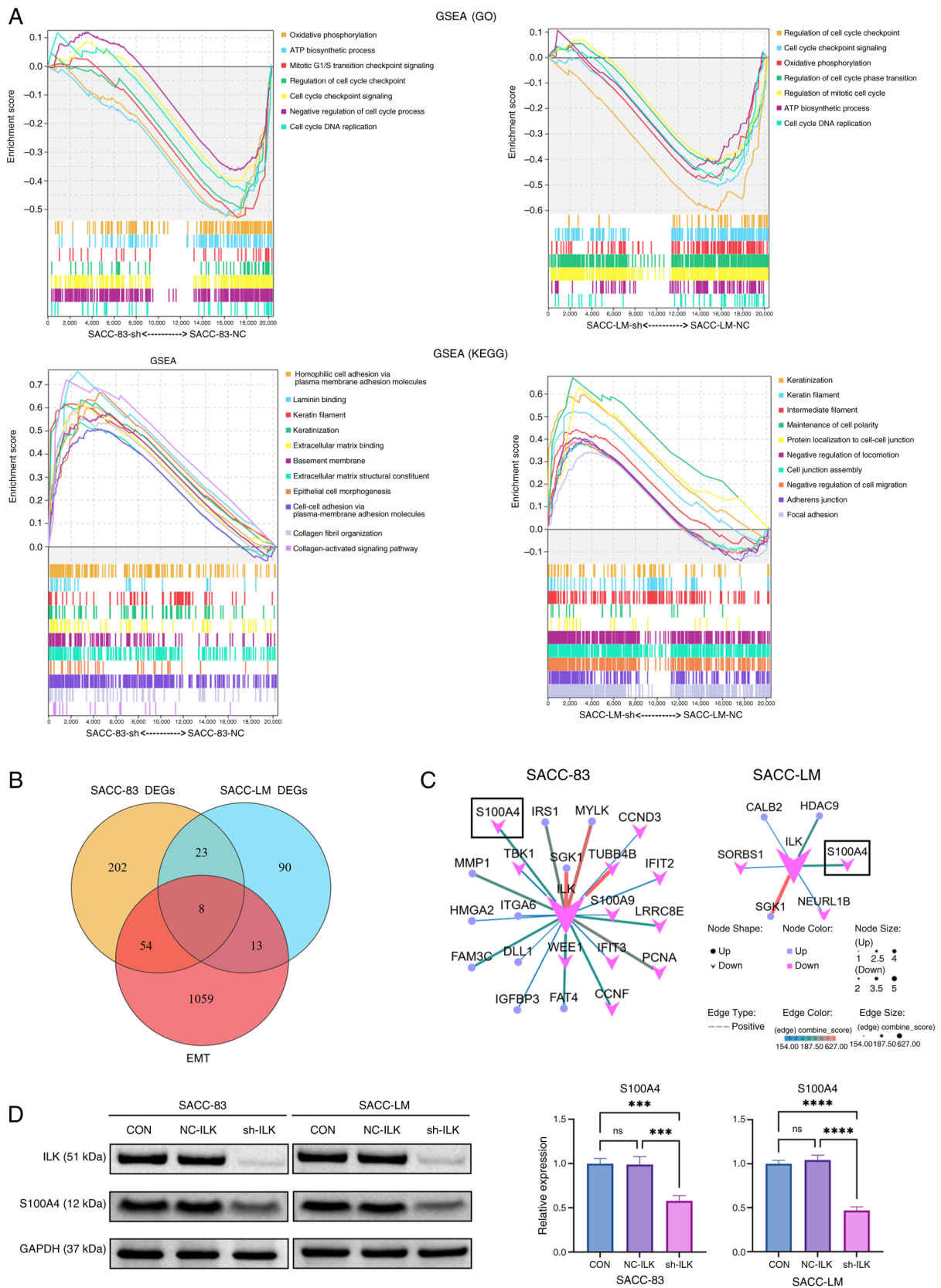


Figure 3. Bioinformatics profiling and western blotting identified S100A4 as a downstream effector of ILK. (A) GSEA ($P < 0.05$, false discovery rate < 0.25) revealed that ILK knockdown downregulated cell cycle pathways while upregulating epithelial phenotype-associated pathways. (B) Venn diagram analysis identified eight overlapping genes between DEGs and 1,153 EMT-core genes from the EMTome database: CFH, CPA4, FST, ANGPTL2, LUM, ADGRF1, ILK and S100A4. (C) Protein-protein interaction network constructed via STRING database highlighted ILK as the central hub node. (D) Western blot analysis validated that ILK knockdown downregulated the protein expression of S100A4 in both cell lines. *** $P < 0.001$, **** $P < 0.0001$. ns not significant; ILK, integrin-linked kinase; GSEA, Gene Set Enrichment Analysis; DEG, differentially expressed gene; CFH, Complement Factor H; CPA, Carboxypeptidase A; FST, Follistatin; ANGPTL, Angiopoietin-like Protein; LUM, Lumican; ADGRF, Adhesion G protein-coupled receptor F; NC, negative control; CON, blank control; sh, short hairpin; EMT, epithelial-mesenchymal transition; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SACC, salivary adenoid cystic carcinoma.

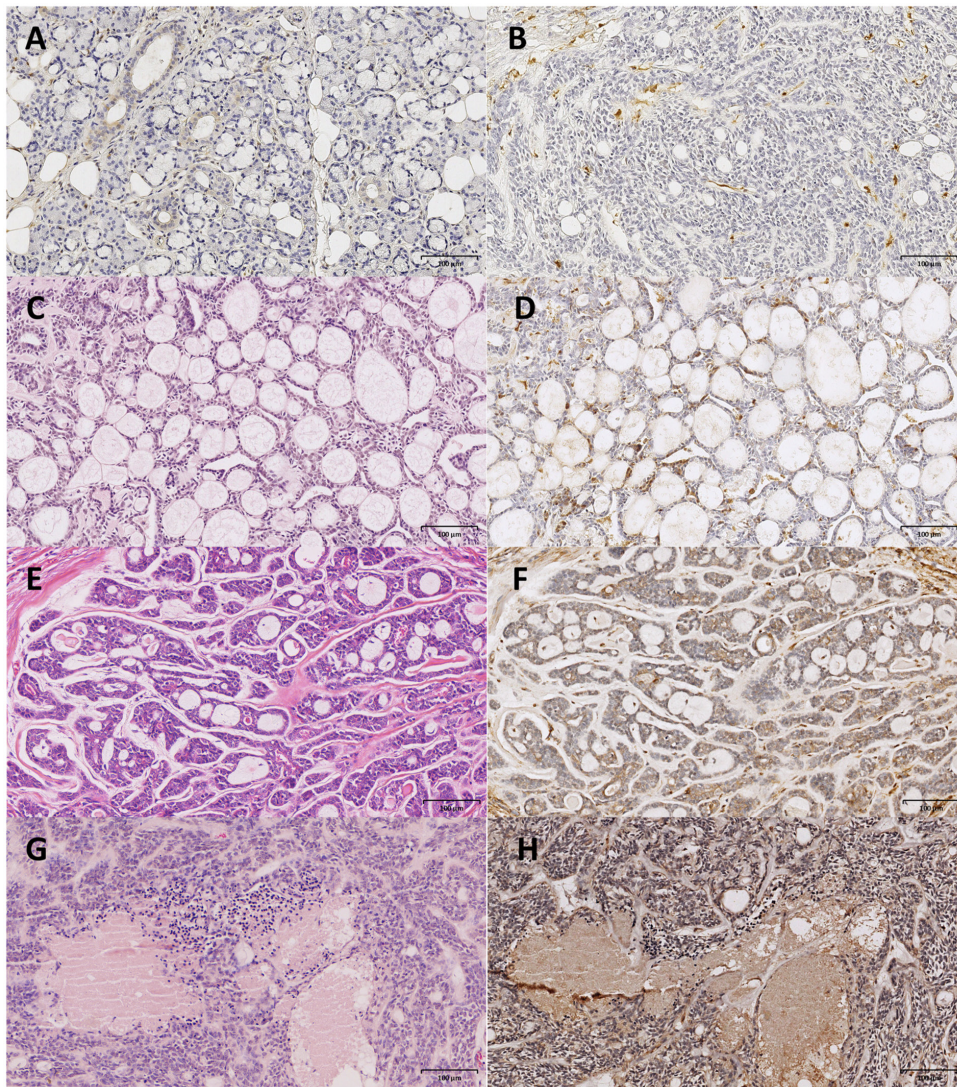


Figure 4. Immunohistochemical staining of S100A4 and HE staining in SACC subtypes and normal salivary gland tissue. (A) S100A4 expression in normal salivary gland tissue. (B) SACC tissue with negative S100A4 expression. (C) HE staining of cribriform adenoid cystic carcinoma. (D) Weak S100A4 expression in cribriform SACC subtype. (E) HE staining of tubular adenoid cystic carcinoma. (F) Moderate S100A4 expression in tubular SACC subtype. (G) HE staining of solid adenoid cystic carcinoma. (H) Strong S100A4 expression in solid SACC subtype. Magnification, x20. HE, hematoxylin-eosin; SACC, salivary adenoid cystic carcinoma.

in SACC cells, shRNA was used to stably knock down ILK expression. The shRNA-3 sequence demonstrated the best knockdown efficiency, as confirmed by western blotting, and was selected for subsequent functional experiments (Fig. 1A). ILK knockdown markedly suppressed cell proliferation, as demonstrated by CCK-8 assay (Fig. 1B), and decreased the colony-forming ability of SACC cells (Fig. 1C), compared with both the CON and NC groups. Flow cytometric analysis of the cell cycle indicated a decreased G1 and an increased S population in ILK-knockdown cells (Fig. 1D), suggesting an enhanced G1 to S transition and an S phase blockade, which hindered cellular DNA replication. In addition, wound healing (Fig. 1E) and Transwell invasion assays (Fig. 1F) showed that ILK-knockdown cells exhibited significantly decreased migratory and invasive capacities. Together, these findings indicated ILK was a key regulator of SACC cell proliferation, colony formation, migration and invasion, highlighting its potential role in promoting tumor progression.

ILK regulates EMT progression in SACC cells via the GSK-3 β /Snail signaling pathway. EMT is characterized by the downregulation of the epithelial marker E-cadherin and upregulation of the mesenchymal marker N-cadherin; this is primarily controlled by transcription factors (6). Building on previous findings demonstrating a correlation between ILK and EMT markers in SACC (14), the present study examined the expression of key EMT markers following ILK knockdown. ILK knockdown significantly increased E-cadherin expression while decreasing N-cadherin and the transcription factor Snail (Fig. 2). GSK-3 β regulates the expression of Snail protein via phosphorylation, thereby modulating EMT (33). ILK knockdown significantly decreased the ratio of p-GSK-3 β to total GSK-3 β . These results suggest that ILK may modulate EMT in SACC cells via the GSK-3 β /Snail pathway, and that its inhibition could effectively block this process.

ILK as a key regulator of EMT in SACC. RNA sequencing was conducted on ILK-knockdown SACC cells. Principal

Table I. Association between S100A4 expression and clinicopathological features of patients with salivary adenoid cystic carcinoma.

Clinicopathological feature	Cases (n=52)	Mean S100A4 expression	P-value
Age, years			
≤60	29	148.0±48.4	0.463
>60	23	158.8±56.3	
Sex			
Male	21	151.2±54.0	0.861
Female	31	153.8±51.1	
Tumor site			
Minor salivary gland	25	151.5±52.5	0.863
Major salivary gland	27	154.0±52.1	
Tumor diameter, cm			
≤2	18	161.4±37.3	0.207
>2, ≤4	23	140.1±42.4	
>4	11	165.2±61.5	
Clinical stage			
I+II	27	134.1±42.9	0.002
III+IV	25	173.0±41.1	
Histological subtype			
Tubular ^{a,b}	17	140.7±37.7	0.023
Cribriform ^c	21	143.7±45.8	
Solid	14	181.1±46.1	
Perineural invasion			
Positive	24	185.4±37.6	<0.001
Negative	28	124.8±32.1	

^aP=0.013 vs. solid; ^bP=0.839 vs. cribriform; ^cP=0.016 vs. solid.

component analysis confirmed high reproducibility among biological replicates (Fig. S1A). Analysis of differential gene expression revealed 288 DEGs in SACC-83 cells and 134 in SACC-LM cells (Fig. S1B). The successful knock-down of ILK was confirmed in both cell lines (Fig. S1C). Additionally, a heatmap was generated to visualize the hierarchical clustering of the top 50 DEGs, as ranked by FDR (Fig. S1D).

GSEA revealed that ILK knockdown led to the down-regulation of cell cycle-associated pathways and enrichment of pathways associated with epithelial phenotypes in both SACC-83-SH and SACC-LM-SH cells (Fig. 3A). Notably, the upregulated pathways showed negative correlations with EMT signatures, providing direct evidence of EMT suppression. In addition, pathways involving negative regulators of cell migration and motility were enriched in SACC-LM-SH cells. These coordinated transcriptomic changes suggested that ILK depletion enhanced intercellular adhesion and suppressed cell motility, consistent with *in vitro* findings.

S100A4 is a key downstream effector of ILK in EMT regulation. To elucidate the molecular mechanisms underlying ILK-mediated EMT in SACC, the present study integrated DEGs from two distinct SACC cell lines with

1,153 experimentally validated EMT core genes from the EMTome database (34). Venn diagram analysis revealed eight overlapping candidate genes: Complement Factor H), CPA4 (Carboxypeptidase A4), FST (Follistatin), ANGPTL2 (Angiopoietin-like Protein 2), LUM (Lumican), ADGRF1 (Adhesion G protein-coupled receptor F1), ILK and S100A4 (Fig. 3B). PPI network analysis using the STRING database highlighted S100A4 as a significantly co-downregulated gene in ILK-knockdown cells (Fig. 3C). These findings were validated by western blot analysis, which confirmed that S100A4 expression markedly decreased following ILK knockdown in both SACC cell lines (Fig. 3D).

S100A4 expression is associated with aggressive clinicopathological features and ILK expression in SACC. S100A4 expression was nearly undetectable in the acinar cells of normal salivary glands (Fig. 4A). Among 52 SACC specimens, 39 cases exhibited S100A4 positivity, while 13 cases demonstrated no observable S100A4 expression (Fig. 4B). By contrast, S100A4 expression was observed in both tumor-derived myoepithelial (Fig. 4C and D) and ductal epithelial cells (Fig. 4E and F), particularly in the solid subtype of SACC, which is associated with the worse prognosis (Fig. 4G and H). S100A4 expression showed no significant

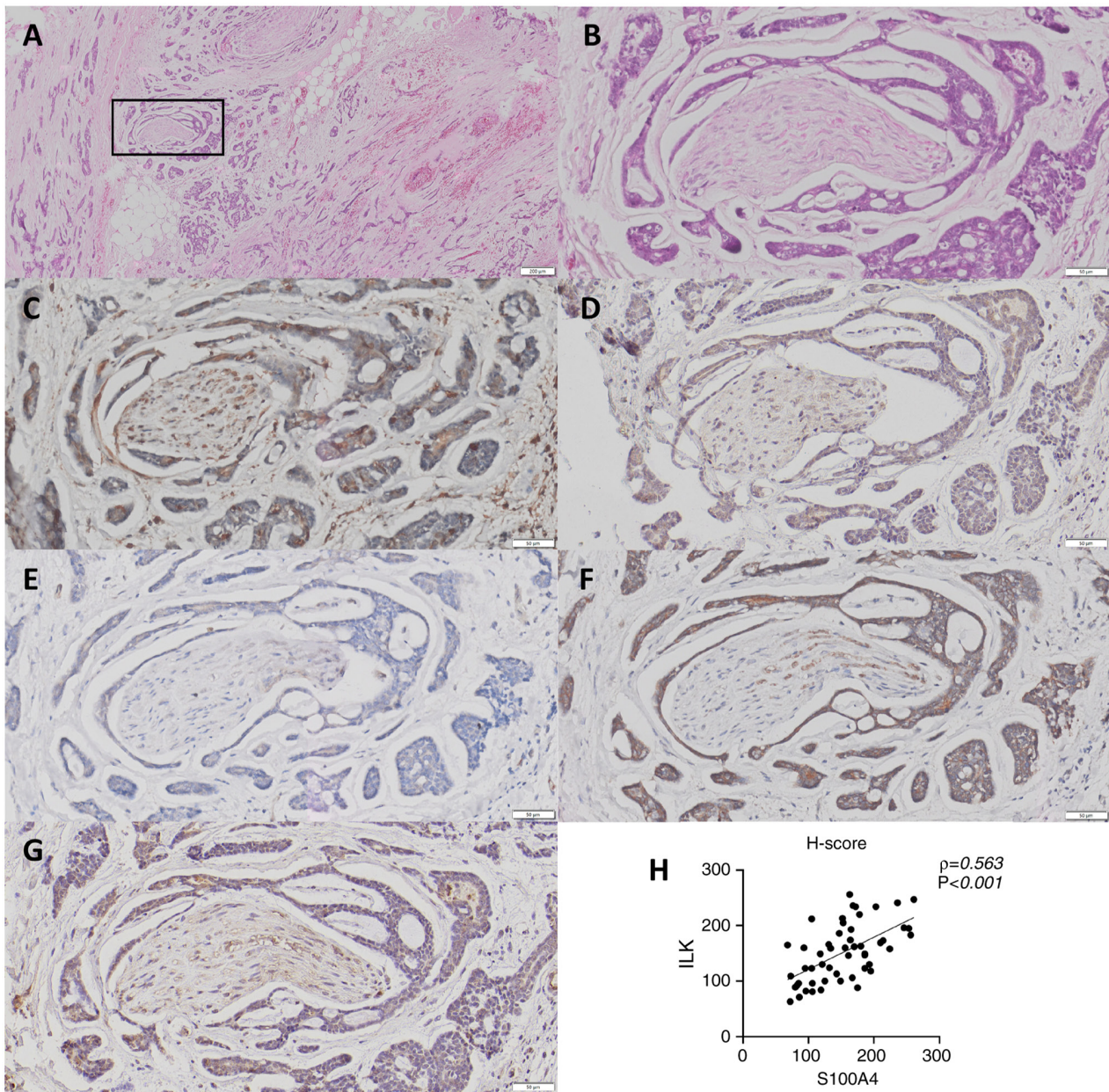


Figure 5. Interaction analysis of ILK and S100A4 in perineural invasion of SACC. HE staining demonstrated perineural invasion in SACC at (A) low (4X) and (B) high (40X) magnification. (C) Dense S100A4 expression is observed in the perineural invasion focus. (D) Strong ILK expression is detected at the perineural invasion site. (E) Loss of membranous E-cadherin in tumor cells in the region of neural invasion. (F) Positive N-cadherin expression in tumor cells in the perineural invasion region. (G) Intense Snail expression in tumor cells at perineural invasion site. Magnification, x40. (H) Significant positive correlation between ILK and S100A4 protein expression levels (quantified by H-score) in SACC specimens (Spearman's $\rho=0.563$, $P<0.001$). ILK, integrin-linked kinase; SACC, salivary adenoid cystic carcinoma.

association with patient age, sex, primary tumor location or maximal tumor diameter (Table I). By contrast, S100A4 levels were significantly associated with clinicopathological markers of aggressive disease. Tumors classified as advanced stage (III/IV) demonstrated significantly higher S100A4 expression compared with early-stage (I/II) cases. Similarly, the solid histological subtype exhibited stronger S100A4 positivity than either tubular or cribriform subtypes. Furthermore, cases with perineural invasion presented more intense staining of S100A4. These findings demonstrated an association between S100A4 expression and adverse clinicopathological features, underscoring its potential value as a prognostic biomarker for assessing tumor aggressiveness in SACC.

ILK expression was undetectable in normal salivary gland tissue, with the exception of partial immunoreactivity in ductal epithelial cells (17). By contrast, S100A4 and ILK expression was observed in the cytoplasm of tumor cells as well as in surrounding stromal cells of SACC tissue. Immunohistochemical analysis revealed that tumor cells at the invasive front surrounding nerves frequently exhibited a spindle-shaped or fusiform morphology (Fig. 5A and B), accompanied by enhanced expression of S100A4 (Fig. 5C), ILK (Fig. 5D), N-cadherin (Fig. 5F) and Snail (Fig. 5G), along with decreased expression of E-cadherin (Fig. 5E). Immunohistochemical evaluation using H-score assessment demonstrated a significant positive correlation between

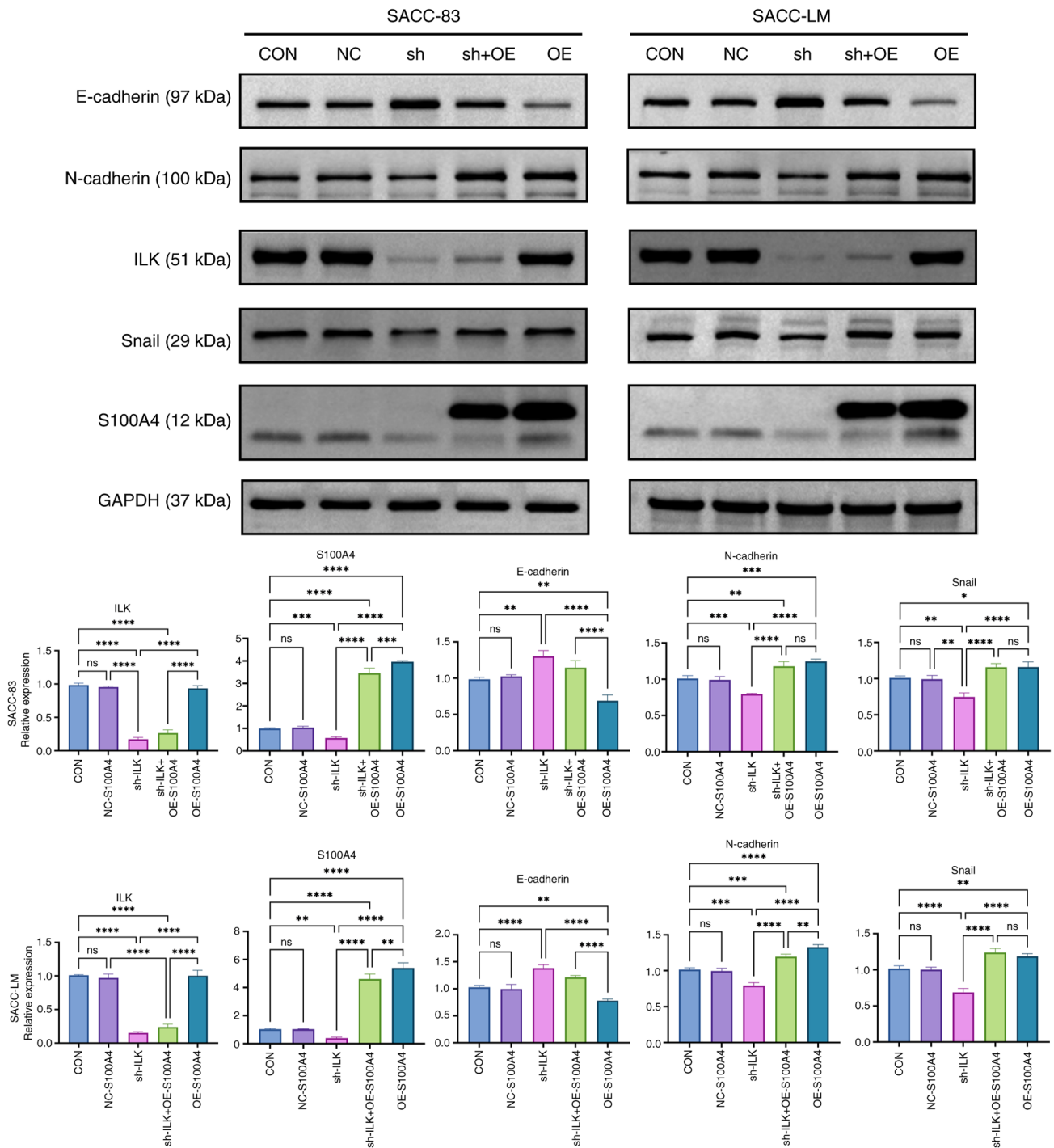


Figure 6. S100A4 OE rescues the suppression of epithelial-mesenchymal transition caused by ILK knockdown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns not significant; ILK, integrin-linked kinase; OE, overexpression; sh, short hairpin; NC, negative control; CON, blank control.

S100A4 and ILK expression in tumor tissue ($\rho = 0.563$; Fig. 5H).

S100A4 mediates ILK-regulated EMT progression via Snail restoration. To elucidate the regulatory association between ILK and S100A4, the present study established cell models with combined ILK knockdown and S100A4 overexpression. Western blot analysis of EMT markers demonstrated that S100A4 overexpression led to decreased E-cadherin and elevated N-cadherin levels, effectively reversing the suppression of EMT caused by ILK knockdown (Fig. 6). Notably, the

expression of Snail protein was restored, suggesting S100A4 may regulate Snail expression. Functional assays demonstrated that S100A4 overexpression enhanced both the migratory and invasive capabilities of SACC cells and effectively reversed the inhibitory effects caused by ILK depletion (Fig. 7A and B). In summary, ILK knockdown may inhibit the phosphorylation of GSK-3 β , leading to downregulation of S100A4 and Snail expression, which decreases N-cadherin and increases E-cadherin levels. Overexpression of S100A4 restored Snail expression and re-induced the EMT phenotype, thereby attenuating the inhibitory effect of ILK knockdown on EMT progression.

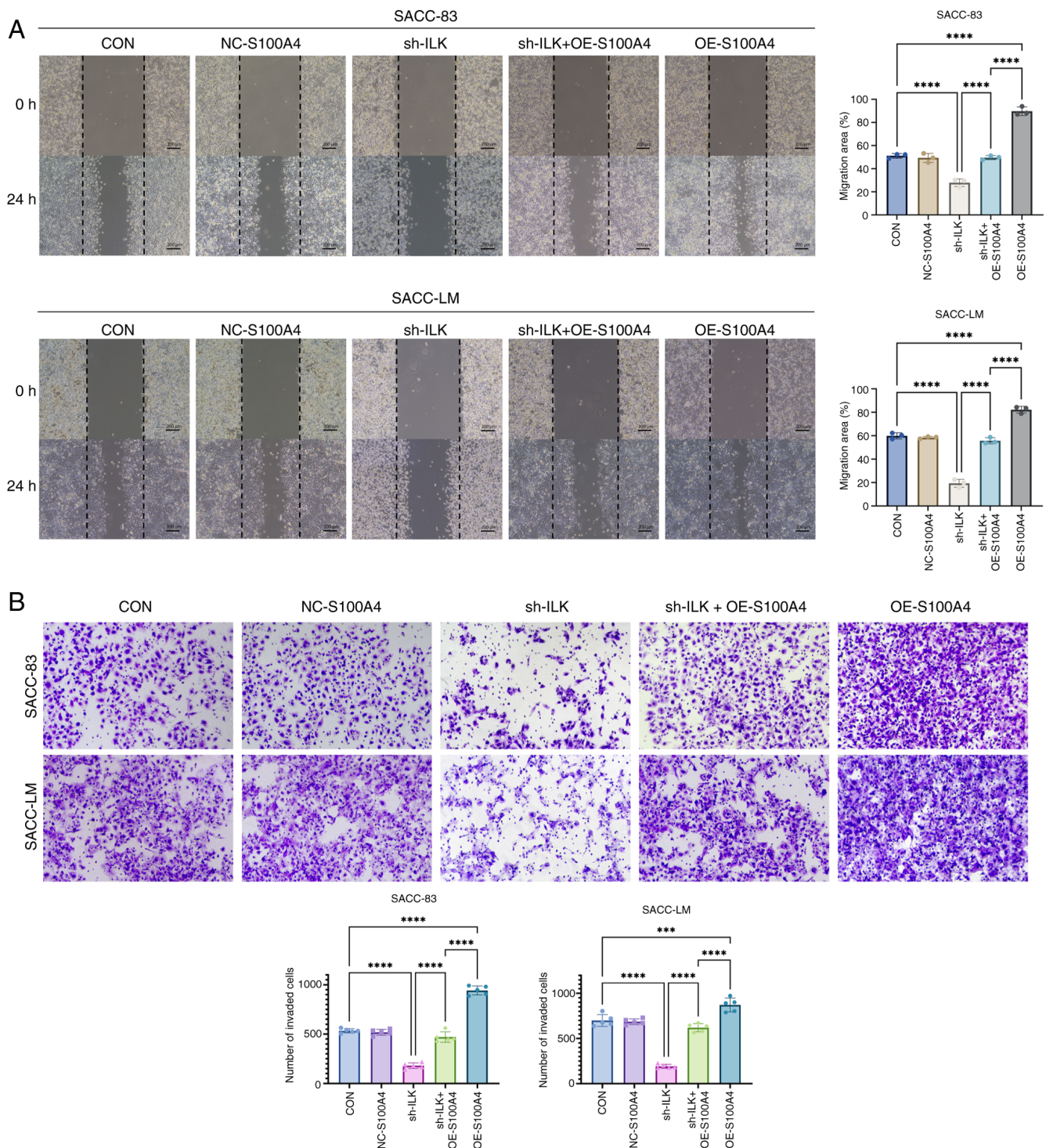


Figure 7. S100A4 OE rescues the inhibitory effects of ILK knockdown on the migration and invasion of adenoid cystic carcinoma. (A) Wound healing assays (10X magnification) demonstrate that S100A4 OE restores migratory ability of SACC cells, with sh + OE cells showing closure rates comparable with NC. (B) Transwell assays revealed restoration of invasive capacity in sh + OE cells suggests a reversal of ILK knockdown-induced inhibition. *** $P < 0.001$, **** $P < 0.0001$. ns, not significant; OE, overexpression; ILK, integrin-linked kinase; SACC, salivary adenoid cystic carcinoma; sh, short hairpin; NC, negative control; CON, blank control.

Discussion

Salivary adenoid cystic carcinoma accounts for ~1% of all head and neck malignancies (35,36). Although it typically exhibits an indolent growth pattern, the long-term prognosis for SACC remains poor (37). Once metastasis occurs, the 10-year survival rate is 10%. EMT in SACC is associated with

solid subtype (38), tumor progression, perineural invasion, recurrence and distant metastasis (39). Although it is challenging to define EMT features in SACC using HE staining alone, the present study demonstrated that tumor cells at the perineural invasion front frequently exhibited a spindle-shaped morphology, accompanied by decreased E-cadherin expression and increased expression of N-cadherin and Snail. The

present study further demonstrated that ILK knockdown significantly attenuated the migratory and invasive capabilities of SACC cells, concurrent with upregulated E-cadherin expression and downregulated N-cadherin and Snail levels. These changes were consistent with the results of our previous clinicopathological study (17). These findings suggested that ILK may serve a key role in promoting EMT in SACC.

To identify the mechanism by which ILK participates in EMT, the present study conducted transcriptomic screening and identified S100A4 as a key target. *In vitro* results were supported by GSEA. Although previous studies have demonstrated the involvement of S100A4 in the invasion and metastasis of numerous types of cancer (40,41), to the best of our knowledge, its specific role in SACC has not been reported. The present clinicopathological analysis revealed that high S100A4 expression was significantly associated with advanced clinical stage, solid histological subtype and perineural invasion, suggesting its potential as a molecular marker of aggressive tumor behavior. While both ILK and S100A4 were nearly undetectable in normal salivary gland tissue, consistent with previous reports (17,42), their expression in SACC tumors was implicated in the promotion of local and perineural invasion.

To clarify the role of S100A4 in ILK-driven EMT, the present study examined the expression of S100A4 in ILK-knockdown cell lines. S100A4 expression was significantly downregulated in ILK-knockdown cells. When S100A4 expression was induced in ILK-knockdown cells, the expression of Snail and N-cadherin was markedly enhanced, while E-cadherin expression was downregulated. The impaired invasion and migration phenotypes of SACC cells were restored. These findings suggested that S100A4 serves a key role in ILK-driven EMT. Studies have indicated that ILK promotes the stabilization and nuclear translocation of β -catenin via the GSK-3 β pathway (43,44). β -catenin that enters the nucleus forms a complex with T cell factor 4; this complex specifically binds the promoter region of the S100A4 gene, thereby enhancing its expression (45). Other studies have revealed that the expression of S100A4 in cancer is significantly associated with Snail family proteins (46) and regulates their expression (47). ILK may regulate the expression of S100A4 and Snail by modulating the phosphorylation of GSK-3 β , thereby influencing EMT.

The most prominent molecular characteristic of SACC is the aberrant activation of the MYB proto-oncogene. A total of ~86% of cases harbor the characteristic MYB-nuclear factor IB gene fusion (48). Research has shown that MYB upregulation contributes to EMT, thereby promoting invasion and metastasis in SACC (49). Although the association between MYB and ILK remains unclear, MYB Proto-Oncogene Like 1 carried by small extracellular vesicles upregulates the expression of integrin proteins ITGB3 (Integrin Subunit Beta 3) and ITGAV (Integrin Subunit Alpha V) (50). As a key molecule in the integrin signaling pathway, ILK may also be regulated by MYB. MYB and ILK modulate the expression of the adhesion molecule ICAM-1 (Intercellular Adhesion Molecule 1) (50,51). ICAM-1 serves a crucial role in mediating EMT, highlighting its role in tumor metastasis (52). In summary, the integrin signaling pathway may serve as a key bridge connecting the functions of MYB and S100A4, and the

synergistic mechanism between MYB and the ILK-S100A4 axis requires further exploration.

Through clinicopathological correlation analysis and *in vitro* experiments, the present study demonstrated the functional role of the ILK-S100A4-Snail signaling axis in promoting EMT in SACC. However, the specific molecular mechanisms by which ILK regulates S100A4/Snail require further validation through *in vivo* models and pharmacological approaches. Furthermore, the prognostic value of ILK and S100A4 for survival in patients with SACC remains to be elucidated.

In conclusion, S100A4 represents a potential biomarker for predicting aggressive behavior in SACC. ILK may facilitate tumor invasion and metastasis by modulating S100A4/Snail expression via GSK-3 β , leading to EMT. These findings suggest dual targeting of ILK and S100A4 may offer synergistic therapeutic benefits and represent a promising strategy to inhibit tumor invasion in SACC.

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Availability of data and materials

The data generated in the present study may be found in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1320937 or at the following URL: www.ncbi.nlm.nih.gov/sra/PRJNA1320937.

Authors' contributions

YY conceived the study, designed and performed experiments and wrote the manuscript. JL designed the experiments. LY constructed figures and performed experiments. YL analyzed data. KG performed the experiments. DZ conceived the study and edited the manuscript. YY and DZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board and Ethics Committee of The Affiliated Stomatological Hospital of Southwest Medical University (approval no. 20211129004; Luzhou, China). Informed consent was obtained in writing from all patients before the tissue collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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