

KIF4A drives esophageal squamous cell carcinoma via Wnt/ β -catenin signaling and predicts poor prognosis

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Received March 17, 2026; Accepted July 23, 2026

DOI: 10.3892/mmr.2026.14013

Abstract. Esophageal squamous cell carcinoma (ESCC) accounts for >90% of esophageal cancer cases in China, with a 5-year overall survival rate of <30% for advanced disease. Kinesin family member 4A (KIF4A) is implicated in multiple malignancies; however, its functional mechanisms in ESCC remain incompletely elucidated. The present study investigated the prognostic value and tumor-promoting mechanism of KIF4A in ESCC. KIF4A expression was detected in 43 paired ESCC and adjacent non-malignant tissues, as well as in ESCC cell lines. Loss- and gain-of-function assays were performed to evaluate the effects of KIF4A on ESCC cell proliferation, migration and tumorigenesis *in vitro* and *in vivo*. RNA sequencing, co-immunoprecipitation and rescue experiments were conducted to explore its downstream regulatory pathway. The results revealed that KIF4A was significantly upregulated in ESCC tissues and cell lines, and its high expression was closely associated with advanced tumor stage and poor overall survival in patients with ESCC. KIF4A knockdown markedly inhibited ESCC cell proliferation, migration and *in vivo* xenograft tumor growth, whereas its overexpression exerted opposite oncogenic effects. Mechanistically, KIF4A exerted tumor-promoting effects by activating the canonical Wnt/ β -catenin signaling pathway via stabilization of the β -catenin/T-cell factor 7 complex. In conclusion, KIF4A may drive ESCC progression by activating the Wnt/ β -catenin

signaling pathway, and may serve as a promising prognostic biomarker and therapeutic target for ESCC.

Introduction

Esophageal cancer (EC) is the sixth leading cause of cancer-related mortality worldwide, with an estimated 511,054 new cases and 445,391 deaths in 2022, ~50% of which were expected to occur in China (1,2). Histologically, >90% of EC cases in China are esophageal squamous cell carcinoma (ESCC), which has a poor 5-year overall survival rate of 20-30%. Notably, most patients with ESCC are diagnosed at advanced stages, for which effective targeted therapeutic strategies remain limited (3,4). Accumulating evidence has indicated that metabolic reprogramming, especially enhanced aerobic glycolysis (the Warburg effect), is a hallmark of ESCC progression and drives malignant phenotypes including proliferation, invasion and distant metastasis, which represents a promising therapeutic vulnerability (5). Multiple natural small molecule compounds, such as baicalin, have been reported to exert antitumor activity through inhibiting hyperactivated Wnt signaling in epithelial tumors, offering promising candidate agents for tumor intervention (6). The poor prognosis and limited therapeutic options for ESCC drive the urgent need to identify novel prognostic biomarkers and therapeutic targets.

Kinesin family member 4A (KIF4A) is an evolutionarily conserved microtubule motor protein, which is primarily involved in mitotic spindle organization and chromosomal segregation during cell division (7,8). Emerging evidence has validated the oncogenic roles of KIF4A across diverse malignancies, including non-small cell lung cancer, colorectal cancer, breast cancer and hepatocellular carcinoma (9-13). KIF4A modulates multiple malignant phenotypes, including cell proliferation, invasion, stemness maintenance and therapeutic resistance, by regulating diverse oncogenic signaling cascades such as the Akt, TGF- β /Smad3 and DNA damage response pathways (14-19). Clinically, KIF4A upregulation is associated with unfavorable prognosis in multiple types of cancer, including non-small cell lung cancer, colorectal cancer, breast cancer and hepatocellular carcinoma, and has been developed as a potential therapeutic target, with small-molecule inhibitors such as WZ-3146 exhibiting antitumor efficacy in preclinical

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Key words: kinesin family member 4A, esophageal squamous cell carcinoma, prognosis, Wnt/ β -catenin signaling, tumor progression, cell proliferation

models (12,13,16,17,20). Notably, our previous study identified KIF4A as a prognostic biomarker for ESCC (21), but the functional mechanisms and downstream regulatory pathways of KIF4A in ESCC progression remain largely undefined.

The Wnt signaling pathway is an evolutionarily conserved cascade divided into the canonical β -catenin-dependent pathway and non-canonical branches (including the Wnt/ Ca^{2+} and planar cell polarity pathways), which collectively regulate cellular proliferation, differentiation, migration and tissue homeostasis (22–27). Among them, the canonical Wnt/ β -catenin signaling pathway is the most well-characterized branch in tumor biology, with aberrant activation closely implicated in the tumorigenesis and progression of multiple solid malignancies, including breast cancer, liver cancer and gastrointestinal tumors such as ESCC (28–32). In the resting state, β -catenin is phosphorylated and degraded by the destruction complex; upon pathway activation, stabilized β -catenin translocates to the nucleus, forms a complex with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors, and drives the expression of pro-oncogenic target genes such as c-Myc, cyclin D1 and MMP-7 (28,30). A recent study confirmed that the transcription factor E2F1 participates in the malignant progression and sorafenib resistance of EC, and multiple transcription factors, including E2F1, can synergistically interact with the Wnt cascade to remodel tumor cell phenotypes and drug responsiveness (33). Previous studies have reported that abnormal activation of the Wnt/ β -catenin pathway is highly prevalent in ESCC, and that it is associated with tumor progression and poor prognosis (34,35). However, to the best of our knowledge, whether KIF4A exerts its oncogenic effects in ESCC by regulating the Wnt/ β -catenin pathway has not yet been reported.

The current study systematically investigated the expression pattern, prognostic value, biological functions and downstream regulatory mechanisms of KIF4A in ESCC. Accordingly, the present study aimed to systematically investigate the expression profile, clinical prognostic value and biological functions of KIF4A in ESCC, and to further explore whether KIF4A drives ESCC progression by regulating the canonical Wnt/ β -catenin signaling pathway. This work may provide a novel potential prognostic biomarker and therapeutic target for ESCC management.

Materials and methods

Bioinformatics analysis. The Cancer Genome Atlas (TCGA) pan-cancer transcriptomic dataset (2,900 samples) and ESCC transcriptomic dataset (195 samples) with corresponding clinical data were downloaded and analyzed via the cBioPortal (<https://www.cbioportal.org/>) and UALCAN (<https://ualcan.path.uab.edu/>) online platforms. Kaplan-Meier survival analysis and differential expression analysis of KIF4A were performed using the UALCAN database (<http://ualcan.path.uab.edu>) based on the TCGA esophageal carcinoma (ESCA) cohort. The correlation between KIF4A expression and overall survival was evaluated by the log-rank test. Gene expression levels were normalized using the $\log_2(\text{RSEM} + 1)$ transformation. The association between KIF4A expression and pTNM stage grouping (I–IV) in ESCC, as well as the co-expression correlation between KIF4A and CTNGB1

(encoding β -catenin)/TCF7, were analyzed. Heatmap visualization of Wnt signaling pathway-related genes was constructed based on normalized expression values using Heatmapper 2 (version 2.0; <http://www.heatmapper.ca>), a freely available online platform for gene expression heatmap generation.

Clinical tissue specimens. The present study involved two independent sets of in-house human tissue specimens, all obtained from patients with pathologically confirmed esophageal squamous cell carcinoma (ESCC) undergoing routine radical esophagectomy at Xiangyang No. 1 People's Hospital (Xiangyang, China). No patients received neoadjuvant chemoradiotherapy before surgery, and no additional invasive procedures were performed specifically for research purposes. Inclusion and exclusion criteria were as follows: Patients with pathologically confirmed primary ESCC who underwent complete radical esophagectomy with complete clinicopathological data. Exclusion criteria were as follows: Patients with a history of other primary malignancies, confirmed distant metastasis at diagnosis, incomplete follow-up records or severe systemic comorbidities that affect survival endpoints. The main research cohort, comprising 43 matched pairs of fresh ESCC tissues and paired adjacent non-malignant esophageal tissues, was surgically collected between August 2023 and September 2025. Patients in this cohort had a mean age of 63 years (range, 56–74 years) and included 29 males and 14 females. The present study was reviewed and approved by the Institutional Review Board of Xiangyang First People's Hospital (approval no. 2025KY082) in September 2025. The second set of specimens, dedicated to patient-derived organoid culture and functional validation assays, was prospectively collected from December 2025 to March 2026. All specimens were freshly resected tumor tissues obtained from 15 patients with pathologically confirmed primary ESCC undergoing routine radical esophagectomy. This cohort was reviewed and approved under a separate independent ethics approval (approval no. 2025KY142, officially approved in December 2025) by the Institutional Review Board of Xiangyang No. 1 People's Hospital. Sample processing, organoid establishment, routine passaging and relevant follow-up experiments were performed immediately after surgical resection following standardized operating procedures. Written informed consent was obtained from all enrolled patients at the time of specimen collection, and all procedures involving human tissues were conducted in strict compliance with The Declaration of Helsinki Patients were stratified into low- and high-KIF4A expression groups according to KIF4A expression levels. Baseline clinical characteristics including age, sex, clinical stage, drinking history and lymph node metastasis status were compared between the two groups. The detailed clinicopathological characteristics of the main research cohort are summarized in Table SI.

Immunohistochemistry (IHC). Tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin and cut into 4- μm sections. The sections were then baked at 60°C for 2 h, deparaffinized with eco-friendly deparaffinization solution (Wuhan Servicebio Technology Co., Ltd.) for 130 min at room temperature (25°C) and rehydrated through a gradient ethanol series to distilled water. Antigen retrieval was performed using Tris-EDTA buffer under high pressure at 121°C for 20 min and

endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min at room temperature. The sections were then incubated with anti-KIF4A primary antibody (1:200; catalog no. A9080; ABclonal Biotech Co., Ltd.) overnight at 4°C, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The chromogenic reaction was developed with DAB for 1-3 min at room temperature, followed by hematoxylin counterstaining for 2 min at room temperature, gradient ethanol dehydration and xylene clearance. Slides were mounted with neutral resin and images were captured under a light microscope.

KIF4A staining was scored semi-quantitatively by two independent pathologists in a blinded manner, based on staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) and the percentage of positive cells (0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%). The 'staining intensity x positive cell percentage' scoring system (intensity 0-3, positive rate 0-4, total score range 0-12) with a cutoff of 6 for KIF4A IHC (≥ 6 , high; < 6 low) has been widely adopted in multiple published studies on KIF4A in solid tumors, which ensures the comparability of the present results with those of previous reports (36,37).

Cell culture and transfection. Human ESCC cell lines (KYSE-150, KYSE-180, TE-10 and TE-1) and the normal human esophageal epithelial cell line HET-1A were used in the present study. All cell lines were obtained from the American Type Culture Collection, authenticated via short tandem repeat profiling and routinely tested for mycoplasma contamination (negative throughout the study). ESCC cell lines were cultured in RPMI-1640 medium (Pricella; Elabscience Bionovation Inc.) supplemented with 10% fetal bovine serum (FBS; Pricella; Elabscience Bionovation Inc) and 1% penicillin/streptomycin, in a humidified incubator containing 5% CO₂ at 37°C. HET-1A cells were maintained in specialized epithelial cell medium (Shanghai Jinyuan Biotechnology Co., Ltd.) under the same culture conditions.

For KIF4A silencing, KYSE-150 and TE-1 cells were seeded into 6-well plates (5×10^4 cells/well). Four KIF4A-targeting small interfering RNAs (si-KIF4A-1, si-KIF4A-2, si-KIF4A-3 and si-KIF4A-4) were synthesized by Shanghai GeneChem Co., Ltd. Cells were transfected with 50 nM corresponding siRNA using Lipo8000TM transfection reagent (Beyotime Biotechnology) at 37°C in a humidified 5% CO₂ incubator for 6 h, followed by complete medium replacement. The knock-down efficiency at the protein level was verified by western blot, and si-KIF4A-1 with the highest interference efficiency was selected for all subsequent functional experiments. The full sequences of all siRNAs are listed in the supplementary materials. For KIF4A overexpression, KYSE-150 and TE-10 cells (the same lines used for gene silencing) were transfected with 5 μ g of KIF4A overexpression plasmid (GV712 vector backbone; Shanghai GeneChem Co., Ltd.) using the same transfection reagent. Overexpression efficiency was verified by western blotting at 48 h post-transfection. The sequences of KIF4A siRNAs are provided in Table I.

Western blot analysis. Total protein was extracted from KYSE-150 and TE-10 human ESCC cell lines and surgically resected ESCC tissue specimens, using pre-chilled RIPA lysis

buffer (Beyotime Biotechnology) supplemented with a protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (both Beyotime Biotechnology). All samples were fully lysed on ice for 30 min, and the supernatants were collected by high-speed centrifugation for subsequent BCA protein quantification. Total protein was extracted by sonication at a frequency of 20 kHz in an ice bath (4°C), with 3-sec on/5-sec off pulses for a total effective duration of 30 sec, followed by centrifugation at 14,000 x g for 15 min at 4°C. Protein concentration was determined using the BCA protein assay kit (Beyotime Biotechnology). Subsequently, equal amounts of protein (30 μ g/lane) were separated on 10% gels using SDS-PAGE and transferred to polyvinylidene fluoride membranes (Wuhan Servicebio Technology Co., Ltd.) via wet transfer. The membranes were then blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature, then incubated with the following primary antibodies overnight at 4°C: Anti-KIF4A (1:1,000; catalog no. A9080; ABclonal Biotech Co., Ltd.), anti- β -catenin (1:1,000; catalog no. A19657; ABclonal Biotech Co., Ltd.) and anti-TCF7 (1:1,000; catalog no. A20835; ABclonal Biotech Co., Ltd.), and the loading controls anti- α -tubulin (1:10,000; catalog no. 66031-1-Ig; Wuhan Sanying Biotechnology), anti-GAPDH (1:10,000; catalog no. 60004-1-Ig; Wuhan Sanying Biotechnology), anti- β -actin (1:10,000; catalog no. 60008-1-Ig; Wuhan Sanying Biotechnology) and anti-Histone H3 (1:10,000; catalog no. 17168-1-AP; Wuhan Sanying Biotechnology). After three washes with TBST, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using the SuperSignalTM West Pico PLUS ECL kit (Wuhan Servicebio Technology Co., Ltd.) on the ChemoDoc XRS+ system (Bio-Rad Laboratories, Inc.). Semi-quantitative grayscale analysis of protein bands was performed using ImageJ software (version 1.53t; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR) and RNA sequencing. Total RNA was extracted from cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed using the AbScript First Strand cDNA Synthesis Kit (catalog no. RK20300; ABclonal Biotech Co., Ltd.) according to the manufacturer's instructions. qPCR was conducted using the Universal SYBR Green Fast qPCR Mix (ABclonal Biotech Co., Ltd.) on the ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermal cycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 30 sec. Melting curve analysis was performed after amplification to verify the specificity of PCR products. The relative mRNA expression levels were calculated using the 2^{- $\Delta\Delta C_q$} method (38), with GAPDH as the internal reference. Detailed information of all primers used for RT-qPCR, including sequences, amplicon lengths annealing temperatures and NCBI Gene IDs, is summarized in Table SII.

For RNA sequencing, total RNA was extracted from KIF4A-knockdown TE-1 cells and TE-1 cells transfected with negative control siRNA (si-NC), with 1 μ g of high-quality RNA used for library preparation per sample. RNA sequencing was performed by Shanghai Jikai Gene Chemical Technology

Table I. siRNA sequences.

siRNA	Sense (5'-3')	Anti-sense (5'-3')
KIF4A si-1	GCAACAGAAUACCAAGAAATT	UUUCUUGGUAUUCUGUUGCTT
KIF4A si-2	UAUUUGAUCCCUCUACUGATT	UCAGUAGAGGGAUCAAUATT
KIF4A si-3	CAGGUCAGCAAACUUGAAATT	UUUCAAGUUUGCUGACCUGTT
KIF4A si-4	GCUGCUGAAACUUGAAAGATT	UCUUUCAAGUUUAGCAGCTT
si-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

AS, anti-sense; KIF4A, kinesin family member 4A; NC, negative control; S, sense; si, small interfering.

Co., Ltd. Differential expression analysis was performed using DESeq2 v1.16.1 (Bioconductor) (39), with $\log_2(\text{fold change}) \geq 1$ and false discovery rate (FDR) < 0.05 set as the threshold for differentially expressed genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using clusterProfiler v3.4.4 (40), with FDR-adjusted $P < 0.05$ defined as significant enrichment (41-43).

Colony formation assay. Transfected cells were seeded into 6-well plates at a density of 500 cells/well, and cultured for 7-14 days (when visible colonies of proper size formed) at 37°C in an atmosphere containing 5% CO₂, with the medium replaced every 3-4 days. Colonies were fixed with pre-chilled 100% methanol at room temperature for 15 min, stained with 0.1% crystal violet (Shanghai Acme Biochemical Technology Co., Ltd.) at room temperature for 20 min, and then washed with PBS. The number of colonies (≥ 50 cells/colony) was counted under a light microscope. All experiments were performed in triplicate with three biological replicates.

Wound healing assay. Transfected cells were seeded into 6-well plates and cultured to 90% confluence. Subsequently, a straight scratch was made in the cell monolayer using a sterile 200- μ l pipette tip. The cells were then washed with PBS to remove floating cells and cultured in serum-free RPMI-1640 medium. Images of the scratch were captured at 0 and 24 h post-scratching under an inverted light microscope (Olympus IX73; Olympus Corporation). The wound healing rate (%) was calculated as: (0 h wound width-24 h wound width)/0 h wound width $\times 100$. All experiments were performed in triplicate with three biological replicates.

Transwell migration assay. ESCC cells were harvested 48 h post-transfection, resuspended in serum-free RPMI-1640 medium and seeded into the upper chambers of 24-well Transwell plates (pore size, 8 μ m; Shanghai Acme Biochemical Technology Co., Ltd.) at a density of 4×10^4 cells/well. The lower chambers were filled with 600 μ l RPMI-1640 medium supplemented with 20% FBS as a chemoattractant. After 24 h incubation at 37°C, non-migrated cells on the upper surface of the membrane were removed with cotton swabs. Cells that had migrated to the lower surface were fixed with 4% paraformaldehyde at room temperature for 15 min, stained with 0.1% crystal violet at room temperature for 10 min, and images were captured under an inverted light microscope. The

number of migrated cells was counted in five random fields per well. All experiments were performed in triplicate with three biological replicates.

EdU staining assay. Cell proliferation was detected using the EdU-488 Cell Proliferation Imaging Detection Kit (Hubei Pumei Biotechnology Co., Ltd.) according to the manufacturer's instructions. Transfected cells were seeded into 96-well plates (2×10^4 cells/well) and cultured for 24 h. Cells were then incubated with EdU working solution at 37°C for 2 h, fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.5% Triton X-100 at room temperature for 10 min, and stained with the click reaction solution in the dark at room temperature for 30 min. Nuclei were counterstained with DAPI in the dark at room temperature for 5 min. Images were captured under a fluorescence microscope and the percentage of EdU-positive cells was calculated. All experiments were performed in triplicate with three biological replicates.

Co-immunoprecipitation (co-IP) assay. TE-1 and KYSE-150 cells were lysed in pre-chilled non-denaturing lysis buffer (Beyotime Biotechnology) supplemented with protease inhibitor cocktail on ice for 5 min. After centrifugation at 12,000 $\times$ g for 20 min at 4°C, the supernatant was collected as total protein extract. For co-immunoprecipitation assays, two independent sets of reactions were performed with different capture antibodies for distinct verification purposes: Anti-KIF4A antibody was used to capture the KIF4A protein complex to verify the endogenous binding between KIF4A and core Wnt pathway proteins, while anti- β -catenin antibody was used to capture the β -catenin protein complex in negative control and KIF4A-knockdown TE-1 cells to evaluate the effect of KIF4A knockdown on the stability of the β -catenin/TCF7 transcriptional complex, and normal mouse IgG was set as the negative control for both immunoprecipitation systems. For each reaction, 500 μ g of total protein lysate was incubated with the corresponding antibody or isotype control overnight at 4°C with gentle rotation, followed by 2 h of incubation with 20-40 μ l Protein A/G magnetic beads (Abmart Pharmaceutical Technology Co., Ltd.) at 4°C. After incubation, the tubes were briefly centrifuged at 3,000 $\times$ g for 1 min at 4°C to collect beads from the tube wall, and the immune complexes were then isolated using a magnetic stand before the supernatant was discarded. The

magnetic beads were washed three times with pre-chilled lysis buffer; after each wash, beads were collected via brief centrifugation at $3,000 \times g$ for 1 min at 4°C followed by magnetic separation, and the supernatant was completely removed.

The precipitated protein complexes were eluted with 2X SDS loading buffer, boiled at 100°C for 10 min for denaturation, and centrifuged at $10,000 \times g$ for 10 sec at room temperature to collect all eluate to the tube bottom. The supernatant was subsequently analyzed by western blotting. Semi-quantitative grayscale analysis of protein bands was performed using ImageJ software (version 1.53t; National Institutes of Health).

The detailed antibody information for the co-immunoprecipitation assay is listed as follows: Mouse monoclonal anti-KIF4A antibody (cat. no. sc-365144; Santa Cruz Biotechnology), mouse monoclonal anti- β -catenin antibody (cat. no. sc-7963; Santa Cruz Biotechnology) and normal mouse IgG isotype control (cat. no. B900620; Proteintech Group, Inc.). For each immunoprecipitation reaction, $2 \mu\text{g}$ of target antibody or isotype control was added.

ESCC organoid culture. Fresh ESCC surgical specimens were cut into small pieces, and digested in AdDF++++ medium containing collagenase II (Sigma-Aldrich; Merck KGaA) and DNase I (Sigma-Aldrich; Merck KGaA) for 3–4 h at 37°C in a water bath. The cell suspension was filtered through a $70\text{-}\mu\text{m}$ cell strainer and centrifuged at $300 \times g$ for 5 min at 4°C . The cell pellet was washed three times with D-PBS. Red blood cells were lysed using red blood cell lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) when the cell pellet appeared visibly red or abundant erythrocytes were observed under light microscopy. The cell pellet was mixed with Matrigel (Corning, Inc.) at a 1:1 volume ratio, and $50 \mu\text{l}$ droplets of the cell-Matrigel mixture were seeded into pre-warmed 24-well plates. After Matrigel gelation at 37°C for 30 min, $500 \mu\text{l}$ ESCC organoid medium (prepared in-house) was added to each well, with the medium changed every 3 days. Organoids were passaged every 1–2 weeks using TrypLE Express (Gibco; Thermo Fisher Scientific, Inc.). Detailed medium components are provided in Table SIII.

In vivo xenograft tumor model. All animal experiments were performed in strict accordance with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (44,45). The study protocol was approved by the Institutional Animal Care and Use Committee of Xiangyang No. 1 People's Hospital (approval no. XYYYYE20240197). A total of 20 male BALB/c nude mice (aged 4–6 weeks) were purchased from Wuhan Zi Keheng Biotechnology Co., Ltd. All mice were housed in a specific pathogen-free facility at $22\pm 2^{\circ}\text{C}$ and $50\pm 10\%$ relative humidity, with a 12-h light/dark cycle and free access to sterile food and water.

Mice were randomly divided into two groups ($n=10$ per group). A total of 1×10^5 control (si-NC transfected) or KIF4A-knockdown KYSE-150 cells resuspended in $100 \mu\text{l}$ sterile PBS were subcutaneously injected into the right flank of each mouse. Tumor volume was measured every 3 days using a vernier caliper and calculated with the formula: $\text{Volume}=(\text{length} \times \text{width}^2)/2$.

Mice were euthanized 5 weeks after injection via inhalation of an overdose of carbon dioxide at a precise flow rate of 30% chamber volume per minute for at least 5 min, followed by cervical dislocation to confirm death. Subcutaneous xenograft tumors were excised, weighed and subjected to immunohistochemical staining with Ki-67 rabbit polyclonal antibody (catalog no. GB121141; Wuhan Servicebio Technology Co., Ltd.).

Nuclear-cytoplasmic fractionation assay. The subcellular distribution of β -catenin was detected by nuclear-cytoplasmic fractionation using a Nuclear and Cytoplasmic Protein Extraction Kit (cat. no. G3741-50T; Wuhan Servicebio Technology Co., Ltd.) following the manufacturer's standard protocol. In brief, TE-1 cells transfected with siRNA-NC or si-KIF4A were harvested, washed with ice-cold PBS, and resuspended in pre-chilled cytoplasmic extraction buffer supplemented with protease inhibitor cocktail. After incubation on ice for 15 min, samples were centrifuged at $12,000 \times g$ for 5 min at 4°C , and the supernatant was collected as the cytoplasmic protein fraction. The remaining nuclear pellets were resuspended in nuclear extraction buffer, incubated on ice for 30 min with intermittent vortexing, and centrifuged at $12,000 \times g$ for 10 min at 4°C to obtain nuclear protein extracts. β -actin and histone H3 were used as specific loading controls for cytoplasmic and nuclear fractions, respectively, and the protein levels of β -catenin in both fractions were determined by western blotting to evaluate the effect of KIF4A silencing on β -catenin nuclear translocation. Semi-quantitative grayscale analysis of protein bands was performed using ImageJ software (version 1.53t; National Institutes of Health).

Wnt/ β -catenin pathway rescue assay. Rescue experiments were performed to verify that KIF4A exerts its oncogenic effects via the Wnt/ β -catenin signaling pathway, using Wnt agonist 1 as a specific activator of the canonical Wnt pathway. Wnt agonist 1 was purchased from Selleck Chemicals (cat. no. S8178), dissolved in DMSO to prepare a 10 mM stock solution, and stored at -20°C protected from light. An equal volume of DMSO was added to the control groups to eliminate solvent interference. TE-1 cells were allocated into four experimental groups: Negative control group, KIF4A-knockdown group, Wnt agonist 1 single-treatment group and KIF4A-knockdown + Wnt agonist 1 combined-treatment group. For the combined treatment, Wnt agonist 1 was added to the culture medium at a final concentration of $10 \mu\text{M}$ at 6 h after siRNA transfection, and cells were continuously incubated for 48 h before subsequent detection. The protein expression level of β -catenin in each group was determined by western blotting to confirm the molecular activation effect of the agonist. Cell proliferation capacity was assessed via EdU incorporation assay. Briefly, cells in each group were incubated with EdU working solution for 2 h, then fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and subjected to fluorescent staining following the manufacturer's protocol. Nuclei were counterstained with DAPI, and the proportion of EdU-positive cells was quantified under a fluorescence microscope to evaluate cell proliferation activity. Cell migration capacity was evaluated by wound healing assay. Cells from each group were seeded in 6-well plates and cultured until reaching 90–100%

confluence. A uniform linear wound was generated across the cell monolayer using a sterile 200- μ l pipette tip, and floating cells were removed by gentle washing with pre-warmed PBS. Cells were then cultured in serum-free medium, and microscopic images of the identical wound regions were captured at 0 and 24 h after scratching. The wound closure rate was calculated to compare the migration ability among different groups.

Statistical analysis. All *in vitro* experiments were performed in triplicate with three independent biological replicates. Statistical analysis was performed using GraphPad Prism 8.0 (Dotmatics). For normally distributed data, values are presented as the mean $\pm$ standard deviation; comparisons between two independent groups were performed using two-sided independent samples Student's t-test; paired comparisons, such as tumor and matched adjacent non-tumor tissues from the same patient, were analyzed using paired Student's t-test; and pairwise comparisons among multiple groups were performed using one-way analysis of variance followed by Tukey's post hoc test. For non-normally distributed categorical data, such as immunohistochemical scoring results, data are presented as the median (interquartile range); the Mann-Whitney U test was used for comparisons between two independent groups; and the Wilcoxon signed-ranks test was used for paired comparisons. For categorical variables in contingency tables, including clinicopathological characteristics of patients, between-group comparisons were analyzed using two-tailed Fisher's exact test. Survival analysis was performed using the Kaplan-Meier method with log-rank test. The correlation between gene expression levels was analyzed using Pearson correlation coefficient. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

KIF4A is highly expressed in ESCC and is associated with poor prognosis. To explore the expression and genomic alteration patterns of KIF4A in human malignancies, the TCGA pan-cancer dataset was first analyzed. As shown in Fig. 1A, KIF4A mRNA was widely upregulated in 32 out of 33 cancer types, including esophageal carcinoma. Fig. 1B further presents the frequency of genomic alterations of KIF4A (including amplification, mutation and deep deletion) across these cancer types, providing genomic-level evidence for the aberrant activation of KIF4A in tumors. KIF4A expression was tightly regulated by copy number variation, with gene amplification associated with upregulated mRNA expression, and deep deletion associated with transcriptional repression (Fig. 1C).

Further analysis of TCGA ESCC dataset revealed that KIF4A expression was significantly higher in primary ESCC tissues than in adjacent normal esophageal tissues (Fig. 1D). KIF4A expression increased progressively from stage I to stage II, and remained elevated in advanced stages, indicating its potential role in early ESCC tumorigenesis (Fig. 1E). Kaplan-Meier survival analysis based on the TCGA ESCA dataset via the UALCAN platform showed that high KIF4A expression was significantly associated with poorer 5-year overall survival rate in patients with esophageal carcinoma (log-rank $P = 0.045$; Fig. 1F).

KIF4A expression was further validated in the 43-pair clinical ESCC cohort. IHC staining further validated the tumor-specific upregulation of KIF4A in ESCC tissues (Fig. 1G). Western blot analysis confirmed that KIF4A protein was significantly upregulated in ESCC tissues compared with in paired adjacent non-malignant tissues (Fig. 1H). Clinicopathological analysis showed that high KIF4A expression was closely associated with lymph node metastasis and advanced TNM stage (46) in patients with ESCC (Table SI). These results indicated that KIF4A is upregulated in ESCC and may serve as a promising prognostic biomarker.

KIF4A knockdown inhibits ESCC cell proliferation and migration *in vitro*. Consistent with the clinical tissue findings, KIF4A protein expression was first detected in a panel of ESCC cell lines (KYSE-150, KYSE-180, TE-10 and TE-1) and the normal esophageal epithelial cell line HET-1A. The results showed that KIF4A was highly expressed in all ESCC cell lines, with the highest expression in TE-1 and KYSE-150 cells, which were selected for subsequent loss-of-function assays (Fig. 2A).

TE-1 and KYSE-150 cells were transfected with four different KIF4A siRNAs respectively. Western blot analysis confirmed effective knockdown of KIF4A protein expression, among which si-KIF4A-1 showed the highest efficiency and was used for subsequent experiments (Fig. 2B). The EdU staining assay showed that KIF4A knockdown significantly impaired the proliferative capacity of ESCC cells (Fig. 2D). The colony formation assay further confirmed that KIF4A silencing resulted in fewer and smaller colonies in both TE-1 and KYSE-150 cells (Fig. 2F). The wound healing assay showed that KIF4A knockdown significantly reduced the migratory capacity of ESCC cells (Fig. 2C), which was further validated by Transwell migration assay (Fig. 2E). Together, these results demonstrated that KIF4A knockdown inhibits the proliferation and migration of ESCC cells *in vitro*.

KIF4A overexpression promotes ESCC cell proliferation and migration *in vitro*. Conversely, to further validate the oncogenic role of KIF4A in ESCC, a KIF4A overexpression model was established in KYSE-150 cells, a classic well-characterized ESCC cell line with stable transfection efficiency and consistent genetic background with the knockdown model to ensure result comparability. The overexpression efficiency confirmed via western blotting (Fig. 3A). The EdU staining assay showed that KIF4A overexpression significantly enhanced the proliferative capacity of ESCC cells (Fig. 3C). The colony formation assay showed that KIF4A-overexpressing cells formed significantly more and larger colonies than control cells (Fig. 3E). Furthermore, the wound healing and Transwell migration assays consistently showed that KIF4A overexpression significantly accelerated the migration of ESCC cells (Fig. 3B and D). These results confirmed that KIF4A overexpression promotes the proliferation and migration of ESCC cells *in vitro*.

Identification of the Wnt/ β -catenin pathway as a downstream target of KIF4A. Having confirmed the oncogenic phenotypes of KIF4A *in vitro*, the current study next explored its downstream regulatory mechanism by performing RNA

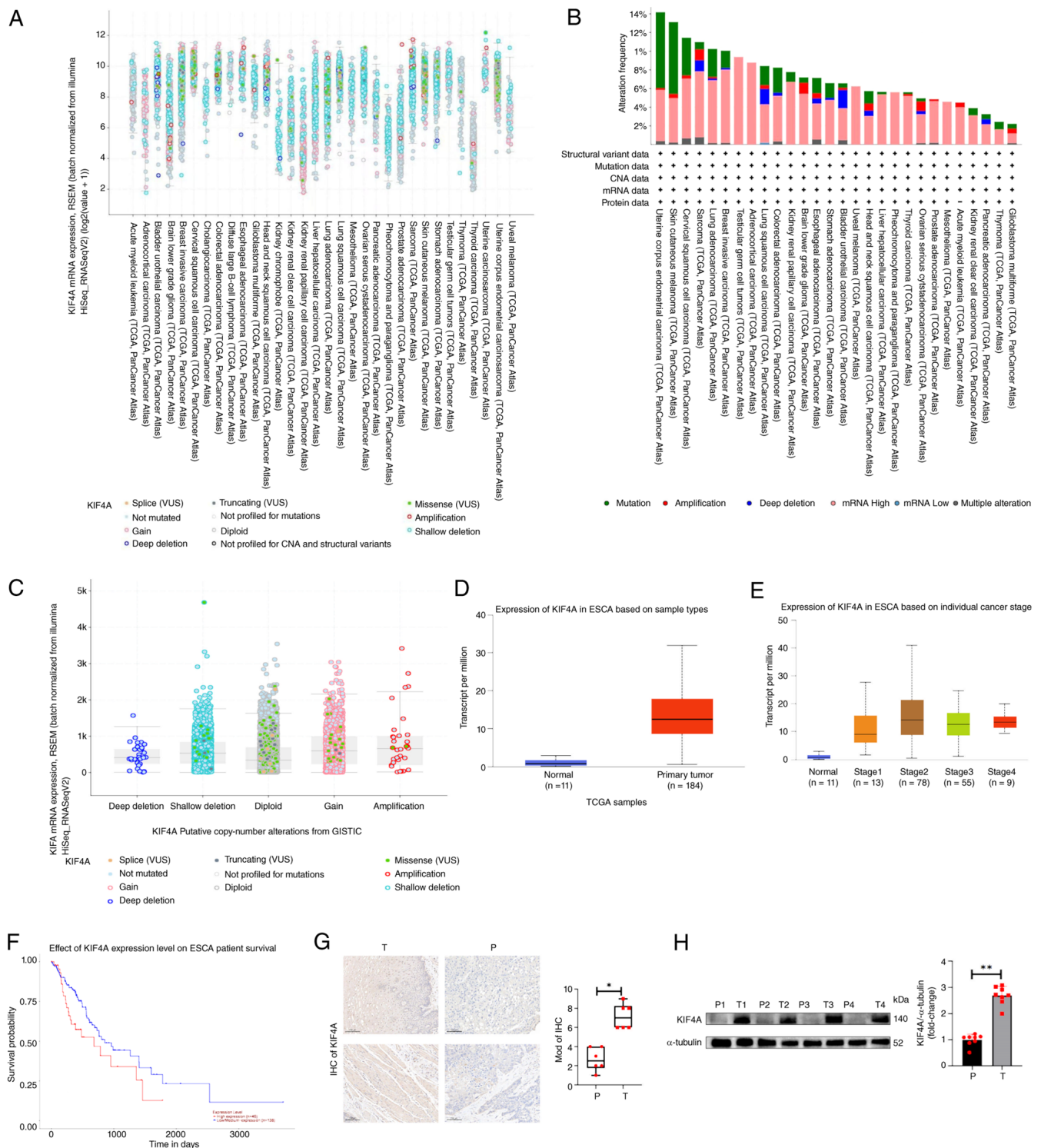


Figure 1. KIF4A is highly expressed in ESCC and is associated with poor prognosis. (A) mRNA expression levels of KIF4A across multiple cancer types from TCGA pan-cancer dataset (cBioPortal). (B) Genomic variant characteristics of KIF4A across multiple cancer types (cBioPortal). (C) Association between KIF4A copy number variation and its mRNA expression (cBioPortal). (D) KIF4A mRNA expression in normal esophageal tissues and primary ESCC tissues. (E) KIF4A expression across different tumor stages in ESCC (UALCAN). (F) Kaplan-Meier survival analysis of overall survival in patients with ESCC stratified by KIF4A expression (UALCAN, log-rank $P=0.045$). (G) IHC staining of KIF4A in ESCC and adjacent non-malignant tissues, with semi-quantitative statistical analysis. Scale bar, 100 μm , MOD, semi-quantitative immunohistochemical score, calculated as the product of staining intensity score and positive cell proportion score. (H) Western blot analysis of KIF4A protein expression in four paired ESCC (T) and adjacent non-malignant (P) tissues. * $P<0.05$, ** $P<0.01$. ESCC, esophageal squamous cell carcinoma; ESCA, esophageal carcinoma; IHC, immunohistochemistry; KIF4A, kinesin family member 4A; TCGA, The Cancer Genome Atlas.

sequencing on KIF4A-knockdown and control TE-1 cells. A total of 11,068 coexpressed genes were identified in both groups (Fig. 4A). Differential expression analysis identified

1,697 significant DEGs, including 647 upregulated and 1,050 downregulated genes in the KIF4A-knockdown group (Fig. 4B and C).

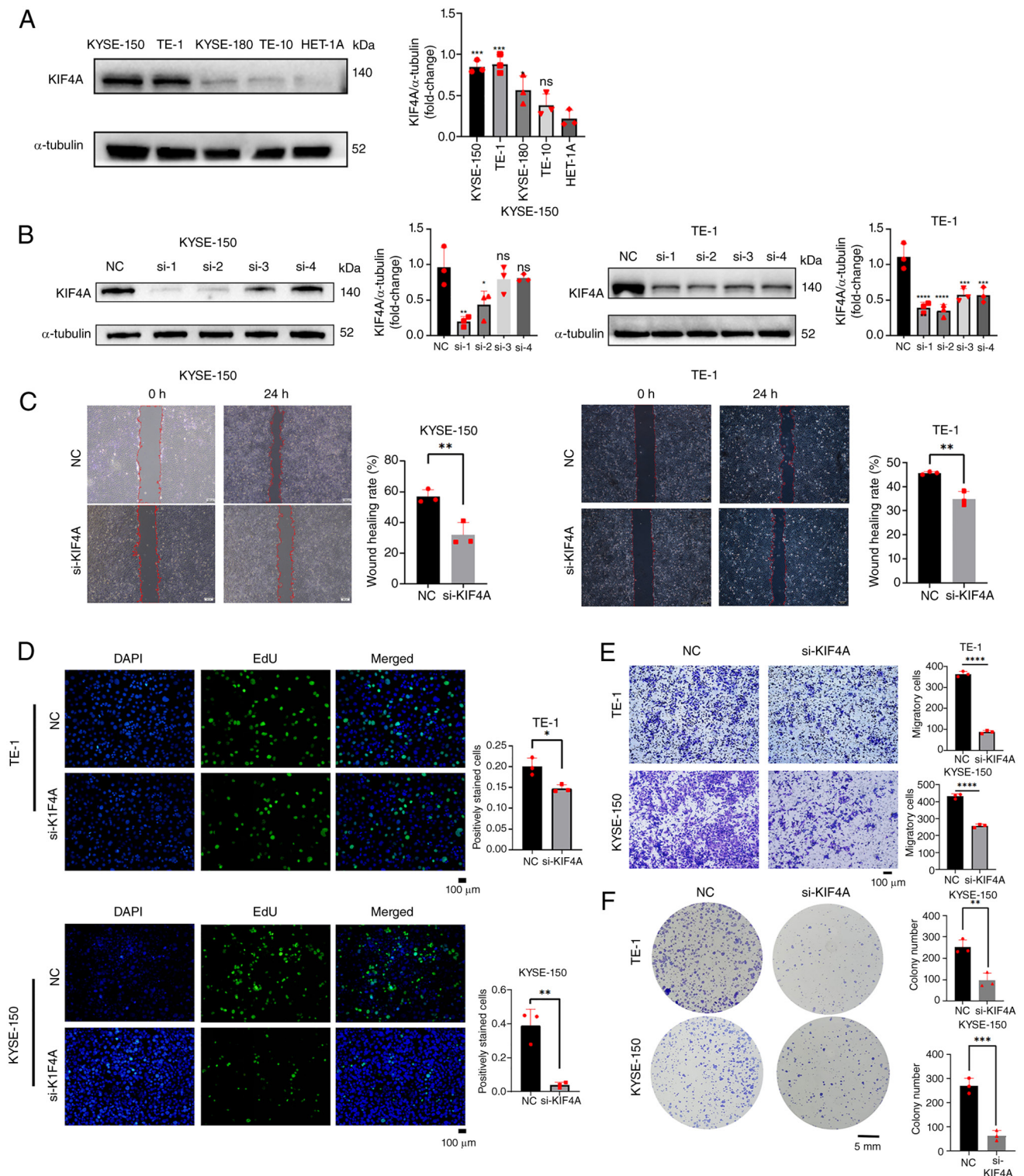


Figure 2. KIF4A knockdown inhibits ESCC cell proliferation and migration *in vitro*. (A) Western blot analysis of KIF4A protein expression in ESCC cell lines (KYSE-150, KYSE-180, TE-10, TE-1) and the normal esophageal epithelial cell line HET-1A. (B) Western blot analysis of KIF4A knockdown efficiency in TE-1 and KYSE-150 cells transfected with KIF4A siRNAs. (C) Wound healing assay evaluating the effect of KIF4A knockdown on ESCC cell migration, with quantitative analysis of wound healing rate. Scale bar, 200 μ m. (D) EdU staining assay evaluating the effect of KIF4A knockdown on ESCC cell proliferation, with quantitative analysis of EdU-positive cell rate. Scale bar, 100 μ m. (E) Transwell migration assay evaluating the effect of KIF4A knockdown on ESCC cell migration, with quantitative analysis of the number of migratory cells. Scale bar, 100 μ m. (F) Colony formation assay evaluating the effect of KIF4A knockdown on ESCC cell clonogenic capacity. Scale bar, 5 mm. * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001 vs. the NC group; ns, not significant. ESCC, esophageal squamous cell carcinoma; KIF4A, kinesin family member 4A; NC, negative control; si, small interfering RNA.

Gene Ontology (GO) enrichment analysis showed that the downregulated DEGs were mainly enriched in the following biological process terms: 'Regulation of transcription,

DNA-templated', 'protein complex assembly' and 'regulation of cell cycle' (Fig. 4D). KEGG pathway enrichment analysis of down-regulated DEGs revealed that the 'basal cell carcinoma' pathway,

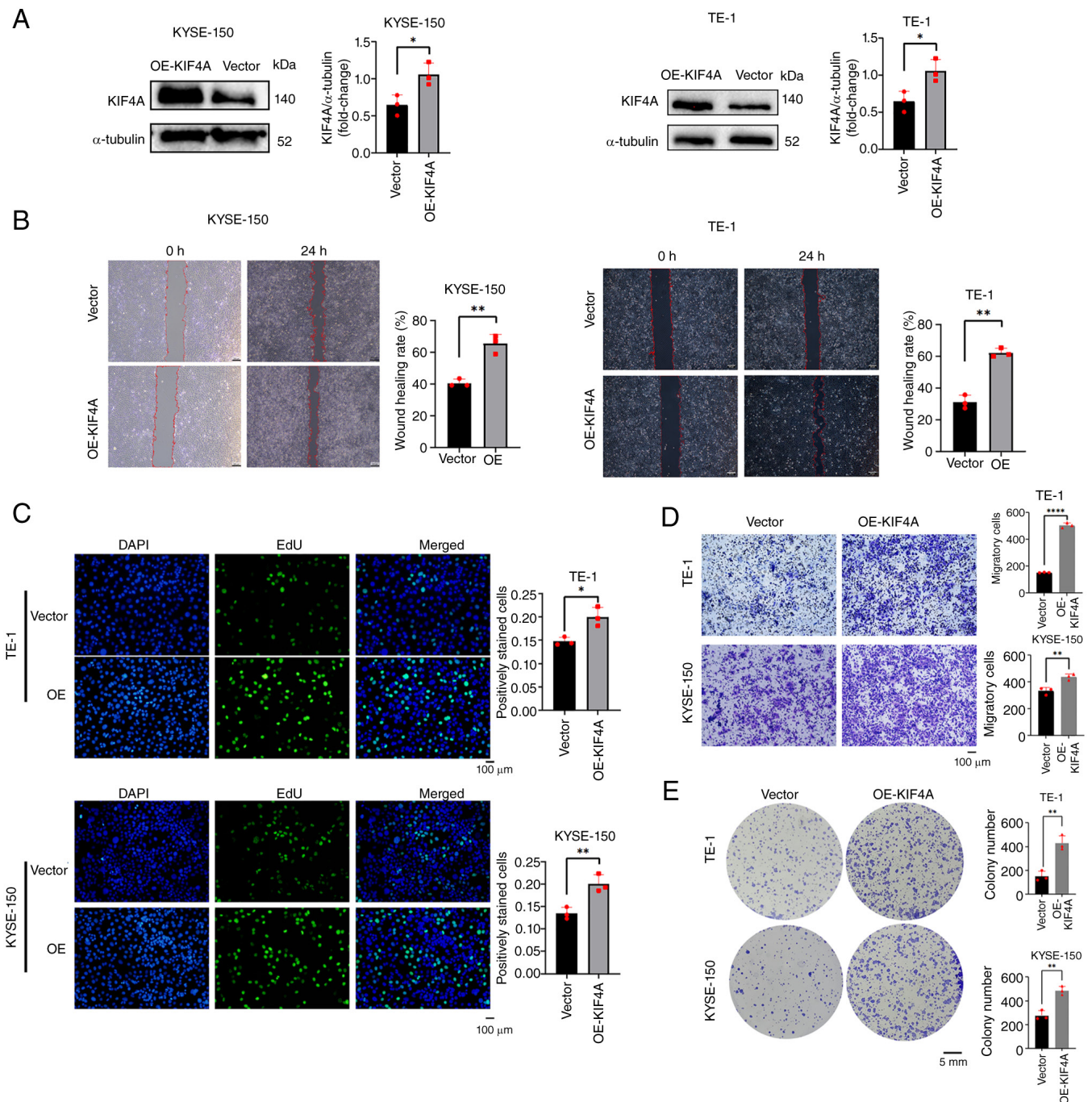


Figure 3. KIF4A OE promotes ESCC cell proliferation and migration *in vitro*. (A) Western blot analysis of KIF4A OE efficiency in KYSE150 cells. (B) Wound healing assay evaluating the effect of KIF4A overexpression on ESCC cell migration, with quantitative analysis of wound healing rate. Scale bar, 200 μ m. (C) EdU staining assay evaluating the effect of KIF4A overexpression on ESCC cell proliferation, with quantitative analysis of EdU-positive cell rate. Scale bar, 100 μ m. (D) Transwell migration assay evaluating the effect of KIF4A overexpression on ESCC cell migration, with quantitative analysis of the number of migratory cells. Scale bar, 100 μ m. (E) Colony formation assay evaluating the effect of KIF4A overexpression on ESCC cell clonogenic capacity. Scale bar, 5 mm. * P <0.05, ** P <0.01, **** P <0.0001. ESCC, esophageal squamous cell carcinoma; KIF4A, kinesin family member 4A; OE, overexpression.

which is tightly associated with the canonical Wnt signaling cascade, was among the top enriched pathways (Fig. 4E) (38,40). Key components of the Wnt/ β -catenin pathway, including WNT2B, WNT5B, WNT8B, TCF7 and FZD2, were significantly downregulated upon KIF4A knockdown. Heatmap visualization further confirmed that most Wnt pathway-related genes were positively associated with KIF4A expression (Fig. 4F). These results indicated that KIF4A may exert its oncogenic effects in ESCC by regulating the Wnt/ β -catenin signaling pathway.

KIF4A activates the Wnt/ β -catenin pathway by stabilizing the β -catenin-TCF7 complex. To validate the regulatory effect of KIF4A on the Wnt/ β -catenin pathway, western blot analysis was performed. The results showed that KIF4A knockdown significantly reduced the protein expression of β -catenin and TCF7 in ESCC cells (Fig. 5A). In paired clinical ESCC specimens, the protein levels of KIF4A, β -catenin and TCF7 were consistently elevated in tumor tissues compared with adjacent normal tissues (Fig. 5B),

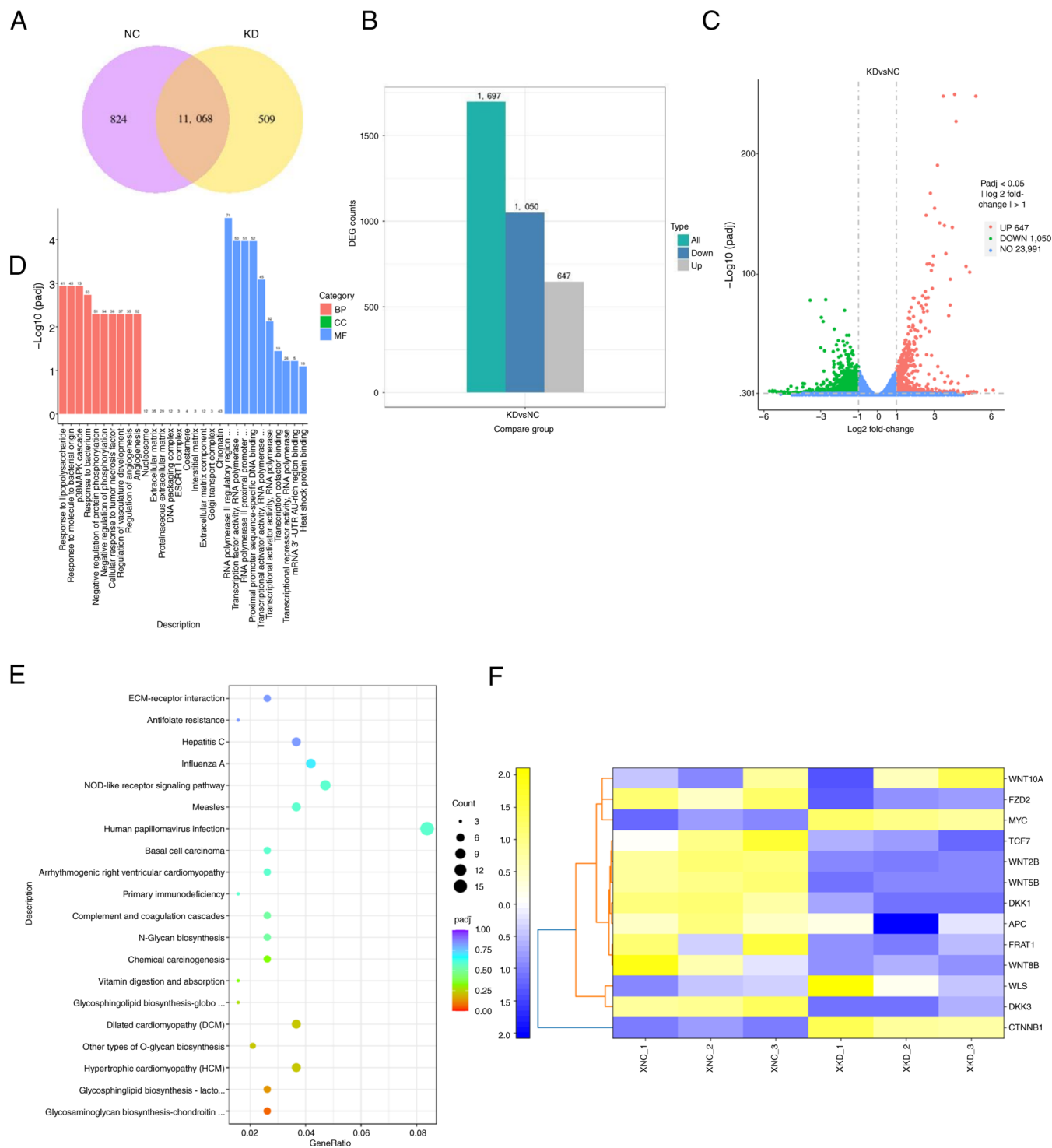


Figure 4. RNA sequencing identifies the Wnt/ β -catenin pathway as a downstream target of KIF4A. (A) Venn diagram of coexpressed genes in KIF4A-KD and NC TE-1 cells. (B) Histogram and (C) volcano plot of DEGs between the two groups. (D) GO enrichment analysis of downregulated DEGs upon KIF4A KD. (E) KEGG pathway enrichment analysis of downregulated DEGs upon KIF4A KD. (F) Heatmap of Wnt/ β -catenin pathway-related genes among the DEGs. BP, biological process; CC, cellular component; DEG, differentially expressed gene; GO, Gene Ontology; KD, knockdown; KEGG, Kyoto Encyclopedia of Genes and Genomes; KIF4A, kinesin family member 4A; MF, molecular function; NC, negative control.

which further corroborated the positive association between KIF4A and Wnt pathway activation at the clinical level. RT-qPCR analysis further confirmed that KIF4A silencing significantly downregulated the mRNA expression levels of β -catenin, TCF7, and the canonical Wnt pathway downstream target genes, including AXIN2, MMP-7, cyclin D1 and c-Myc (Fig. 5C).

Correlation analysis using TCGA ESCC dataset showed that KIF4A expression was positively but weakly correlated with the expression of β -catenin ($r=0.39$, $P=4.1 \times 10^{-8}$) and TCF7 ($r=0.27$, $P=0.00026$) in ESCC tissues (Fig. 5D). A co-IP assay confirmed that KIF4A directly interacted with TCF7 in ESCC cells (Fig. 5E). Furthermore, the co-IP assay showed that KIF4A knockdown markedly

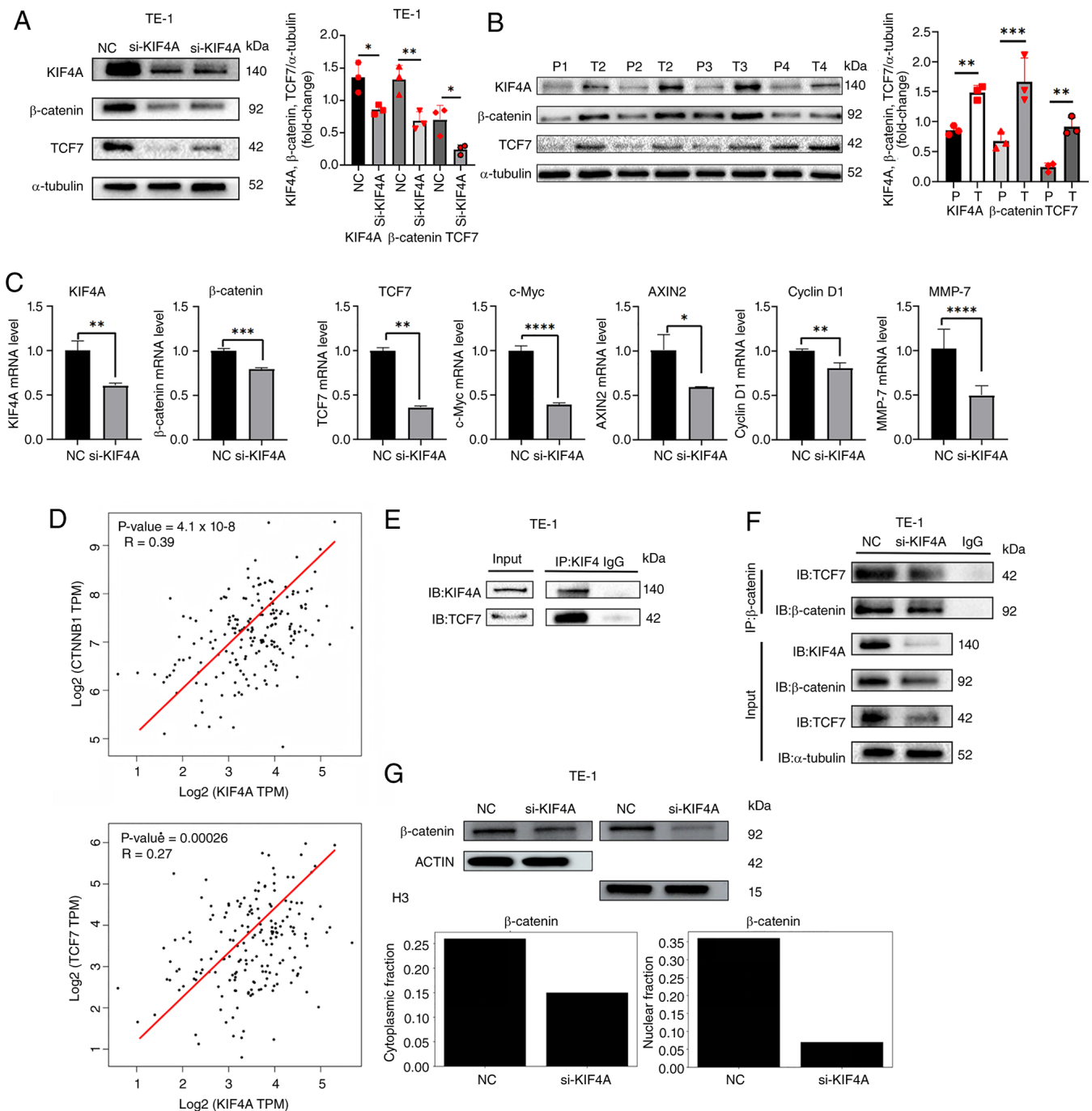


Figure 5. KIF4A activates the Wnt/β-catenin pathway by stabilizing the β-catenin-TCF7 complex. Western blot analysis of β-catenin and TCF7 protein expression in (A) KIF4A-knockdown ESCC cells and (B) paired ESCC/adjacent tissues. (C) Reverse transcription-quantitative PCR analysis of the mRNA expression levels of Wnt/β-catenin pathway components and downstream target genes in KIF4A-knockdown ESCC cells. (D) Pearson correlation analysis between KIF4A and CTNNB1/TCF7 expression in ESCC tissues (The Cancer Genome Atlas dataset). (E) Co-IP assay confirming the direct interaction between KIF4A and TCF7 in TE-1 cells. (F) Co-IP assay showing that KIF4A knockdown disrupts the interaction between β-catenin and TCF7 in TE-1 cells. (G) Relative protein levels of β-catenin in nuclear and cytoplasmic fractions of ESCC cells with or without KIF4A silencing. The nuclear-to-cytoplasmic ratio of β-catenin was markedly reduced upon KIF4A knockdown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs. the NC group. CTNNB1, β-catenin; ESCC, esophageal squamous cell carcinoma; IB, immunoblot; IP, immunoprecipitation; KIF4A, kinesin family member 4A; NC, negative control; si, small interfering; TCF7, T-cell factor 7; T, tumor tissue; P, peritumoral normal tissue.

disrupted the interaction between β-catenin and TCF7 in ESCC cells (Fig. 5F). Nuclear-cytoplasmic fractionation showed that KIF4A knockdown markedly reduced nuclear β-catenin (5.1-fold downregulation) and moderately decreased cytoplasmic β-catenin (1.7-fold reduction). The nuclear-to-cytoplasmic ratio of β-catenin was decreased by 65.9% upon KIF4A silencing, indicating that KIF4A

promotes β-catenin nuclear translocation and canonical Wnt pathway activation (Fig. 5G). Based on these findings, it may be hypothesized that KIF4A facilitates activation of the Wnt/β-catenin pathway, at least in part, by stabilizing the β-catenin-TCF7 transcription complex via direct interaction with TCF7. However, the possibility that KIF4A may also indirectly modulate this complex through other intermediate

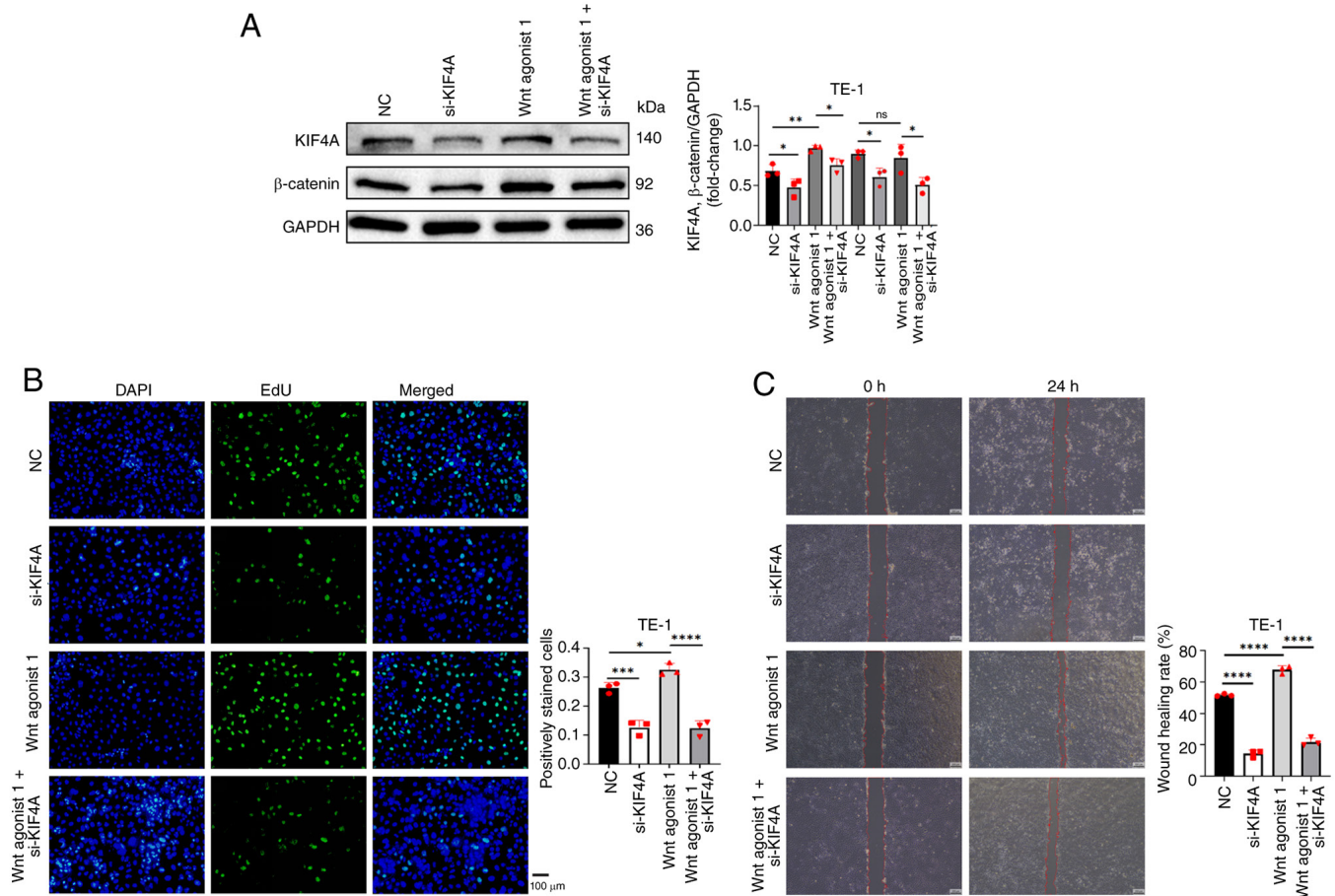


Figure 6. KIF4A is essential for the pro-tumor effects of Wnt/ β -catenin pathway activation. (A) Western blot analysis of β -catenin expression in KIF4A-knockdown TE-1 cells treated with Wnt agonist 1. (B and C) EdU staining assay and Wound healing assay showed that Wnt agonist 1 markedly enhanced the proliferation and migration abilities of control cells, but could not significantly reverse the inhibitory effects induced by KIF4A knockdown. Scale bar, 100 μ m (EdU staining assay); scale bar, 200 μ m (wound healing assay). * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. KIF4A, kinesin family member 4A; NC, negative control; ns, not significant; si, small interfering.

proteins or post-translational modifications cannot be excluded; therefore, this requires further validation.

KIF4A is essential for the pro-tumor effects of Wnt/ β -catenin pathway activation. To further confirm the functional association between KIF4A and the Wnt/ β -catenin pathway, rescue experiments were performed using a Wnt pathway agonist (Wnt agonist 1). Western blot analysis showed that Wnt agonist 1 significantly upregulated β -catenin expression in control TE-1 cells, but failed to effectively restore β -catenin protein level in KIF4A-knockdown cells; no statistically significant difference was detected between the si-KIF4A group and the Wnt agonist 1 + si-KIF4A group (Fig. 6A). Functional assays showed consistent results: Wnt agonist 1 markedly enhanced the proliferation and migration abilities of control cells, but could not significantly reverse the inhibitory effects induced by KIF4A knockdown (Fig. 6B and C). These results demonstrated that KIF4A is an essential regulator required for full activation of the canonical Wnt/ β -catenin signaling pathway, and thus drives the malignant phenotype of ESCC cells.

KIF4A promotes ESCC xenograft tumor growth in vivo. To validate the *in vivo* oncogenic role of KIF4A, a subcutaneous

xenograft tumor model was established in nude mice. The results showed that KIF4A knockdown significantly inhibited the growth of ESCC xenograft tumors, with markedly reduced tumor volume and weight in the KIF4A-knockdown group compared with the control group (Fig. 7A-C). Furthermore, IHC staining showed that the expression of Ki-67, a proliferation marker, was significantly reduced in xenograft tumors from the KIF4A-knockdown group (Fig. 7E). In addition, IHC staining of ESCC patient-derived organoids showed that KIF4A expression was positively associated with tumor stage progression (Fig. 7D). These results confirmed that KIF4A promotes ESCC tumorigenesis *in vivo*.

Discussion

ESCC is the predominant histological subtype of EC in China, with high morbidity and mortality rates, and limited targeted therapeutic options (47,48). The identification of novel prognostic biomarkers and therapeutic targets is critical to improve the clinical outcomes of patients with ESCC. In the present study, the expression, prognostic value, biological functions and molecular mechanisms of KIF4A were systematically investigated in ESCC. The findings demonstrated that KIF4A was upregulated in ESCC,

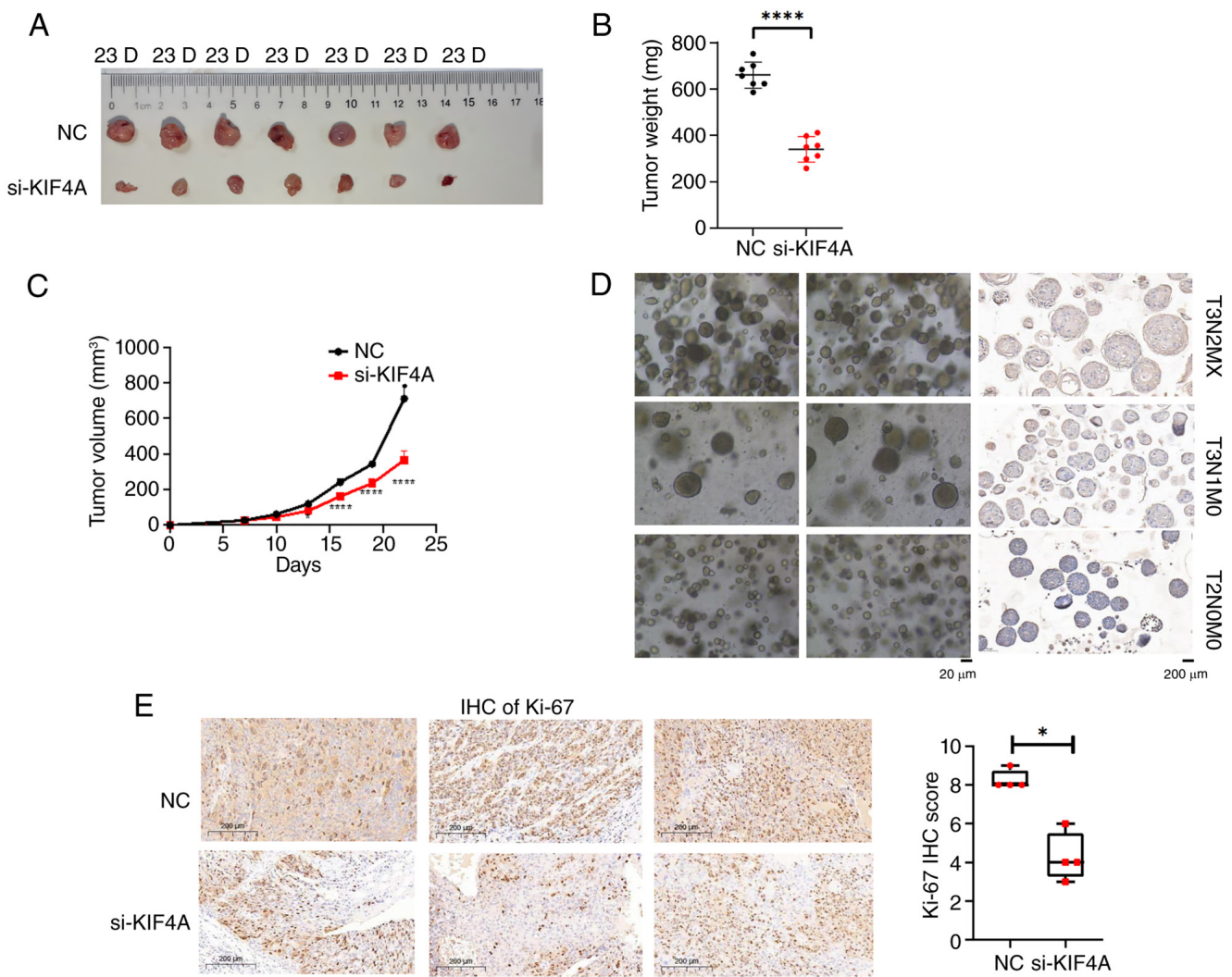


Figure 7. KIF4A promotes ESCC xenograft tumor growth *in vivo*. (A) Representative images of subcutaneous xenograft tumors from the control and KIF4A-knockdown groups. (B) Quantitative analysis of tumor weight in the two groups. (C) Tumor growth curves of the two groups. (D) IHC staining of KIF4A in ESCC patient-derived organoids from different tumor stages. Scale bar, 20 μ m. (E) IHC staining of Ki-67 in xenograft tumor tissues from the two groups. Scale bar, 200 μ m. * P <0.05, **** P <0.0001. ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry; KIF4A, kinesin family member 4A; NC, negative control; si, small interfering.

predicted a poor prognosis, and drove ESCC cell proliferation and migration by activating the canonical Wnt/ β -catenin signaling pathway.

In terms of molecular function, KIF4A is a member of the kinesin superfamily, which serves critical roles in mitotic progression and intracellular transport (7,8,13,49). Previous studies have validated the oncogenic roles of KIF4A in multiple malignancies. For example, KIF4A drives hepatocellular carcinoma proliferation by activating the Akt signaling pathway (13), promotes endometrial cancer progression by regulating TPX2 protein degradation (11), and enhances colorectal cancer cell invasion by upregulating MMP2/MMP9 expression (17). KIF4A was identified in our previous study (50) as a prognostic biomarker for ESCC, but its functional mechanisms in ESCC remain largely unknown. In the current study, it was confirmed that KIF4A was significantly upregulated in ESCC tissues and cell lines, and its high expression was associated with advanced tumor stage and poor overall survival in patients with ESCC. Loss- and gain-of-function assays demonstrated that KIF4A may exert strong oncogenic effects,

promoting ESCC cell proliferation, migration and *in vivo* xenograft tumor growth, which is consistent with its oncogenic roles in other malignancies.

Moving beyond functional phenotypes, the current study further elucidated the downstream regulatory mechanism of KIF4A in ESCC via RNA sequencing analysis, which revealed that the Wnt/ β -catenin signaling pathway was the key downstream target of KIF4A. The canonical Wnt/ β -catenin pathway is a well-characterized oncogenic cascade, which is aberrantly activated in 30-50% of ESCC cases, and is associated with tumor progression, therapeutic resistance and poor prognosis (34,35). In the canonical Wnt pathway, stabilized β -catenin translocates to the nucleus, forms a transcription complex with TCF/LEF family members, and drives the expression of pro-oncogenic target genes, such as c-Myc, cyclin D1 and MMP-7 (51,52). In the present study, it was demonstrated that KIF4A knockdown significantly downregulated the expression of β -catenin, TCF7 and the downstream target genes of the Wnt/ β -catenin pathway, whereas KIF4A overexpression exerted the opposite effects. Rescue experiments further demonstrated that the

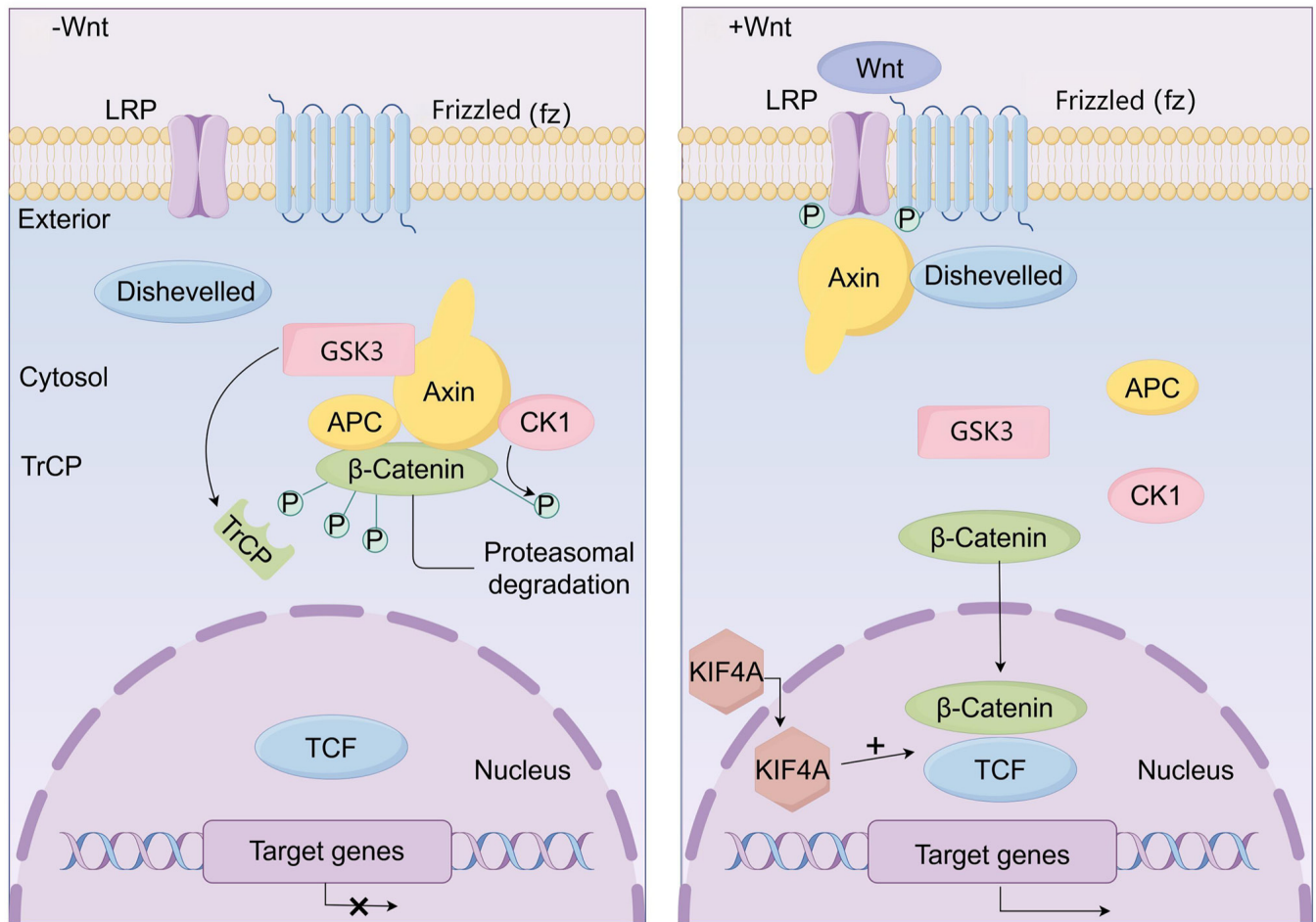


Figure 8. Schematic diagram of the mechanism by which KIF4A drives esophageal squamous cell carcinoma progression via activating the canonical Wnt/ β -catenin signaling pathway. KIF4A stabilizes the β -catenin-TCF7 transcription complex via direct interaction with TCF7, thereby enhancing the transcriptional activity of the Wnt/ β -catenin pathway and promoting the expression of downstream pro-oncogenic target genes, ultimately driving ESCC cell proliferation, migration and tumorigenesis. KIF4A, kinesin family member 4A; TCF, T-cell factor. APC, adenomatous polyposis coli; CK1, casein kinase 1; GSK3, glycogen synthase kinase 3; LRP, low-density lipoprotein receptor-related protein; TrCP, β -transducin repeat-containing protein.

pro-proliferative and pro-migratory effects of Wnt/ β -catenin pathway activation are largely dependent on KIF4A expression in ESCC cells., indicating that KIF4A drives ESCC progression via activating the Wnt/ β -catenin pathway.

Notably, the mRNA-level correlation between KIF4A and core Wnt pathway molecules (β -catenin and TCF7) in the TCGA ESCC cohort was at a weak-to-moderate level. This is consistent with the universal characteristics of solid tumor transcriptome studies: Clinical tumor tissues are highly heterogeneous and influenced by multiple confounding factors, including individual genetic background, tumor microenvironment, pathological stage and differentiation status. In solid tumor research, where intratumor heterogeneity may dilute correlation signals observed from single-biopsy sampling, modest correlation coefficients with statistical significance may still reflect biologically relevant relationships, although the threshold for 'biological meaning' remains context-dependent and requires independent validation (53,54). Mechanistically, KIF4A, as a kinesin family protein, regulates the Wnt/ β -catenin pathway mainly at the post-translational level rather than directly driving the transcription of CTNNB1 and TCF7.

Notably, the present study further revealed the mechanistic basis of KIF4A-mediated Wnt pathway activation. The co-IP

assay confirmed that KIF4A directly interacted with TCF7, and KIF4A knockdown disrupted the interaction between β -catenin and TCF7. As summarized in Fig. 8, these findings collectively support a model in which KIF4A stabilizes the β -catenin-TCF7 transcription complex via direct binding to TCF7, thus enhancing the transcriptional output of the Wnt/ β -catenin pathway. Notably, this mechanism does not rule out potential indirect regulatory effects mediated by additional cofactors, and the exact structural basis underlying this regulation remains to be fully elucidated. This finding provides novel insights into the regulatory mechanism of the Wnt/ β -catenin pathway in ESCC, and establishes the KIF4A-Wnt/ β -catenin axis as a key oncogenic cascade in ESCC progression.

From a clinical perspective, the current study has two important translational values for ESCC management. First, KIF4A was validated as a promising prognostic biomarker for ESCC. High KIF4A expression was closely associated with advanced tumor stage and poor overall survival, which may help clinicians stratify high-risk ESCC patients at initial diagnosis, optimize personalized follow-up strategies and guide adjuvant treatment decisions. Second, the KIF4A-Wnt/ β -catenin axis was identified as a potential therapeutic target for ESCC. Small-molecule inhibitors targeting KIF4A, such

as WZ-3146, have shown significant antitumor efficacy in preclinical glioma models (20), which provides a preclinical basis for the development of KIF4A-targeted therapies for ESCC. For patients with ESCC, and with KIF4A upregulation and aberrant Wnt/ β -catenin activation, combination therapy with KIF4A inhibitors and Wnt pathway inhibitors may improve therapeutic efficacy and overcome drug resistance, which warrants further preclinical and clinical investigation. However, the clinical translation of KIF4A inhibitors faces potential specificity challenges. Given that KIF4A is also expressed in actively dividing normal cells and shares structural homology with other kinesin family members, off-target effects on normal proliferative tissues and non-specific inhibition of other kinesins may occur. Further structural optimization of the ATP-binding pocket of inhibitors and the development of ESCC-specific delivery strategies (such as antibody-drug conjugates) are needed to minimize off-target toxicity in future studies.

The current study has several limitations that need to be addressed in future research. First, although the co-IP assay showed that KIF4A knockdown reduced the amount of TCF7 co-precipitated with β -catenin, we acknowledge that the total protein level of TCF7 in the input was also decreased after KIF4A silencing. Therefore, the reduced TCF7 in the immunoprecipitated complex cannot be solely attributed to the weakened interaction between β -catenin and TCF7; the downregulation of total TCF7 expression may also contribute to this observation. The exact effect of KIF4A on the binding affinity between β -catenin and TCF7, independent of total protein expression changes, needs to be further validated via *in vitro* pull-down assays with normalized protein input and yeast two-hybrid systems. Notably, the current work has not fully delineated whether KIF4A modulates β -catenin protein stability through regulating its ubiquitination degradation, and the *in situ* spatial colocalization of KIF4A with β -catenin/TCF7 in clinical ESCC tissues remains to be visualized at the single-cell level. These post-translational regulatory details and histological spatial evidence will be systematically investigated in our follow-up studies. Second, the prognostic value of KIF4A was validated in a single-center cohort, and further large-scale, multi-center clinical studies are needed to confirm its clinical application value. Third, the *in vivo* antitumor efficacy of KIF4A inhibitors needs to be validated in patient-derived xenograft models and ESCC organoid models to lay a more solid foundation for clinical translation.

In conclusion, the present study systematically indicated that KIF4A is significantly upregulated in ESCC, and its high expression is associated with advanced tumor stage and poor overall survival in patients with ESCC. Functional experiments confirmed that KIF4A can drive ESCC cell proliferation, migration and *in vivo* tumorigenesis. Mechanistically, the present findings suggested that KIF4A exerts its oncogenic effects in ESCC, at least in part, through activation of the canonical Wnt/ β -catenin signaling pathway, potentially via stabilization of the β -catenin-TCF7 transcription complex. These findings identify KIF4A as a promising prognostic biomarker and therapeutic target for ESCC, and provide novel insights into the molecular mechanisms of ESCC progression.

Acknowledgements

The authors would like to thank Professor Shanshan Liang (Zhongshan Hospital of Dalian University, Dalian, China) for providing technical guidance and support.

Funding

This work was supported by the Educational Commission of Hubei Province of China (grant no. B2023106), the Natural Science Foundation of Hubei Province for Young Scholars (grant no. 2022CFB930), the Natural Science Foundation of Hubei Province (grant no. 2026AFB087) and the Key Project of Science and Technology Bureau of Xiangyang City (grant no. 2022YL28A).

Availability of data and materials

The sequencing data generated in the present study may be found in the Open Archive for Miscellaneous Data (OMIX), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number OMIX018645 or at the following URL: <https://ngdc.cnbi.ac.cn/omix/preview/cWRNNvxO>. Access to the data is subject to restrictions due to privacy. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

LZ and XS conceptualized the study and designed the overall research framework. JC and TW developed the research methodology, including the design and optimization of experimental protocols for cellular function assays and molecular detection. JC and DW performed the formal data analysis and statistical interpretation. JC, TW and DW conducted the experimental investigations. WL, CL and YY participated in the conception and design of the clinical research protocol, performed the acquisition and pathological verification of clinical tissue specimens, analyzed and interpreted the clinical patient data, and critically reviewed and revised the clinical content of the manuscript for important intellectual content. They also provided essential research resources, including the clinical tissue biobank and experimental platform support. JC and DW drafted the original manuscript. LZ, XS, WL, CL and YY critically reviewed and edited the manuscript. LZ and XS supervised the whole study and acquired the research funding. LZ and XS confirm the authenticity of all the raw data, and WL, CL and YY confirm the authenticity and integrity of the clinical data and tissue specimens. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All studies involving human participants were reviewed and approved by the Institutional Review Board of Xiangyang No.1 People's Hospital (approval nos. 2025KY082 and 2025KY142). Written informed consent was obtained from all enrolled participants, and all experiments involving human tissues were performed in accordance with The Declaration of Helsinki. All animal experiments were reviewed and approved by the

Institutional Animal Care and Use Committee of Xiangyang No.1 People's Hospital (approval no. XYYYYE20240197), and performed in strict accordance with the ARRIVE guidelines and national guidelines for the care and use of laboratory animals.

Patient consent for publication

Written informed consent for the publication of clinical data, pathological images and related research results was obtained from all enrolled participants.

Competing interests

The authors declare that they have no competing interests.

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