Overcoming Linsitinib intrinsic resistance through inhibition of nuclear factor-κB signaling in esophageal squamous cell carcinoma

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Abstract
The aim of this study is to evaluate the efficacy of insulin-like growth factor 1 receptor (IGF-1R) inhibitor Linsitinib, in esophageal squamous cell carcinoma (ESCC), and to characterize special biomarker to screen Linsitinib-sensitive patients as well as explore the molecular-resistant mechanism to Linsitinib in ESCC. Our study evaluated the sensitivity of insulin-like growth factor 1 receptor (IGF-1R) inhibitor, Linsitinib in ESCC cells with MTT assay. After Linsitinib treatment, the expressions of downstream signaling molecules and apoptosis pathways were measured by western blot. And the antitumor effect of Linsitinib and JSH-23, an inhibitor of nuclear factor-κB transcriptional activity, was analyzed both as single agent and in combination in ESCC. Apoptosis, cell viability, and clonogenic survival analysis were also investigated. The sensitivity of Linsitinib was relatively variable in patient-derived primary ESCC cells as well as in human commercial cell lines. And the downstream AKT/mTOR and ERK signaling pathways were inhibited by Linsitinib, while phosphorylation level of NF-κB p65 was obviously activated to reduce apoptosis effect in Linsitinib-resistant cell lines. Most importantly, blockage of NF-κB activity by JSH-23 could sensitize resistant cells to Linsitinib treatment. Results from this study demonstrated that the intrinsic resistance to Linsitinib was predominantly mediated by NF-κB activation in ESCC. Moreover, combination of Linsitinib and JSH-23 as therapy provides a novel strategy to overcome resistance to Linsitinib in ESCC.
Insulin-like growth factor-1 receptor (IGF-1R) signaling pathway has been implicated in the carcinogenesis and progression of multiple cancer sites, including ESCC [7, 8]. Studies of ESCC demonstrated that upon binding to its ligands IGF-1 or IGF-2, IGF-1R is autophosphorylated and the phosphorylation activates the downstream pathways of PI3K/AKT/mTOR and Ras/Raf/MEK/MAPK [9, 10], which promote tumor cell proliferation, invasion, metastasis, and evasion of apoptosis [10, 11]. Moreover, elevated levels of IGF-1R expression are common in 60–80% ESCC [12, 13], and patients with higher expression of IGF-1R are more likely to have shorter overall survival [7]. Thus, inhibition of the IGF-1R pathway may offer a promising strategy for ESCC treatment.

Recently, around 30 compounds targeting IGF-1R have been tested in phase II/III clinical trials for the treatment of several types of cancer including ESCC. [8, 14–16]. Among them, Linsitinib (also named OSI-906) is a selective and orally bioavailable IGF-1R/insulin receptor (IR) inhibitor [17], which has been shown to block ligand-induced activation of pAkt, pERK1/2, and p-p70S6K [15]. However, clinical trials involving Linsitinib showed varied response rates [18–20]. Two phase I trials showed an overall objective response rate of about 30% in advanced solid tumors [18, 19], with some patients obtaining durable benefit from the IGF-1R blockade [18, 19]. However, a phase III clinical trial of adrenocortical carcinoma indicated that Linsitinib had no effect in comparison to the placebo group [21]. The different responses may be partly due to the innate drug resistance or activation of compensatory pathways allowing for continued growth [15, 22]. To deal with these challenges, we need to elucidate the mechanisms that underlie Linsitinib resistance in ESCC and identify biomarkers that can screen Linsitinib-sensitive patients with ESCC.

In this study, we investigated the mechanisms underpinning the sensitivity and resistance of Linsitinib in ESCC, and found an intrinsic Linsitinib resistance mediated through the nuclear factor-xB (NF-xB) pathway. Our experiments suggest that Linsitinib administration in combination with NF-xB inhibitor JSH-23 may have synergy in ESCC treatment.

Methods

Ethics approval

This study was approved by the institutional review board of Zhejiang Cancer Hospital. All patients signed an informed consent before surgery.

Cell lines and cell culture

Human commercially available ESCC cell lines were bought from Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences (Shanghai, China). The patient-derived primary cancer cells were isolated and cultured from solid tumors of ESCC patients. Reduction esophagectomy tissue samples were mechanically dissociated and then incubated with collagenase (Roche Life Science, Indianapolis, IN) and hyaluronidase (Sigma-Aldrich, St. Louis, MO) at 37°C for 2 h. Primary cancer cells thus obtained were tested for drug response between passage 3 to 5 generations. All tissue specimens used in our study were obtained from the tissue bank of Zhejiang Cancer Hospital. All patients signed an informed consent before surgery. This study was approved by the institutional review board of Zhejiang Cancer Hospital.

Primary ESCC cells were cultured in DMEM/F12 (Life technologies, Gaithersburg, MD). DMEM/F12 medium was supplemented with 10% fetal bovine serum (FBS, Gibco, Life technologies), 1% penicillin-streptomycin (Gibco, Life technologies), and 1% MEM nonessential amino acids (Gibco, Life technologies). All other cells were cultured in RPMI 1640 (Life technologies), supplemented with 10% FBS. All the cells were cultured under the standard conditions (5% CO₂ at 37°C).

Antibodies and reagents

Linsitinib and JSH-23 were purchased from Selleckchem Co. (Houston, TX). Stock solutions with a concentration of 10 mM were prepared and stored at −20°C.

Antibodies against phospho-IGF1R (CST-3918), total-IGF1R (CST-9750), total-PI3K (CST-5536), total-mTOR (CST-2983), total-p65 (CST-3033), phosphorylated-PI3K (CST-2927), total-Akt (CST-1085), phospho-mTOR (CST-5536), total-mTOR (CST-2983), pERK1/2 (CST-3918), total-p70S6K (CST-2927), and total-Akt (CST-1085) were purchased from Cell Signaling Technology (Danvers, MA, USA). Cleaved Caspase-3 (25546-1-AP) were purchased from Protein Technology (Tucson, AZ) and Tubulin (ARH4207) were purchased from AR (San Diego, CA). HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Santa Cruz Biotechnology (Dallas, TX). MTi [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] was obtained from Sigma-Aldrich.

Cell viability analysis

A colorimetric MTT assay was performed to quantify the effect of drugs on cell viability. Cells were seeded in 96-well plates at a density of 3000 cells/well, and were grown for over 24 h before being incubated with the respective
compound for 72 h. Controls were treated with DMSO only. Four hours prior to the end of the culture period, 50 μL MTT solution (5 mg/mL in PBS) was added to each well. Each reaction was stopped by adding 150 μL DMSO. The absorbance was measured at a wavelength of 570 nm.

Cell lysis and western blot
Cells were lysed to extract proteins with a lysis buffer using the standard method. The extracted protein samples were analyzed using 8% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Each membrane was blocked with 5% nonfat dry milk or bovine serum albumin in TBS-Tween-20 (TBS-T) for 1 h, followed by incubation with primary antibody at 4°C overnight. The membrane was then incubated with HRP-conjugated secondary antibody before it was detected with enhanced chemiluminescence (ECL, Millipore).

RNA extraction and quantitative real-time PCR
Total RNA was extracted from cells with the TRIzol reagent and cDNA was synthesized using a Prime-Script RT Reagent kit (Takara Bio, Inc. Otsu, Shiga, Japan). Targeted cDNA were amplified using a SYBR Premix Ex Taq kit (Takara Bio, Inc. Otsu, Shiga, Japan). Targeted cDNA were further examined for drug resistance. Since incomplete blockade of the IGF-1R pathway could confer Linsitinib resistance, we evaluated the downstream effectors of the MEK and PI3K pathways (ERK1/2, AKT and mTOR) with western blot. As shown in Figure 2, IGF-1R phosphorylation was inhibited after treatment of Linsitinib at 0.1 or 1.0 μmol/L for 72 h. Moreover, compared to the controls, phosphorylation of ERK1/2 was down-regulated by Linsitinib in both sensitive (TE-13)

Flow cytometric analysis of apoptosis cell
Cells (1 × 10^5/mL) were seeded in 6-well plates and incubated overnight. Cells were then treated with Linsitinib (1.0 or 10.0 μmol/L) and JSH-23 (20 μmol/L) alone or in combination for at least 10 days. At the end of the incubation period, cells were fixed with methanol and stained with 0.05% crystal violet solution. Colonies containing >50 cells were counted.

Statistical analysis
Statistical analyses were performed using SPSS (version 18.0, Chicago, IL). Data from the experiments were expressed as mean ± SD which was based on a minimum of three independent experiments. Differences between groups were compared using the two-way ANOVA, followed by the Newman–Keuls test, and a P < 0.05 was considered significant.

Results
Effect of Linsitinib on ESCC cells
To investigate the effect of Linsitinib on the viability of ESCC cells, a panel of 16 primary ESCC cells was exposed to different concentrations of Linsitinib (0.1–100 μmol/L), and the cell viability was then measured with MTT assay (Fig. 1A). The test results showed that four of 16 primary cells were almost completely resistant to Linsitinib, while four displayed high sensitivity. In addition, we evaluated the sensitivity of Linsitinib with concentrations of 0.1–80 μmol/L in a panel of six commercially available ESCC cell lines (Eca-109, EC-9706, KYSE-510, KYSE-410, TE-1, and TE-13). As shown in Figure 1B, TE-13 was the most sensitive cell line and the remaining five were much resistant. The sensitive (TE-13) and resistant cell lines (TE-1 and KYSE-510) were further examined for drug resistance.

Inhibition of ERK and PI3K signaling by Linsitinib
Since incomplete blockade of the IGF-1R pathway could confer Linsitinib resistance, we evaluated the downstream effectors of the MEK and PI3K pathways (ERK1/2, AKT and mTOR) with western blot. As shown in Figure 2, IGF-1R phosphorylation was inhibited after treatment of Linsitinib at 0.1 or 1.0 μmol/L for 72 h. Moreover, compared to the controls, phosphorylation of ERK1/2 was down-regulated by Linsitinib in both sensitive (TE-13)
Reduced apoptosis in Linsitinib-resistant ESCC cells

To examine if Linsitinib resistance affects cell apoptosis, we analyzed the expression of cleaved PARP and activated Caspase-3 protein with western blot. As indicated in Figure 3, after treatment of Linsitinib at 0.1 or 1.0 μmol/L for 72 h, the expression of cleaved PARP and activated Caspase-3 were increased in a sensitive cell line (TE-13), but decreased in resistant cell lines (TE-1 and KYSE-510).

These results suggested that Linsitinib-resistant cells had a reduced capacity of apoptosis.

Activation of NF-κB pathway in Linsitinib-resistant ESCC cells

NF-κB pathway may play a critical role in drug resistance of ESCC cells. We evaluated whether NF-κB pathway was activated after Linsitinib treatment. Figure 4A shows a dose-dependent elevation of p-p65 levels in Linsitinib-resistant cell lines (TE-1 and KYSE-510), with little or no change in total p65 levels. Expression of p-p65 was inhibited in TE-13, the Linsitinib-sensitive cell line. Additionally, as the transcriptional targets of p65, IL-6 and IL8 mRNA levels were also altered following the trends of p-p65, up-regulation in resistant cell lines, and down-regulation in the sensitive cell line (Fig. 4B). Taken together, our experiment suggested that NF-κB pathway was activated in resistant ESCC cells after Linsitinib treatment.

Enhanced apoptosis following combined treatment of Linsitinib and NF-κB inhibitor in Linsitinib-resistant cells

To test if NF-κB inhibitor could be used in combination with IGF-1R blocker in treating ESCC, we performed flow cytometry analysis of cell apoptosis in Linsitinib-sensitive and -resistant cells (Fig. 5). Interestingly, after exposing cells to Linsitinib (1.0 or 10.0 μmol/L) and JSH-23 (20 μmol/L) alone or in combination for 48 h, cells treated with the combined regimens showed statistically significant induction of programmed cell death when compared to single-regimen treatment in Linsitinib-resistant cells (P < 0.01, Fig. 5). However, this effect was not observed in Linsitinib-sensitive cell TE-13 (P > 0.05, Fig. 5). This difference suggests that reduction in apoptosis may be an important mechanism in tumors resistant to Linsitinib.

Combined treatment of Linsitinib and NF-κB inhibitor affected cell viability and colony formation in Linsitinib-resistant cells

We next measured cell viability and colony formation ability after exposing the cells to Linsitinib (1.0 or 10.0 μmol/L) and JSH-23 (20 μmol/L) alone or in combination (Fig. 6A). As depicted in Fig. 6A, compared to Linsitinib monotherapy, the combination of Linsitinib and JSH-23 had a statistically significant effect on growth inhibition of Linsitinib-resistant cells (TE-1 and KYSE-510, P < 0.01). However, no inhibitory effect was observed in Linsitinib-sensitive cell TE-13 (P > 0.05, Fig. 5). Similarly, the addition of JSH-23 could reverse ESCC cells from...
Linsitinib resistant to Linsitinib sensitive with regard to their colony formation.

Discussion

Recent clinical trials of IGF-1R inhibitors have demonstrated variable antitumor effects [20, 23], which may reflect either the lack of patient selection strategies and/or little understanding of drug-resistant mechanisms. Consistent with the findings of a previous study of Linsitinib in colorectal cancer [24], our study also showed that the sensitivity of Linsitinib was variable not only in primary cells but also in commercial cell lines. This observation indicates the presence of intrinsic resistance to Linsitinib in ESCC. Possible mechanisms explaining the intrinsic resistance include limited effect on downstream signaling of IGF-1R, existence of subclones resistant to the drug, and alternative compensatory pathway [25, 26]. Previous studies showed that cell lines with active downstream molecules MAPK/MEK [27, 28] or AKT/mTOR/p70S6K [24, 29] had intrinsic resistance to IGF-1R inhibitor. Moreover, using Linsitinib in combination with a MEK inhibitor to treat colorectal cancer cells with active MAPK demonstrated synergistic antitumor effects on the Linsitinib-resistant cell lines [27]. In our study, we found that the AKT/mTOR and ERK1/2 pathways were inhibited by Linsitinib in both sensitive and resistant cell lines. On the basis of this observation, we hypothesized that failure to Linsitinib treatment was not due to the activities of IGF-1R downstream molecules, but rather resulted from a compensatory mechanism that counteracted the effect of a single regimen which targeted only upstream molecule.

As previously proposed, IGF-1R inhibitors could induce apoptosis, inhibit tumor growth, as well as sensitize cells to chemotherapy in esophageal carcinoma cells [29, 30]. Programmed cell death can be suppressed by the nucleus localization of nuclear factor-κB (NF-κB) [31], which induces the expression of antiapoptotic factors such as the IAPs, the TRAFs, and Bcl-1 [32]. NF-κB plays a
critical role in chemotherapy resistance due to its ability to reduce apoptosis [33–37]. Apoptotic pathways are related with the sensitivity of target drugs [38–40], and Linsitinib resistance in ESCC may be related to apoptosis. So we investigated the expression of cleaved PARP, activated Caspase-3, and phosphorylated NF-κB p65, as well as its transcriptional targets IL-6 and IL-8. Interestingly, our results demonstrated that the apoptotic effect was decreased, while NF-κB p-p65 was significantly increased in Linsitinib-resistant cells. Meanwhile, the opposite trend was observed in Linsitinib-sensitive cells.

To further confirm these results, we investigated the combined effects of Linsitinib and JSH-23, a molecule that inhibits the transcriptional activity of NF-κB, on ESCC cell growth. JSH-23 has been found to reduce the resistance to TRAIL-induced apoptosis in acute myeloid leukemia [41]. In addition, JSH-23 has been proven to reverse the radioresistance in breast cancer [42]. We investigated the apoptotic activities of both Linsitinib-resistant and -sensitive cell lines treated with Linsitinib and JSH-23 alone or in combination. As expected, a single-regimen therapy of JSH-23 did not work well, but a combination therapy of both Linsitinib and JSH-23 demonstrated a significant synergy in induction of apoptosis, as well as effective reduction in cell viability and colony formation in Linsitinib-resistant cell lines. However, no difference
Figure 6. Combined treatment of Linsitinib and nuclear factor-κB (NF-κB) inhibitor affected cell viability and colony formation ability in Linsitinib-resistant cells. TE-13, TE-1, and KYSE-510 cells were exposed to Linsitinib 1.0 μmol/L for (A), 10.0 μmol/L for (B), and JSH-23 (20 μmol/L) alone or in combination. Then MTT assay was performed to measure the cell viability, and data from each cell line represent mean growth inhibition compared to DMSO control cells for three independent experiments. Furthermore, colony formation analysis was also investigated. Representative images are shown in (C) and results from three independent experiments are summarized in (D). NS indicates $P > 0.05$. *$P < 0.01$
was found in Linsitinib-sensitive cells when they were treated with single or double regimens. Treatment with both Linsitinib and JSH-23 exhibited increased efficacy of Linsitinib in Linsitinib-resistant cells, indicating that targeting on both IGF-1R and NF-kB may generate a promising therapeutic effect on ESCC.

To sum up, our study suggests that the intrinsic resistance of ESCC to Linsitinib may be mediated by NF-kB activation. A combined therapy that targets both IGF-1R and NF-kB may provide a novel strategy to overcome the ESCC’s resistance to Linsitinib.

Conclusions

The intrinsic resistance of ESCC to Linsitinib may be mediated by NF-kB activation. A combined therapy that targets both IGF-1R and NF-kB provides a novel strategy to overcome resistance to Linsitinib in ESCC.

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Conflict of Interest

The authors declare that they have no competing interests.

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