Blocking FGFR4 exerts distinct anti-tumorigenic effects in esophageal squamous cell carcinoma

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Abstract

Background: The FGFR family can be activated by FGFs and plays important roles in regulating cell growth, differentiation, migration, and angiogenesis. Recent studies have suggested that FGFR4 could regulate several processes, including tumor progression. Esophageal squamous cell carcinoma (ESCC) is a malignancy with high global occurrence. However, the molecule mechanism and the potential roles of FGFR4 in ESCC remain unknown.

Methods: Immunohistochemistry and Western blotting were used to detect FGFR4 expression in ESCC samples and cell lines. Cell counting kit-8, and clonogenic, transwell, flow cytometric, and tumor xenograft in nude mice assays were utilized to determine the effect of blocking FGFR4 in proliferation, invasion, migration, and apoptosis of ESCC cells.

Results: FGFR4 is frequently overexpressed in ESCC tissue and cell lines. in vitro assays have shown that blocking FGFR4 by a specific blocker, H3B-6527, significantly decreases proliferation, invasion, and migration, and alters epithelial-mesenchymal transition markers in ESCC cells. In addition, FGFR4 blockade is associated with the induction of apoptosis and affects PI3K/Akt and MAPK/ERK pathways. Moreover, FGFR4 blockade could significantly inhibit the growth of xenograft tumors in vivo.

Conclusion: Our findings suggest that blocking FGFR4 significantly suppresses the malignant behaviors of ESCC and indicate that FGFR4 is a potential target for the treatment of ESCC.

Introduction

Esophageal cancer is one of the most common cancers worldwide and the sixth leading cause of cancer-related mortality. More than 90% of esophageal cancers are esophageal squamous cell carcinoma (ESCC). Despite advances in multidisciplinary treatment strategies and diagnostic imaging modalities, esophageal cancer remains a devastating malignancy because of rapidly aggressive progression, and only 15–25% of patients survive five years after their diagnosis. It is therefore a formidable task to find novel biological molecules related to the malignant behavior of the carcinoma and elucidate the underlying therapeutic targets for ESCC patients.

Previous studies have identified FGFR4 as a novel, promising therapeutic target and prognostic biomarker in a wide spectrum of tumors. FGFR4 is one of a family of highly conserved tyrosine kinase receptors, which consists of a cellular ligand domain, a single transmembrane helix domain, and a cytoplasmic domain with tyrosine kinase activity. FGF can combine with FGFR and induce dimerization and phosphorylation; activate various downstream signal transduction cascades; and plays an important part in regulating cellular functions including cell growth, differentiation, migration, and angiogenesis. In cancer, FGFR4 genomic aberrations dysregulate downstream signaling pathways of FGFR4 protein. This leads to sustained cell proliferation and contributes to tumor development. Because of its contribution to tumor development, FGFR4, which is known as CD334, is reported to interfere with signaling events and its expression level may be associated...
with some human tumors, such as breast cancer, colorectal cancer, human rhabdomyosarcoma, lung adenocarcinoma, and melanoma.14–18

However, previous studies on ESCC have focused on other members of the FGFR family, such as FGFR1, FGFR2, and FGFR3.19–21 Few studies have investigated whether FGFR4 protein expression is involved in pathogenesis and progression in ESCC. In this study, we selected a covalent inhibitor, H3B-6527, which targets the unique hinge cysteine in the FGFR4 kinase domain that specifically inhibits FGFR4, and largely spares other FGFRs to play a blocking role.22 Thus, we explored the function of FGFR4 in the malignant growth of ESCC and investigated whether FGFR4 aberrations could serve as predictive biomarkers and potential therapeutic targets.

Methods

Patients

Forty pairs of ESCC and paracancerous tissues (> 5 cm from the margin of tumor) from patients who underwent esophagectomy at the Department of Thoracic Surgery at the Provincial Hospital affiliated with Shandong University were collected and collated from January 2015 to December 2016. The inclusion criteria were as follows: stage I–III squamous cell carcinoma of the middle thoracic esophagus diagnosed by postoperative pathology; no preoperative chemotherapy or radiotherapy had been administered; no contraindications to surgery; and all patients had undergone esophagectomy with complete resection: no residual tumor cells were present on the upper or lower cutting edge as verified by pathology, and no residual focus on lateral margins to the naked eye. Tumor node metastasis staging was determined by the criteria established by the International Union Against Cancer in 2009. Written informed consent was obtained from all patients, and the study protocol was approved by the Institutional Ethics Committee of the Provincial Hospital affiliated with Shandong University according to the Guide for Chinese Ethics Review Committees.

Cell lines and culture

Human ESCC cell lines (TE-1, Eca9706, Eca109, KYSE150, and KYSE450) and one normal human esophageal epithelial cell line (HET-1A) were provided by the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in RPMI 1640 enriched with 1% penicillin/streptomycin (Sigma, Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% l-glutamine. Cell culture plates were maintained at 37°C in a 5% CO₂ incubator. H3B-6527 (Selleckchem, Houston, TX, USA) was prepared by dissolving stock solution (3 mM) in dimethyl sulfoxide (DMSO, ≥ 99.7%; Sigma-Aldrich) and then diluting to the corresponding concentration using phosphate buffered saline (Gibco).

Immunohistochemistry

Immunohistochemistry (IHC) studies of FGFR4 were performed using the streptavidin peroxidase method. Formalin-fixed, paraffin-embedded tissues were cut into sections that were then deparaffinized and incubated with hydrogen peroxide. Rabbit anti-FGFR4 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were diluted to 1:100 and incubated at 4°C overnight. The subsequent steps were performed following the instructions of the secondary biotinylated antibody kit (Zhongshan Biotech, Beijing, China). Two blinded, independent observers scored the stained slides.

Protein extraction and Western blot

Proteins were extracted from the tissue samples and cell lines, and the total protein concentration was determined using a Pierce bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (30 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene fluoride membrane filters (EMD Millipore, Billerica, MA, USA). Next, 10% non-fat dry milk was used to block nonspecific binding. The membranes were incubated with primary antibodies overnight. Following washing, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase anti-mouse/rabbit immunoglobulin G (Boster Biological Technology Co. Ltd., Pleasanton, CA, USA). Finally, the protein levels were quantified using an enhanced chemiluminescence detection system (Amersham imager 600; General Electric, Fairfield, CT, USA). The antibodies used in this study were as follows: FGFR4 (1:1000; Santa Cruz Biotechnology); N-cadherin, Vimentin, Snail, ERK, p-ERK, p-FGFR4, and Bcl-2 (1:1000; Abcam, Cambridge, MA, USA); E-cadherin (1:1000; Affibiotec, Cincinnati, OH, USA); Claudin-1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and Bax (1:1000; Proteintech, Beijing, China); β-actin (1:1000; Boster Biological Technology Co. Ltd.); and AKT and p-AKT (1:1000; Omimabs, Alhambra, CA, USA).

Colony formation assays

Cells were seeded in six-well plates for 24 hours and then treated with H3B-6527 (Selleckchem) or pure culture
media every three days. The plates were incubated at 37°C in a 5% CO₂ incubator for 10 days. The colonies were then stained with crystal violet, and those with > 50 cells were scored as surviving colonies. Cloning efficiency was calculated by dividing the average number of colonies/dish by the number of plated cells. This experiment was performed in triplicate.

Cell counting kit-8 assay

Cell proliferation was detected by using cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). The logarithmically growing cells were seeded in 96-well plates and incubated with H3B-6527 or pure culture media, respectively, for 24, 48, and 72 hours. At different time intervals, the cells were incubated with CCK-8 reagent for one hour at 37°C. The absorbance of each well was measured using Thermo Scientific Varioskan Flash (Vantaa, Finland). This experiment was performed in triplicate.

Wound-healing assay

Cells were treated separately with H3B-6527 or pure culture media in plates until 95% confluence. A straight “wound” was made by scratching with a plastic pipette tip. After culture in fetal bovine serum (FBS)-free medium for 12 and 24 hours, cell migration was analyzed by using light microscopy. The residual area of the scratch at the same position was measured using ImageJ software (NIH, Bethesda, MD, USA). The migration rate was calculated using the following formula:

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\text{Migration rate} = \frac{\text{the area of initial wound} - \text{the area of wound after 12/24 hours}}{\text{the area of the initial wound}} \times 100.
\]

Cell apoptosis detection

Flow cytometric assays were conducted using the PE Annexin-V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s instructions. Cells from the two groups (treated with H3B-6527 or pure culture media) were harvested by trypsinization and resuspended in 1X binding buffer. After double staining with P-phycoerythrin (PE) and 7-Amino-actinomycin D (7-AAD), the samples were analyzed by flow cytometry and FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cell migration and invasion assay

For both migration and invasion assays, the cells treated each day with 1 μM H3B-6527 or pure culture media for three days were precultured in FBS-free medium for 12 hours. The uncoated transwells were used in the migration assay, while Matrigel (1:4; BD Biosciences) was precoated to the upper surface of transwells in the invasion assay. Starved cells were then seeded into the upper chamber of 24-well transwells (8 mm pore size polycarbonate membrane; Merck KGaA, Darmstadt, Germany) with FBS-free medium, while medium with 10% FBS was added to the low chamber. The migration assay was allowed to incubate for 24 hours, and the invasion assay was allowed to proceed for 48 hours. The cells on the lower surface of the membrane were then fixed and stained. The cell numbers were calculated from three independent visual fields under light microscopy.

Esophageal squamous cell carcinoma (ESCC) tumor xenograft treatment studies

Six-week old female BALB/c nude were injected with 2 × 10⁶ ESCC cells subcutaneously in the flank. When the tumors reached ~150 mm³, the mice were pair-matched into two groups of six mice each and treated by the control vehicle or 10 mg/kg H3B-6527 administered intraperitoneally (IP) each day. We measured the tumor size every 4 days and weighed the tumors after 20 days. Tumor volumes were calculated by using the formula \( V = L \times W^2/2 \), where \( L \) and \( W \) are the larger and smaller diameters, respectively. All procedures relating to animal handling, care, and treatment were performed in strict accordance with the regulations on the management of experimental animals, approved by the State Council of the People’s Republic of China on 31 October 1988 and promulgated by Decree No. 2 of the State Science and Technology Commission on 14 November 1988. The ethics committee of the Provincial Hospital to Shandong University approved the protocol.

Statistical analysis

Quantitative data were expressed as mean ± standard deviation. \( P \) values were generated using the Student’s \( t \)-test. Values were considered significant if \( P < 0.05 \). All statistical procedures were performed using SPSS version 20.0 (IBM Corp, Armonk, NY, USA).

Results

FGFR4 is frequently overexpressed in ESCC and noncancerous tissues

The results of IHC staining of the 40 sample pairs showed that FGFR4 was weakly expressed in the normal esophageal epithelium but frequently overexpressed in ESCC.
specimens (Fig 1a). Approximately 42.5% (17/40) of patients tested positive for FGFR4 expression. Consistent with IHC staining results, Western blot analysis revealed that FGFR4 protein was present at a higher level in tumor tissues compared to corresponding noncancerous tissues (Fig 1b,c). In the positive group of tumor samples based on IHC staining, FGFR4 expression was significantly increased (FGFR4/β-actin: 0.927 ± 0.15 vs. 1.279 ± 0.17; \( P < 0.001 \)). In the negative group, there was no statistical difference in the FGFR4 expression level (FGFR4/β-actin: 0.975 ± 0.19 vs. 0.992 ± 0.16; \( P > 0.05 \)) (Fig 1c). Furthermore, compared to normal esophageal epithelial cells (FGFR4/β-actin: 0.652 ± 0.12 in HET-1A), different degrees of FGFR4 overexpression in ESCC cell lines were detected (FGFR4/β-actin: 1.238 ± 0.11, \( P < 0.01 \) in TE-1; 1.404 ± 0.05, \( P < 0.01 \) in Eca9706; 2.259 ± 0.14, \( P < 0.001 \) in KYSE150; 1.805 ± 0.05, \( P < 0.001 \) in Eca109; and 1.918 ± 0.06, \( P < 0.001 \) in KYSE450) (Fig 1d,e). The levels of FGFR4 in KYSE150 and KYSE450 cell lines were higher, which provided the basis for selecting cell lines for further study.

**Blocking FGFR4 decreases ESCC cell line proliferation**

To determine if blocking FGFR4 could suppress ESCC cell proliferation, clonogenic assay and CCK-8 were conducted. The colony formation assays showed that clonogenic

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**Figure 1** FGFR4 expression in esophageal squamous cell carcinoma (ESCC) sample and cell lines. (a) FGFR4 expression in ESCC tissues detected by immunohistochemistry (x200). (b) Bands of FGFR4 and β-actin in six representative tissue sample pairs. (c) Quantitative analysis of FGFR4 in 40 pairs of tissue specimens normalized to β-actin. (d) FGFR4 expression in ESCC cell lines or normal esophageal epithelial cells by Western blot analysis. (e) Quantitative analysis of FGFR4 in ESCC cell lines or normal esophageal epithelial cells normalized to β-actin. **\( P < 0.01 \).**
survival was suppressed in KYSE150 and KYSE450 cells treated with H3B-6527 compared to cells treated with pure culture media (Fig 2a). Survival rates decreased by 30.6% ($P < 0.001$) in KYSE150 and 20.9% ($P < 0.001$) in KYSE450 when treated with the blocker (Fig 2b). A similar result was obtained from the CCK-8 assay. The optical density values (450 nm) of the KYSE150 cells treated with H3B-6527 decreased by 0.141 (0.845 ± 0.06 vs. 0.704 ± 0.03; $P < 0.05$), 0.374 (1.156 ± 0.10 vs. 0.782 ± 0.08; $P < 0.01$), and 1.174 (2.150 ± 0.24 vs. 0.976 ± 0.14; $P < 0.01$) at 24, 48, and 72 hours, respectively, compared to the cells left untreated (Fig 2c). In KYSE450 cells treated with H3B-6527, the optical density values (450 nm) decreased by 0.355 (1.115 ± 0.11 vs. 0.760 ± 0.14; $P < 0.01$) and 0.538 (1.711 ± 0.15 vs. 1.173 ± 0.18; $P < 0.05$) at 48 and 72 hours, respectively (Fig 2c).

**Blocking FGFR4 inhibits migration, invasion, and the inducement of epithelial-mesenchymal transition in ESCC cells**

To evaluate the migration and invasion abilities of ESCC cells, which are signs of the degree of malignancy, wound-healing and transwell assays were performed. With respect to wound-healing assay, closure of the wounds was slower in ESCC cells treated with H3B-6527 (Fig 3a). The migration rates of the KYSE150 cells treated with H3B-6527 decreased by 17.0% ($P < 0.01$) and 31.3% ($P < 0.001$) after 12 and 24 hours, respectively, compared to cells left untreated. In KYSE450 cells treated with H3B-6527, the migration rates decreased by 26.7% ($P < 0.01$) and 30.6% ($P < 0.01$) at 48 and 72 hours, respectively (Fig 3b). In the transwell assay, compared to the control group, the number of ESCC cells treated with H3B-6527 that could specifically block FGFR4 was significantly lower (Fig 3c,e). The number of migration cells in visual fields decreased by 95 (187 ± 26 vs. 92 ± 10; $P < 0.01$) in KYSE150 and 129 (276 ± 24 vs. 147 ± 41; $P < 0.05$) in KYSE450 cells when treated with H3B-6527 (Fig 3d). The number of invasion cells also decreased by 99 (192 ± 28 vs. 93 ± 16, $P < 0.01$) in KYSE150 and 165 (319 ± 58 vs. 154 ± 24, $P < 0.05$) in KYSE450 cells when treated with the blocker (Fig 3f). These results indicated that blocking FGFR4 by H3B-6527 could suppress the migration and invasion of ESCC cells.

Because epithelial-mesenchymal transition (EMT) is involved in the migration and invasive capacity of epithelial cells, we examined whether blocking FGFR4 in ESCC cells was associated with EMT. The results of Western blotting showed that FGFR4 blocking caused a significant increase of epithelial marker E-cadherin (E-cadherin/...
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Figure 3 Legend on next page.
GAPDH: 0.143 ± 0.06 vs. 0.418 ± 0.03, P < 0.01 in KYSE150; 0.370 ± 0.07 vs. 0.634 ± 0.08, P < 0.05 in KYSE450) and Claudin-1 levels (Claudin-1/GAPDH: 0.359 ± 0.05 vs. 0.679 ± 0.08, P < 0.01 in KYSE150; 0.557 ± 0.05 vs. 0.799 ± 0.02, P < 0.01 in KYSE450) and a decrease of mesenchymal marker N-cadherin (N-cadherin/GAPDH: 1.048 ± 0.06 vs. 0.590 ± 0.03, P < 0.01 in KYSE150; 0.874 ± 0.06 vs. 0.658 ± 0.05, P < 0.01 in KYSE450), Vimentin (Vimentin/GAPDH: 0.957 ± 0.10 vs. 0.696 ± 0.05, P < 0.05 in KYSE150; 1.051 ± 0.08 vs. 0.746 ± 0.07, P < 0.01 in KYSE450), and Snail levels (Snail/GAPDH: 0.986 ± 0.05 vs. 0.640 ± 0.06, P < 0.01 in KYSE150; 1.010 ± 0.11 vs. 0.767 ± 0.08, P < 0.05 in KYSE450) in ESCC cells (Fig 3g,h).

Blocking FGFR4 promotes apoptosis in ESCC cells

In order to determine whether apoptosis resulted in the inhibition of proliferation, a flow cytometric apoptosis assay was performed. The data showed that in contrast to the control groups, after four days of treatment with H3B-6527, the number of apoptotic KYSE150 and KYSE450 cells increased (Fig 4a) by 5.9% (P < 0.01) in KYSE150 and 5.8% (P < 0.01) in KYSE450 cells (Fig 4b). Western blot was used to test two apoptotic-related factors, including Bcl-2 and Bax. We found that blocking FGFR4 not only significantly suppressed the levels of anti-apoptotic protein Bcl-2 (Bcl-2/β-actin: 0.913 ± 0.12 vs. 0.382 ± 0.08, P < 0.01 in KYSE150; 1.248 ± 0.11 vs. 0.648 ± 0.09, P < 0.01 in KYSE450), but also increased the levels of pro-apoptotic protein Bax (Bax/β-actin: 0.473 ± 0.07 vs. 0.886 ± 0.09, P < 0.01 in KYSE150; 0.744 ± 0.06 vs. 0.931 ± 0.04, P < 0.05 in KYSE450) (Fig 4c,d). Based on these results, we concluded that blocking FGFR4 promotes apoptosis of KYSE150 and KYSE450 cells.

Effects of blocking FGFR4 on signaling pathways in ESCC cell lines

To assess the effects of blocking FGFR4 on signaling pathways in ESCC cell lines, we first determined the level of p-FGFR4 in KYSE150 and KYSE450 cells after treatment with the indicated blocker (H3B-6527). Western blot analysis revealed that H3B-6527 could significantly inhibit the phosphorylation of FGFR4 in ESCC cells compared to the control group (p-FGFR4/FGFR4: 0.624 ± 0.05 vs. 0.365 ± 0.08, P < 0.01 in KYSE150; 0.539 ± 0.05 vs. 0.342 ± 0.03, P < 0.01 in KYSE450) (Fig 5). That is, H3B-6527 could efficiently block FGFR4. Additionally, in accordance with the inhibition of tumorigenic effects, blocking FGFR4 could significantly decrease the levels of p-AKT (p-AKT/AKT: 1.043 ± 0.07 vs. 0.608 ± 0.07, P < 0.01 in KYSE150; 1.362 ± 0.09 vs. 0.975 ± 0.05, P < 0.01 in KYSE450) and p-ERK (p-ERK/tERK: 0.981 ± 0.12 vs. 0.531 ± 0.06, P < 0.01 in KYSE150; 1.050 ± 0.07 vs. 0.753 ± 0.09, P < 0.05 in KYSE450) in ESCC cells (Fig 5). Collectively, our data revealed that blocking FGFR4 by H3B-6527 may affect AKT and ERK signaling in ESCC cells.

The FGFR4 blocker, H3B-6527, could inhibit tumor growth in mice

The effects of H3B-6527 on inhibiting tumor growth in vivo were also evaluated by zoopery. Compared to the control group, the average volumes of tumors in mice treated by H3B-6527 were decreased (Fig 6a). The average volumes of tumors reduced by 1963.8 mm³ (3115.8 ± 825.8 vs. 1152.0 ± 121.9 mm³; P < 0.001) in KYSE150 and 1365.7 mm³ (2466.0 ± 313.4 vs. 1100.3 ± 166.3 mm³; P < 0.001) in KYSE450 when treated with the blockade (Fig 6b). That is, growth velocity in the experimental group was significantly decreased. The weight of the tumors in the mice treated with H3B-6527 was also significantly decreased. The weight of the tumors in the mice treated with H3B-6527 was also significantly decreased.
Figure 4  Blocking FGFR4 promotes apoptosis in esophageal squamous cell carcinoma (ESCC) cells. (a) The cells treated each day with 1 μM H3B-6527 or left untreated for four days were incubated with PE/7-AAD and analyzed by flow cytometry. The percentage of PE and/or7-AAD positive cells is included in the panels. (b) The results are expressed as the percentage (mean ± standard deviation [SD] of three repeated experiments) of apoptotic/necrotic cells. (c) The cell apoptosis-related factors in KYSE150 and KYSE450 were detected by Western blotting. (d) Quantitative analysis of apoptosis-related factors in KYSE150 and KYSE450 cells normalized to β-actin. The average values ± SD were calculated from three separate experiments. *P < 0.05, **P < 0.01.

Figure 5  The effect of blocking FGFR4 on protein phosphorylation in esophageal squamous cell carcinoma (ESCC) cells. (a) KYSE150 and KYSE450 cells were treated with 1 μM H3B-6527 or left untreated for four hours, and lysates were immunoblotted with indicated antibodies. (b) Quantitative analysis of phosphorylated proteins in KYSE150 and KYSE450 cells normalized to the total protein, respectively. *P < 0.05, **P < 0.01.
attenuated (7.00 ± 0.80 vs. 2.29 ± 0.37 g, P < 0.001 in KYSE150; 6.37 ± 0.50 vs. 2.30 ± 0.37 g, P < 0.001 in KYSE450) (Fig 6c). In contrast to H3B-6527 activity, treatment with physiological saline had no effect on ESCC tumor growth (Fig 6a–c). The results illustrate that H3B-6527 could inhibit tumor growth in vivo.

**Discussion**

Despite significant improvements in therapeutic regimens, surgical resection is still considered the first choice of treatment for early-stage and localized ESCC. However, ESCC is often resistant to conventional therapeutic agents. To date, no inhibitors have been shown to be effective for ESCC. Thus, biological targets that may be helpful in treatments are becoming the hot spot to improve prognosis in patients with ESCC.

Studies have reported that FGFs can bind to FGFRs, which activates FGF signaling to induce a variety of cellular processes. The mutation and abnormal expression of FGFRs cause diverse pathologies and affect multiple solid tumors, including breast and lung cancers. FGFR4, belonging to the FGFR family, differs from other FGFRs in genomic structure, ligand binding, and signal transduction. Although FGFR4 is associated with multiple kinds of tumors, the functions and mechanisms of FGFR4 in ESCC have not been fully elucidated.

In the current study, we confirmed the frequent overexpression of FGFR4 in ESCC tissue and cell lines by IHC and Western blot. This data was consistent with the findings of previous studies, which have confirmed the overexpression of FGFR4 in ovarian cancer, mouth and oropharynx carcinoma, and cholangiocarcinoma. Thus, FGFR4 might contribute to ESCC progression.

To explore the effects and mechanisms of blocking FGFR4 in ESCC, we selected two cell lines, KYSE150 and KYSE450, with relatively high FGFR4 expression levels for further study. H3B-6527 is a highly selective and covalent small-molecule inhibitor of FGFR4, which has been reported to be effective in inhibiting cell proliferation in vitro and in xenografts models of hepatocellular carcinoma. Our results indicate that blocking FGFR4 by H3B-6527 could significantly inhibit ESCC cell proliferation in vitro. In additional, we found that FGFR4 blockade attenuated the migration and invasion ability of ESCC cells. We speculate that this outcome may be a result of EMT being affected in ESCC. EMT is an important process in cancer metastasis, with 90% of tumors exhibiting different degrees of EMT during tumor development. During this process, the epithelial cells attenuate the expression of epithelial characteristics and gain mesenchymal phenotype properties. In this study, we surprisingly found that blocking FGFR4 suppressed EMT in ESCC cells, including the upregulation of epithelial markers
cadherin and Claudin-1) and the downregulation of mesenchymal markers (N-cadherin, Vimentin, and Snail). Thus, we posit that FGFR4 is correlated with ESCC metastasis. This result is consistent with findings in colorectal cancer and nasopharyngeal carcinoma. Moreover, many kinds of tumors, including ovarian cancer and FGFR4 overexpression is associated with poor prognosis in ESCC cells, which was consistent with the Western blot results of apoptotic-related factors.

The PI3K/Akt and MAPK/ERK pathways are two vital signaling pathways in tumors. They play an important role in regulating cell survival, cell cycle, angiogenesis, metastasis, and metabolism. Several studies have demonstrated that the PI3K/Akt and MAPK/ERK signaling pathways are activated in the progression of ESCC. Additionally, FGFR4 overexpression can promote cell malignant growth, migration, invasion, and suppress apoptosis via the PI3K/Akt and MAPK/ERK pathways in colorectal cancer, rhabdomyosarcoma and ovarian cancer. In this study, we found that blocking FGFR4 could significantly inhibit its phosphorylation and further reduce the levels of phosphorylated AKT and ERK. This was consistent with the biological results we obtained in vitro. Therefore, we infer that FGFR4 blockade may affect cell growth, migration, invasion, and apoptosis via PI3K/Akt and MAPK/ERK signaling pathways.

Furthermore, ESCC tumors in nude mice responded to H3B-6527 treatment with an apparent slowing of growth. At 20 days of H3B-6527 treatment, the speed of ESCC tumor growth was reduced and the tumor volume was significantly smaller. On the contrary, the control group treated with physiological saline exhibited no significant inhibitory effects. These findings indicate that FGFR4 blocking is effective in inhibiting the growth and survival of ESCC cancer cells in xenograft models. We suggest that a dose-response effect of tumor regression may be achieved with higher doses of H3B-6527 administered for longer periods (20 days). The effectiveness of H3B-6527 in ESCC tumor xenografts may be linked to the blocking of FGFR4 in human ESCC cancers. Therefore, FGFR4 may become a potential target and H3B-6527 may be useful for ESCC cancer patients with FGFR4 overexpression.

Although studies have shown that blocking FGFR4 by H3B-6527 could attenuate the biological characteristics of ESCC cells in vivo and in vitro, we only used one blocking method in this study. In future studies, we will build FGFR4-overexpressed and FGFR4-silencing cell lines using other agents, such as short hairpin RNA, small interfering RNA, and plasmid, to further explore the role of FGFR4. FGFR4 overexpression is associated with poor prognosis in many kinds of tumors, including ovarian cancer and nasopharyngeal carcinoma. Therefore, there is a high probability that FGFR4 overexpression in ESCC is associated with poor prognosis and reduced chemosensitivity. We will confirm this in future studies.

Based on our results, we conclude that blocking FGFR4, which impacts AKT and ERK signaling, can inhibit the cellular proliferation, migration, invasion, and survival of ESCC cells. In addition, blocking FGFR4 by H3B-6527 can lead to tumor regression in esophageal xenograft models. Hence, FGFR4 may become a potential target and an FGFR4 blocker may become a new agent for treating ESCC patients.

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Disclosure
No authors report any conflict of interest.

References
1 Abnet CC, Arnold M, Wei WQ. Epidemiology of esophageal squamous cell carcinoma. Gastroenterology 2018; 154: 360–73.
2 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7–30.
3 Chen W, Zheng R, Baade PD et al. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115–32.
4 Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. Gut 2015; 64: 381–7.
5 Ohashi S, Miyamoto S, Kikuchi O, Goto T, Amanuma Y, Muto M. Recent advances from basic and clinical studies of esophageal squamous cell carcinoma. Gastroenterology 2015; 149: 1700–15.
6 Sasaki Y, Kato K. Chemoradiotherapy for esophageal squamous cell cancer. Jpn J Clin Oncol 2016; 46: 805–10.
7 Dieci MV, Arnedos M, Andre F, Soria JC. Fibroblast growth factor receptor inhibitors as a cancer treatment: From a biologic rationale to medical perspectives. Cancer Discov 2013; 3: 264–79.
8 Xu YF, Yang XQ, Lu XF et al. Fibroblast growth factor receptor 4 promotes progression and correlates to poor prognosis in cholangiocarcinoma. Biochem Biophys Res Commun 2014; 446: 54–60.
9 Friesel R, Maciag T. Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. *Thromb Haemost* 1999; 82: 748–54.

10 Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer* 2000; 7: 165–97.

11 Lin BC, Desnoyers LR. FGF19 and cancer. *Adv Exp Med Biol* 2012; 728: 183–94.

12 Turner N, Grose R. Fibroblast growth factor signalling: From development to cancer. *Nat Rev Cancer* 2010; 10: 116–29.

13 Haugsten EM, Wiedlocha A, Olssen S, Wesche J. Roles of fibroblast growth factor receptors in carcinogenesis. *Mol Cancer Res MCR* 2010; 8: 1439–52.

14 Spinola M, Leoni VP, Tanuma J et al. FGF4R Gly388Arg polymorphism and prognosis of breast and colorectal cancer. *Oncol Rep* 2005; 14: 415–9.

15 Taylor JG, Cheuk AT, Tsang PS et al. Identification of FGFR4-activating mutations in human rhabdomyosarcomas that promote metastasis in xenotransplanted models. *J Clin Invest* 2009; 119: 3395–407.

16 Spinola M, Leoni V, Pignatiello C et al. Functional FGFR4 Gly388Arg polymorphism predicts prognosis in lung adenocarcinoma patients. *J Clin Oncol Off J Am Soc Clin Oncol* 2005; 23: 7307–11.

17 da Costa Andrade VC, Parise O Jr, Hors CP, de Melo Martins PC, Silva AP, Garicochea B. The fibroblast growth factor receptor 4 (FGFR4) Arg388 allele correlates with survival in head and neck squamous cell carcinoma. *Exp Mol Pathol* 2007; 82: 53–7.

18 Streit S, Mestel DS, Schmidt M, Ullrich A, Berking C. FGFR4 Arg388 allele correlates with tumour thickness and FGFR4 protein expression with survival of melanoma patients. *Br J Cancer* 2006; 94: 1879–86.

19 Shi Y, Liu X, Fredimoses M et al. FGFR2 regulation by picrasidine Q inhibits the cell growth and induces apoptosis in esophageal squamous cell carcinoma. 2018; 119: 2231–9.

20 Kwon D, Yun JY, Keam B, Kim YT, Jeon YK. Prognostic value of FGFR4 role in epithelial-mesenchymal transition and its therapeutic value in colorectal cancer. *PloS One* 2013; 8: e63695.

21 Zaid TM, Yeung TL, Thompson MS et al. Identification of FGFR4 as a potential therapeutic target for advanced-stage, high-grade serous ovarian cancer. *Clin Cancer Res Off J Am Assoc Cancer Res* 2013; 19: 809–20.

22 Vainikka S, Partanen J, Bellotta P et al. Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. *EMBO J* 1992; 11: 4273–80.

23 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; 139: 871–90.

24 Kwon D, Kim YT, Jeon YK. Prognostic implications of FGFR4 and MYC status in esophageal squamous cell carcinoma. *World J Gastroenterol* 2016; 22: 9803–12.

25 Mariette C, Piessen G, Triboulet JP. Therapeutic strategies in oesophageal carcinoma: Role of surgery and other modalities. *Lancet Oncol* 2007; 8: 545–53.

26 Jang JH, Shin KH, Park JG. Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers. *Cancer Res* 2001; 61: 3541–3.

27 Chin K, DeVries S, Fridlyand J et al. Genomic and transcriptional aberrations linked to breast cancer pathophysiology. *Cancer Cell* 2006; 10: 529–41.

28 Behrens C, Lin HY, Lee JJ et al. Immunohistochemical expression of basic fibroblast growth factor and fibroblast growth factor receptors 1 and 2 in the pathogenesis of lung cancer. *Clin Cancer Res Off J Am Assoc Cancer Res* 2008; 14: 6014–22.
40 Manning BD, Cantley LC. AKT/PKB signaling: Navigating downstream. *Cell* 2007; 129: 1261–74.
41 Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E. PI3K/AKT signaling pathway and cancer: An updated review. *Ann Med* 2014; 46: 372–83.
42 De Luca A, Maiello MR, D’Alessio A, Pergameno M, Normanno N. The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: Role in cancer pathogenesis and implications for therapeutic approaches. *Expert Opin Ther Targets* 2012; 16 ((Suppl 2)): S17–27.
43 Yoshioka A, Miyata H, Doki Y et al. The activation of Akt during preoperative chemotherapy for esophageal cancer correlates with poor prognosis. *Oncol Rep* 2008; 19: 1099–107.
44 Tasioudi KE, Saetta AA, Sakellariou S et al. pERK activation in esophageal carcinomas: Clinicopathological associations. *Pathol Res Pract* 2012; 208: 398–404.
45 Crose LE, Etheridge KT, Chen C et al. FGFR4 blockade exerts distinct antitumorigenic effects in human embryonal versus alveolar rhabdomyosarcoma. *Clin Cancer Res Off J Am Assoc Cancer Res* 2012; 18: 3780–90.