

Cetuximab inhibits cisplatin-induced activation of EGFR signaling in esophageal squamous cell carcinoma

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Abstract. A malignant esophageal cancer, squamous cell carcinoma is one of the most prevalent cancers. Despite the use of present surgical techniques combined with various treatment modalities, the overall 5-year survival rate remains at 15-34%. Epidermal growth factor receptor (EGFR) is the most well studied receptor in various cancers and EGFR overexpression is detected in 40% of esophageal squamous cell carcinomas (ESCCs) and ESCC cell lines. To examine the EGFR antibody combination effect, we used treatment with cisplatin and cetuximab in ESCC cell lines, TE-4 and TE-8. Combination of cetuximab and cisplatin resulted in a growth inhibition only in the EGFR overexpressed TE-8 cell line. Furthermore, we confirmed that cisplatin-induced EGFR activation was inhibited by cetuximab in TE-8 but not in TE-4 cells. Our data suggest that cetuximab combined with cisplatin exerts antitumorigenic effects *in vitro* and *in vivo* via suppression of EGF signaling, which can be applied toward targeted ESCC treatments.

Introduction

Esophageal squamous cell carcinoma (ESCC) is an aggressive disease characterized by high mortality rates. Epidemiological studies indicate that ESCC is the fifth most common cause of cancer-related mortality in men (1). Despite significant improvements in diagnosis and treatment including surgery, chemotherapy and radiotherapy, patient prognosis remains poor, with a 5-year survival rate of 15-34% (2-4). Systemic chemotherapy with cisplatin and 5-fluorouracil (5-FU) is the most commonly used treatment regimen for advanced ESCC. However, response rates are low at 15-45% and median survival

is generally <8 months (5,6). Therefore, novel therapeutic agents are urgently needed to improve clinical outcomes.

Epidermal growth factor receptor (EGFR) is the most extensively studied of the ErbB receptors in relation to cancer. EGFR is overexpressed in 60-70% of ESCC cases and EGFR gene amplification was detected in approximately 28% of tumors by fluorescence *in situ* hybridization (7). Activated EGFR signals via the AKT, ERK and RAS pathways and has an essential role in the control of many fundamental cellular processes. As such, the inhibition of EGFR signaling has emerged as an important antitumor treatment strategy. Cetuximab (also known as Erbitux or C225) is a recombinant chimeric human-murine monoclonal antibody and is among the most promising and clinically effective of these agents (8-10). Cetuximab binds EGFR with high affinity and prevents receptor activation, thereby suppressing proliferation and angiogenesis and promoting antibody-dependent cellular toxicity (11). When administered in conjunction with the platinum-based chemotherapy drug cisplatin, treatment with cetuximab resulted in a longer overall survival time in patients with recurrent or metastatic head and neck SCC (HNSCC) (12). However, despite several clinical trials for cetuximab and cisplatin, the precise role of these agents in the regulation of the EGFR signaling pathway is unclear.

The present study investigated the molecular mechanisms of cetuximab and cisplatin in two ESCC cell lines in the regulation of EGF signaling and assessed the potential for combination therapy *in vitro* and *in vivo*.

Materials and methods

Reagents and antibodies. The humanized mouse anti-human EGFR antibody cetuximab was purchased from Merck (Dietikon, Switzerland) and cisplatin was obtained from Dong-A PharmTech Co., Ltd. (Seoul, Korea). Primary antibodies against EGFR, phosphorylated p-EGFR, AKT, p-AKT, ERK and p-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Boston, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody was from Santa Cruz Biotechnology.

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Cell culture. Human ESCC cell lines TE-1, -2, -4, -5, -6, -8, -9, -10, -11, -14 and -15 were obtained from RIKEN (Tsukuba, Japan) and the human primary esophageal epithelial cell line Het-1A was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C under 5% CO₂ and 95% air.

Cell viability assay. Cells were seeded at 4x10³/well in 96-well plates in 0.1 ml medium for 24 h before treatment. Cells were exposed to a range of concentrations of cetuximab, cisplatin, or both in the presence of 0.5% FBS. At the indicated time points, CellTiter 96 AQueous (MTS) solution (Promega, Madison, WI, USA) in serum-free medium was added to each well. After 2 h, the colored MTS product in the supernatant was measured using a microplate reader (BioTek, Winooski, USA) at 490 nm absorbance.

Western blot analysis. Protein was extracted from cells and frozen tissues using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitors (Roche, Basel, Switzerland). Lysates containing equivalent amounts of protein were resolved by 10% SDS-PAGE. Samples were transferred to nitrocellulose membranes, which were blocked in 5% skim milk in TBS containing 0.1% Tween-20. Membranes were probed overnight at 4°C with primary antibodies against EGFR (1:3,000), p-EGFR (1:1,000), AKT, p-AKT (both 1:1,000), ERK (1:1,000) and p-ERK (1:1,000); the following day, HRP-conjugated secondary antibody (1:5,000) was applied for 1 h at room temperature. Protein was visualized using the enhanced chemiluminescence detection system (Thermo Fisher Scientific).

Immunohistochemistry. Formalin-fixed, paraffin-embedded tumor tissue samples were sectioned at a thickness of 4 μ m and mounted on glass slides. Endogenous peroxidase was blocked by treating sections with 0.3% H₂O₂ for 30 min. Antigen retrieval was performed in a steamer with citrate buffer for 30 min. Immunostaining was performed using a Lab Vision Autostainer (Thermo Scientific, Bremen, Germany) and primary antibodies against EGFR (1:500) and Ki67 (1:300), followed by treatment with the Lab Vision HRP polymer detection system (Thermo Scientific) according to the manufacturer's protocol. The TUNEL reaction was performed according to the manufacturer's instructions. Stained sections were visualized using a light microscope (Olympus, Tokyo, Japan).

Subcutaneous xenograft model. Animals were housed and treated in accordance with institutional guidelines for animal care and use. Female athymic mice (BALB/c-nu/nu; 5-6 weeks old; 17-23 g) were injected subcutaneously with TE-8 cells (1.5x10⁷) in the right flank. Tumors were allowed to grow to a volume of approximately 100 mm³, at which time mice were randomly assigned to one of four groups (10 animals each). One group received intraperitoneal (i.p.) injections of cisplatin at doses of 40 μ g/head per injection three times a week. A second group was administered cetuximab intravenously at 0.5 mg/head per week. In the third group, both cisplatin and cetuximab were administered using the same schedule

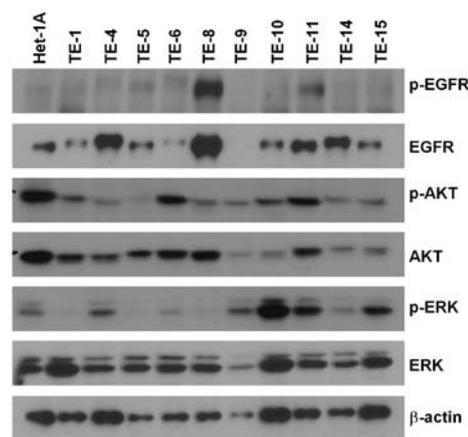


Figure 1. Relative levels of EGFR, p-EGFR, AKT, p-AKT, ERK and p-ERK expression in ESCC cell lines (TE-1, -2, -4, -5, -6, -8, -9, -10, -11, -14 and -15) and a normal esophageal epithelial cell line (Het-1A), as determined by western blot analysis. β -actin was used as a loading control.

for each drug as for single treatments. The fourth group, the control group, received i.p. injections of saline. The tumor volume and weight of each animal were assessed every other day for 4 weeks. Tumor volumes (V) were estimated using the formula: $V = \text{length} \times \text{width}^2 / 2$.

Statistical analysis. Data were obtained from at least three independent experiments and are expressed as mean \pm standard deviation. Mean differences were analyzed using the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression and phosphorylation status of EGFR, AKT and ERK in ESCC cell lines. The expression of EGFR, p-EGFR, AKT, p-AKT, ERK, p-ERK was examined in 10 ESCC and control Het-1A cell lines by western blotting (Fig. 1). TE-8 and -11 cells showed the highest expression of EGFR and p-EGFR, whereas activated EGFR (i.e., p-EGFR) levels were low in the other cell lines, including TE-4. Levels of p-AKT and p-ERK did not differ significantly between cell lines. Two cell lines, TE-4 and -8, were selected for subsequent experiments to examine the correlation between EGFR and p-EGFR expression and combined drug treatment.

Cisplatin-induced cytotoxicity is enhanced by cetuximab in EGFR-activated cells. The effects of cisplatin and cetuximab administered in isolation or in combination were assessed in two ESCC cell lines, TE-4 and -8, expressing low and high endogenous levels of EGFR, respectively. A range of concentrations of both agents were tested (cisplatin: 0, 1, 2, 5, 10 and 20 μ M for 3 days; cetuximab: 0, 10, 50, 100, 200 and 400 μ g/ml for 7 days). TE-8 had greater sensitivity to cisplatin than TE-4 cells, with IC₅₀ values of 2.06 and 16.79, respectively (Fig. 2A). There was no difference in sensitivity to cetuximab between the two cell lines (IC₅₀ = 232.65 in TE-4 vs. 230.35 in TE-8) (Fig. 2B). Cell viability was examined in cells treated with either or both agents. A dose-dependent, overall decrease in cell viability was observed; combined treatment with cisplatin

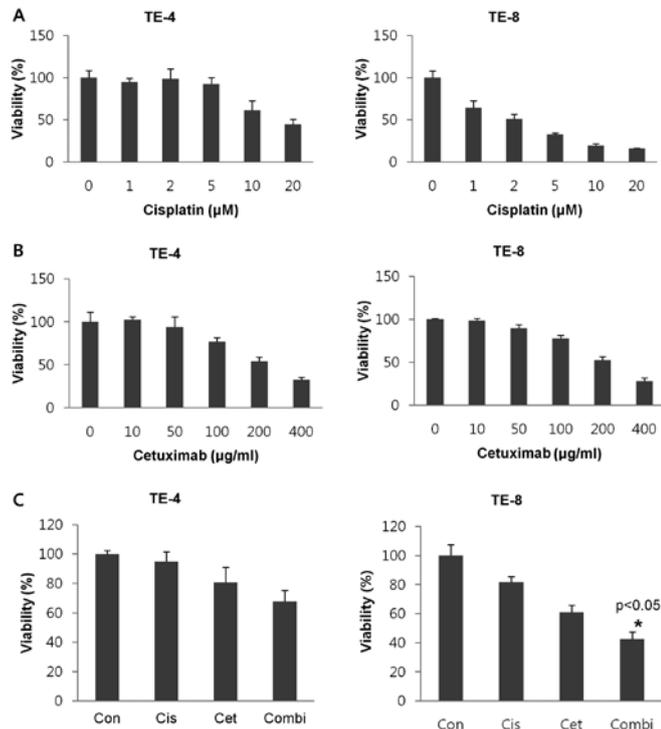


Figure 2. Effects of cisplatin, cetuximab and a combination of both on ESCC cell growth, as determined by a colorimetric cell viability assay. TE-4 and -8 cells were treated with the indicated concentrations of (A) cisplatin, (B) cetuximab, or (C) 1 μ M cisplatin and 400 μ g/ml cetuximab.

and cetuximab had an additive cytotoxic effect compared to single treatments ($P < 0.05$) in the EGFR-overexpressing TE-8 but not in TE-4 cells (Fig. 2C).

Activation of EGF signaling by cisplatin in EGFR-expressing cells. To determine the mechanism underlying cisplatin- and cetuximab-induced inhibition of ESCC cell growth, the expression and phosphorylation status of EGFR and AKT were examined in TE-4 and -8 cells by western blotting. Expression of EGFR, p-EGFR and p-AKT was upregulated by cisplatin treatment in a dose-dependent manner in TE-8 but not TE-4 cells (Fig. 3A), indicating an activation of EGF signaling. In contrast, treatment of TE-8 cells with cetuximab led to a dose-dependent decrease in EGFR, p-EGFR and p-AKT expression (Fig. 3B). The cisplatin-induced increases in EGFR, p-EGFR and p-AKT expression in TE-8 cells were abrogated in the presence of cetuximab (Fig. 2C). In TE-4 cells, cisplatin had no effect on EGFR, p-EGFR and p-AKT levels; treatment with cetuximab, or a combination of both agents, led to a decrease in p-AKT expression but had no effect on the expression or phosphorylation of EGFR.

Antitumor effects of cisplatin combined with cetuximab in an ESCC xenograft model. The effects of cisplatin and cetuximab on tumor growth were investigated in an ESCC mouse model. The volume of TE-8 cell-derived tumors was reduced by 13.5 and 39.1% upon treatment with cisplatin and cetuximab, respectively, compared to saline-treated controls (Fig. 4A), demonstrating that cetuximab has an anti-tumor effect *in vivo*. Combined treatment with cisplatin and

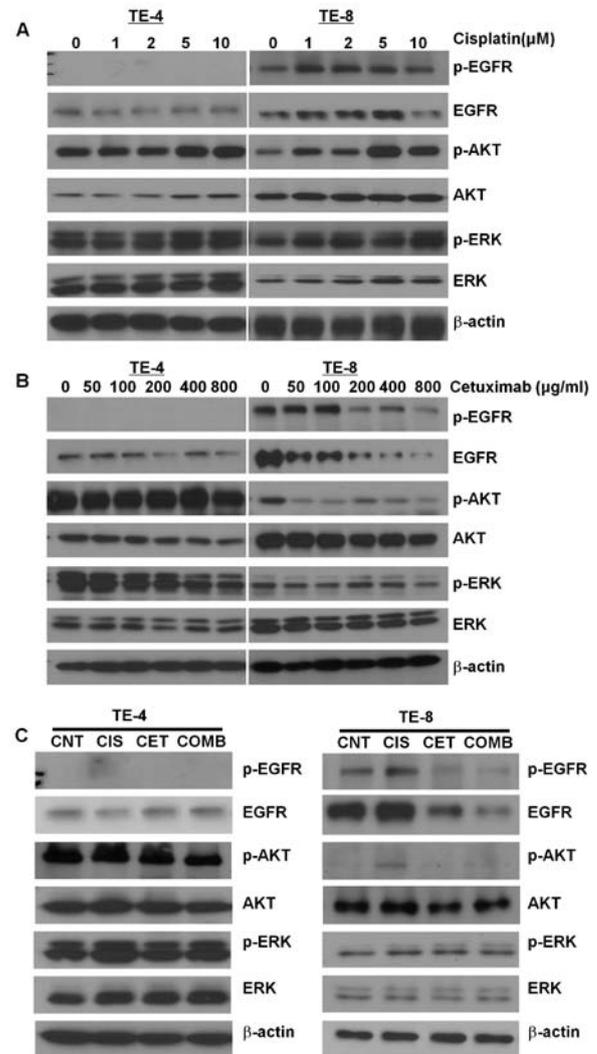


Figure 3. Modulation of cisplatin-induced EGFR activation by cetuximab in ESCC cells, as determined by western blotting. (A) EGFR and AKT were activated in a dose-dependent manner by cisplatin in TE-8 but not TE-4 cells, as assessed by increased expression of p-EGFR and p-AKT, respectively. (B) Activation of EGFR and AKT was suppressed in a dose-dependent manner by cetuximab in TE-8 cells. (C) Cisplatin-induced expression and activation of EGFR and AKT was inhibited by cetuximab in TE-8 but not TE-4 cells.

cetuximab decreased tumor volume by 63.2%, suggesting that the treatment of cetuximab and cisplatin as a combination therapy may be promising in a TE-8 cell-derived ESCC tumor model.

EGFR expression and phosphorylation were examined in tumor tissue samples by western blotting (Fig. 4B). Consistent with *in vitro* observations, EGFR and p-EGFR levels were higher in tumors from the cisplatin treatment group, while levels were downregulated in the combination treatment group, effects that were confirmed by immunohistochemistry (Fig. 4C). EGFR expression was reduced in necrotic areas of cetuximab- or combination-treated tumor tissue samples. Moreover, the Ki67-expressing proliferative fraction was decreased after treatment with cisplatin (by 29% vs. control; $P < 0.001$), cetuximab (by 38% vs. control; $P < 0.001$), or both (by 66% vs. control; $P < 0.001$). No differences in the fraction of apoptotic cells were observed between the various treatment groups.

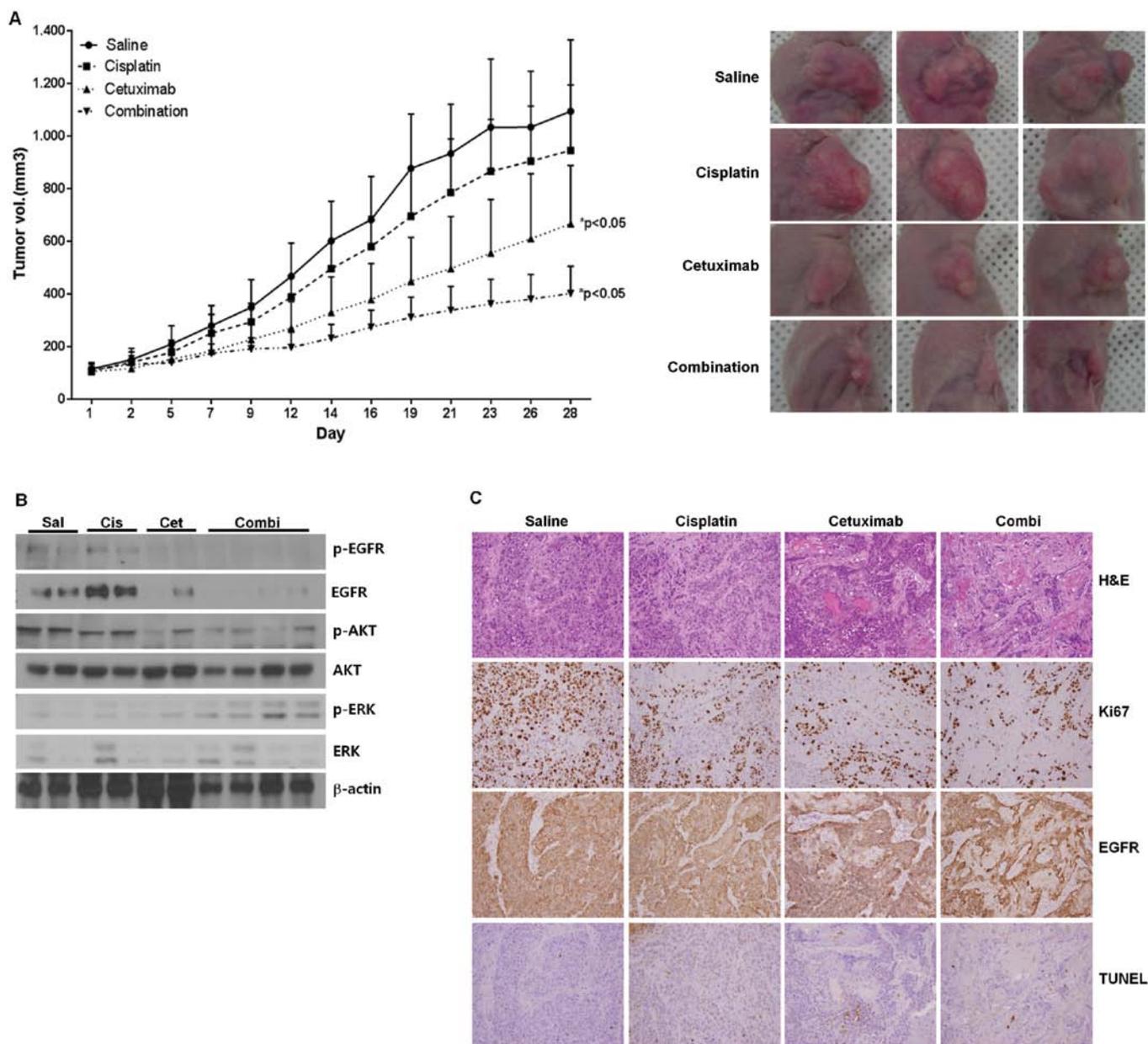


Figure 4. Effect of cisplatin, cetuximab and a combination treatment on tumor growth *in vivo*. Immunocompromised mice with TE-8 cell-derived xenografts (100 mm³) were treated with cisplatin (40 μ g/head), cetuximab (0.5 mg/head), or both, while control animals were injected with saline. (A) Average tumor volume was assessed every other day for 4 weeks in the four treatment groups. (B) EGFR expression and activation were assessed in tumor tissue samples by western blotting. Tumors in the combined cisplatin/cetuximab treatment group expressed decreased levels of EGFR and p-EGFR. (C) A decrease in EGFR expression in tumors derived from cetuximab and combination treatment groups was observed by immunohistochemistry. Low levels of tumor cell proliferation were detected by Ki67 immunostaining in cisplatin, cetuximab and combination treatment groups.

Discussion

Esophageal cancer is one of the leading causes of cancer mortality worldwide and is the fifth most common cause of cancer-related mortality in men (1). Approximately half of patients diagnosed with esophageal cancer present with overt metastatic disease and chemotherapy is the treatment of choice for advanced stages. ESCC patients often receive a combination of drugs such as cisplatin, 5-FU, etoposide and paclitaxel (13). Despite the widespread use of combination chemotherapy, there is no solid evidence for significant improvements in overall survival rate using this strategy. Previous studies have examined the effects of treatment using

a combination of an anti-EGFR antibody (e.g., cetuximab) and a conventional drug (e.g., cisplatin) on cell lines derived from colon cancer, non-small cell lung cancer and cervical cancer (14,15). However, little is known about the effects of this combination of agents in ESCC cells.

Results from recent clinical trials using a combination of cetuximab, cisplatin, irinotecan and radiotherapy to treat ESCC patients revealed significant adverse side-effects, such as diarrhea and dehydration, which were indicators of increased toxicity, without parallel increases in treatment efficacy (16,17). Similarly, SCOPE1 trials in patients in the UK did not show any benefits to adding cetuximab to a standard chemo-/radiotherapy treatment regimen. However, in a multi-

center phase II trial in Chinese patients with non-resectable, locally advanced ESCC, it was found that cetuximab can be safely used concurrently with chemo- and radiotherapy and may actually increase the clinical response rate (18,19). The reasons for these conflicting results of cetuximab combination therapy remain unclear, but recent data point to the influence of mutations in, or amplification of, specific genes; for instance, combination therapy had different survival outcomes for patients depending on the presence of EGFR or MET gene amplifications, or mutations in EGFR, KRAS, or PI3CA (20,21). The results of the present study, showing that the combined treatment effects are predominantly observed in an ESCC cell line overexpressing EGFR, support the theory that the choice and efficacy of treatment methods depend on the specific genotype of each patient.

The results of this study also underscore the complexity of the effects produced by combined treatment with cetuximab and cisplatin. For instance, the cytotoxic effects of the two agents used in combination were additive in TE-8 cells (Fig. 2), suggesting that it is promising as a treatment strategy. However, cisplatin-induced increases in EGF signaling were reversed by cetuximab treatment (Fig. 3). Given the role of phosphorylated EGFR in stimulating proliferation of some cancer cell types, this antagonistic interaction could be advantageous in ESCC treatment. Further studies on the downstream effects of EGF signaling in ESCC cells and a closer examination of the genotypic differences between different ESCC cell lines that could explain the differential responses to combined drug treatment, are required for the development of personalized and effective treatment regimens that are tailored to individual patients.

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