Tumor Growth Inhibition by Simultaneously Blocking Epidermal Growth Factor Receptor and Cyclooxygenase-2 in a Xenograft Model

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Abstract

Purpose: Our previous study revealed that simultaneously targeting epidermal growth factor receptor (EGFR) tyrosine kinase and cyclooxygenase-2 (COX-2) additively or synergistically inhibited growth of squamous cell carcinoma of the head and neck (SCCHN) *in vitro*. However, an *in vivo* efficacy of this combined treatment in SCCHN has not been studied.

Experimental Design: Nude mice were pretreated with control (1% Tween 80), ZD1839 (50 mg/kg) alone, celecoxib (50 mg/kg) alone, or a combination of ZD1839 and celecoxib at the same dosages for 7 days before injection of a human SCCHN cell line Tu212. The animals were continuously treated with the agents 5 days a week for about 11 weeks.

Results: Tumor growth in the combined treatment was significantly inhibited compared with the control ($P < 0.001$), ZD1839 ($P = 0.005$), or celecoxib ($P < 0.001$). At the same time, a dramatic delay of tumor progression was observed in the combined treatment compared with all other three groups. Molecular analysis showed that the combined treatment significantly decreased prostaglandin E metabolite production. The cooperative effect of these two agents in combination was also associated with down-regulation of phosphorylated EGFR, phosphorylated extracellular signal-regulated kinase, and phosphorylated signal transducers and activators of transcription 3 levels and reduction of vascular endothelial growth factor and Ki-67 expression. Specifically, gene silencing of both EGFR and COX-2 by small interfering RNA further confirmed the cooperative antitumor effect.

Conclusion: The current results strongly suggest that a cooperative effect of the combined treatment on tumor progression is mediated through blocking both EGFR- and COX-2-related pathways. This combination regimen may provide a promising strategy for cancer therapy and chemoprevention in SCCHN.

Squamous cell carcinoma of the head and neck (SCCHN) is a serious health problem in the United States and worldwide. Despite advances in conventional surgical procedures, radiotherapy, and chemotherapy, the overall survival rate for SCCHN has not been significantly improved in the past two decades (1, 2). Identification of effective novel approaches to prevent or treat the progression of cancer is desirable to reduce the incidence or mortality of SCCHN.

Accumulating evidence indicates that targeting molecules that are crucial for cancer cell proliferation and survival provides promising approaches to impede or delay the development of cancer. One of such targets is epidermal growth factor receptor (EGFR) and its signaling pathways in cell growth. EGFR is a transmembrane glycoprotein with intrinsic tyrosine kinase activity that regulates cell growth in response to binding of its ligands. EGFR is highly expressed in a variety of solid tumors, including 80% to 90% of SCCHN (3–6). In particular, EGFR expression dramatically increased as dysplasia progressed to squamous cell carcinoma (5). Overexpression of EGFR was consistently correlated with disease progression, poor survival, poor response, and resistance to cytotoxic agents (7).

Cyclooxygenase 2 (COX-2), an inducible prostaglandin G/H synthase catalyzing the synthesis of prostaglandins from arachidonic acid, is also considered as a promising target for cancer prevention and therapy. There are two isoforms in the COX family. COX-1 is expressed constitutively in most tissues and seems responsible for the production of prostaglandins that control normal physiologic functions (8). In contrast, COX-2 is not detected in most normal tissues. It can be induced by a variety of proinflammatory stimuli and growth factors (9–11). COX-2 is overexpressed in many human premalignant and malignant lesions (for review, see ref. 12), including SCCHN (13), establishing its role in carcinogenesis (for review, see ref. 14). Currently, selective COX-2 inhibitors

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have been extensively studied for cancer chemotherapy and/or chemoprevention (15).

Emerging evidence suggests a direct interaction between EGFR signaling and COX-2 activity. Recent reports have shown that prostaglandin E_2 (PGE_2), a major product from COX-2-catalyzed reaction, transactivates EGFR by induction of phosphorylation of EGFR and mitogen-activated protein kinase in colon cancer cell lines (16, 17). On the other hand, EGF and transforming growth factor-α also induce COX-2 expression and PGE_2 production (18, 19). Therefore, simultaneously targeting both EGFR and COX-2 in SCCHN may be more effective by cooperatively blocking both pathways and their downstream targets.

Our previous study showed that a combined treatment of a selective EGFR tyrosine kinase inhibitor (TKI) ZD1839 with a COX-2 inhibitor celecoxib, *in vitro*, has synergistic or additive inhibitory effect of cell growth on SCCHN. This efficacy was associated with inhibition of cell growth, induction of G_1 arrest and apoptosis, and suppression of angiogenesis. Furthermore, this combination synergistically reduced the level of phosphorylated EGFR (p-EGFR), phosphorylated extracellular signal-regulated kinase (p-ERK), and phosphorylated Akt in SCCHN cells (20). These results prompt us to investigate *in vivo* study using a xenograft model. The current study evaluates the chemopreventive or therapeutic efficacy of this combination regimen on a SCCHN xenograft model and its effect on the immediate targets of the two drugs, phosphorylation of EGFR and COX-2 activity, and the downstream molecules of EGFR- and COX-2-mediated signal transduction pathways. Meanwhile, we used a gene-silencing technology specifically knocking down both EGFR and COX-2 expression *in vitro* to further confirm the cooperative inhibitory effect by simultaneously blocking EGFR- and COX-2-mediated pathways.

Materials and Methods

Reagents and cell line. The EGFR-selective TKI ZD1839 was provided by AstraZeneca (Cheshire, England). The COX-2 inhibitor celecoxib was obtained by Pharmacia Corp./G.D. Searle and Co. (Chicago, IL). 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* study, respectively.

SCCHN human cell line Tu212 used for this study was established from a primary hypopharyngeal tumor. It was obtained from Dr. Gary L. Clayman's laboratory (The University of Texas M.D. Anderson Cancer Center, Houston, TX). The tumor cell line was grown in DMEM/F12 (1:1) with supplemented 10% fetal bovine serum.

Development of nude mice xenografts. All animal experiments were approved by the Animal Care and Use Committee of The University of Pittsburgh. Twenty-five nude mice (Athymic *nu/nu*, Taconic, NY), ages 4 to 6 weeks (about 20 g of weight) were randomly divided into four groups. The mice in each group were orally gavaged with the vehicle control (1% Tween 80, $n = 6$), ZD1839 (50 mg/kg, $n = 6$), celecoxib (50 mg/kg, $n = 6$), or combination ($n = 7$) of ZD1839 (50 mg/kg) and celecoxib (50 mg/kg) for 7 days as a pretreatment. Each mouse was then injected s.c. with 2×10^6 Tu212 cells. The animals were continuously given with the agents 5 days a week. The tumor size was measured thrice a week. Growth curve was plotted using average tumor size within each experimental group at the set time points. The mean time from the injection of tumor cells until the average tumor size (V) reaching 500 mm^3 in each group was estimated from the Kaplan-Meier curve. The tumor volume was measured using the formula: $V = \pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as reported previously (21). The whole group of mice was sacrificed once the size of any tumor in that group reached 2 cm in diameter. Blood was drawn by heart puncture for prostaglandin E metabolite (PGEM) assay after the mouse was sacrificed. The fresh tumor tissues were collected for immunoblotting assay and paraffin-embedded tissues were used for immunohistochemical analyses.

Enzyme immunoassay. PGE_2 is rapidly converted *in vivo* to its metabolites, with >90% of circulating PGE_2 cleared by a single passage through the lungs of the mice. To estimate the actual PGE_2 production more reliably, the PGEM in the mice blood were measured using PGEM EIA kit following the standard manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). Blood was drawn from each mouse immediately after they were sacrificed and stored at −80°C until use. All of the PGE_2 were converted to PGEM before measurement. The PGEM concentration was calculated using a standard curve generated from PGEM standards provided by the manufacturer. The experiment was repeated thrice.

Immunoblotting analyses. Immunoblotting analyses were used to study expression levels of the proteins, which were potentially modulated by ZD1839, celecoxib, or in combination. These proteins include p-EGFR, total EGFR, COX-2, and the downstream molecules of EGFR-mediated signaling pathways. Polyclonal antibodies against p-EGFR, total EGFR, and total ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against p-ERK, phosphorylated signal transducer and activator of transcription 3 (p-STAT3), and total STAT3 were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-COX-2 antibody was purchased from RDI (Flanders, NJ). Anti-β-actin antibody was purchased from Sigma Chemical for an internal control.

Protein (100 μg) from nude mouse xenograft for each sample was separated by 8% to 16% gradient SDS-PAGE gel (Bio Whittaker Molecular Applications, Inc., Rockland, ME), transferred onto a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA), and blotted with corresponding antibodies. The antibody binding signals were detected using enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Silencing of gene expression with small interfering RNA. Gene silencing by small interfering RNA (siRNA) technology uses a small double-strand RNA (i.e., the siRNA) that triggers degradation of target mRNA. High-purity control siRNA oligonucleotides that target the sequence 5′-AATTCTCCGAACGTGTCACGT-3′ were purchased from Qiagen (Valencia, CA). This scrambled sequence does not match any human genome sequence. EGFR siRNA duplexes that target the sequences 5′-AACACAGTGGAGCGAATTCCT-3′ as described previously (22) was synthesized by Qiagen. SMARTpool COX-2 siRNA which contains four individual duplexes was purchased from Dharmacon (Lafayette, CO).

Transfection of siRNA was conducted with RNAiFect transfection reagent (Qiagen) according to the manufacturer's instructions. Briefly, Tu212 cells were maintained in DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum. The cells were seeded into 24-well plate at concentration of 8×10^4 per well in triplicates for 24 hours before siRNA transfection. The cells were then transfected with EGFR (1 μg) or COX-2 (1 μg) siRNA. After 24 hours, the transfection medium was replaced by complete medium containing either celecoxib (25 μmol/L) or ZD1839 (1 μmol/L), respectively. The cells were incubated in the presence of the drugs for another 48 hours. For siRNA cotransfection study, the cells were transfected with COX-2 siRNA (0.5 μg) alone, EGFR siRNA (0.5 μg) alone, or the combination of COX-2 siRNA (0.5 μg) and EGFR siRNA (0.5 μg). The cells were incubated in the transfection complexes for 48 hours.

At the end of the experiment, the cells were trypsinized and the cell number was determined using a hemocytometer for cytotoxic assay. Fifty micrograms of the whole cell lysates for each sample were also made for immunoblotting analysis as described above to detect gene-silencing efficiency. All experiments were repeated thrice.

Immunohistochemistry. Immunohistochemistry analysis on formalin-fixed, paraffin-embedded nude mouse xenografts tissue was done using Cell and Tissue Staining Kit following the standard manufacture's protocol (R&D Systems, Minneapolis, MN). The primary antibodies used for this study were rabbit anti-human COX-2 (1:100, Cayman Chemical) and rabbit anti-human vascular endothelial growth factor (VEGF, 1:30, Santa Cruz Biotechnology). The incubation time for the primary antibodies was overnight at 4°C. The slides were stained with R&D 3,3′-diaminobenzidine and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA).

Mouse anti-human monoclonal antibody Ki-67 (Prediluted, Biomeda Corp., Foster, CA) was also employed for immunohistochemical analysis using the same specimens as above. R.T.U. Vectastain kit was used for this staining according to the standard procedure from the manufacture (Vector Laboratories). The incubation time for primary antibody was overnight at 4°C.

The intensity of immunohistochemical staining was measured using a numerical scale (0 = no expression, 1^+ = weak expression, 2^+ = moderate expression, and 3^+ = strong expression). The staining was quantified as weight index [WI = % positive staining (>0) in tumor × intensity score].

Statistical analyses. A linear mixed model with random intercept was fitted to the log transformed data to test for the treatment effects on tumor growth (23). Times to reach 500 mm^3 in different treatment groups were compared by the log-rank test (24). Kaplan-Meier curves for each treatment group were also provided (25). Pairwise comparisons of PGEM concentration and expression levels of p-EGFR as well as its downstream molecules were done using a Wilcoxon test (26). The Kruskal-Wallis and Wilcoxon tests were adopted to test the overall and pairwise weight index of VEGF and Ki-67 expression in four treatment groups, respectively (26).

Results

Effect of treatment with ZD1839, celecoxib, or their combination on the growth of mouse xenografts. Our previous study has shown that a combined treatment of ZD1839 with celecoxib has synergistic or additive inhibitory effects on growth of several SCCHN cell lines *in vitro* (20). To evaluate the antitumor efficacy of this combination regimen on SCCHN *in vivo* and to mimic an intrinsically preventive environment in the mouse body, we pretreated each group of mice with vehicle as a control, ZD1839 alone, celecoxib alone, or the combination of the two drugs for 7 days before s.c. injection of Tu212 tumor cells. The animals were continuously given with agents after tumor injection. The antitumor activities were analyzed in different treatments when the first group of mice (control group) was sacrificed (Fig. 1A). A linear mixed model with random intersect was fitted to the log-transformed data to compare the tumor growth over time in different treatment groups. The results indicated that although ZD1839 alone moderately inhibited tumor growth, no significant difference was found compared with the control (P = 0.17) and celecoxib (P = 0.41) groups. Celecoxib alone resulted in similar tumor growth pattern to the control group (P = 0.57). However, the combined treatment of ZD1839 with celecoxib significantly inhibited tumor growth compared with the control (P < 0.001), ZD1839 (P = 0.005), or celecoxib alone (P < 0.001). A representative Tu212 xenograft tumor in four treatment groups is presented in Fig. 1B. One mouse in the combination group did not develop tumor at all. One mouse in ZD1839 treatment group was inadvertently sacrificed early in the experiment; thus, only five mice in the ZD1839 group were available for analysis. Two mice in the ZD1839 group and one in the combination group showed mild skin rashes during the treatment that fully resolved in 1 month. No other adverse events were recorded during the treatment.

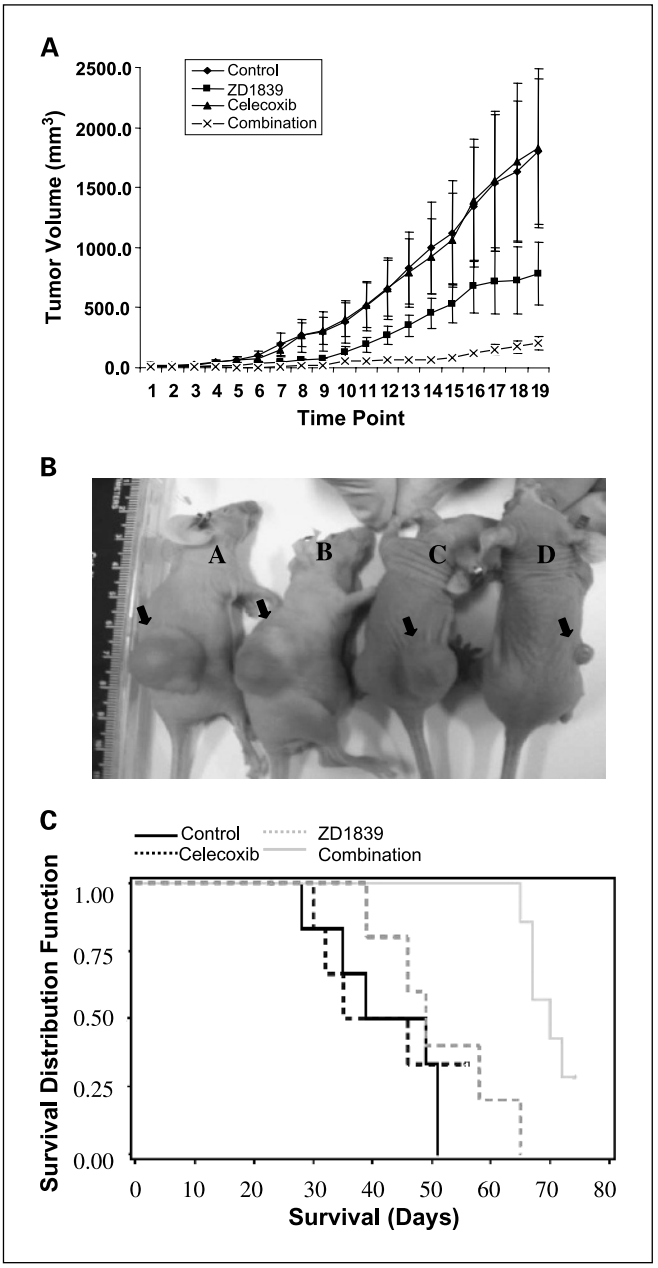

Fig. 1. Tumor growth effects of the treatment with ZD1839, celecoxib, and in combination on SCCHN xenografts. Four group of animals were pretreated with control (1% Tween 80, n = 6), ZD1839 (50 mg/kg, n = 5), celecoxib (50 mg/kg, n = 6), and in combination (same dosages, n = 7) for 7 days, respectively. The mice were then injected s.c. with 2×10^6 of Tu212 cells. The animals were continuously gavaged with the agents 5 days a week as described in Materials and Methods. *A, points,* mean tumor volume over time; *bars,* ±SE. Tumor volume was measured thrice per week as indicated as time points. Tumor growth curve was shown during the period from first xenograft appeared until first group of mice (*control*) was sacrificed. ZD1839 alone moderately inhibited tumor growth, but celecoxib as a single agent was almost ineffective. A dramatic and sustained inhibitory effect was achieved by the combined treatment with ZD1839 and celecoxib at the same dosages. *B,* representative Tu212 xenograft tumors in the four treatment groups. *C,* Kaplan-Meier curves showed time to reach 500 mm^3 in tumor size over time. The average of time (mean ± SE days) from tumor cells injection to tumor reached a volume of 500 mm^3 was significantly delayed in combination treatment (69 ± 1.1) compared with control (42.2 ± 3.9), ZD1839 (51.4 ± 4.6), or celecoxib treatment alone (39.2 ± 3.3).

***Combination of ZD1839 with celecoxib significantly delayed tumor progression* in vivo.** To evaluate whether the combined treatment of ZD1839 with celecoxib delayed tumor progression in mouse xenografts, time (days) from the date of tumor cell injection until the date when tumor size reached 500 mm^3 was also recorded. Kaplan-Meier curves showed the proportion of mice whose tumor size did not reach 500 mm^3 over time in each treatment (Fig. 1C). The mean time (mean ± SE days) to reach a tumor volume of 500 mm^3 was delayed in combined treatment (69.3 ± 1.1) compared with control (42.2 ± 3.9), ZD1839 (51.4 ± 4.6), or celecoxib treatments (39.2 ± 3.3). Log-rank tests showed dramatic time delay effects in the combined treatment group compared with the control (P = 0.0003), ZD1839 (P = 0.0007), or celecoxib treatment groups (P = 0.01). Although ZD1839 alone also postponed tumor progression, no statistically significant difference was obtained compared with the control (P = 0.24) and celecoxib (P = 0.49) treatment group. The proportion of mice that did not reach 500 mm^3 in tumor size over time in the Celecoxib group was similar to that of the control group (P = 0.61).

Effect of ZD1839, celecoxib, and combined treatment on blood prostaglandin E metabolite level in nude mice xenografts. PGE_2 is one of the major products of COX-2-catalyzed reaction. PGE_2 is rapidly converted *in vivo* to its metabolites (PGEM) with >90% of circulating PGE_2 cleared by a single passage through the lungs. We measured PGEM levels (mean ± SE pg/mL) in nude mice blood to estimate the actual PGE_2 production. Our results showed that both ZD1839 (1,553 ± 420.5 pg/mL, n = 5) and celecoxib (1,074 ± 170.9 pg/mL, n = 6) reduced the PGEM levels, whereas the combined treatment (757 ± 84 pg/mL, n = 7) further decreased PGEM production compared with the control group (1,791 ± 154.5 mg/mL, n = 6; Fig. 2). Although no significant difference was obtained in the combined treatment compared with ZD1839 (P = 0.07) or celecoxib (P = 0.18), significant differences were found between the combined treatment group and the control group (P = 0.001), and between the celecoxib and the control group (P = 0.03). A significant difference was not shown between ZD1839 and celecoxib treatment (P = 0.43) nor between the ZD1839 treatment and the control group (P = 0.43).

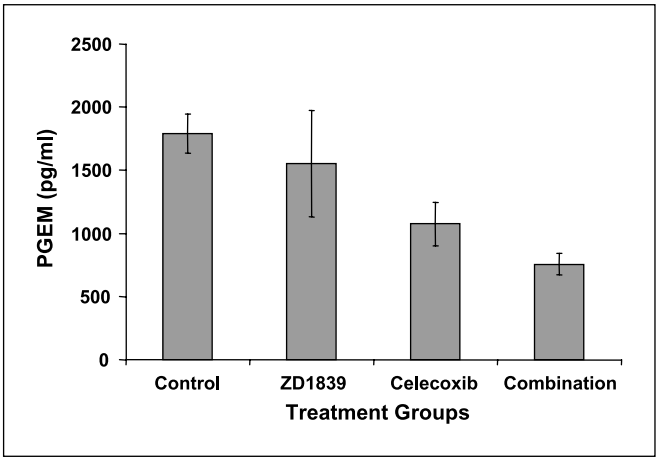

Fig. 2. Effect of different treatment on blood levels of PGEM in SCCHN xenograft model. Four groups of animals were treated with control (1% Tween 80, n = 6), ZD1839 (50 mg/kg, n = 5), celecoxib (50 mg/kg, n = 6), and in combination (same dosages, n = 7) as described in Materials and Methods. Blood was drawn by heart puncture from each mouse for PGEM assay. PGEM production in each group (mean ± SE pg/mL): control = 1,791 ± 154.5 pg/mL, ZD1839 = 1,553 ± 420.5 pg/mL, celecoxib = 1,070 ± 170.9 pg/mL, combination = 757 ± 84 pg/mL.

***Combination of ZD1839 with celecoxib reduced levels of phosphorylated epidermal growth factor receptor, phosphorylated extracellular signal-regulated kinase, and phosphorylated signal transducer and activator of transcription 3* in vivo.** To elucidate the molecular mechanisms of the treatment effects with ZD1839, celecoxib, and their combination on mouse SCCHN xenograft, Western blotting was done to detect expression levels of p-EGFR and its downstream molecules in different settings. Our results showed that whereas celecoxib alone had a minimum effect on p-EGFR expression, ZD1839 alone effectively reduced p-EGFR expression level. Interestingly, the combined treatment of ZD1839 with celecoxib almost abolished p-EGFR level, whereas total EGFR expression was not affected (Fig. 3A). Two-sided Wilcoxon tests were used to analyze the intensity of densitometeric image data. The results indicated that expression level of p-EGFR in the combination group was significantly down-regulated compared with that in the control group (P = 0.002), ZD1839 (P = 0.03), or celecoxib alone (P = 0.002). Significant differences of expression of p-EGFR were also observed between ZD1839 and the control group (P = 0.004) and between ZD1839 and celecoxib group (P = 0.004). No statistically significant difference was found between the celecoxib and control group (P = 0.82).

Similarly, p-ERK was also down-regulated by ZD1839 treatment. Celecoxib alone showed similar expression level of p-ERK to the control group. Importantly, the combined treatment of ZD1839 with celecoxib further down-regulated p-ERK level (Fig. 3B). Densitometeric image analysis showed that the combined treatment significantly reduced p- ERK level compared with the control group (P = 0.04). No significant difference was obtained in the comparisons between the combined treatment group and the single treatment groups (P = 0.08 and 0.09 for ZD1839 group and celecoxib group, respectively).

STAT3 is another important signaling transducer to regulate growth of tumor cells and is involved in comparable pathways including EGFR signaling pathway. Our results showed that celecoxib as single agent down-regulated p-STAT3 expression in mouse xenografts; ZD1839 did not significantly interfere with its expression. The combined treatment of ZD1839 with celecoxib further decreased p-STAT3 expression (Fig. 3C). However, no significant difference of p-STAT3 expression was obtained in combined treatment group when compared with control (P = 0.1), ZD1839 alone (P = 0.25), or celecoxib alone (P = 0.48).

Cell growth inhibition by specifically knocking down EGFR and COX-2 genes using small interfering RNA. To investigate whether the cell growth inhibition by ZD1839 and celecoxib was specifically mediated by EGFR- and COX-2-related signaling transduction pathways, we employed siRNA technology to specifically knock down target genes. After transfection of EGFR or COX-2 siRNA into Tu212 cells, the expression of EGFR and COX-2 was significantly suppressed (Fig. 4A, B, C), indicating that the gene silencing by siRNA was successful. We initially knocked down *EGFR* gene, and then the cells were continuously incubated with celecoxib (25 μmol/L) for 48 hours. Interestingly, the addition of celecoxib further reduced EGFR phosphorylation compared with that in simply EGFR siRNA-transfected cells (Fig. 4A). Similarly, in COX-2 knockdown

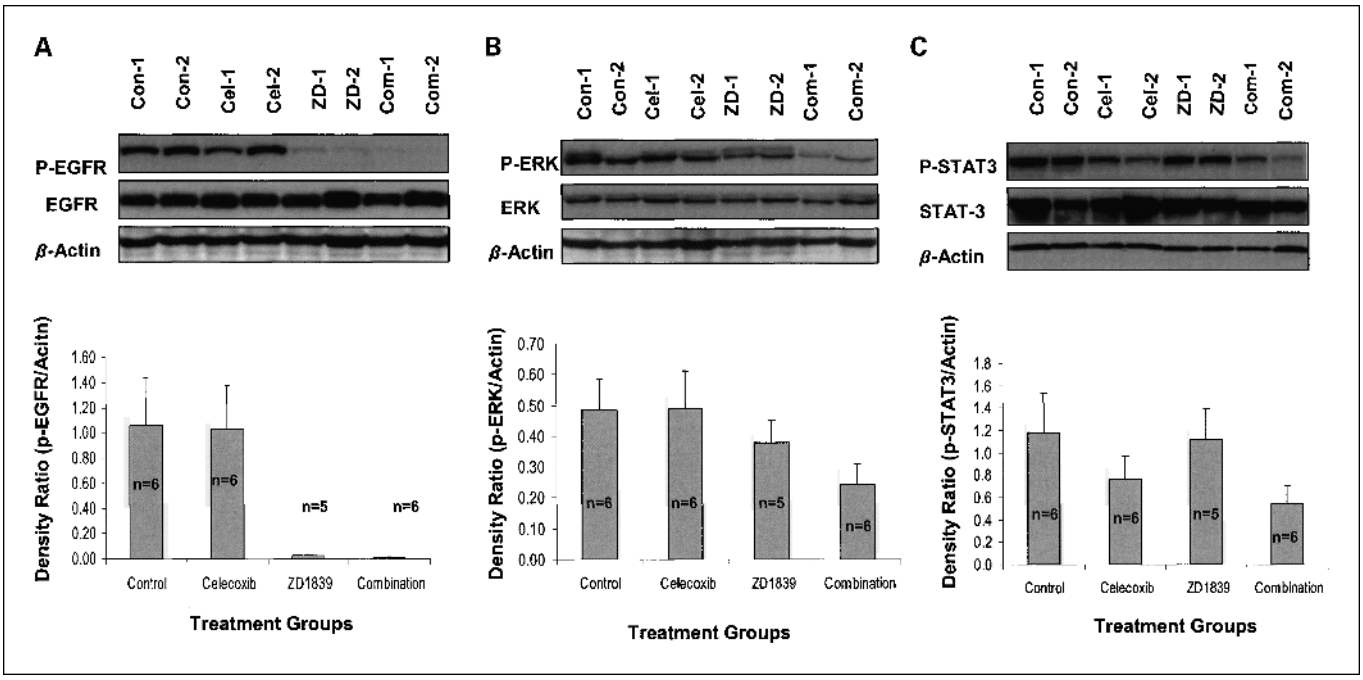

Fig. 3. Regulation of expression levels of p-EGFR and its downstream molecules by the different treatments in SCCHN mouse xenograft tissues. Proteins were extracted from each mouse xenografts. Protein (100 μg) for each sample was used for Western blot as described in Materials and Methods. *A*, expression of p-EGFR and its total level. *B*, p-ERK and its total level and (*C*) p-STAT3 and its total level in the different treatment group. *Top,* representative Western blot results for two samples from each group; *bottom,* statistical results for all samples using densitometeric image analyses for Western blot.

cells, the addition of ZD1839 (1 μmol/L) further suppressed p-EGFR expression (Fig. 4B). We then extended to cotransfect cells with both EGFR and COX-2 siRNA. Only half amount of each single siRNA was used in this study to reduce the toxicity of transfection. However, the cotransfection of EGFR and COX-2 siRNA not only more efficiently knocked down EGFR expression but also further reduced p-EGFR level compared with each siRNA transfection alone (Fig. 4C). The transfection of COX-2 siRNA did significantly knock down COX-2 expression. However, either addition of ZD1839 or cotransfected with EGFR siRNA did not further reduce COX-2 expression (Fig. 4B-C).

In addition, effect of the gene silencing on cell growth was observed. Transfection of single EGFR siRNA inhibited cell growth by 30%; celecoxib alone inhibited cell growth by 50%. However, the addition of celecoxib in EGFR knockdown cells reduced the cell growth up to 65% (Fig. 5A). Although knocking down COX-2 by siRNA did not apparently affect cell growth (only about 10% of control siRNA group), the addition of ZD1839 in COX-2 knockdown cells showed significantly cytotoxic activity compared with each single treatment (Fig. 5B). The similar effect of cell growth inhibition was also observed in the siRNA-cotransfected cells, although the efficacy was not as good as that in the other two groups (Fig. 5C).

Effect of combination treatment of ZD1839 and celecoxib on COX-2, vascular endothelial growth factor, and Ki-67 expressions. Immunohistochemistry was done to examine expression of VEGF and Ki-67, important biomarkers for angiogenesis and cell proliferation, respectively, in tumor xenograft tissues. The expression levels were presented as WI (mean ± SE). The expression of VEGF was decreased in the combined treatment (72.5 ± 7.0) compared with the control (92 ± 13.9), ZD1839 alone (93 ± 9.7), and celecoxib alone (91 ± 11.9). However, the omnibus test of equality of VEGF expression in all four groups was not statistically significant (P = 0.1, Fig. 6A-B).

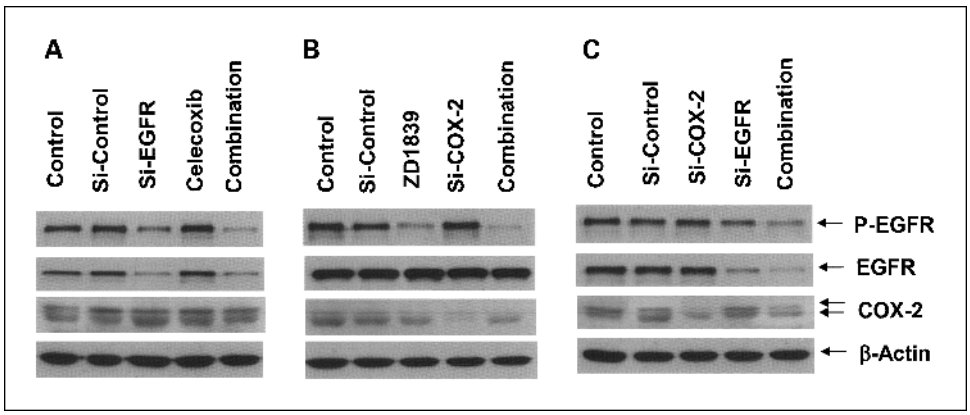

Fig. 4. Silencing of gene expression with siRNA in Tu212 cells. Immunoblotting analysis was used to examine levels of EGFR and COX-2 proteins after treatments with the relevant siRNAs. Fifty micrograms of the whole cell lysate were collected from for each treatment. *A,* cells were initially transfected with EGFR siRNA (1 μg) for 24 hours and then treated with or without celecoxib (25 μmol/L) for another 48 hours. *B,* after the cells were transfected with COX-2 siRNA (1 μg) for 24 hours, they were continuously treated with or without ZD1839 (1 μmol/L) for another 48 hours. *C,* cells were either transfected with EGFR siRNA (0.5 μg) alone, COX-2 siRNA (0.5 μg) alone, or cotransfected with both of the siRNAs at the same concentration as used alone for 48 hours.

Similarly, the two drug combination inhibited Ki-67 expression (86 ± 14.2) compared with the control (127 ± 7.3), ZD1839 (115 ± 15.3), or celecoxib treatment alone (128 ± 18.8). Although no statistical difference among all four groups was found (P = 0.38 by the omnibus test; Fig. 6A-C), a pairwise comparison using the Wilcoxon rank sum test showed a significant difference between the control and the combination treatment (P = 0.02).

Immunohistochemistry was also used to detect COX-2 expression in mouse xenograft tissues. COX-2 was stained in cytoplasm of tumor cells of all four groups. The expression levels of COX-2 in the four groups did not reach any significant difference (data not shown).

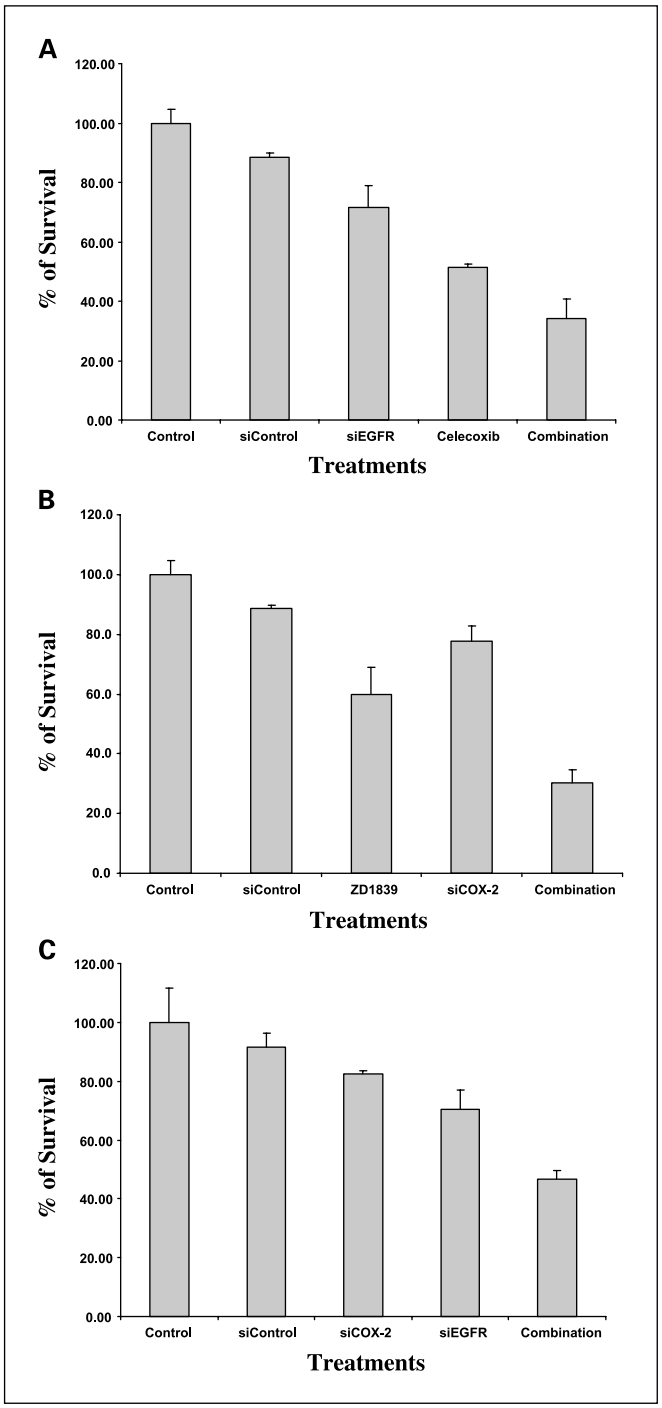

Fig. 5. Effects of gene silencing on cell proliferation in Tu212 cells. Inhibitory effects of the EGFR and COX-2 siRNAs on tumor cell proliferation were examined in three groups. *A,* cells were initially transfected with EGFR siRNA (1 μg) for 24 hours. The transfected cells were then treated with or without celecoxib (25 μmol/L) for another 48 hours. *B,* after the cells were transfected with COX-2 siRNA (1 μg) for 24 hours, they were continuously treated with or without ZD1839 for another 48 hours. *C,* cells were transfected with either EGFR siRNA (0.5 μg) alone, COX-2 siRNA (0.5 μg) alone, or cotransfected with both of them at the same amount of siRNA for 48 hours. At the end of the treatment, the cells were trypsinized and the cell number was determined using a hemocytometer for cytotoxic assay.

Discussion

A growing body of evidence shows that both EGFR signaling and COX-2 activity play key roles in developing premalignant and malignant diseases, and these two pathways have been considered as attractive targets for anticancer therapy and cancer chemoprevention (14, 27, 28). Both EGFR TKIs and COX-2-selective inhibitors have been used as agents for cancer treatment and chemoprevention (15, 29). Furthermore, combination of either EGFR TKIs or COX-2-selective inhibitors with cytotoxic agents or radiotherapy achieved additive or synergistic antitumor effects in a variety of human cancers (30–32). Recently, a direct interaction between EGFR signaling and COX-2 activity has been suggested by several researchers (16–19), which led us to speculate that simultaneous targeting both EGFR and COX-2 may be a more effective strategy to abrogate both signal transduction pathways and their downstream molecules.

In our previous study, a combined treatment of EGFR TKI (ZD1839) with COX-2 inhibitor (celecoxib) showed an additive/synergistic growth inhibition of SCCHN cell *in vitro* (20). The cell growth inhibition was associated with cell cycle arrest at G_1 phase, induction of apoptosis, and reduction of EGFR- and COX-2-mediated signal transducers, including phosphorylated EGFR, ERK, and Akt (20). To confirm our *in vitro* observation, we did an *in vivo* study using a nude mice xenograft model. We pretreated mice with four different settings of the agents, including the vehicle control, celecoxib, or ZD1839 alone, and the combination of ZD1839 with celecoxib for 1 week before injection of SCCHN cells Tu212 into the mice. The purpose of this pretreatment was to create a suitable internal environment to prevent from tumor growth in nude mice. Our result showed that single-agent celecoxib (50 mg/kg) did not affect tumor growth, whereas ZD1839 alone (50 mg/kg) did moderately reduce the tumor volume. Importantly, the combined treatment of ZD1839 with celecoxib at same dosages of single agents dramatically inhibited tumor growth. Immunohistochemical study on mouse xenograft tumors showed that the expression level of a cell proliferation marker Ki-67 matched the tumor growth pattern in each group. Moreover, as expected, the time period from tumor cells injection to average tumor size reaching 500 mm^3 in the combined treatment group was significantly delayed compared with the control, ZD1839, or celecoxib treatment alone. This result is not unexpected because overexpressions of EGFR and COX-2 have been illustrated in premalignant lesion of SCCHN (13, 33). Overactivation of these two proteins may be an early event for carcinogenesis in oral cavity. Therefore, inhibiting activation of them in both SCCHN tumor and the environment for the tumor growth should prevent tumor development.

One of the main functions of these two drugs is blocking EGFR/mitogen-activated protein kinase signaling pathway. In our study, we measured the levels of phosphorylated EGFR and ERK in mouse xenograft tumors. ZD1839, but not celecoxib, effectively reduced expression of both p-EGFR and p-ERK. Furthermore, the combined treatment with the two drugs significantly inhibited p-ERK and almost completely abolished p-EGFR expression. These results were consistent with our previous *in vitro* observation (20), strongly suggesting a cooperative effect of this combined treatment on suppressing EGFR-mediated mitogen-activated protein kinase pathway.

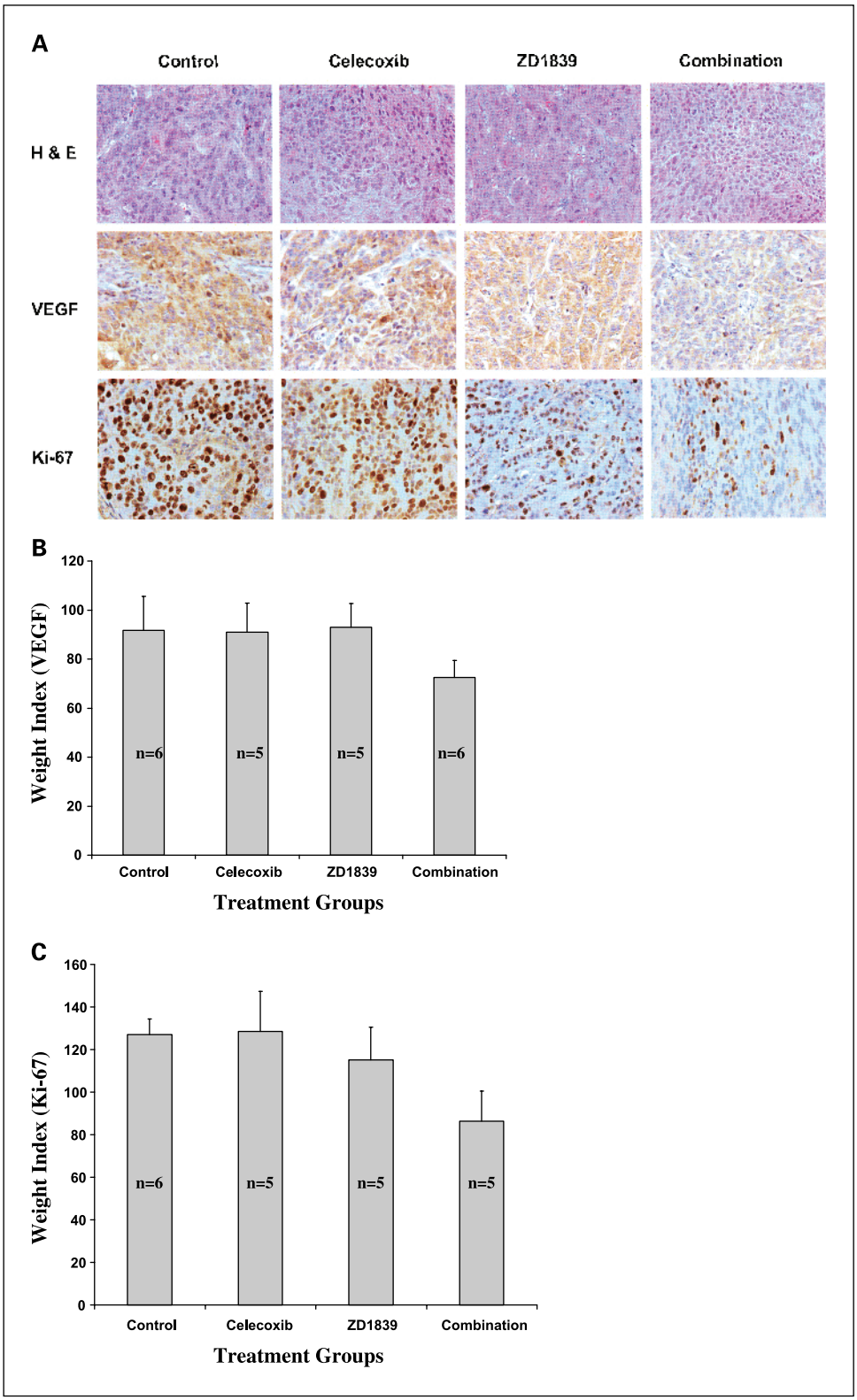

Fig. 6. Expression of VEGF and Ki-67 in different treatment group in SCCHN mouse xenograft tissues. Immunohistochemistry was used to determine expression levels of VEGF and Ki-67 in mouse xenograft tissues by the different treatments as described in Materials and Methods. *A,* a representative sample from each group was stained in the picture. *Top,* H&E staining for each sample (× 400). *Middle,* expression of VEGF in combined treatment was decreased compared with that in other three groups (× 400). *Bottom,* ZD1839 in single agent down-regulated expression of Ki-67 protein, whereas the combined treatment further reduced its expression (× 400). The sample shown in each group is the same sample for H&E, VEGF, and Ki-67 staining. *B, columns,* mean VEGF expression level of weight index in each group; bars, ±SE. *C, columns,* mean Ki-67 expression level of weight index in each group; *bars,* ±SE.

Cumulative evidence showed that STAT3 is also a crucial downstream signaling mediator of transforming growth factor-α/EGFR autocrine pathway (34, 35). Our results showed that celecoxib as single agent more effectively inhibited p-STAT3 expression than ZD1839. Because phosphorylation of STAT3 is also regulated by PGE_2 through an interleukin receptor (36), it may be affected directly by celecoxib through reduction of PGE_2 rather than through modulation of EGFR-mediated pathway. Importantly, the combination of celecoxib with ZD1839 showed further down-regulation of the p-STAT3 level, although no statistical significance in expression level of p-STAT3 among each group had been reached yet due to limited sample size, implying that cooperative inhibitory efficacy of the two drugs in combination on SCCHN growth may also function through affecting STAT3-related signaling transduction pathway.

In this study, we also compared PGEM levels in mice plasma in each group. As consistent with our *in vitro* observations and other reports (20, 37, 38), celecoxib or ZD1839 as single agent could reduce PGEM levels. However, both agents in combination further decreased PGEM production. The current *in vivo* results strongly suggested that the cooperative inhibitory effect of the combination on tumor growth was at least partially resulted from reduction of COX-2 activity in mice carrying SCCHN tumors. Our *in vitro* study and others showed both EGFR and COX-2 inhibitors can down-regulate COX-2 expression (17, 20, 38). However, we did not observe significant reduction of COX-2 expression in xenograft tumors by treatment of ZD1839 or celecoxib alone, or the combination treatment compared with the control using immunohistochemical analysis. One possible explanation is that the dosage for ZD1839 or celecoxib used in this study may not be high enough to reduce COX-2 expression levels in tumor cells. The interaction of xenograft tumor with *in vivo* environment of the mice may also potentially influence gene expression. On the other hand, celecoxib only suppresses COX-2 activity instead of changing the expression level of COX-2.

It was also reported that the antitumor effects of both EGFR-TKIs and COX-2 selective inhibitors are target independent instead of the target-dependent mechanism in some cancer cells. Campiglio et al. found that inhibition of proliferation and induction of apoptosis in breast cancer cells by ZD1839 is independent on EGFR expression level (39). The celecoxib-mediated growth inhibition may also be independent on COX-2 expression (40–42). To further investigate whether the activity of cell growth inhibition by ZD1839 and celecoxib in our study was mediated through targeting EGFR- and COX-2-mediated signaling transduction pathways, we transfected target siRNAs into Tu212 cells to specifically knock down EGFR and COX-2 gene expression. Our results showed that introduction of EGFR siRNA not only effectively knocked down EGFR expression but also significantly reduced its phosphorylation. Interestingly, the addition of celecoxib in the EGFR knockdown cells further decreased p-EGFR expression. On the other hand, the addition of ZD1839 in COX-2 knockdown cells also further suppressed p-EGFR level compared with ZD1839 treatment alone. When we cotransfected both EGFR and COX-2 siRNA into Tu212 cells, half amount of each siRNA was used to avoid the toxicity of the transfection reagent. It is expected that the cotransfection efficiency was not as good as that in single siRNA transfection. Although COX-2 siRNA alone did not affect EGFR expression in this experiment, the cotransfection of both EGFR and COX-2 siRNA still more effectively inhibited EGFR expression and its phosphorylation compared with EGFR siRNA single transfection.

More importantly, either addition of celecoxib in the EGFR knockdown cells, addition of ZD1839 in the COX-2 knockdown cells, or cotransfection of both EGFR and COX-2 siRNAs into the cells, all achieved cooperative effects of cell growth inhibition. These results strongly indicated that an interaction between EGFR and COX-2 signaling exists in SCCHN. Simultaneously targeting EGFR and COX-2 may cooperatively block EGFR- and COX-2-mediated signaling transduction pathways and in turn, inhibit tumor cell growth.

Many studies have shown that both EGFR signaling and COX-2 activity contribute to tumor angiogenesis (for review, see refs. 43, 44). Tumor angiogenesis is effectively suppressed by EGFR- or COX-2-targeting strategies through inhibiting expression of VEGF and other angiogenesis factors (45, 46). Analysis of the mouse xenograft tumors also showed a potent reduction of VEGF expression in the combination treatment compared with the control or treatments with the single agents. Our result suggested the two drugs in combination may more potently inhibit angiogenic pathway than any of the single drugs. Although it is not clear why each single agent did not change the expression level of VEGF. This may be also due to relative low dosages of each agent used in our experiment.

Both ZD1839 and celecoxib are orally active, noncytotoxic selective agents targeting specific molecules involving in crucial signaling transduction pathways for cancer cells proliferation. Agents suitable for chemoprevention and long-term cancer control should have mild and differing toxicity patterns as well as simple administration route. In agreement with these requirements, we only recorded that three mice had mild rashes and were spontaneously recovered within a few weeks. No any other adverse events were observed during the experiment, supporting that these two agents are suitable for chemoprevention. Our results using a xenograft mouse model as well as previous *in vitro* study in SCCHN provides a promising support for using this combined treatment with an EGFR TKI and a COX-2 inhibitor for chemoprevention and cancer therapy. Our observation is supported by a study showing that using a combination of an EGFR TKI (EKB-569) and a COX-2 inhibitor (Sulindac) significantly reduced intestinal polyps in $APC^{min/+}$mice compared with the use of single agents alone (47). Tortora et al. has recently reported that combination of an EGFR TKI (ZD1839), a COX-2I (SC-236), and a protein kinase A antisense molecule achieved significant antitumor and antiangiogenic effects (48). Two most recent reports also illustrated the cooperated inhibitory effect of EGFR-TKI and COX-2 inhibitor on growth of breast, lung, pancreas, colon, and gastric carcinomas (49, 50). Therefore, the combination of EGFR-TKI and COX-2 inhibitors deserves attention in future clinical studies.

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