

Epigenetic silencing of *JAM3* promotes laryngeal squamous cell carcinoma development by inhibiting the Hippo pathway

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Abstract. Laryngeal squamous cell carcinoma (LSCC), which represents a significant proportion of head and neck squamous cell carcinoma cases, is often diagnosed at advanced stages, underscoring the urgent need for effective biomarkers and therapeutic targets. Junctional adhesion molecule 3 (*JAM3*) is implicated in various types of cancer; however, its role in LSCC remains unclear. Therefore, the present study aimed to investigate the epigenetic regulation and tumor-suppressive functions and mechanisms of *JAM3* in LSCC. Bioinformatics analysis and 5-Aza-2'-deoxycytidine treatment, which restored *JAM3* expression as confirmed by reverse transcription-quantitative PCR and western blotting, revealed that aberrant hypermethylation of the *JAM3* promoter was associated with reduced *JAM3* expression and poorer clinical outcomes in patients with LSCC. *In vitro* experiments, including Cell Counting Kit 8, colony formation and Transwell assays, demonstrated that *JAM3* overexpression inhibited LSCC cell proliferation, migration and invasion. Western blotting and immunofluorescence analysis revealed that the tumor-suppressive function of *JAM3* was mediated through activation of the Hippo pathway. By contrast, both *in vitro* and *in vivo* experiments showed that *JAM3* knockdown enhanced these oncogenic behaviors by inhibiting the Hippo pathway, suggesting its critical tumor-suppressive role. In conclusion, the results of the present study indicated that *JAM3* may be epigenetically down-regulated and could function as a novel tumor suppressor gene through the Hippo pathway in LSCC, offering insights into developing targeted treatments and diagnostics.

Introduction

Head and neck cancer includes squamous cell carcinoma that originates in the oral cavity, sinonasal cavity, pharynx or larynx (1,2). These types of cancer are frequently diagnosed at advanced stages with distant metastasis, leading to a poor prognosis (3). In 2022, there were 188,960 new cases of laryngeal squamous cell carcinoma (LSCC) worldwide, and it was the second most prevalent type of head and neck cancer, with 891,453 new cases globally (4).

Traditionally, research on LSCC has primarily focused on genetic factors (5,6); however, the role of epigenetic modifications in cancer development is being increasingly recognized (7). Epigenetic modifications, including DNA methylation, histone modification and regulation by non-coding RNAs, frequently occur and significantly affect gene expression (8,9). In mammals, DNA methylation, particularly at CpG dinucleotides in gene promoters (10,11), can lead to the silencing of genes, especially of tumor suppressor genes (TSGs), thereby increasing the risk of carcinogenesis (12,13). Given the variability of DNA methylation patterns with age and cancer type, these epigenetic markers offer promising avenues for diagnostic and therapeutic interventions (14).

Junctional adhesion molecules (JAMs), located at the intercellular junctions of endothelial and epithelial cells, serve diverse roles in cancer (15). *JAM3*, a member of this family, is implicated in several types of cancer, including renal carcinoma, colorectal cancer and cholangiocarcinoma (16-18); however, its roles in LSCC has not been thoroughly investigated, necessitating further assessment. The present study aimed to investigate the epigenetic regulation of *JAM3*, and its impact on cell proliferation, migration and invasion in LSCC, with the goal of exploring its potential as a targeted therapeutic approach and diagnostic marker.

Materials and methods

Tissue samples. LSCC specimens were obtained from the Department of Pathology, The First Hospital, Shanxi Medical University (Taiyuan, China) and were pathologically diagnosed as squamous cell carcinoma between May 2018 and December 2020. A total of 38 archived formalin-fixed paraffin-embedded

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LSCC tissues and paired adjacent normal mucosa (ANM) tissues were used in the present study for immunohistochemistry (IHC). The age of patients ranged between 43 and 86 years old (mean $\pm$ SD age, 63.39 $\pm$ 9.51 years), and the sex ratio of was 1:12 female/male. No patients had undergone chemotherapy or radiotherapy prior to surgical resection. The present study received ethical approval (approval no. KYLL-2023-180) from the Ethics Committee of The First Hospital, Shanxi Medical University and was conducted in accordance with the committee's guidelines.

Cell lines and culture. The LSCC cell line FD-LSC-1 [provided by Professor Liang Zhou, Department of Otolaryngology-Head and Neck Surgery, Eye, Ear, Nose and Throat Hospital, Fudan University, Shanghai, China (19)], the head and neck squamous cell carcinoma (HNSCC) cell lines FaDu (cat. no. TCHu132; The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) and HN30 (provided by Professor Qiancheng Shen, Medicinal Chemistry and Bioinformatics Center, Shanghai Jiao Tong University, School of Medicine, Shanghai, China) and the normal human epidermal cell line HaCaT (cat. no. GDC0106; China Center for Type Culture Collection) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.). The LSCC cell line AMC-HN-8 (cat. no. HZ-5240HC; Shanghai Huzhen Industrial Co., Ltd.) was maintained in RPMI-1640 (Pricella). Notably, the HaCaT cell line underwent authentication via short tandem repeat profiling to confirm its identity (Table SI). All media were supplemented with 10% fetal bovine serum (FBS; Shanghai ExCell Biology, Inc.), 100 IU/ml penicillin and 100 μ g/ml streptomycin. Cell cultures were maintained at a temperature of 37°C in an atmosphere containing 5% CO₂.

Plasmids and small interfering (si)RNAs. The *JAM3* coding region sequence was inserted into p3xFlag-CMV-10 (Promega Corporation) to construct an overexpression vector (p3xFlag-CMV-10-*JAM3*), and its functionality was confirmed by Sanger sequencing. The empty p3xFlag-CMV-10 vector served as the negative control (NC). In addition, siRNA targeting *JAM3* (si-*JAM3*-1, sense: 5'-GAGAGACUCAGCCCUUUA UTT-3', antisense: 5'-AUAAAGGGCUGAGUCUCUCTT-3'; si-*JAM3*-2, sense: 5'-CUGUACCAGUAGGCAAGAU-3', antisense: 5'-AUCUUGCCUACUGGUACAGTT-3') and NC siRNA (si-NC, sense: 5'-UUCUCCGAACGUGUCACG UTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3') were designed and synthesized by Shanghai GenePharma Co., Ltd. The transfection was conducted according to the manufacturer's instructions of Lipofectamine[®] 3000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The AMC-HN-8 and FD-LSC-1 cell lines were cultured to 70-80% confluence in 6-well plates. Plasmids were transfected at a final concentration of 1.5 μ g/ml, and siRNA was transfected at a concentration of 37.5 nM. Transfection was performed at room temperature for 30 min, followed by media replacement 6 h post-transfection. Further analyses were carried out 24 h post-transfection.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from HaCaT, AMC-HN-8, FD-LSC-1, FaDu and HN30 cells using TRIzol[®]

(Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was generated using a kit (cat. no. AU341; TransGen Biotech Co., Ltd.) according to the manufacturer's protocol on a ProFlex[™] 3x32-well PCR System (Thermo Fisher Scientific, Inc.). qPCR was performed using PerfectStart[®] Green qPCR SuperMix (TransGen Biotech Co., Ltd.) on a LightCycler[®] 96 instrument (Roche Diagnostics). The relative mRNA expression levels were calculated using the 2^{- $\Delta\Delta$ C_q} method (20). The *18S* ribosomal RNA was used as an internal control. All procedures (initial denaturation at 94°C for 30 sec, followed by 40 cycles of denaturation at 94°C for 5 sec, annealing at 60°C for 5 sec and extension at 72°C for 10 sec) were performed according to the manufacturer's instructions. The primer sequences were as follows: *JAM3* forward, 5'-TCCAGCAATCGAACCCAG-3' and reverse, 5'-CTTGTCTGCGAATCCGTAATGAT-3'; and *18S* forward, 5'-CCTGGATACCGCAGCTAGGA-3' and reverse, 5'-GCGGCGCAATACGAATGCC-3'.

5-Aza-2'-deoxycytidine (5-Aza) treatment. The AMC-HN-8 and FD-LSC-1 cells were treated with 5 μ M 5-Aza (MilliporeSigma) for 72 h at 37°C, and were then collected for further analysis, including RT-qPCR and western blotting.

DNA isolation and bisulfite conversion. Genomic DNA from AMC-HN-8 and FD-LSC-1 cell lines was isolated using a TIANamp Genomic DNA Kit (cat. no. DP304; Tiangen Biotech Co., Ltd.) and was then bisulfite-treated with an EZ DNA Methylation-Gold kit (cat. no. D5006; Zymo Research Corp.) according to the manufacturer's instructions.

Bisulfite sequencing PCR (BSP). Bisulfite-treated DNA was amplified using primers specific for bisulfite sequencing PCR. The following primer sequences targeting a CpG island within the *JAM3* promoter were used: Forward primer, 5'-GTTTAT TGAAAGAGAATTTATGTGT-3'; reverse primer, 5'-AAA CAACCCCTAAAAACAACAAC-3'. CpG island region prediction and BSP primer design were performed using MethPrimer 1.0 software (21), with the *JAM3* sequence ranging from -2,000 to +500 from the transcription start site input into the software. PCR was performed using TransTaq HiFi PCR SuperMix I (TransGen Biotech, Co., Ltd.) with an initial denaturation at 94°C for 5 min; followed by 40 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; and a final extension step at 72°C for 1 min. The products were analyzed using 1.5% agarose gel electrophoresis, and the gel was visualized with DuRed staining (cat. no. R21868; 1:10,000; Shanghai Saint-bio Biotechnology Co., Ltd.). Images were captured using a gel imaging system (Azure Biosystems, Inc.). With the aid of a blue LED transilluminator (Sangon Biotech Co., Ltd), the target DNA bands were excised from the gel and were subsequently recovered using SanPrep Column DNA Gel Extraction Kit (cat. no. B518131; Sangon Biotech Co., Ltd). The recovered PCR products were cloned into the T1 cloning vector (TransGen Biotech Co., Ltd.). The cloned products were transformed and plated onto Luria-Bertani culture plates containing 100 μ g/ml penicillin; the plate surface had already been coated with a mixture of 8 μ l 500 mM isopropyl β -D-1-thiogalactopyranoside and 40 μ l 20 mg/ml X-gal. After overnight incubation, 10 white colonies were selected and sent to Sangon Biotech Co., Ltd for Sanger sequencing.

Sequencing outcomes were analyzed using BiQ Analyzer v2.02 software (22).

Cell Counting Kit 8 (CCK8) assay. After a 24-h transfection, 4,000 transfected AMC-HN-8 or FD-LSC-1 cells were seeded per well in a 96-well plate. Subsequently, the cells were incubated with medium containing 10% CCK8 assay reagent (Shanghai Yeasen Biotechnology Co., Ltd.) for 1 h at 37°C and detected at 450 nm using a multi-mode microplate reader (SpectraMax i3x; Molecular Devices, LLC) at 0, 24, 48, 72 and 96 h.

Colony formation assay. Transfected AMC-HN-8 or FD-LSC-1 cells were seeded at 1,000 cells/well in a 6-well plate and incubated for 10-14 days. The cells were then fixed with 4% paraformaldehyde (Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min and stained with 0.1% crystal violet (Amresco, LLC) for 10 min at room temperature, and the number of colonies (defined as groups containing >50 cells) was counted using ImageJ 1.53k analysis software (National Institutes of Health).

Transwell migration and invasion assays. AMC-HN-8 or FD-LSC-1 cells transfected with p3xFlag-CMV-10 or p3xFlag-CMV-10-JAM3, and si-NC or si-JAM3 were suspended in serum-free medium and adjusted to 8×10^5 cells/ml, after which a 200- μ l suspension was added to the upper chamber (PET membrane; pore size, 8 μ m; Corning, Inc.) in a 24-well plate. DMEM or RPMI-1640 supplemented with 20% FBS was added to the lower chamber. For the invasion assay, each membrane was coated with Matrigel (Corning, Inc.) for 6 h at 37°C, and the cell density was adjusted to 10×10^5 cells/ml. After incubation at 37°C for 48 h, the chambers were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 10 min at room temperature. The number of cells that migrated to or invaded the lower chamber was counted in eight light microscopic fields (magnification, x100).

Western blot analysis. Proteins were extracted from the AMC-HN-8 and FD-LSC-1 cells using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Inc.). The protein concentration was determined using the BCA protocol (Shanghai Yeasen Biotechnology Co., Ltd.). SDS-PAGE loading buffer (Shanghai Yeasen Biotechnology Co., Ltd.) was added to the protein solution and heated at 100°C for 5 min. Subsequently, 30-60 μ g proteins were separated by SDS-PAGE on 10% gels, and transferred onto PVDF membranes. The membranes were then blocked in 10% non-fat milk (BD Biosciences) for 1.5 h at room temperature to prevent non-specific binding, and were incubated with primary antibodies targeting Flag (cat. no. F1804; 1:1,000; mouse; MilliporeSigma), JAM3 (cat. no. bs-11086R; 1:1,000; rabbit; BIOSS), large tumor suppressor kinase 1 (LATS1; cat. no. 17049-1-AP; 1:1,000; rabbit; Proteintech Group, Inc.), phosphorylated (p)-LATS1 (Thr1079) (cat. no. 28998-1-AP; 1:5,000; rabbit; Proteintech Group, Inc.), yes-associated protein 1 (YAP1; cat. no. 13584-1-AP; 1:5,000; rabbit; Proteintech Group, Inc.), p-YAP1 (Ser127) (cat. no. 13008S; 1:1,000; rabbit; Cell Signaling Technology, Inc.) and β -actin

(cat. no. HC201-02; 1:2,000; mouse; TransGen Biotech Co., Ltd.) overnight at 4°C. After primary antibody incubation, the membranes were washed and subsequently incubated with appropriate HRP-conjugated secondary antibodies [anti-rabbit (cat. no. HS101-01; 1:5,000) and anti-mouse (cat. no. HS201-01; 1:5,000); both from TransGen Biotech Co., Ltd.] for 2 h at room temperature, based on the primary antibodies used. Following secondary antibody incubation, protein bands were visualized using enhanced chemiluminescence detection reagents (cat. no. K-12045-D50; Advansta Inc.) and images were captured using a MiniChem 610 instrument (SinSage Technology, Co., Ltd.). Band intensity was semi-quantified by densitometry using ImageJ 1.53k software to assess relative protein levels.

In vivo assay. Animal experiments were conducted in accordance with the guidelines approved (approval no. DWLL-2024-027) by the Research Ethics Committee for Animal Experimentation at The First Hospital, Shanxi Medical University. Six specific pathogen-free female BALB/c nude mice (age, 4-6 weeks; average weight, 13.15 ± 0.17 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were housed under controlled conditions. The housing environment was maintained at a temperature of 22-24°C and a relative humidity of 50-60%. Mice had *ad libitum* access to food and water and were maintained under a 12-h light/dark cycle. Following a 7-day acclimation period, 5×10^6 AMC-HN-8 cells were subcutaneously injected into both flanks of each mouse. Tumor dimensions were measured twice daily. Once tumors reached 5x5 mm, si-NC was administered into the left tumor, and si-JAM3 into the right tumor at the same interval; both types of siRNA were prepared at a concentration of 0.1 μ g/ μ l. The siRNA solution was injected directly into the tumor mass at a volume of 100 μ l per injection. This treatment was repeated every other day for a total of seven injections. After seven injections, the nude mice were anesthetized with isoflurane at an induction concentration of 4-5% in oxygen for 1-3 min. Following the induction phase, the isoflurane concentration was reduced to 1-2% for an additional 1-2 min to ensure deep anesthesia. Once the loss of the righting reflex and a lack of response to a toe pinch confirmed deep anesthesia, cervical dislocation was promptly and competently performed by trained personnel. The tumors were then removed and weighed. The excised tumors were processed for further analysis, including RT-qPCR and IHC. Tumor volume was calculated using the following formula: Tumor volume=(length x width²)/2 (23).

Hematoxylin and eosin (H&E) staining. Formalin-fixed (10% neutral buffered formalin at room temperature for 24 h), paraffin-embedded 4- μ m tissue sections were deparaffinized in xylene and rehydrated through a graded series of alcohol. The sections were then stained with hematoxylin for 1 min, washed in water, differentiated in 1% acid alcohol for 30 sec. After a rinse in water, the sections were stained with eosin for 1 min, dehydrated in increasing concentrations of alcohol, cleared in xylene and mounted with a resinous medium. The stained slides were examined under a light microscope to evaluate tissue morphology, ensuring clarity of nuclear and cytoplasmic details.

IHC staining. Formalin-fixed (10% neutral buffered formalin at room temperature for 24 h), paraffin-embedded 4- μ m tissue sections were mounted on charged slides. Sections were deparaffinized in xylene, rehydrated through a graded series of alcohol, and submerged in 10 mM citrate buffer (pH 6.0) for antigen retrieval using a pressure cooker for 2 min. After cooling and rinsing in phosphate-buffered saline (PBS), endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min at room temperature. Sections were then blocked with 5% bovine serum albumin (BSA; Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature to reduce non-specific binding, then incubated overnight at 4°C with primary antibodies against Ki-67 (cat. no. RMA-0731; Fuzhou Maixin Biotechnology Development Co., Ltd.), *JAM3* (cat. no. bs-11086R; 1:200; rabbit; BIOSS), YAP1 (cat. no. 13584-1-AP; 1:500; rabbit; Proteintech Group, Inc.), E-Cadherin (cat. no. 20874-1-AP; 1:2,000; rabbit; Proteintech Group, Inc.), N-Cadherin (cat. no. 22018-1-AP; 1:2,000; rabbit; Proteintech Group, Inc.) and Vimentin (cat. no. 5741S; 1:600; rabbit; Cell Signaling Technology, Inc.). Following primary antibody incubation, slides were washed with PBS and incubated with a biotinylated secondary antibody (cat. no. PV-6000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) for 20 min at 37°C. Detection used an avidin-biotin complex method with diaminobenzidine (cat. no. ZLI-9019; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) as the chromogen, and sections were then counterstained with hematoxylin for 1 min, differentiated in 1% acid alcohol for 30 sec and rinsed in 0.1% ammonia water for another 30 sec at room temperature. Slides were then dehydrated, cleared and mounted. Staining intensity and cell positivity were independently evaluated by two pathologists under a light microscope, with results semi-quantified to assess protein expression levels. Images were acquired using the PANORAMIC SCAN II (3DHISTECH, Ltd.), the results were analyzed using CaseViewer version 2.3 and the expression levels were recorded as H-Score (24).

Immunofluorescence staining. Immunofluorescence staining was performed on LSCC cells cultured on glass coverslips. Cells were fixed and permeabilized with cool methanol at -20°C for 10 min and blocked with 5% BSA for 1 h at room temperature to prevent non-specific binding. The cells were then incubated overnight at 4°C with primary antibodies targeting YAP1 (cat. no. 13584-1-AP; 1:100; rabbit; Proteintech Group, Inc.) diluted in the blocking solution. After washing with PBS, cells were incubated with a CY3-conjugated secondary antibody (cat. no. BA1032; 1:250; goat; Wuhan Boster Biological Technology, Ltd.) for 1 h at room temperature in the dark. Nuclei were stained with DAPI (Wuhan Boster Biological Technology, Ltd.) for 2 min at room temperature, and coverslips were mounted with an anti-fade mounting medium to preserve fluorescence. Fluorescence microscopy (Leica Microsystems GmbH) was used to examine and capture images of the cells, focusing on protein expression and localization. Analysis of fluorescence intensity and subcellular distribution was performed using ImageJ 1.53k to semi-quantify expression levels and patterns.

Bioinformatics analysis. The present study employed comprehensive bioinformatics analyses to investigate gene expression

and methylation patterns associated with LSCC and HNSCC. For gene expression, sequencing data from GSE216664, and microarray data from GSE59102 (25) and GSE51985 (26) specific to laryngeal tissues were analyzed using GEO2R (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>). To investigate the association between *JAM3* expression and its methylation status, microarray and corresponding methylation data were examined from GSE33205 (27) and GSE33202 (27) for HNSCC through GEO2R. This relationship was further validated by analyzing data from The Cancer Genome Atlas (TCGA; <https://www.cancer.gov/tcga>) (28) using the DNA Methylation Interactive Visualization Database (DNMIVD; <http://119.3.41.228/dnmivd/>) (29). The methylation status of each CpG site was quantified by calculating the β value, defined as the ratio of the fluorescent signal from the methylated allele to the sum of the signals from both the methylated and unmethylated alleles. The β value ranges from 0 (completely unmethylated) to 1 (completely methylated), providing a continuous measure of DNA methylation levels (30). Protein expression data were analyzed using The University of Alabama at Birmingham Cancer data analysis Portal (UALCAN; <https://ualcan.path.uab.edu/index.html>), with data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) and the International Cancer Proteogenome Consortium (ICPC) (31). The protein expression values were normalized to Z-values, which represent standard deviations from the median across samples for the given cancer type (31). Gene expression associations were assessed via The cBio Cancer Genomics Portal (<https://www.cbioportal.org/>) (32) and survival analysis focusing on CpG sites of the *JAM3* promoter was conducted through MethSurv (<https://biit.cs.ut.ee/methsurv/>) (33). These tools facilitated a detailed examination of the genetic and epigenetic factors contributing to tumor biology in LSCC and HNSCC.

Statistical analysis. All *in vitro* experiments were repeated three times. Differences between two groups were determined using an unpaired two-tailed Student's t-test or paired two-tailed Student's t-test. For comparisons involving more than two groups, one-way ANOVA followed by Tukey's Honest Significant Difference test were utilized. Pearson and Spearman correlation analyses were employed to evaluate gene co-expression and the relationship between gene expression and DNA methylation across the samples. Survival analyses were conducted using Cox proportional-hazards models, as performed by the MethSurv web tool, with model fit assessed using log likelihood-ratio and Wald tests (33). Differences in survival rates were visualized using Kaplan-Meier plots, with hazard ratio and log-likelihood ratio test P-value included in the plots (33). All data were analyzed using GraphPad Prism 7.0 (Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of *JAM3* in LSCC. Analysis of Gene Expression Omnibus (GEO) datasets, including GSE216664, GSE59102 and GSE51985, demonstrated a significant reduction in *JAM3* mRNA expression in LSCC tumor tissues compared with that in non-tumor counterparts (Fig. 1A). For a broader understanding of *JAM3* expression, further investigation into

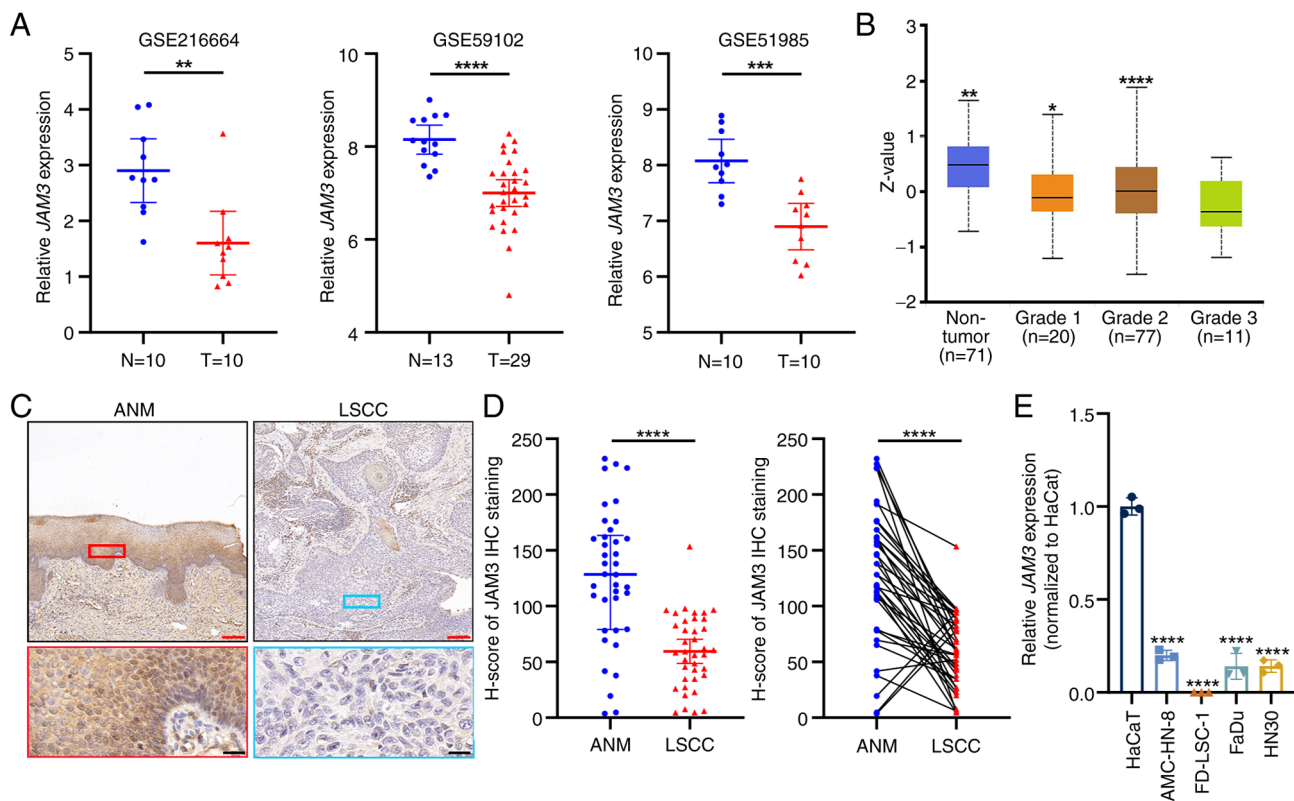


Figure 1. *JAM3* is downregulated in LSCC tissues and samples at both mRNA and protein levels. (A) GSE216664, GSE59102 and GSE51985 were used to analyze the different expression levels of *JAM3* between N and LSCC T tissues. For GSE216664 and GSE51985, N and T tissues were from the same LSCC patients; for GSE59102, N tissues were ANM tissues from different patients with LSCC. Data are presented as the mean with 95% CI indicated by error bars. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. (B) Protein levels of *JAM3* in the Clinical Proteomic Tumor Analysis Consortium and the International Cancer Proteogenome Consortium datasets among different grades of HNSCC tissues. Data are presented as the mean and range. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ vs. Grade 3. (C) Representative images of *JAM3* IHC staining of LSCC tissues and paired ANM tissues. Red scale bar, 150 μm ; Black scale bar, 20 μm . (D) IHC results of clinical tissues were analyzed by paired two-tailed Student's t-test. Data are presented as the mean with 95% CI. **** $P < 0.0001$. (E) mRNA expression levels of *JAM3* in HaCaT, AMC-HN-8, FD-LSC-1, FaDu and HN30 cell lines. Data are presented as the mean $\pm$ SD of three independent experiments. **** $P < 0.0001$ vs. HaCaT. ANM, adjacent normal mucosa; IHC, immunohistochemistry; *JAM3*, junctional adhesion molecule 3; LSCC, laryngeal squamous cell carcinoma; N, non-tumor; T, tumor.

its protein levels using data from the CPTAC and the ICPC accessed through UALCAN revealed *JAM3* was expressed at lower levels in HNSCC tumor tissues compared with those in normal tissues, although the difference was not statistically significant (Fig. S1). Notably, the difference in *JAM3* protein expression was more pronounced and statistically significant in high-grade tumors when compared with both normal and lower-grade tumors (Fig. 1B).

To validate these findings at the protein level, IHC was conducted on LSCC tissues and ANM tissues. The staining highlighted the diminished expression of *JAM3* in the tumor tissues (Fig. 1C), with semi-quantification supported by statistical analysis using paired two-tailed Student's t-tests (Fig. 1D). Additionally, further analysis of IHC H-scores did not reveal any significant differences in *JAM3* expression when comparing tumor samples based on patient age (>60 years), presence of lymph node metastasis, tumor grade or differentiation level (Fig. S2). These results suggested that while *JAM3* expression was generally lower in tumor tissues, its levels were not significantly associated with these clinical parameters in patients with LSCC; this may be due to the limited number of clinical specimens. Another comparative mRNA expression analysis in the HaCaT normal epidermal cell line, LSCC cell lines (AMC-HN-8 and FD-LSC-1) and HNSCC cell lines

(FaDu and HN30) consistently showed a decrease in *JAM3* expression in tumor cells compared with that in HaCaT cells (Fig. 1E). In summary, these findings indicated a consistent downregulation of *JAM3* at both mRNA and protein levels in LSCC, highlighting its potential role in the pathology of this cancer type across diverse datasets and sample types.

Aberrant hypermethylation of the JAM3 promoter is associated with reduced expression and a poor prognosis in LSCC. To investigate the underlying causes of *JAM3* downregulation in LSCC, the present study extended the analysis to include broader TCGA and GEO datasets of HNSCC tissues. This approach allowed for the use of larger datasets to strengthen the understanding of epigenetic influences across related types of cancer. Analysis of GEO datasets GSE33202 and GSE33205 revealed that lower *JAM3* expression in tumor tissues was significantly negatively correlated with higher methylation levels (Spearman's $\rho = -0.31$; Pearson's $r = -0.34$; Fig. 2A). This trend was corroborated by data from TCGA, where similar negative correlations between *JAM3* expression and methylation levels were noted (Spearman's $\rho = -0.34$; Pearson's $r = -0.33$), as analyzed using the DNMIIVD online tool (Fig. 2B). Furthermore, prognosis analysis using MethSurv identified four hyper-methylated CpG sites

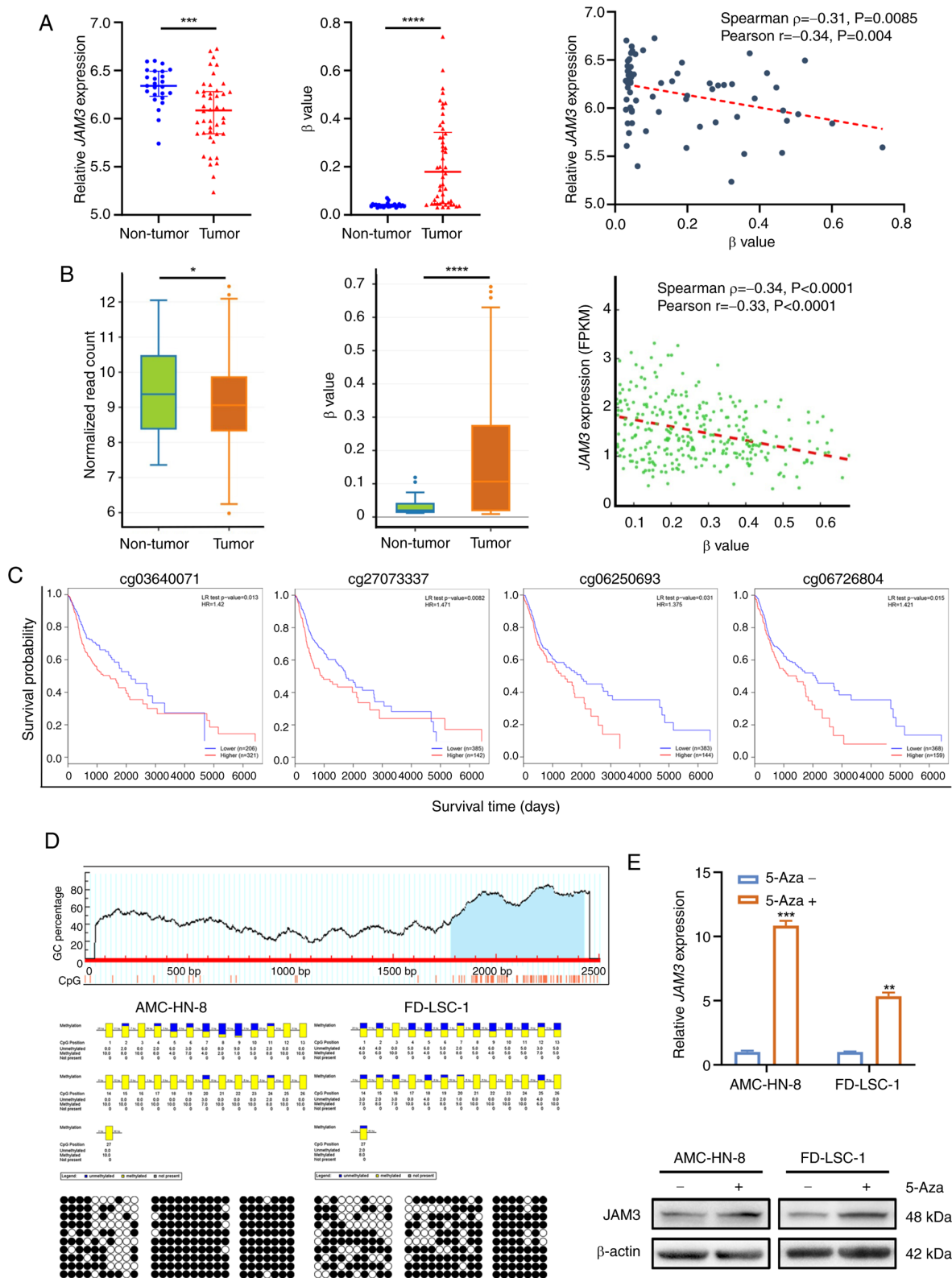


Figure 2. Aberrant hypermethylation in the *JAM3* promoter is related to low expression of *JAM3*. (A) Analysis of *JAM3* expression and methylation levels, as well as their correlation using the Gene Expression Omnibus datasets GSE33202 and GSE33205. β value indicates the methylation level of the CpG site. Data are presented as the mean with 95% CI. *** $P < 0.001$, **** $P < 0.0001$. (B) Analysis of *JAM3* expression and methylation levels, as well as their correlation in The Cancer Genome Atlas HNSCC samples. β value indicates the methylation level of the CpG site. Data are presented as the mean and range. * $P < 0.05$, **** $P < 0.0001$. (C) Kaplan-Meier plots, generated using the MethSurv webtool highlighted the relationship between methylation levels at certain *JAM3* sites and overall survival rates in patients with HNSCC. (D) CpG island (blue area) predicted by MethPrimer tool were detected by bisulfite sequencing PCR in AMC-HN-8 and FD-LSC-1 cell lines. Each row represents an individual cloned allele. Black circles show methylated CpG sites and white circles show unmethylated CpG sites. The percentage methylation rate of each CpG site is shown by the blue-yellow columns; blue indicates unmethylated and yellow indicates methylated sites. (E) Reverse transcription-quantitative PCR and western blotting showing restored expression of *JAM3* in both AMC-HN-8 and FD-LSC-1 cells treated with 5-Aza (5 μ M) for 72 h. Data are presented as the mean $\pm$ SD of three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. the 5-Aza-group. 5-Aza, 5-Aza-2'-deoxycytidine; HNSCC, head and neck squamous cell carcinoma; *JAM3*, junctional adhesion molecule 3; FPKM, fragments per kilobase million; LR test, log-likelihood ratio test; HR, hazard ratio.

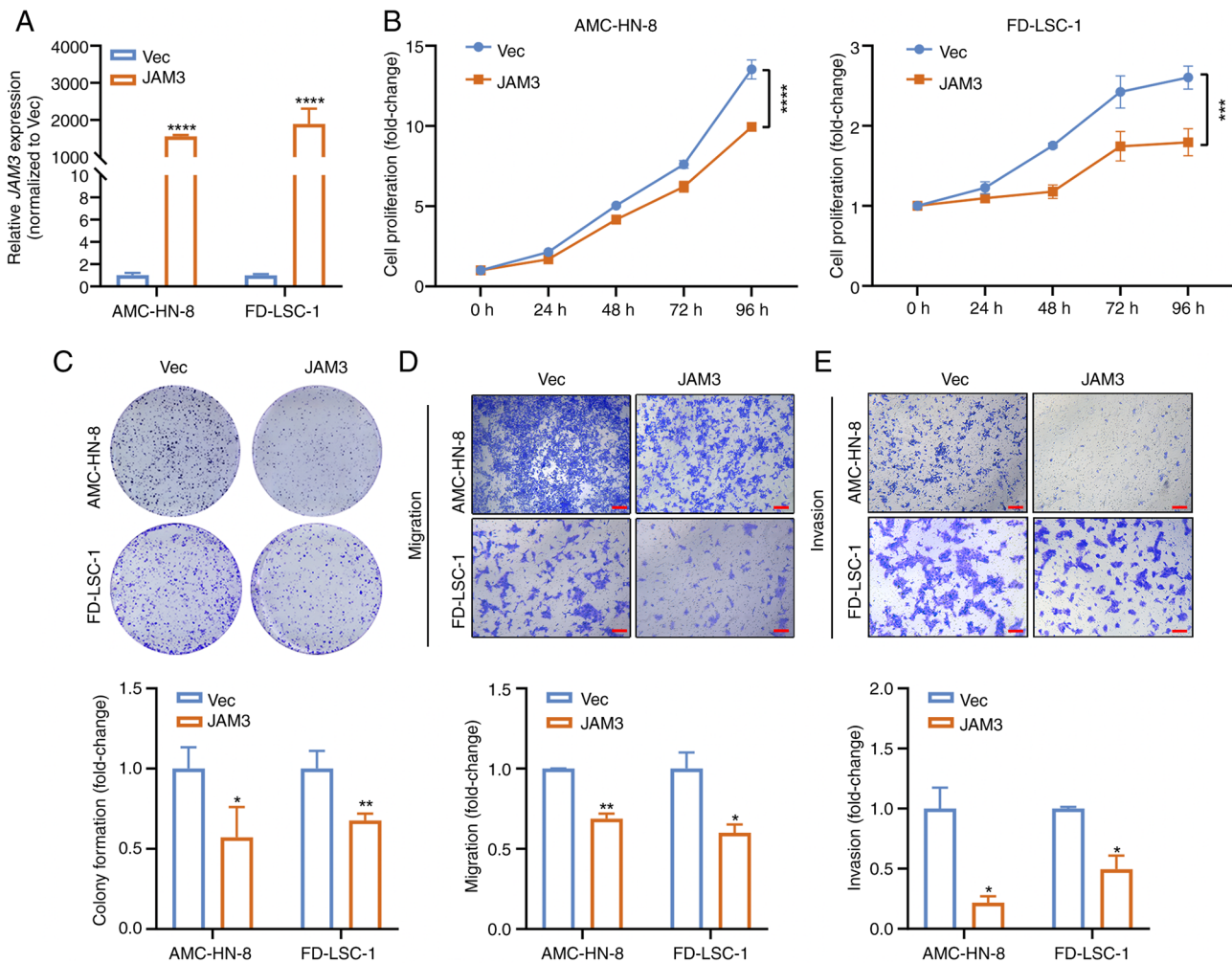


Figure 3. Overexpression of *JAM3* suppresses laryngeal squamous cell carcinoma cell proliferation, migration and invasion. (A) AMC-HN-8 and FD-LSC-1 cells were transfected with *JAM3* overexpression plasmid or Vec. Expression of *JAM3* was examined by reverse transcription-quantitative PCR. Cell proliferation was detected by (B) Cell Counting Kit 8 and (C) colony formation assays. (D) Migration and (E) invasion abilities of AMC-HN-8 and FD-LSC-1 cells after transfection were determined by Transwell assays. Scale bar, 200 μ m. Data are presented as the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. Vec. *JAM3*, junctional adhesion molecule 3; Vec, p3xFlag-CMV-10 empty vector.

within the *JAM3* gene promoter (cg03640071, cg06250693, cg06726804 and cg27073337), each significantly associated with poorer survival outcomes in HNSCC (Fig. 2C). These findings suggested that epigenetic mechanisms contributing to *JAM3* downregulation in HNSCC could be relevant to LSCC, given the shared pathophysiological characteristics of these types of cancer.

Further validation specific to LSCC involved MethPrimer (21) analysis, which identified a CpG-rich region (among -216 to +425 bp from transcription start site) within the *JAM3* promoter. Sequencing of 10 clones from the T1 cloning vector containing the CpG island region of AMC-HN-8 and FD-LSC-1 cell lines demonstrated extensive methylation. In the AMC-HN-8 cell line, 16 of the 27 cytosine sites showed complete methylation, with others showing partial methylation; the FD-LSC-1 cell line exhibited similar patterns (Fig. 2D). Furthermore, treatment with 5-Aza, a known DNA promoter methylation reverser, restored *JAM3* expression at both the mRNA and protein levels in both LSCC cell lines (Fig. 2E).

Collectively, these results indicated that low *JAM3* expression may originate from aberrant hypermethylation of the

JAM3 promoter, impacting survival outcomes in HNSCC. By integrating findings from broader HNSCC analyses and the similar modulation of expression by methylation of *JAM3* promoter in LSCC cell lines, the potential of *JAM3* methylation patterns to inform on LSCC was identified, reinforcing its significance as a prognostic biomarker and a target for therapeutic interventions.

Impact of *JAM3* overexpression and knockdown on LSCC cell behavior. To elucidate the function of *JAM3* in the tumorigenesis of LSCC, experiments were conducted to assess its impact on cancer cell behavior. AMC-HN-8 and FD-LSC-1 cells were transfected with p3xFlag-CMV-10-*JAM3* to induce overexpression of the gene, or with p3xFlag-CMV-10 empty vector as a control. *JAM3* overexpression was confirmed by RT-qPCR (Fig. 3A). Cells overexpressing *JAM3* exhibited a significant reduction in cell proliferation compared with those transfected with the empty vector, as demonstrated using a CCK8 assay (Fig. 3B). This suppressive effect on cell proliferation was further supported by results from the colony formation assay (Fig. 3C). Additionally, *JAM3*-overexpressing cells displayed

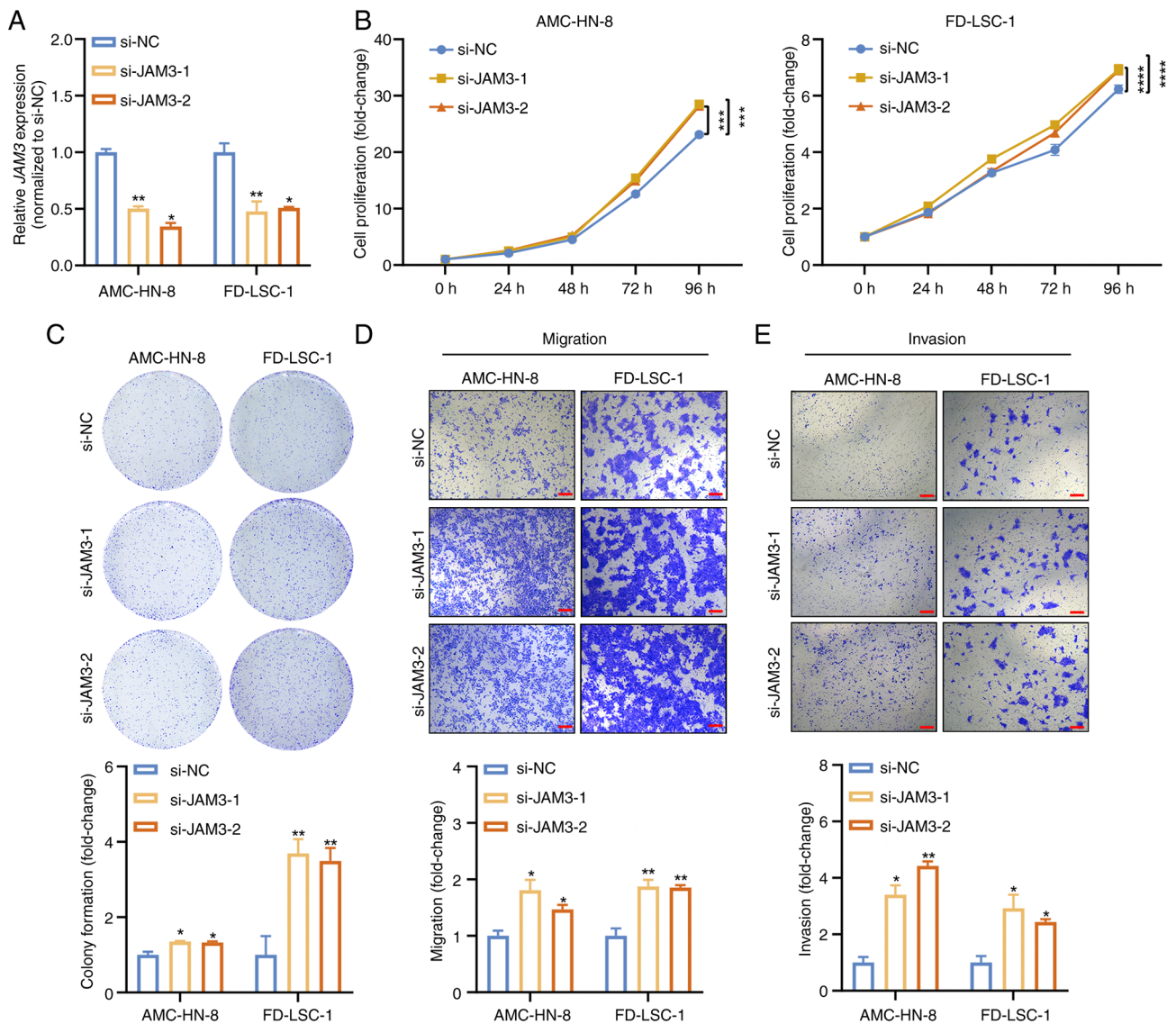


Figure 4. Knockdown of *JAM3* promotes laryngeal squamous cell carcinoma cell proliferation, migration and invasion. (A) AMC-HN-8 and FD-LSC-1 cells were transfected with siRNAs targeting *JAM3* (si-JAM3-1, si-JAM3-2) or si-NC. Expression of *JAM3* was examined by reverse transcription-quantitative PCR. Cell proliferation was detected by (B) Cell Counting Kit 8 and (C) colony formation assays. (D) Migration and (E) invasion abilities of AMC-HN-8 and FD-LSC-1 cells after transfection were determined by Transwell assays. Scale bar, 200 μ m. Data are presented as the mean $\pm$ SD of three independent experiments. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001 vs. the si-NC group. *JAM3*, junctional adhesion molecule 3; NC, negative control; si, small interfering.

decreased migration and invasion compared with that in the control group, as determined using Transwell assays (Fig. 3D). These findings indicated that *JAM3* overexpression significantly inhibited the proliferation, colony formation, migration and invasion of LSCC cells, suggesting its potential role as a TSG in LSCC.

Conversely, knockdown experiments using siRNAs targeting *JAM3* (si-JAM3-1 and si-JAM3-2) highlighted its critical regulatory role. Efficient knockdown was achieved, as verified by RT-qPCR (Fig. 4A). Cells with reduced *JAM3* expression displayed enhanced proliferative, migratory and invasive capabilities, as confirmed by CCK8, colony formation and Transwell assays (Fig. 4B-E). These results suggested that reduced *JAM3* expression significantly augmented the proliferation, migration and invasion of LSCC cells, further establishing *JAM3* as a pivotal tumor suppressor in LSCC.

Collectively, the present data indicated that *JAM3* served as a potent modulator of tumorigenic processes in LSCC, where its expression levels directly influenced tumor cell behavior. The dual experimental approach of overexpression and knockdown elucidated the suppressive impact of *JAM3* on LSCC progression, offering valuable insights for potential therapeutic strategies.

JAM3 modulates LSCC tumorigenesis via the Hippo pathway.

The investigation into the involvement of the Hippo pathway in HNSCC was prompted by initial analyses conducted on the cBio Cancer Genomics Portal platform, which revealed a weak positive correlation between *JAM3* and *LATS1*, a key component of the Hippo pathway, in HNSCC (Fig. S3). These findings suggested that *JAM3* may interact with or influence the Hippo pathway, a critical regulator of cell proliferation

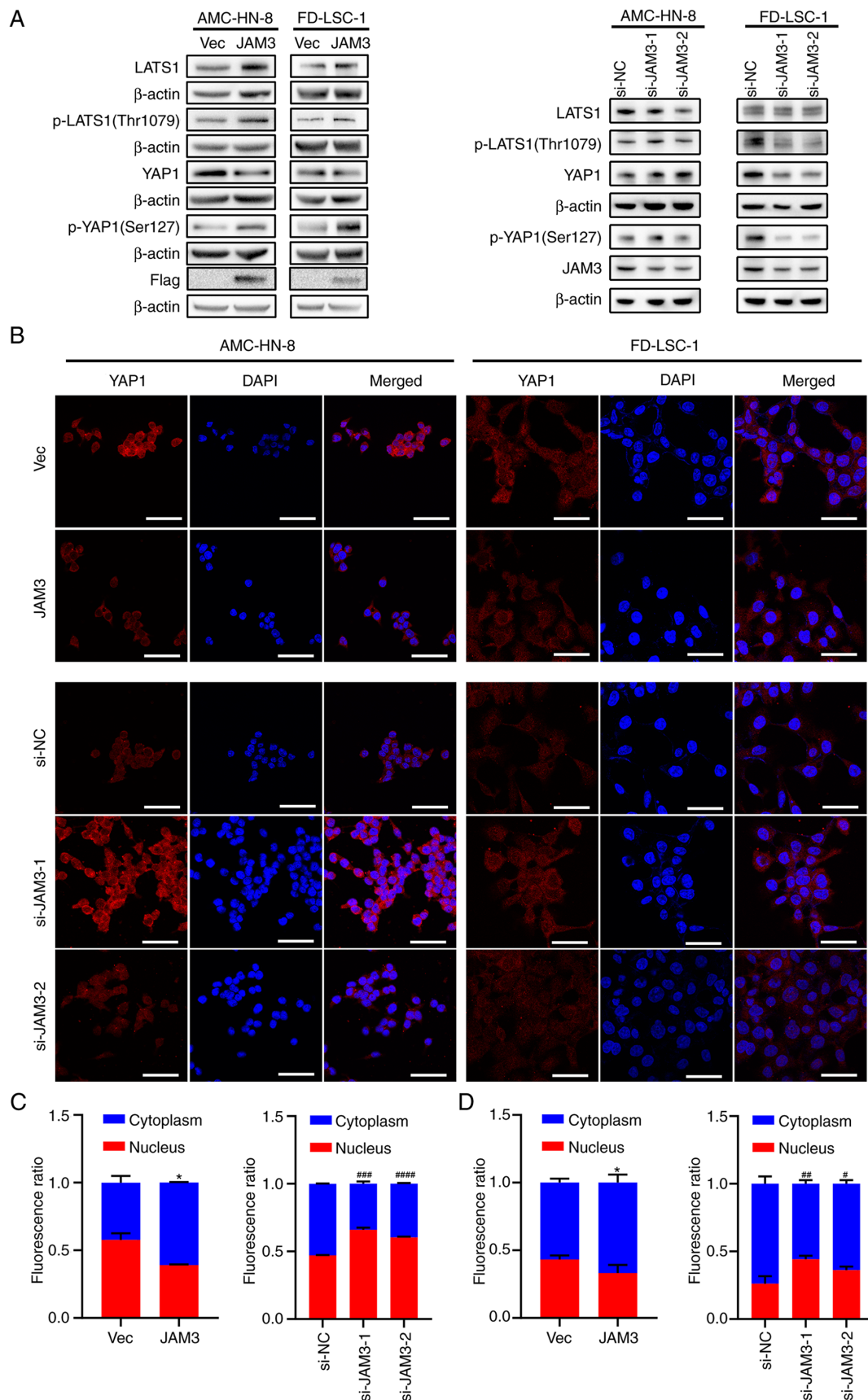


Figure 5. JAM3 mediates laryngeal squamous cell carcinoma tumorigenesis through the Hippo pathway. (A) Protein levels of Flag, JAM3, p-LATS1 (Thr1079), LATS1, p-YAP1 (Ser127), YAP1 and β -actin in AMC-HN-8 and FD-LSC-1 cells with overexpression or knockdown of JAM3 were detected by western blotting. (B) Expression of YAP1 in AMC-HN-8 and FD-LSC-1 cells after transfection with the JAM3 overexpression plasmid or si-JAM3 were detected by confocal microscopy. Scale bar, 50 μ m. Fluorescence ratio of YAP1 in the cytoplasm and nucleus of (C) AMC-HN-8 and (D) FD-LSC-1 cells was calculated by ImageJ and analyzed by two-tailed Student's t-test or one-way ANOVA. Data are presented as the mean $\pm$ SD of three independent experiments. * P <0.05 vs. Vec; ** P <0.05, *** P <0.01, **** P <0.001, ***** P <0.0001 vs. si-NC group. JAM3, junctional adhesion molecule 3; LATS1, large tumor suppressor kinase 1; NC, negative control; p-, phosphorylated; si, small interfering; Vec, p3xFlag-CMV-10 empty vector; YAP1, yes-associated protein 1.

and apoptosis (34). To elucidate the molecular mechanisms by which *JAM3* influences LSCC tumorigenesis, the present study further investigated its impact on key proteins within the Hippo signaling pathway. This approach aimed to uncover how changes in *JAM3* expression affect pathway dynamics and, consequently, tumor behavior.

Western blotting was performed on AMC-HN-8 and FD-LSC-1 cells with either *JAM3* overexpression or knock-down. The protein levels of Flag, *JAM3*, p-LATS1 (Thr1079), total LATS1, p-YAP1 (Ser127) and total YAP1 were examined, with β -actin serving as a loading control. In cells with *JAM3* overexpression, there was a noticeable increase in total LATS1, p-LATS1 and p-YAP1, indicating activation of the Hippo pathway (Fig. 5A). Conversely, *JAM3* knockdown resulted in reduced total LATS1, p-LATS1 and p-YAP1 expression, suggesting that YAP1 activity and downstream signaling were inhibited (Fig. 5A).

To further elucidate the intracellular dynamics of YAP1 following genetic manipulation of *JAM3*, immunofluorescence assays were performed. Using confocal microscopy (Fig. 5B), the subcellular localization of YAP1 was observed in transfected cells. Semi-quantitative analysis conducted with ImageJ 1.53k software revealed a significant decrease in the nuclear accumulation of YAP1 in cells overexpressing *JAM3*, suggesting Hippo pathway activation (Fig. 5C and D). Conversely, cells with *JAM3* knockdown displayed predominant YAP1 localization in the nucleus, indicating suppression of the pathway (Fig. 5C and D).

These findings underscored the role of *JAM3* as a regulatory element in the Hippo pathway, influencing both the phosphorylation status and the subcellular localization of YAP1 in LSCC cells. These results suggested that *JAM3* may serve as a crucial modulator of cell proliferation and motility through its effects on this signaling pathway.

JAM3 knockdown enhances the tumorigenicity of LSCC cells *in vivo*. To evaluate the tumor suppressor function of *JAM3* in an *in vivo* model, experiments were conducted using nude mice. AMC-HN-8 cells were subcutaneously injected into the mice, after which, si-*JAM3* or si-NC was injected to observe the effects on tumor growth and aggressiveness.

In these experiments, mice injected with si-*JAM3* cells developed larger tumors than those in the si-NC group (Fig. 6A). This finding was confirmed by the significant increase in tumor volume and weight in the si-*JAM3* group, indicating a marked enhancement in tumorigenic capacity associated with the suppression of *JAM3* (Fig. 6B and C).

Further molecular analyses confirmed the knockdown efficacy, with the RT-qPCR results showing significantly reduced *JAM3* expression in the tumors derived from the si-*JAM3* group (Fig. 6D); this result suggested effective gene silencing was achieved. H&E staining demonstrated structural changes in the xenograft tumors, including an increase in LSCC tumor cell density after *JAM3* knockdown (Fig. 6E). Further analysis through IHC clarified the cellular and molecular impacts of *JAM3* knockdown. A reduction in *JAM3* expression was detected, accompanied by elevated levels of YAP1 and the proliferation marker Ki-67 in the xenograft tumors (Fig. 6F). Additionally, changes in epithelial-mesenchymal transition (EMT) markers were evident; N-cadherin and Vimentin levels

were higher, whereas E-cadherin expression was reduced in xenograft tumors from the si-*JAM3* group (Fig. 6G). These findings collectively supported the hypothesis that *JAM3* silencing may promote the growth and aggressiveness of LSCC cells *in vivo* by disrupting Hippo pathway signaling.

Discussion

The present findings demonstrated that *JAM3* expression was suppressed through the hypermethylation of its promoter, and this was revealed to be associated with poorer patient outcomes. Moreover, the overexpression of *JAM3* inhibited tumor-related behaviors by activating the Hippo pathway. Conversely, *JAM3* silencing promoted these oncogenic behaviors *in vitro* and *in vivo*. The present study provides deeper insights into the understanding of the role of *JAM3* as a TSG in the development and progression of LSCC, and underscores its potential as a diagnostic and prognostic biomarker. Overall, the current study aimed to elucidate the mechanisms by which *JAM3* modulates tumor dynamics, offering promising directions for future therapeutic strategies.

Over the past 30 years, despite a decrease in overall incidence, the survival rate of LSCC has decreased from 66 to 61% in the United States (35). This concerning trend is largely due to the fact that the majority of patients are diagnosed at a late stage (3) and underscores the need to improve the understanding of the molecular mechanisms underlying LSCC tumorigenesis. This improved understanding may enable early diagnosis and increase the accuracy of treatment, improving the quality of life of patients.

JAM proteins, part of the immunoglobulin (Ig) superfamily, contain two extracellular Ig-like domains and one intracellular PDZ-binding motif (36), which are integral to cell-cell contact and migration (37,38), processes crucial for early tumor metastasis (39). Research has identified *JAM3* as a TSG, often silenced by methylation in cancer, such as esophageal and colorectal cancer (17). *JAM3* has also been identified as a potential DNA methylation marker in cholangiocarcinoma and cervical lesions (39,40). Although *JAM3* has been reported and characterized in several tumors, its role in LSCC remains unclear. The present study observed a pronounced downregulation of *JAM3* in LSCC tumor tissues, both at the mRNA and protein levels, which was corroborated by public dataset analyses and IHC analysis of LSCC clinical specimens. Notably, according to public dataset analyses, lower *JAM3* protein levels were observed in higher-grade HNSCC tumors compared with those in normal and lower-grade tumors, suggesting a potential link between *JAM3* downregulation and poor tissue differentiation. However, in the LSCC clinical specimens assessed, IHC confirmed a general downregulation of *JAM3* in tumor tissues compared with that in normal tissues, but did not show a significant difference across different tumor grades. This observation underscores the need for further exploration of the role of *JAM3* expression across various stages of LSCC progression, especially because of the limited sample size of the present study. Furthermore, a negative correlation between *JAM3* mRNA expression and promoter methylation indicated that high methylation levels may contribute to *JAM3* depletion in LSCC. This was supported by extensive methylation observed in BSP data and the successful restoration of *JAM3*

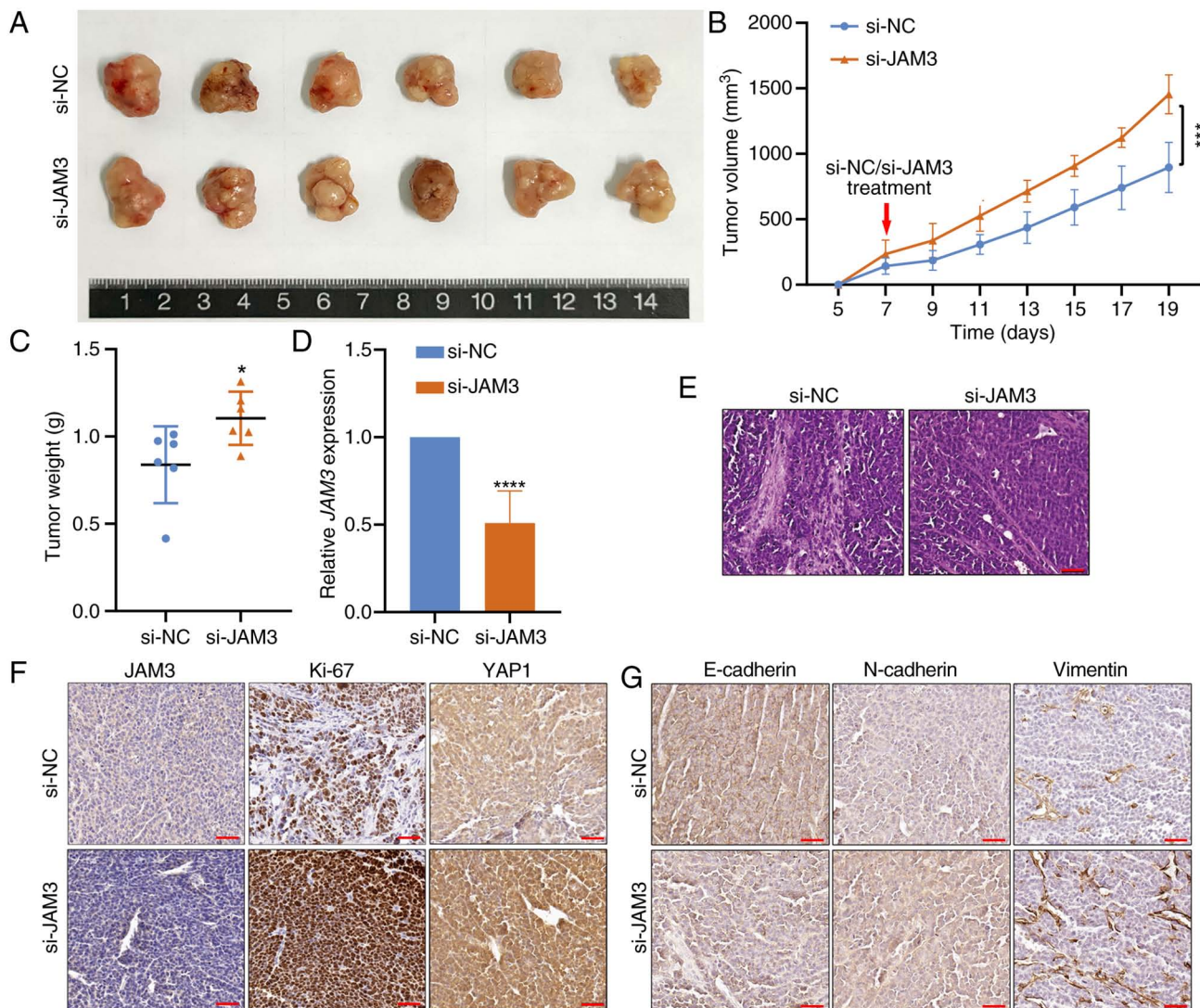


Figure 6. Knockdown of *JAM3* promotes tumorigenicity of laryngeal squamous cell carcinoma cells *in vivo*. (A) Representative images of tumors in nude mice after subcutaneous injection of AMC-HN-8 cells transfected with si-NC and si-JAM3. (B) Tumor growth curve was plotted using xenograft tumor volume data. ***P<0.001 vs. si-NC. (C) Tumor weight was measured after tumor excision. *P<0.05 vs. si-NC group. (D) Relative expression levels of *JAM3* in xenograft tumors as determined by reverse transcription-quantitative PCR analysis. Data are presented as the mean $\pm$ SD of three independent experiments. ****P<0.0001 vs. si-NC. (E) Hematoxylin and eosin staining of xenograft tumors. Immunohistochemistry staining of (F) *JAM3*, Ki-67 and YAP1, and (G) E-cadherin, N-cadherin and Vimentin in xenograft tumors. Scale bar, 20 μ m. *JAM3*, junctional adhesion molecule 3; NC, negative control; si, small interfering; YAP1, yes-associated protein 1.

expression following treatment with the demethylating agent 5-Aza. Additionally, higher methylation at several CpG sites within the *JAM3* promoter was associated with poorer patient outcomes, underscoring the importance of monitoring *JAM3* methylation as a potential early diagnostic and prognostic biomarker in HNSCC. These findings also suggested that *JAM3* methylation may act as a biomarker in LSCC, which deserves further exploration.

To deeply understand the function of *JAM3* in LSCC, the present study conducted comprehensive *in vivo* and *in vitro* experiments. The findings indicated that *JAM3* overexpression inhibited LSCC cell proliferation, migration and invasion, whereas its knockdown promoted these oncogenic behaviors. Specifically, *in vivo* results demonstrated enhanced proliferation, invasion and migration of AMC-HN-8 cells with *JAM3* knockdown, as evidenced by increased Ki-67, N-cadherin and Vimentin staining, as well as decreased E-cadherin staining

in xenograft tumors. These results collectively suggested that *JAM3* may function as a TSG in LSCC.

The Hippo pathway is critical in cancer development, with previous studies highlighting the amplification of *YAP1* and *TAZ* in 14% of HNSCC cases, and their association with adverse clinical outcomes, including tumor recurrence and resistance to therapy (41,42). In this context, the present study revealed that *JAM3* was positively correlated with *LATS1*, a key regulator of *YAP1/TAZ*, which can promote the phosphorylation of the downstream protein *YAP1*, and reduce the activation of its target genes related to proliferation, EMT and other hallmarks of cancer (43,44). This association was important because it implies that *JAM3* may exert tumor-suppressive effects through modulation of the Hippo pathway. Further molecular investigations revealed that *JAM3* overexpression in LSCC cells increased *LATS1* levels and its phosphorylation, and enhanced phosphorylation of *YAP1*, indicating that

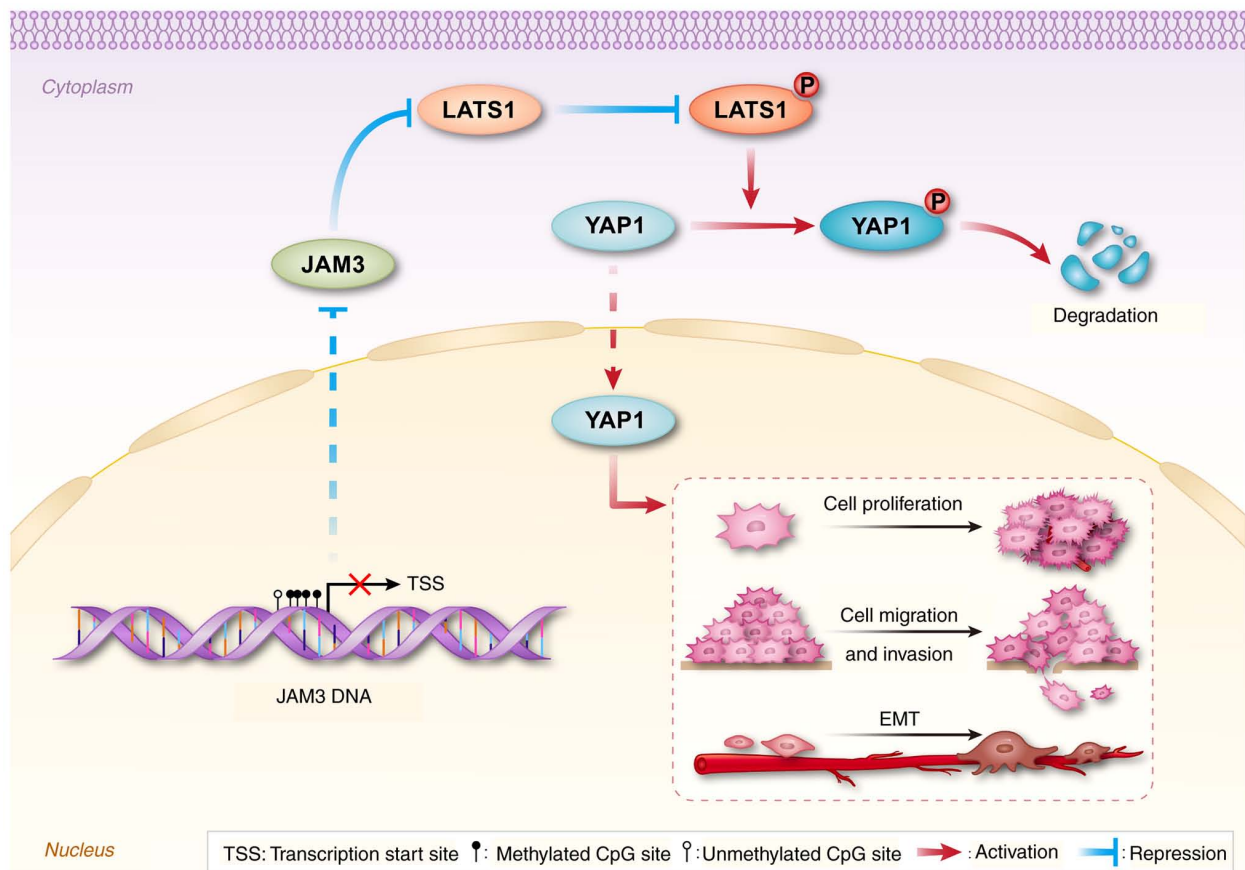


Figure 7. Mechanism by which epigenetic silencing of *JAM3* promotes LSCC development by inhibiting the Hippo pathway. *JAM3*, junctional adhesion molecule 3; *LATS1*, large tumor suppressor kinase 1; *YAP1*, yes-associated protein 1; p, phosphorylated; EMT, epithelial-mesenchymal transition.

the Hippo pathway was activated. *JAM-A*, a member of the *JAM* family, has been shown to activate the Hippo pathway by sensing cell-cell contact and promoting *LATS1* activation (45). *JAM3* likely enhances these processes by improving cell-cell adhesion and supporting the role of *JAM-A* in organizing Hippo pathway components. By facilitating the activity of *JAM-A*, *JAM3* overexpression could stabilize *LATS1* expression and enhance its phosphorylation, further promoting Hippo pathway activation. Conversely, *JAM3* knockdown led to reduced phosphorylation of these proteins and increased nuclear accumulation of *YAP1*, suggesting suppression of the Hippo pathway. IHC analysis in mouse models reinforced these findings, showing elevated *YAP1* expression following *JAM3* silencing, which aligned with decreased pathway activity. This mechanism is supported by similar observations in breast cancer research, where *JAM3* depletion has been shown to lead to increased *YAP/TAZ* nuclear translocation and activation (46), highlighting a potential universal role for *JAM3* in regulating the Hippo pathway across various types of cancer. Despite the significant findings, the present study has some limitations, particularly the lack of direct exploration into whether *JAM3* expression influences tumor differentiation and its potential as a methylation biomarker in LSCC. Further research in this area is essential, not only to improve understanding of the role of *JAM3*, but also to establish its potential as a prognostic biomarker in LSCC. This would require more detailed mechanistic studies to elucidate the specific pathways through which *JAM3* exerts its effects.

In conclusion, the present study substantiated the role of *JAM3* in the pathogenesis of LSCC. Aberrant hypermethylation of the *JAM3* promoter was demonstrated to be associated with decreased *JAM3* expression and poorer clinical outcomes in patients with LSCC. The present findings suggested that *JAM3* may function as a TSG, inhibiting tumor growth and progression, potentially through its regulatory effects on the Hippo pathway in LSCC (Fig. 7). These interactions highlight *JAM3* not only as a key player in tumor dynamics but also as a promising prognostic biomarker for LSCC. Further investigations into the mechanisms underlying the effects of *JAM3* could provide deeper insights into its tumor-suppressive activities and pave the way for novel therapeutic approaches aimed at enhancing *JAM3* expression to mitigate LSCC progression. This pioneering study on the role of *JAM3* in LSCC expands possibilities for developing targeted treatments, which could significantly improve prognosis and patient outcomes.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YJ, CZ and HH contributed to the conceptualization of the study. YJ designed and performed most of the experiments, with assistance from JL, JS, XW, LZ, YL and XG. YJ, JL and JS sorted and analyzed the data. YJ, XW, LZ and YL contributed to the animal experiments. CZ and HH designed the experiments and supervised the study. YJ wrote the manuscript, and HH reviewed and edited it. YJ and HH confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study involving clinical samples received ethics approval (approval no. KYLL-2023-180) from the Ethics Committee of The First Hospital, Shanxi Medical University and was conducted in strict accordance with the committee's guidelines. Due to the retrospective nature of the study and the use of archived samples, the requirement for informed consent was waived by the Ethics Committee, in line with The Declaration of Helsinki. All patient data were thoroughly anonymized to ensure confidentiality. Animal experiments (approval no. DWLL-2024-027) were conducted according to the Health Guide for the Care and Use of Laboratory Animals and were approved by the Research Ethics Committee for Animal Experimentation at The First Hospital, Shanxi Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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