

Synergistic inhibitory effects of cetuximab and curcumin on human cisplatin-resistant oral cancer CAR cells through intrinsic apoptotic process

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Abstract. Cetuximab, an epidermal growth factor receptor (EGFR)-targeting monoclonal antibody (mAb), is a novel targeted therapy for the treatment of patients with oral cancer. Cetuximab can be used in combination with chemotherapeutic agents to prolong the overall survival rates of patients with oral cancer. Curcumin is a traditional Chinese medicine, and it has been demonstrated to have growth-inhibiting effects on oral cancer cells. However, information regarding the combination of cetuximab and curcumin in drug-resistant oral cancer cells is lacking, and its underlying mechanism remains unclear. The purpose of the present study was to explore the oral anticancer effects of cetuximab combined with curcumin on cisplatin-resistant oral cancer CAR cell apoptosis *in vitro*. The results demonstrated that combination treatment synergistically potentiated the effect of cetuximab and curcumin on the suppression of cell viability and induction of apoptosis in CAR cells. Cetuximab and curcumin combination induced apoptosis and dramatically increased caspase-3 and caspase-9 activities compared with singular treatment. Combination treatment also markedly suppressed the protein expression levels of EGFR and mitogen-activated protein kinases (MAPKs) signaling (phosphorylation of ERK, JNK and p38).

The results demonstrated that co-treatment with cetuximab and curcumin exerts synergistic oral anticancer effects on CAR cells through the suppression of the EGFR signaling by regulation of the MAPK pathway.

Introduction

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase in the cell transmembrane that serves an essential role in proliferation, metastasis, angiogenesis and chemo-resistance of oral cancer cells (1,2). More than 80% patients with oral cancer and oral cancer cell lines exhibit overexpression of EGFR (3-6). High expression of EGFR protein in oral cancer is associated with poor prognosis, decreased survival time and increased metastatic potential (7,8). Inhibition of EGFR and regulation of downstream signaling represents a novel approach for oral cancer therapy (9,10). Various strategies have been developed to disrupt EGFR function and to interfere with downstream signaling (11,12). Anti-EGFR mAbs and EGFR inhibitors have been investigated the most extensively (3,5,13).

Cetuximab is an EGFR-targeting mAb and the first novel targeted agent for oral cancer treatment to obtain Food and Drug Administration approval in the United States (11,13). In combination with chemotherapeutic agents, cetuximab has been demonstrated to increase the overall survival rate of patients with oral cancer, and to have less toxicity (7,10,14). In oral cancer therapy, combination of cetuximab with cisplatin, 5-fluorouracil (5-FU), docetaxel (Taxotere) or paclitaxel (Taxol) has become the new standard advanced treatment (8,9,15,16).

Curcumin is a traditional Chinese medicine isolated from the rhizome of *Curcuma longa* (17-20). Curcumin has been shown to exert anti-inflammatory, anti-oxidant, and anticancer effects, it is pharmacologically safe and has minimal toxicity (17,19,20). The anticancer activities of curcumin are attributable to its anti-proliferative, anti-angiogenic, anti-metastatic, pro-apoptotic and autophagic characteristics (21-25). *In vitro* studies reported that curcumin inhibited cell proliferation in various oral cancer

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cell lines, including CAL 27, 1483, SCC-1, SCC-9, KB, SAS and SCC15 (26). Curcumin also suppressed EGFR expression and its downstream signaling molecules (NF- κ B, JNK, p38 and ERK) which are vital for oral cancer pathogenesis (27-29). Furthermore, curcumin enhanced cisplatin cytotoxicity in PE/CA-PJ15 cells *in vitro* (30). The combination of 5-FU, doxorubicin or cisplatin with curcumin exhibited inhibited proliferation and induced apoptotic cell death of NT8e oral squamous cell carcinoma cells (31). However, the molecular mechanism of the suppression of cell proliferation and apoptotic induction of drug-resistant oral cancer cells following co-incubation with cetuximab and curcumin remains poorly understood. Herein, the synergistic effects and underlying molecular mechanism of the effect of combined treatment of cetuximab and curcumin in cisplatin-resistant oral cancer CAR cells was explored.

Materials and methods

Chemicals and reagents. Erbitux (the active ingredient of cetuximab) was provided by Hualien Tzu Chi Hospital (Taiwan) and originally purchased from Merck KGaA (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin solution were purchased from HyClone (GE Healthcare, Logan, UT, USA). Caspase-3 and Caspase-9 colorimetric assay kits were sourced from R&D Systems (Minneapolis, MN, USA). All primary antibodies and anti-mouse/-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibodies were purchased from GeneTex (Hsinchu, Taiwan). Curcumin, Thiazolyl Blue Tetrazolium Bromide (MTT) and other reagents were of analytical grade from Sigma-Aldrich (Merck KGaA, Darmstadt Germany), unless otherwise stated.

Cell culture. The human oral cancer cell line, CAL 27, was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cisplatin-resistant subline of CAL 27, CAR, was generated in our laboratory, as previously described (32-34) and exposed to increasing concentrations of cisplatin to generate a stable subline with resistance to $\geq 80 \mu\text{M}$ cisplatin. CAR cells were maintained in an environment of 5% CO₂ at 37°C in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 Units/ml penicillin and 80 μM cisplatin. Cetuximab was diluted with cultured medium (DMEM with supplementation as described above), and curcumin was dissolved in dimethyl sulfoxide (DMSO).

Cytotoxicity assay. Cell viability was estimated by MTT assay. In brief, CAR cells (1×10^4 cells/well) were plated in 96-well tissue culture plates and treated with curcumin (10, 20, 40 or 50 μM), cetuximab (10, 20, 40 or 50 $\mu\text{g}/\text{ml}$) or 20 $\mu\text{g}/\text{ml}$ cetuximab and 10, 20 or 40 μM curcumin for 24 h. Following exposure and removal of the medium, the cells were cultured with 0.5 mg/ml MTT for an additional 2 h. The blue formazan product was dissolved in 100 μl DMSO and spectrophotometrically measured at a wavelength of 570 nm using an ELISA plate reader (Anthos Labtec Instruments GmbH, Salzburg, Austria), as previously described (35). The percentage of living cells was calculated, and the ratio of optical density of the experimental wells and control wells was calculated as %

of control. Combination index (CI) was determined using the Chou-Talalay method, as previously described (36). A value < 1.0 indicated a synergistic effect.

Morphological determination. CAR cells (1×10^5 cells per well) were seeded into a 24-well plate and treated with 20 $\mu\text{g}/\text{ml}$ cetuximab and 10, 20 or 40 μM curcumin for 24 h. The cells were visualized using a phase-contrast microscope to check for apoptotic characteristics and photographed, as previously described (37).

Caspase-3 and -9 activity measurement. CAR cells were seeded at a density of 5×10^6 cells per 75T flask and incubated with 20 $\mu\text{g}/\text{ml}$ cetuximab, 40 μM curcumin, or 20 $\mu\text{g}/\text{ml}$ cetuximab and 40 μM curcumin for 24 h. The cell lysate was collected, and the cell fraction was analyzed for caspase-3/-9 activity using Caspase-3 and Caspase-9 Colorimetric Assay kits (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol.

Western blot analysis. CAR cells (5×10^6 cells per 75T flask) were treated with either 20 $\mu\text{g}/\text{ml}$ cetuximab, 40 μM curcumin or both for 24 h. Then, the cells were harvested and lysed with PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea). The protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 40 μg protein was loaded per lane of 8-10% SDS-PAGE gels. The protein was thereafter transferred into Immobilon-P Transfer Membranes (Merck Millipore, Billerica, MA, USA). Each membrane was blocked in 5% non-fat dry milk in phosphate-buffered saline with Tween-20 (PBST; 8 mM Na₂HPO₄, 0.15 M NaCl, 2 mM KH₂PO₄, 3 mM KCl, 0.05% Tween-20, pH 7.4) for 1 h. The membranes were then incubated at 4°C overnight with primary antibodies against p-EGFR (cat. no. GTX61353), EGFR (cat. no. GTX100448), p-ERK (cat. no. GTX59568), ERK (cat. no. GTX59618), p-JNK (cat. no. GTX52326), JNK (cat. no. GTX52360), p-p38 (cat. no. GTX48614), p38 (cat. no. GTX110720) (all 1:1,000 dilution), and β -actin (cat. no. GTX109639) (1:5,000 dilution) (GeneTex). Following washing with PBST, the membrane was incubated with appropriate anti-mouse (cat. No. GTX213111-01)/-rabbit (cat. no. GTX213110-01) HRP-conjugated secondary antibodies (1:10,000 dilution) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate; Merck Millipore) and using the LAS-4000 imaging system (Fuji, Tokyo, Japan), as previously described (38-40). The density of the immunoblots was analyzed using ImageJ (version 1.47; National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. The values are presented as the mean \pm standard deviation of 3 independent experiments. Comparisons between the drug-treated and -untreated groups were made using one-way analysis of variance followed by Dunnett's test using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). $P < 0.001$ was considered to indicate a statistically significant difference.

Results

Effects of curcumin, cetuximab and combination treatment on the viability of cisplatin-resistant oral cancer CAR cells. The cytotoxicity of curcumin and cetuximab on CAR cells. The cells were cultured with various concentrations of curcumin (10, 20, 40 or 50 μ M), or cetuximab (10, 20, 40 or 50 μ g/ml), or 20 μ g/ml cetuximab combined with 10, 20 or 40 μ M curcumin, for 24 h. Cell viability was evaluated by MTT assay. The results demonstrated that curcumin markedly decreased the viability of CAR cells in a concentration-dependent manner, and the viability at 20, 40 and 50 μ M was 87.1, 48.5 and 12.3%, respectively (Fig. 1A). It was also revealed that 20, 40 and 50 μ g/ml cetuximab reduced CAR-cell viability to 85.8, 72.8 and 67.4%, respectively, another concentration-dependent effect (Fig. 1B). However, 10 μ g/ml cetuximab demonstrated no significant inhibition. Thus, CAR cells were more sensitive to curcumin than that to cetuximab. The cells were treated with a combination of 20 μ g/ml cetuximab and 0, 10, 20 and 40 μ M curcumin for 24 h, and significant potentiation of cytotoxicity and synergy of CAR cells was demonstrated by viabilities of 83.7, 75.7, 70.3 and 38.6%, respectively (Fig. 1C). The combination index (CI) was 0.9, 0.8 and 0.6 at treatments of 20 μ g/ml cetuximab and 10, 20 and 40 μ M of curcumin, respectively, indicating the synergistic effects of cetuximab and curcumin. The results imply that a significant increase in cytotoxic effects was achieved with simultaneous administration of cetuximab and curcumin to CAR cells.

Effects of curcumin and cetuximab, alone or combined, on the morphology of CAR cells. Photomicrographs demonstrated that combined treatment resulted in cell shrinkage, cytoplasmic membrane blebbing and cell death, compared with exposure to 20 μ g/ml cetuximab alone and untreated control (Fig. 2). Furthermore, combined treatment resulted in increased inhibition in viability of CAR cells compared with single-drug (cetuximab) treatment and untreated control (Fig. 2). These data indicated that concurrent exposure to cetuximab and curcumin synergistically induced apoptosis and reduced proliferation of CAR cells.

Effects of curcumin and cetuximab, alone or in combination, on caspase-3/-9-dependent apoptosis of CAR cells. To further examine whether the observed suppression of cell viability involved in apoptotic machinery, the cells were treated with 20 μ g/ml cetuximab, or 40 μ M curcumin, or both, for 24 h prior to determination of caspase-3 and caspase-9 activities. Individual treatment with cetuximab and curcumin induced 1.6- and 4.9-fold increases in caspase-3 activity compared with control, whereas combination treatment stimulated a 6.8-fold increase in the activity of caspase-3 of CAR cells (Fig. 3A). Similarly, caspase-9 activity was synergistically increased in CAR cells when treated with cetuximab and curcumin (6.0-fold increase; Fig. 3B). However, either curcumin or cetuximab alone stimulated more minor effects on caspase-9 activity, causing 1.5- and 3.6-fold increases in CAR cells. These results indicate the synergistic cytotoxicity of curcumin and cetuximab, and that the apoptotic mechanism was caspase-3/-9-dependent in CAR cells.

Effect of curcumin and/or cetuximab treatment on EGFR and MAPKs-regulated molecular signaling in CAR cells. Cetuximab was reported to inhibit tumor growth, invasion, angiogenesis and metastasis by binding to the extracellular domain of EGFR by regulating the MAPKs pathway (12,13). Furthermore, it has been documented that colorectal cancer-cell resistance to cisplatin chemotherapy may involve MAPK signaling (41). The present study further analyzed the expression levels of EGFR and the downstream MAPK pathway in CAR cells prior to cetuximab or curcumin treatment, alone or in combination, using western blot analysis. It was demonstrated that treatment combination treatment effectively inhibited the phosphorylation of EGFR, but no effect on total EGFR protein level was observed in CAR cells (Fig. 4). The levels of phosphorylated MAPKs (ERK, JNK and p38) were also synergistically decreased compared with cetuximab or curcumin treatment alone. However, there was no effect on total ERK, JNK or p38 protein expression levels in any treatment group (Fig. 4). Taken together, these results suggest that combined use of cetuximab and curcumin triggered a dramatic increase in CAR-cell apoptosis by suppressing EGFR and MAPKs signaling (Fig. 5), suggesting that the synergic effects resulted in enhanced oral anticancer activity compared with single-drug treatment in resistant oral cancer cells.

Discussion

Surgery and brachytherapy are the major therapies for oral cancer in the T1, T2 and artificial T3 groups (Tumor-Node-Metastasis classification) in clinical practice guidelines for head and neck cancer in Japan (42). Platinum-based chemotherapy (cisplatin or carboplatin) is used for advanced stage cancer (42,43). In 1978, cisplatin was approved by the Food and Drug Administration (FDA) for oral cancer treatment (44). Cisplatin is used for oral cancer chemotherapy and functions via direct reaction with cellular nucleophiles to achieve inter- and intra-stand DNA cross-links and protein cross-links with DNA and RNA (45). However, oral cancer cells have gained resistance to chemotherapeutic agents (46,47). Several molecular mechanisms are involved in cisplatin-resistance: i) Increased activity of transporter protein function (MDR1 or p-glycoprotein); ii) activated drug metabolism activity by enzymes; iii) decreased drug binding to DNA; iv) promoted ROS production; v) stimulated DNA repair; vi) increased tolerance to DNA damage; vii) altered transcription of target genes; viii) changes in cell cycle-associated events and ix) inhibition of cell death (46-48). Recently, EGFR has been demonstrated as an important therapeutic target in oral cancer, and it is expressed more highly in oral cancer tissue than in normal tissues (7,10). In addition, a correlation between high EGFR expression and radio-resistance was demonstrated in patients with oral cancer (49). Kuroda *et al* (50) demonstrated that cisplatin-resistance is associated with EGFR-mediated signaling in lung cancer A549 cells. Chemo-sensitivity to cisplatin was restored by an EGFR-selective tyrosine kinase inhibitor (AG1478) in A549 cells, suggesting that the EGFR inhibitor may be a therapy for cisplatin-resistance (50). To the best of our knowledge, this is the first study to report

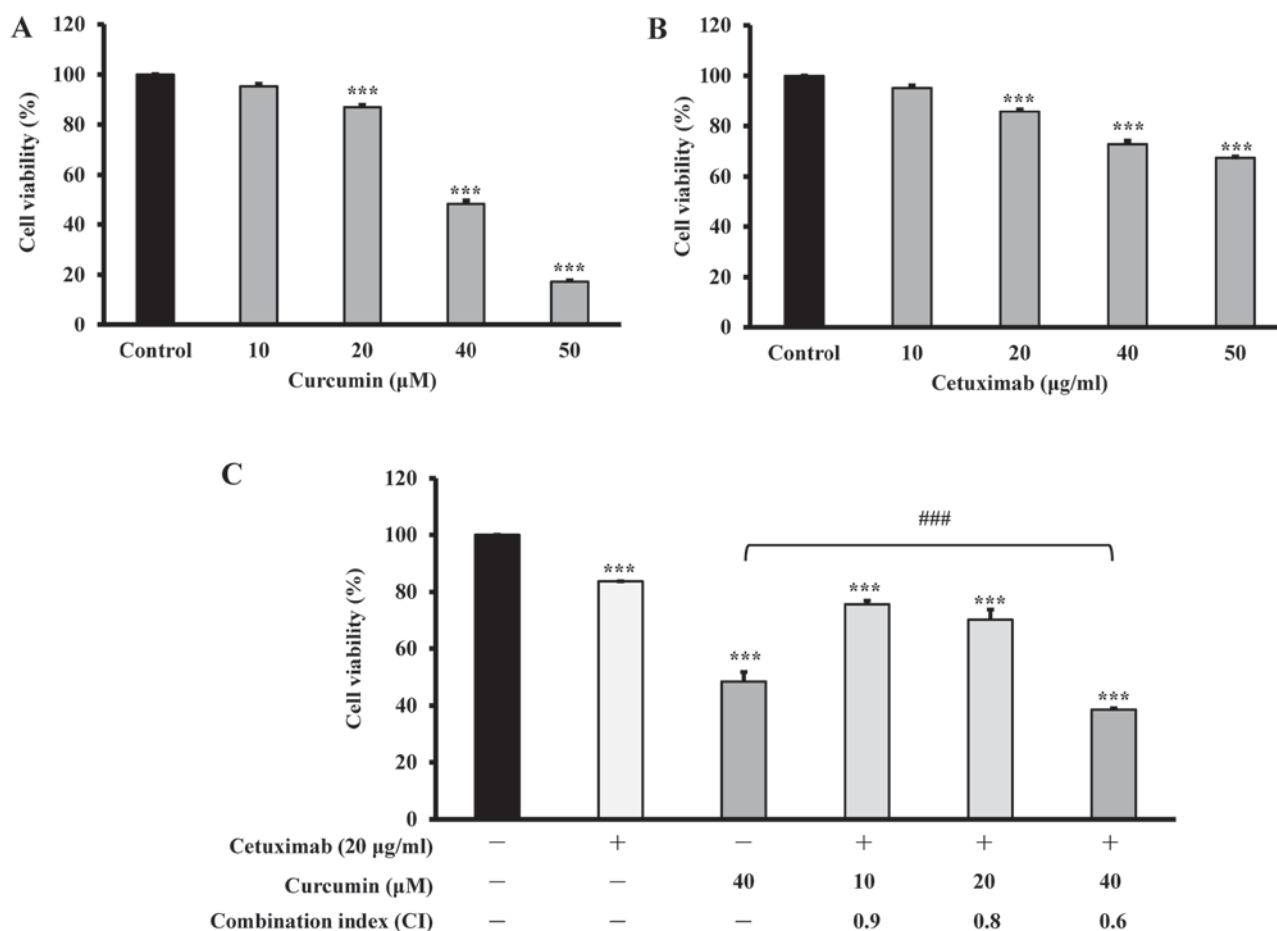


Figure 1. Cytotoxic effects of curcumin and cetuximab on CAR cells. The concentration-response curves of (A) curcumin, (B) cetuximab and (C) combined treatment. Cell viability was measured by MTT assay. Each data point is presented as the mean \pm standard deviation of 3 independent experiments. *** $P < 0.001$ vs. untreated control. ### $P < 0.001$ vs. curcumin (40 μM) treatment only group.

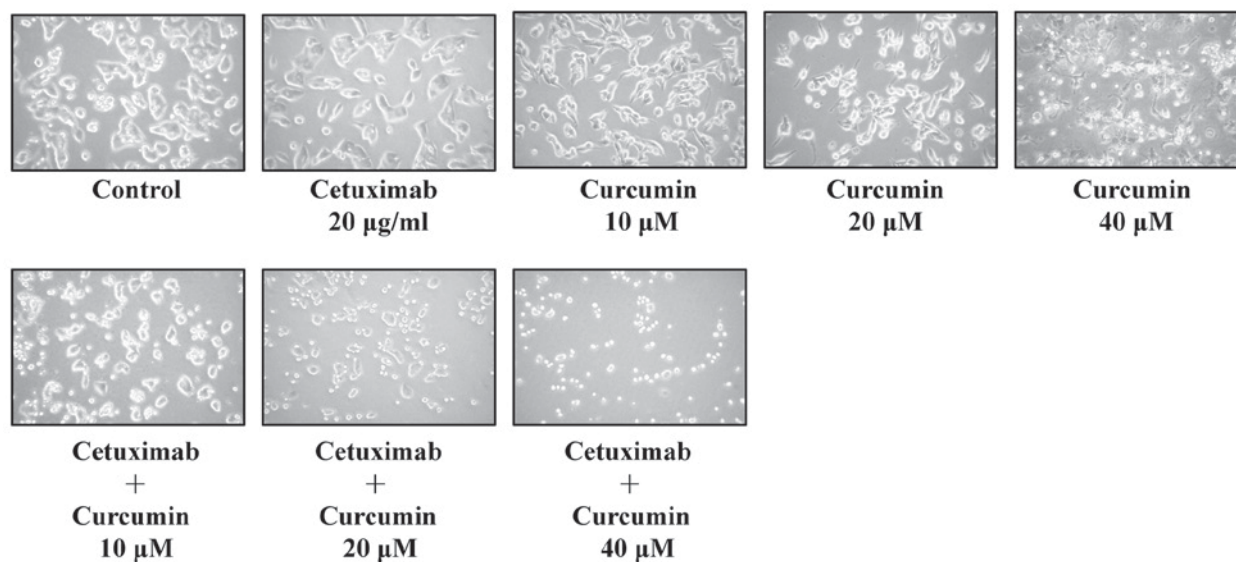


Figure 2. Effects of curcumin and cetuximab on CAR cell morphology. The phase contrast microscopy images represent the results from 1 of 3 independent experiments at x200 magnification.

the synergistic inhibitory effect of cetuximab (an EGFR inhibitor) and curcumin in cisplatin-resistant human oral cancer cells.

Evidence indicates that the molecular mechanisms of cetuximab anticancer activity take 2 forms (51,52). Firstly, EGF binding to the EGFR extracellular domain to inhibit

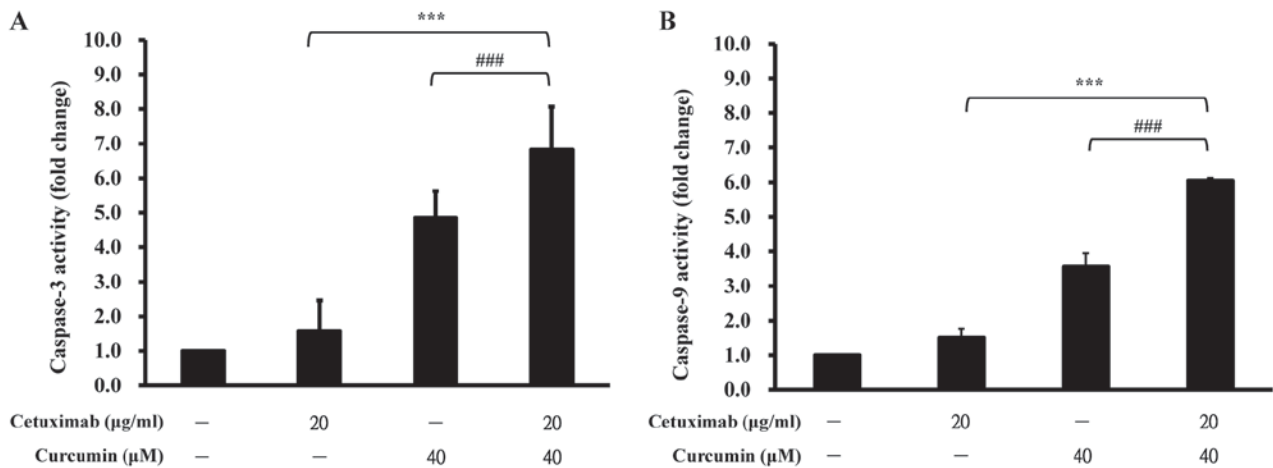


Figure 3. Effects of curcumin and cetuximab on caspase-3 and caspase-9 activities in CAR cells. (A) Caspase-3 and (B) caspase-9 activities were detected by colorimetric assay. Results are represented as the mean \pm standard deviation of 3 independent experiments. *** $P < 0.001$ vs. untreated control. ### $P < 0.001$ vs. curcumin (40 μ M) treatment only group.

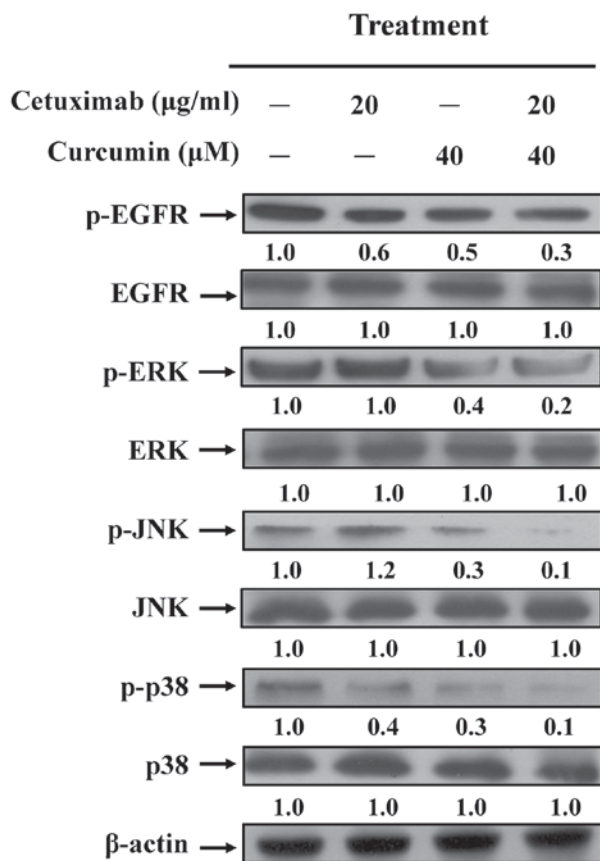


Figure 4. Effects of curcumin and cetuximab on EGFR and MAPK-regulated signaling in CAR cells. Protein expression of p-EGFR, EGFR, p-ERK, ERK, p-JNK, JNK, p-p38 and p38 were determined, and a β -actin control was applied to ensure equal loading. Representative images were taken from 3 independent experiments. EGFR, epidermal growth factor receptor; MAPK, mitogen activated protein kinase; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

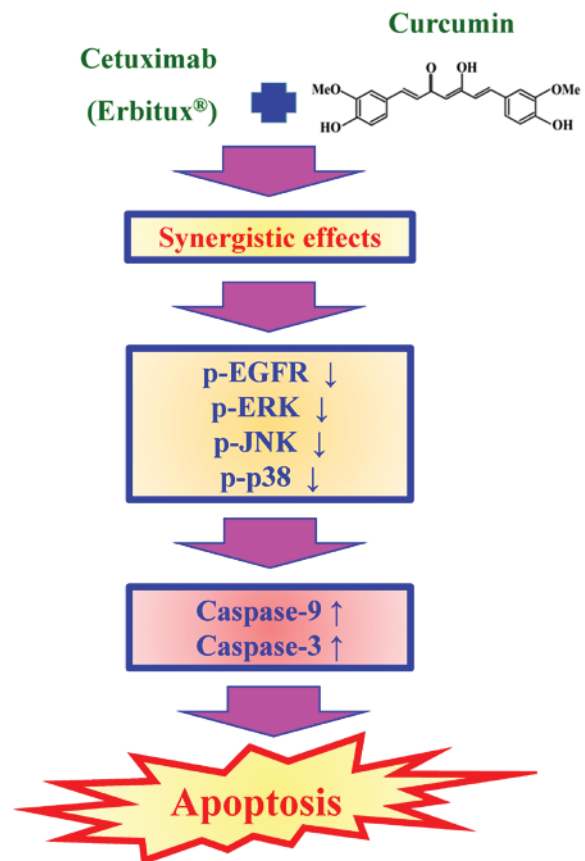


Figure 5. The underlying molecular mechanisms of combinational curcumin and cetuximab treatment. EGFR/MAPK signaling provokes a pro-apoptotic mechanism in human cisplatin-resistant oral cancer CAR cells. EGFR, epidermal growth factor receptor; MAPK, mitogen activated protein kinase; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

subsequent receptor dimerization/activation and to induce EGFR degradation is inhibited. Secondly, antibody-dependent cellular cytotoxicity or complement-dependent cell-mediated cytotoxicity (51,52). Curcumin has been

historically used in traditional Chinese medicine, and its anticancer effects on various types of solid cancers, such as colon cancer, multiple myeloma and pancreatic cancer have reached phase II and III clinical trials (53,54). Curcumin is also a potential therapeutic agent for the treatment of

diabetic patients (55,56). It has been reported that curcumin is able to directly but partially inhibit the enzymatic activity of the EGFR intracellular domain. Inhibition of EGFR phosphorylation and induction of EGFR ubiquitination then block the EGFR signaling pathway in cancer cells (57). However, it has not been reported whether combined cetuximab with curcumin synergistically inhibit drug-resistant oral cancer-cell proliferation. The present results demonstrated that a combined treatment of cetuximab and curcumin synergistically inhibited cell viability (Figs. 1B and 2), induced cell death (Fig. 2), and stimulated caspase-3 and caspase-9 activities (Fig. 3). Furthermore, curcumin dramatically enhanced cetuximab-suppressed phosphorylation of EGFR, ERK, JNK, and p38 levels in CAR cells (Fig. 4). A previous study by Son *et al* (58) demonstrated the effect of cetuximab combined with cisplatin on colon cancer growth. Cetuximab significantly decreased phosphorylation of EGFR and phosphorylated p38/p38 ratio at 30 $\mu\text{g/ml}$, but there was no effect on the phosphorylated ERK/ERK ratio in colon cancer HCT116 cells. The present study also demonstrated that cetuximab treatment decreased the protein level of phosphorylated EGFR and phosphorylated p38. In addition, Li *et al* (59) demonstrated that the EGFR monoclonal antibody, cetuximab, mildly evoked apoptosis of human vulvar squamous carcinoma A431 cells. This is consistent with the present finding that cetuximab triggered a non-significant increase in caspase-3 and caspase-9 activities in CAR cells. However, the activities of caspase-3 and caspase-9 were dramatically enhanced in CAR cells prior to treatment with cetuximab in combination with curcumin; or with exposure only to curcumin. The present results are also consistent with previous studies (26,31) showing that curcumin is effective against various types of cancer via intrinsic apoptotic function. Although curcumin possesses powerful biological activities, it does not reach the criteria of a good drug candidate because it lacks adequate water solubility and high bioavailability, and undergoes rapid *in vivo* metabolism (60). To overcome these limitations, novel forms of curcumin targeting, including nanoparticles, liposomes, cyclodextrin encapsulation, micelles and phospholipid complexes, have been synthesized and tested in recent years (61,62).

In conclusion, combined cetuximab and curcumin treatment is a novel therapeutic option for oral cancer treatment, exhibiting synergistic anti-proliferative activity. The mechanism results in a decreased activated EGFR level in cisplatin-resistant oral cancer cells. With the results presented in the present study, curcumin could be used as an adjuvant drug, and the combination of cetuximab and curcumin may be a strategy to pursue in clinical trials.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CFC, HJH and TDW conceived and designed the experiments; CFC, CCL, JHC, HYC, JSY, and CYL performed the experiments. CFC, CCL, JHC, and JSY analysed the data; CFC, HJH and TDW wrote and modified the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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