

Overexpression of the Long Noncoding RNA FOXD2-AS1 Promotes Cisplatin Resistance in Esophageal Squamous Cell Carcinoma Through the miR-195/Akt/mTOR Axis

Huasong Liu,*¹ Jun Zhang,*¹ Xiangyu Luo,* Min Zeng,* Liqiang Xu,* Qunxian Zhang,* Hua Liu,* Jialong Guo,* and Lanlan Xu†

*Department of Cardiothoracic Surgery, Taihe Hospital Affiliated to Hubei University of Medicine, Shiyan, Hubei, P.R. China

†Hubei University of Medicine, Hubei, P.R. China

Emerging evidence has demonstrated that long noncoding RNAs (lncRNAs) mediate the development of esophageal squamous cell carcinoma (ESCC) via various pathophysiological pathways. This study explored the impact of the lncRNA FOXD2-AS1 on cisplatin resistance in ESCC and its possible mechanisms. Upregulation of FOXD2-AS1 was detected in patients with ESCC and ESCC cells that are resistant to cisplatin. In an in vitro assay, knockdown of FOXD2-AS1 noticeably inhibited cell invasion and growth, triggered cell death, and repressed the stimulation of the Akt/mTOR axis in cisplatin-resistant ESCC cells (TE-1/DDP). Conversely, the overexpression of FOXD2-AS1 remarkably increased cell invasion and growth, repressed cell death, and triggered the stimulation of the Akt/mTOR axis in TE-1/DDP cells. These findings, along with bioinformatics and validation tests, showed that FOXD2-AS1 targeted miR-195 by acting as a competing endogenous RNA. FOXD2-AS1/miR-195/Akt/mTOR axis plays a crucial role in resistance to cisplatin in ESCC cells, offering an innovative strategy to treat ESCC.

Key words: FOXD2-AS1; Esophageal squamous cell carcinoma (ESCC); Cisplatin resistance; miR-195; Akt/mTOR signal pathway

INTRODUCTION

Esophageal carcinoma (EC) is one of the most frequent malignancies in the gastrointestinal tract, and it is associated with poor clinical outcomes, even with early diagnosis¹. The main EC subtype in Asia is esophageal cell carcinoma (ESCC)². Despite remarkable progress in surgical technologies and perioperative management, its clinical outcome is still far from satisfactory due to local relapse and distant metastasis³. The majority of patients with ESCC receive their diagnosis in the terminal stage, making only chemotherapy, adjuvant therapy, and radiotherapy viable treatment options⁴. Cisplatin (CDDP) is a platinum agent commonly applied for ESCC, and its application, with and without other agents, has led to a noticeable improvement in prognosis⁵. Nevertheless, acquired resistance to chemotherapy is a frequent cause of treatment failure^{6,7}. Consequently, avoiding resistance is crucial for prognosis^{8,9}. Therefore,

identifying useful markers of resistance and determining the basic mechanisms influencing treatment response are urgently needed.

It is commonly accepted that more than 90% of the transcripts arising from the human genome do not encode proteins¹⁰, and long noncoding RNAs (lncRNAs) are crucial RNAs that consist of more than 200 nucleotides¹¹. lncRNAs are frequently expressed in various cells and tissues and make essential contributions to multiple cell functions, such as expression, differentiation, proliferation, cell cycle modulation, and apoptosis^{12–14}. Emerging evidence has indicated that abnormal lncRNA expression in malignancies is crucial for the initiation and development of various malignancies^{15–17}.

The lncRNA FOXD2 adjacent opposite strand RNA 1 (FOXD2-AS1) is 257 nucleotides in length and is located at chromosome 1p33¹⁸. FOXD2-AS1 upregulation was first discovered in gastric cancer (GC)¹⁹ and was subsequently shown to be related to worse clinical outcomes in

¹These authors provided equal contribution to this work and should be considered as equal first coauthors.

Address correspondence to Jialong Guo, Department of Cardiothoracic Surgery, Taihe Hospital Affiliated to Hubei University of Medicine, No. 30 Renmin South Road, Shiyan, Hubei, P.R. China. Tel: +86-0719-8801710; E-mail: gjl9988@163.com or Lanlan Xu, Hubei University of Medicine, No. 30 Renmin South Road, Hubei, P.R. China. Tel: +86-0719-8891111; E-mail: 215518542@qq.com

nasopharyngeal carcinoma, colorectal cancer, non-small cell lung cancer (NSCLC), and bladder cancer (BC)^{18,20–22}. FOXD2-AS1 modulates cell death, cell proliferation, and the invasion and migration of malignant cells, indicating that it could serve as a marker or promising target for treating malignancies²³. However, our current understanding of the relationship between the expression of FOXD2-AS1 and therapy resistance in ESCC is insufficient. Therefore, our study explored the activities of lncRNA FOXD2-AS1 in ESCC cells that are resistant to cisplatin and shed light on the possible mechanisms underlying the initiation of ESCC.

MATERIALS AND METHODS

Clinical Samples

Tumor specimens and matched adjacent nonmalignant tissues (ANCTs) were acquired from 20 patients with ESCC who were treated by esophagectomy. Diagnoses were confirmed by two pathologists according to World Health Organization (WHO) criteria. ANCTs were acquired greater than 5 cm apart from the malignant fringe. All participants (including 15 men and 5 women) were less than 75 years old. They were newly diagnosed and had received no previous therapy. Patient data and samples were anonymous and were obtained in accordance with ethical and legal guidelines. Informed consent was obtained from all study participants, and the study was approved by the ethics committee of Taihe Hospital Affiliated to Hubei University of Medicine.

Cell Lines

The human ESCC cell line TE-1 was purchased from the Chinese Academy of Science cell bank. Normal esophageal epithelial cells (HEp-2) were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were cultivated in RPMI-1640 at 37°C with 5% CO₂. All reagents used for cell cultivation were purchased from GIBCO (St. Louis, MO, USA).

Generating Cisplatin-Resistant Cells

Cisplatin-resistant ESCC cells (TE-1/DDP) were generated by step-by-step exposure to increased concentrations of cisplatin (Sigma-Aldrich, St. Louis, MO, USA) as follows. The initial concentration of cisplatin was 2 mmol/L. The cells were then cultured in medium without cisplatin for 3 days. If confluence was achieved at the previous concentration, the cells were cultured with a higher concentration (1.5- to 2.0-fold higher) of cisplatin. The cisplatin concentration was increased step-by-step every few passages until the concentration reached 35 mmol/L over a 2-month period. The TE-1/DDP cells were 5.8-fold more resistant to cisplatin than the parent TE-1 cells. The IC₅₀ values for TE-1 and TE-1/DDP were 8.1 and 41.3 mmol/L, respectively.

Transfection

For the knockdown (KD) assay, specific small interfering RNAs (siRNAs) directed toward FOXD2-AS1 (GCTTCCAGGTATGTGGGAA), a control siRNA (si-NC, AGCCGCGAGCACACGAAGG), a miR-195 inhibitor, and corresponding mimics were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Vectors for overexpression of FOXD2-AS1 and a negative control vector were obtained from the Ribo Bio Corporation (Guangzhou, P.R. China). Cells were transfected with the plasmid or oligonucleotide by utilizing Lipofectamine 2000 (Invitrogen, St. Louis, MO, USA).

Colony Formation Test

The cells were trypsinized (0.25%) and seeded into six-well plates at 500 cells per well (250/ml). The cells were then fixed with anhydrous ethanol for 10 min and stained with crystal violet (0.1%) for 30 min. Colonies with 50 cells were evaluated and reported as the ratio of the quantity of colonies in the transfection group to that of the control group, multiplied by 100.

Chemotherapy Resistance Test

TE-1 cells (1×10^4 cells) were seeded in 96-well plates for the response investigation. Then the cells were incubated with various concentrations of DDP for 48 h, and cell survival was assessed by the CCK-8 assay. After a 1-h incubation with 10 μ l of CCK-8 solution, the absorbance was measured. Percent survival was assessed by multiplying the calculated value by 100. Proliferation was reported according to the optical density (OD).

Flow Cytometry (FC)

Cells (1×10^6) were isolated from each specimen, suspended in binding buffer, and stained with the Annexin-V-FITC/Propidium Iodide kit (BD Pharmingen, San Diego, CA, USA). FC was performed to quantify positive cells 48 h after transfection.

Cell Invasion Test

Transwell chambers containing 24 wells with polycarbonate membranes (8- μ m pore size; BD Biosciences, San Diego, CA, USA) were used for the invasion test. Matrigel (BD Biosciences)-coated Transwell chambers were used for the assay. Cells in 200 μ l of DMEM were placed in the upper chamber, while 800 μ l of DMEM was placed in the bottom chamber. After a 24-h incubation, nonmigrating cells that remained in the upper chamber were removed with a cotton swab, while invading cells in the bottom were fixed with 4% paraformaldehyde and stained with crystal violet (0.1%). The cells present in six randomly selected fields of every well were counted, and the relative number of invading cells is shown.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells and tissues using the ReliaPrep RNA Miniprep Systems according to the manufacturer's instructions (Promega, Madison, WI, USA). The extracted RNA was quantified, and the quality was assessed by measuring the A_{260}/A_{280} with an ultraviolet spectrophotometer. The RNA was reverse transcribed to generate cDNA with the Primer-Script One-Step RT-PCR kit. The sequences of the primers used are FOXD2-AS1: forward (F), 5'-TGT CTT ACA CTA CAG CCC ATT TCC TT-3' and reverse (R), 5'-CTG ATA CAA GTG AAG CCA CAT GCG-3'; miR-195: F, 5'-ACA GAC CGG TAC AAG TGC AGA-3' and R, 5'-GGT CGG CAT ACA GCT AAT ACA-3'; and GAPDH: F, 5'-TCC ACC ACC CTG TTG CTGTA-3' and R, 5'-ACC ACA GTC CAT GCC ATC AC-3'. Relative expression was quantified by SYBR green (Applied Biosystems, Billerica, MA, USA) using a 500 Real-Time PCR System (Applied Biosystems), and expression was normalized to GAPDH using the 2^{-C_t} method.

Luciferase Reporter Test

Two recombinant luciferase reporter gene constructs, pmir-GLO-FOX D2-AS1-WT and pmir-GLO-FOX D2-AS1-MUT, were generated using a plasmid pmirGLO vector (Promega, Beijing, P.R. China). The reporter constructs and miR-195 mimics were cotransfected into 293T cells. After 48 h of incubation, luciferase activity was assessed with the Dual-Luciferase Assay System (Promega, Beijing, P.R. China) and normalized to Renilla luciferase activity.

Xenograft Malignancy Models

BALB/c nude mice were provided by the Institute of Laboratory Animal Sciences, Peking Union Medical College (Beijing, P.R. China). Mice were maintained under specific sterile conditions. The mice received a subcutaneous injection of TE-1 cells (5×10^6 in 0.1 ml) in their back. The tumor volume was assessed every 3 days in the FOXD2-AS1-, Vector-, siFOX D2-AS1-, and siNC-transfected groups ($n=6$ each). Tumor volume was determined based on the following formula: volume = $0.5 \times \text{length} \times \text{width} \times \text{width}$. Mice were sacrificed 21 days after injection, and the tumors were isolated from all of the animals and weighed, and prepared for assay. All procedures related to the animals were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of Taihe Hospital Affiliated to Hubei University of Medicine.

Western Blotting (WB)

The proteins were quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA) prior to separation by

standard SDS-PAGE. The separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. The protein blots were incubated overnight with primary antibodies against MMP-9, AKT, mTOR, p-AKT, p-mTOR, and β -actin (all from Cell Signaling Technology, Beverly, MA, USA) in TBST at 4°C. Next, the blots were incubated with the appropriate secondary antibodies (conjugated to horseradish peroxidase, HRP). Enhanced chemiluminescence (ECL) plus detection reagent (Pierce, Rockford, IL, USA) was used to detect immunoreactive bands, which were analyzed using the Omega 16ic Chemiluminescence Imaging System (Ultra-Lum, Claremont, CA, USA).

Statistical Analysis

Data are reported as mean \pm SEM. Differences between groups were evaluated with a two-tailed unequal variance Student's *t*-test or ANOVA prior to Tukey's post hoc analysis. A value of $p < 0.05$ was considered to be significant.

RESULTS

FOX D2-AS1 Expression Was Increased and miR-195 Expression Was Decreased in ESCC Cells and Cisplatin-Resistant ESCC Cells

FOX D2-AS1 expression was examined in ESCC cells and tissues. FOXD2-AS1 transcription was assessed through qRT-PCR in 20 pairs of matched ESCC specimens and surrounding normal tissues. The results showed that FOXD2-AS1 transcription was remarkably higher in the ESCC specimens than in the corresponding control tissues (Fig. 1A). In addition, FOXD2-AS1 expression was also compared in normal esophageal cells, ESCC cells, and cisplatin-resistant ESCC cells (TE-1/DDP). FOXD2-AS1 expression was upregulated in TE-1/DDP cells compared to the parent TE-1 cells (Fig. 1C). In addition, qRT-PCR analysis showed that miR-195 expression was repressed in ESCC cells, regardless of cisplatin resistance (Fig. 1B and D). The above findings demonstrate that the expression of the lncRNA FOXD2-AS1 was increased in ESCC cells regardless of cisplatin resistance, indicating that FOXD2-AS1 expression is conversely related to miR-195 expression.

FOX D2-AS1 Functions as a Molecular Sponge for miR-195

Previous research has shown that lncRNAs can function as ceRNAs, promoting transcription by binding to microRNAs (miRNAs)²⁴. Bioinformatics method prediction showed that miR-195 was able to bind to FOXD2-AS1 (Fig. 2A), and qRT-PCR showed that repression of FOXD2-AS1 expression remarkably upregulated miR-195, while an increase in FOXD2-AS1 expression noticeably downregulated miR-195 (Fig. 2B and C), suggesting that FOXD2-AS1 represses miR-195 expression.

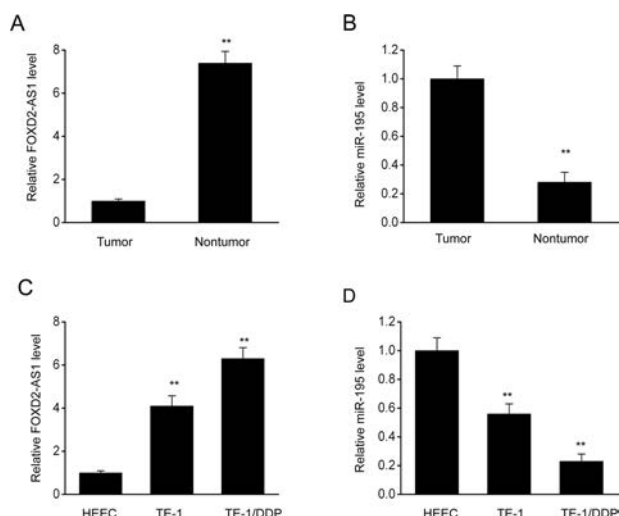


Figure 1. FOXD2-AS1 expression is increased, whereas miR-195 expression is inhibited in esophageal squamous cell carcinoma (ESCC) cells and cisplatin-resistant ESCC cells. (A) Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the expression of FOXD2-AS1 in 20 pairs of ESCC specimens and matched surrounding non-malignant tissues. (B) qRT-PCR was performed to assess the expression of miR-195 in 20 pairs of ESCC specimens and matched surrounding nonmalignant tissues. The results are shown as means \pm SEM, $n=20$, $**p < 0.01$ versus tumor specimens. (C) qRT-PCR was performed to assess FOXD2-AS1 expression in normal esophageal cells (HEEC), ESCC cells (TE-1), and cisplatin-resistant ESCC cells (TE-1/DDP). (D) qRT-PCR was used to assess the expression of miR-195 in HEEC cells, TE-1 cells, and TE-1/DDP cells. The results are shown as means \pm SEM. $**p < 0.01$ versus the expression in HEEC cells.

Luciferase assays were performed in TE-1/DDP cells to verify the association between miR-195 and FOXD2-AS1. Transfection with a miR-195 mimic remarkably repressed FOXD2-AS1 luciferase activity, while transfection with a miR-195 inhibitor noticeably increased FOXD2-AS1 luciferase activity (Fig. 2D and E), suggesting that FOXD2-AS1 functions as a molecular sponge for miR-195.

FOXD2-AS1 Increased Cisplatin Resistance in ESCC Cells In Vitro

Our previous assays showed that FOXD2-AS1 was highly expressed in TE-1/DDP cells. Therefore, gain-of-function and loss-of-function assays were performed to investigate the complicated etiology of cisplatin resistance. Knockdown (KD) of FOXD2-AS1 markedly inhibited colony formation in TE-1/DDP cells (Fig. 3A and B). In contrast, overexpression of FOXD2-AS1 remarkably increased colony formation by TE-1/DDP cells (Fig. 3C and D). A CCK-8 survival assay showed that cell proliferation was dose-dependently inhibited by DDP. FOXD2-AS1 KD markedly inhibited the growth

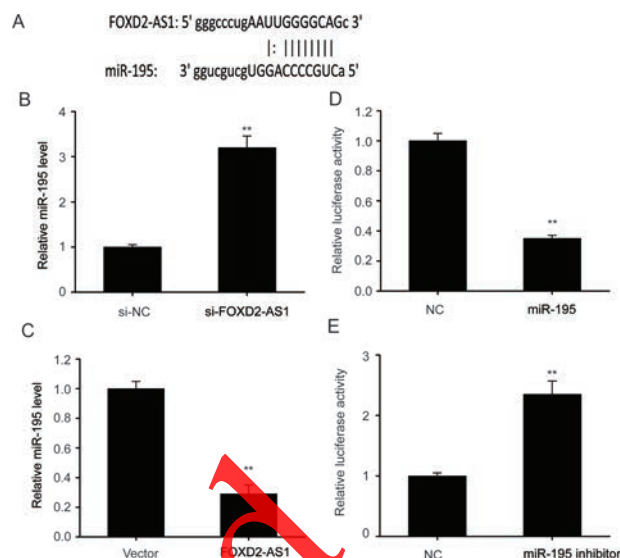


Figure 2. FOXD2-AS1 functions as a molecular sponge for miR-195. (A) Bioinformatics analysis showed the potential interaction between FOXD2-AS1 and miR-195. (B) qRT-PCR was performed to assess the expression of miR-195 in TE-1/DDP cells that were transfected with a FOXD2-AS1-specific siRNA (si-FOXD2-AS1) or a negative control siRNA (si-NC). (C) qRT-PCR was performed to assess expression of miR-195 in TE-1/DDP cells that were transfected with a FOXD2-AS1 expression vector (FOXD2-AS1) or a control vector (vector). (D, E) A luciferase reporter assay revealed that miR-195 bound to the 3'-UTR of FOXD2-AS1. The results are shown as the means \pm SEM. $**p < 0.01$ versus the control (vector) cells.

of DDP-supplemented cells when compared with control siRNA-transfected DDP-supplemented cells. Cells that overexpressed FOXD2-AS1 showed better survival (Fig. 3E and F). The above findings indicated that FOXD2-AS1 aggravated cisplatin resistance in ESCC cells.

FOXD2-AS1 Inhibited the Death of ESCC Cells

Cells were transfected with a FOXD2-AS1 expression vector to overexpress FOXD2-AS1 or a FOXD2-AS1-siRNA vector to KD FOXD2-AS1 expression to explore the contribution of FOXD2-AS1 to cisplatin resistance. The results showed that cell death was obviously altered after transfection of the FOXD2-AS1 expression vector or FOXD2-AS1-siRNA. Overexpression of FOXD2-AS1 inhibited cell death, while FOXD2-AS1 KD increased cell death, both in the early and late stage (Fig. 4).

FOXD2-AS1 Increased the Invasiveness of ESCC Cells

A Transwell assay was performed to evaluate the effect of FOXD2-AS1 expression on cell invasion. Repression of FOXD2-AS1 expression inhibited the invasiveness of TE-1/DDP cells when compared with control cells, whereas FOXD2-AS1 overexpression increased its invasive ability (Fig. 5A–D). We also investigated the

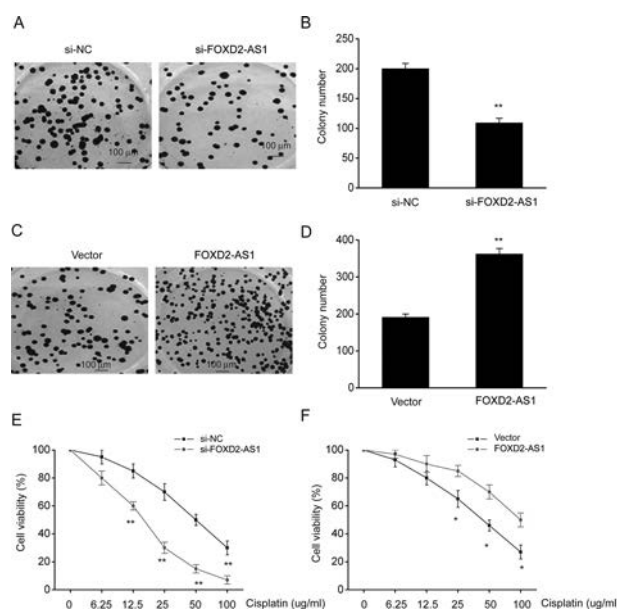


Figure 3. FOXD2-AS1 increased cisplatin resistance in ESCC cells in vitro. (A) Cells were stained with crystal violet to observe colony formation. (B) Quantitative assessment of colony formation in TE-1/DDP cells transfected with FOXD2-AS1 siRNA (si-FOX2-AS1) or negative control siRNA (si-NC). (C) The cells were stained with crystal violet to observe colony formation. (D) Quantitative assessment of colony formation in TE-1/DDP cells transfected with a FOXD2-AS1 expression vector (FOX2-AS1) or a control vector (vector). (E) CCK-8 assay was performed to evaluate the survival of TE-1/DDP cells transfected with si-FOX2-AS1 or si-NC. (F) CCK-8 assay was performed to evaluate the survival of TE-1/DDP cells transfected with FOXD2-AS1 or a control vector. The results are reported as means \pm SEM. * $p < 0.05$, ** $p < 0.01$ versus the vector control.

effect of FOXD2-AS1 on MMP-9 expression because of the role of MMP-9 in invasion. WB showed that KD of FOXD2-AS1 markedly inhibited MMP-9 expression in TE-1/DDP cells, whereas the overexpression of FOXD2-AS1 upregulated MMP-9 (Fig. 5E and F).

FOX2-AS1 Promoted Stimulation of the AKT/mTOR Axis in ESCC Cells

Emerging research has focused on the AKT/mTOR axis due to its increased stimulation in resistant malignancies²⁵. Our research explored various major target genes in the AKT/mTOR axis, such as p-AKT, p-mTOR, AKT, and mTOR, to investigate whether FOXD2-AS1 modulated resistance to cisplatin via the AKT/mTOR axis. FOXD2-AS1 KD remarkably downregulated p-AKT and p-mTOR in TE-1/DDP cells, whereas overexpression of FOXD2-AS1 upregulated p-AKT and p-mTOR (Fig. 6). The above findings suggested that FOXD2-AS1 upregulated the AKT/mTOR axis in TE-1/DDP cells, which could be the mechanism underlying FOXD2-AS1-mediated resistance to cisplatin in ESCC cells.

FOX2-AS1 Increased Resistance to Cisplatin in ESCC Cells In Vivo

We examined whether KD of FOXD2-AS1 increases cisplatin sensitivity in vivo. Nude mice were administered a subcutaneous injection of TE-1 cells with either high expression of FOXD2-AS1 (transfected with the FOXD2-AS1-vector) or KD of FOXD2-AS1 expression (transfected with FOXD2-AS1-siRNA), and the resulting xenograft tumors were examined. Mice were then

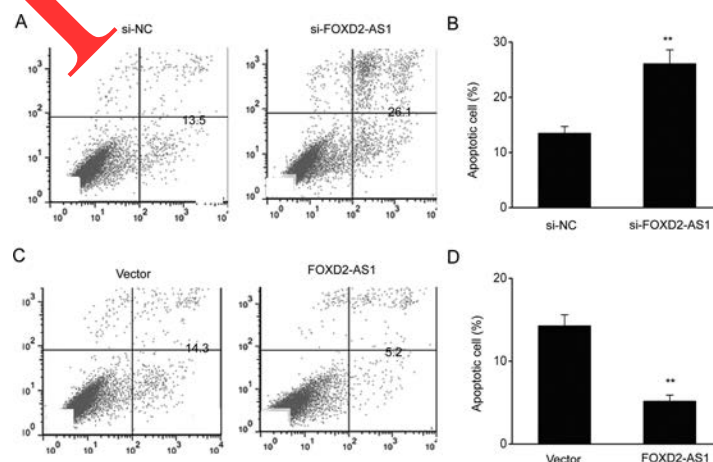


Figure 4. FOXD2-AS1 inhibited the death of ESCC cells. (A) Annexin V-PI staining and flow cytometry were performed to assess cell death in TE-1/DDP cells. (B) Quantitative assessment of cell death in TE-1/DDP cells transfected with FOXD2-AS1 siRNA (si-FOX2-AS1) or negative control siRNA (si-NC). (C) Annexin V-PI staining and flow cytometry were performed to assess cell death in TE-1/DDP cells. (D) Quantitative assessment of cell death in TE-1/DDP cells transfected with the FOXD2-AS1 expression vector (FOX2-AS1) or a control vector (vector). The results are shown as the means \pm SEM. ** $p < 0.01$ versus NC (vector) group.

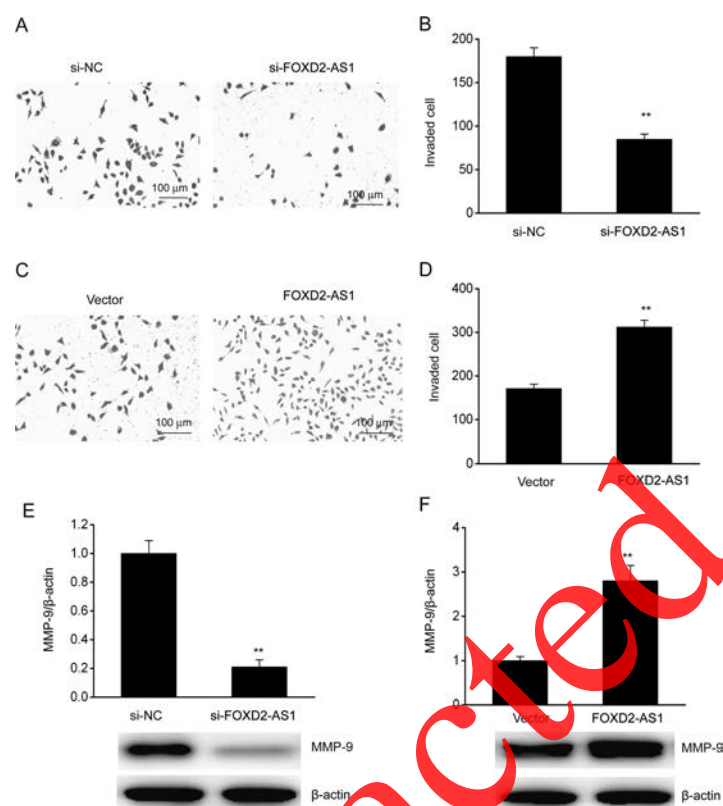


Figure 5. FOXD2-AS1 promoted the invasion of ESCC cells. (A) A Transwell assay was performed to evaluate invasion in TE-1/DDP cells. (B) Quantitative assessment of invading TE-1/DDP cells transfected with FOXD2-AS1 siRNA (si-FOX2-AS1) or negative control siRNA (si-NC). (C) A Transwell assay was performed to evaluate invasion in TE-1/DDP cells. (D) Quantitative assessment of invading TE-1/DDP cells transfected with the FOXD2-AS1 expression vector (FOX2-AS1) or a control vector (vector). (E) Representative immunoblot and quantitative evaluation of MMP-9 in TE-1/DDP cells transfected with si-FOX2-AS1 or si-NC. (F) Representative immunoblot and quantitative evaluation of MMP-9 in TE-1/DDP cells transfected with FOXD2-AS1 or the control vector. The results are shown as the means ± SEM. ** $p < 0.01$ versus the NC vector group.

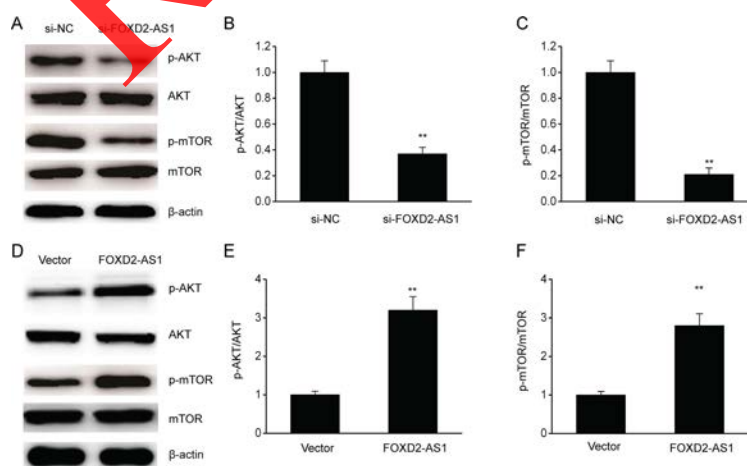


Figure 6. FOXD2-AS1 promoted the invasion of ESCC cells. (A–C) Representative immunoblots (A) and quantitative assessment of p-AKT (B) and p-mTOR (C) in TE-1/DDP cells transfected with FOXD2-AS1 siRNA (si-FOX2-AS1) or the negative control siRNA (si-NC). (D–F) Representative immunoblots (D) and quantitative assessment of p-AKT (E) and p-mTOR (F) in TE-1/DDP cells transfected with the FOXD2-AS1 expression vector (FOX2-AS1) or control vector (vector). The results are shown as the means ± SEM. ** $p < 0.01$ versus NC (vector) group.

administered intraperitoneal injections of cisplatin (2 mg/kg) twice weekly for 4 weeks after the 10-day inoculation. The malignancy-counteracting impact was assessed by examining the tumor volume and weight after treatment. Tumor growth markedly decreased in the FOXD2-AS1-siRNA group treated with cisplatin (Fig. 7A–C), which was similar to the *in vitro* findings. In contrast, overexpression of FOXD2-AS1 increased the tumor volume and weight.

DISCUSSION

EC is a frequent digestive malignancy, with high incidence rates around the world²⁶; in China, ESCC is the most common subtype. Due to its therapeutic effects, step-by-step chemoradiation before surgery has been used to treat ESCC. Response to therapy is crucial to the clinical outcome of patients who are treated with surgery²⁷, as resistance to cisplatin often hinders successful ESCC treatment⁸. lncRNAs are a family of RNAs that do not encode proteins and have been shown to contribute to various pathological conditions, particularly malignancies^{28,29}. Previous studies have shown that the lncRNA FOXD2-AS1 is overexpressed in breast cancer (BC) and increases the proliferation, migration, and invasion of BC cells via positive feedback with E2F1 and Akt³⁰. Therefore, our study was focused on exploring the pathological impact of the lncRNA FOXD2-AS1 on cisplatin resistance in ESCC.

Previous research has demonstrated that lncRNAs contribute to transcriptional and posttranscriptional regulation in ESCC^{31,32}. Chemotherapy is one of the most frequently applied approaches for treating terminal malignancies. For ESCC, combination treatments, including agents such as cisplatin, are essential to therapy³³. In our

study, overexpression of FOXD2-AS1 was detected in ESCC tissues, and cell proliferation of cisplatin-resistant ESCC cells was inhibited in a dose-dependent manner. We confirmed, via gain-of-function and loss-of-function assays, that FOXD2-AS1 overexpression increased the inhibitory impact of cisplatin, increased migration, and repressed cell death in ESCC cells, whereas FOXD2-AS1 KD reduced the inhibitory impact of cisplatin, repressed migration, and triggered cell death. KD of FOXD2-AS1 obviously reduced tumorigenicity as well as tumor volume and weight, and overexpression of FOXD2-AS1 markedly increased tumor volume and weight. Consequently, the above findings suggest that FOXD2-AS1 mediates resistance to cisplatin in ESCC, not only *in vitro* but also *in vivo*.

According to the National Comprehensive Cancer Network (NCCN) guidelines, cisplatin is the most appropriate treatment option for distant metastasis of EC. Nevertheless, the use of cisplatin is greatly hampered by resistance³⁴. Our research findings demonstrated that KD of FOXD2-AS1 increased survival *in vitro*, remarkably repressed malignancy, and had a synergistic impact with cisplatin in xenograft murine models. Nevertheless, our current understanding of how FOXD2-AS1 induces chemoresistance is insufficient. It is widely accepted that irregularities in the AKT/mTOR axis contribute to cisplatin resistance in ESCC cells^{35,36}, and our research showed that FOXD2-AS1 triggers resistance to cisplatin in ESCC mainly through the AKT/mTOR axis.

The dominant mode of action for lncRNAs on malignant phenotypes is modulation of miRNA activity by functioning as a ceRNA, and emerging research has shown that lncRNAs can interact with miRNAs by acting as a sponge and modulating their expression and

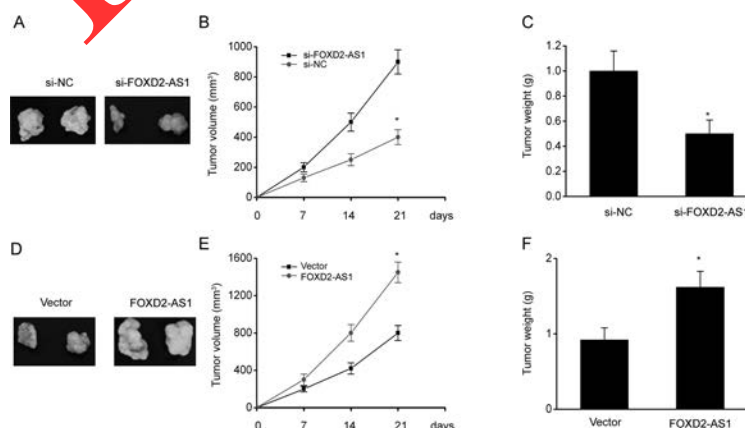


Figure 7. FOXD2-AS1 promoted the invasion of ESCC cells. (A–C) Representative image (A) and the tumor volumes (B) and weights (C) in xenograft mice after injection of TE-1/DDP cells transfected with FOXD2-AS1 siRNA (si-FOXD2-AS1) or negative control siRNA (si-NC) for 21 days. (D–F) Representative image (D) and the tumor volumes (E) and weights (F) in xenograft mice after injection of TE-1/DDP cells transfected with FOXD2-AS1 expression vector (FOXD2-AS1) or a control vector (vector) for 21 days. The results are shown as the means \pm SEM, $n=6$. * $p<0.05$ versus the NC (vector) group.

functions^{37–39}. Our research demonstrated that the lncRNA FOXD2-AS1 sponged miR-195, as it was shown that miR-195 targets FOXD2-AS1 and miR-195 was down-regulated in ESCC cells and tissues. Previous research has shown that miR-195 inhibits malignancy in pancreatic cancer and adrenocortical cancer^{40,41}. Expression of miR-195, which inhibited the survival, invasion, colony generation, and migration of malignant cells, was down-regulated in ESCC in vitro^{42,43}. Consequently, we hypothesized that the FOXD2-AS1/miR-195 axis is a crucial biological system.

In conclusion, this study explored the effect of the lncRNA FOXD2-AS1 on cisplatin resistance in ESCC. Our results showed that FOXD2-AS1 modulates the AKT/mTOR axis and miR-195, indicating its importance during the development of ESCC and suggesting an innovative strategy to treat ESCC.

ACKNOWLEDGMENTS: This work was supported by the Youth Talent Project of Health Commission of Hubei Province (WJ2019Q014). The authors declare no conflicts of interest.

REFERENCES

- Wang BY, Hung WH, Wu SC, Chen HC, Huang CL, Lin CH, Chen HS. Comparison between esophagectomy and definitive chemoradiotherapy in patients with esophageal cancer. *Ann Thorac Surg*. 2019;107(4):1060–7.
- Jiang D, He Z, Wang C, Zhou Y, Li F, Pu W, Zhang X, Feng X, Zhang M, Yecheng X, Xu Y, Jin L, Guo S, Wang J, Wang M. Epigenetic silencing of ZNF132 mediated by methylation-sensitive Sp1 binding promotes cancer progression in esophageal squamous cell carcinoma. *Cell Death Dis*. 2018;10(1):1.
- Zhao JJ, Zhou ZQ, Wang P, Chen CL, Liu X, Pan QZ, Zhu Q, Tang Y, Weng DS, Xia JC. Orchestration of immune checkpoints in tumor immune contexture and their prognostic significance in esophageal squamous cell carcinoma. *Cancer Manag Res*. 2018;10:6457–68.
- Yu J, Ouyang W, Li Y, Hu J, Xu Y, Wei Y, Liao Z, Liu Y, Zhang J, Xie C. Value of radiotherapy in addition to esophagectomy for stage II and III thoracic esophageal squamous cell carcinoma: Analysis of surveillance, epidemiology, and end results-Medicare data. *Cancer Med*. 2019;8(1):21–7.
- Guo X, Fang W, Li Z, Yu Z, Rong T, Fu J, Han Y, Tan L, Chen C, Liu S, Liao Y, Xiao G, Wei Y, Zhu C, Li H, Luo J, Xing W. Adjuvant radiotherapy, chemotherapy or surgery alone for high-risk histological node negative esophageal squamous cell carcinoma: Protocol for a multicenter prospective randomized controlled trial. *Thorac Cancer*. 2018;9(12):1801–6.
- Lyu J, Li T, Wang Q, Li F, Diao P, Liu L, Li C, Lang J. Outcomes of concurrent chemoradiotherapy versus chemotherapy alone for stage IV esophageal squamous cell carcinoma: A retrospective controlled study. *Radiat Oncol*. 2018;13(1):233.
- Xu C, Guo Y, Liu H, Chen G, Yan Y, Liu T. TUG1 confers cisplatin resistance in esophageal squamous cell carcinoma by epigenetically suppressing PDCD4 expression via EZH2. *Cell Biosci*. 2018;8:61.
- Che Y, Wang J, Li Y, Lu Z, Huang J, Sun S, Mao S, Lei Y, Zang R, Sun N, He J. Cisplatin-activated PAI-1 secretion in the cancer-associated fibroblasts with paracrine effects promoting esophageal squamous cell carcinoma progression and causing chemoresistance. *Cell Death Dis*. 2018;9(7):759.
- Hsiao YT, Li SH, Wang YM, Kuo HK. Successful outcome after radiotherapy in esophageal squamous cell carcinoma with choroidal metastasis: A case report and review of literature. *Indian J Ophthalmol*. 2018;66(8):1215–7.
- Fanucchi S, Fok ET, Dalla E, Shibayama Y, Borner K, Chang EY, Stoychev S, Imakaev M, Grimm D, Wang KC, Li G, Sung WK, Mhlanga MM. Immune genes are primed for robust transcription by proximal long noncoding RNAs located in nuclear compartments. *Nat Genet*. 2019;51(1):138–50.
- McKinsey TA, Vondriska TM, Wang Y. Epigenomic regulation of heart failure: Integrating histone marks, long noncoding RNAs, and chromatin architecture. *F1000Res*. 2018;7.
- Li J, Li K, Gao J, Guo X, Lu M, Li Z, Li D. Endogenously synthesized n-3 polyunsaturated fatty acids in pregnant fat-1 mice decreases mammary cancer risk of female offspring by regulating expression of long noncoding RNAs. *Mol Nutr Food Res*. 2018;20:e1801150.
- Xu Q, Yang Q, Zhou Y, Yang B, Jiang R, Ai Z, Teng Y. A long noncoding RNAs signature to improve survival prediction in endometrioid endometrial cancer. *J Cell Biochem*. 2019;120(5):8300–10.
- Zhou Y, Gu C, Li J, Zhu L, Huang G, Dai J, Huang H. Aberrantly expressed long noncoding RNAs and genes in Parkinson's disease. *Neuropsychiatr Dis Treat*. 2018;14:3219–29.
- Denaro N, Merlano MC, Lo NC. Long noncoding RNAs as regulators of cancer immunity. *Mol Oncol*. 2019;13(1):61–73.
- Fanelli GN, Gasparini P, Coati I, Cui R, Pakula H, Chowdhury B, Valeri N, Loupakakis F, Kupcinskis J, Cappellesso R, Fassan M. Long-noncoding RNAs in gastroesophageal cancers. *Noncoding RNA Res*. 2018;3(4):195–212.
- Song X, Fan J, Jia R, Zhou Y, Ge S, Zhang G, Wang H, Fan X. Identification and regulation pattern analysis of long noncoding RNAs in meibomian gland carcinoma. *Epigenomics*. 2019;11(4):381–400.
- Yang X, Duan B, Zhou X. Long non-coding RNA FOXD2-AS1 functions as a tumor promoter in colorectal cancer by regulating EMT and Notch signaling pathway. *Eur Rev Med Pharmacol Sci*. 2017;21(16):3586–91.
- Xu TP, Wang WY, Ma P, Shuai Y, Zhao K, Wang YF, Li W, Xia R, Chen WM, Zhang EB, Shu YQ. Upregulation of the long noncoding RNA FOXD2-AS1 promotes carcinogenesis by epigenetically silencing EphB3 through EZH2 and LSD1, and predicts poor prognosis in gastric cancer. *Oncogene*. 2018;37(36):5020–36.
- An Q, Zhou L, Xu N. Long noncoding RNA FOXD2-AS1 accelerates the gemcitabine-resistance of bladder cancer by sponging miR-143. *Biomed Pharmacother*. 2018;103:415–20.
- Chang Y, Zhang J, Zhou C, Qiu G, Wang G, Wang S, Chang X, Li X, Fan L. Long non-coding RNA FOXD2-AS1 plays an oncogenic role in hepatocellular carcinoma by targeting miR206. *Oncol Rep*. 2018;40(6):3625–34.
- Chen G, Sun W, Hua X, Zeng W, Yang L. Long non-coding RNA FOXD2-AS1 aggravates nasopharyngeal carcinoma

- carcinogenesis by modulating miR-363-5p/S100A1 pathway. *Gene* 2018;645:76–84.
23. Ren W, Zhu Z, Wu L. FOXD2-AS1 correlates with the malignant status and regulates cell proliferation, migration, and invasion in cutaneous melanoma. *J Cell Biochem*. 2019;120(4):5417–23.
 24. Yu S, Zhao Y, Lai F, Chu M, Hao Y, Feng Y, Zhang H, Liu J, Cheng M, Li L, Shen W, Min L. LncRNA as ceRNAs may be involved in lactation process. *Oncotarget* 2017;8(58):98014–28.
 25. Liu J, Xing Y, Rong L. miR-181 regulates cisplatin-resistant non-small cell lung cancer via downregulation of autophagy through the PTEN/PI3K/AKT pathway. *Oncol Rep*. 2018;39(4):1631–9.
 26. Chen Z, Hu X, Wu Y, Cong L, He X, Lu J, Feng J, Liu D. Long non-coding RNA XIST promotes the development of esophageal cancer by sponging miR-494 to regulate CDK6 expression. *Biomed Pharmacother*. 2019;109:2228–36.
 27. Sadeghpour S, Ghorbani S. Evaluation of the potential clinical prognostic value of lncRNA-BANCR gene in esophageal squamous cell carcinoma. *Mol Biol Rep*. 2019;46(1):991–5.
 28. Gong J, Lu X, Xu J, Xiong W, Zhang H, Yu X. Coexpression of UCA1 and ITGA2 in pancreatic cancer cells target the expression of miR-107 through focal adhesion pathway. *J Cell Physiol*. 2019;234(8):12884–96.
 29. Lin Y, Zhang CS, Li SJ, Li Z, Sun FB. LncRNA LOC554202 promotes proliferation and migration of gastric cancer cells through regulating p21 and E-cadherin. *Eur Rev Med Pharmacol Sci*. 2018;22(24):8690–97.
 30. Su F, He W, Chen C, Liu M, Liu H, Xue F, Bi J, Xu D, Zhao Y, Huang J, Lin T, Jiang C. The long non-coding RNA FOXD2-AS1 promotes bladder cancer progression and recurrence through a positive feedback loop with Akt and E2F1. *Cell Death Dis*. 2018;9(2):233.
 31. Kang K, Huang YH, Li HP, Guo SM. Expression of UCA1 and MALAT1 long-chain non-coding RNAs in esophageal squamous cell carcinoma tissues is predictive of patient prognosis. *Arch Med Sci*. 2018;14(4):752–9.
 32. Kang M, Ren M, Li Y, Fu Y, Deng M, Li C. Exosome-mediated transfer of lncRNA PART1 induces gefitinib resistance in esophageal squamous cell carcinoma via functioning as a competing endogenous RNA. *J Exp Clin Cancer Res*. 2018;37(1):171.
 33. Qiao Y, Zhang C, Li A, Wang D, Luo Z, Ping Y, Zhou B, Liu S, Li H, Yue D, Zhang Z, Chen X, Shen Z, Lian J, Li Y, Wang S, Li F, Huang L, Wang L, Zhang B, Yu J, Qin Z, Zhang Y. IL6 derived from cancer-associated fibroblasts promotes chemoresistance via CXCR7 in esophageal squamous cell carcinoma. *Oncogene* 2018;37(7):873–83.
 34. Murakami Y, Hamai Y, Emi M, Hihara J, Imano N, Takeuchi Y, Takahashi I, Nishibuchi I, Kimura T, Okada M, Nagata Y. Long-term results of neoadjuvant chemoradiotherapy using cisplatin and 5-fluorouracil followed by esophagectomy for resectable, locally advanced esophageal squamous cell carcinoma. *J Radiat Res*. 2018;59(5):616–24.
 35. Wang HJ, Yang ZX, Dai XT, Chen YF, Yang HP, Zhou XD. Bisdemethoxycurcumin sensitizes cisplatin-resistant lung cancer cells to chemotherapy by inhibition of CA916798 and PI3K/AKT signaling. *Apoptosis* 2017;22(9):1157–68.
 36. Zhang Y, Chen S, Wei C, Rankin GO, Rojanasakul Y, Ren N, Ye X, Chen YC. Dietary Compound Proanthocyanidins from Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) leaves inhibit angiogenesis and regulate cell cycle of cisplatin-resistant ovarian cancer cells via targeting Akt pathway. *J Funct Foods* 2018;40:573–81.
 37. Huang X, Xie X, Liu P, Yang L, Chen B, Song C, Tang H, Xie X. Adam12 and lnc015192 act as ceRNAs in breast cancer by regulating miR-34a. *Oncogene* 2018;37(49):6316–26.
 38. Liang R, Han B, Li Q, Yuan Y, Li J, Sun D. Using RNA sequencing to identify putative competing endogenous RNAs (ceRNAs) potentially regulating fat metabolism in bovine liver. *Sci Rep*. 2017;7(1):6396.
 39. Wang Y, Gao L, Zhu B, Zhu H, Luo Y, Wang Q, Zuo J. Integrative analysis of long non-coding RNA acting as ceRNAs involved in chilling injury in tomato fruit. *Gene* 2018;667:25–33.
 40. Chabre O, Libe R, Assie G, Barreau O, Bertherat J, Bertagna X, Feige JJ, Cherradi N. Serum miR-483-5p and miR-195 are predictive of recurrence risk in adrenocortical cancer patients. *Endocr Relat Cancer* 2013;20(4):579–94.
 41. Zhou B, Sun C, Hu X, Zhan H, Zou H, Feng Y, Qiu F, Zhang S, Wu L, Zhang B. MicroRNA-195 suppresses the progression of pancreatic cancer by targeting DCLK1. *Cell Physiol Biochem*. 2017;44(5):1867–81.
 42. Fu MG, Li S, Yu TT, Qian LJ, Cao RS, Zhu H, Xiao B, Jiao CH, Tang NN, Ma JJ, Hua J, Zhang WF, Zhang HJ, Shi RH. Differential expression of miR-195 in esophageal squamous cell carcinoma and miR-195 expression inhibits tumor cell proliferation and invasion by targeting of Cdc42. *FEBS Lett*. 2013;587(21):3471–9.
 43. Sun N, Ye L, Chang T, Li X, Li X. microRNA-195-Cdc42 axis acts as a prognostic factor of esophageal squamous cell carcinoma. *Int J Clin Exp Pathol*. 2014;7(10):6871–9.