

Aim of the study: FaDu human squamous cell carcinoma (FaDu-hSCC) demonstrated accelerated tumor repopulation during fractionated irradiation with pathological validation in a xenograft model system. Previous studies showed that the selective cyclooxygenase (COX)-2 inhibitor celecoxib can enhance the tumor response to radiotherapy. So we aimed to explore the effect of celecoxib in inducing apoptosis and inhibiting repopulation of FaDu tumors in nude mice during fractionated radiotherapy.

Material and methods: FaDu-hSCC was transplanted into the right hind leg of BALB/C nude mice. Mice were treated with celecoxib and/or fractionated irradiation. Celecoxib (100 mg/kg/day) was administered by daily gavage. Irradiation was delivered with 12 to 18 fractions of 3.0 Gy daily or every second day based on Petersen's repopulation model. At different time points, tumors were excised for immunohistochemistry staining.

Results: Significant tumor repopulation occurred after about 18 days of radiotherapy. On average, Ki-67 and bromodeoxyuridine (BrdUrd) labeling indices (LI) decreased with daily irradiation (both $p < 0.05$) and increased with every-second-day irradiation (both $p > 0.05$), suggesting accelerated repopulation. Ki-67 LI decreased in celecoxib concurrent with radiotherapy for 12 fractions in 24 days and 18 fractions in 36 days compared with irradiated alone ($p = 0.004$ and 0.042 , respectively). BrdUrd LI values were lower in the concurrent groups than irradiated alone ($p = 0.001$ and 0.006 , respectively). Epithelial growth factor receptor (EGFR) expression score decreased in the concurrent groups than irradiated alone ($p = 0.037$ and 0.031 , respectively). Caspase-3 expression scores were higher in the concurrent groups than irradiated alone ($p = 0.05$ and 0.006 , respectively).

Conclusions: Celecoxib concurrent radiotherapy could inhibit tumor repopulation and increase tumor apoptosis during the treatment in FaDu squamous cell carcinoma.

Key words: tumor repopulation, apoptosis, fractionated radiotherapy, cyclooxygenase (COX)-2 inhibitor.

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Effect of celecoxib on inhibiting tumor repopulation during radiotherapy in human FaDu squamous cell carcinoma

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Introduction

Accelerated tumor repopulation describes the continuing proliferation of surviving tumor cells during fractionated radiotherapy, which means that cells have the capacity to regenerate the tumor [1]. Accelerated tumor repopulation generally is considered the main reason for observation of the time factor, i.e. a loss of local tumor control with increasing overall treatment time in squamous cell carcinomas [2–4].

Petersen *et al.* performed a series of experiments elegantly demonstrating a clear-cut time factor of clonogenic tumor cell repopulation in an irradiated human FaDu squamous cell carcinoma (FaDu-hSCC) xenograft model system [5]. In the experiments, irradiation was performed under clamp hypoxia and ambient conditions. After increasing numbers of 3.0-Gy fractions delivered either every 24 or 48 hours, graded top-up doses were given to determine the TCD50 (dose required to control 50% of the tumors). The results under clamp hypoxia were consistent with a biphasic course of clonogenic repopulation with a switch to rapid repopulation after 22 days (95% confidence interval [CI] 13, 30). A similar biphasic course of cell repopulation was observed under ambient conditions. In further experiments, tumor repopulation was assessed with histopathological markers of proliferation [6]. After irradiation under homogeneous hypoxic or ambient conditions, either daily or every second day, tumors were removed at four selected time points of the top-up doses, i.e. two time points before the change in clonogen doubling time (Tclon) was calculated from the TCD50 results [5] and two time points after the switch in Tclon occurred. Tumors were immunohistochemically stained for Ki-67 and bromodeoxyuridine (BrdUrd), with the labeling index (LI) for each generated. The data demonstrated initial decreases in Ki-67 and BrdUrd, followed by increases at later time points during the course of fractionated radiotherapy. These results were in good agreement with the kinetics of clonogenic tumor cell repopulation. Taken together, these studies concluded the following: accelerated repopulation of clonogenic FaDu tumor cells starts after approximately 3 weeks of fractionated radiotherapy; accelerated tumor repopulation is observed under both hypoxic and ambient conditions; and Ki-67 and BrdUrd are excellent markers of accelerated tumor repopulation.

Studies in various *in vitro* and *in vivo* preclinical models showed that a selective cyclooxygenase (COX)-2 inhibitor can enhance the tumor response to radiotherapy, inhibit tumor cell proliferation and improve therapeutic efficacy of radiation [7–10]. After experimental verification of tumor repopulation as described by Petersen *et al.*, we investigated the effect of the COX-2 inhibitor celecoxib in inducing apoptosis and inhibiting repopulation during fractionated radiotherapy in FaDu tumor by comparison to histopathological markers at selected time points after the switch in Tclon.

Material and methods

Cell culture

FaDu, an established hSCC line, was purchased from Chinese Academy of Sciences Shanghai Institute of Cell Bank and was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) and penicillin (100 units/ml), and streptomycin (100 µg/ml). Cultures were kept in a humidified atmosphere of 95% air and 5% CO₂ incubator at 37°C.

Animal model

The experiments were performed using 6- to 8-week-old female BALB/C nude mice from the specific pathogen-free animal breeding facility of Beijing Hua Fukang (approval no. SCXK [Jing] 2009–0008). The animal facility and experiments were approved according to Chinese animal welfare regulations. The microbiologic status of the mice was regularly checked by veterinarians of the facility. The animal rooms provided daylight plus a 12-h light/12-h dark electric cycle (lights on at 0700 hours), a constant temperature of 26°C, and relative humidity of 50–60%. The mice were fed a laboratory animal diet and sterile water *ad libitum*.

FaDu cells were injected subcutaneously as single-cell suspensions in phosphate buffer saline (PBS; 2×10^6 cells in 100 µl) in the right hind leg of nude mice. The lengths and widths of the masses were measured by Vernier calipers every three days and the tumor volumes (in cubic millimeters) were calculated using the following equation: volume (mm³) = length × width² × 1/2. The relative tumor volume (RTV) was calculated as $RTV = V_i/V_0$, where V_i is the tumor volume at any given time and V_0 is the volume at the time of initial treatment. After tumors had grown

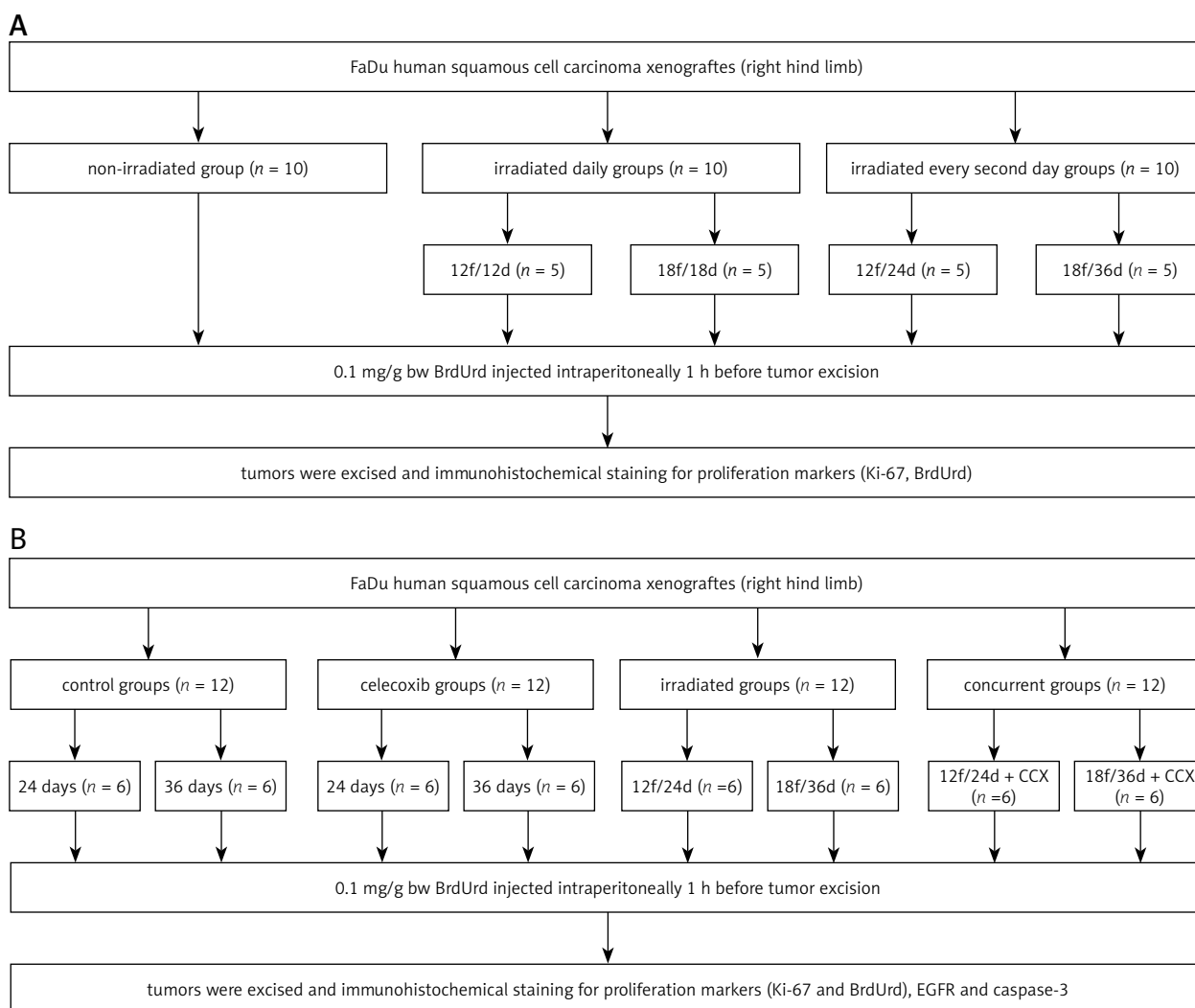


Fig. 1. Experimental protocol. Verification of tumor repopulation during fractionated radiotherapy (**A**), investigation of celecoxib on inhibiting tumor repopulation during radiotherapy (**B**). Celecoxib (100 mg/kg) was given for 24 or 36 consecutive days; Irradiations were administered every second day for 12 or 18 fractions of 3.0 Gy (CCX – celecoxib, f – fractions, d – days)

to a mean diameter of 10 mm (10 ± 2 mm) as measured in two orthogonal diameters, mice were randomized to treatment versus control groups.

Local tumor irradiations and celecoxib treatment

The experimental schema of this study is shown in Fig. 1. Different numbers of 3.0 Gy fractions (12 or 18) were delivered to mice either daily or every second day. The average tumor diameter at the start of irradiation was 10 ± 2 mm. The unanesthetized mice were immobilized using plastic tubes fixed to a wood plate. The tumor-bearing leg was positioned in the radiation field by a foot-holder distal to the tumor. Local irradiation was administered using 6 MeV electrons.

To validate prior studies of tumor accelerated repopulation as described by Petersen *et al.*, a first set of experiments was performed (Fig. 1A). Mice were first randomized to either daily or every second day irradiation, then randomized to either 12 or 18 fractions. A non-irradiated control group was also utilized. After radiation courses were completed, 0.1 mg/g bw BrdUrd (10 mg/ml saline) was injected intraperitoneally 1 h before tumor excision. Tumors were then excised, measured, and evaluated by immunohistochemistry as described later.

A second set of experiments to investigate the role of celecoxib in inhibiting proliferation and inducing apoptosis of FaDu tumors was performed (Fig. 1B). Mice were randomized to controls, celecoxib alone groups (for 24 days and 36 days), irradiation alone groups (12f/24d group and 18f/36d group) and celecoxib concurrent irradiation groups. Celecoxib (trade name Xilebao; SFDA approval no. J20080059) was purchased from Pfizer Pharmaceuticals Co. Ltd (New York, NY, USA). Celecoxib powder was dissolved in dimethyl sulfoxide (DMSO) (Sigma, America) and diluted in phosphate-buffered saline (PBS). The final concentration of celecoxib was 10 mg/

ml. Celecoxib [100 mg/kg (0.2 ml)] was given by daily gavage for 24 or 36 consecutive days. The control groups were treated with DMSO only. Irradiation was performed every second day. For the concurrent groups, irradiations were given within 2 hours after celecoxib gavage. After treatment for every group was completed, 0.1 mg/g bw BrdUrd (10 mg/ml saline) was injected intraperitoneally 1 h before tumor excision. Tumors were then excised, measured, and evaluated by immunohistochemistry as described later.

Immunohistochemistry study

After excision, tumors were fixed overnight in 10% formalin and embedded in paraffin blocks, from which 4- μ m sections were cut for immunohistochemical staining. To measure tumor proliferation, slides were incubated with mouse monoclonal antibodies against Ki-67 MIB-1 (dilution 1 : 200, Dianova, Hamburg, Germany) and BrdUrd (dilution 1 : 100, Dako, Glostrup, Denmark). The fraction of Ki-67 and BrdUrd labeled tumor cells were assessed by examination of stained sections at 400 \times magnification. All samples were blinded for analysis. Five fields of non-necrotic areas were randomly selected in each specimen. In each field, 200 nuclei were scored for Ki-67 and BrdUrd labeling. The number of positively labeled cells was summed from the five scored fields to derive a LI for both Ki-67 and BrdUrd, calculated as the total number of positively labeled cells divided by 1000.

To measure the expression of EGFR and caspase-3, slides were incubated with mouse monoclonal antibodies against EGFR (dilution 1 : 100, Santa Cruz Biotechnology, Texas, USA) and caspase-3 (dilution 1 : 100, Abcam, Cambridge, UK). Estimated glomerular filtration rate staining was predominantly located in the cell membrane and caspase-3 staining was located in the cytoplasm. The proportion of positive tumor cells was graded: 0 if < 10%, 1 if 10–25%, 2 if 26–50%, 3 if 51–75%, and 4 if > 75% [11].

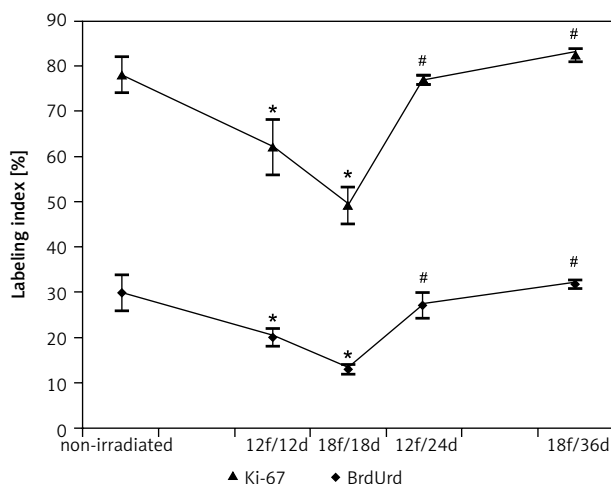


Fig. 2. Labeling index (LI) for the proliferation markers. Changes of Ki-67 LI and BrdUrd LI during fractionated radiotherapy. Bars represent mean (three to five individual tumors) \pm standard deviation. Irradiations were administered daily or every second day for 12 or 18 fractions of 3.0 Gy; * $p < 0.05$, # $p > 0.05$ compared with non-irradiated group

Statistical analysis

Statistics were calculated using statistical software (SPSS for Windows 17.0, SPSS, Inc., Cary, NC, USA). All quantitative data were expressed as mean \pm standard deviation (SD). Comparisons of histological parameters between groups were calculated using one-way ANOVA followed by the Bonferroni *post hoc* test or the Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant.

Results

Tumor repopulation during fractionated radiotherapy detected by pathological proliferation parameters

In the first part, the mean Ki-67 LI for untreated FaDu tumors was 77.9%. A significant decrease to 62.0% and 49.3% was observed after daily irradiation with 12 fractions in 12 days ($p = 0.002$) and 18 fractions in 18 days ($p < 0.001$). After that this increased again to 76.8% and 82.5%, to values not significantly different from the un-

treated controls ($p = 0.553$ and 0.058). The mean BrdUrd LI in untreated FaDu tumors was 29.9%. There was a significant decrease to 19.9% and 12.8% after daily irradiation with 12 fractions in 12 days ($p = 0.006$) and 18 fractions in 18 days ($p < 0.001$). After that it increased again to 27.1% in the 12 fractions/24 days group ($p = 0.323$) and 31.6% in the 18 fractions/36 days group ($p = 0.605$) compared with untreated controls (Fig. 2).

Tumor growth delay effects

The mean volume at the start of treatment showed no significant difference between all groups. In the second set of experiments, the RTVs increased throughout the experimental period (for 24 or 36 days) in the control groups and celecoxib-treated groups, but were lower in celecoxib-treated groups than the control groups ($p = 0.009$ and 0.02 , respectively). The RTVs decreased throughout the experimental period in celecoxib concurrent radiotherapy groups and irradiated alone groups, and were lower in concurrent groups than irradiated alone groups ($p = 0.022$ and $p < 0.001$ respectively) (Fig. 3A, B).

Proliferation inhibition

Compared with control groups, changes of Ki-67 LI and BrdUrd LI in celecoxib alone groups were significant ($p < 0.05$), but not significant in irradiated alone groups ($p > 0.05$). Ki-67 LI decreased in celecoxib concurrent with radiotherapy for 12 fractions in 24 days and 18 fractions in 36 days compared with irradiated alone groups ($p = 0.004$ and 0.042 , respectively). BrdUrd LI values were also lower in the concurrent groups than irradiated alone groups

($p = 0.001$ and 0.006 , respectively) (Fig. 4A, B). Microphotographs of typical FaDu tumors are shown in Fig. 4C.

EGFR expression inhibition

The changes in the proportion of positive cells for EGFR during the treatment are shown in Fig. 5A. Compared with control groups, EGFR expression decreased significantly in celecoxib alone groups ($p < 0.05$), but not significantly in irradiated alone groups ($p > 0.05$). Estimated glomerular filtration rate expression scores were lower in celecoxib concurrent with radiotherapy for 12 fractions in 24 days and 18 fractions in 36 days than irradiated alone groups at the same time points ($p = 0.037$ and 0.031 , respectively).

Tumor apoptosis change

The changes in the proportion of positive cells for caspase-3 during the treatment are shown in Fig. 5B. Compared with control groups, caspase-3 expression increased significantly in celecoxib alone groups ($p < 0.05$), but not significant in irradiated alone groups ($p > 0.05$). Caspase-3 expression score were higher in celecoxib concurrent with radiotherapy for 12 fractions in 24 days and 18 fractions in 36 days than irradiated alone groups; p values are 0.05 and 0.006 respectively.

Discussion

The aim of the present study was to demonstrate the acceleration of tumor repopulation during fractionated radiotherapy and assess the roles of celecoxib in inducing proliferation inhibition and apoptosis of FaDu tumors.

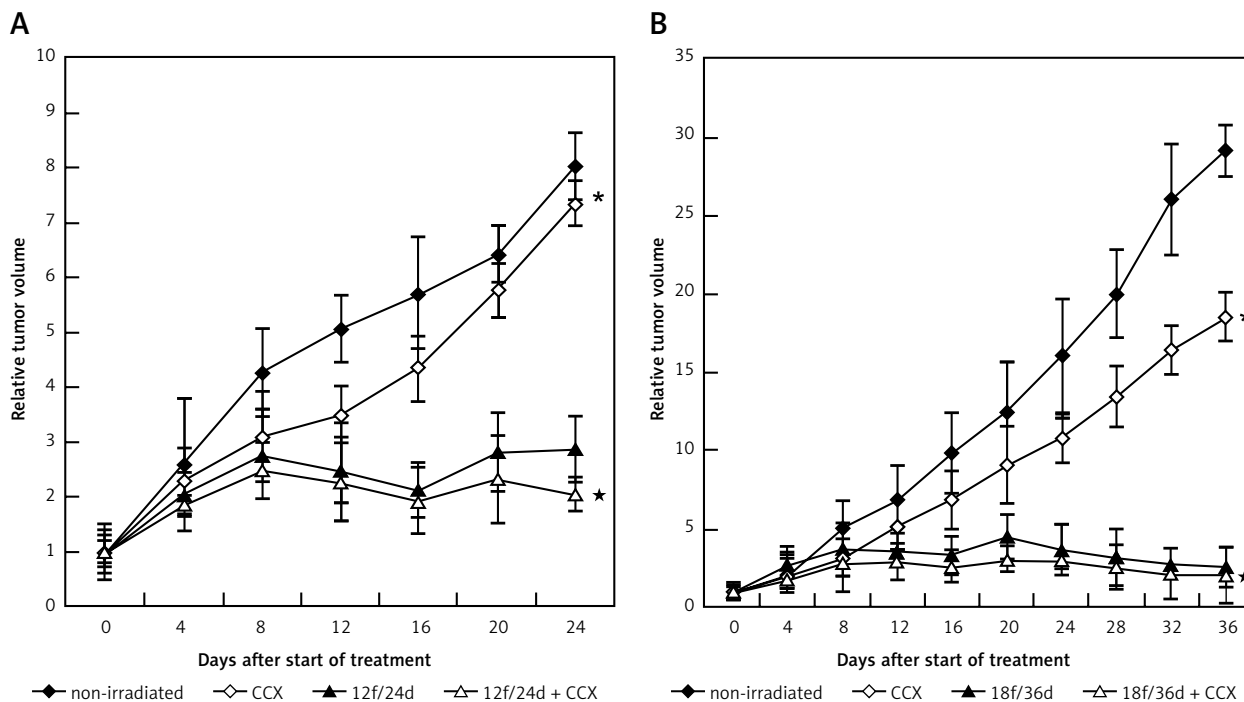


Fig. 3. Tumor growth delay effects of celecoxib of the FaDu tumor models. The changes of relative tumor volumes (RTVs) are shown (A, B) throughout the experimental period. * $p < 0.05$ compared with control groups, * $p < 0.05$ compared with irradiation alone groups. Celecoxib (100 mg/kg) was given by daily gavage for 24 or 36 consecutive days, irradiations were administered every second day for 12 or 18 fractions of 3.0 Gy

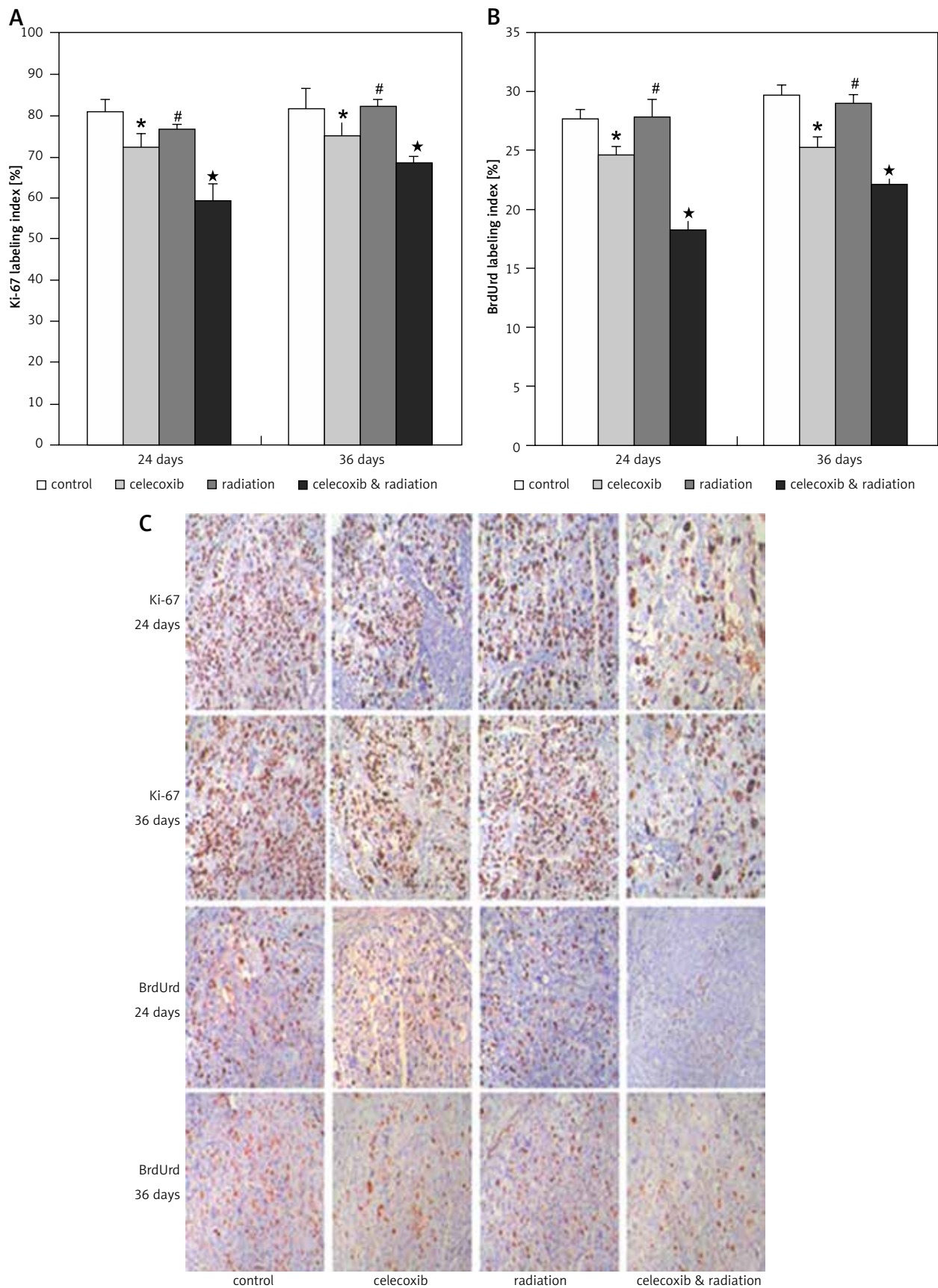


Fig. 4. Proliferation inhibition effects in FaDu-hSCC. The proportion of positive proliferating cells in tumor tissues was detected by Ki67 and BrdUrd immunocytochemistry (**A and B**). Columns, means; bars, standard deviation (SD); $n = 4$ to 6 ; $*p < 0.05$, $^{\#}p > 0.05$ compared with control groups, $*p < 0.05$ compared with irradiation alone groups. The representative pathological pictures of FaDu tumors (100 \times) (**C**). Brown precipitate dense coverage in the nucleus labeled as positive. Selected pictures represent Ki-67 (treatment for 24 and 36 days) and BrdUrd (treatment for 24 and 36 days) expression in tumor tissues. Celecoxib (100 mg/kg) was given by daily gavage for 24 or 36 consecutive days, irradiations were administered every second day for 12 or 18 fractions of 3.0 Gy

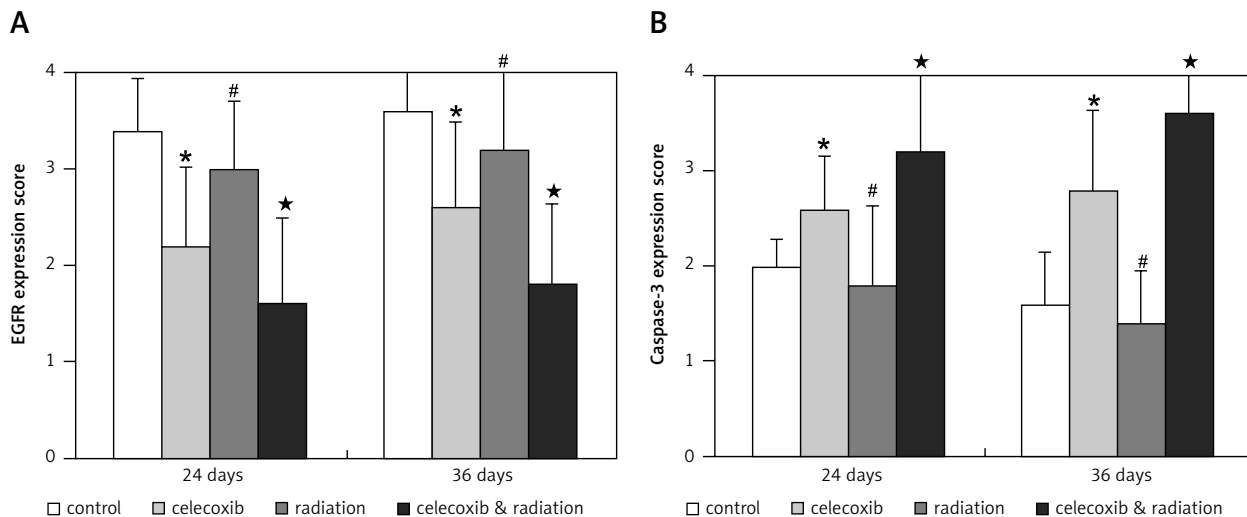


Fig. 5. Immunohistochemistry staining of EGFR (**A**) and caspase-3 (**B**) during the course of treatment. The changes in proportion of positive cells were scored as 0 (< 10%) to 4 (> 75%). Columns, means; bars, standard deviation (SD); $n = 4$ to 6 ; * $p < 0.05$, # $p > 0.05$ compared with control groups, ★ $p < 0.05$ compared with irradiation alone groups. Celecoxib (100 mg/kg) was given daily for 24 or 36 consecutive days, irradiations were administered every second day for 12 or 18 fractions of 3.0 Gy

In the first part of the present study, BrdUrd LI and Ki67 LI decreased during the initial part of fractionated irradiation. At later time points, both indices increased again (Fig. 2). These kinetics of proliferation markers were in remarkably good agreement with the repopulation kinetics of clonogenic cells in FaDu tumors treated with the same irradiation regimen by Petersen *et al.* [6]. In conclusion, we found that a phenomenon of accelerated tumor repopulation happened at every-second-day irradiation (that is 12 fractions in 24 days and 18 fractions in 36 days).

Several clinical trials have demonstrated that a safe combination of cyclooxygenase inhibitors (celecoxib) and radiotherapy is feasible, and celecoxib can enhance tumor response to radiotherapy, inhibit tumor cell proliferation and improve therapeutic efficacy of radiation [12–14]. Based on the verification of tumor repopulation using the same irradiation protocol proposed by Petersen *et al.* [6], we performed the second part of the experiment; we added celecoxib to the two time points (12 fractions in 24 days and 18 fractions in 36 days) after the switch in Tcon occurred to investigate the roles of celecoxib on tumor repopulation during radiotherapy.

In the second part, the RTVs increased throughout the experimental period (for 24 or 36 days) in the control groups and celecoxib-treated groups, but were lower in celecoxib-treated groups, indicating that celecoxib treatment had an effect on the growth of FaDu tumors. This anti-tumorigenic effect of COX-2 inhibition by measuring tumor volumes was similar to the studies of several investigators [15, 16]. The explanation of celecoxib's anti-tumoral effect on various cancers may be that COX-2 inhibition leads to a reduction of tumor-cell proliferation. Irradiation alone or concurrent celecoxib treatment led to a significant decrease in tumor volumes, and the RTVs of combined groups were lower than irradiation alone groups, indicating that the addition of celecoxib to the radiotherapy (RT) regimen may lead to enhancement (Fig. 3).

In this study, celecoxib combined RT enhanced tumor growth delay both at day 24 and 36 when compared to RT alone. However, RTV in celecoxib concurrent RT for 36 days (the right figure in Fig. 3) was not as significant as that in celecoxib concurrent RT for 24 days (the left figure in Fig. 3). This may be due to the small number of nude mice in each group or the changes in tumor volume could not reflect the treatment efficacy sensitively.

Recently, celecoxib has been reported to decrease Ki-67 expression in cervical cancer [17], lung cancer [18] and human medullary thyroid cancer [15]. We evaluated the action of celecoxib supplementation on tumor cell division by immunostaining of the human Ki-67 protein and bromodeoxyuridine (BrdUrd). In our study, celecoxib alone decreased Ki-67 and BrdUrd proliferative indices, while radiation alone showed no significant changes compared to the control (Fig. 4A, B), which is suggestive of tumor repopulation, and these results are consistent with the results of the preliminary experiment in the present study. Celecoxib concurrent irradiation significantly decreased both indices compared to irradiation alone, which demonstrated inhibition of proliferation by celecoxib alone or concurrent irradiation and further indicated additional enhancement of the radiotherapy regimen by celecoxib. Our findings provide supporting evidence that celecoxib concurrent radiotherapy may be capable of inhibiting tumor repopulation measured by proliferation parameters Ki-67 and BrdUrd in FaDu-hSCC.

Schmidt-Ullrich *et al.* [19] previously showed that irradiation could activate EGFR and other members of the ErbB family of tyrosine kinases and lead to activation of mitogen-activated protein kinase (MAPK) pathways and the stimulation of cellular proliferation. Dittmann *et al.* [7] found that celecoxib enhanced radiosensitivity by inhibition of EGFR-mediated mechanisms of radioresistance, a signaling that was independent of COX-2 activity. In the data presented here, EGFR expression score decreased in

celecoxib alone groups compared with controls, and were lower in concurrent groups than radiation alone groups (Fig. 5A), suggesting an additive effect of celecoxib in combination with radiotherapy in this FaDu tumor model. This result is consistent with the results of the proliferation inhibition detected by Ki-67 and BrdUrd. However, the mechanisms underlying this tumor model need to be further studied later.

The *in vitro* study of Wu *et al.* [20] indicated that inhibition of proliferation and induction of apoptosis in human cholangiocarcinoma cells by the cyclooxygenase-2 specific inhibitor celecoxib may be involved in COX-dependent mechanisms and the prostaglandin E₂ (PGE₂) pathway, and data also indicated that celecoxib inhibits proliferation and induces apoptosis by an accumulation of cells in the G0/G1 phase and the inhibition of G0/G1 phase transition to S phase. Recent data obtained by Quidville *et al.* [15] suggested that the anti-proliferative effect of celecoxib is exerted by a pathway that does not implicate COX-2 inhibition and/or PGE₂ reduction, but results from the inhibition of cell division and/or the induction of apoptosis. Caspase-3 is involved in the 'execution' phase of cellular apoptosis and is a marker of apoptosis. In the data presented here, compared with the controls, celecoxib alone could increase caspase-3 expression, while radiation alone showed no significant changes (Fig. 5B). The combined groups (12f/24d group and 18f/36d group) showed a higher apoptosis index than irradiation alone groups (Fig. 5B). The higher caspase-3 expression in tissue level may suggest higher cellular apoptosis, and this is in agreement with our proliferation inhibition findings detected by Ki-67 and BrdUrd.

In the study of Bucci *et al.* [21], they observed an antiproliferative effect using fractionated doses (4 × 5 Gy) and showed apoptosis induced by the fractionated irradiation treatment proceeded through a process involving caspase-3 activation. De Heer *et al.* [22] evaluated the use of biochemical detection of caspase-3 activity as a simple and quantitative technique to measure apoptosis in tissue samples; they found that higher caspase-3 activity indicated higher apoptosis and lower local recurrence rates. In the study of Kim *et al.* [23], *in vivo* immunohistochemistry staining showed that combination therapy yielded over a 100% increase in caspase-3 activity (apoptosis) and cell proliferation (Ki-67 staining) was reduced by 77% ($p = 0.001$) compared with radiation alone. However, other studies contradicted these viewpoints. Huang *et al.* [24] made an unexpected discovery that caspase-3, is a key regulator of growth promoting signals to stimulate the repopulation of tumors undergoing radiotherapy. One downstream effector that caspase-3 regulates is prostaglandin E₂ (PGE₂), which can potently stimulate growth of surviving tumor cells. They first pointed out the mechanism of activation of the caspase-3-iPLA-AA-PGE₂ signaling pathway. In the study of Kim *et al.* [25], the Ki-67 index showed > 5-fold reduction of tumor proliferation in the combination therapy group despite the reduced levels of apoptosis and they found that a caspase-3 inhibitor increased the radiosensitizing effect. To our knowledge, both radiation and celecoxib can induce apoptosis in tumor cells. Primari-

ly, radiation inactivates tumor cells through double-strand DNA breakage, while celecoxib may be induced by COX-dependent or COX-2-independent mechanisms, and further investigation is warranted.

The limitations of this study include the small sample size in each group, the fact that the irradiation dose applied (3.0 Gy) was different from a conventional fractionated radiation dose (1.8–2.0 Gy), and prolonged treatment time led to a smaller number of mice that survived.

In conclusion, celecoxib concurrent with radiotherapy can significantly delay tumor growth, inhibit tumor proliferation and increase tumor apoptosis during the treatment. However, the results suggest that further preclinical and clinical investigations are necessary to explore the effect of the COX-2 inhibitor celecoxib during fractionated radiotherapy.

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The authors declare no conflict of interest.

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