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ORIGINAL ARTICLE

Basic Study

Role of deubiquitinase JOSD2 in the pathogenesis of esophageal squamous cell carcinoma

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Abstract

BACKGROUND

Esophageal squamous cell carcinoma (ESCC) is a deadly malignancy with limited treatment options. Deubiquitinases (DUBs) have been confirmed to play a crucial role in the development of malignant tumors. JOSD2 is a DUB involved in controlling protein deubiquitination and influencing critical cellular processes in cancer.

AIM

To investigate the impact of JOSD2 on the progression of ESCC.

METHODS

Bioinformatic analyses were employed to explore the expression, prognosis, and enriched pathways associated with JOSD2 in ESCC. Lentiviral transduction was utilized to manipulate JOSD2 expression in ESCC cell lines (KYSE30 and

KYSE150). Functional assays, including cell proliferation, colony formation, drug sensitivity, migration, and invasion, were performed, revealing the impact of JOSD2 on ESCC cell lines. JOSD2's role in xenograft tumor growth and drug sensitivity *in vivo* was also assessed. The proteins that interacted with JOSD2 were identified using mass spectrometry.

RESULTS

Preliminary research indicated that JOSD2 was highly expressed in ESCC tissues, which was associated with poor prognosis. Further analysis demonstrated that JOSD2 was upregulated in ESCC cell lines compared to normal esophageal cells. JOSD2 knockdown inhibited ESCC cell activity, including proliferation and colony-forming ability. Moreover, JOSD2 knockdown decreased the drug resistance and migration of ESCC cells, while JOSD2 overexpression enhanced these phenotypes. *In vivo* xenograft assays further confirmed that JOSD2 promoted tumor proliferation and drug resistance in ESCC. Mechanistically, JOSD2 appears to activate the MAPK/ERK and PI3K/AKT signaling pathways. Mass spectrometry was used to identify crucial substrate proteins that interact with JOSD2, which identified the four primary proteins that bind to JOSD2, namely USP47, IGKV2D-29, HSP90AB1, and PRMT5.

CONCLUSION

JOSD2 plays a crucial role in enhancing the proliferation, migration, and drug resistance of ESCC, suggesting that JOSD2 is a potential therapeutic target in ESCC.

Key Words: Esophageal squamous cell carcinoma; JOSD2; Ubiquitination; Biomarker; Targeted therapy; Drug resistance

Core Tip: JOSD2, a deubiquitinating enzyme, is a key player in the aggressive pathogenesis of esophageal squamous cell carcinoma (ESCC). Elevated JOSD2 expression in ESCC tissues is associated with poor prognosis. Functional analyses, including *in vivo* xenograft assays, highlight JOSD2's role in promoting tumor proliferation and drug resistance. Mechanistically, JOSD2 activates the MAPK/ERK and PI3K/AKT signaling pathways. Mass spectrometry identified key interacting proteins, including USP47, IGKV2D-29, HSP90AB1, and PRMT5. This study underscores the potential role of JOSD2 as a therapeutic target in ESCC.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) ranks among the deadliest malignancies worldwide, posing a significant public health concern[1]. Despite advances in early detection and therapeutic interventions, ESCC remains a formidable challenge due to its mostly late-stage diagnosis and limited treatment options. To explore the potential therapeutic targets of ESCC, there has been an increasing focus on the genetic underpinnings of this aggressive cancer. Deubiquitinases (DUBs), responsible for cleaving ubiquitin chains from their protein targets, are crucial for controlling protein ubiquitination and preserving protein homeostasis. DUBs influence important cellular processes such as tumor cell proliferation, drug resistance, distant metastasis, and immune evasion by stabilizing the expression of key cancer proteins[2-6]. The research on DUBs provides new avenues for developing treatment options for cancers lacking effective therapeutic strategies. However, the role of DUBs in malignant tumors is far from fully elucidated. Currently, an increasing number of small molecule inhibitors targeting DUBs are being developed and reported, with preclinical and clinical trials underway, demonstrating significant potential in this research field[7].

JOSD2, also known as Josephin domain-containing 2, is a member of the Machado-Joseph disease protein family. It consists of 188 amino acids and contains only one highly conserved catalytic Josephin domain, possessing enzymatic activity. Several recent studies have shed light on the involvement of JOSD2 in some malignant tumors[8-12]. JOSD2 has been found to interact with key signaling pathways, such as the Hippo pathway, Wnt/β-catenin pathway, and DNA repair mechanisms[8,9,11]. Dysregulation of JOSD2 expression has been implicated in cancer initiation, tumor growth, and resistance to chemotherapy[8,11]. There is a lack of relevant research on the association between JOSD2 and ESCC. The elucidation of the function of JOSD2 in ESCC will be helpful to identify individuals at higher risk and devise personalized treatment strategies. Therefore, we aimed to explore the function of JOSD2 in ESCC, shedding light on its potential as a promising avenue for further investigation and clinical applications.

MATERIALS AND METHODS

Analyses of JOSD2 expression, prognosis and enriched pathways in ESCC tissue

The University of Alabama at Birmingham CANcer database (https://ualcan.path.uab.edu/) was used to analyze the differential expression of JOSD2 mRNA between ESCC and normal esophageal tissues[13,14]. The online survival database Kaplan–Meier Plotter (https://kmplot.com/analysis/) was used to assess the impact of high *vs* low JOSD2 mRNA expression on the survival prognosis of patients with ESCC[15]. Clinical and RNA-seq data from ESCC patients were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/), and a nomogram predicting the 1-, 3- and 5-year survival probability of ESCC patients was constructed and visualized using the "survival" and "rms" packages in R (4.2.1).

The top 500 genes that have a similar expression pattern to JOSD2 in ESCC were downloaded from the Gene Expression Profiling Interactive Analysis 2 database (http://gepia2.cancer-pku.cn/#index) and listed in Supplementary Table 1. The Search Tool for the Retrieval of Interacting Genes/Proteins database (https://string-db.org/) was used to plot a protein-protein interaction (PPI) network of the top 100 genes associated with JOSD2.

Gene Ontology (GO) (Supplementary Table 2) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Supplementary Table 3) enrichment analyses of the top 500 genes that have a similar expression pattern to JOSD2 were conducted using the R packages "clusterProfiler", "enrichplot", and "ggplot2". The differentially expressed genes between the high and low JOSD2 expression groups based on TCGA database were determined using the R package "DESeq2" and listed in Supplementary Table 4. A Gene Set Enrichment Analysis (GSEA) was then conducted using the R packages "clusterProfiler" and "ggplot2" (Supplementary Table 5), based on the C2. CP. KEGG.v7.2 gene sets from the Molecular Signatures Database (MSigDB). Single-cell sequencing data from GSE160269 dataset was also analyzed to determine JOSD2 expression of different cell types (including B cell, CD4 T cell, CD8 T cell, DC, endothelial cell, fibroblasts, malignant cell, mast cell, mono/macro cell, pericytes, plasma cell, T proliferation cell, and Treg cell) using the Tumor Immune Single-Cell Hub 2 online tool (http://tisch.comp-genomics.org/home/).

Cell culture

A normal esophageal epithelial cell line, Het-1A, was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, United States) supplemented with 10% fetal bovine serum (FBS) (Sigma, United States), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) (Sigma, United States). Four ESCC cell lines (KYSE30, KYSE140, KYSE150, and KYSE410) were obtained from the Chinese Academy of Sciences Cell Bank and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific, United States) supplemented with 10% FBS (Sigma, United States), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (Sigma, United States). These cells were cultured at 37°C in a 5% CO_2 incubator.

Lentiviral packaging and transduction

Two independent small hairpin RNA (shRNA) sequences targeting JOSD2 (sh1: CGATGAGATCTG-CAAGAGGTT; sh2: GTGTCTACTACAACCTGGACT) were designed, cloned into a PSIH1 vector (GenePharma, China), and used for lentivirus packaging in 293T cells. The lentiviral supernatant was collected. KYSE150 cells (which had the highest JOSD2 expression among the ESCC cell lines) were transduced with the lentivirus and selected with puromycin 72 h later.

The JOSD2 gene was cloned into the lentiviral expression vector pLVX-IRES-Neo to create the overexpression plasmid pLVX-G418 JOSD2-Flag (GenePharma, China). The lentivirus was packaged and used to infect KYSE30 cells (which had the lowest JOSD2 expression among the ESCC cell lines), which were then selected with G418 72 h later.

RNA extraction and real-time fluorescent quantitative polymerase chain reaction

The total RNA was extracted using an RNApure Tissue/Cell Kit (Cwbiotech, China). The isolated RNA was used as a template for reverse transcription reaction using a HiFiScript cDNA Synthesis Kit (Cwbiotech, China). Real-time fluorescent quantitative polymerase chain reaction (RT-qPCR) was performed using SYBR Fast qPCR Mix (TaKaRa, Japan) and a CFX96 Real-Time System (Bio-Rad, United States). The primer sequences for JOSD2 were as follows: Forward: 5′-CCCACCGTGTACCACGAAC-3′; reverse: 5′-CTCCTGGCTAAAGAGCTGCTG-3′. The primer sequences for GAPDH were as follows: Forward: 5′-GATTCCACCCATGGCAAATTC-3′; reverse: 5′-CTGGAAGATGGTGATGGGATT-3′.

Cell protein extraction and western blotting

Approximately 1 × 10^6 cells were placed in each well of a 6-well culture plate. The cells were lysed by adding radioimmunoprecipitation assay buffer (10 μL) and a phosphatase inhibitor (1 μL) for every 100000 cells. The protein lysate was centrifuged at 17000g for 30 min to obtain the supernatant. The protein concentration was measured using a bicinchoninic acid assay. A mixture containing 10 μg of protein was then boiled at 95 °C for 10 min to denature the proteins.

Gel electrophoresis and transfer were carried out using a Mini Gel Tank chamber system (Thermo Fisher Scientific, United States) following the detailed procedures and reagents provided in the manufacturer's instructions. Gel electrophoresis was performed with constant voltage, starting at 70 V for 20 min, followed by an adjustment to 100 V for 50 min. The proteins were then transferred to a polyvinylidene fluoride membrane using a constant voltage of 10 V for 50 min.

The membrane was blocked with 5% skim milk for 1 h. The membrane was then incubated with one of the following primary antibodies overnight at 4 °C: JOSD2 antibody (sab2103354, 1:500, Sigma-Aldrich, United States), phosphorylated p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (9101, 1:1000, Cell Signaling Technology, United States), p44/42 MAPK (Erk1/2) (L34F12) antibody (4696, 1:1000, Cell Signaling Technology, United States), phosphorylated Akt (Ser473)

(D9E) antibody (4060, 1:2000, Cell Signaling Technology, United States), phosphorylated Akt (Thr308) antibody (13038, 1:1000, Cell Signaling Technology, United States), or Akt (pan) (40D4) antibody (2920, 1:2000, Cell Signaling Technology, United States). The membrane was then incubated with one of the following secondary antibodies at room temperature for 1 h: Anti-rabbit (7074, 1:1000, Cell Signaling Technology, United States) or anti-mouse (7076, 1:1000, Cell Signaling Technology, United States) horseradish peroxidase-linked antibody. The membrane was subjected to enhanced chemiluminescence (ECL) detection using SignalFire ECL reagent (Cell Signaling Technology, United States), and images were captured and saved using an automated imaging system. Anti-β-actin antibody (4967, 1:1000, Cell Signaling Technology, United States) was then added and incubated at room temperature for 1 h, followed by detection and image capture.

Cell proliferation assays

The Cell Counting Kit-8 (CCK-8) assay (Solarbio, China) was performed according to the manufacturer's instructions. Cells were seeded in a 96-well culture plate with approximately 1000 cells per well, and incubated at 37 °C in a 5% CO_2 incubator for 24, 48, 72, or 96 h. Subsequently, 10 μL of CCK-8 reagent was added to the cells, and the absorbance at 450 nm was measured using a microplate reader.

Colony formation assay

After exposure to shRNA1, shRNA2, or negative control, 1000 cells in the logarithmic growth phase were suspended in RPMI-1640 medium with 10% FBS and seeded in each well of 6-well plates. Following a 12-d incubation, the cells were fixed with methanol for 15 min and then stained with 0.5% crystal violet for 3 min at room temperature. After three washes with distilled water, the plates were air-dried and the cell colonies were manually counted. A positive colony was defined as a cluster containing at least 50 cells.

Drug sensitivity assay

Cells in the logarithmic growth phase, with knocked-down or overexpressed JOSD2, and their respective control cells, were divided into different groups and seeded into 96-well plates with each well containing 1 × 10^4 cells. After cell adhesion, cisplatin (Med-ChemExpress, United States) was added to each well at concentration gradients of 0, 10, 20, 40, 60, and 80 μg/mL in JOSD2 knock-down cell group. For the JOSD2 overexpression cell group, cisplatin was added at concentration gradients of 0, 20, 40, 60, 80, and 100 μg/mL. After 48 h, the sensitivity of the tumor cells to cisplatin was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (Sangon Biotech, China).

Migration assay

Transwell chambers (Corning, United States) were preloaded with 5 × 10^4 cells suspended in 100 μL of RPMI-1640 medium without FBS. The chambers were then placed in a 24-well plate, with each well containing 500 μL of RPMI-1640 medium supplemented with 10% FBS, and incubated in a 37 °C, 5% CO_2 incubator. After 24 h, the medium in the chambers was removed, and the cells in the chambers were gently wiped away with a cotton swab. The cells on the chambers were fixed on a new 24-well plate with 4% paraformaldehyde for 20 min. Next, the chambers were removed, followed by crystal violet staining for 20 min. Excess crystal violet solution was washed off with phosphate-buffered saline (PBS), and cell images were captured using a microscope.

Invasion assay

Approximately 60 μL of diluted Matrigel (1:8, Becton, Dickinson and Company, United States) was added to Transwell chambers. The chambers were incubated in a 37 °C, 5% CO_2 incubator for 3 h, excess liquid was removed from the chambers, and 100 μL of RPMI-1640 medium without FBS was added. The chambers were then placed in the 37 °C, 5% CO_2 incubator for 30 min to hydrate the basement membrane. Subsequently, 100 μL of cell suspension comprising 5 × 10^4 cells in FBS-free RPMI-1640 medium was added to the chambers. The chambers were then placed in a 24-well plate, with each well containing 500 μL of RPMI-1640 medium with 10% FBS, and incubated in the 37 °C, 5% CO_2 incubator. After 24 h, the medium in the chambers was removed, and the Matrigel and the cells in the chambers were gently wiped away with a cotton swab. As in the Transwell migration assays, a new 24-well plate with 4% paraformaldehyde was used to fix the cells on the chambers for 20 min, the chambers were removed, crystal violet staining was performed for 20 min, excess crystal violet solution was rinsed off with PBS, and cell images were captured using a microscope.

Xenograft and drug sensitivity assays

Xenograft assays were conducted by subcutaneously injecting JOSD2-knockdown KYSE150 cells and JOSD2-overexpressing KYSE30 cells under the armpits of BALB/c nude mice. Tumor dimensions, including length and width, were assessed using a vernier caliper every 3 d. Tumor volume was determined as 0.52 × length × $width^2$, and growth curves were plotted. From subcutaneous injection until tumor growth on the 19^{th} day, the tumor tissues were harvested and their weights were recorded.

Additionally, the role of JOSD2 in cisplatin sensitivity *in vivo* was studied using xenograft and drug sensitivity assays. JOSD2-knockdown KYSE150 cells or JOSD2-overexpressing KYSE30 cells were injected under the armpits of BALB/c nude mice. When the tumor volume reached 10 mm^3, cisplatin was intraperitoneally injected (6 mg/kg, every 3 d for 15 d) and the tumor volume was measured at the same time. At approximately 2 wk after the first administration of cisplatin, the tumor tissues were excised and weighed.

Mass spectrometry

To explore the proteins that interact with JOSD2, KYSE30 cells with Flag-tagged JOSD2 (Flag-JOSD2 sequence: GATTACAAGGATGACGACGATAAG) were lysed with protein lysis buffer to obtain the total proteins. Flag-JOSD2 was then enriched by immunoprecipitation. After obtaining the protein precipitate interacting with Flag-JOSD2, the protein complex was subjected to SDS-PAGE, followed by silver staining for band visualization. Specific bands were then subjected to mass spectrometry analysis (Beijing Protein Innovation Co., Ltd., China).

Statistical methods

ImageJ software was used to quantify the protein expression levels in Western blot analysis. Graphs were constructed and statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, Inc., United States). A P value less than 0.05 was considered statistically significant. Student's t-test was used to determine the significance of differences between two groups, while analysis of variance was employed to compare differences among more than two groups.

RESULTS

JOSD2 is highly expressed in ESCC tissues, which is associated with poor prognosis

JOSD2 expression was significantly higher in ESCC tissues than normal esophageal tissues ($P < 0.0001$) (Figure 1A). Additionally, ESCC patients with high JOSD2 expression had a worse prognosis than those with low expression ($P = 0.025$), providing a basis for predicting the prognosis of ESCC (Figure 1B and C).

To determine the biological functions of JOSD2, GO and KEGG enrichment analyses were performed. The top five most enriched biological process, cellular component, and molecular function (MF) terms are shown in Figure 1D (only one MF term was enriched), and the top five most enriched KEGG pathways are shown in Figure 1E. The top five gene sets in the GSEA, comprising CROMER Tumorigenesis Up, UROSEVIC Response to Imiquimod, MOSERLE IFNA Response, GNATENKO Platelet Signature, and RADAEVA Response to IFNA1 Up, are shown in Figure 1F. A PPI diagram based on the top 100 genes associated with JOSD2 is depicted in Supplementary Figure 1.

JOSD2 expression in ESCC cells

To determine whether JOSD2 is expressed in ESCC cells, single-cell sequencing data in GSE160269 was utilized and JOSD2 was highly expressed in ESCC cells (Figure 2). Additionally, JOSD2 mRNA and protein expression in a normal esophageal epithelial cell line (Het-1A) and ESCC cell lines (KYSE30, KYSE140, KYSE150, and KYSE410) was assessed using RT-qPCR and western blotting, respectively. The results showed that both JOSD2 mRNA (Figure 3A) and protein (Figure 3B) expression were consistently upregulated in ESCC cell lines compared to the normal esophageal cell line.

JOSD2 knockdown inhibits activity of ESCC cells

To confirm the function of JOSD2 in ESCC cells, two shRNAs targeting JOSD2 were designed and knockdown assays in the KYSE150 cell line (which had the highest JOSD2 expression among the ESCC cell lines) were conducted. RT-qPCR results showed that both shRNAs achieved effective knockdown (Figure 4A).

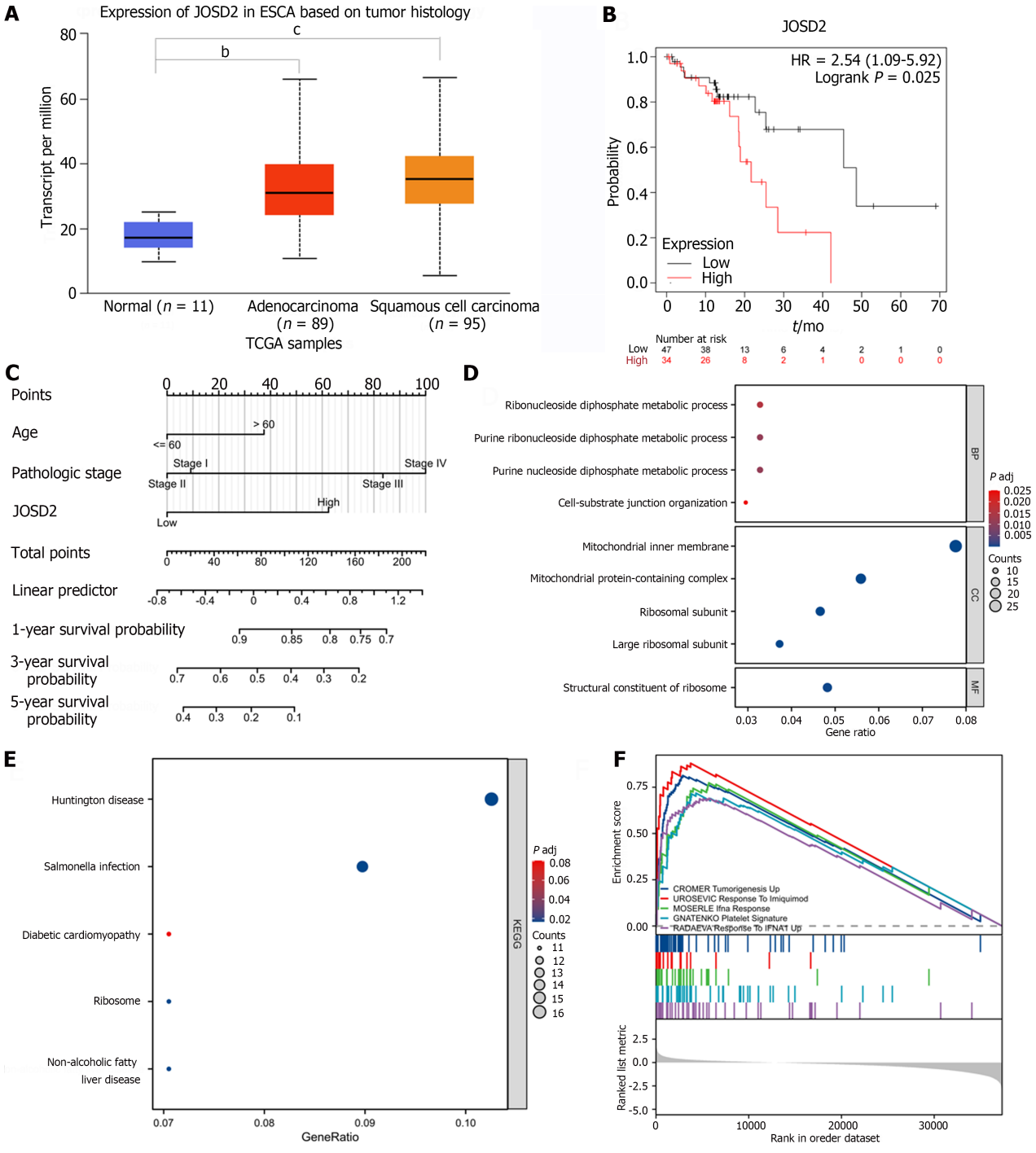
Subsequently, CCK-8 cell viability assays were performed to study the influence of JOSD2 knockdown on cell proliferation. The results indicated that both shRNAs significantly inhibited the proliferation of KYSE150 cells ($P < 0.0001$ for both) (Figure 4B). Colony formation assays were conducted to investigate the effect of JOSD2 knockdown on tumor-forming ability. JOSD2 knockdown significantly suppressed the colony-forming ability of KYSE150 cells (Figure 4C).

JOSD2 enhances ESCC cell proliferation, drug resistance, and migration in vitro

Chemoresistance and distant metastasis are major contributors to the poor prognosis of ESCC patients. Therefore, the next step was to investigate whether JOSD2 also affects the drug resistance and migratory ability of ESCC cells. JOSD2 knockdown increased the sensitivity of KYSE150 cells to 48-h treatment with various concentrations of cisplatin ($P < 0.0001$ for both) (Figure 5A). Transwell migration and invasion assays also demonstrated that JOSD2 knockdown significantly inhibited the migratory ($P < 0.01$) and invasion ($P < 0.0001$) ability of KYSE150 cells (Figure 5B). To validate the promoting role of JOSD2 in the development of ESCC cells, exogenous JOSD2 was overexpressed in KYSE30 cells (which had the lowest JOSD2 expression among the ESCC cell lines). The results showed that JOSD2 overexpression significantly promoted the proliferation ($P < 0.0001$), drug resistance ($P < 0.0001$), migration ($P < 0.01$), and invasion ($P < 0.001$) capability of KYSE30 cells (Figure 5C-E).

JOSD2 promotes ESCC cell proliferation and drug resistance in vivo

Xenograft assays were conducted in nude mice using JOSD2-knockdown KYSE150 cells, JOSD2-overexpressing KYSE30 cells, and their respective control cells. The mice in each group were divided into subgroups with and without cisplatin treatment to study the effects of JOSD2 on ESCC cell proliferation and drug sensitivity *in vivo*. JOSD2-knockdown KYSE150 cells exhibited significantly slower tumor growth and a more pronounced reduction in tumor volume under cisplatin treatment compared to control cells ($P < 0.001$ for tumor volume, $P < 0.0001$ for tumor weight) (Figure 6A and B). On the other hand, JOSD2-overexpressing KYSE30 cells not only had faster tumor growth but also exhibited significantly increased resistance to cisplatin ($P < 0.0001$ for tumor volume, $P < 0.0001$ for tumor weight) (Figure 6C and D). These findings suggested that JOSD2 has a vital role in promoting the development of ESCC.



DOI: 10.3748/wjg.v30.i6.565

Figure 1 Expression level, survival analysis, and enrichment analyses of JOSD2 in esophageal squamous cell carcinoma tissues. A: Boxplots of JOSD2 expression in esophageal squamous cell carcinoma (ESCC) tissues and normal esophageal tissues based on University of Alabama at Birmingham CANcer database; B: Kaplan-Meier plot showing the survival difference between ESCC patients with high *vs* low JOSD2 expression based on the Kaplan-Meier Plotter database; C: Nomogram of JOSD2 expression predicting 1-, 3- and 5-year survival probability of ESCC patients; D-F: Gene Ontology (D), Kyoto Encyclopedia of Genes and Genomes (E) and Gene Set Enrichment Analysis (F) enrichment analyses of JOSD2. $^{b}P < 0.001$; $^{c}P < 0.0001$.

JOSD2 facilitates activation of cancer-related pathways in ESCC cells

To explore the molecular mechanisms by which JOSD2 promotes ESCC, the effects of JOSD2 on the phosphorylation pathways MAPK/ERK and PI3K/AKT, which play important roles in carcinogenesis, were assessed. Western blotting results showed that JOSD2 knockdown in ESCC cells inhibited the phosphorylation levels of ERK1/2 and AKT (Figure 7A), while JOSD2 overexpression in ESCC cells led to the activation of MAPK/ERK and PI3K/AKT signaling pathways (Figure 7B). These results indicated that the activation of the MAPK/ERK and PI3K/AKT signaling pathways serves as a pivotal downstream mechanism in facilitating the oncogenic function of JOSD2.

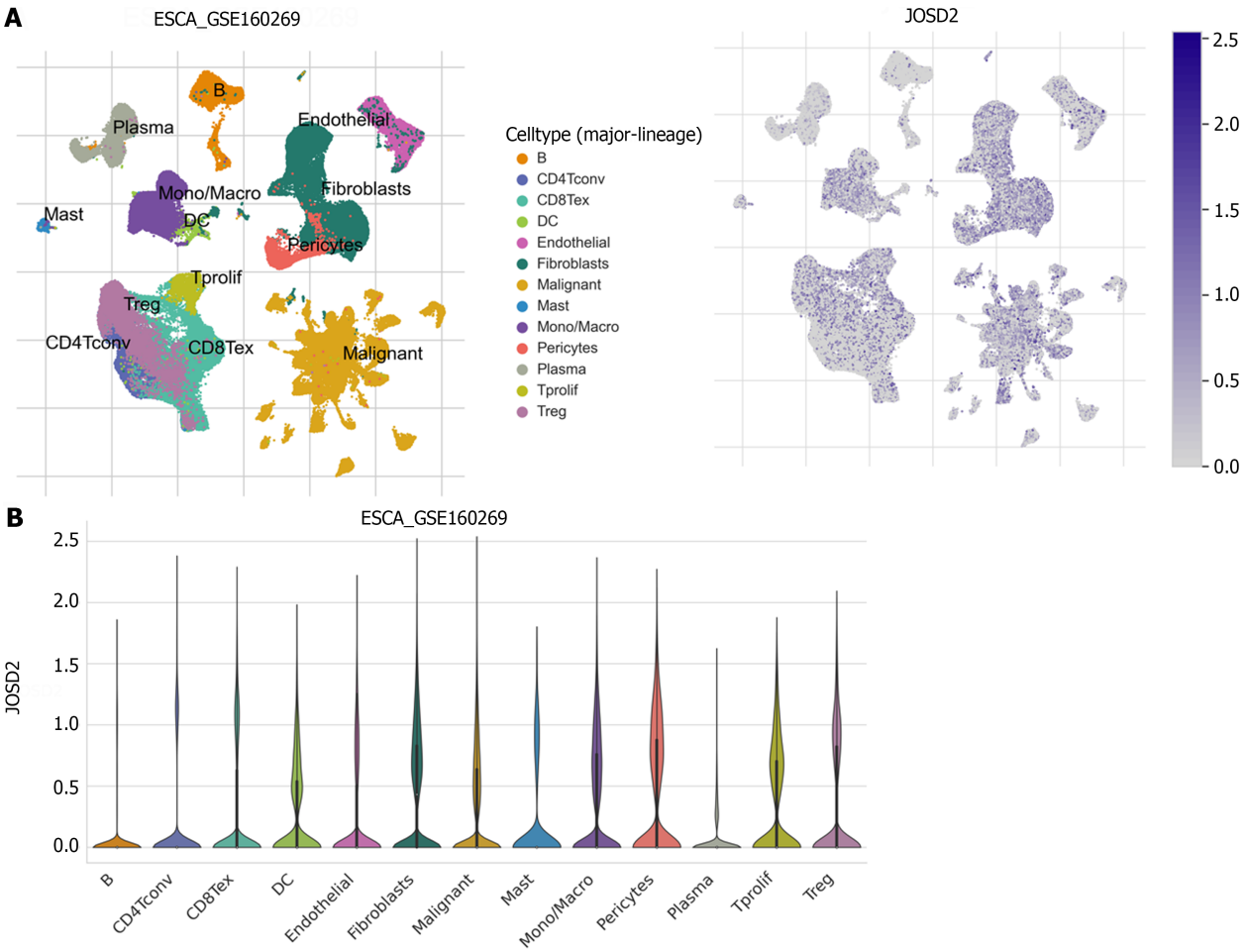


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Figure 2 Single-cell sequencing data of esophageal squamous cell carcinoma in GSE160269. A: Uniform manifold approximation and projection plots showing the grouping of different cell types (left) and the expression profile of JOSD2 (right) in different cell types; B: Violin plots showing JOSD2 expression in different cell types.

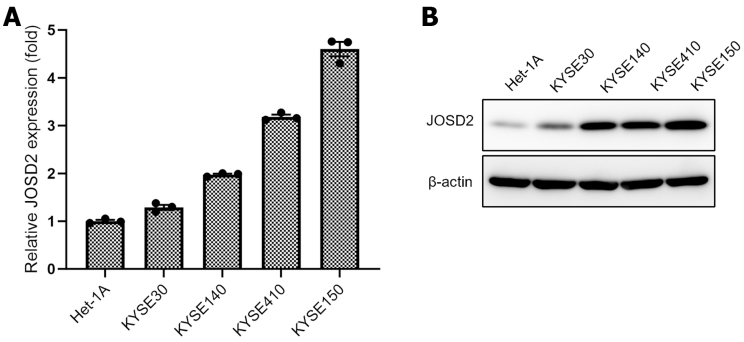

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Figure 3 JOSD2 expression in esophageal squamous cell carcinoma cell lines. Real-time fluorescence quantitative polymerase chain reaction and western blotting results showing JOSD2 mRNA (A) and protein (B) expression in esophageal squamous cell carcinoma cell lines and a normal esophageal epithelial cell line. A: JOSD2 mRNA; B: Protein.

Proteins potentially binding to JOSD2 were identified by mass spectrometry

To identify key substrate proteins interacting with JOSD2, anti-Flag magnetic beads were used to enrich Flag-JOSD2 protein and its interacting proteins in KYSE30 cells with exogenous Flag-JOSD2 overexpression. As indicated by the blue arrows in Figure 8A, silver staining of the SDS-PAGE gel revealed numerous protein bands in the overexpression group but not in the control group, indicating that these proteins were specifically immunoprecipitated along with Flag-JOSD2. Mass spectrometry showed that JOSD2 had the highest score (validating the reliability of its enrichment) and the other top-ranked proteins were USP47, IGKV2D-29, HSP90AB1, and PRMT5 (Figure 8B), which indicated that these proteins

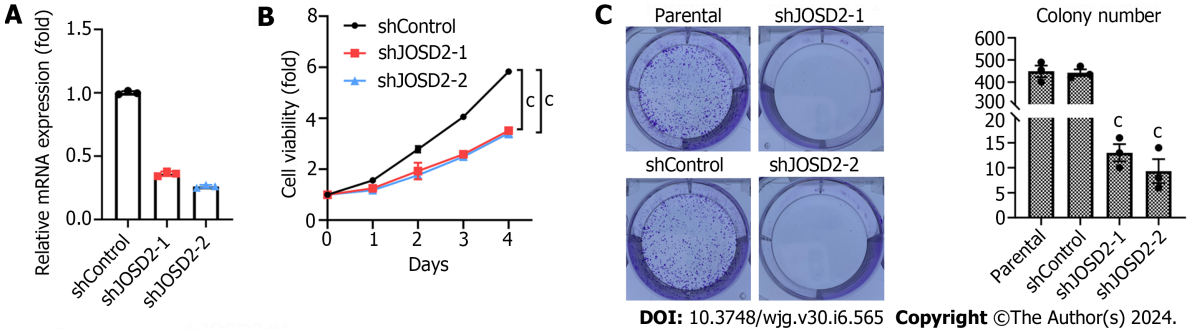

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Figure 4 JOSD2 knockdown significantly suppresses the activity of esophageal squamous cell carcinoma cells. A: JOSD2 was successfully knocked down using shRNA1/2 directed against JOSD2 in KYSE150 cells; B: JOSD2 knockdown significantly inhibited cell proliferation; C: JOSD2 knockdown significantly inhibited the ability of the cells to form colonies. $^{c}P < 0.0001$.

may be the significant substrates that bind to JOSD2.

DISCUSSION

In recent years, an increasing number of studies have shown that DUBs play a crucial role in the development of malignant tumors[11,16]. There has been a substantial amount of research on DUBs in five common malignant tumors, namely non-small cell lung cancer, hepatocellular cancer, gastric cancer, colorectal cancer, and breast cancer[8,17-20]. For instance, USP9X, DUB3, and USP7 have been identified in these malignant tumors[21-23]. There has been less research on DUBs in ESCC, and this research has mostly focused on the impact of the known DUBs on the metastatic process of ESCC [24-27]. PSMD14, OTUB1, USP26, and EIF3H, by stabilizing Snail, promote the occurrence of metastasis[24-27]. This redundancy in the regulatory mechanism makes using individual DUBs as effective therapeutic targets challenging.

This study is the first to report on the role of JOSD2 in ESCC. The preliminary findings indicated that JOSD2 is not only highly expressed in ESCC tissues, but its high expression is significantly associated with a poor prognosis. Subsequent analyses revealed that JOSD2 significantly enhanced the proliferation, migration, and drug resistance of ESCC cells. The *in vivo* results confirmed that altering JOSD2 expression, either by overexpression or knockdown, modulates the resistance of ESCC to the chemotherapy drug cisplatin (one of the primary chemotherapy drugs used to treat ESCC). This highlights the potential significance of using JOSD2 as a therapeutic target in order to overcome cisplatin resistance in ESCC.

Norberg *et al*[28] reported the role of JOSD2 in lung adenocarcinoma. They analyzed the metabolic profile of lung adenocarcinoma and found that PHGDH, a critical rate-limiting enzyme in serine synthesis, was highly expressed in a subgroup with poor prognosis[28]. Tumors with high PHGDH expression exhibited rapid proliferation and migration. Subsequently, the authors discovered that the protein expression of PHGDH is regulated by the ubiquitin proteasome system pathway. The authors screened for DUBs that stabilize PHGDH by using a siRNA library targeting 99 DUBs for transient knockdown. Targeting the DUB JOSD2, among these 99 DUBs, led to the largest significant reduction (> 80%) in PHGDH protein expression. Thus, JOSD2 affected the metabolism of lung adenocarcinoma by stabilizing PHGDH and promoting tumor growth. The same research team further explored and revealed the relationship between JOSD2 and metabolism[29]. They found that both *in vitro* and *in vivo*, JOSD2 directly regulated the metabolic enzyme complex comprising aldolase A, phosphofructokinase 1, and PHGDH. Wild-type JOSD2, but not its enzymatic mutant, stabilized this complex *via* deubiquitination, enhancing its activity, and thereby increasing the glycolytic rate of cancer cells. The absence of JOSD2 inhibited various cancer cells (including non-small cell lung cancer, breast cancer, and ovarian cancer cells) and reduced glycolysis. In summary, JOSD2 effectively integrated glycometabolism and serine metabolism by stabilizing the metabolic enzyme complex. For cancer types that are highly reliant on glycolysis for their energy supply, increased JOSD2 expression significantly promoted cell proliferation and growth. This finding suggests that JOSD2 is a potential therapeutic target in cancer cells that are dependent on glycolytic metabolism.

Qian *et al*[11] reported the role of JOSD2 in the progression of cholangiocarcinoma (CCA)[11]. They identified JOSD2 as a crucial regulator that stabilizes Yes-associated protein/Transcriptional co-activator with PDZ-binding motif (YAP/TAZ), which are involved in the malignant progression of CCA. Depleting JOSD2 led to the degradation of YAP/TAZ and thereby significantly inhibited CCA proliferation both *in vitro* and *in vivo*. Additionally, there was a positive association between JOSD2 and YAP abundance in CCA patient samples, suggesting that JOSD2 is a potential target for treatment in patients with YAP/TAZ-related CCA. Moreover, Huang *et al*[9] identified JOSD2 as a novel prognostic indicator for individuals with hepatocellular cancer and identified CTNNB1 as a significant collaborator and downstream protein targeted by JOSD2[9]. However, Lei *et al*[10] reported that, in acute myeloid leukemia, JOSD2 is a tumor suppressor and PKM2 is a newfound JOSD2-interacting partner, which suggests that JOSD2 has different functions and mechanisms in different malignant tumors[10].

The MAPK/ERK and PI3K/AKT signaling pathways are crucial for key cancer characteristics, including cell proliferation, differentiation, migration, and genomic stability[30,31]. Therefore, we assessed the effects of JOSD2 on these pathways. The results revealed that the phosphorylation activation of MAPK/ERK and PI3K/AKT was a critical

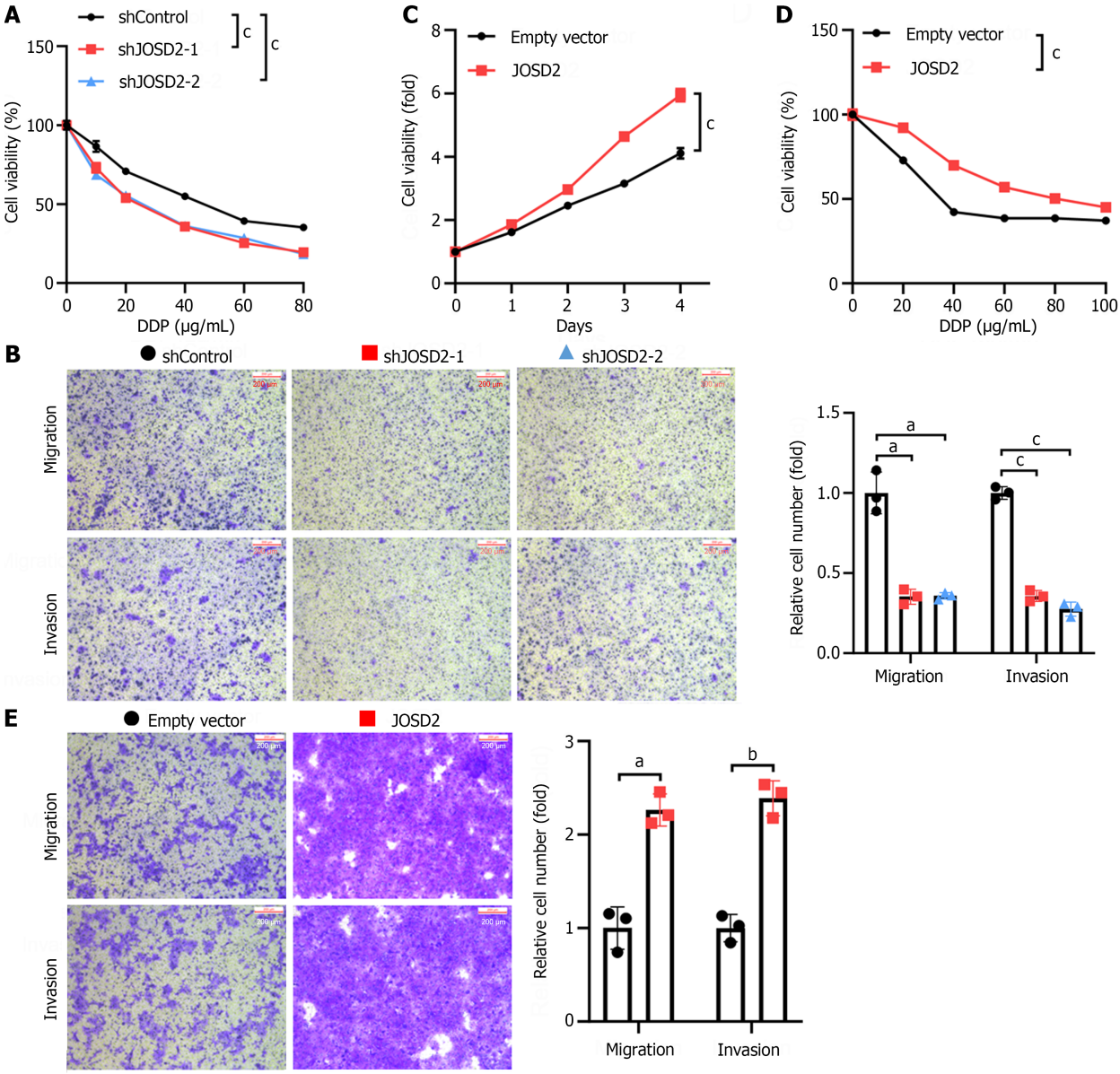

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Figure 5 JOSD2 contributes to the proliferation, drug resistance, and metastatic capability of esophageal squamous cell carcinoma cells.
A: JOSD2-knockdown KYSE150 cells exhibited increased sensitivity to 48-h treatment with various concentrations of cisplatin; B: JOSD2-knockdown KYSE150 cells exhibited significantly decreased migration and invasion in Transwell migration and invasion assays; C: JOSD2-overexpressing KYSE30 cells exhibited significantly increased cell growth in cell proliferation assays; D: JOSD2-overexpressing KYSE30 cells exhibited increased resistance to 48-h treatment with various concentrations of cisplatin; E: JOSD2-overexpressing KYSE30 cells exhibited significantly increased migration and invasion in Transwell migration and invasion assays. $^{a}P < 0.01$; $^{b}P < 0.001$; $^{c}P < 0.0001$.

downstream event mediating the oncogenic function of JOSD2.

Regarding the key substrate proteins interacting with JOSD2, we found that USP47, IGKV2D-29, HSP90AB1, and PRMT5 were the top four binding proteins of JOSD2 and may also be substrates for JOSD2's activity. USP47, a DUB, can counteract the functions of E3 ubiquitin ligases, playing a role in cell growth and survival processes[32]. Several studies have provided evidence that USP47 is involved in the advancement of diverse cancer types[33-35]. There is limited research on the IGKV2D-29 gene, but polymorphism in this gene was shown to lower the recombination frequency in B cells and to be especially important for immune responses to *Haemophilus influenzae* type b polysaccharide[36]. HSP90AB1 is a crucial participant in oncogene activity and the preservation of cancer cell viability[37]. This is due to its chaperone mechanism in cancer cells, safeguarding significant amounts of mutated and excessively expressed oncogenic proteins from undergoing misfolding and degradation[37]. Lastly, PRMT5 plays a crucial oncogenic role in various malignancies and has been a key target in recent cancer therapies[38,39]. However, there have been no studies reporting its deubiquitination modification. PRMT5's role in various malignancies implies that it is likely a key substrate protein for JOSD2's oncogenic function, and JOSD2's deubiquitination of PRMT5 may have significant implications for the treatment of ESCC.

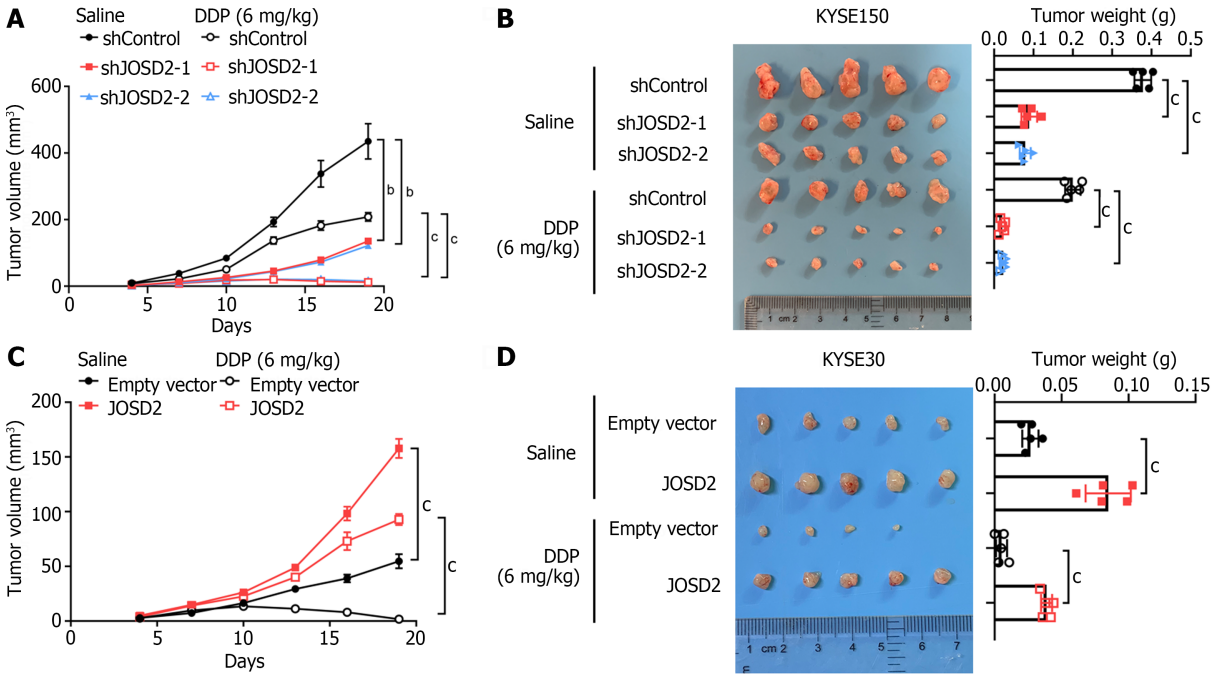

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Figure 6 JOSD2 promotes *in vivo* esophageal squamous cell carcinoma cell proliferation and cisplatin resistance. A and B: JOSD2-knockdown cisplatin-treated KYSE150 cells exhibited significantly decreased tumor growth in both volume (A) and weight (B) compared to cisplatin-treated control cells; C and D: JOSD2-overexpressing cisplatin-treated KYSE30 cells exhibited significantly increased tumor growth in both volume (C) and weight (D) compared to cisplatin-treated control cells. $^{b}P < 0.001$; $^{c}P < 0.0001$.

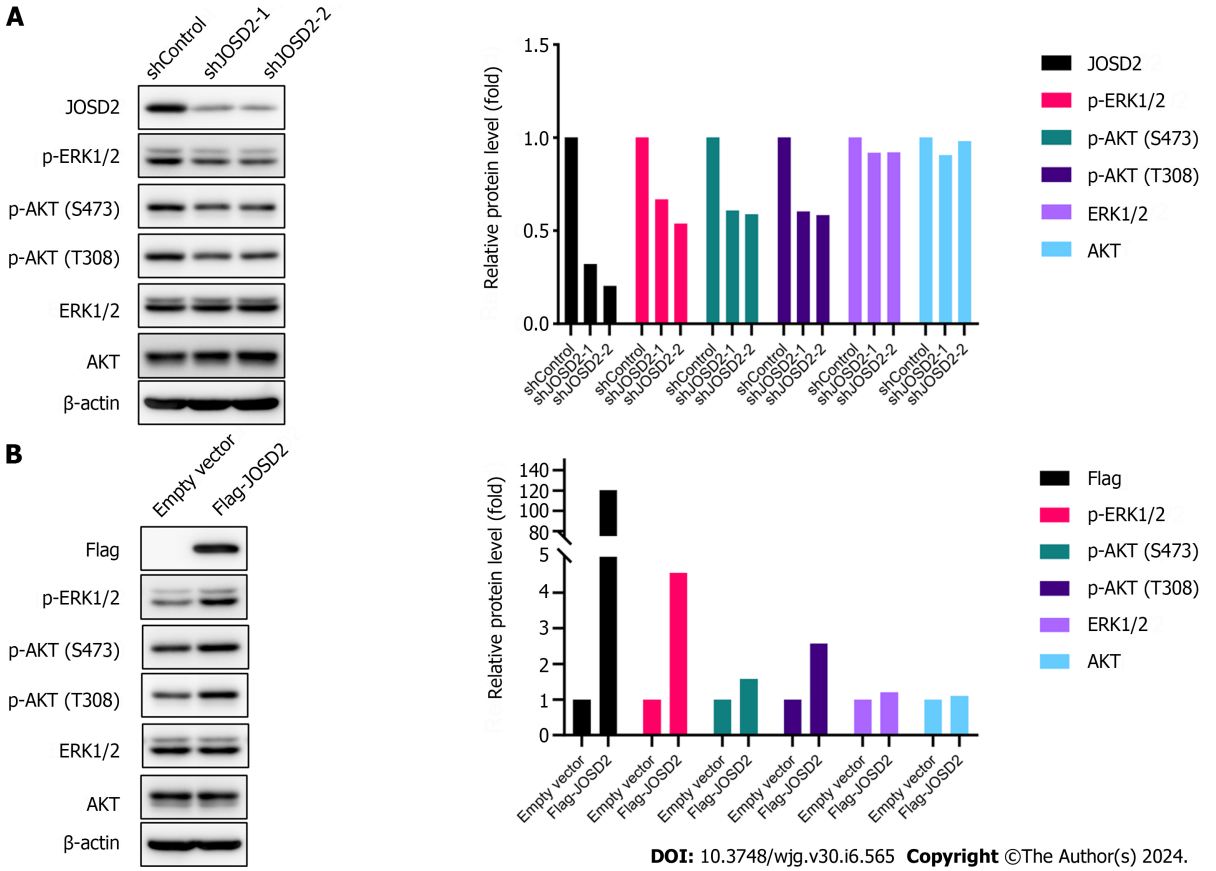

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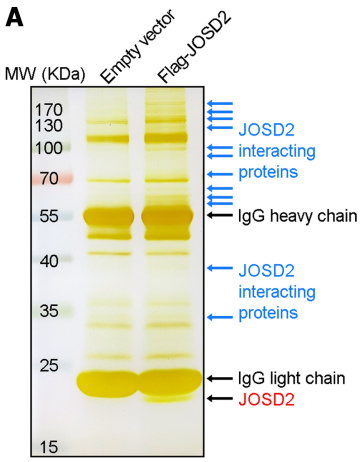
Figure 7 JOSD2 enhances the activation of phosphorylation pathways in esophageal squamous cell carcinoma. A: Western blotting showing that JOSD2 knockdown in esophageal squamous cell carcinoma (ESCC) cells decreased the phosphorylation of ERK1/2 and AKT; B: Western blotting showing that JOSD2 overexpression in ESCC cells activated the MAPK/ERK and PI3K/AKT signaling pathways.



B

Top 5 proteins identified by MS

Proteins	Unique peptide numbers	MS score
JOSD2	25	3476
USP47	57	1450
IGKV2D-29	4	1371
HSP90AB1	20	897
PRMT5	40	857

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Figure 8 Mass spectrometry analysis of proteins that bind to JOSD2 protein. A: Protein bands after SDS-PAGE gel silver staining; B: Proteins that potentially interact with JOSD2.

CONCLUSION

In conclusion, this study reveals the tumorigenic role of JOSD2 in the advancement of ESCC. In terms of the mechanism, JOSD2 influences the phosphorylation activation of MAPK/ERK and PI3K/AKT. USP47, IGKV2D-29, HSP90AB1, and PRMT5 are the four primary proteins that interact with JOSD2 and may serve as substrates for JOSD2's functional activity, especially PRMT5. In 2019, Grasty *et al*[40] elucidated the molecular structure of the JOSD2 protein, which will facilitate the development of molecular targeted inhibitors of JOSD2. However, there are currently no report on JOSD2 inhibitors. Consequently, there is a need for further exploration of the effects of specific and potent JOSD2 inhibitors on the clinical outlook for ESCC patients.

ARTICLE HIGHLIGHTS

Research background

Esophageal squamous cell carcinoma (ESCC) is a highly lethal malignancy with limited treatment options. Deubiquitinases (DUBs), crucial for maintaining protein homeostasis, are emerging as key players influencing vital cellular processes in ESCC, offering new treatment avenues. In addition, the ongoing development of small molecule inhibitors targeting DUBs shows significant promise, with several preclinical and clinical trials underway.

Research motivation

Recognizing the crucial involvement of DUBs in malignant tumor development, JOSD2, a specific DUB, has been identified as playing a pivotal role in controlling protein deubiquitination and impacting essential cellular processes in cancer. Nevertheless, the function of JOSD2 in ESCC remains uncertain.

Research objectives

The objective of this study was to explore the impact of JOSD2 on the progression of ESCC.

Research methods

Bioinformatics analyses were used to investigate the expression patterns, prognosis, and enriched pathways of JOSD2 in ESCC tissues. Manipulation of JOSD2 expression in ESCC cell lines (KYSE30 and KYSE150) was achieved through lentiviral transduction. Comprehensive functional assays, encompassing cell proliferation, colony formation, drug sensitivity, migration, and invasion assays, were conducted to unveil the influence of JOSD2 on ESCC cell lines. Additionally, the effects of JOSD2 on xenograft tumor growth and drug sensitivity *in vivo* were assessed. Proteins interacting with JOSD2 were determined by mass spectrometry.

Research results

The initial results suggested that JOSD2 was highly expressed in ESCC tissues and was associated with a poor prognosis. Subsequent investigations revealed upregulation of JOSD2 in ESCC cell lines compared to normal esophageal cells. JOSD2 knockdown inhibited various ESCC cell activities, including proliferation, colony formation, and migration, as well as reducing drug resistance. Conversely, JOSD2 overexpression enhanced these phenotypes. *In vivo* xenograft assays confirmed that JOSD2 promoted tumor proliferation and drug resistance in ESCC. Mechanistically, JOSD2 appears to activate the MAPK/ERK and PI3K/AKT signaling pathways. Mass spectrometry identified four primary proteins

interacting with JOSD2: USP47, IGKV2D-29, HSP90AB1, and PRMT5.

Research conclusions

JOSD2 promotes cell proliferation, migration, and drug resistance in ESCC.

Research perspectives

JOSD2 is a promising therapeutic target for the treatment of ESCC.

FOOTNOTES

Co-first authors: Wen-Peng Wang and Dan Shi.

Co-corresponding authors: Jun-Feng Wang and Da-Lu Kong.

Author contributions: Wang WP and Shi D contributed equally to this work; Kong DL and Wang JF were co-corresponding authors; The study was conceptualized and designed by Kong DL, Wang JF, and Wang WP; Shi D and Yun D were in charge of acquiring public data; Bioinformatic and statistical analyses were conducted by Wang WP, Shi D, Yun D, Hu J, Wang JF, and Liu J; Wang WP, Shi D, Yun D, Yang YP, and Li MR carried out both *in vitro* and *in vivo* experiments; Figures and tables were prepared, and the initial draft of the manuscript was written by Wang WP, Shi D, Wang JF and Yun D; Manuscript revisions were performed by Kong DL, Wang JF, and Wang WP; All authors have reviewed and approved the final version of the manuscript for publication.

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