Original Article

FOXF2 may inhibit esophageal squamous cell carcinoma growth and metastasis by regulating the *EZR-ERBB2* axis

Zhen-Yu Hu[1,2#], Wei-Hao Deng[2,3#], Wei-Jie Cai[1,2#], Xian-Yu Qin[1,2], Hao-Sheng Zheng[1,2], Jian Tan[1,2], Xiao-Long Jiang[1,2], Yu-Zhen Zheng[1,2], Hong-Ying Liao[1,2]

[1]Department of Thoracic Surgery, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; [2]Biomedical Innovation Center, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; [3]Department of Pathology, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Contributions: (I) Conception and design: YZ Zheng; (II) Administrative support: HY Liao; (III) Provision of study materials or patients: YZ Zheng, HY Liao; (IV) Collection and assembly of data: ZY Hu, WH Deng, WJ Cai, XY Qin, HS Zheng, J Tan; (V) Data analysis and interpretation: ZY Hu, WH Deng, WJ Cai, XL Jiang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Yu-Zhen Zheng, MD; Hong-Ying Liao, MD. Department of Thoracic Surgery, The Sixth Affiliated Hospital, Sun Yat-sen University, No. 26 Erheng Road, Yuancun, Tianhe District, Guangzhou 510655, China; Biomedical Innovation Center, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. Email: zhengyzh26@mail.sysu.edu.cn; liaohy2@mail.sysu.edu.cn.

Background: FOXF2, a member of the transcription factor FOX family proteins, plays a key role in tumorigenesis and tumor aggressiveness. However, the potential molecular mechanism of FOXF2 in esophageal squamous cell carcinoma (ESCC) remains largely unknown. Exploring its role and mechanism in ESCC progression may help identify new diagnostic markers and therapeutic targets. The aim of this study is to investigate the potential functions of the *FOXF2* gene within the context of ESCC and to elucidate the underlying molecular pathways involved.

Methods: Using the GoMiner database, GeneCard database, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, and the COMPARTMENTS subcellular localization database, we identified the most likely downstream molecule of the *FOXF2* gene, EZR; the subcellular locations of FOXF2 and EZR; the possible biological pathways [Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)]; and the protein interactions networks of the *EZR* gene enriched from the OMICS datasets via Metascape. We also used The Cancer Genome Atlas database to analyze the correlation between EZR and ERBB signaling pathway. In addition, we verified the RNA and protein expression of the target genes using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Furthermore, we employed Western blot analysis and plasmid transfection and lentiviral infection techniques to gene edit *FOXF2* and *EZR* in different EC cells to obtain stable overexpression or knockdown of the cell lines. This was followed by *ex vivo* and *in vivo* experiments including migration assay, cell scratch assay, clone formation assay, and a xenotransplantation mouse model to validate the functional phenotype of the gene-edited cells.

Results: We found that knockdown of *FOXF2* expression significantly enhanced the growth, invasion, and metastasis of ESCC cells both *in vitro* and *in vivo*. Moreover, we demonstrated that FOXF2 was predominantly expressed in the nucleus and directly interacted with EZR, thereby inhibiting *EZR* transcriptional expression, resulting in suppressed *ERBB2* signal function, ultimately halting ESCC growth and metastasis.

Conclusions: Taken together, these results reveal the tumor-suppressive functions of FOXF2 in inhibiting EZR-mediated ERBB2 activation, suggesting that FOXF2 could serve as a potential novel predicting prognostic biomarker for ESCC.

Keywords: *FOXF2*; *EZR*; esophageal squamous cell carcinoma (ESCC); ERBB2 signaling pathway

Submitted Nov 26, 2024. Accepted for publication Dec 20, 2024. Published online Dec 27, 2024.
doi: 10.21037/tcr-2024-2365
View this article at: https://dx.doi.org/10.21037/tcr-2024-2365

Introduction

Esophageal cancer (EC) is the seventh most common cancer worldwide, with more than 50% of patients being of Chinese ethnicity (1). In China, more than 90% of the EC cases are esophageal squamous cell carcinoma (ESCC) (2). In addition, patients with ESCC are usually diagnosed at a late stage and do not benefit from targeted therapies due to the lack of biomarkers, which facilitate early diagnosis and precise treatment, leading to a poor prognosis. Despite significant advances in surgical technique, ESCC has a poor outlook, and the 5-year survival rate is less than 30% (3). Therefore, there is an urgent need to develop novel prognostic biomarkers and therapeutic targets for ESCC.

FOXF2 is a member of the transcription factor FOX family proteins. In recent years, several studies have indicated that *FOXF2* may play a critical role in tumorigenesis, progression, and therapeutic resistance. For example, in breast cancer, a deficiency in FOXF2 activates the VEGF-C/VEGFR3 signaling pathway and facilitates lymphangiogenesis and metastasis (4). Higashimori *et al.* found that FOXF2 expression is lower in gastric cancer than in normal mucosa and that the knockdown of *FOXF2* expression promotes proliferation and migration ability of gastric cancer cell line (5). Moreover, FOXF2 may be critical for Hedgehog/Wnt feedback signaling and for maintaining intestinal homeostasis, with suppressed function of *FOXF2* being associated with the tumorigenesis of colorectal cancer (6). Similar results have been reported for other human cancers (7,8). While the *FOXF2* gene has been implicated in various cancer types, the precise mechanisms underlying its action remain to be fully elucidated. In the context of breast cancer, its role appears to be contingent upon the specific tumor subtype (9). Current research predominantly concentrates on the gene's biological function as a transcription factor. Nevertheless, the intricate mechanisms by which *FOXF2* exerts its tumor-inhibitory effects, particularly within the diverse tumor microenvironments, warrant further in-depth investigation. In our previous study, we observed that FOXF2 had a lower expression in ESCC. In addition, patients with ESCC with lower FOXF2 expression were associated with a more advanced stage of disease and shorter survival (10).

We subsequently conducted this study, in which knockdown of *FOXF2* expression significantly enhanced the growth, invasion, and metastasis of ESCC cells both *in vitro* and *in vivo*. We further identified *EZR* as a downstream gene of *FOXF2*. FOXF2 may bind to the promoter of *EZR* and inhibit its transcriptional expression, resulting in the suppressed ERBB2 signal function and ultimately halting ESCC growth and metastasis. Our research addresses a significant gap by providing an experimental exploration of the *FOXF2* gene's biological function in the context of ESCC. Notably, our study introduces a novel finding that the *EZR* gene potentially participates in the modulation of the ERBB2 signaling cascade, a role previously attributed to the *FOXF2* gene. This involvement suggests a critical contribution to the pathogenesis and progression of ESCC. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available at https://tcr.amegroups.com/article/view/10.21037/tcr-2024-2365/rc).

Highlight box

Key findings

- Our study revealed that *FOXF2* plays a tumor-suppressing role in esophageal cancer (EC) and inhibits the *ERBB2* signaling pathway through the inhibition of the downstream signaling molecule *EZR*.

What is known and what is new?

- FOXF2, one of the family proteins of the transcription factor FOX, has been demonstrated to have a role in promoting tumor development and inducing therapeutic resistance in several malignancies, such as breast, gastric, and colorectal cancers, when its gene expression is reduced or absent.
- We validated the functional role of *FOXF2* in EC cell lines, identified its downstream direct-acting molecules, and clarified the possible relationship between *FOXF2* and the *EZR-ERBB2* signaling pathway axis.

What is the implication, and what should change now?

- FOXF2 may serve as a potential novel biomarker for predicting the prognosis of patients with EC. Moreover, detection of its own and downstream signaling molecules may improve the long-term survival of patients with this disease.

Methods

Data collection

A range of open-source, publicly accessible databases were utilized in our research. For instance, we accessed RNA-seq data related to esophageal cancer patients from The Cancer Genome Atlas (TCGA) and the University of California Santa Cruz (UCSC) databases. Additionally, we employed the R packages "TFBSTools", "JASPAR", and "PROMO" (R version 4.4.0) for our analysis. To further analyze protein localization, subcellular localization, function, and interactions, we utilized the GoMiner (11), GeneCards

(https://www.genecards.org/), Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://string-db.org/), and COMPARTMENTS subcellular localization databases (https://compartments.jensenlab.org). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell lines

Ten different ESCC cell lines, including KYSE30, KYSE140, KYSE410, KYSE510, HK, TE1, KYSE180, EC18, EC109, and CE81t, were obtained from Sun Yat-sen University Cancer Center, Guangdong Esophageal Cancer Institute, Guangzhou, China. All cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 IU/mL of penicillin and 100 mg/mL of streptomycin (Gibco) and incubated at 37 ℃ in a humidified incubator containing 5% CO_2.

RNA isolation and real-time quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated using Revert Aid First Strand cDNA Synthesis (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using a Power SYBR Green PCR Master Mix Kit (Applied Biosystems Inc., Thermo Fisher Scientific, Waltham, MA, USA) on a real-time PCR system. The relative expression of messenger RNA (mRNA) in EC cells (fold) was calculated using the $2^{-\Delta Ct}$ method and normalized to GAPDH.

Western blot analysis

The cells were subjected to lysis using a lysis buffer and were followed by centrifugation at 4 ℃ to isolate and retrieve the proteins present in the supernatant fraction. Protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For Western blot analysis, equal amounts of protein extract were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Pall Corp., Port Washington, NY, USA). The PVDF membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween20 for 1 hour and incubated overnight with primary antibodies according to the manufacturer's instructions. Antibodies of anti-EZR (1:1,000; CST, 3145), antibodies of anti-FOXF2 (1:1,000; Abcam, ab198283) were purchased and used.

Plasmid transfection

The *FOXF2* expression of each ESCC cell line was analyzed by qRT-PCR and Western blot analysis (*Figure 1A*). The ESCC cells with higher FOXF2 expression (CE81t) were transfected with lentivirus carrying *FOXF2* short hairpin RNA (shRNA) and FOXF2 control (CON) vectors (GeneChem Co., Shanghai, China) to knockdown the expression of *FOXF2*; ESCC cells with lower *FOXF2* expression (KYSE30) were transfected with lentivirus carrying *FOXF2*-ORF (open reading frame) and *FOXF2*-CON vectors (GeneChem Co.) to overexpress *FOXF2*. Stably transfected cell lines, namely KYSE30-ORF, KYSE30-CON, CE81t-SH, and CE81t-CON, were established via the addition of puromycin.

To evaluate the interaction between FOXF2 and EZR, CE81t-FOXF2SH was transfected with a lentivirus carrying *EZR*-shRNA and *EZR*-CON vector (GeneChem Co.) to knockdown the expression of *FOXF2*. Stably transfected cell lines, namely CE81t-FOXF2SH-EZRCON and CE81t-FOXF2SH-EZRSH, were established via the addition of chloramphenicol.

Cell Counting Kit-8 analysis

The ESCC cells were seeded onto 96-well plates and incubated for 48 hours. Subsequently, Cell Counting Kit-8 (10 μL) was added into the culture medium for 4 hours, and the optical density (OD) value at 450 nm was detected using a microplate reader (Elx808; BioTek Instruments, Winooski, VT, USA).

Colony formation assay

A total of 2,000 viable ESCC cells were seeded into six-well plates and incubated for 10 days to allow for growth. After incubation, the colonies were fixed with phosphate-buffered saline (PBS) and stained with 1% crystal violet for 30 minutes.

Scratch assay

A scratch assay was performed to study cell migration

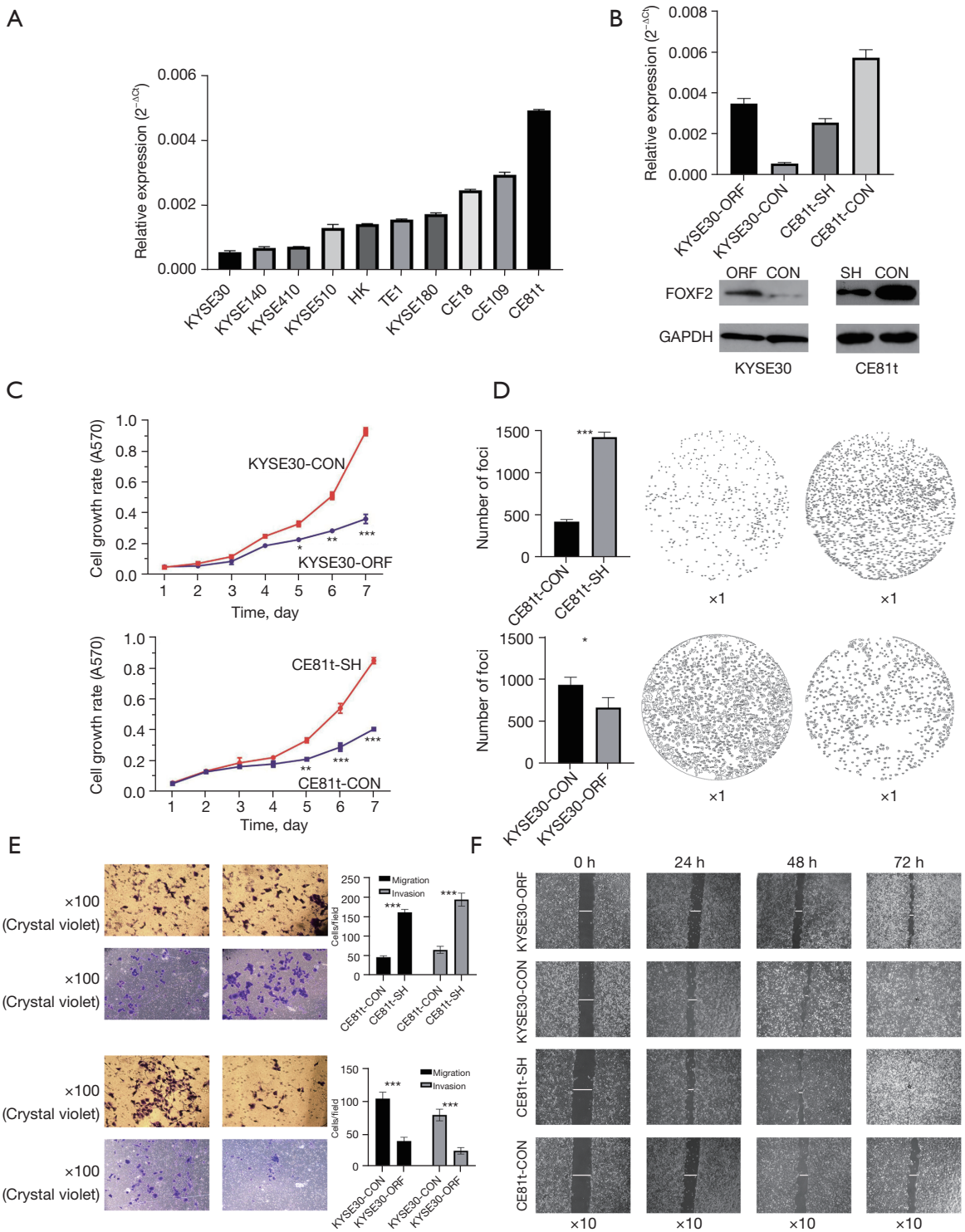

Figure 1 FOXF2 inhibited ESCC cell proliferation, invasion, and metastasis. (A) FOXF2 expression in different EC cell lines. (B) KYSE30 was transfected with FOXF2-ORF or control vector, CE81t was transfected with FOXF2-SH or control vector, and the expression of FOXF2 was detected by qRT-PCR and Western blotting. (C-F) Cells with lower FOXF2 expression demonstrated enhanced cell growth, invasion, and metastasis. (D) Clone formation and (F) wound-healing assay. *, P<0.05; **, P<0.01; ***, P<0.001. ORF, open reading frame; CON, control; SH, short hairpin RNA; ESCC, esophageal squamous cell carcinoma; EC, esophageal cancer; qRT-PCR, real-time quantitative reverse-transcription polymerase chain reaction.

changes. Trypsinized cells were seeded into six-well plates and grown until full confluence. A straight scratch was created with a sterile pipette tip. Subsequently, the cells were incubated with fresh complete medium. The area of the wound closed was calculated at indicated time points.

Migration and invasion assay

For the migration or invasion assay, 2,000 cells were incubated in the upper chamber with solid growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), with the lower chamber filled with complete medium. Cells were incubated for 12 hours at 37 ℃ to allow for migration. Next, the cells that had migrated through the filter pores from the underside of the membrane were fixed and stained.

Luciferase assay

Cells were seeded into six-well plates, and then *FOXF2* overexpression plasmid and *EZR* promoter-driven luciferase plasmids were simultaneously cotransfected into the cells via Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA). After 48 hours of transfection, a luciferase assay kit was applied to examine the luciferase expression in the cells according to the manufacturer's instructions of Lipofectamine 2000.

Functional enrichment analysis

Metascape (http://metascape.org) is a free, efficient, effective, user-friendly gene-list analysis tool for gene annotation and analysis (12) and based on enriched biological pathways and protein complexes contained within the OMICs datasets. Metascape integrates functional enrichment, interactome analysis, and gene annotation, serving as a comprehensive tool to elucidate shared and distinct pathways across various independent target-discovery studies. In this study, Metascape was used to conduct pathway and process enrichment analysis of the top 100 genes positively correlated with *EZR* in multiple types of cancer. Gene Ontology (GO) terms for biological processes, cellular component, and molecular function categories, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, were enriched using the Metascape online tool.

Xenograft mouse model

Four-week-old male C57BL/6 nude mice were purchased for this experiment (Cyagen, Guangzhou, China). In the subcutaneous xenograft model, 0.5×10^7 stable ESCC cells were subcutaneously injected into the abdomen of nude mice. All nude mice were harvested on the 21st day after tumor inoculation and euthanized on the 28th day. A protocol was prepared before the study without registration. Animal experiments were performed under a project license (No. L2021ZSLYEC-106) granted by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University, in compliance with the national guidelines for the care and use of animals.

Statistical analysis

Experiments were repeated three times, and data are presented as the mean ± standard deviation. Data were analyzed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.0 (GraphPad Software, Boston, MA, USA). One-way analysis of variance followed by the Tukey post hoc test was used to analyze differences between multiple groups, while the Student *t*-test was applied to assess differences between two groups. $P<0.05$ indicated a statistically significant difference.

Results

FOXF2 promoted ESCC growth, metastasis, and drug resistance

The *FOXF2* expression of each ESCC cell line was analyzed via qRT-PCR and Western blot analysis (*Figure 1A*). CE81t cells exhibited the highest expression level of *FOXF2* among the ESCC cell lines, while KYSE30 exhibited the slowest expression level. We knocked down the *FOXF2* expression in CE81t (CE81t-SH and CE81t-CON) cells and overexpressed *FOXF2* in KYSE30 (KYSE30-ORF and KYSE30-CON) cells. The efficiency of *FOXF2* transfection was assessed via qRT-PCR and Western blot analysis (*Figure 1B*). In the cell viability assay, ESCC cells with a relative low level of FOXF2 expression (KYSE30-CON and CE81t-SH) had a greater proliferative (*Figure 1C*), clone formation (*Figure 1D*), migration and invasion ability (*Figure 1E*), and wound-healing ability (*Figure 1F*).

EZR as a potential downstream gene of FOXF2

From TCGA database, we extracted the genome and transcriptome information of 184 cases of ESCC and analyzed the potential factor coexpression with *FOXF2*. A positive association with *FOXF2* expression was observed in 1,518 genes, and a negative association with *FOXF2* expression was observed in 1,270 genes (*Figure 2A*). Through the GoMiner database and GeneCard database, we identified the localization and function of the protein products of these candidate genes. Using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, we evaluated the gene-protein interaction relationship and selected *EZR* as the key downstream gene of FOXF2 (*Figure 2B,2C*).

FOXF2 could inhibit the transcription through biding the promoter of EZR

Based on the COMPARTMENTS subcellular localization database (13), we predicted the subcellular locations of FOXF2 and EZR. In *Figure 3A*, the confidence of the evidence is color coded, ranging from light green [1] for low confidence to dark green [5] for high confidence, while white [0] indicates an absence of localization evidence. The FOXF2 protein was primarily located in the nucleus, and the EZR protein was enriched in the cytosol, plasma membrane, and nucleus.

The sequence of the *EZR* promoter region was evaluated by a review of the PubMed database and verified in the UCSC database. Prediction of transcription factors binding sites in the *EZR* promoter region was examined with the "TFBSTools", "JASPAR", and "PROMO" packages. As shown in *Figure 3B*, FOXF2 can bind to the promoter region of *EZR*.

To further determine whether FOXF2 negatively regulates the *EZR* gene, an *FOXF2* overexpression plasmid was transfected into HEK293T cells. We performed a dual-luciferase reporter (DLR) assay to determine whether FOXF2 could regulate the promoter of the *EZR* gene. The luciferase relative activity of firefly/Renilla indicated that the FoxF2 protein was a negative regulator of the *EZR* promoter, confirming that FOXF2 inhibits the transcription of EZR by anchoring at its promoter (*Figure 3C*). Western blot and qRT-PCR analysis indicated that *FOXF2* expression was negatively correlated with *EZR*, which is accordance with the aforementioned relationship (*Figure 3D,3E*).

ERBB2 maybe the downstream signaling pathway of EZR

We further analyzed the downstream signaling pathway of EZR through the database and found that there was a significant correlation between the ERBB signaling pathway and EZR (*Figure 4A*). Further analysis of the mRNA levels in the EC database in TCGA showed that this relationship was particularly evident in *ERBB2*; that is, *EZR* was negatively correlated with *FOXF2* and positively correlated with *ERBB2* (*Figure 4B*). Western Blot analysis based on the constructed *EZR* stable knockdown cell line and its control group (CE81t-FOXF2SH-EZRSH *vs.* CE81t-FOXF2SH-EZRCON) showed that the cell line with the higher *EZR* expression level had a higher *HER2* expression level (*Figure 4C*). Moreover, the phosphorylation levels of PI3K and Akt protein were high, suggesting that EZR may exert its effect by activating HER2 and its downstream PI3K/Akt signaling pathway.

Xenograft mouse model confirmed the impact of the FOXF2/EZR signal pathway on tumor progression

To determine the effect of FOXF2-EZR on tumorigenicity of ESCC *in vivo*, an EC xenograft model was constructed (*Figure 5A*). The tumors formed by the *FOXF2*-knockdown EC cell line (CE81t-FOXF2SH) were much larger and heavier than were those in the control group (CE81t-FOXF2CON) (*Figure 5B,5C*). Inhibition of EZR protein expression reversed the trend of tumorigenesis in the low FOXF2 protein expression group. The tumors formed by the *EZR*-knockdown EC cell line (CE81t-FOXF2SH-EZRSH) were much smaller and lighter than were those with normal *EZR* expression (CE81t-FOXF2SH) (*Figure 5B,5C*).

Discussion

In this study, we found that *FOXF2* would suppress the growth of ESCC by inhibiting *EZR* expression and by downregulating the related *HER2* pathways.

FOXF2 has been demonstrated to be a tumor-suppressor gene in various human cancers. For example, Fang *et al.* found that suppressed *FOXF2* expression is often observed in thyroid cancer and is associated with epithelial-mesenchymal transition (EMT) and metastasis (14). Jia *et al.* showed that augmenting lung stromal Foxf2 mediates an immunosuppressive milieu and inhibits lung colonization of prostate cancer (15). The diminished expression of

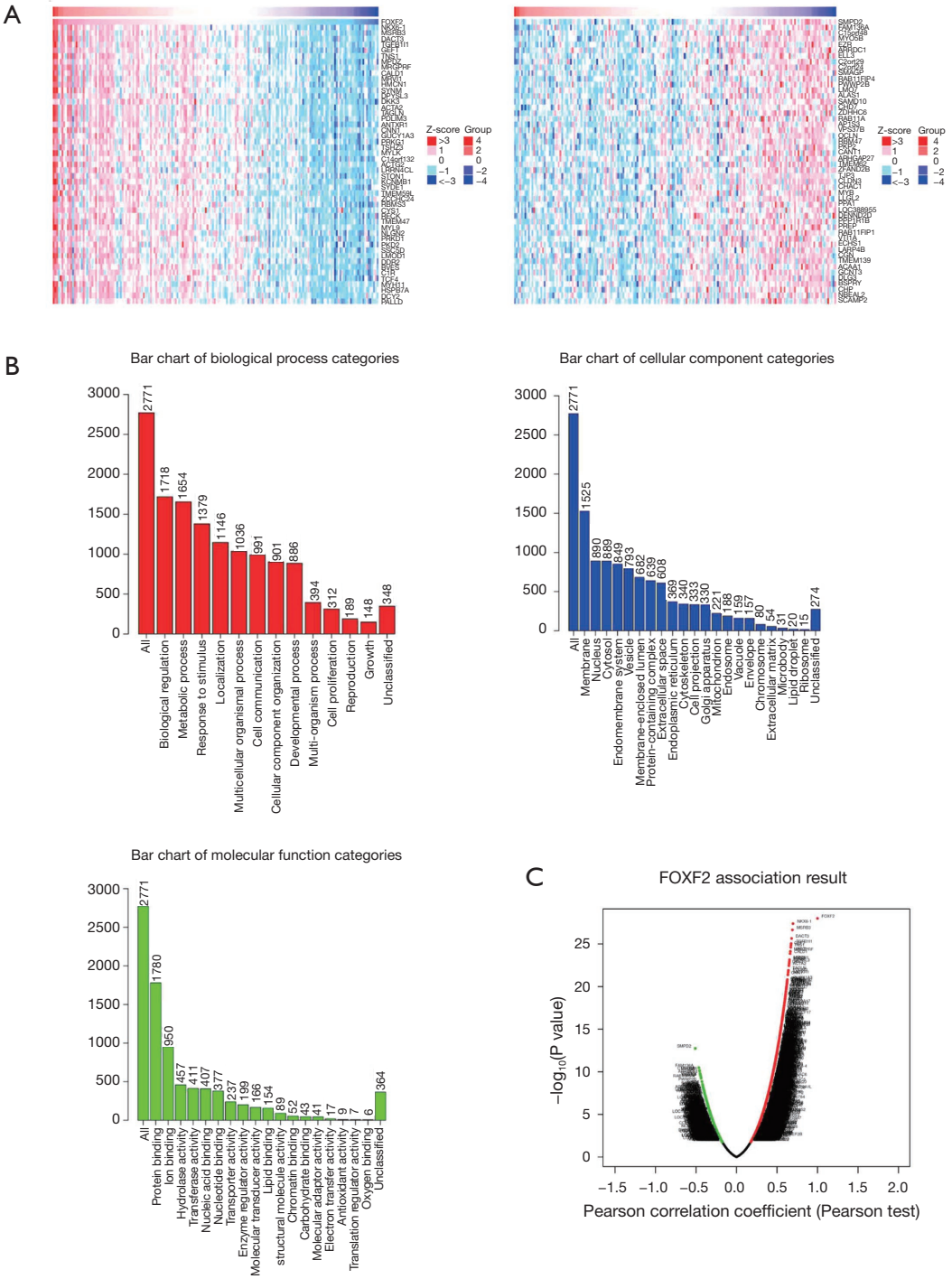

Figure 2 *FOXF2* gene may be involved in the occurrence and development of tumors through various regulatory modes. (A) The color represents the Z-score, with blue indicating low expression and red indicating high expression, which provides a comparison of how the expression of each gene changes under different conditions. (B) Gene classifications into categories of biological processes (colored red), cellular components (colored blue), and molecular functions (colored green) are presented. In these classifications, the height of each bar corresponds to the number of genes involved. (C) Scatter plot of *FOXF2* association results with Pearson correlation coefficient on the horizontal axis and $-\log_{10}$(P value) on the vertical axis. The red dots denote statistically significant positive correlations with the FOXF2 gene, whereas the green dots indicate statistically significant negative correlations.

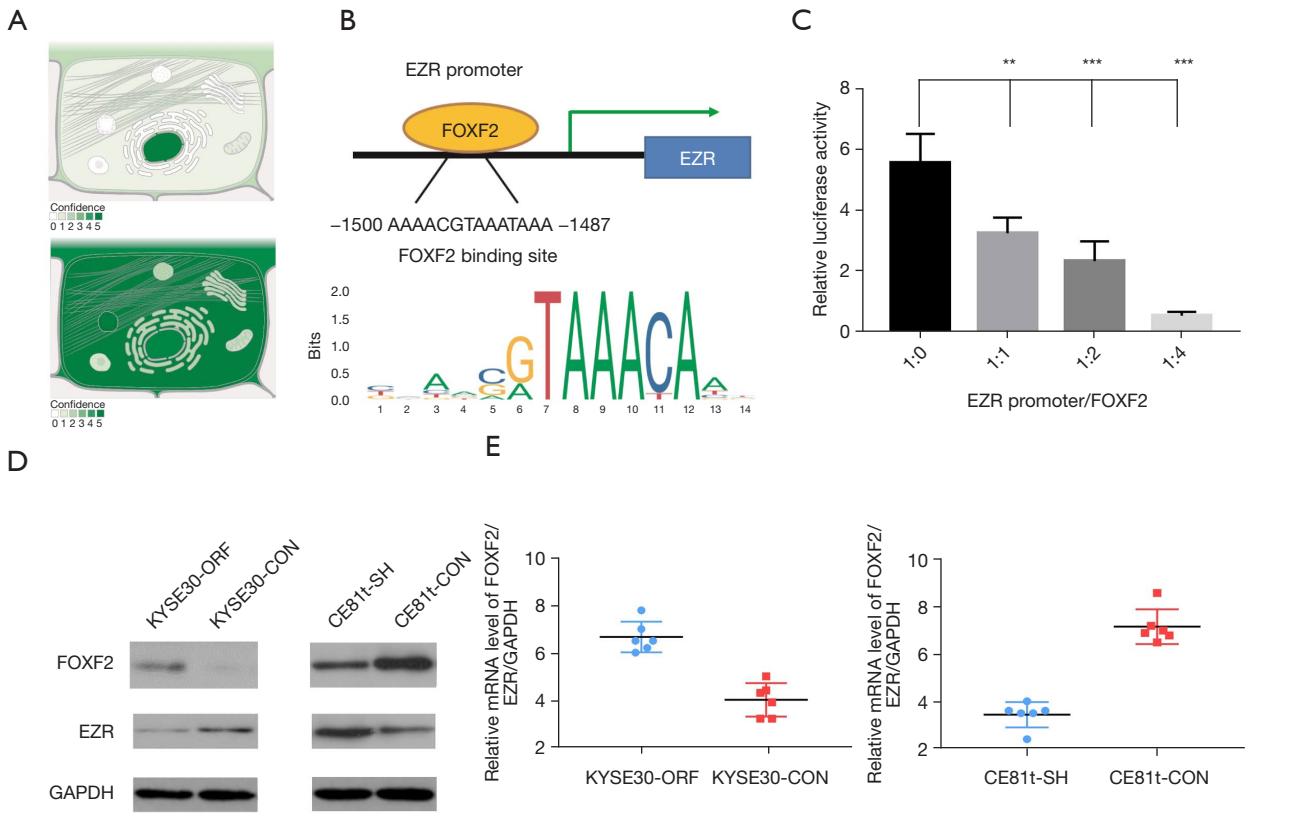

Figure 3 FOXF2 inhibited transcription through biding the promoter of EZR. (A) Subcellular locations of the FOXF2 and EZR. (B) FOXF2 could bind to the promoter region of EZR. The x-axis represents the sequence positions from the 5' to 3' end on the DNA. (C) The luciferase relative activity of firefly/Renilla indicated that the FOXF2 protein was a negative regulator of the EZR promoter, which verified the direct transcriptional inhibition of EZR by FOXF2. (D,E) According to the Western blotting and qRT-PCR analysis, FOXF2 expression was negatively correlated with EZR. **, P<0.01; ***, P<0.001. ORF, open reading frame; CON, control; SH, short hairpin RNA; qRT-PCR, real-time quantitative reverse-transcription polymerase chain reaction.

FOXF2 protein presents novel opportunities for tumor development. However, the precise factors contributing to this reduction remain to be comprehensively identified. Extensive research has demonstrated that the methylation of gene promoters can lead to the suppression of gene expression. The relationship between the downregulation of the *FOXF2* gene in ESCC and its promoter methylation status is a subject of ongoing debate. Some researchers have suggested that *FOXF2*, classified as a tumor suppressor gene, when hypermethylated at the promoter level, may contribute to oncogenesis and tumor progression. For instance, Tian *et al.* revealed that methylation within the CpG islands of the *FOXF2* proximal promoter region plays a role in the regulation of *FOXF2* isoform-specific expression in breast cancer cells (16). Chen *et al.* examined the methylation status of the *FOXF2* gene promoter in tumor tissue from 135 patients with esophageal squamous carcinoma and found that higher levels of methylation showed a poor prognosis (17). These studies underscore the significance of the *FOXF2* gene in tumorigenesis and development. Nevertheless, the intricate mechanisms by which *FOXF2* is involved in the initiation, progression, and metastasis of ESCC are yet to be fully elucidated. In our previous study, we performed qRT-PCR analysis in 188 resected ESCC samples, including 33 pairs of tumor and non-tumor tissues (10). We found that the *FOXF2* mRNA was downregulated in tumor tissues compared to paired non-tumor tissues. Moreover, a lower level *FOXF2* mRNA expression was an independent predictor of lymph node metastasis and shorter survival, even after adjustments

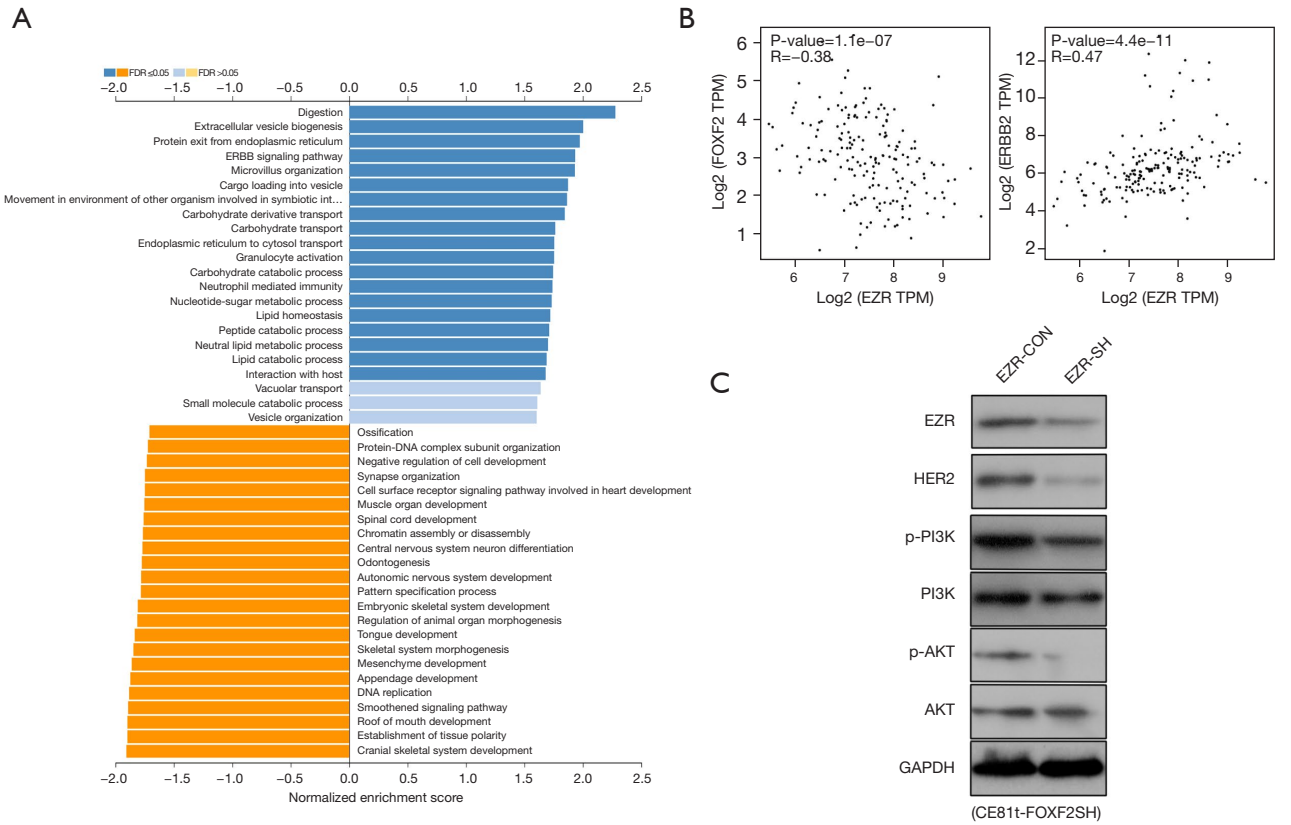

Figure 4 EZR upregulation activated the ERBB2 signaling pathway. (A) There was a significant correlation between the ERBB signaling pathway and the EZR expression matrix in TCGA database. (B) EZR was negatively correlated with FOXF2 but positively correlated with ERBB2 in the esophageal cancer database of TCGA. (C) Western blot analysis was performed on the stable EZR-knockdown cell line and its control group. FDR, false discovery rate; TPM, transcripts per kilobase of exon model per million mapped reads; CON, control; SH, short hairpin RNA; TCGA, The Cancer Genome Atlas database.

were made for other confounders. These results were in high accordance with the present study, as ESCC with suppressed *FOXF2* expression was associated with enhanced cell proliferation, colony formation, migration, and invasion. Our studies suggest that further research to clarify the role of *FOXF2* is warranted.

EZR is a cytoskeletal protein belonging to ezrin-radixin-moesin (ERM) family and contains three major domains: the amino-terminal FERM domain, the α-helical domain, and the carboxy-terminal ERM association domain (C-ERMAD) (18,19). In an inactive closed status, EZR is localized in the cytoplasm where the FERM and C-ERMAD domains bind to each other (20). In an active open status, EZR is recruited to the plasma membrane where the C-ERMAD domain binds to filamentous actin (F-actin) and the FERM domain binds with cell membrane lipids, transmembrane proteins, and other membrane-associated proteins (21). Therefore, EZR is prime bridging protein, regulating various cytoskeleton-membrane interactions and modulating a diverse range of physiological processes (22,23). Overexpression of *EZR* is frequently observed in various human cancers and is associated with cancer progression and metastasis. Using an *in vitro* experiment, Zhu *et al.* found that the overexpression of *EZR* led to the formation of more metastatic foci in nude mice with liver metastasis (24). Moreover, Xie *et al.* demonstrated that *DOCK1* overexpression can lead to greater aggression in endometrial cancer, with the underlying mechanism potentially involving the upregulation of *EZR* expression (25). The pivotal role of *EZR* in modulating cancer progression and metastasis in ESCC has also been indicated in a series of studies. For example, Deng *et al.*

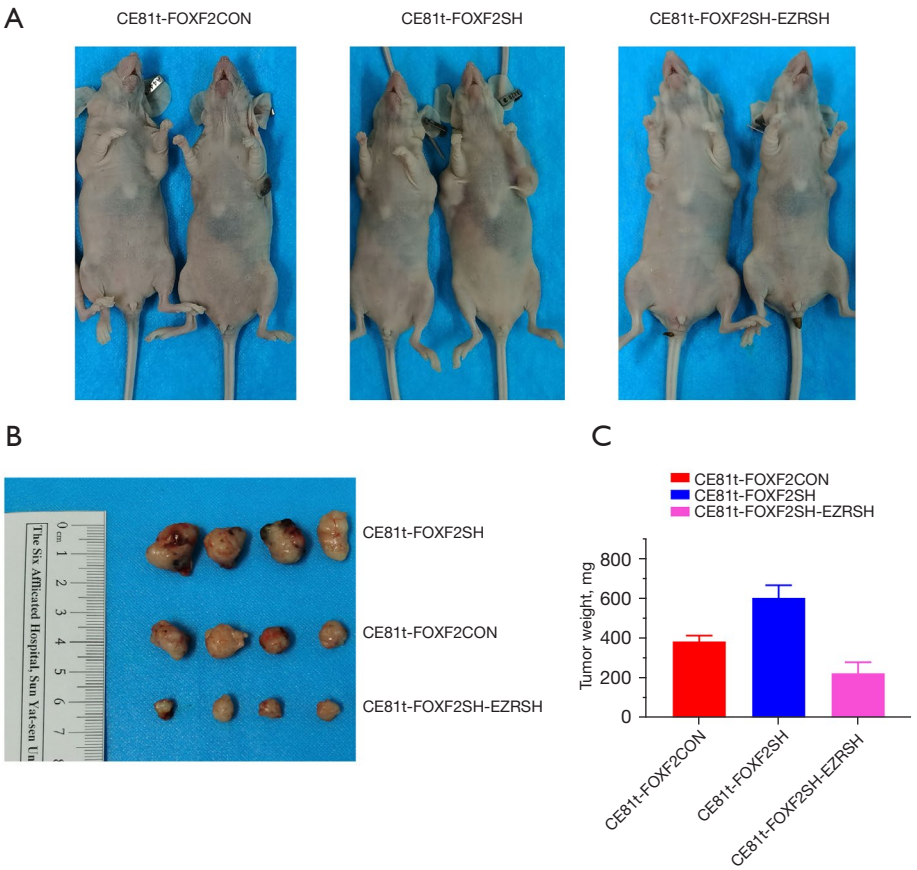

Figure 5 The xenograft mouse model confirmed the effect of the FOXF2/EZR signaling pathway on tumor progression. (A) Establishment of the xenograft mouse model with different expressions of FOXF2-EZR. (B,C) The accelerated tumor progression caused by the decrease in FOXF2 expression was inhibited by decreased EZR expression.

found that although *PALM2* overexpression could enhance cell migration in ESCC, knockout of *EZR* expression could reduce invasiveness (26). In addition, *L1CAM* was found to upregulate the expression of *EZR* to enhance the cell growth, migration, and invasiveness of ESCC (27). In this study, we found that *FOXF2* could influence the invasiveness of ESCC by regulating the transcriptional expression of *EZR*. Overall, these findings suggest that *EZR* might be a potential and promising therapeutic target for ESCC.

ERBB2 is overexpressed and/or amplified in almost 20% of patients with EC (28,29). Anti-ERBB2 therapeutic antibody has long been used in combination with cytotoxic therapy as the standard therapeutic approach for ERBB2-positive gastroesophageal cancer but is associated with poor prognosis in patients with ESCC (30,31). On this basis, future studies should focus on two key areas. The first is clarifying the interaction mechanism between EZR and ERBB2. A recent study has indicated that EZR might interact with ERBB2 through some intermediate element (32). The other is to evaluate the efficacy of the combined application of anti-EZR and anti-ERBB2 therapy for ESCC through *in vivo* and *in vitro* experiments.

Conclusions

In conclusion, suppression of *FOXF2* expression is associated with enhanced cell growth, migration, and invasion in ESCC.

Acknowledgments

Funding: This study was supported by the National Natural Science Foundation of China (No. 82102955 to Y.Z.Z.).

Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-2024-2365/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-2024-2365/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-2024-2365/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-2024-2365/coif). Y.Z.Z. reports funding support from the National Natural Science Foundation of China (No. 82102955). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Animal experiments were performed under a project license (No. L2021ZSLYEC-106) granted by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University, in compliance with the national guidelines for the care and use of animals

References

1. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. CA Cancer J Clin 2016;66:115-32.
2. Veeramachaneni NK, Zoole JB, Decker PA, et al. Lymph node analysis in esophageal resection: American College of Surgeons Oncology Group Z0060 trial. Ann Thorac Surg 2008;86:418-21; discussion 421.
3. Zheng YZ, Zhao W, Hu Y, et al. Aggressive surgical resection does not improve survival in operable esophageal squamous cell carcinoma with N2-3 status. World J Gastroenterol 2015;21:8644-52.
4. Cai J, Tian AX, Wang QS, et al. FOXF2 suppresses the FOXC2-mediated epithelial-mesenchymal transition and multidrug resistance of basal-like breast cancer. Cancer Lett 2015;367:129-37.
5. Higashimori A, Dong Y, Zhang Y, et al. Forkhead Box F2 Suppresses Gastric Cancer through a Novel FOXF2-IRF2BPL-β-Catenin Signaling Axis. Cancer Res 2018;78:1643-56.
6. van Dop WA, Heijmans J, Büller NV, et al. Loss of Indian Hedgehog activates multiple aspects of a wound healing response in the mouse intestine. Gastroenterology 2010;139:1665-76, 1676.e1-10.
7. Wang A, Jin C, Li H, et al. LncRNA ADAMTS9-AS2 regulates ovarian cancer progression by targeting miR-182-5p/FOXF2 signaling pathway. Int J Biol Macromol 2018;120:1705-13.
8. Xu JL, Hua T, Ding J, et al. FOXF2 aggravates the progression of non-small cell lung cancer through targeting lncRNA H19 to downregulate PTEN. Eur Rev Med Pharmacol Sci 2019;23:10796-802.
9. Lo PK, Lee JS, Liang X, et al. The dual role of FOXF2 in regulation of DNA replication and the epithelial-mesenchymal transition in breast cancer progression. Cell Signal 2016;28:1502-19.
10. Zheng YZ, Wen J, Cao X, et al. Decreased mRNA expression of transcription factor forkhead box F2 is an indicator of poor prognosis in patients with resected esophageal squamous cell carcinoma. Mol Clin Oncol 2015;3:713-9.
11. Zeeberg BR, Feng W, Wang G, et al. GoMiner: a resource for biological interpretation of genomic and proteomic data. Genome Biol 2003;4:R28.
12. Zhou Y, Zhou B, Pache L, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun 2019;10:1523.
13. Binder JX, Pletscher-Frankild S, Tsafou K, et al. COMPARTMENTS: unification and visualization of protein subcellular localization evidence. Database (Oxford) 2014;2014:bau012.
14. Fang H, Fu K, Shi P, et al. Forkhead box F2/ Lysyl oxidase like 1 contribute to epithelial-mesenchymal transition and angiogenesis in thyroid cancer. Cell Signal 2024;113:110956.

15. Jia D, Zhou Z, Kwon OJ, et al. Stromal FOXF2 suppresses prostate cancer progression and metastasis by enhancing antitumor immunity. Nat Commun 2022;13:6828.
16. Tian HP, Lun SM, Huang HJ, et al. DNA Methylation Affects the SP1-regulated Transcription of FOXF2 in Breast Cancer Cells. J Biol Chem 2015;290:19173-83.
17. Chen X, Hu H, Liu J, et al. FOXF2 promoter methylation is associated with prognosis in esophageal squamous cell carcinoma. Tumour Biol 2017;39:1010428317692230.
18. Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 2002;3:586-99.
19. Bretscher A, Reczek D, Berryman M. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. J Cell Sci 1997;110 (Pt 24):3011-8.
20. Pearson MA, Reczek D, Bretscher A, et al. Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain. Cell 2000;101:259-70.
21. Fehon RG, McClatchey AI, Bretscher A. Organizing the cell cortex: the role of ERM proteins. Nat Rev Mol Cell Biol 2010;11:276-87.
22. Xu WT, Shi LL, Xu J, et al. Ezrin expression in female reproductive tissues: A review of regulation and pathophysiological implications. Front Cell Dev Biol 2023;11:1125881.
23. Zhao S, Luo J, Hu J, et al. Role of Ezrin in Asthma-Related Airway Inflammation and Remodeling. Mediators Inflamm 2022;2022:6255012.
24. Zhu Y, Zhang X, Chen Y, et al. Ezrin's role in gastric cancer progression: Implications for immune microenvironment modulation and therapeutic potential. Heliyon 2024;10:e27155.
25. Xie S, Jin Y, Wang J, et al. DOCK1 regulates the malignant biological behavior of endometrial cancer through c-Raf/ERK pathway. BMC Cancer 2024;24:296.
26. Deng DX, Li CY, Zheng ZY, et al. Prenylated PALM2 Promotes the Migration of Esophageal Squamous Cell Carcinoma Cells Through Activating Ezrin. Mol Cell Proteomics 2023;22:100593.
27. Guo JC, Xie YM, Ran LQ, et al. L1CAM drives oncogenicity in esophageal squamous cell carcinoma by stimulation of ezrin transcription. J Mol Med (Berl) 2017;95:1355-68.
28. Augustin JE, Soussan P, Bass AJ. Targeting the complexity of ERBB2 biology in gastroesophageal carcinoma. Ann Oncol 2022;33:1134-48.
29. Rong L, Zhao H, Li Y, et al. Expression and correlation of PD-L1 and HER2 in oesophageal squamous cell carcinoma. J Clin Pathol 2024;77:233-8.
30. Bang YJ, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010;376:687-97.
31. Kim HS, Kwon HJ, Jung I, et al. Phase II clinical and exploratory biomarker study of dacomitinib in patients with recurrent and/or metastatic squamous cell carcinoma of head and neck. Clin Cancer Res 2015;21:544-52.
32. Penchev VR, Chang YT, Begum A, et al. Ezrin Promotes Stem Cell Properties in Pancreatic Ductal Adenocarcinoma. Mol Cancer Res 2019;17:929-36.

(English Language Editor: J. Gray)

Cite this article as: Hu ZY, Deng WH, Cai WJ, Qin XY, Zheng HS, Tan J, Jiang XL, Zheng YZ, Liao HY. *FOXF2* may inhibit esophageal squamous cell carcinoma growth and metastasis by regulating the *EZR-ERBB2* axis. Transl Cancer Res 2024;13(12):6970-6981. doi: 10.21037/tcr-2024-2365

