Paclitaxel in combination with cetuximab exerts antitumor effect by suppressing NF-κB activity in human oral squamous cell carcinoma cell lines

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Abstract. In the present study, we examined the antitumor effect of paclitaxel (PTX) in combination with cetuximab in oral squamous cell carcinoma (OSCC) and the mechanism of its enhanced antitumor activity. Treatment of OSCC (HSC2, HSC3 and HSC4) cells with PTX (0.02 μg/ml) and cetuximab (1 μg/ml) combination resulted in a significant inhibition of cell growth *in vitro* compared to either agent alone. Moreover, it was found by Hoechst 33258 staining that DNA fragmentation markedly occurred in OSCC cells treated with PTX and cetuximab combination treatment. Furthermore, PTX and cetuximab combination treatment reduced the expression of p65 (NF-κB) protein in OSCC cells. In our *in vivo* experiment, HSC2 tumor-bearing nude mice were treated with PTX (20 mg/kg/day, twice/week, 3 weeks) and/or cetuximab (20 mg/kg/day, twice/week, 3 weeks). Tumor growth was significantly suppressed by PTX and cetuximab combined treatment when compared to PTX or cetuximab alone, or the untreated control. TUNEL-positive cells were upregulated in HSC2 tumors treated with PTX and cetuximab. In addition, immunohistochemical staining revealed that expression of p65 was downregulated in HSC2 tumors treated with PTX and cetuximab. Our results indicate that cetuximab may enhance the effect of PTX in OSCC through the downregulation of PTX induced p65 expression. Therefore, the combination of PTX and cetuximab might be a promising option for OSCC treatment.

Introduction

Oral squamous cell carcinoma (OSCC) is the 8th most common cancer in humans, which accounts for ~2% of all carcinomas in women and 4% in men worldwide (1,2). Over 300,000 new cases of OSCC are diagnosed annually with ~11,000 new cases in Japan (3). Surgery, chemotherapy and radiotherapy are the standards for the treatment of head and neck squamous cell carcinomas (HNSCCs) including OSCC. However, despite recent advances in cancer diagnosis, surgery, chemotherapy, radiotherapy and other treatment methods, the overall survival rate of OSCC is ~50% in the advanced stage of the disease (4,5). Almost 60% of HNSCC patients are diagnosed with locally advanced disease at presentation (6). Therefore, it is important to establish more promising therapeutic strategies.

Cetuximab (Erbitux®; formerly IMC-C225) is a chimeric (mouse/human) IgG1 monoclonal antibody that targets the extracellular ligand-binding domain of EGFR with high affinity and inhibits tumor growth, invasion, angiogenesis and metastasis (7,8). Cetuximab causes G1 phase cell cycle arrest by decreasing cyclin-dependent kinase 2 (CDK2) and increasing $p27^{Kip1}$ levels in tumor cell lines (9). Paclitaxel (PTX) is a diterpenoid isolated from the bark of the Pacific yew, *Taxus brevifolia* (10). PTX induces mitotic arrest and cell death by binding to microtubules, promoting microtubule assembly, and stabilizing tubulin polymers against depolymerization affecting cells in the G2/M-phase (11,12).

Most of the available reports on HNSCC demonstrated the effect of cetuximab in combination with cisplatin/platinum-based drugs or radiotherapy (7,13,14). However, cetuximab with paclitaxel combination therapy has been proven to be effective in HNSCC including several other cancers (15-19). For last few years the number HNSCC patients treated with PTX and cetuximab combined therapy has been increased gradually. This combination therapy could be a promising regimen for patients with advanced head and neck cancer after failure of platinum-based therapy (15).

The transcription factor NF-κB plays an important role in regulating various genes involved in inflammatory and immune responses as well as in cell survival (20-24). A major form of NF-κB is composed of a dimer of p50 and p65 subunits, and this complex is retained in the cytoplasm by inhibitory molecules (IκBs) (25). When the IκB-α is phosphorylated through the stimulation of radiation or anticancer agents, it becomes degraded through the ubiquitin-proteasome pathway which leads to the nuclear translocation of NF-κB to stimulate the expression of its target genes (21). The high constitutive levels of NF-κB activity is seen in Hodgkin's disease tumor cells, in breast cancer cells, and in head and

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neck cancer cells, and may contribute to the abnormal survival of these cells (22,23,26). Briefly, cancer cells can manage to defend themselves from radiation or anticancer agents by the activation of NF-κB (21-23). Therefore, suppression of NF-κB activity in cancer cells may be crucial to induce marked cell death by chemotherapeutic agents. However, it is reported that some anticancer drugs, including vinblastine, vincristine, daunomycin, doxorubicin, etoposide, and PTX can upregulate the expression of NF-κB (27-30). Hence the efficacy of PTX-cetuximab combination therapy might be dependent upon the ability of cetuximab to control the PTX influenced upregulation of NF-κB.

In this study, we investigated the antitumor efficacy of PTX in combination with cetuximab against OSCC both *in vitro* and *in vivo*. We also examined whether cetuximab can regulate the expression of p65 NF-κB induced by PTX in OSCC cells.

Materials and methods

Cell lines and nude mice. Human lung fibroblast cell line (Wi-38) and Human tongue squamous cell carcinoma (HSC2, HSC3 and HSC4) cell lines were purchased from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan) respectively. These cell lines were cultured in 100-mm culture dish (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Waltham, MA, USA), 5% of antibiotic-antimycotic solution (Thermo Fisher Scientific). In all experiments, cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Four-week-old female Balb/c nu/nu athymic nude mice (average weight 15.0 g) were purchased from CLEA Japan, Co., Ltd. (Tokyo, Japan). The mice were provided with sterile water and food *ad libitum*, and maintained under pathogen-free conditions in accordance with the Guidelines for Animal Experimentation of Yamaguchi University.

Agents. Cetuximab was purchased from Merck Serono (Dermstadt, Germany) and PTX was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Western blot analysis. Untreated Wi-38, HSC2, HSC3 and HSC4 cells were used for the detection of EGFR expression. PTX and/or cetuximab treated (0, 3, 6, 12, 24 and 48 h) HSC2, HSC3 and HSC4 cells were used for the analysis of p65 NF-κB expression. Whole cell lysates from control and treated cells were prepared with RIPA buffer (Thermo Fisher Scientific) and the amount of protein in the cell lysates were quantified with NanoDrop 1000 (Thermo Fisher Scientific). Cell lysates containing 50 μg protein/sample were subjected to electrophoresis on 10% SDS-polyacrylamide gels, and then transferred to a PVDF membrane. The membranes were blocked and treated with the anti-EGFR rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-p65 NF-κB rabbit polyclonal antibody (Santa Cruz). All antibodies were detected using Western Breeze chromogenic immunodetection system (Thermo Fisher Scientific) according to the manufacturer's instructions. Anti-actin monoclonal antibody (Santa Cruz) was used for normalization of western blot analysis.

In vitro cell growth inhibition assay. Wi-38, HSC2, HSC3 and HSC4 cells ($5x10^3$ cells/well) were seeded on 96-well plates (Becton-Dickinson Labware) in DMEM supplemented with 10% FBS. Twenty-four hours later, the cells were treated with different concentrations of cetuximab (0, 0.1, 1 and 10 μg/ml) or PTX (0, 0.01, 0.02 and 0.05 μg/ml) alone or in combination to determine the suitable concentration of these drugs in cell growth inhibition. After 48 h, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 25 μl/well) was added to the 96-well plate and incubated for 4 h. The blue dye taken up by cells was dissolved in dimethyl sulfoxide (100 μl/well), and the absorbance was measured with a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm. Growth inhibitory effects were compared among the groups. All assays were run in triplicate.

Hoechst staining. Cells ($5x10^5$ cells/well) were cultured on cover glasses in 6-well plates (Becton-Dickinson Labware) in DMEM with 10% FBS alone or with PTX and/or cetuximab. Forty-eight hours later, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and stained with 2 μg/ml Hoechst 33258 (Dojindo Laboratories, Kumamoto, Japan), a fluorescent DNA binding dye, for 30 min at 37˚C. Fluorescence of Hoechst 33258 was observed under a fluorescent microscope (FLoid® Cell Imaging Station, Thermo Fisher Scientific). Apoptotic cells were identified by their typical morphological appearance, including chromatin condensation, nuclear fragmentation, formation of membrane blebs and apoptotic bodies. The mean percentages of apoptotic cells were estimated by counting 500 cells/area from at least three different areas/sample.

In vivo tumor growth inhibition assay. HSC2 cells ($1x10^6$) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of 5-week old nude mice using a 27-gauge needle. When the estimated tumor volume (0.5 x length x $width^2$) reached 100-150 mm^3, the tumor-bearing mice were allocated randomly into control and treatment groups (5 mice/group). HSC2 tumors were treated for 3 weeks with PTX (20 mg/kg, twice/week) and/or cetuximab (20 mg/kg, twice/week) dissolved in 0.5% hydroxypropyl methylcellulose (HPMC). Control group received HPMC (0.5%) only. Tumor size and body weight were monitored and measured every three days. Antitumor effects and body weight changes were compared among the groups. All mice were sacrificed at the end of 3 weeks/21 days.

Immunohistochemical staining. HSC2 tumors harvested at autopsy were embedded in paraffin blocks. Four-micrometer-thick sections were prepared from the blocks and mounted on slides. These sections were processed for immunostaining using the anti-p65 NF-κB rabbit polyclonal antibody (Santa Cruz), and appropriate peroxidase conjugated anti-rabbit IgG second antibody. Negative controls were done using PBS instead of the primary antibody. The blocking and immunostaining were performed using Dako Envision kit/HRP (Dako, Glostrup, Denmark). Slides were counterstained with hematoxylin. The

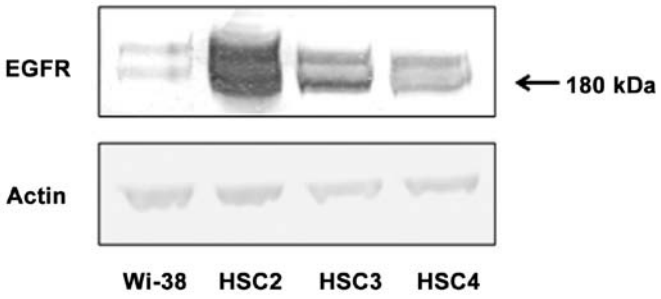

Figure 1. EGFR expression. Western blotting was performed to investigate expression levels of EGFR protein. HSC2 cells showed high EGFR expression and Wi-38 showed low EGFR expression.

slides were then examined under a bright-field microscope. A positive reaction was detected as reddish-brown precipitates.

TUNEL (terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling) assay. To detect apoptotic cells in mouse tumor tissues, TUNEL assay using the Apoptosis *In Situ* Detection kit (Wako) was performed, labeling 3'-OH DNA ends generated by DNA fragmentation. Four-micrometer-thick paraffin sections of tumor were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Tissue sections were incubated in 20 μg/ml proteinase K (Dako) for 15 min. After sections were rinsed in distilled water, endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide in PBS for 5 min. After washing with PBS, the sections were incubated with equilibration buffer and then TdT enzyme in a humidified chamber at 37˚C for 60 min. They were subsequently put into prewarmed stop wash buffer for 10 min. After rinsing in PBS, the sections were incubated with antidigoxigenin-peroxidase conjugate for 30 min. Peroxidase activity in each section was demonstrated by the application of diaminobenzidine (Peroxidase Substrate kit; Vector Laboratories, Burlingame, CA, USA). Hematoxylin was used as a counterstain. At least 1,000 cells were counted under a microscope in several random fields of each section. The number of apoptotic cells was divided by the total number of cells counted and the result was expressed as a percentage.

Statistical analyses. All data are expressed as mean ± SD. The significance of the experiment results was determined by the Mann-Whitney's U test or one-way ANOVA. The differences were considered statistically significant when $P<0.05$.

Results

Analysis of EGFR expression in cells. To evaluate the expression pattern of EGFR in Wi-38, HSC2, HSC3 and HSC4 cells, western blotting was performed. Wi-38 cells showed relatively weak expression of EGFR compared to the other three cell lines. HSC2 cells showed high EGFR expression compared to HSC3 and HSC4 cells (Fig. 1).

Effect of PTX and cetuximab on cell growth inhibition of oral squamous cell carcinoma cell lines in vitro. The growth inhibitory effect of PTX and cetuximab on Wi-38, HSC2, HSC3 and HSC4 cells was analyzed by the MTT assay. Cells were treated with different concentrations of PTX (0.01, 0.02 and 0.05 μg/ml) or cetuximab (0.1, 1 and 10 μg/ml) alone for 48 h. PTX inhibited the growth of Wi-38, HSC2, HSC3 and HSC4 cells in a dose-dependent manner (Fig. 2B). Similar result was observed after cetuximab treatment in all cells except Wi-38 cell (Fig. 2A). As combination treatment, we selected the concentrations of 0.02 μg/ml PTX and 1 μg/ml cetuximab as these concentrations significantly inhibited the growth of all cell lines (Fig. 2A and B). As shown in Fig. 2C, 48-h treatment with PTX and cetuximab combination significantly inhibited the growth of HSC2, HSC3 and HSC4 cells compared to PTX or cetuximab alone, or the untreated control.

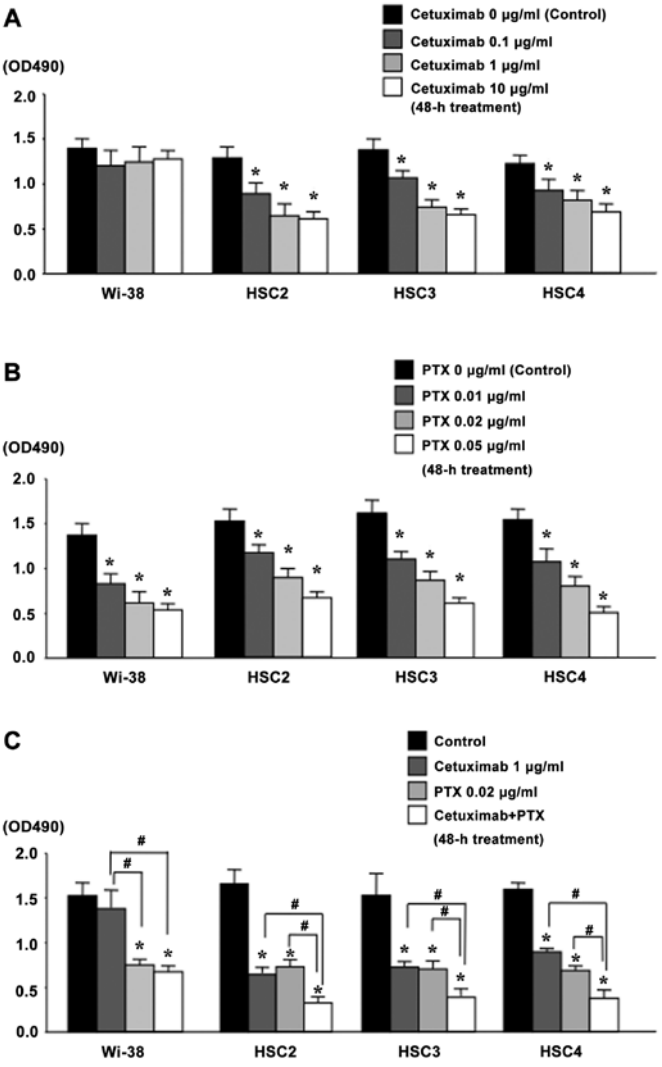

Figure 2. Cell growth inhibition assay *in vitro*. Inhibition of cell growth was evaluated by MTT assay. (A) Cetuximab inhibited the growth of HSC2, HSC3 and HSC4 cells in a dose-dependent manner. (B) PTX inhibited the growth of Wi-38, HSC2, HSC3 and HSC4 cells in a dose-dependent manner. (C) PTX and cetuximab combined treatment significantly inhibited the growth of HSC2, HSC3 and HSC4 cells compared to PTX or cetuximab alone or the untreated control. Error bars represent the standard deviation of the mean of six independent experiments. *$P<0.01$ when compared to that of control (Mann-Whitney's U test). #$P<0.01$ when compared to that of each agent alone (Mann-Whitney's U test).

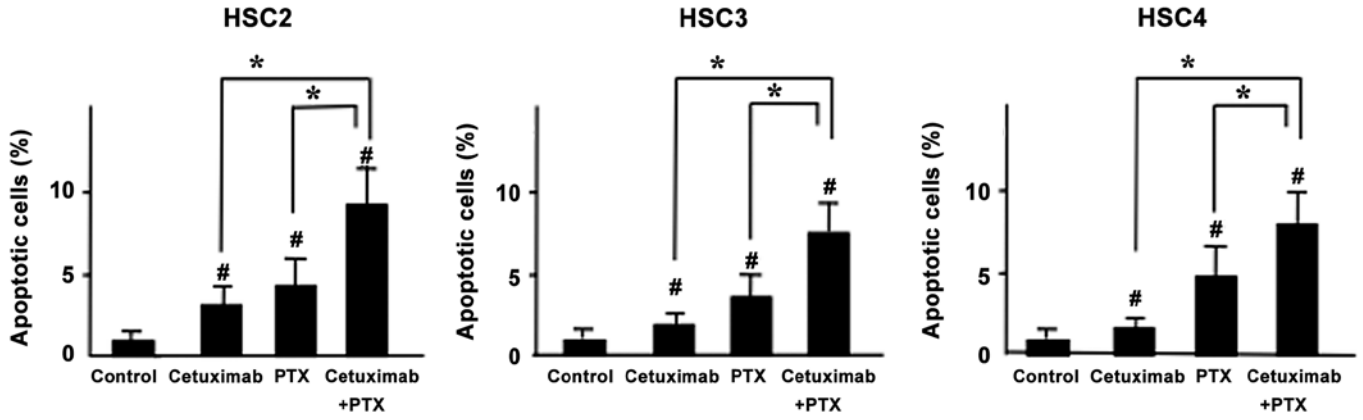

Figure 3. Hoechst staining. Hoechst staining was performed to confirm the apoptotic inducing activity of PTX and cetuximab combined treatment. The number of apoptotic cells was significantly increased after PTX and cetuximab combined treatment compared to treatment with either agent alone. Error bars represent the standard deviation of the mean of six independent experiments. $^{\#}$P<0.01 when compared to that of control (Mann-Whitney's U test). *P<0.01 (Mann-Whitney's U test).

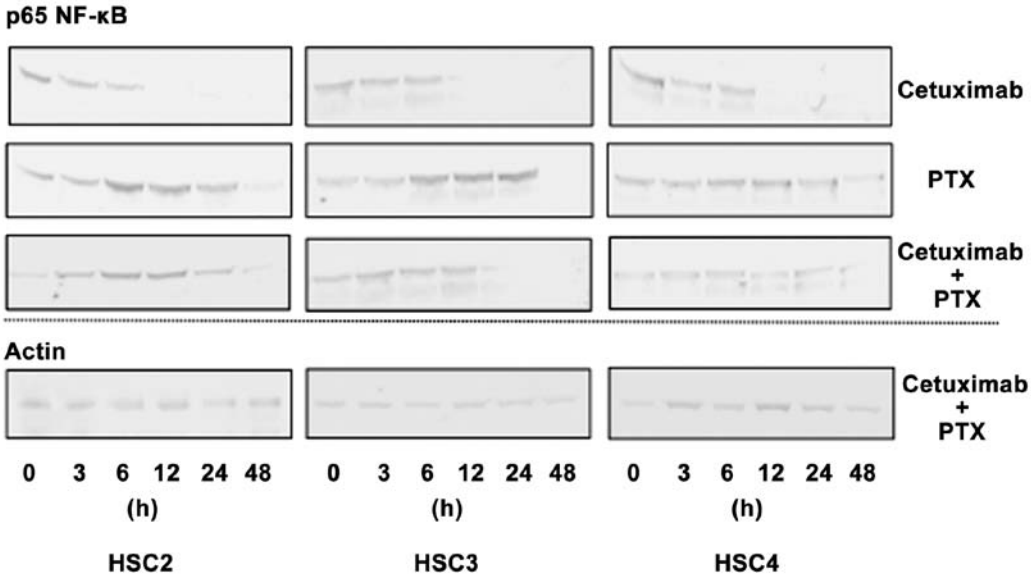

Figure 4. p65 NF-κB expression assay. Western blotting was performed to investigate the mechanisms behind the apoptosis inducing activities of cetuximab and PTX combination treatment. Cetuximab treatment markedly reduced the expression of p65, and PTX treatment induced the expression of p65. Combined treatment with PTX and cetuximab also reduced the expression of p65.

Effect of PTX and cetuximab on induction of apoptosis in vitro. To understand whether the enhanced cell growth inhibitory effect of PTX and cetuximab combined treatment was due to apoptosis, we performed Hoechst staining to detect DNA fragmentation and chromatin condensation in treated cells. The numbers of apoptotic cells were significantly increased after PTX and cetuximab combined treatment compared to treatment with either agent alone (Fig. 3).

Effect of PTX and cetuximab on the expression of p65 in vitro. To clarify the mechanisms behind the apoptosis inducing activities of cetuximab and PTX combination treatment, we examined the expression of p65 NF-κB in cells by western blotting. Cetuximab reduced the expression of p65 in a time-dependent manner, while the highest reduction of p65 expression was observed after 12 h of treatment. On the other hand, PTX treatment induced the expression of p65 after 6 h of treatment. Combined treatment with PTX and cetuximab induced p65 expression slightly, and reduced it after 24 h of treatment (Fig. 4).

Effect of PTX and cetuximab on tumor growth inhibition in vivo. Nude mice with HSC2 tumor xenografts were used to examine the antitumor activity of PTX and cetuximab single/combination treatment. Control group received 0.5% HPMC only, while treatment groups were treated with either PTX (20 mg/kg/day, twice/week) or cetuximab (20 mg/kg/day, twice/week) alone, or in combination for 3 weeks. Fig. 5 shows the result of the *in vivo* experiment. All the treatment groups significantly inhibited tumor growth compared to the untreated control. Antitumor effect of cetuximab alone was in the same range as PTX alone. However, the maximum reduc-

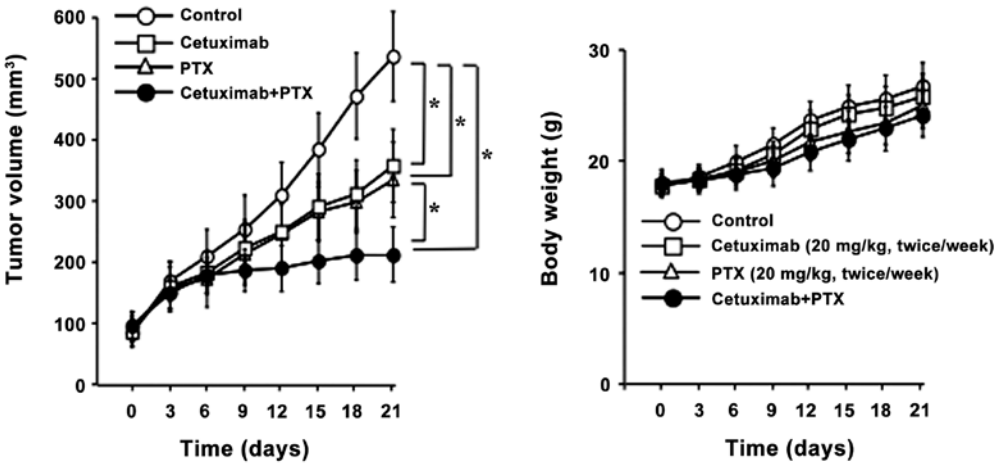

Figure 5. Tumor growth assay. Cells ($1x10^6$) were inoculated into the backs of nude mice. When the tumors reached 100-150 mm^3 in volume, they were treated with either PTX (20 mg/kg/day, twice/week) or cetuximab (20 mg/kg/day, twice/week) alone, or in combination for 3 weeks. Tumor volume and body weight was measured every two days. Error bars represent the standard error of the mean from five mice results (n=5). *P<0.01 (one-way ANOVA).

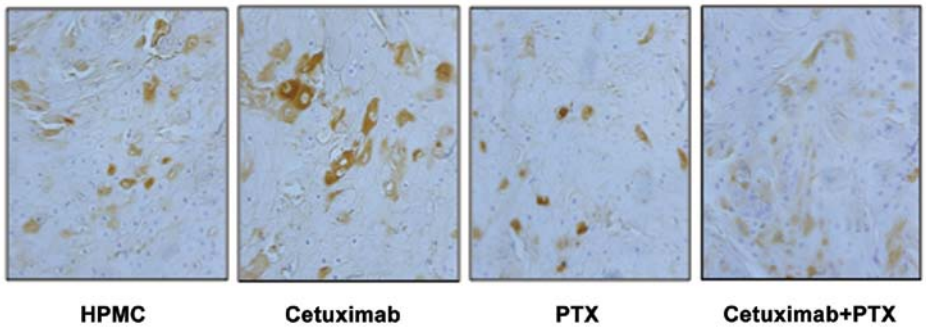

Figure 6. p65 NF-κB expression assay. Immunohistchemical staining was performed to investigate the localization of p65 NF-κB expression. p65 expression was detected in cytoplasm of cetuximab single treatment tumors and the expression of p65 was detected in nucleus of PTX single treatment tumors. The expression of p65 was also decreased and was mainly detected in the cytoplasm of PTX and cetuximab combined treatment tumors.

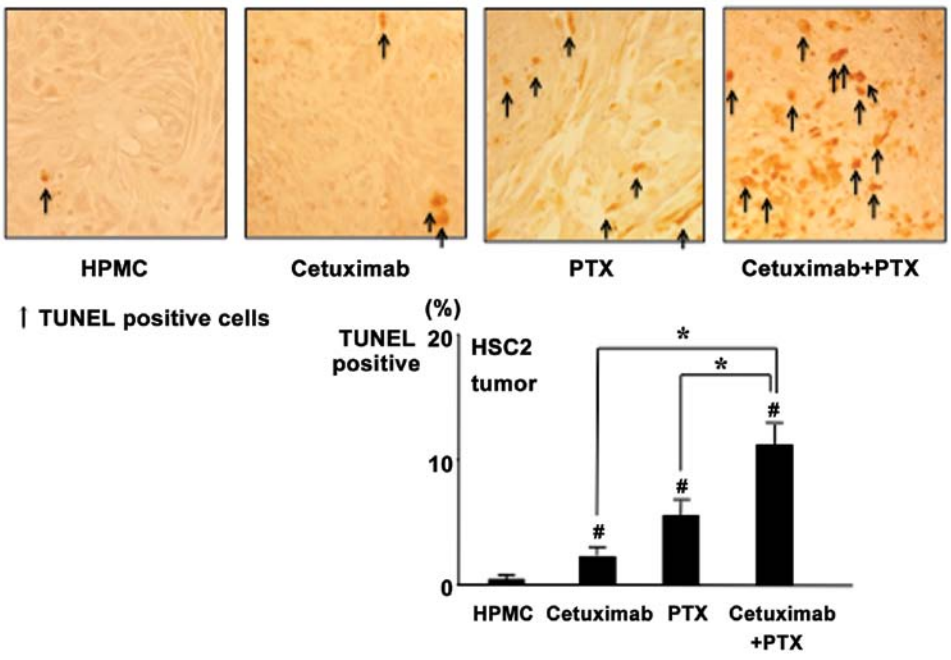

Figure 7. TUNEL assay. A TUNEL assay was performed to quantify the number of apoptotic cells in the treated mouse tumors. Cells stained brown after TUNEL staining, which indicates that apoptosis occurred in tumors treated with PTX and/or cetuximab. The TUNEL assay showed significantly increased number of apoptotic cells in PTX and cetuximab combined treatment tumors. Error bars represent the standard error of the mean from five mice (n=5). #P<0.01 when compared to that of HPMC (Mann-Whitney's U test). *P<0.01 (Mann-Whitney's U test).

tion of tumor growth was observed with PTX and cetuximab combination treatment, which is significantly different than treatment with either agent alone. Compared to the control, mice in all treatment groups showed no toxicity or significant weight loss during the treatment.

Effect of PTX and cetuximab on the expression of p65 in vivo. We examined the expression of p65 in mouse tumors by immunohistochemistry. The expression of p65 was detected in both the nucleus and the cytoplasm of untreated HSC2 tumor cells. However, p65 expression was mainly detected in cytoplasm of cetuximab treated tumors, while it was detected in the nucleus of PTX treated tumor cells. Moreover, the expression of p65 was decreased and was mainly detected in the cytoplasm of tumors that received PTX and cetuximab combined treatment (Fig. 6).

Effect of PTX and cetuximab on induction of apoptosis in vivo. To detect the degree of apoptosis induced by PTX and/or cetuximab *in vivo*, tumors were removed from mice after treatment and the number of apoptotic cells was quantified by the TUNEL assay. Although treatment with PTX or cetuximab alone moderately induced apoptosis in mice tumors compared to the untreated control, PTX and cetuximab combined treatment significantly upregulated the expressions of TUNEL-positive cells in mice tumors than all other treatment groups or control (Fig. 7).

Discussion

In this study, we have shown the efficacy of PTX in combination with cetuximab on OSCC both *in vitro* and *in vivo*. In addition, the results suggest that cetuximab may enhance the effect of PTX on OSCC through the downregulation of p65 NF-κB.

Cetuximab has attracted attention over the years because it is thus far the most effective drug shown to improve survival when combined with cisplatin and 5-fluorouracil (5-FU) in recurrent and/or metastatic squamous cell carcinoma of the head and neck (R/M-SCCHN) (31). Briefly, adding cetuximab to platinum/5-FU as first-line treatment of R/M-SCCHN significantly improved median overall survival (OS) by 2.7 months versus chemotherapy alone, without adversely impacting patients' quality of life (31,32). Based on these reports, we consider that the current first-line standard treatment approach for R/M-SCCHN must be the combination of platinum/5-FU and cetuximab. However, it has been reported recently that the weekly PTX-cetuximab combination might be an effective first-line treatment option for patients in a poor state of health and for patients with R/M-SCCHN, particularly where platinum-based chemotherapy is contraindicated and there are few treatment options (33,34). However, the mechanism of antitumor effect of PTX in combination with cetuximab is still unclear.

First of all, we examined whether EGFR protein was consistently expressed in all OSCC cell lines studied. Our results revealed that expression of EGFR proteins varied among different OSCC cell lines. HSC2 cells had the highest levels of EGFR expression, while HSC3 cells expressed moderate levels of EGFR protein and HSC4 contained the lowest level of EGFR protein. This variable EGFR expression in different cell lines might be related with the characteristics of primary tumor tissues. Moreover, we detected very low EGFR proteins expression in Wi-38 (Fig. 1).

In accordance with our expectation, cetuximab exerted the strongest growth inhibitory effect in HSC2 cells, while it exhibited the lowest growth inhibitory effect in HSC4. In addition, cetuximab did not suppress the cell growth of Wi-38 (Fig. 2A). The activity of cetuximab may be related to the level of EGFR protein expression in OSCC cells. On the other hand, PTX exerted the strongest growth inhibitory effect in HSC4 cell, and it also suppressed the cell growth of Wi-38 (Fig. 2B). Interestingly, PTX in combination with cetuximab showed strong growth inhibitory effect in all three OSCC cell lines. Therefore, it appears that the level of EGFR expression alone cannot be considered sufficient as a predictive marker of PTX-cetuximab combined activity. Janmaat *et al* reported similar observations in non-small cell lung cancer cells, where the EGFR expression level showed no correlation with sensitivity to gefitinib and cetuximab (34).

Our Hoechst staining data showed that, apoptosis was induced almost equally in all three OSCC cell lines after PTX-cetuximab combination treatment (Fig. 3). Therefore, we assumed that the growth inhibitory effect of PTX and cetuximab combined treatment was due to apoptosis. Cetuximab might be acting as a enhancer of the PTX-induced apoptosis in PTX-cetuximab combination treatment, as cetuximab alone could not exert strong apoptosis inducing activity on OSCC cells (Fig. 3). PTX is reported to exert antitumor effects on cancer cells by inhibiting cell division, however, it can also upregulate NF-κB activity, which might lead to defend cancer cells from chemotherapeutic agents (30). We observed similar results in case of PTX single treatment on OSCC (Fig. 4). Therefore, we examined whether cetuximab can regulate the expression of p65 NF-κB induced by PTX. As we expected, the expression of p65 NF-κB was reduced after PTX-cetuximab combination treatment both *in vitro* and *in vivo*, which suggests that cetuximab can regulate PTX-induced p65 NF-κB activity in OSCC cells (Figs. 4 and 6). The precise mechanism responsible for the antitumor effects of PTX and cetuximab combined treatment in OSCC is still unclear and will require further study. Nevertheless, the combination of PTX and cetuximab could be a promising therapeutic strategy for OSCC patients with poor prognosis. Future studies should aim at defining the most appropriate dose and schedule of administration of this combination treatment.

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