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## Chemoprevention of Head and Neck Cancer by Simultaneous Blocking of Epidermal Growth Factor Receptor and Cyclooxygenase-2 Signaling Pathways: Preclinical and Clinical Studies

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### Abstract

**Purpose**—We investigated the efficacy and underlying molecular mechanism of a novel chemopreventive strategy combining epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) with cyclooxygenase-2 inhibitor (COX-2I).

**Experimental Design**—We examined the inhibition of tumor cell growth by combined EGFR-TKI (erlotinib) and COX-2I (celecoxib) treatment using head and neck cancer (HNC) cell lines and a preventive xenograft model. We studied the antiangiogenic activity of these agents and examined the affected signaling pathways by immunoblotting analysis in tumor cell lysates and immunohistochemistry (IHC) and enzyme immunoassay (EIA) analyses on the mouse xenograft tissues and blood, respectively. Biomarkers in these signaling pathways were studied by IHC, EIA, and an antibody array analysis in samples collected from participants in a phase I chemoprevention trial of erlotinib and celecoxib.

**Results**—The combined treatment inhibited HNC cell growth significantly more potently than either single agent alone in cell line and xenograft models, and resulted in greater inhibition of cell cycle progression at G1 phase than either single drug. The combined treatment modulated the

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EGFR and mTOR signaling pathways. A phase I chemoprevention trial of combined erlotinib and celecoxib revealed an overall pathologic response rate of 71% at time of data analysis. Analysis of tissue samples from participants consistently showed downregulation of EGFR, pERK and pS6 levels after treatment, which correlated with clinical response.

**Conclusion**—Treatment with erlotinib combined with celecoxib offers an effective chemopreventive approach through inhibition of EGFR and mTOR pathways, which may serve as potential biomarkers to monitor the intervention of this combination in the clinic.

## Introduction

There are more than 52,000 new cases of squamous cell carcinoma of the head and neck (SCCHN) in the United States per year, with approximately 12,000 deaths (1). The survival rate for patients with SCCHN remains poor despite advances in detection and management (2). An effective preventive approach that could be implemented before the development of invasive cancer would be highly desirable to reduce the incidence of SCCHN (3).

One of the main etiologic factors that contributes to SCCHN development is tobacco carcinogen exposure throughout the upper aerodigestive tract. Extensive campaigns to educate the public about the risk of cigarette smoking have resulted in a substantial reduction in the percentage of adults who actively smoke in the United States (4, 5). However, several studies have demonstrated that former smokers in addition to active smokers remain at a high risk of cancer development in the tobacco carcinogen-exposed field (6, 7), likely resulting from the accumulation of cellular and genetic alterations, resulting in activation of several signaling pathways that support cell proliferation (7, 8). Blocking one or several of these signaling pathways may impede or delay the development of cancer (9). The opportunity to prevent cancer formation has been demonstrated by previous studies involving precancerous and cancerous lesions of the head and neck, including oral premalignant lesions (i.e. leukoplakia, erythroplakia, and/or erythroleukoplakia) (10-12). However, a standard approach to chemoprevention of head and neck cancer has not been established (7, 13, 14). Thus, new strategies are needed to prevent the development of premalignant lesions into invasive cancer.

EGFR overexpression has been documented extensively in a wide variety of malignant tumors, including SCCHN (15-17). Overexpression of EGFR and its ligand TGF- $\alpha$  was observed in 80 to 90% of SCCHN specimens (18). Importantly, we previously demonstrated that EGFR was upregulated in normal human oral epithelium adjacent to tumor and remained elevated throughout the phenotypic progression from hyperplasia to dysplasia to carcinoma *in situ* (15). EGFR expression was dramatically increased when dysplasia progressed to squamous cell carcinoma, suggesting that EGFR may serve as an excellent target for chemoprevention. Erlotinib (Tarceva, OSI-774), is an orally bioavailable EGFR-tyrosine kinase inhibitor (TKI) that is a low-molecular weight quinazoline derivative. Erlotinib has been widely studied as a chemotherapeutic agent in a variety of cancer types including SCCHN (19). Erlotinib is well tolerated, although common toxicities include a characteristic acneiform rash and diarrhea (20). The ongoing erlotinib prevention of oral cancer trial (EPOC) with placebo control is expected to be completed by the end of 2014 (NCT00402779, see website: [clinicaltrials.gov](http://clinicaltrials.gov)).

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandin (PG) from arachidonic acid. COX-2 is overexpressed in many human cancers [for review, see reference (21)]. In SCCHN, COX-2 expression is found to be upregulated at both the mRNA and protein levels. This upregulation is also seen in normal epithelium adjacent to SCCHN, and in premalignant lesions such as leukoplakia and dysplasia (22-26). Thus, selective COX-2 inhibitors (COX-2Is) are considered as promising agents for chemoprevention. Treatment

with COX-2Is, such as celecoxib, in a cancer chemoprevention trial reduced the risk of developing familial adenomatous polyposis (FAP), some cases of which would inevitably progress to fully-fledged colon cancer (27). Though the infrequent cardiovascular side effects of these agents raise concerns about their use in the preventive setting (18, 29), celecoxib as a single agent has been used in more than twenty trials for the chemoprevention of colon, oral, bladder, breast, and prostate cancers (29).

We and others have proposed blocking the interaction loop between the EGFR and COX-2 signaling pathways as a strategy for chemoprevention [for review, see (30-32)]. We suggested that a combination of two agents for prevention and treatment of SCCHN, namely an EGFR-selective TKI and a COX-2I, act on different biological targets, phosphorylated EGFR and COX-2, respectively, but might affect the same signaling pathways. Therefore, we hypothesized that these two agents would function synergistically to inhibit tumorigenesis of the head and neck, which was supported by our preclinical studies using EGFR-TKI gefitinib and COX-2I celecoxib (33, 34). Recently, we have enrolled patients with premalignant lesions of the head and neck in a phase I chemoprevention trial using erlotinib and celecoxib. We observed an preliminary overall response rate of 71% (35). Tissue specimens from this clinical trial were obtained and examined for the modulation of biomarkers by the combined treatment. Along with preclinical studies using SCCHN cell line models *in vitro* and *in vivo*, we identified several biomarkers for which alterations in expression level correlated with clinical response.

## Materials and Methods

### Material and cell lines

The EGFR-selective TKI erlotinib was provided by Genentech (San Francisco, CA). The COX-2 inhibitor celecoxib was obtained from G.D. Searle & Co. (Chicago, IL) and Pfizer Inc. (New York, NY). Both drugs were dissolved in DMSO (Sigma Chemical, St. Louis, MO) or Tween 80 (Sigma Chemical) in appropriate concentrations for *in vitro* or *in vivo* studies, respectively. Human SCCHN cell lines Tu212 and Tu686 were established from a primary SCCHN as described in our previous studies (33). The genotyping of these two cell lines confirmed their human origins (IDEXX RADIL Lab Animal and Biological Materials Diagnostic Testing, Columbia, MO). The cell lines were grown in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD) and cultured in EGM-2 medium (Lonza).

### Analysis of cell growth, cell cycle progression, and apoptosis

Cell growth inhibition was measured by determining cell density with sulforhodamine B assay (36) 72 hours after addition of the drugs as described in our previous studies (33). The drugs were added in a range of concentrations as single agents [erlotinib (0–10  $\mu\text{mol/L}$ ) and celecoxib (0–40  $\mu\text{mol/L}$ )] and in combinations. Percentage inhibition was determined by comparison of cell density in the drug-treated cells with that in the untreated cell controls in the same incubation period (percentage inhibition =  $1 - \text{cell density of a treated group} / \text{cell density of the control group}$ ). All experiments were repeated three times. Combination Index (CI) was determined using CalcuSyn software (Biosoft Inc, Ferguson, MO). A CI value of  $>1$  is defined as antagonism, equal to 1 as additive and  $<1$  as synergy.

The effects of each of the single agents and the combination on cell cycle arrest were analyzed in Tu212 and Tu686 cell lines by PI/RNase staining (BD PharMingen, San Diego, CA). Cells were treated with erlotinib (1.0  $\mu\text{M}$ ), celecoxib (10  $\mu\text{M}$ ), and the combination for 24, 48, and 72 hours. The concentration of erlotinib used is similar to the achievable level in

patients' blood (400 µg/L, 75 mg QD) observed in the pharmacokinetic study of the phase I clinical trial, while the celecoxib concentration used is less than a double dose of the achievable blood level (2000 µg/L, 400 mg BID) (35). The experiments were repeated at least 3 times independently.

Annexin V-phycoerythrin staining was used to examine apoptosis induced by treatment with erlotinib and celecoxib in Tu212 and Tu686 cell lines. The cells were then resuspended in 1× Annexin binding buffer (BD PharMingen), and stained with Annexin V-phycoerythrin (Annexin V-PE; BD PharMingen) and 7-AAD (BD PharMingen) for 15 min at room temperature. The stained samples were measured using a fluorescence-activated cell sorting caliber bench-top flow cytometer (Beckman Coulter, Miami, FL).

### Immunoblotting analysis

Whole cell lysates were extracted from drug-treated cells using lysis buffer and immunoblotted with specific antibodies as described previously (33, 34), except the antibody against phosphorylated ribosomal protein S6 (pS6) that was purchased from Cell Signaling technology (Danvers, MA). Mouse anti-β-actin antibody (Sigma Chemical) was used as a sample loading control. Immunostained protein bands were detected with an enhanced chemiluminescence kit (Thermo Scientific, Rockfield, IL).

### Endothelial capillary tube formation assay

To perform the capillary tube formation assay, 24-well plates were coated with Matrigel (250 µM/well; BD Bioscience, Bedford, MA). HUVEC cells suspended in EGM-2 medium (Clonetics Co., San Diego, CA) containing DMSO (control), erlotinib (1 µM), celecoxib (10 µM), or the combination of erlotinib (1 µM) and celecoxib (10 µM) were added to each Matrigel-coated well (40,000 cells/well) and incubated overnight. The status of capillary tube formation by HUVECs was recorded at 2× using an Olympus inverted microscope (CKX40; Olympus, New York, NY). Experiments were repeated twice.

### Matrigel invasion assay

The invasion chamber was precoated with 27.2µg Matrigel (BD Bioscience, Bedford, MA). HUVEC cells were suspended in serum-free medium containing 0.1% BSA with or without DMSO (control), erlotinib (1 µM), celecoxib (10 µM), or the combination of erlotinib (1 µM) and celecoxib (10 µM) in triplicate for 8 h before being placed in the lower chamber as described previously (37). After 36-40 h of incubation, cells at the upper membrane were removed with a cotton swab, and the membrane was stained with the Hema-3 kit (Fisher Scientific, Pittsburgh, PA). The number of invaded cells was expressed as the sum of 10 random fields under 200X magnification. Experiments were repeated twice.

### Nude mouse xenograft model

The animal experiments were approved by the Animal Care and Use Committee of Emory University. Before inoculation of  $5 \times 10^6$  Tu212 cells by s.c. injection into the right flank, nude mice (athymic *nu/nu*, Taconic, NY) aged 4-6 weeks (about 20 g weight) were randomly assigned to four groups. Animals received oral gavage with vehicle control (0.1% Tween 80 and 0.5% methylcellulose, n=6), erlotinib (75 mg/kg, n=6), celecoxib (50mg/kg, n=7), or the combination (n=8) of erlotinib (75 mg/kg bodyweight) and celecoxib (50 mg/kg bodyweight) using a blunt tipped 20G 1.1/2 needle (Popper and Sons, New Hyde Park, NY). The dose selection was based on our previous publication (34), and is about 1/2 to 2/3 of the dose level when the agents were used as single drugs. The tumor size and bodyweight were measured thrice or twice weekly, respectively. The tumor volume was

calculated using the formula:  $V = \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$  as reported previously (34).

### Immunohistochemistry (IHC) and enzyme immunoassay (EIA)

Staining of Ki67, EGFR, phosphorylated-extracellular signal regulated kinase (pERK), pS6, pAKT, COX-2, CD34, and TUNEL was performed with formalin-fixed, paraffin-embedded tumor tissues as previously described in detail (34, 38). The antibodies against Ki67, EGFR, pERK, pAKT, and COX-2 were described in our previous studies. Antibody against pS6 was the same as used in immunoblotting. Antibody against CD34 was purchased from AbCam (Dilution 1:25). For Ki67 and TUNEL, the absolute number of labeled nuclei per 1,000 cells was counted in 10 randomly selected areas from each slide. Necrotic areas were excluded for analysis. The intensity of EGFR, pERK, pS6 and COX-2 staining was measured using a numerical scale (0-3 with 3 as the strongest staining) and quantified as Weighted Index [WI = % positive staining (> 0) in tumor  $\times$  intensity score]. Positive CD34 signals were counted in 10 random fields at 100X. Microvessels were quantified as described previously (39).

Prostaglandin E2 (PGE2) is rapidly converted *in vivo* to its metabolites, with >90% of circulating PGE2 cleared by a single passage through the lung. To estimate the actual PGE2 production more reliably, the PGE2 metabolite (PGEM) in blood was measured using a PGEM EIA kit following the standard manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). Blood from human or mouse was processed within 30 min after being drawn and plasma samples were stored at -80°C until use. The PGEM concentration was calculated using a standard curve generated from PGEM standards provided by the manufacturer. The experiment was repeated three times.

### Antibody array analysis

To each cell pellet, 100  $\mu$ l extraction buffer was added and the tubes were placed at 4°C for 30 min with occasional shaking. Tubes were spun at 10,000 rpm for 15 min at 4°C. Supernatants were transferred to fresh tubes and the protein level estimated using the BCA kit from Promega. Cell lysates were labeled with Cy3 and applied to the microarray slides for 2 hours according to the manufacturer's instructions (Cancer/Apoptosis phosphorylation antibody array, Full Moon Biosystems, Sunnyvale, CA). Signals obtained from the arrays were normalized to the controls spotted on each array. Levels of phosphorylated proteins were normalized to the total proteins. To correct for differences due to exposure between the 2 slides and protein loading (normalization to housekeeping gene), two factors were introduced in the data analysis following the manufacturer's introductions: a baseline correction factor (= average of the signal from each empty well - the median of the averages of the empty well signals from 2 slides) and a scaling factor (= average of  $\beta$ -actin signal - average of signal from empty wells).

### Patients and tissue samples

We had access to tissue samples, cytobushed cells, and blood prospectively collected from patients who had premalignant lesions (moderate to severe dysplasia and carcinoma *in situ*) and participated in a phase I chemoprevention trial conducted at the Emory University Winship Cancer Institute. The clinical trial was approved by the Institutional Review Board and continues to accrue patients. Tissue samples from participating patients were obtained and evaluated pathologically at 3, 6 and 12 months of therapy. All patients received celecoxib 400 mg orally BID and erlotinib was dose escalated at 3 dose levels of 50, 75 and 100 mg orally once a day continuously for 6 months. Pathologic complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) were defined as CR: complete disappearance of dysplasia from the epithelium, PR: improvement of dysplasia at

least two degrees (i.e., severe dysplasia becomes mild dysplasia) of dysplasia after treatment, SD: minor focal improvement without change of degree of dysplasia (i.e., focal improvement from moderate dysplasia to mild dysplasia with overall still moderate dysplasia) or no pathologic changes after treatment, and PD: worsening of at least two degrees of dysplasia (i.e., mild dysplasia to severe dysplasia) or development of invasive cancer after treatment, accordingly. This clinical trial was first reported at the American Society of Clinical Oncology annual meeting 2010 (35).

### Statistical analyses

A linear mixed model with random intercept was fitted to the log transformed data to test for the effects of treatment on tumor growth. The Kruskal-Wallis and Wilcoxon tests were adopted to test the pairwise percentage of positive Ki67 expression, weighted index of pS6, and microvessel density (MVD) in four treatment groups, respectively. Pairwise comparisons of PGEM concentration were performed using a Wilcoxon test.

Spearman's correlation coefficient was then estimated to measure the relationship between the change in the value of each gene/protein with the clinical response of patient, assuming that the pathologic response in increasingly worse order was CR<PR<SD<PD. T-test was further employed to test whether the Spearman's correlation coefficient for each gene/protein was significant. All 122 genes/proteins were sorted by their Spearman's correlation coefficient in a descending order. The top 27 genes/proteins were plotted in a second heat map and analyzed with cluster analysis. Genes/proteins at the significance level of 0.1 were further plotted in a third heat map and analyzed with cluster analysis again. The SAS statistical package V9.2 (SAS Institute, Inc., Cary, North Carolina) was used for data management and Spearman's correlation coefficient analyses. The heat map and cluster analysis were conducted with R language.

## Results

### Synergistic/additive inhibition of SCCHN cell growth *in vitro* by combined treatment with erlotinib and celecoxib

To understand the effect of erlotinib and celecoxib on the growth of SCCHN, we examined two SCCHN cell lines (Tu212 and Tu686), which express comparable amounts of activated and total EGFR and COX-2 proteins at basal level (data not shown). Cells were treated with erlotinib (0 to 40  $\mu$ M) and/or celecoxib (0 to 20  $\mu$ M) for 72 hours. A cell growth inhibition assay showed that both erlotinib and celecoxib as single agents inhibited growth of both SCCHN cell lines in a dose-dependent manner (Figure 1A and 1B).

To evaluate the effect of the combined treatment with erlotinib and celecoxib on growth of SCCHN cells, a combination index was determined through a series of 2-fold reductions of erlotinib and celecoxib concentrations. The same concentration range used for the single drug was applied for the combination. The combination indices ranged from 0.29 to 0.48, indicating that the two-drug combination of erlotinib and celecoxib synergistically inhibited cell growth in the tested SCCHN cell lines, T212 and Tu686.

### Effects of the combined treatment on cell cycle progression and apoptosis

To understand the underlying mechanisms of cell growth inhibition induced by the combination of erlotinib and celecoxib, we further investigated the effects of the combination on cell cycle progression in Tu212 and Tu686 cells. As shown in Figure 2A and 2B, cells were treated for 24, 48, and 72 hours with 1.0  $\mu$ M erlotinib and 10  $\mu$ M celecoxib. Compared with the control group, erlotinib alone induced significant cell cycle arrest at the G0/G1 phase in both Tu212 and Tu686 cells as early as 24 hours after treatment

( $p = 0.0002$  and  $0.0017$ , respectively). Celecoxib alone exerted limited or no effect on cell cycle arrest. The combined treatment induced a higher level of cell cycle arrest at 72 hours in both Tu212 and Tu686 cells than celecoxib ( $p = 0.01$  and  $p < 0.0001$ , respectively) or erlotinib ( $p = 0.04$  and  $p = 0.002$ , respectively) alone. The two drug combination did not affect cell cycle changes at either S or G2/M phase (data not shown). Consistent with the G0/G1 arrest, we observed upregulation of the cell cycle regulators p21 and p27 at 48 hours after treatment in these cells (Figure 2C).

To examine whether growth inhibition of SCCHN cells by erlotinib, celecoxib, and their combination is also attributable to programmed cell death, we evaluated the apoptotic effect of the combination on Tu212 and Tu686 cells. Both cell lines were treated with erlotinib and celecoxib as either single agents or the combination for 72 and 96 hours. Approximately 25–30% apoptosis was observed 96 hours after treatment with erlotinib alone and the combination. Treatment with celecoxib did not induce apoptosis under the current conditions (data not shown).

### **Multiple proteins downstream of EGFR were affected by erlotinib and celecoxib treatment**

To elucidate the molecular functions by which the combination of erlotinib and celecoxib acts in SCCHN, we examined whether these two drugs could modulate activation of EGFR and its downstream AKT and ERK proteins, and S6 as a readout for mTOR signaling. SCCHN cell lines Tu212 and Tu686 cells maintained in medium with 5% serum were treated with celecoxib (10  $\mu\text{M}$ ), erlotinib (1.0  $\mu\text{M}$ ), or their combination (10  $\mu\text{M}$  + 1.0  $\mu\text{M}$ ) for 24, 48 and 72 hours. Immunoblotting analyses showed that at the concentration used, erlotinib as a single agent inhibited the phosphorylation of EGFR, AKT, ERK, and S6 as early as 24 hours after treatment, while celecoxib alone did not affect these pathways (Figure 3). Compared with the single drugs in both cell lines, the combination had an effect similar to erlotinib alone.

### **Blockage of capillary tube formation of endothelial cells and invasion**

To assess potential antiangiogenesis activity, the effect of the combination of erlotinib and celecoxib on vascularization was assessed in HUVEC cells. Compared with control group, treatment with celecoxib alone or the combination significantly suppressed the level of tubular formation ( $p < 0.05$ ), however, this process was not affected by erlotinib treatment (Figure 4A). In addition, the ability of HUVEC cells to invade into tumor cells was significantly inhibited by the combination treatment but not by either single agent alone ( $p < 0.05$ , Figure 4B), supporting the antiangiogenesis function of the combined treatment.

### **Effects of the combined treatment on SCCHN xenograft tumor development, tumor cell proliferation, and blood vasculature formation**

In this study, mice were given the chemopreventive agents erlotinib, celecoxib, and the combination approximately one week before SCCHN cell inoculation. All 3 treatments suppressed tumor growth significantly compared to the vehicle control ( $p < 0.0001$ , Figure 5A). Importantly, the combination treatment showed a marked advantage over either erlotinib or celecoxib alone ( $p < 0.0001$ ). The slightly greater potency of erlotinib over celecoxib in suppressing tumor growth was not significant.

All 3 treatments significantly reduced the expression of pS6 and microvessel density when compared with the staining in tumor tissues treated with the vehicle control ( $p < 0.05$ ). Cell proliferation, reflected by Ki67 staining, was significantly inhibited by all treatment groups ( $p < 0.0001$ ), and while the combination treatment reduced Ki67 expression more effectively than either single agent alone ( $p < 0.01$ ), there was no significant difference between treatment with erlotinib and celecoxib (Figure 5B and 5C).



The combined treatment tended to reduce the expression of EGFR, however, this effect was not statistically significant (data not shown). In addition, the treatments only slightly reduced the level of plasma PGEM collected at the time of termination (Figure 5C). Consistent with the *in vitro* observation, apoptosis detected by TUNEL assay was not significantly higher in the combined treatment group than in the vehicle control (data not shown).

### **Correlation between clinical response and alterations in biomarker expression in tissue specimens from patients participating in a chemoprevention clinical trial**

A total of 11 patients received treatment with erlotinib and celecoxib. Seven had both baseline and follow up biopsies and cytobruished cells from oral mucosa and sera and were evaluable for response using the last documented histologic response at 3, 6 or 12 months. Among them, based on the pathologic evaluation of biopsy samples by pathologists, 3/7 achieved a complete pathologic response (CR 42%), 2/7 a partial response (PR 29%), and 2/7 progressive disease (PD 29%) with an overall response rate (RR) of 71%. The mean duration of response for the 7 evaluable patients defined as the interval from the time of enrollment to the last pathologic evaluation, or last follow up visit without evidence of progression, was 20 months (range is 6 to 36 months).

We explored whether changes in the levels of several biomarkers which were affected by erlotinib and celecoxib *in vitro*, including EGFR, COX-2, pERK, pS6, and pAKT, before and after the treatment correlated with clinical response (Figure 6A). IHC staining was quantified as described in the Methods section. Assuming a pathologic response in an increasingly worse order of CR<PR<SD<PD, we noted that a decrease in the expression level of EGFR and pERK at the patient's last clinical response was significantly and positively associated with clinical response ( $p=0.019$  and  $p=0.006$  for EGFR and pERK, respectively) (Figure 6B). In addition, decreasing pS6 expression was associated with clinical response though this was not statistically significant ( $p = 0.14$ ), perhaps due to the small sample size. Using the same analysis, there was no significant relationship between change in the expression level of COX-2 or pAKT at the patient's last clinical response from baseline and actual clinical response (data not shown).

In addition, proteins isolated from the cytobruished cells of the oral mucosa before and 3 months after treatment were used to screen potential biomarkers using an antibody array. A total of 7 patients were included in this analysis. Figure 6C shows a heat map of the 6 phospho-proteins that were found to have a significance level of 0.1 among all 7 patients (Table S1). Based on the overall expression of these 6 proteins, patients with PD were completely separated from patients with other types of clinical response by the cluster analysis.

## **Discussion**

Numerous preclinical studies have flourished ever since the concept of chemoprevention was proposed by Dr. Sporn in 1976 (40). However, limited studies have been translated into therapeutic options as standard care for patients with premalignant lesions. Furthermore, to date there have been 1741 studies related to "prognostic markers" in head and neck cancer since 1983 in the PubMed database, but no biomarkers are recommended or approved by the Food and Drug Administration (FDA) for routinely diagnosing premalignant lesions. Limiting factors for the development of premalignant biomarkers include: 1) paucity of preventive clinical trials; 2) biostatistical challenges in biomarker validation (41); 3) the need for repeated biopsies for pharmacodynamic biomarker study; 4) biomarker development is often not in sync with targeted drug development, making it difficult to integrate and translate into clinical practice; 5) the demand for large resources for validation

trials, which have often been conducted far later than the drug intervention study has been completed.

Hence, to promote the integration of biomarker development into individualized targeted therapy (42) we propose adopting an incremental approach, by which we seek every drug development clinical trial as an opportunity for biomarker identification and validation, bypassing this need for large and time costly separate validation studies. In light of the success with the Dx/Rx model in breast cancer (pairing the HercepTest with Trastuzumab) and recent guidelines issued by the FDA promoting companion diagnostics (43), we propose that biomarker identification and incremental validation should be carried out in parallel in all clinical trial phases. Taking advantage of our phase I chemoprevention trial, we used three different patient tissue sources to examine biomarkers and identify those that correlate with clinical response to the combination treatment, i.e., exfoliated cells of the oral mucosa collected by cytobrush before and 3 months after treatment, patient plasma samples at different time points, and patient biopsies at baseline and after treatment. In order to make comparisons across clinical trials in the future, we stratified the changes seen in biomarker expression levels based on pathologic response. In addition, we explored the involvement of these biomarkers in the mechanism of action of the combined treatment via *in vitro* and *in vivo* studies.

In the past few years, we have provided evidence to support our hypothesis that EGFR-TKI and COX-2I can act synergistically to inhibit tumorigenesis of the head and neck by showing gefitinib and celecoxib either additively or synergistically inhibited the growth of five SCCHN cell lines (33, 34). We have also confirmed these findings by combining a similar EGFR-TKI, erlotinib, with celecoxib in the current study in which we examined alterations of some proteins which are directly and indirectly affected by the two targeted agents in both SCCHN cell lines and xenograft models. The results confirmed the synergetic/additive inhibitory effects of combined erlotinib and celecoxib treatment on SCCHN cell growth. The phase I chemoprevention trial exploring this combination in patients with premalignant lesions of the head and neck is ongoing and near completion (thus, the full clinical study will be reported separately). The same proteins examined in the preclinical study were explored as the biomarkers in the phase I trial. The correlations between pathologic responses and levels of these biomarkers were evaluated in serial samples obtained from patients, including tissue biopsy and cytobrushed oral mucosa cells.

Our results from the biopsy tissues by IHC identified that downregulation of EGFR and pERK (Figure 6), but not pAKT, was significantly correlated with the patients' responses to treatment with erlotinib and celecoxib, suggesting that EGFR/ERK pathway is more sensitive to the combined treatment than the AKT pathway. The *in vitro* data consistently support the targeting effect of erlotinib on EGFR and its downstream pathways, although the findings from cell lines did not match the clinical observations for every biomarker. In the SCCHN cell line study, erlotinib showed strong reduction of pAKT (Figure 3), but we did not observe a significant correlation of the change in expression of this protein with either clinical outcome or xenograft tumor inhibition. One potential explanation for this is that in addition to EGFR, AKT is also regulated by a complicated upstream network involving G-protein-coupled receptors (GPCRs) and insulin growth factor 1 receptors (IGF-1R), the activation of which depends on autocrine and paracrine signals (44, 45). It is also possible that a larger sample size is required to observe whether pAKT modulation can serve as a biomarker for the combined treatment due to its complicated regulatory nature. From another perspective, we should be aware of the limitations of the use of cell lines and xenograft models as guides for the selection of biomarkers in a clinical trial since there will inevitably be some inconsistent observations between cultured cells/xenograft tumors and human tissues due to different biological environments. However, human cell lines and

xenograft tumor models are still and the most commonly used systems for preclinical evaluation of therapeutic agents and their relevant biomarkers. In this study, the use of cell lines and xenograft mouse models serves as a simplified system to provide evidence and a starting point to support the clinical investigation. In light of an excellent review describing why the translation of cancer biomarkers into clinical practice is a highly failure-prone process (46), our consistent findings across three systems should be recognized.

As an exploratory study, we performed phosphorylation antibody array analysis using proteins isolated from the cytobrushes mucosa cells before and 3 months after treatment from 7 patients. Changes in the expression levels of six proteins were identified to discriminate progressive disease from the responsive cases (Table S1). Among these 6 phospho-proteins, 3 are in the downstream part of the EGFR signaling pathway and 1 is in the downstream section of the mTOR pathway, both major signaling pathways that dominated our biomarker identification in the phase I chemoprevention trial. The other 2 phospho-proteins are a breast cancer suppressor and an NF- $\kappa$ B regulator, which both deserve further investigation. The 6 significant phospho-proteins found in the antibody assay study have potential to be signature proteins for clinical response. However, we should acknowledge that since there were only 6 samples, no conclusion can be made from this study. With a larger sample size, we expect to find significant proteins that can predict clinical response.

Our observation of downregulation of pS6 (Figure 6), a commonly used readout for mTOR activation (47, 48), in premalignant tissues after the combined treatment brought the mTOR pathway to our attention. The modulation of pS6 by the combined treatment was supported by our preclinical studies in both SCCHN cell lines (Figure 3) and the SCCHN xenograft model (Figure 5C). The contribution of the mTOR pathway to the early stage of head and neck carcinogenesis was reported by Gutkind and colleagues in an oral-specific chemical carcinogenesis model using the mTOR inhibitor rapamycin for prevention of SCCHN (49, 50). Based on the previous studies, inhibition of the mTOR pathway may result from the inhibition of its upstream suppressor TSC1/2 by pERK and pAKT (48). Since the roles of the mTOR pathway in the early development of SCCHN have not been well elucidated, our finding deserves further investigation.

The observation of PGEM downregulation in mouse blood is mainly attributed to the COX-2I celecoxib through it is not significant. It seems that celecoxib does not play a role in most of the pathways examined in our study. However, consistent with previous reports from our group and others (33, 51), we observed that celecoxib inhibited microtubular and mouse vasculature formations both *in vitro* (Figure 4) and *in vivo* (Figure 5B and 5C), respectively, indicating the antiangiogenesis activity of this agent. Celecoxib also contributed to regulation of proliferation as evidenced by the induction of p21 and p27 *in vitro* (Figure 1 and 2C) and reduction of Ki67 *in vivo* (Figure 5C). Additionally, blockage of EGFR and its downstream mTOR pathways by erlotinib also showed an antiangiogenesis effect *in vitro* and *in vivo* (Figure 4A and 5C, respectively). Therefore, it was expected that the combination of celecoxib and erlotinib would achieve greater growth inhibition than either single agent both *in vitro* and *in vivo* (Figure 1A/B and Figure 5A, respectively).

The observations of our biomarker studies are supported by recent publications from Kao's group who reported both a preclinical study and a phase I trial of concurrent erlotinib, celecoxib, and reirradiation therapy for recurrent head and neck cancer (52, 53). Their studies suggest that combination therapy with erlotinib and celecoxib can also be used as a radiation sensitizing approach in the treatment of recurrent head and neck cancer. For treatment of recurrent and metastatic SCCHN, Wirth *et al* also conducted a phase I clinical trial using a combination of gefitinib and celecoxib, which achieved a partial response rate

of 22% (54). In addition, Ferris's group recently reported a pilot randomized trial in a neoadjuvant setting using erlotinib plus sulindac, a non-specific inhibitor targeting COX1/2. The major goal of this study was to study modulation of serum proteins upon the treatment (55).

In summary, we observed correlations between changes in the activated protein levels of the EGFR signaling pathway with the combined therapy using erlotinib and celecoxib in both cell lines and a preventive xenograft model. As expected, the changes in some protein markers were significantly correlated with the clinical outcome of patients on a phase I chemoprevention trial, although the sample size is small. Our preclinical studies not only confirm the clinical findings but also provide insight into the mechanisms of action of combined treatment with erlotinib and celecoxib in head and neck cancer prevention. Together, our investigation combining both preclinical studies and the biomarker-driven phase I clinical trial serves as a proof of principle to justify future chemoprevention trials which target multiple signal transduction pathways involved in carcinogenesis of head and neck cancer.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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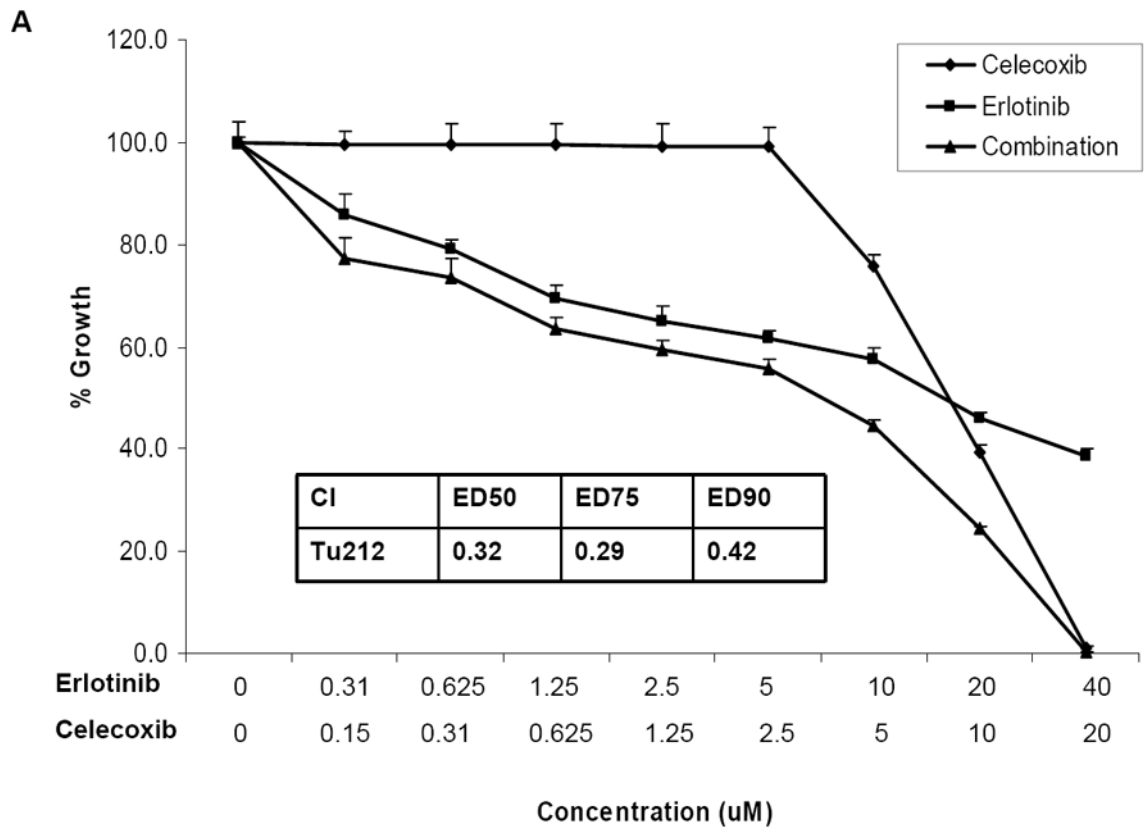
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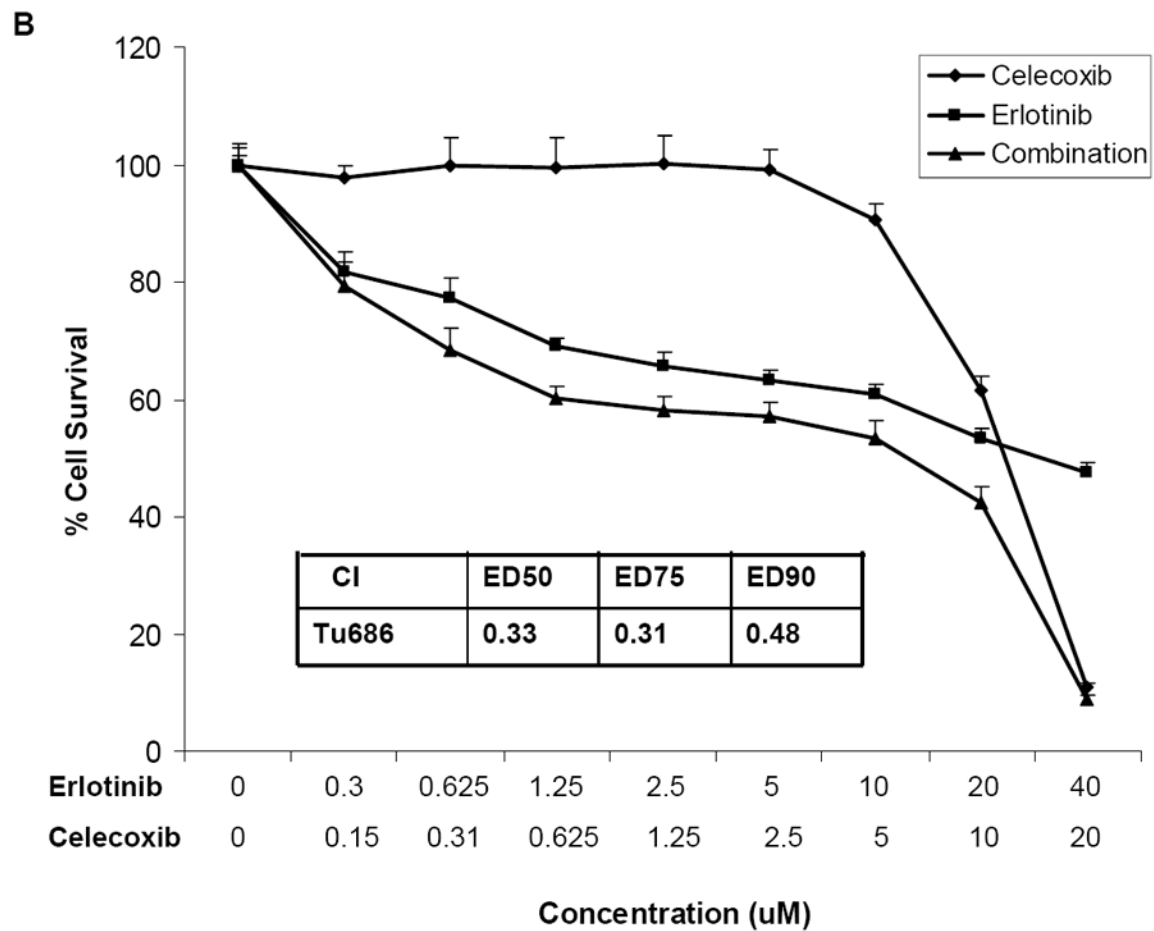
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### Translational Relevance

Epidermal growth factor receptor (EGFR) and cyclooxygenase 2 (COX-2) contribute to tumorigenesis in head and neck squamous cell carcinoma (SCCHN). In this study, we examined the efficacy and underlying molecular mechanism of a novel chemopreventive strategy combining the EGFR tyrosine kinase inhibitor, erlotinib, with the COX-2 inhibitor, celecoxib. Preclinical studies confirmed that the combined treatment inhibited SCCHN cell growth significantly more potently than either agent alone in both cell line and xenograft models. Current data from a phase I chemoprevention trial using this combination revealed an overall pathologic response rate of 71%. Analysis of tissue samples from the trial participants consistently showed downregulation of EGFR, pERK and pS6 levels after the treatment, which were found to correlate with clinical response. These results support clinical trials using combinations of targeting agents for the prevention of SCCHN. Such chemoprevention strategies may help to reduce SCCHN incidence in patients at high risk.







**Figure 1. Effects of erlotinib and celecoxib on growth of SCCHN cell lines**

SCCHN cell lines Tu212 (A) and Tu686 (B) were treated with 1:2 serial dilutions of erlotinib (0-40  $\mu$ M) and celecoxib (0-20  $\mu$ M) as single agents and in combination as described in the Methods section. After incubation for 72 hours, SRB assay was used to determine the percentage of survival relative to the untreated cells. CIs at effective doses which resulted in 50% (ED50), 75% (ED75), and 90% (ED90) inhibitory rates (1 - survival rate) were calculated using CalcuSyn software. A CI value of >1 is antagonism, = 1 is additivity, and <1 is synergy.

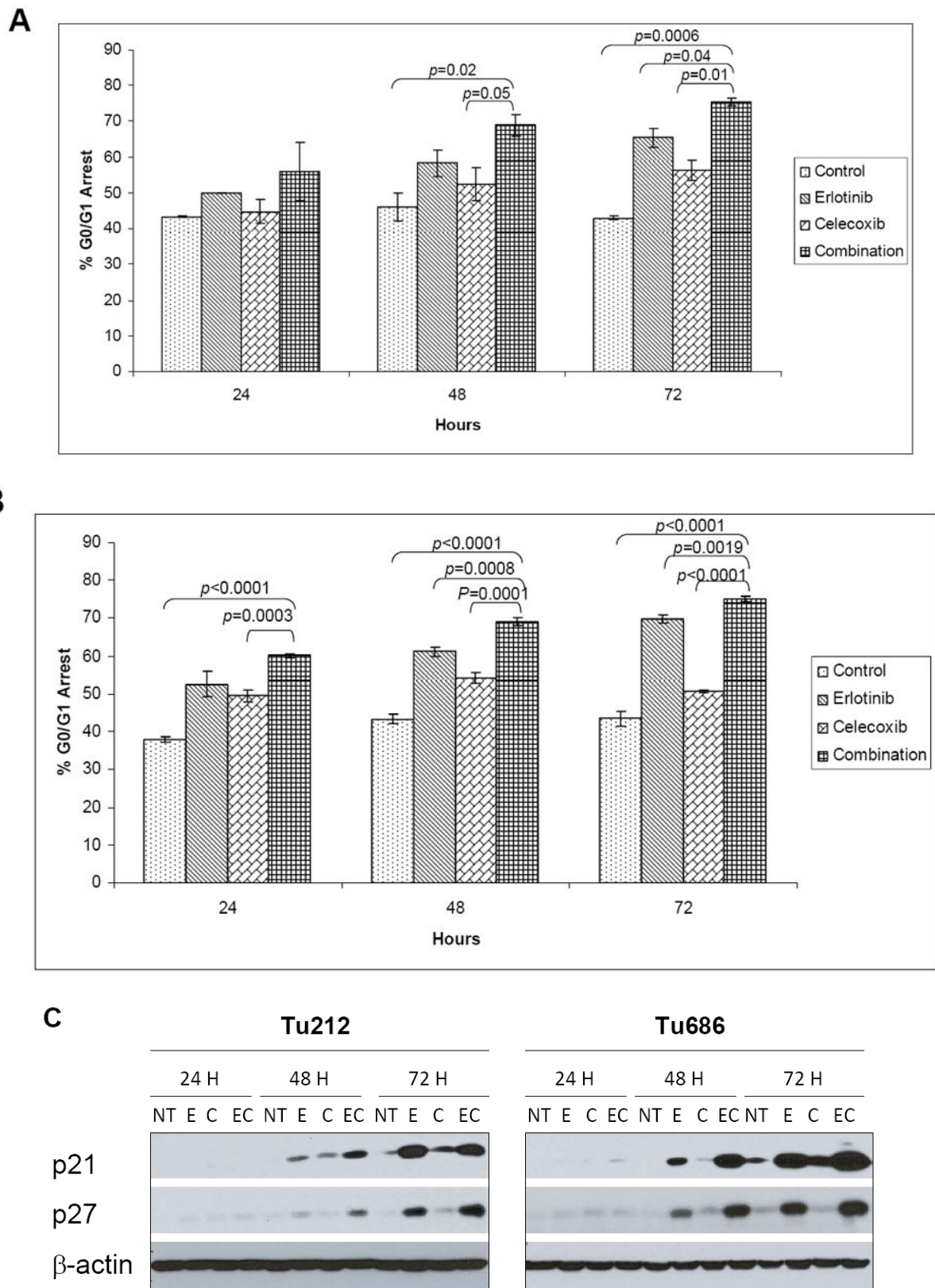
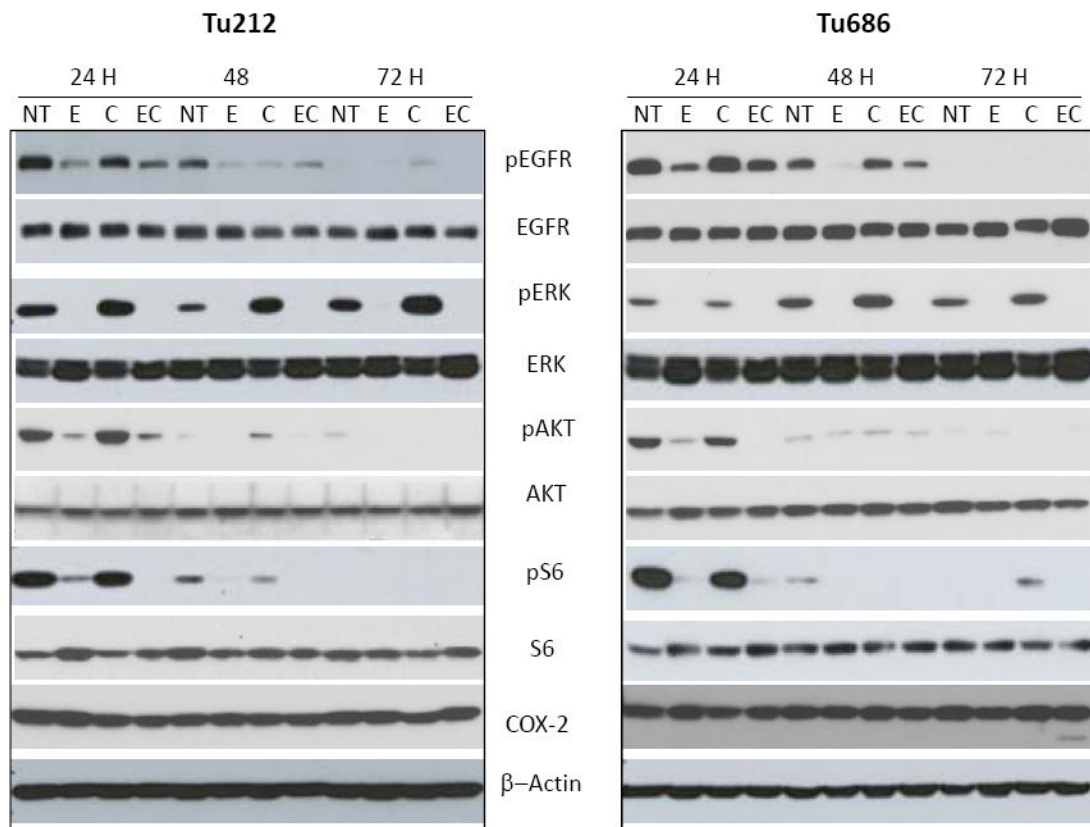
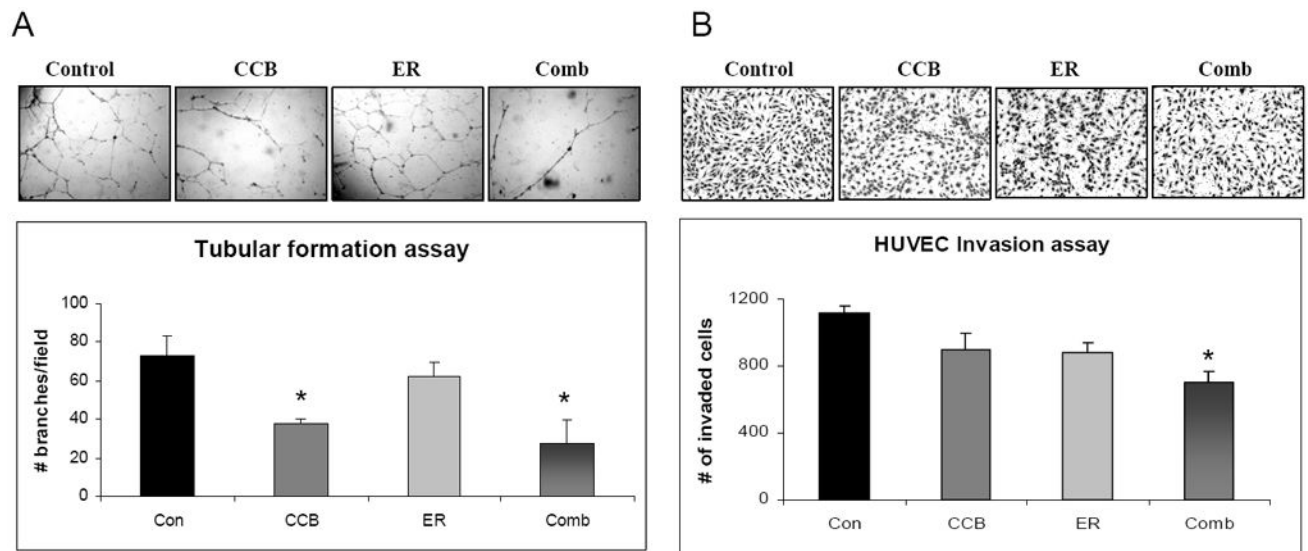


Figure 2. Induction of G0/G1 arrest by erlotinib and celecoxib in SCCHN cells

SCCHN cell lines Tu212 (A) and Tu686 (B) were treated with erlotinib (1  $\mu$ M), celecoxib (10  $\mu$ M), and their combination for 24, 48, and 72 hours. Cell cycle analysis was performed by flow cytometry as described in the Methods section. The average percentages of the cell population arrested at G0/G1 at each time point are presented with standard deviation from three repeated experiments. Significant differences ( $p < 0.05$ ) in comparison of the combination treatment with the control and each of the single treatments at all time points are shown. (C) Western blot analyses of cell cycle regulatory proteins in both Tu212 and Tu686 cells treated for 24, 48, and 72 hours. The experiments were repeated three times.



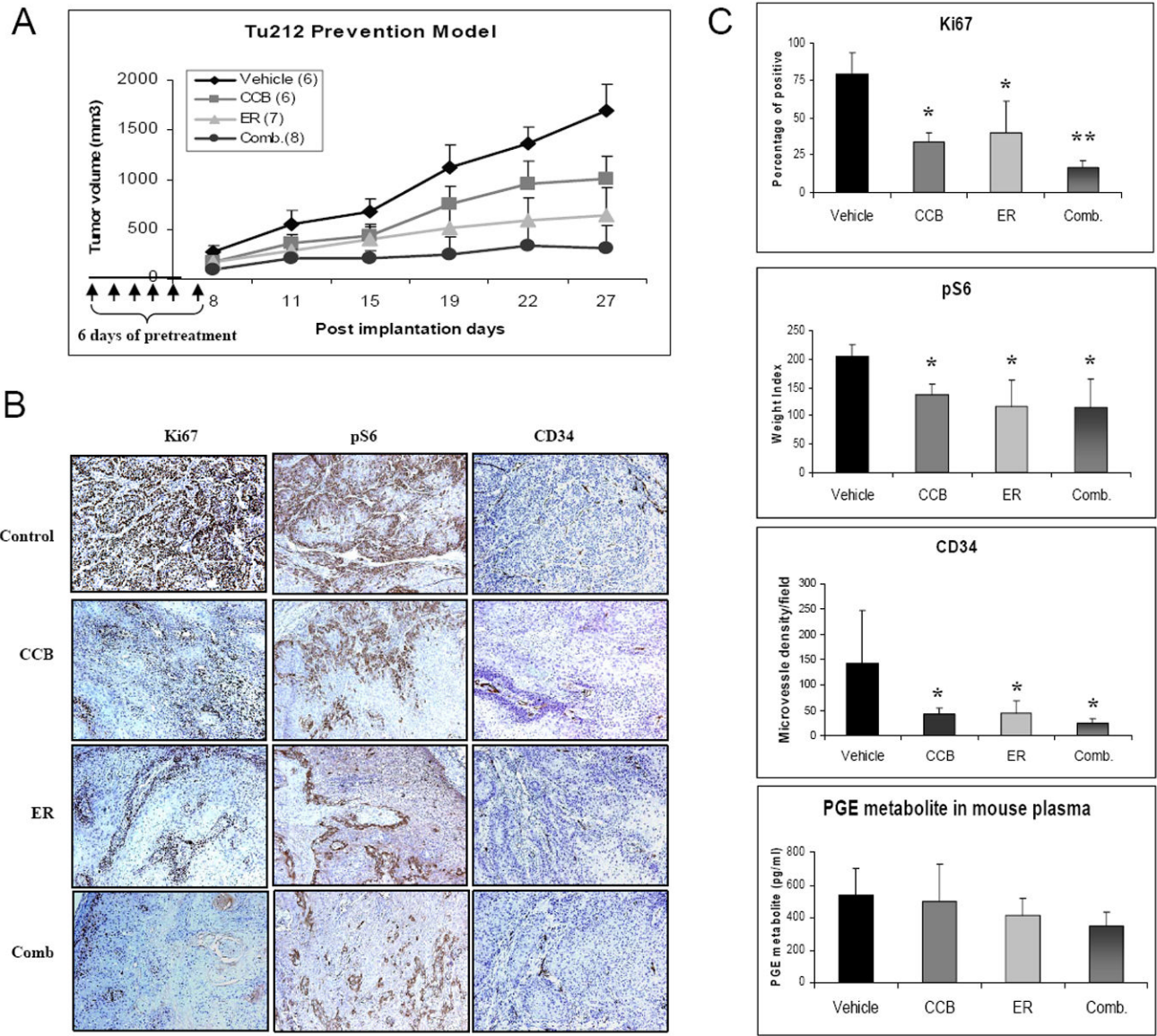
**Figure 3. Signal transduction pathways affected by erlotinib and celecoxib in SCCHN cells**  
 Cell lysates were collected from SCCHN cell lines Tu212 and Tu686 after treatment with erlotinib (E: 1  $\mu$ M), celecoxib (C: 10  $\mu$ M), and their combination (EC) for 24, 48, and 72 hours. Untreated cells (NT) were used as a control at each time point. Western blot analyses were performed on total protein extracts from each of the time points to detect the expression levels of proteins involved in EGFR, AKT, mTOR, and COX-2 pathways.  $\beta$ -actin served as a loading control.



**Figure 4. Inhibitory effects of erlotinib and celecoxib on HUVEC tubular formation and Matrigel invasion**

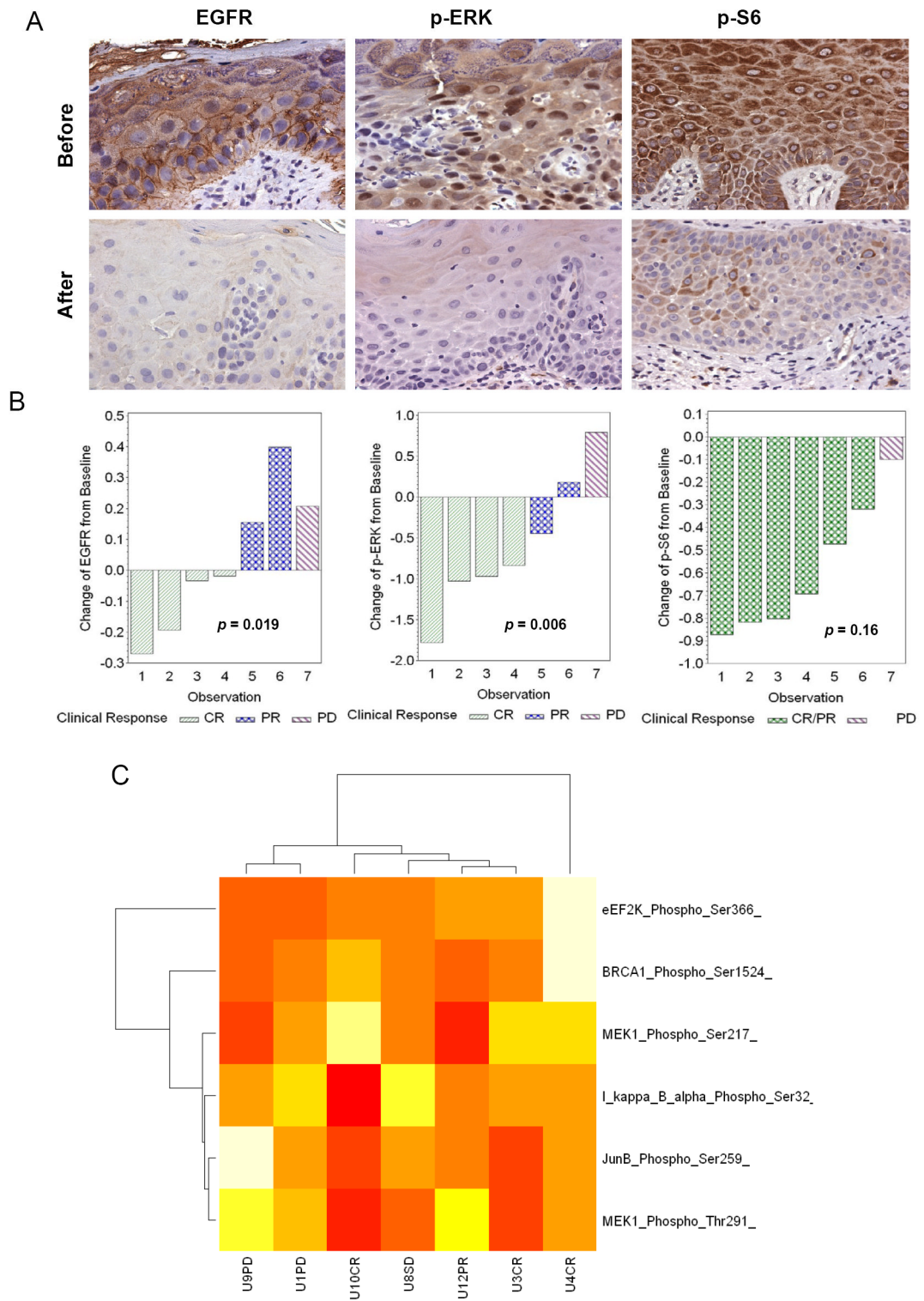
(A) Effects of erlotinib and celecoxib on capillary formation by HUVECs were examined *in vitro*. HUVECs were pretreated with vehicle DMSO (Control), celecoxib (CCB: 10  $\mu$ M), erlotinib (ER: 1  $\mu$ M), and their combination (Comb) for 12 hours, followed by inoculation in 24-well plates pre-coated with Matrigel and incubation overnight. A representative image is shown in the figure. The average number of HUVEC capillary tube branches in 10 fields was counted using an Olympus inverted microscope (CKX40; Olympus, New York, NY).

(B) HUVECs suspended in serum-free medium containing 0.1% BSA with or without DMSO (control), celecoxib (CCB: 10  $\mu$ M), erlotinib (ER: 1  $\mu$ M), or combination of erlotinib and celecoxib (Comb: 1  $\mu$ M and 10  $\mu$ M, respectively) in triplicate were seeded in the invasion chamber. After 36-40 hours of incubation, the number of invaded cells was counted and quantified as the sum of 10 random fields under the microscope with 200X magnification. Experiments were repeated twice. \* indicates statistical significance ( $p < 0.05$ ) of the treatment compared with control.



**Figure 5. Effect of erlotinib and celecoxib on Tu212 xenograft tumor growth**  
 Four groups of mice were orally gavaged with control (0.1% Tween 80 and 0.5% methylcellulose), erlotinib (75 mg/kg), celecoxib (50mg/kg), or the combination (n=8) of erlotinib (75 mg/kg) and celecoxib (50 mg/kg) for 6 days prior to a subcutaneous inoculation of  $2 \times 10^6$  Tu212 cells. The animals were continuously gavaged with the agents 5 days a week for a total of 4 weeks. (A) Tumor volume was measured at the indicated time points. (B) Immunohistochemistry analyses shown as representative H&E staining ( $\times 200$ ) were performed for expression of proliferation marker Ki-67, mTOR substrate p-S6, and endothelium marker CD34. (C) Quantification of these biomarkers. \* indicates statistical significance ( $p < 0.05$ ) of the treatment compared with control and \*\* indicates significant difference between the combination and either single agent.

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**Figure 6. Biomarker alterations upon treatment with the combination of erlotinib and celecoxib in patient tissue samples**

(A) Immunohistochemical staining of EGFR, pERK, and p-S6 before and after the combined treatment. (B) Correlation of the change in expression of EGFR, pERK, and p-S6



with patients' responses at the last clinical time point. Seven patients were included in the analysis. (C) Heat map of significant proteins ( $p < 0.1$ ) associated with clinical response.