의학박사 학위논문

The role of reactive oxygen species in combination therapy of 5-FU and celecoxib in head and neck squamous cell carcinoma

두경부 편평상피세포암에 대한 5-FU와 celecoxib 병합치료의 효과에서 활성산소의 역할에 대한 연구

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의학과 이비인후과학 전공

이 도 영

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두경부 편평상피세포암에 대한 5-FU 와 celecoxib 병합치료의 효과에서 활성산소의 역할에 대한 연구

The role of reactive oxygen species in combination therapy of 5-FU and celecoxib in head and neck squamous cell carcinoma

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The Department of Otorhinolaryngology
Head and Neck Surgery,
Seoul National University
College of Medicine
Doh Young Lee

ABSTRACT

Introduction: The role of celecoxib in preventing and treating tumors has attracted broad attention in recent years because of its selective and specific inhibition of COX-2 activity. We investigated the inhibitory effects and mechanisms of celecoxib combined with 5-FU on proliferation of squamous cell carcinoma cells in vivo and in vitro.

Methods: SNU-1041 and SNU-1076 squamous cell lines and an orthotopic tongue cancer mouse model were used to study growth inhibition with 5-FU enhanced by celecoxib. Sensitivity of cells to drug treatment was analyzed by the MTT assay, and generation of reactive oxygen species (ROS) was measured using dichlorofluorescein diacetate (DCFH-DA). Phosphorylation of AKT was detected by Western blotting. Survival analysis in the mouse model was assessed according

to combination treatment with 5-FU and celecoxib.

Results: ROS production in vitro was highest when celecoxib was administered 48 hours after 5-FU treatment. 5-FU-induced inhibition of cell proliferation was enhanced when combined with celecoxib, which was positively correlated with ROS production. Antioxidant treatment reversed 5-FU-inhibited cell proliferation by up to 60%. Co-treatment with celecoxib and 5-FU partially blocked AKT phosphorylation, although no significant changes in total AKT protein levels were detected. An increased survival time was observed in an orthotopic mouse model treated with a combination of celecoxib and 5-FU compared to treatment with either agent alone.

Conclusions: Celecoxib may have an enhanced anticancer effect in combination with 5-FU. ROS production may be a key mechanisms in this combination therapy by inhibiting the AKT

pathway.

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Keywords: celecoxib, 5-FU, AKT pathway, reactive oxygen species, head and neck cancer, squamous cell carcinoma
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INTRODUCTION

The combination of cisplatin and 5-fluorouracil (5-FU) for induction treatment of head and neck squamous cell carcinoma (HNSCC) has been acknowledged as the gold standard for decades. Substantial progress has been made in treating the disease's advanced stages, mainly with the optimal combination of chemoradiotherapy in the induction phase of treatment.[1,2] Facilitated transport mechanism enhances rapid entrance of 5-FU into cells, and 5-FU makes anticancer effect by suppression of DNA replication through thymidylate synthase inhibition. Acquired resistance against 5-FU remains an important issue in 5-FU-based cancer chemotherapy, while there are several molecular mechanisms of acquired 5-FU resistance to be elucidated.[3]

Cyclooxygenase (COX)-2 is overexpressed in HNSCC and is believed to be correlated with decreased apoptosis and increased angiogenesis and invasiveness of cancer cells.[4]

Consequently, inhibiting COX-2 is considered to be an alternative option in treating HNSCC, and celecoxib has been shown to be effective in suppressing tumor growth, angiogenesis, and metastasis. In previous studies, we reported possible anticancer mechanisms of celecoxib when combined with cisplatin.[5,6] Clinical data have also shown an improved response to chemotherapy when celecoxib is added to conventional regimens.[7,8]

Given that celecoxib can have an effect in combination with other chemotherapeutic agents and because 5-FU is one of the most important drugs in combination chemotherapy for advanced HNSCC, evaluating the combination effects of celecoxib and 5-FU has potential clinical impacts. Until recently, no studies have assessed the outcomes or mechanisms of combined 5-FU and celecoxib therapy. We showed that increased reactive oxygen species (ROS) production during chemotherapy is an important mechanism in the anticancer effects of this combination treatment.[9] Changes in ROS production during HNSCC treatment with 5-FU,

celecoxib, or the combination of both agents may correlate with chemotherapy outcomes. In this study, we examined the anticancer effects of 5-FU on HNSCC cell lines enhanced by celecoxib in vivo and in vitro. Possible mechanisms of anticancer effects involving ROS production are also discussed.

MATERIALS AND METHODS

Cell culture

SNU-1041 and SNU-1076 HNSCC cell lines were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). SNU-1041 and 1076 was derived from the squamous cell carcinoma of pharynx and larynx, respectively. Cultures were maintained in an air atmosphere of 5% CO_2 in air at 37 ° C, and regularly subcultured using trypsin-EDTA (0.25% w/v). All the reagents using for cell culture were obtained from Gibco BRL (Grand Island, New York, USA).

Chemicals

Celecoxib (COX-2 inhibitor) was a gift from Pharmacia Korea (Seoul, Korea). The antioxidant glutathione (GSH) was obtained from Cayman Chemical (Ann Arbor, MI). All chemicals were used according to published protocols (IC50 and

references).

Cell proliferation assay

Prior to the treatment of specific drugs, cells were incubated for 24 h at 37° C seeded in 96-well plates. After drug treatment, Cell Counting Kit-8 (Dojindo Lab., Tokyo, Japan) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide (Sigma) were used to measure cell proliferation according to the manufacturer's instructions.

Production of reactive oxygen species

Dichlorofluorescein diacetate (DCFH-DA) assay was performed to measure ROS generation. Modified Hank's buffered salt solution was used and each ROS values were modified to eliminate the effect of cell numbers. Cell monolayers were prepared in black, 96-well, flat-bottom microtiter plates, and intracellular ROS production was

measured using a Fluoroskan Ascent FL microplate reader (Labsystems, Sweden). Cells in complete medium were incubated with the indicated drugs for 18 hours. After the pretreatment period, cells were incubated with 5 μM DCFH-DA at 37° C in the culture medium for 30 minutes, and fluorescence was monitored with excitation wavelength at 480 nm and emission wavelength at 530 nm.

Western blot analysis

Extracts were prepared with resolution in 4-12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes (Schleicher and Schuell, Dachen, Germany) for 30 min at 350 mA in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol, pH 8.3). The membrane was blotted with primary antibody (anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-p-AKT (Ser473) and anti-pan-AKT (Cell Signaling, Danvers, MA)) or monoclonal anti-a-tubulin (Sigma) for 2 hours at room temperature or

overnight at 4° C. Membranes were washed with Tris-buffered saline and incubated with secondary antibody (Pierce, Rockford, Illinois, USA) for 1 hour. Blots were visualized by development with Lumi-light western blotting substrate (Roche Diagnostics GmbH, Mannheim, Germany) and subsequent exposure in a LAS-3000 (Fuji Film Co., Tokyo, Japan).

Orthotopic mouse model studies

Animal studies were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (IACUC No. 14-0193-S1A0) and all the procedures were carried out in accordance with institutional guidelines. First, $1x10^6$ cells in 15 μL of PBS were injected into the lateral tongue of 6- to 8-week-old nude mice as previously described.[10] After tumor growth on the tongue was established, the nude mice were randomly divided into following four groups (10 animals in each group); 1) celecoxib group, 2) 5-FU group, 3) celecoxib + 5-FU group,

and 4) control group. Dose and route of administration of celecoxib and 5-FU were 200mg/kg/day by gastric gavage and 100mg/kg/w by intraperitoneal injection, respectively. Control group was treated with intraperitoneal injection of 0.9% sodium chloride and oral sterile distilled water administration. When body weight had decreased by more than 30% of the original weight or when the tumor in the neck measured more than 1.5 cm in size, the mice were sacrificed.

Statistical analysis

Data are presented as the mean ± standard deviation of triplicate results, reflecting average results from three separate experiments. Significance levels between the treated and untreated groups were measured with two-sided Student's t-test. Survival curves were compared using a log-rank test. All statistical analyses were performed using SPSS V20.0 (IBM SPSS, New York, NY, USA). Statistical significance was defined as $p<0.05$.

RESULTS

Combination of 5-FU and celecoxib enhanced anticancer effects on head and neck squamous cell carcinoma by increasing production of reactive oxygen species

Among various NSAIDs, celecoxib showed highest ROS production when treated to head and neck cancer cell line (Fig. 1). ROS production was independently induced by treatment with celecoxib and 5-FU (Fig. 2, 3), and SNU-1041 cell lines treated with celecoxib showed the highest production. We combined 5-FU and celecoxib in various sequential experiments. ROS production was highest when celecoxib treatment was applied 48 hours after 5-FU treatment (Fig. 4, 5). 5-FU and celecoxib independently inhibited cell proliferation in HNSCC SNU-1041 and SNU-1076 cell lines. 5-FU-induced inhibition was enhanced when combined with celecoxib, particularly in the SNU-1041 cell line (Fig. 6).

Inhibition of cell proliferation was positively correlated with ROS production.

Antioxidants significantly reversed inhibition of cell proliferation

We confirmed the role of increased ROS leading to inhibition of cell proliferation by combining treatment with the antioxidant GSH. GSH reversed 5-FU-inhibited cell proliferation up to 60%, depending on the cell line (Fig. 7). Together with the results shown in Figure 3, this finding confirms that ROS induced by combined 5-FU and celecoxib treatment may play a significant role in inhibiting HNSCC cell proliferation.

Celecoxib potentiates 5-FU-induced inhibitory effects by inhibiting AKT phosphorylation

To investigate the possible role of the AKT pathway in potentiating the effects of celecoxib, we evaluated

phosphorylated AKT and total protein levels. pAKT was not suppressed when exposed to celecoxib or 5-FU alone. Co-treatment with celecoxib and 5-FU partially blocked AKT phosphorylation, although no significant changes in total AKT protein levels were detected (Fig. 8). In addition, AKT phosphorylation was recovered when cells were exposed to the antioxidant GSH. These results indicate that inhibiting AKT phosphorylation has very important anticancer effects.

Effects of celecoxib with 5-FU on survival in an orthotopic mouse model

The SNU-1041 cell line was used for the in vivo model, as the SNU-1076 cell line was toxic and mice could not survive the experiment schedule. Ten days after cell injection, tongue tumors were observed in all mice, and intravenous drug injection was initiated beginning on day 13. When the orthotopic mouse model was treated with a combination of celecoxib and 5-FU, changes in serial body weight were minimal (Fig. 9A)

and more significant growth inhibition of HNSCC was observed than using either agent alone (Fig. 9B, 10). Similarly, survival was longest when treated with combined 5-FU and celecoxib ($p=0.042$) (Fig. 9C). A Western blot of harvested specimens revealed that phosphorylation of AKT was significantly inhibited by combination treatment with celecoxib and 5-FU (Fig. 11).

DISCUSSION

Combined therapies with multiple anticancer drugs have been used in clinical practice in an effort to improve the efficacy of treatment. Because of the positive correlation between COX-2 expression and the resistance of HNSCC to anticancer drugs, COX- 2 inhibition played an important role in increasing the chemoresponse of tumor cells.[11,12] Our study demonstrated that celecoxib could enhance the inhibition effects of 5-FU by elevating the ROS production and inhibiting the AKT pathway. To the best of our knowledge, this was the first study to elucidate the effects of celecoxib on 5-FU treatment of HNSCC and the mechanisms of the effects.

Celecoxib is known to have a protective effect on normal cells and a synergistic effect on cancer cells.[13-16] The protective effect may be achieved by antagonizing the cytotoxic effect of a chemotherapeutic agent by decreasing intracellular accumulation or inhibiting the DNA damage. In contrast, the

synergistic effects of celecoxib have been shown to work through both COX-2-dependent and COX-2-independent mechanisms.[17] In fact, COX-2 is one of the overexpressed markers in HNSCC that correlates with a poor prognosis.[18] Thus, combining celecoxib twice daily was well tolerated in a phase I trial for patients who were suffering from recurrent locoregional and/or distant metastatic HNSCC. Another suggested important mechanism is ROS production. Although intracellular ROS is normally produced under aerobic conditions, unaerobic stress can influence and elevate the ROS level within the cell. Oxidative injury by increased intracellular ROS results in breakage of lipids and proteins in cell membrane.[19] Increased ROS within cells also cause extensive chemical modifications of DNA and nucleoproteins, breakage of DNA strands, and subsequent activation of the p53 pathway.[20,21]

Moreover, protein kinase pathways including MAPK/ERK pathway and PI3K/AKT pathway are also vulnerable to oxidative stress and following attack of free radicals in most cell types.[22,23] Our results showed the participation of AKT in

the combination effects of celecoxib and 5-FU, and celecoxib induced ROS accumulation in HNSCC in a dose-dependent manner. To further evaluate the importance of ROS, the antioxidant GSH was employed to ascertain the role of the oxidative stress signal in inducing apoptosis. Our results showed that blocking the signal with antioxidants effectively prevented cell apoptosis (Fig. 7). In addition, owing to the blocked ROS signal, inhibited AKT phosphorylation induced by 5-FU and celecoxib were almost completely reversed. These results suggested that ROS acted as an essential upstream molecular messenger in the enhanced antitumor effect of celecoxib with 5-FU, which is in accordance with previous studies that showed anticancer activities of celecoxib as an anti-oxidant.[24]

AKT is a key mid-stream molecule in protein kinase pathway which is significantly related to p53 activation, and PI3K/AKT pathway have been continuously suggested and evaluated as promising targets of anticancer drugs because of its role in growth and progression of malignant tumor.[25,26] Among the

COX-2-independent mechanisms of celecoxib, several studies have shown that AKT suppression results from inhibiting phosphoinositide-dependent kinase-1.[27-29] The activated AKT regulates cell proliferation, motility, invasion, and apoptosis.[30] It has been shown that in cancer cells, phosphorylated AKT not only stimulates cell proliferation and invasion but also triggers anti-apoptotic signals.[31] Therefore, AKT pathway is frequently activated in cancer cells, resulting in enhanced resistance to apoptosis through multiple mechanisms.[22] The exact points of mutation of AKT in SNU-10411 and SNU-1076 cell lines are important for evaluating the relevance of chemotherapeutic targets and the mechanisms of tumorigenesis. SNU-1076 cell line is known to have mutation in PI3K/AKT pathway, and SNU-1041 has not.[32] Therefore, we think that the difference in inhibition of cell or tumor growth may result from this mutation status of cell lines. Further study, including the analysis of mutation status of AKT and other genes in the PI3K/AKT pathway, may be needed to clearly define the roles of celecoxib and ROS related to proliferation inhibition. We think that our

results can be applied to other types of cancers, and it is expected that the significant results of combination treatment of celecoxib and 5-FU can be achieved in PI3K/AKT wild type cell line or tumor.

Our results indicated that celecoxib has a potent anticancer effect on HNSCC cells and that its receptor-independent increase of ROS mediates its effect on cancer cell death. In addition, an orthotopic tongue cancer model showed better survival and less weight reduction during the treatment (Fig. 9). Although our animal model was advantageous in mimicking the real human disease, the tumors were relatively small for serial measurement. However, we also observed that tumors regressed during the treatment, especially treatment with celecoxib and 5-FU combined. Survival was also longest in the combination treatment group.

Although 5-FU is not the main drug in single therapy in HNSCC because of its short half-life and cytotoxicity after frequent administration, our study indicated that celecoxib can be a good adjuvant in combination chemotherapy with 5-FU. The

sequence of celecoxib treatment and timing was important in our study: the enhanced antitumor effect was best when it was administered 48 hours after the 5-FU. Although we could not reach a clear conclusion, pretreatment with 5-FU may be important considering that celecoxib abrogates the drug-induced cell cycle arrest, enhancing premature entry into mitosis with damaged DNA; this then increases apoptosis, resulting in synergism.[33] Moreover, celecoxib may induce more ROS under circumstances of oxidative stress induced by 5-FU. Currently, in vivo imaging of hydrogen peroxide is possible in a mouse tumor model.[34] In the near future, we believe that the ROS production by combination treatment with celecoxib and 5-FU can be evaluated in this in vivo model and that the serial assessment of ROS production is possible using visual analysis. Moreover, exact source of ROS, such as mitochondrial or cytoplasmic ROS, should be evaluated in the further study. Because mechanistic investigations revealed that celecoxib exerts different molecular effects with different chemotherapeutic agents and in different cells, its combination

with other drugs should be tailored to the tumor type, drug, and drug administration schedule. In addition, it should be in consideration that the dosage used in in-vitro analysis is higher than used in in-vivo analysis and real clinical setting. We think the more evidences are needed for tailoring the drug combination in other tumor types.

Although the anticancer effects of celecoxib have been well established, they are not yet fully linked with specific molecular targets in cancer cells. In particular, no report has shown a enhanced antitumor effect of celecoxib on treatment with 5-FU. Our findings suggest that the extent of higher ROS accumulation correlates with this combination effect, and these findings could have future implications for clinical trials of combination chemotherapy for HSNCC patients.

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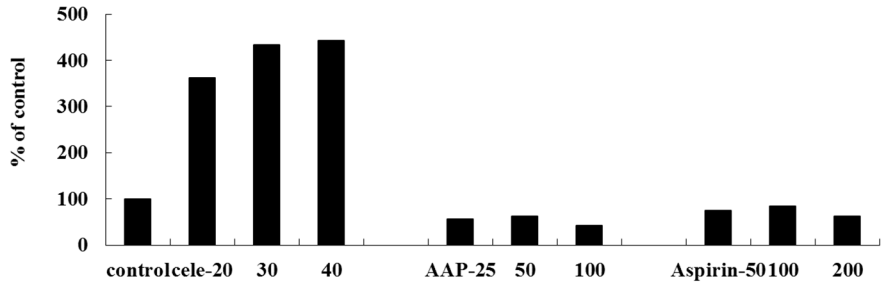
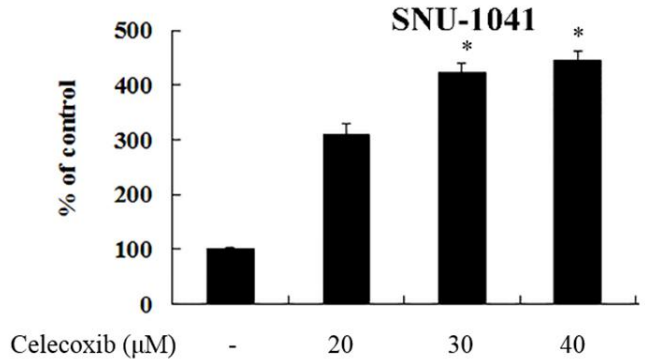


Figure 1. ROS production by celecoxib, acetaminophen (AAP), and aspirin

Celecoxib showed significantly higher ROS production than other chemicals when treated to head and neck squamous carcinoma cell line.

(A)



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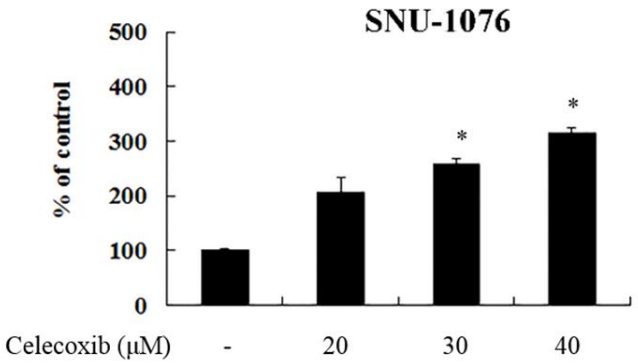
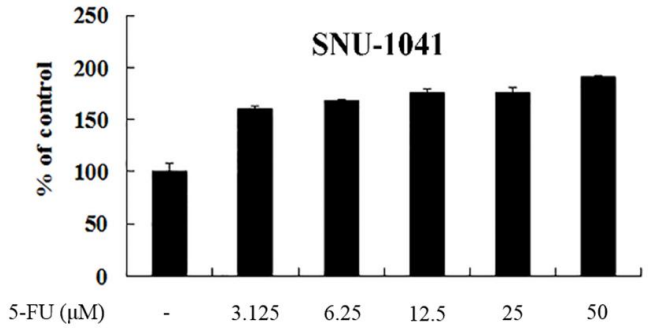


Figure 2. ROS production by celecoxib

ROS production by celecoxib in SNU-1041 (A) and SNU-1076 cells (B). SNU-1041 cell lines treated with celecoxib showed the highest production.

*p-value<0.05.

(A)



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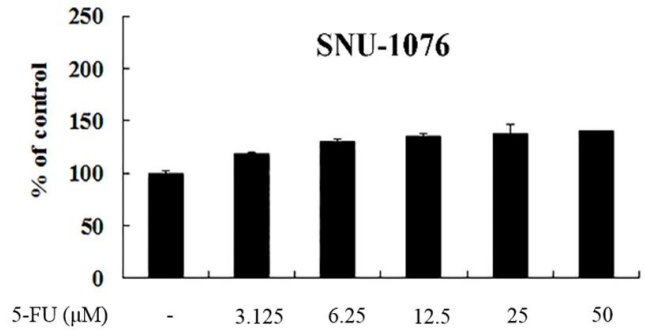

Figure 3. ROS production by 5-FU

ROS production by 5-FU in SNU-1041 (A) and SNU-1076 cells (B).

*p-value<0.05.

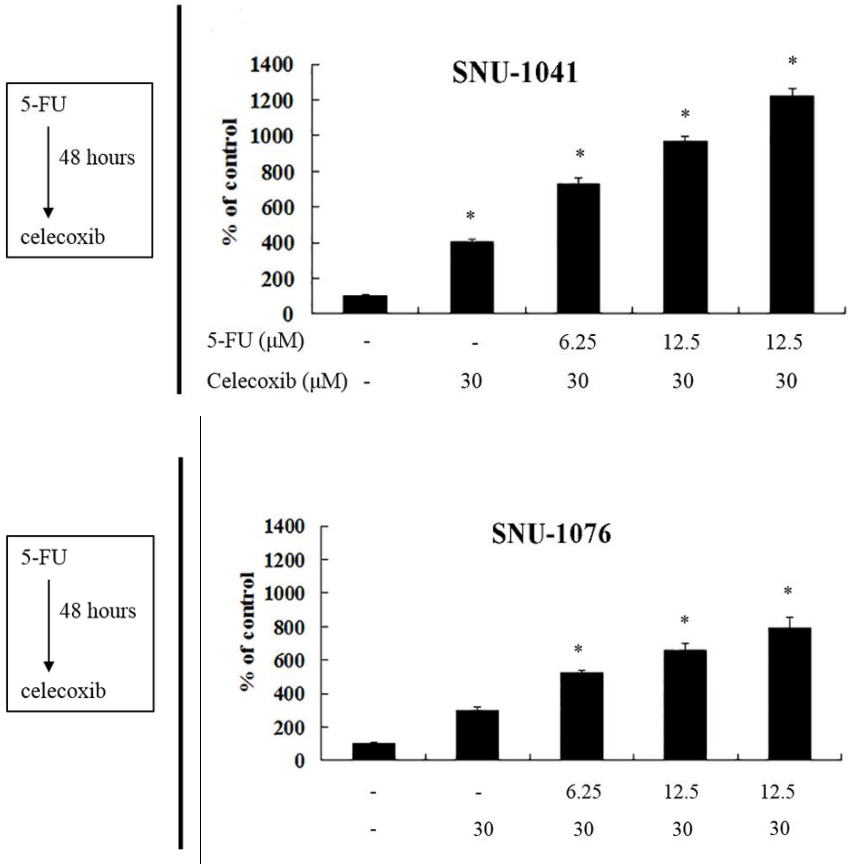

Figure 4. ROS production following 5-FU and celecoxib treatment (1)

ROS production analysis in SNU-1041 and SNU-1076 cells with 5-FU treatment followed by 30 μM celecoxib 48 hours later. ROS production was highest when celecoxib treatment was applied 48 hours after 5-FU treatment.

*p-value<0.05.

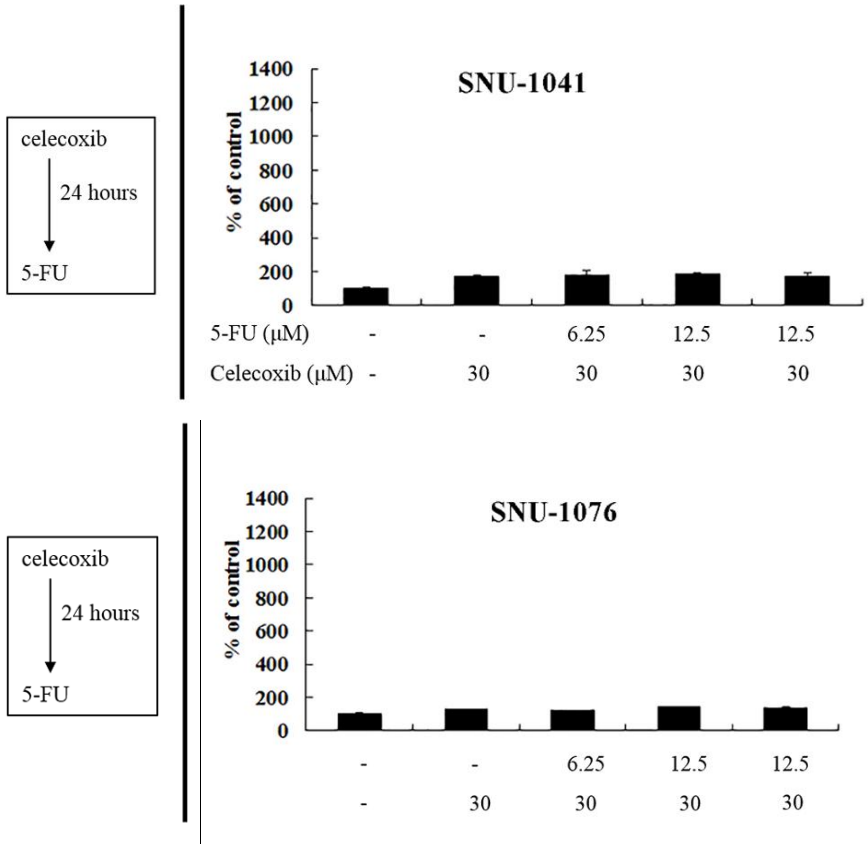

Figure 5. ROS production following 5-FU and celecoxib treatment (2)

ROS production analysis in SNU-1041 and SNU-1076 cells with celecoxib treatment followed by serial concentrations of 5-FU 24 hours later.

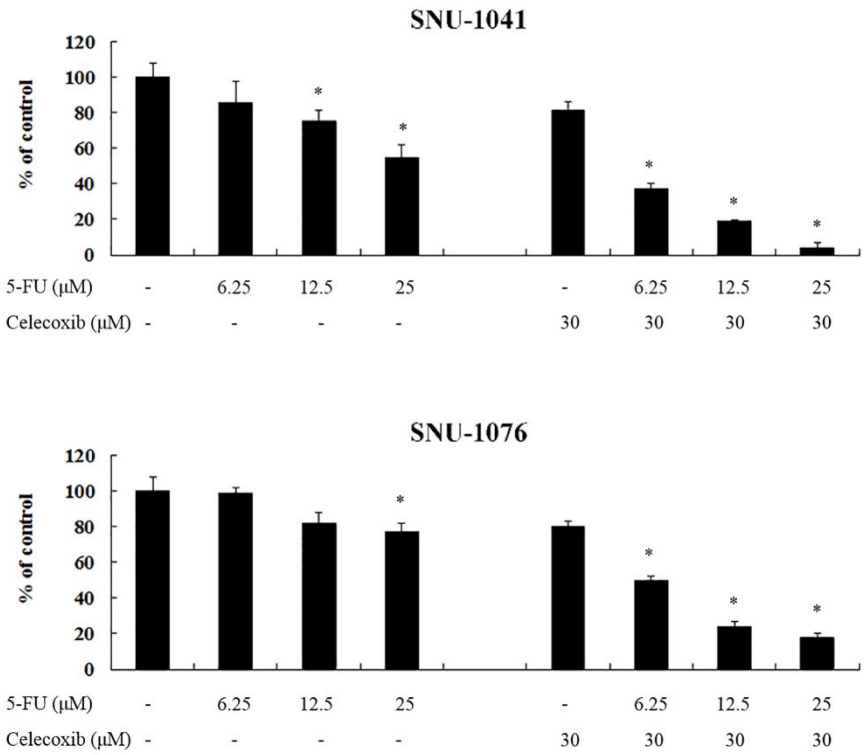

Figure 6. Cell proliferation assay after treatment with 5-FU and combined 5-FU and celecoxib

Cell proliferation assay following celecoxib and 5-FU treatment in SNU-1041 and SNU-1076 cells. 5-FU-induced inhibition was enhanced when combined with celecoxib, particularly in the SNU-1041 cell line.

*p-value<0.05.

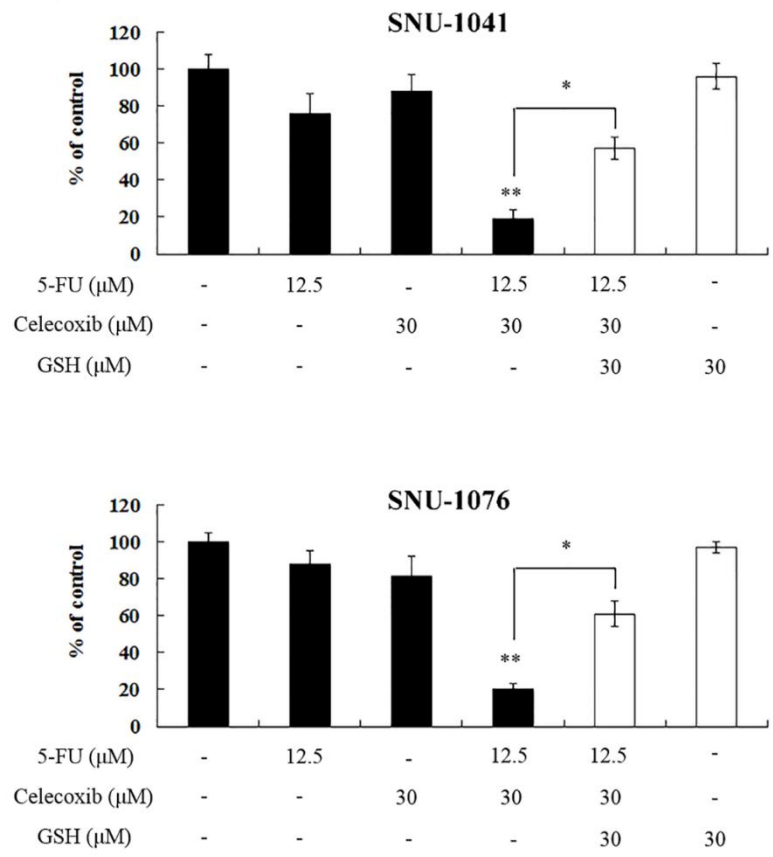

Figure 7. Cell proliferation assay after antioxidant (GSH) treatment

Up to 60% of the inhibition of cell proliferation caused by combined treatment with celecoxib and 5-FU was reversed by GSH, depending on the cell line.

*p-value<0.05; **compared with control (p-value<0.05)

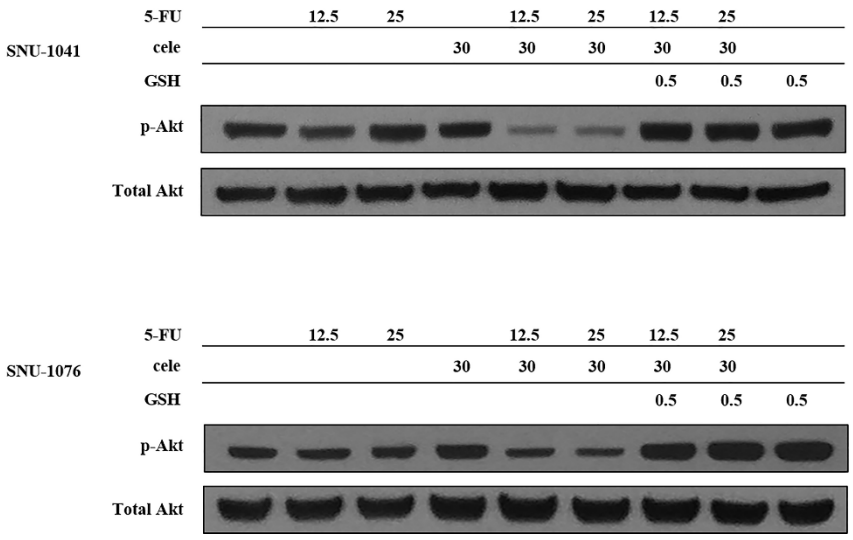

Figure 8. Changes in p-AKT after 5-FU with celecoxib treatment and GSH

Co-treatment with celecoxib and 5-FU partially blocked AKT phosphorylation, although no significant changes in total AKT protein levels were detected.

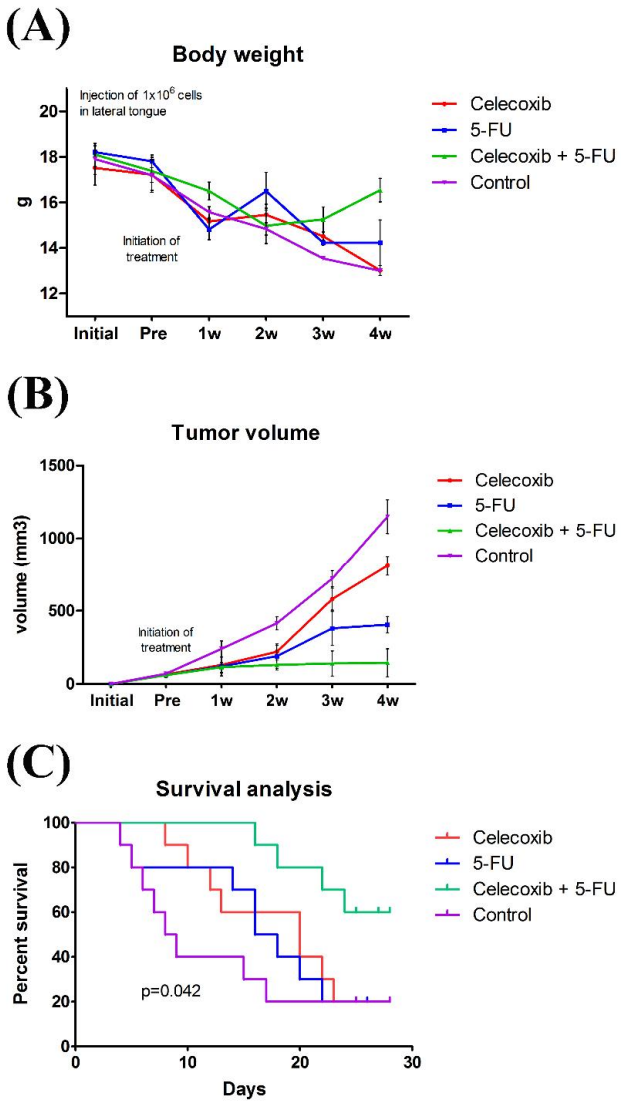

Figure 9. Effects of treatment in an orthotopic tongue cancer mouse model

(A) Body weight changes. (B) Tumor volume changes, (C) Survival analysis.

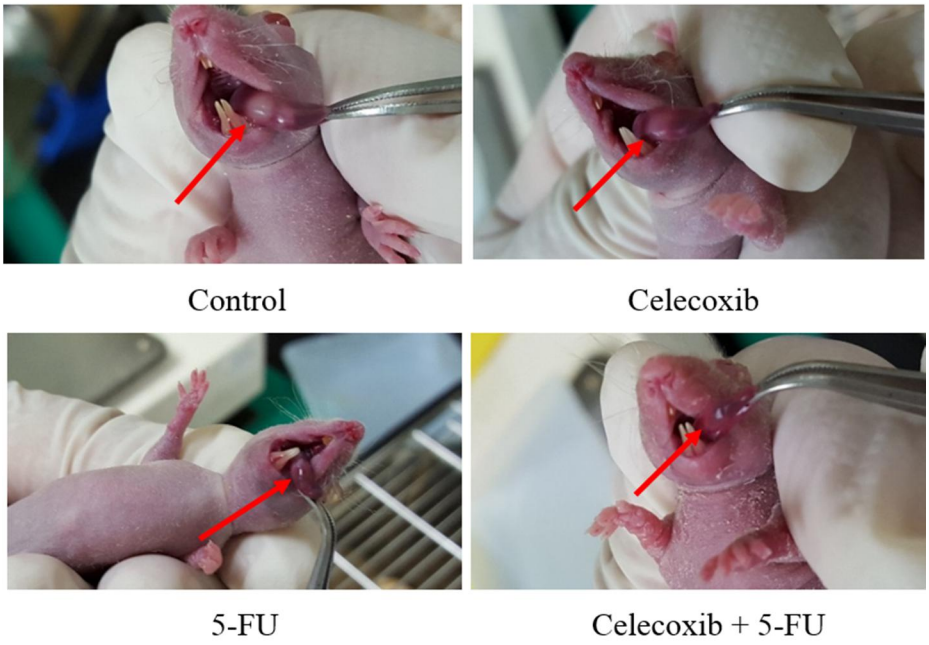

Figure 10. Tumor volume at post-treatment 4 weeks.

Growth of tumor was significantly more inhibited in celecoxib + 5-FU group than other groups in grow inspection

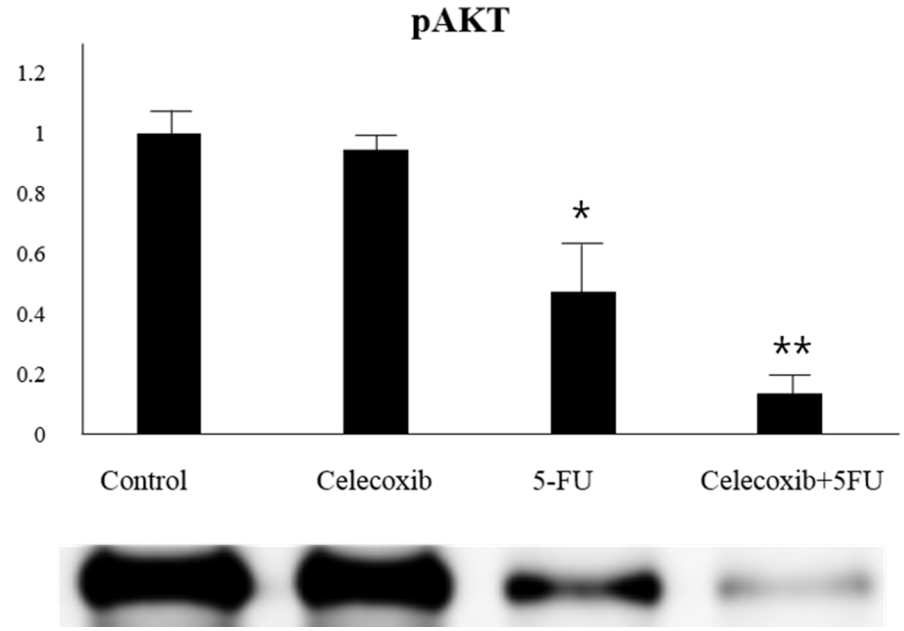

Figure 11. Difference of p-AKT in harvested specimen

A Western blot of harvested specimens revealed that phosphorylation of AKT was significantly inhibited by combination treatment with celecoxib and 5-FU

*p-value<0.05, **p-value<0.01, compared with control

국문 초록

서론: 셀레콕시브는 다양한 암을 치료하고 재발을 방지하는 등의 역할을 보이는 것으로 알려져 있다. 본 연구에서는 셀레콕시브와 5-FU 병합 요법이 두경부 편평세포암종에서 어떠한 효과를 보이는지 알아보고자 하였다.

방법: 5-FU 의 항암효과에 셀레콕시브가 어떠한 영향을 미치는지 알아보고자 SNU-1041, SNU-1076 편평상피세포주와 설암 동물 모델을 이용하여 연구하였다. 세포 증식의 변화를 관찰하기 위해 MTT assay 를 시행하였고 활성산소 발생을 측정하기 위해 dichlorofluorescein diacetate (DCFH-DA) assay 를 시행하였다. AKT pathway 에서 AKT 의 인산화에 미치는 영향을 Western blot assay 를 통해 분석하였다. 동물 모델을 다양한 병합 요법을 통한 생존 분석을 비교 분석하였다.

결과: 활성산소는 셀레콕시브를 5-FU 치료 48 시간이 지난 후 투여하였을 때 가장 많이 발생하였다. 또한 5-FU 에 의한 세포 증식 억제는 셀레콕시브 병합 요법에 의해 강화되었는데 이는 활성산소

의 발생과 비례하였다. 항산화제를 투여하였을 때 세포 증식이 많게는 60%까지 회복되어 셀레콕시브 병합 요법에 의한 활성산소 발생이 세포 억제에 주효한 기전임을 알 수 있었다. AKT pathway 분석을 통해 전체 AKT 의 양은 변화가 없었으나 AKT 의 인산화가 셀레콕시브 병합 요법으로 유의하게 감소하는 것을 관찰하였고, 이 역시 항산화제를 투여하였을 때 회복되는 것을 관찰하였다. 설암동물모델에서는 셀레콕시브와 5-FU 병합요법이 생존 기간이 가장 긴 것을 확인하였고, 적출된 검체에서 AKT 의 인산화가 유의하게 감소되어 있음을 확인하였다.

결론: 셀레콕시브는 5-FU 와 병합요법을 통해 항암 효과를 증진시키는 것으로 생각되며 활성산소가 AKT 의 인산화를 억제하여 세포 증식을 억제하는 주요한 기전으로 생각된다.

주요어 : 셀레콕시브, 5-FU, AKT 대사경로, 활성산소, 두경부암, 편평세포암종

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