FBXW7 inhibits the Progression of ESCC by directly inhibiting the Stemness of Tumor Cells

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Abstract

Background

F-box and WD repeat domain containing 7 (FBXW7), is an aboriginal and high frequency mutant gene associated with esophageal squamous cell carcinoma (ESCC). This study was designed to determine the clinical value and molecular mechanisms of FBXW7 in the development of ESCC.

Methods

The clinical significance of FBXW7 was analyzed in ESCC from TCGA data. The effects of FBXW7 on proliferation, colony formation, migration and invasion, angiogenesis and apoptosis were tested in ESCC cells. PCR-array, sphere formation assay, quantitative real-time polymerase chain reaction (qPCR) were used to explore the mechanism of FBXW7.

Results

FBXW7 was a significantly mutated gene in ESCC. It was an independent and potential predictor for survival in ESCC patients. In addition, FBXW7 overexpression significantly inhibited ESCC cell proliferation, migration, invasion, angiogenesis, and promoted cell apoptosis. PCR-array revealed that FBXW7 overexpression leads to a significant change of genes expression associated with angiogenesis, cell senescence and DNA damage and repair. Sphere formation assay and qPCR showed FBXW7 was associated with ESCC stem cell formation.

Conclusions

Our results suggest that FBXW7 may act as a tumor suppressor by repressing cancer stem cell formation and regulating tumor angiogenesis, cell senescence, DNA damage and repair in ESCC.

Introduction

Esophageal cancer was an important killer that threatened human health. Its morbidity and mortality rank the eighth and sixth among all malignant tumors, respectively[1]. ESCC was the main pathology type of esophageal cancer in China, gathered in Fujian and Guangdong, Xinjiang and Shanxi[2]. At present, the treatment strategy for esophageal cancer patients was surgery combined with radiotherapy and chemotherapy. However, for patients with tumor progression and metastasis, the treatment effect was poor, and the 5-year survival rate ranged from 15–25%[3, 4]. Therefore, exploring the biomarkers for diagnosis and treatment of esophageal squamous carcinoma was essential to improve the clinical status[5].
In our previous whole genome sequencing (WGS) and whole exome sequencing (WES) study of 104 ESCC cases, FBXW7 was identified as a significantly mutated gene (SMG) associated with ESCC [6]. Moreover, FBXW7 was also reported as one of the SMGs in other ESCC genomic studies [7–10]. It suggested that FBXW7 may be a driver gene in ESCC development. FBXW7, a substrate of the SCF (SKP1-CUL1-F-box)-type ubiquitin ligase (E3) complex that played a key role in cell division, growth and differentiation by targeting proteins (including c-Myc, Notch1, Notch4, c-Jun and cyclin E) for phosphorylation-dependent ubiquitination degradation [11]. Recent studies have reported that FBXW7 as a tumor suppressor gene in various malignant tumors, such as gastric cancer, colon cancer, ovarian cancer and breast cancer [12, 13]. In gastric cancer, FBXW7 promoted gastric cancer cell apoptosis, growth arrest and inhibited EMT by inducing RhoA ubiquitination and proteasome degradation [12]. In colon cancer, FBXW7 functioned as a tumor suppressor gene by negatively regulating ENO1 activity via the ubiquitin/proteasome pathway in a GSK3β-dependent manner [14]. In ovarian cancer, FBXW7 inhibited tumor growth and progression by degrading YTHDF2 to enhance the stability of the pro-apoptotic gene BMF [15]. FBXW7 inhibits breast cancer proliferation and promotes apoptosis by targeting MTDH for degradation [16]. MiR-27a-3p promotes ESCC cell proliferation by inhibiting FBXW7 [17]. However, the mechanism and clinical significance of FBXW7 in ESCC remain unclear. Therefore, in this study, we explored the clinical significance of FBXW7 in ESCC and clarified its function and mechanism, which was expected to provide reliable molecular markers and therapeutic targets for early diagnosis and precise treatment of ESCC.

Materials And Methods

Cell lines and culture conditions

All esophageal squamous cell carcinoma cell lines in this study: KYSE150, KYSE180, KYSE450, TE1, TE5, TE6, TE9, TE14 and normal esophageal squamous epithelial cell HET-1A were preserved in the Translational Medicine Center, Shanxi Medical University (Taiyuan, China). All esophageal squamous cell carcinoma cell lines were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Sigma-Aldrich), and normal esophageal squamous epithelial cell lines were cultured in DMEM high glucose medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ incubator.

Plasmid transfection

KYSE450 and TE5 esophageal cancer cell lines were seeded in 6-well plates, and when the cell confluence reached 70%, PCDNA3-FBXW7 wt plasmid was transfected. The PCDNA3-FBXW7 wt plasmid was purchased from GeneChem company. The efficiency of overexpression was detected by qPCR.

Real-time quantitative PCR (qPCR)

The mRNA expression level of FBXW7 in ESCC cell lines was detected by qPCR. Total RNA was extracted from cells using RNA extraction reagent (RNAiso Plus, Takara, Bio Inc, Japan). qPCR was performed as
instructed using the Takara SYBR Premix Ex TaqTM (Takara Bio Inc, Japan) kit for qPCR. All qPCR reactions were replicated using Applied Biosystems (ABI, Foster City, CA, USA). GAPDH gene as an internal reference, and the results were calculated using \( \Delta \Delta Ct \). Each experiment was repeated at least three times.

**Vascular mimicry**

50µl matrigel was added into 96-well plate and cured at 37°C for 30min. During this period, \( 1 \times 10^4 \) cells/ml single cell suspension (including 200µl fetal bovine serum, 4µl hydrocortisone, 40µl hFGF-B, 10µl VEGF, 10µl R3-IGF-1, 10µl ascorbic acid, 10µl hEGF, 10µl GA-1000, 10µl heparin in 10ml DMEM medium) was prepared. And 50µl cell suspension was added to 96-well plates with matrix and incubated at 37°C for 12h. The tube formation was observed under the microscope, and the images were photographed and recorded, and the number of the tubes were measured by ImageJ software. Repeat 3 independent experiments.

**MTT assay**

The different cells were seeded into 96-well plates at a concentration of \( 5 \times 10^3 / \text{well} \) and cultured in 5% CO\(_2\) at 37°C. 20µl 5 mg/ml MTT (Invitrogen) was added at 24h, 48h, 72h and 96h, and incubated at 37°C for 4h. The cell culture medium was gently discarded and 150µl of dimethyl sulfoxide (DMSO) was added to dissolve the crystals. The absorbance at 490nm was detected by a microplate reader, and the growth curve was drawn according to the OD value to observe the proliferation of cells in each group. Each group contained five multiple holes, and the experiment was repeated at least three times.

**Cell migration and invasion assays**

Cell migration and invasion abilities were detected by transwell assay. In the migration assay, \( 5 \times 10^4 \) cells were seeded in basic medium without fetal bovine serum in a 24-well plate chamber, and 600µl of 10% fetal bovine serum medium was added to the bottom chamber. Set up 3 replicate wells for each group. Cells were incubated in 5% CO\(_2\) at 37°C for 24h. 4% paraformaldehyde was added to the upper chamber to fix the cells, and 0.1% crystal violet solution was used for staining. When the samples were observed under a microscope (Olympus, Japan), 8 areas were randomly selected to count the cell number. In the invasion assay, the chambers were precoated with 50µl Matrigel (1:6 mixed with serum-free medium, BD Biosciences, Heidelberg, Germany) and performed as described above. Each group included 3 repeated holes, and the experiment was repeated 3 times independently.

**Colony formation assay**

Cells were seeded in 6-well plates at a density of 1000 cells/well and cultured in 5% CO\(_2\) at 37 °C for 2 weeks. The cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The number of effective clones formed by cells in each group was observed and counted under a microscope, and the clonal ability of cells in each group was compared. Each experiment consisted of 3 replicates with at least 3 experiments.
Flow cytometry apoptosis detection

ESCC cells were digested with EDTA-free trypsin, centrifuged to collect cell precipitation, washed with PBS, resuspended with 500µl binding buffer, mixed with 5µl of AnnexinV-FITC, and mixed with 10µl of Propidium Iodide. After incubation in dark at room temperature for 20 min, the cells were detected by flow cytometry.

Stem cell formation

The logarithmic phase cells were seeded in DMEM-F12 medium(GIBCO, USA) containing 20ng/mL human epidermal growth factor (hEGF), 20ng/mL basic fibroblast growth factor (bFGF), 1×B27, 5µg/mL insulin at a density of 5×10³/mL-1 and cultured in 5%CO₂ 37℃. Add 1ml fresh culture medium every 3 days, when cells proliferated to the suspension spheres (more than 50µm was counted as tumor stem cell balls), the size and number of suspension spheres were recorded under inverted microscope.

PCR Array

To screen for differentially expressed genes associated with FBXW7, we performed PCR array using the Cancer Pathway PCR Array Kit provided by Shanghai QIAGEN Biotechnology Company. According to the operation instructions: Total RNA extracted by Trizol, and then reverse transcription into cDNA with qPCR kit (PrimeScript RTMasterMix, Takara, Shiga, Japan). The cDNA template was diluted and added to the real-time quantitative PCR reaction mixture, and then 25µl reaction solution was added to each well of the RT2 Profiler PCR Array containing gene-specific primers for real-time quantitative PCR reaction. The results were analyzed using online analysis software (https://geneglobe.qiagen.com/cn/).

Statistical analysis

The protein-protein interaction network was analyzed based on Differently Expressed Genes (DEGs). SPSS 26.0 software was used for statistical analysis. The experiment was divided into three replicates and the data were expressed as mean ± SEM. Rank sum test and chi-square test (χ²) were used to analyze the relationship between FBXW7 mutation and clinical pathological characteristics. Non-paired t test was used for the data of the two groups, and one-way ANOVA was used for the data above the two groups. p < 0.05 was considered statistically significant.

Results

Low FBXW7 expression reflected poor prognosis in ESCC patients.

Previously, we performed WGS/WES in 104 ESCC cases, and identified 8 significant mutated genes (SMGs) (FDR < 0.178, p < 0.0001). Of them, the mutation frequency of FBXW7 was 7.7% (8/104), covering nonsense mutations 2/8 (25%), insertion/deletion 1/8 (12.5%), and missense mutations 5/8 (62.5%) (Fig. 1A)[18, 19]. In addition, the RNA-seq results from 154 ESCC cases showed that the FBXW7 mRNA level in tumor tissues were significantly lower than those in adjacent normal tissues (Fig. 1B).
In order to clarify the clinical significance of \( FBXW7 \) in ESCC, rank sum and Chi-square (\( \chi^2 \)) tests were applied in the ESCC cohort (95 cases) from TCGA database. According to the expression of \( FBXW7 \) and the survival information of ESCC patients, we drew the ROC curve and determined the critical value of \( FBXW7 \) expression (cutoff = 324.78), and divided ESCC patients into low expression group (\( \leq 324.78 \)) and high expression group (> 324.78). Then the correlation between \( FBXW7 \) expression and ESCC clinical variables was analyzed, and it was found that \( FBXW7 \) expression was correlated with survival status (\( p = 0.031 \)) (Table 1). And survival analysis showed that patients with low \( FBXW7 \) expression in ESCC had a worse prognosis than those with high \( FBXW7 \) expression (\( p < 0.05 \), Fig. 1C). In addition, further stratified analysis showed that the prognosis of ESCC patients with low \( FBXW7 \) expression was poor in patients \( \leq 55 \) years old, without drinking history and T3 stage (\( p < 0.05 \), Fig. 1D). These results suggested that the low expression of \( FBXW7 \) may be an independent prognostic factor for poor prognosis of ESCC patients.
**Table 1**

Correlation analysis between *FBXW7* mRNA levels in ESCC and clinicopathological variables

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 95)</th>
<th>FBXW7 expression [n%]</th>
<th>Chi-square</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Low(T ≤ 324.7751) n = 23</td>
<td>High(T &gt; 324.7755) n = 72</td>
<td>$\chi^2$</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 55</td>
<td>40</td>
<td>11(27.500)</td>
<td>29(72.500)</td>
<td>0.407</td>
</tr>
<tr>
<td>≥ 55</td>
<td>55</td>
<td>12(21.818)</td>
<td>43(78.182)</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>81</td>
<td>21(25.926)</td>
<td>60(74.074)</td>
<td>0.361</td>
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<tr>
<td>female</td>
<td>14</td>
<td>2(14.286)</td>
<td>12(85.714)</td>
<td></td>
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<td><strong>Smoking</strong></td>
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<td></td>
<td></td>
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<tr>
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<td>11</td>
<td>6(54.545)</td>
<td>5(45.455)</td>
<td>0.451</td>
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<tr>
<td>Yes</td>
<td>84</td>
<td>57(67.857)</td>
<td>27(32.143)</td>
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<tr>
<td><strong>Drinking</strong></td>
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<tr>
<td>No</td>
<td>69</td>
<td>16(23.188)</td>
<td>53(76.812)</td>
<td>0.342</td>
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<td>26</td>
<td>9(34.615)</td>
<td>17(65.385)</td>
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<tr>
<td>+</td>
<td>62</td>
<td>16(23.188)</td>
<td>46(74.194)</td>
<td>0.116</td>
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<tr>
<td>+</td>
<td>33</td>
<td>9(27.273)</td>
<td>24(72.727)</td>
<td></td>
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<tr>
<td><strong>T stage</strong></td>
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</tr>
<tr>
<td>1 + 2</td>
<td>39</td>
<td>10(25.641)</td>
<td>29(74.359)</td>
<td>0.030</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>15(26.786)</td>
<td>41(73.214)</td>
<td></td>
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<tr>
<td><strong>Prognosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>survival</td>
<td>63</td>
<td>11(17.460)</td>
<td>52(82.540)</td>
<td>4.644</td>
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<tr>
<td>dead</td>
<td>32</td>
<td>2(37.500)</td>
<td>20(62.500)</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05

*FBXW7* inhibited cell proliferation, cell migration and cell invasion, as well as promoted apoptosis in ESCC
We detected FBXW7 mRNA expression level in nine ESCC cell lines (Fig. 2A) and found that KYSE450 and TE5 with low endogenous FBXW7 mRNA expression. Therefore, we transiently transfected the pcDNA3-FBXW7wt plasmid into KYSE450 and TE5 cells respectively to explore the gene function. The over-expression efficiency was confirmed by qPCR(p<0.05, Fig. 2B). We observed that FBXW7 overexpression significantly inhibited the proliferation of KYSE450 and TE5 cells by MTT and colony formation assays(p < 0.05, Fig. 2C,D). Moreover, the results of transwell experiments showed that FBXW7 overexpression was able to inhibit the invasion and migration ability of ESCC cells (p < 0.05, Fig. 2E,F). And, the apoptosis of FBXW7 overexpressed cells was detected by Annexin V-FITC/PI double staining using flow cytometry, and it was found that the number of apoptosis cells significantly increased (Fig. 2G). These results indicated that FBXW7 may be a tumor suppressor gene in ESCC development.

**Altered pathways and gene-interaction networks affected by FBXW7 over-expressed in ESCC cells**

On the basis of clarifying the function of FBXW7, we performed PCR array experiments with FBXW7wt and control cells to explore the mechanism of FBXW7 in ESCC. The results showed that there were 16 genes whose expression changes were more than two times, namely ANGPT1, SERPINB2, SOD1, DDB2, DDIT3, ERCC3, ERCC5, POLB and PPP1R15A, FLT1, KDR, PGF, TEK, BMI1, IGFBP5 and TBX2. And they were mainly concentrated in angiogenesis, DNA damage repair and cell senescence related signaling pathways (Fig. 3A,B), suggesting that FBXW7 might be related to these pathways in ESCC. And this result was also verified by qPCR experiment (Fig. 3C).

Additionally, we also analyzed the gene interaction network between FBXW7 and these genes using cystoscope software. As shown in Fig. 4A, in the angiogenesis network, in addition to the central FBXW7 gene, there were other core genes, including up-regulated ANGPT1 and down-regulated FLT1, KDR, PGF, and TEK. In the cell senescence network, core genes also include up-regulated SOD1, SERPINB2, and down-regulated IGFBP5(Fig. 4B). In the DNA damage repair network, the core genes also include up-regulated DDIT3, PPP1R15A, POLB, ERCC5, and DDB2(Fig. 4C). Overall, above results showed that FBXW7 may play a tumor suppressor role via angiogenesis, cell senescence, DNA damage and repair signaling pathways in ESCC.

**FBXW7 regulated angiogenesis, cell senescence and DNA damage repair pathways by inhibiting tumor stemness in ESCC**

It has been reported that cancer stem cells (CSCs) play a crucial role in the regulation of tumor angiogenesis and cell senescence, and they coordinate pathological angiogenesis by secreting angiogenic factors during tumor progression[20]. The stemness marker ALDH1A1 promotes tumor angiogenesis through retinoic acid/HIF-1α/VEGF signaling in MCF-7 breast cancer cells[21, 22]. Hydrogen reduces cellular senescence by regulating ROS/p53/p21 pathways in the mesenchymal stem cell of the bone marrow[23, 24]. Therefore, we propose the hypothesis that whether the effect of FBXW7 on angiogenesis and cell senescence-related pathways in ESCC cells was related to tumor stemness. We performed vascular mimicry assay to examine the effect of FBXW7 on angiogenesis in FBXW7wt and control cells. The results also showed that compared with the control group, the number of tubular
structures in FBXW7wt group was significantly reduced, suggesting that FBXW7 could inhibit angiogenesis in ESCC (Fig. 5A). Furthermore, we collected KYSE450 and TE5 stem cells, and found that compared with the parental cells, the FBXW7 expression was decreased in ESCC stem cells, while the stemness markers ALDH1A and LLF4 were significantly increased (Fig. 5B). In addition, FBXW7wt and control group ESCC cells were also suspended and cultured. The number and size of floating cell sphere were counted under the microscope (more than 50µm was counted as tumor stem cell balls). It was found that compared with the control group, the volume of cell spheres formed in the KYSE450-FBXW7wt group was smaller, and the number of cell spheres formed in the TE5-FBXW7wt group was less ($p < 0.05$) (Fig. 5C). These results suggested that the negative regulation of FBXW7 on stemness might be a universal mechanism in ESCC.

**Discussion**

In this study, we identified a significant mutation gene FBXW7 in ESCC by analyzing our previous genomic sequencing data, and uncovered its potential prognostic value for ESCC patients. We applied in vitro methods to reveal the tumor suppressor role of FBXW7 and a possible novel mechanism of regulating ESCC angiogenesis, cell senescence and DNA damage repair by affecting the stemness of ESCC cells (Fig. 6).

F-box and WD repeat domain containing 7 (FBXW7), also known as Sel10, hCDC4 or hAgo, is a member of the F-box protein family, which functions as the substrate recognition component of the SCF E3 ubiquitin ligase [25]. Previous whole-genome or -exome sequencing study revealed a series of mutated genes of ESCC. FBXW7 was identified as one of SMGs in our cohort. Subsequently, our expanded cohort study of 508 ESCC samples showed that the mutation frequency of FBXW7 was 8.66% [6]. We analyzed the genomic sequencing data from 4 ESCC cohorts and found FBXW7 was mutated frequently in ESCC. In Peina Du’s cohort [9], in 490 tumor samples and matched non-tumor samples, the mutation rate of FBXW7 is approximately 5.31%. In Yibo Gao’s cohort [7], in 113 tumor samples and matched non-tumor samples, the mutation rate of FBXW7 is approximately 5%. In Nishant Agrawal’s cohort [26], in 41 tumor samples and matched non-tumor samples, the mutation rate of FBXW7 is approximately 6%. In Genta Sawada’s cohort [8], in 144 tumor samples and matched non-tumor samples, the mutation rate of FBXW7 is approximately 5.6%. The RNA-seq results of 154 pairs of ESCC showed that the expression level of FBXW7 in tumor samples was lower than that in matched normal samples. Clinically, we observed that low FBXW7 expression were associated with poor prognosis in ESCC, and individuals with low FBXW7 expression had much worse prognosis than individuals with high FBXW7 expression in young, non-drinking, and later stage populations. Consistent with our analysis, the clinical significance of FBXW7 prognosis in 75 ESCC patients was evaluated and the loss of FBXW7 copy number in 45 ESCC patients was related to the prognosis [27]. The 5-year survival rates of the 90 patients with high and low FBXW7 expression were 67.6% and 39.3%, respectively ($\chi^2 = 6.699, P = 0.01$) [28].

It has been reported that FBXW7 is a suppressor gene in gastric cancer, colon cancer, ovarian cancer, breast cancer, liver cancer and other malignant tumors [15, 29, 30]. Those were consistent with our
functional results that *FBXW7* also functions as a suppressor gene in ESCC, inhibiting ESCC cell proliferation, invasion, migration and promoting apoptosis. Accordingly, our PCR-array results and validation tests indicated that *FBXW7* may contribute to ESCC tumorigenesis via angiogenesis, DNA damage repair and cell senescence. We deduced that *FBXW7* interacts with sequence-specific transcription factors and suppresses activation of angiogenesis and DNA damage repair, promotes activation of cell senescence relevant genes, whereby participating in the tumorigenesis and development of ESCC. The changed genes in angiogenesis in *FBXW7* overexpression ESCC cells included the down-regulation of *FLT1, KDR, PGF*, and *TEK*, and up-regulation of *ANGPT1*. *FLT1*, the receptor of VEGF/PIGF, is associated with cancer angiogenesis and tumorigenesis[31]. *KDR* is not only expressed in endometrial carcinoma cells, but also actively participates in the biological process of the tumor[32]. Abnormal expression of *PGF* in cervical carcinoma[33]. *TEK* promotes cell apoptosis by regulating AKT phosphorylation, thereby inhibiting cell proliferation[34]. The expression of *ANGPT1* is significantly down regulated in patients with lung squamous cell carcinoma and lung adenocarcinoma[35]. These findings were consistent with the trend of expression change of these genes in our study indicating the inhibition effect of *FBXW7* on angiogenesis. In the DNA damage repair network, the core genes also include up-regulated *DDIT3, PPP1R15A, POLB, ERCC5*, and *DDB2*. *DDIT3* enhanced gastric cancer cell proliferation, colony formation, spheroid formation and CSC dryness[36]. *PPP1R15A* plays a vital role in promoting cell death and the unfolded protein response (UPR)[37]. *POLB* regulates the growth of oral squamous cell carcinoma cells by regulating cell cycle and chromosome instability[38]. *ERCC5* polymorphisms may be associated with metastasis and recurrence of gastric cancer[39]. *DDB2* plays a role as a transcription inhibitor and can eliminate the characteristics of ovarian CSC by down regulating the expression of ALDH1A1[40]. These reports are consistent with the change trend of gene expression in our study, indicating that *FBXW7* can activate DNA damage repair. In the cell senescence network, core genes also include up-regulated *SOD1, SERPINB2*, and down-regulated *IGFBP5*. Inhibition of *SOD1* induces cell death by apoptosis[41]. *SERPINB2* reduces survival rate and increases lymph node metastasis in breast cancer patients[42]. *IGFBP5* regulates radiosensitivity of prostate cancer through PI3K-AKT pathway[43]. These reports are consistent with the change trend of gene expression in our study, indicating that *FBXW7* can promote the activation of genes related to cell aging.

It was well known that angiogenesis, DNA damage repair and cell senescence were biological processes that participate in the tumorigenesis. The coenzyme Q10 analog decylubiquinone (DUb) inhibits angiogenesis by regulating ROS/p53/BAI1 signaling pathway, thereby inhibiting the occurrence and development of breast cancer[44]. The pathways of DNA damage repair determining cell fate are intertwined and play a key role in the occurrence and development of cancer[45]. In cancer, cell senescence is an effective barrier to prevent the occurrence of tumors and can also drive proliferative diseases[46]. Therefore, we further analyzed the interaction network between *FBXW7* and related pathway genes. Our results revealed that *FBXW7* might be a considerable factor in angiogenesis, DNA damage and cell senescence pathway, forming a complex regulatory network and playing an important role in ESCC. Corroborate with our findings, Lihua Hu’s study have shown that *FBXW7* overexpression reduces angiogenesis in retinal tissues of mice with diabetic retinopathy[47]. Therefore, we applied the vascular
mimicry experiments to determine the the effect of FBXW7 on angiogenesis of ESCC cells, and realized that FBXW7 could inhibited the angiogenesis ability of ESCC cells. In response to DNA damage, phosphorylated FBXW7 could play a role in maintaining genomic stability[48]. And under the action of radiation or oxidative stress, FBXW7 binds to telomeric protective protein 1 (TPP1) and triggering telomere uncapping to mediate cellular senescence[49]. The above results confirmed that FBXW7 may inhibit the proliferation and metastasis of ESCC by regulating angiogenesis, DNA damage repair, and cell senescence related signaling pathways.

However, many studies have reported that angiogenesis, DNA damage and cell senescence pathway were involved in CSCs formation in many malignant tumors[50]. In gastric cancer, tumor stem cells show greater tumor formation and angiogenesis than cancer cells[51]. Tumor stem cells are responsible for the inhibition of angiogenesis caused by Notch signaling pathway in breast cancer[52]. As for cell senescence, colorectal cancer stem cells can promote apoptosis and cellular senescence by regulating the FasR/FasL signaling pathway. And tumor stem cells could also effectively regulate DNA damage repair. Hematopoietic stem cells were affected by the ubiquitination enzyme USP16, which removes ubiquitin from H2A and H2AX and thereby impairs DNA damage repair[53]. Therefore, we investigated the effect of FBXW7 on stemness of ESCC cells. Compared with the parental cells, the expression of stem cell surface markers ALDH1A1 and KLF4 was increased, while the expression of FBXW7 was decreased. Further studies showed that overexpression of FBXW7 could inhibit the formation and proliferation of ESCC stem cells.

Combined with our results, in summary, our functional and clinical analysis suggests that FBXW7 may regulate the occurrence and development of ESCC by inhibiting the formation of ESCC stem cells, and then affecting the signaling pathways related to ESCC angiogenesis, DNA damage repair and cell aging, as well as low expression of FBXW7 associated with poor prognosis. These findings are important for understanding the mechanisms that drive the development and progression of ESCC, providing new clues to unravel the mechanisms of tumorigenesis and tumor progression, and opening up new ideas for the clinical treatment of ESCC.

**Declarations**

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**Conflict of interest** The authors declare that they have no conflict of interests.

**Ethical approval** Not applicable.

**Ethics approval and consent to participate:** Not applicable
Consent for publication: Not applicable

Availability of data and materials: Not applicable

Competing interests: The authors declare that they have no competing interests

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Authors’ contributions: BY and YHB designed the experiments, supervised data analysis and edited the manuscript. YFY, YHB, CXC, and PST conceived the study and analyzed the data. YFY, CXC, PST, HX, FY, and WWW performed experiments. FJY and WWW performed bioinformatics and statistics analyses. YHB, CXC and BY provided clinical samples, coordinated and performed pathology review. YFY wrote the manuscript. All authors had access to the study data and reviewed and approved the final manuscript.

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References


Figures
Low expression of FBXW7 was associated with poor prognosis in ESCC patients. A Mutation site distribution of FBXW7 gene in 104 ESCC samples. B Analysis of the mRNA expression of FBXW7 in 154 pairs of ESCC tissues. C FBXW7 expression level and overall Kaplan-Meier survival analysis of ESCC patients. D Kaplan-Meier survival curves of patients with different FBXW7 genotypes in different age, gender, smoking history, drinking history, tumor grade, and pathological T patients.
Figure 2

Function of FBXW7 in ESCC. A FBXW7 mRNA expression levels in 9 ESCC cell lines. B The overexpressed efficiency of FBXW7 in KYSE450 and TE5 cells was detected by qPCR. C Overexpression of FBXW7 inhibited the proliferation of ESCC cells. D Overexpression of FBXW7 inhibited colony formation of ESCC cells. E Overexpression of FBXW7 inhibited the migration of ESCC cells. F Overexpression of FBXW7 inhibited the invasion of ESCC cells. G Overexpression of FBXW7, the proportion of apoptosis was significantly increased*P < 0.05, **P < 0.01.
Figure 3

Key cancer pathway components are altered in *FBXW7* overexpressing cells. **A** PCR arrays were used to detect gene expression affected by *FBXW7*. Blue-white-red scales represent fold change and fold regulation values for genes. **B** PCR-Array was used to show the key cancer pathway correlating with *FBXW7*. **C** q-PCR was used to detect the mRNA level of genes in KYSE450NC and KYSE450-*FBXW7* wt. GAPDH was used as a loading control. All data are presented as the mean ± standard deviation and three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 4

Gene interaction network analysis of *FBXW7*. **A** The network of *FBXW7* gene interactors is mainly concentrated in the angiogenic pathway. **B** The network of *FBXW7* gene interactors is mainly focused on the signaling pathways of cellular senescence. **C** The network of *FBXW7* gene interactors is mainly concentrated in the DNA damage repair pathway. Up-regulated genes are indicated in red, down-regulated genes are indicated in green, and blue indicates no significant difference. The size of the circle represents the change in folding.
Figure 5

The effect of *FBXW7* on ESCC stemness. **A** Left panel: Representative images of vasculogenic mimicry experiments. Right panel: Quantitative results of tube formation using ImageJ software. **B** Differential expression of *FBXW7* between parent cells and stem cells and differential expression of stem cell markers between adherent cells and stem cells by qPCR. **C** Left panel: Representative images of *FBXW7* and ESCC cell spheroid formation experiments. Right panel: Quantitative results of the quantification experiment. *p < 0.05, **p < 0.01.*
Figure 6

Figure legend not available with this version.