

Original Article

Selective EP2 and Cox-2 inhibition suppresses cell migration by reversing epithelial-to-mesenchymal transition and Cox-2 overexpression and E-cadherin downregulation are implicated in neck metastasis of hypopharyngeal cancer

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Abstract: Cyclooxygenase-2 (Cox-2) has been shown to promote cancer initiation and progression through pleiotropic functions including induction of epithelial-to-mesenchymal transition (EMT) via its predominant product prostaglandin E₂ that binds to the cognate receptor EP2. Hence, pharmacological inhibition at the level of EP2 is assumed to be a more selective alternative with less risk to Cox-2 inhibition. However, little is known regarding the anti-cancer effect of an EP2 antagonist on the malignant properties of cancers including hypopharyngeal squamous cell carcinoma (HPSCC). The present study found that both the Cox-2 inhibitor celecoxib and the EP2 antagonist PF-04418948 upregulated CDH-1 expression, restored membranous localization of E-cadherin, and reduced vimentin expression, by downregulating the transcriptional repressors of E-cadherin in BICR6 and FaDu cells. Such Cox-2 or EP2 inhibition-induced EMT reversal led to repressed migration ability in both cells. Immunohistochemical analysis of surgical HPSCC specimens demonstrated an inverse relationship in expression between Cox-2 and E-cadherin both in the context of statistics ($P = 0.028$) and of reciprocal immunolocalization *in situ*. Multivariate logistic regression revealed that overexpression of Cox-2 ($P < 0.001$) and downregulation of E-cadherin ($P = 0.016$) were both independently predictive of neck metastasis. These results suggest that suppression of cell migration ability via reversing EMT by inhibiting the Cox-2/EP2 signaling may contribute to preventing the development and progression of lymphatic metastasis. Collectively, targeting Cox-2/EP2, especially using EP2 antagonist, can be a promising therapeutic strategy by exerting an anti-metastatic effect via EMT reversal for improving the treatment outcomes of patients with various cancers including HPSCC.

Keywords: Cyclooxygenase-2, EP2, E-cadherin, epithelial-to-mesenchymal transition, hypopharyngeal squamous cell carcinoma, neck metastasis

Introduction

Hypopharyngeal squamous cell carcinoma (HPSCC) is a relatively rare entity that affects 0.8-1.3 per 100,000 persons per year in the

US, constituting 3-6.5% of all head and neck squamous cell carcinomas (HNSCCs) [1-3]. Despite great advances in overall oncological treatments including combined multi-disciplinary management, the prognosis of HPSCC

remains the worst among all HNSCCs with a reported 5-year overall survival rate of approximately 15-45% [3-5]. Because of a plentiful lymphatic network that develops extensively in the pharynx, HPSCC readily leads to neck lymph node metastasis even in the early phase, which is one of the most critical factors underlying its unfavorable prognosis as well as that of other HNSCCs [6, 7]. Thus, devising more effective strategies based on a better understanding of the molecular mechanisms contributing to lymphogeneous metastasis is of utmost importance.

Cyclooxygenase-2 (Cox-2), an isoform of Cox enzymes responsible for the biosynthesis of various prostanoids including prostaglandin E_2 (PGE_2), is inducibly upregulated only in response to certain stimuli like cytokines, mitogens, and growth factors, thereby playing essential roles in the crosstalk between chronic inflammation and cancer development [8-15]. Increased expression of Cox-2 in tumor tissues as well as its clinicopathological and prognostic significance have been reported in a variety of human malignancies [9-13, 16, 17] including HNSCC with a meta-analysis [18]; however, the relevance of Cox-2 in patients with HPSCC remains unknown. A wide range of mechanisms by which Cox-2 contributes to tumor initiation and progression has been unraveled, which comprises activating carcinogens; promoting cell proliferation, migration, invasiveness, survival, and resistance to apoptosis; regulating angiogenesis; and creating immunosuppressive microenvironments [9-15]. Some of these functions constitute the sequential process of eventual metastasis that closely involves acquisition of epithelial-to-mesenchymal transition (EMT), wherein cells lose epithelial characteristics including cell-to-cell adhesion and gain mesenchymal features including cell motility [19-21]. Upon EMT induction, CDH-1, a gene encoding human E-cadherin, is downregulated by diverse transcriptional repressors such as snail, DeltaEF1/ZEB1, SIP1/ZEB2, twist, and slug [19-21]. Among various upstream mechanisms regulating EMT, Cox-2-dependent downregulation of E-cadherin was first revealed in non-small cell lung cancer (NSCLC) [22], and later in other cancers including HNSCC [23, 24], suggesting the implication of Cox-2 as a key promoter in EMT-driven metastasis.

Because of the above-mentioned pleiotropic functions by which Cox-2 endows cells with a malignant phenotype and metastatic ability, Cox-2 has been recognized as a promising preventive and therapeutic target for various cancers [9-15, 25-27]. Cox-2 inhibitor-induced EMT reversal with restored E-cadherin expression has been observed in subsets of several cancer cells [22, 23, 28-32], including oral SCC (OSCC) cells as shown in our previous study [33]. However, little is known regarding the suppressive effect of selective Cox-2 inhibition on malignant properties including EMT in HPSCC cells. In clinical practice, meta-analysis of numerous epidemiologic studies revealed that regular inhibition of Cox-2 reduces the risk of carcinogenesis in the breast, colon, prostate, and lung, with greater preventive effects in selective Cox-2 inhibitors compared to other nonsteroidal anti-inflammatory drugs (NSAIDs) [34-37]. Nevertheless, because selective Cox-2 inhibitors as well as NSAIDs concurrently reduce Cox-2-derived anti-thrombotic prostacyclin (PGI_2) [38-40], these drugs have been linked with elevated cardiovascular risk across a large number of population-based observational studies [41, 42], which has precluded extensive use of these drugs and resulted in the withdrawal of selective Cox-2 inhibitors except for celecoxib [37, 38, 40]. Such issues associated with Cox-2 inhibition encourage exploration for alternative and more specific therapeutic targets downstream of Cox-2.

PGE_2 , a predominant metabolic product of Cox-2, can affect multiple mechanisms involved in diverse physiological and pathological functions, thereby playing a prime role in malignant tumorigenesis and its progression as well as in chronic inflammation [43-48]. Similar to Cox-2, elevated levels of PGE_2 have also been observed in a wide variety of epithelial malignancies [10, 47, 48] including HNSCC [49-51]. PGE_2 exerts its biological effects in an autocrine or paracrine fashion by binding to four distinct G-protein-coupled receptors on the cell membrane, i.e., EP1, EP2, EP3, and EP4, which can each activate different downstream signaling pathways. Among these, the EP2 receptor has been principally implicated in cancer development and progression [47, 48, 52-55]. Hence, pharmacological suppression of PGE_2 activity at the level of its cognate recep-

tors such as EP2 is assumed to retain the anti-cancer benefits of Cox-2 inhibition while evading the risk of adverse reactions. Recently, selective EP2 antagonists have been developed to study the role of EP2 in animal models of human diseases; among these, PF-04418948 was shown to possess over 2000-fold higher selectivity for EP2 than other EP subtypes *in vivo* [48, 56, 57]. However, the anti-cancer effect of an EP2 antagonist, a possibly more selective inhibitor of Cox-2/PGE₂ signals, on human cancers, including HNSCC, largely remains to be elucidated.

We conducted the present study to examine whether selective Cox-2 inhibitor, as well as EP2 antagonist, suppresses cell migration via reversal of EMT by restoring E-cadherin expression in HPSCC cells. We also aimed to elucidate whether Cox-2 and E-cadherin expression in tumor cells in surgical specimens is correlated with clinicopathological variables, especially with neck metastasis, in patients with HPSCC.

Methods

Cell culture

We used eight cell lines established from human HNSCC: BICR6, FaDu, and Detroit-562 derived from the hypopharynx; SAS, HSC-3, and HSC-4 from the tongue; and HSC-2 and HO1U1 from the floor of the mouth. The human fibrosarcoma cell line HT-1080 was used as the negative control for E-cadherin/CDH-1 expression. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BICR6, FaDu, Detroit-562, HSC-2, HSC-3, and HSC-4), a mixture of DMEM and Ham's F-12 (SAS and HO1U1), or minimal essential medium (HT-1080), supplemented with 10% fetal bovine serum (FBS) in a humidified incubator (37°C, 5% CO₂).

Inhibition of Cox-2 and EP2 using the specific inhibitor or antagonist

BICR6 and FaDu cells were seeded in six-well plates at a density of 2×10^5 cells per well and incubated overnight in medium containing 10% FBS. The cells were then treated with a selective Cox-2 inhibitor: 50 µM of celecoxib (Toronto Research Chemicals) or a selective EP2 antagonist: 1 µM of PF-04418948 (Cayman CHEMICAL). These concentrations of the reagents were each found to be optimal with

no toxic effect on cell viability up to at least 48 h based on our preliminary experiments. Treatments with only dimethyl sulfoxide (DMSO) (Nacalai Tesque, Japan) used as a solvent for the reagents were set as controls. To evaluate alterations in gene expression associated with Cox-2 or EP2 inhibition, total RNA was extracted after a 12 h incubation. The experiment in each condition was performed at least three times to assess the consistency of response.

Quantitative real-time PCR

Total RNA from cell lines was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) was performed using the 7500 Fast Real-Time PCR system instrument and software (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Specific primers and probes were purchased from Applied Biosystems as TaqMan® Gene Expression Assays, with the following IDs: Cox-2/PTGS2, Hs01-573471_m1; human E-cadherin/CDH-1, Hs0-0170423_m1; intermediate filament/vimentin, Hs00958111_m1; Snail/SNAI1, Hs001955-91_m1; zinc finger E-box binding homeobox 1/ZEB1, Hs00232783_m1; twist/TWIST1, Hs-01675818_s1; and BRWS1/ACTB, Hs0106-0665_g1. The PCR amplification conditions were as follows: 20 s at 95°C followed by 40 cycles of 3 s denaturation at 95°C and 30 s annealing at 60°C. We quantified the relative gene expression levels using the standard curve method, and compared the levels after normalization to the value of ACTB used as an endogenous control.

Immunofluorescence staining

For immunofluorescence staining of E-cadherin, BICR6 and FaDu cells were seeded in slide chambers (IWAKI, Japan) and treated with 50 µM of celecoxib, 1 µM of PF-04418948, or DMSO alone for 24 h. After washing the cells extensively with phosphate-buffered saline (PBS), the cells were fixed with cold methanol for 10 min at -20°C. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated anti-E-cadherin antibody (Santa Cruz Biotechnology, Dallas, TX) at 1:200

dilution in PBS for 1 h. The nuclei were visualized by staining with Hoechst 33258 (Sigma-Aldrich). Stained cells were then mounted with Prolong Gold Antifade Reagent (Invitrogen). The fluorescence images were obtained using a fluorescence microscope (Keyence, Japan).

In vitro cell proliferation assay

The effect of Cox-2 and EP2 inhibition on the proliferation of BICR6 and FaDu cells was assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, WI) according to the manufacturer's instructions. Briefly, cells were plated in a 96-well plate at a density of 1000 cells per well and incubated in culture medium containing 5% FBS with 50 μ M of celecoxib, 1 μ M of PF-04418948, or DMSO alone, for 24 h at 37°C. Twenty microliters of the reagent containing a tetrazolium compound and phenazine ethosulfate were added to each well, and the plate was incubated for 4 h at 37°C. Viable cells were quantified by measuring the optical density (OD) values of absorbance at 490 nm using a microplate reader. The experiment was performed three times and run in sextuplicate each time.

In vitro migration assay

Cell migratory ability was examined using 24-well Transwell inserts (polycarbonate filters) with 8 μ m pores (BD Biosciences). The sorted BICR6 and FaDu cells suspended in serum-free medium were plated onto the Transwell inserts at 2.5×10^4 cells per well. Medium containing 10% FBS was added to the bottom of the wells as a chemoattractant. The inserts were incubated in culture medium with 50 μ M of celecoxib, 1 μ M of PF-04418948, or DMSO alone, for 24 h at 37°C. The filters were removed, and then, cells on the lower surface of the filters were fixed and stained with a Diff-Quick kit (Sysmex Corp., Japan) according to the manufacturer's instructions. The migratory capacities were quantified as total cell numbers counted in five random fields for each insert under a light microscope at 200 \times magnification. The assay was conducted three times and performed in triplicate each time.

Patients and tissue samples

We reviewed the medical records of patients with histologically verified HPSCC who under-

went transoral videolaryngoscopic surgery (TOVS) with or without neck dissection as the primary treatment with curative intent at the Department of Otorhinolaryngology-Head and Neck Surgery, Keio University Hospital (Tokyo, Japan) between 2007 and 2013. The indication for TOVS and its procedures was reported in detail previously [58]. Tumor stages were classified according to the American Joint Committee on the Cancer TNM staging system (2010, 7th edition). Formalin-fixed and paraffin-embedded (FFPE) surgical specimens eligible for the histopathological study were obtained from 54 patients, among whom 31 patients had neck lymph node metastasis and 23 patients had no neck metastasis. The protocols for the use of the clinical materials were approved by the Institutional Ethics Review Board of the Ethics Committee of Keio University School of Medicine (reference numbers: 2010-013 and 2010-013-2). The requirement for informed consent from patients was waived owing to the retrospective nature of the analysis. All procedures for clinical tissues were performed in accordance with the ethical standards of the institutional research committee and with the principles of the 1964 Helsinki Declaration and its later amendments.

Histopathological evaluation

The FFPE HPSCC specimens were sliced into 4- μ m-thick serial sections. A pathologist who was blinded to the clinical information evaluated the histopathological characteristics, including differentiation (histological grade), vascular invasion, and lymphatic invasion, by reviewing all slides from each patient stained with hematoxylin and eosin.

Immunohistochemical analysis

Immunohistochemical detection of protein expression was performed using the Ventana Discovery XT automated staining system (Ventana Medical Systems, AZ, USA) following the manufacturer's instructions with proprietary reagents. For each case, specimens serially sliced to a 4- μ m thickness at the central or maximum cross-section were selected. Slides were deparaffinized and rehydrated on an automated system with EZ Prep solution (Ventana). Following heat-induced antigen retrieval and quenching of endogenous peroxidase activity, the sections were incubated with

each of the following primary antibodies for 60 min: rabbit anti-Cox-2 (1:20 dilution, clone SP21, Roche Diagnostics) or mouse anti-E-cadherin antibody (1:25 dilution, clone 36, Roche Diagnostics). The antibodies on the sections were visualized using the DAB (3,3'-diaminobenzidine tetrahydrochloride) Map kit (Ventana) and then counterstained with Harris' hematoxylin. Slides were then mounted with a cover glass and evaluated under a light microscope. Immunohistochemical expression was defined as positive if staining was observed in more than 10% of the tumor cells.

Statistical analysis

The data repeatedly obtained in the *in vitro* assays are presented as the mean \pm standard deviation of three or more independent experiments. Differences in data between each condition and control were examined using a two-tailed Student's t-test. The correlation between Cox-2 and E-cadherin expression, as well as the association between these immunohistochemical expressions and clinicopathological variables, were evaluated using Fisher's exact test. Risk factors that affect neck lymph node metastasis were also examined using Fisher's exact test for univariate analysis. The independent significance of the variables that were considered significant in univariate analysis was further assessed by multivariate analysis using a multiple logistic regression model with backward stepwise selection. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS Ver. 23.0.

Results

Baseline mRNA expression levels of Cox-2 and CDH-1 in HNSCC cells

We assessed the mRNA expression levels of Cox-2 and E-cadherin transcripts (CDH-1) in various HNSCC cell lines using quantitative real-time PCR. **Figure 1A** shows the relative expression levels of each gene, which were normalized by dividing each value by that of BICR6 cells as a calibrator for the sake of convenience. Based on these baseline mRNA expression levels, we selected the following HPSCC-derived cells for *in vitro* experiments: BICR6 cells expressing a relatively high level of Cox-2 and a low level of CDH-1, and FaDu cells expressing a

relatively low level of Cox-2 and a middle level of CDH-1.

Alterations in CDH-1 and vimentin mRNA expression by Cox-2 and EP2 inhibition

We examined the effect of the selective Cox-2 inhibitor celecoxib and the EP2 antagonist PF-04418948 on the mRNA expression of CDH-1 and vimentin in BICR6 and FaDu cells. Because we observed neither dose-dependent nor time-dependent effects on the regulation with each reagent in our preliminary experiments, the results were shown with the doses and exposure times considered optimal for each reagent and each purpose. Celecoxib upregulated the CDH-1 expression compared to DMSO treatment as the control, with increases of 1.63- and 1.22-fold in BICR6 and FaDu cells, respectively (**Figure 1B**). Furthermore, PF-04418948 more noticeably upregulated CDH-1 expression, with increases of 1.75- and 1.72-fold in BICR6 and FaDu cells, respectively (**Figure 1B**). In contrast, vimentin expression was downregulated by celecoxib compared to the control, with decreases of 0.34- and 0.85-fold in BICR6 and FaDu cells, respectively (**Figure 1C**). In addition, PF-04418948 more markedly downregulated vimentin expression, with decreases of 0.23- and 0.47-fold in BICR6 and FaDu cells, respectively (**Figure 1C**). These results suggest that the extent of the effect of Cox-2 and EP2 inhibition on these molecules may differ depending on the cell type, and presumably on the baseline expression levels of both Cox-2 and CDH-1 in each cell.

Restoration of intercellular E-cadherin expression by Cox-2 and EP2 inhibition

Because the function of E-cadherin in intercellular adhesion is maintained through its membranous localization, alteration in the cellular localization of E-cadherin expression in BICR6 and FaDu cells was evaluated by immunofluorescence staining. As displayed in **Figure 2A** and **2B**, treatment with celecoxib, as well as PF-04418948, led to enhanced membranous expression of E-cadherin compared to the control, in both cells. Furthermore, the cellular morphology was observed using a phase contrast microscope. In line with the aforementioned results, treatment with celecoxib, as well as PF-04418948, restored the epithelial morphol-

EP2 and Cox-2 inhibition reverses EMT in hypopharyngeal cancer

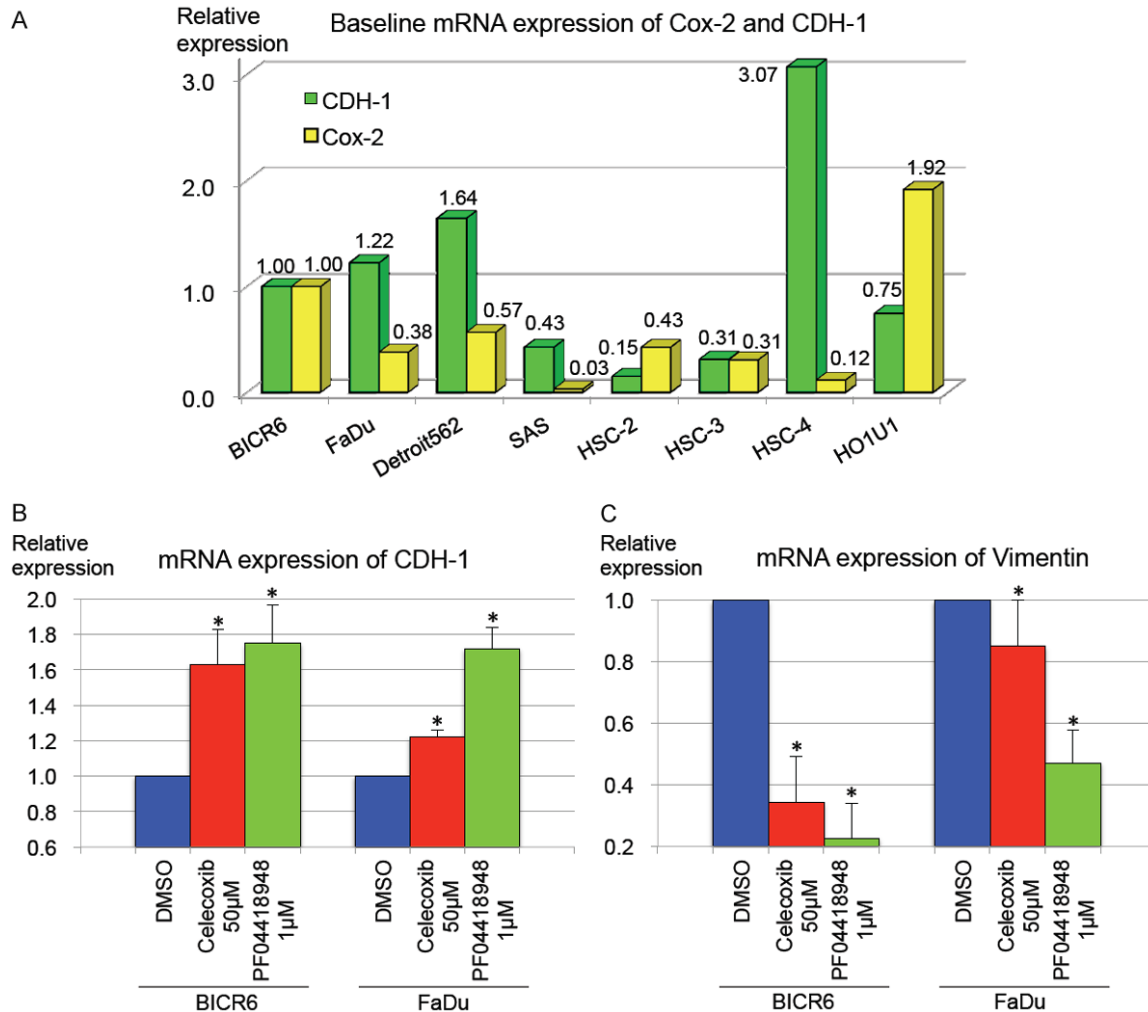


Figure 1. Baseline mRNA expression levels of Cox-2 and CDH-1, and the effects of Cox-2 and EP2 inhibition on CDH-1 and vimentin mRNA expression. The mRNA expression levels of each gene were assessed by quantitative real-time PCR. The relative expression levels of genes were compared after normalization using those of ACTB. (A) Baseline mRNA expression of Cox-2 and CDH-1 in HNSCC cells. Relative expression levels were calibrated by dividing each value with that of BICR6 cells for the sake of convenience. (B and C) Alterations in the mRNA expression levels of CDH-1 (B) and vimentin (C) in BICR6 and FaDu cells after a 12-h incubation with the Cox-2 inhibitor celecoxib (50 μ M) or EP2 antagonist PF-04418948 (1 μ M). The data are presented as the fold increase or decrease in the relative expression levels of each gene compared with the respective controls. In both BICR6 and FaDu cells, celecoxib upregulated CDH-1 expression, and PF-04418948 led to its upregulation more noticeably (B). By contrast, vimentin expression was downregulated by celecoxib, and PF-04418948 induced its downregulation more markedly in both cells (C). The values represent the mean \pm standard deviation. Differences between each condition and control were statistically analyzed using a two-tailed t-test: *, $P < 0.05$.

ogy to a polygonal shape in both cells (**Figure 2A and 2B**).

Alterations in the mRNA expressions of transcriptional repressors of E-cadherin by Cox-2 and EP2 inhibition

Additionally, the inhibitory effects of celecoxib and PF-04418948 were examined on the mRNA expressions of the representative transcriptional repressors of E-cadherin: *snai1*,

ZEB1, and *twist* (**Figure 3A and 3B**). In BICR6 cells, celecoxib clearly downregulated all three repressors, with decreases of 0.45-, 0.63-, and 0.81-fold in *snai1*, *ZEB1*, and *twist*, respectively. In contrast, in FaDu cells, only *snai1* was suppressed by celecoxib, decreasing by 0.50-fold, whereas no alteration was observed in *ZEB1* and *twist*. Moreover, in BICR6 cells, PF-04418948 distinctly reduced *snai1* and *ZEB1*, with decreases of 0.39- and 0.26-fold, respectively, whereas no effect was found on

EP2 and Cox-2 inhibition reverses EMT in hypopharyngeal cancer

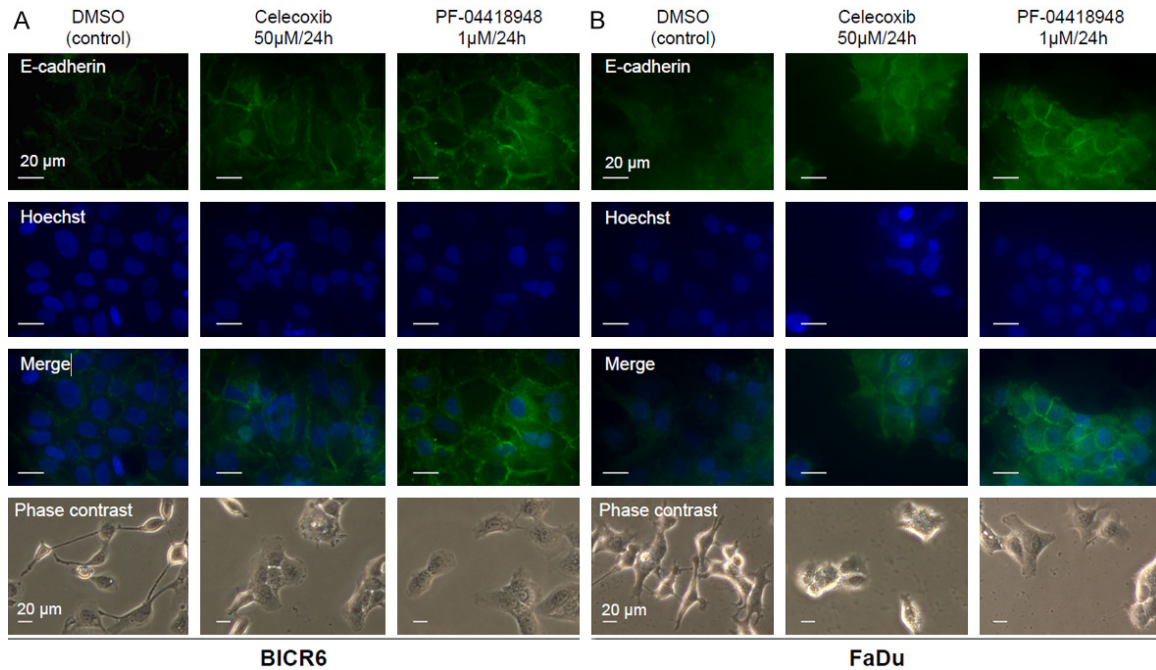


Figure 2. Restoration of intercellular E-cadherin expression and epithelial cell morphology in HPSCC cells by Cox-2 and EP2 inhibition. Alteration in the cellular localization of E-cadherin expression in BICR6 cells (A) and FaDu cells (B) after a 24 h incubation with celecoxib (50 μM) or PF-04418948 (1 μM) was evaluated by immunofluorescence staining. Nuclei were stained with Hoechst 33258. Alteration in cellular morphology was also observed using a phase contrast microscope. The Cox-2 and EP2 inhibition enhanced the membranous expression of E-cadherin, as well as restored the epithelial morphology to a polygonal shape in both cells. Scale bar: 20 μm.

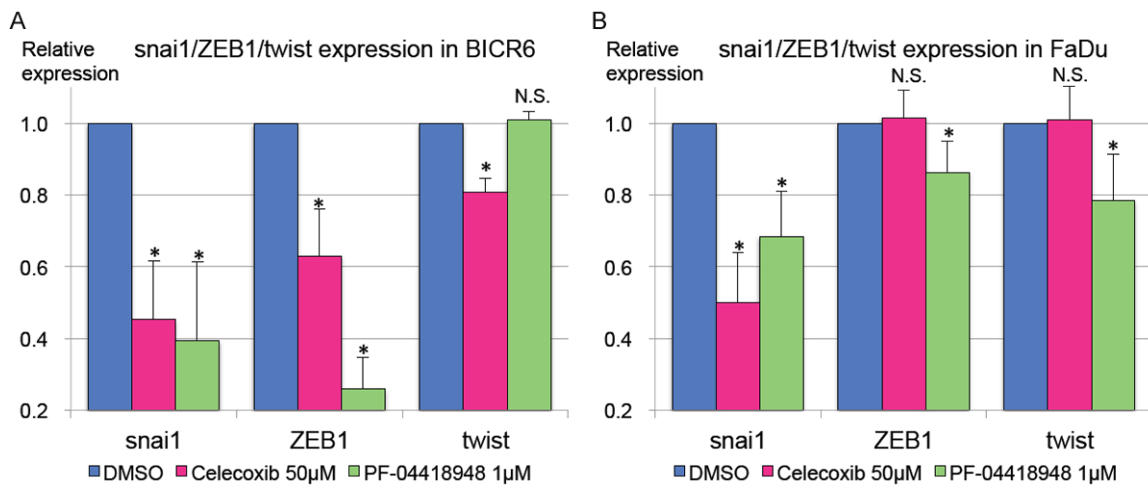


Figure 3. Effects of Cox-2 and EP2 inhibition on the mRNA expression of transcriptional repressors of E-cadherin in HPSCC cells. Alterations in the mRNA expression of snai1, ZEB1, and twist in BICR6 cells (A) and FaDu cells (B) after a 12 h incubation with celecoxib (50 μM) or PF-04418948 (1 μM) were assessed by quantitative real-time PCR. The data are presented as the fold decrease in the relative expression levels of each gene compared with the respective controls. In BICR6 cells, celecoxib clearly downregulated all three repressors (A), whereas in FaDu cells, only snai1 was suppressed by celecoxib (B). On the other hand, PF-04418948 distinctly reduced snai1 and ZEB1, but not twist in BICR6 cells (A), whereas it attenuated all three transcriptional repressors in FaDu cells (B). The values represent the mean ± standard deviation. Differences between each condition and control were statistically analyzed using a two-tailed t-test: *, P < 0.05; N.S., not significant.

twist. However, in FaDu cells, all three transcriptional repressors were attenuated by

PF-04418948, decreasing by 0.69-, 0.86-, and 0.79-fold in snai1, ZEB1, and twist, respective-

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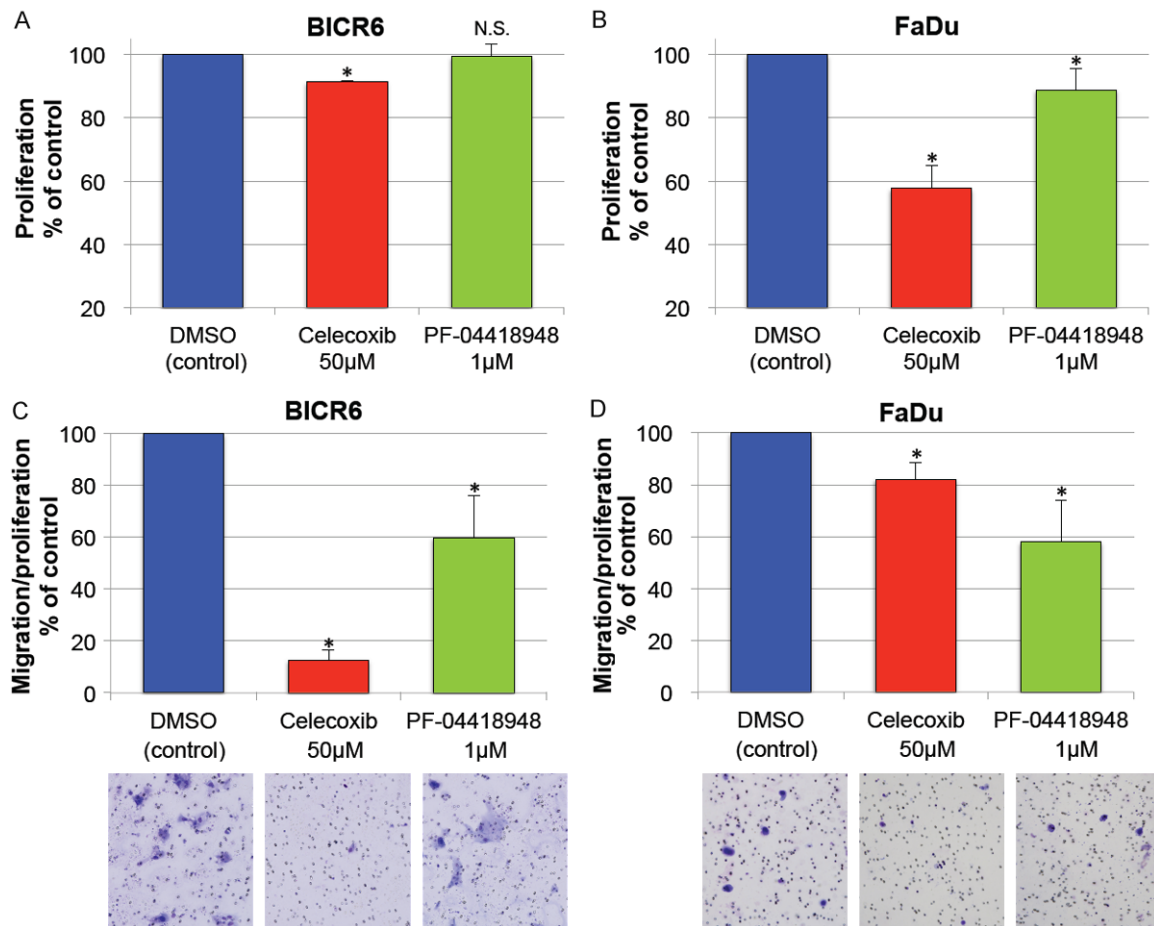


Figure 4. Effects of Cox-2 and EP2 inhibition on the cell proliferation activity and migration ability of HPSCC cells. Alterations in cell proliferation activity and migratory ability after a 24 h incubation with celecoxib (50 μM) or PF-04418948 (1 μM) were examined using BICR6 cells (A and C) and FaDu cells (B and D). (A and B) An *in vitro* cell proliferation assay was conducted for which the data are presented as the fold decrease in the OD values of absorbance measured at 450 nm compared with the respective controls. In BICR6 cells (A), celecoxib showed a slight suppressive effect on proliferation, whereas PF-04418948 displayed no effect. In FaDu cells (B), celecoxib obviously attenuated proliferation, whereas PF-04418948 showed a modest effect. (C and D) An *in vitro* migration assay was performed for which the results are presented as the fold decrease in the number of migrated cells compared with the respective controls. To eliminate the possible influence of differences in proliferation activity, each value was normalized according to the corresponding ratio of the proliferation rate observed in the same condition. Celecoxib repressed migration in BICR6 cells (C) much more obviously than in FaDu cells (D), whereas PF-04418948 reduced migration in BICR6 (C) and FaDu cells (D) almost equally. Values represent the mean \pm standard deviation. Differences between each condition and control were statistically analyzed using a two-tailed t-test: *, $P < 0.05$; N.S., not significant.

ly. These results suggest that the extent of the effects of Cox-2 and EP2 inhibition on these transcriptional repressors varies largely depending on the cell type but is not exactly associated with their effects on CDH-1 and vimentin, in both cells.

Effects of Cox-2 and EP2 inhibition on cell proliferation activity

Alterations in cell proliferation activity owing to Cox-2 and EP2 inhibition were examined by an

in vitro cell proliferation assay. The data are presented as the fold decrease in the OD values compared with the respective controls. In BICR6 cells, celecoxib slightly suppressed proliferation activity, with a decrease of 0.91-fold, whereas PF-04418948 showed no effect on cell proliferation (**Figure 4A**). Moreover, in FaDu cells, celecoxib obviously attenuated proliferation activity, with a decrease of 0.58-fold, whereas PF-04418948 showed modest suppression, decreasing by 0.89-fold (**Figure 4B**).

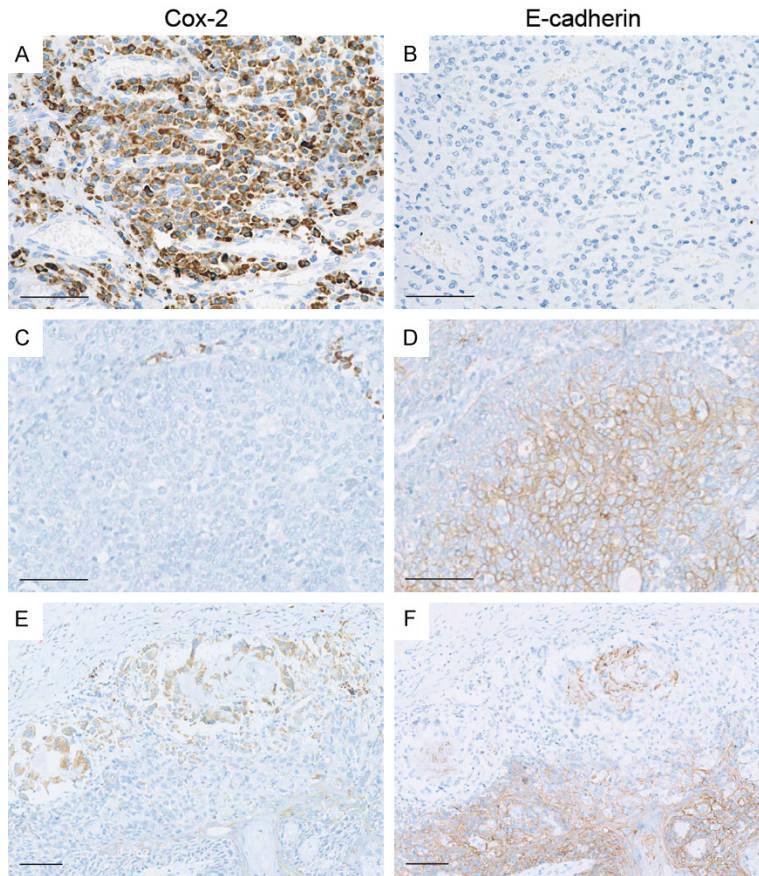


Figure 5. Immunohistochemistry of Cox-2 and E-cadherin in surgical specimens of HPSCC. Immunohistochemical staining was performed for Cox-2 (A, C, and E) and E-cadherin (B, D, and F). In representative cases with positive staining in the tumor cells, cytoplasmic expression was observed for Cox-2 (A), whereas membranous expression was visualized for E-cadherin (D). Notably, Cox-2 and E-cadherin tended to display an inverted expression pattern *in situ*, especially in the tumor cell clusters, as shown in each pair of serial sections (A vs B, and C vs D). In cases where both proteins were positive, Cox-2 and E-cadherin exhibited reciprocal immunolocalization with an apparent contrast, in which Cox-2 was expressed in E-cadherin-negative cells (E) and vice versa (F). Original magnification: $\times 200$. Scale bar: 100 μm .

Table 1. Correlation between immunohistochemical expression of Cox-2 and E-cadherin

Immunohistochemical markers		E-cadherin		P value
		Positive (n = 39)	Negative (n = 15)	
Cox-2	Positive (n = 23)	13	10	0.028*
	Negative (n = 31)	26	5	

*Statistically significant according to Fisher's exact test.

Effects of Cox-2 and EP2 inhibition on cell migration ability

Alterations in cell migration ability owing to Cox-2 and EP2 inhibition were assessed by an

in vitro migration assay. The results are presented as the fold decrease in the number of migrated cells compared with the respective controls. To eliminate the possible influence of differences in proliferation activity, each value was normalized according to the corresponding ratio of the proliferation rate observed in the same condition (**Figure 4C** and **4D**). Celecoxib repressed the migration ability in BICR6 cells much more obviously than in FaDu cells, with decreases of 0.13- and 0.82-fold, respectively. In contrast, PF-04418948 reduced the migration ability in BICR6 and FaDu cells almost equally, decreasing by 0.60- and 0.58-fold, respectively.

Immunohistochemical analysis of Cox-2 and E-cadherin in HPSCC tissues

Of the 54 cases examined, positive immunohistochemical staining of Cox-2 was demonstrated in 23 cases (42.6%) and E-cadherin in 39 cases (72.2%). As shown in the representative cases, cytoplasmic expression was observed for Cox2 (**Figure 5A**), whereas membranous expression was visualized for E-cadherin (**Figure 5D**). Intriguingly, from the viewpoint of relative localization, Cox-2 and E-cadherin tended to exhibit an inverted staining pattern in the tumor cell clusters, as shown in each pair of serial sections (**Figure 5A-D**) with an obvious contrast, especially in cases where both proteins were positive

(**Figure 5E, 5F**), suggesting an inverse correlation *in situ* between these molecules in terms of their cellular localization. The association of expression between Cox-2 and E-cadherin regarding the incidence of immu-

Table 2. Association between Cox-2 and E-cadherin immunohistochemical expression and clinico-pathological variables

Variable			n	Cox-2			E-cadherin						
				Positive (n = 23)		Negative (n = 31)		P value	Positive (n = 39)		Negative (n = 15)		P value
				n	(%)	n	(%)		n	(%)	n	(%)	
Age	≥ 70	20	9	(45.0)	11	(55.0)	0.503	15	(75.0)	5	(25.0)	0.491	
	< 70	34	14	(41.2)	20	(58.8)		24	(70.6)	10	(29.4)		
Sex	Men	49	23	(46.9)	26	(53.1)	0.054	35	(71.4)	14	(28.6)	0.572	
	Women	5	0	(0.0)	5	(100.0)		4	(80.0)	1	(20.0)		
T classification	T1	19	4	(21.1)	15	(78.9)	0.018*	18	(94.7)	1	(5.3)	0.006	
	T2-3	35	19	(54.3)	16	(45.7)		21	(60.0)	14	(40.0)		
Neck metastasis	Negative	23	1	(4.3)	22	(95.7)	< 0.001*	22	(95.7)	1	(4.3)	0.001*	
	Positive	31	22	(71.0)	9	(29.0)		17	(54.8)	14	(45.2)		
Differentiation	Well	19	4	(21.1)	15	(78.9)	0.018*	18	(94.7)	1	(5.3)	0.006*	
	Moderately or poorly	35	19	(54.3)	16	(45.7)		21	(60.0)	14	(40.0)		
Vascular invasion	Negative	41	17	(41.5)	24	(58.5)	0.506	32	(78.0)	9	(22.0)	0.977	
	Positive	13	6	(46.2)	7	(46.2)		7	(46.2)	6	(46.2)		
Lymphatic invasion	Negative	34	13	(38.2)	21	(61.8)	0.287	28	(82.4)	6	(17.6)	0.033*	
	Positive	20	10	(50.0)	10	(50.0)		11	(55.0)	9	(45.0)		

*Statistically significant according to Fisher's exact test.

nopositivity was examined by Fisher's exact test. In accordance with the above-mentioned observation, the expression of these two molecules was inversely correlated significantly ($P = 0.028$, **Table 1**).

Correlation of Cox-2 and E-cadherin immunohistochemical expression with clinicopathological variables

The association between Cox-2 and E-cadherin immunohistochemical expression in tumor cells and the clinicopathological variables was examined in 54 patients with HPSCC who underwent TOVS. As summarized in **Table 2**, Cox-2 expression was significantly correlated with advanced T-classification ($P = 0.018$), neck metastasis ($P < 0.001$), and differentiation ($P = 0.018$), whereas E-cadherin expression showed a significant inverse correlation with advanced T-classification ($P = 0.006$), neck metastasis ($P = 0.001$), differentiation ($P = 0.006$), and lymphatic invasion ($P = 0.033$). Neither of the molecules was associated with other variables including age, sex, and vascular invasion.

Univariate and multivariate analyses of risk factors affecting neck metastasis

To elucidate the risk factors affecting neck metastasis, we further examined the associa-

tion of neck metastasis with other clinicopathological variables. As summarized in **Table 3**, univariate analysis exhibited that less differentiation was significantly correlated with neck metastasis ($P = 0.001$), whereas sex ($P = 0.097$), T-classification ($P = 0.083$), and vascular invasion ($P = 0.093$) also showed marginal significance in correlation with neck metastasis. A multiple logistic regression model was applied to determine the independent significance of variables that were correlated with neck metastasis in the aforementioned univariate analyses. As shown in **Table 4**, the expression of Cox-2 (odds ratio [OR] = 53.49, $P < 0.001$) and E-cadherin (OR = 0.06, $P = 0.016$) were found to be independent risk factors that affect neck metastasis in this cohort.

Discussion

In addition to the issues of increased cardiovascular risk as described in the Introduction section, Cox-2 inhibitors have faced tough challenges in clinical trials. Regarding the chemopreventive effects on post-treatment patients with cancer, regular intake of NSAIDs significantly reduced recurrence risk and/or mortality in breast cancer patients [59-61], while that of aspirin or Cox-2 inhibitor significantly improved survival in colon cancer patients [62]. However, concerning patients with premalignant lesions,

Table 3. Univariate analysis of risk factors affecting neck metastasis

			Neck metastasis				
Variable		n	Positive (n = 31)		Negative (n = 23)		P value
			n	(%)	n	(%)	
Age	≥ 70	20	10	(50.0)	10	(50.0)	0.287
	< 70	34	21	(61.8)	13	(38.2)	
Sex	Men	49	30	(61.2)	19	(38.8)	0.097
	Women	5	1	(20.0)	4	(80.0)	
T classification	T1	19	8	(42.1)	11	(57.9)	0.083
	T2-3	35	23	(65.7)	12	(34.3)	
Differentiation	Well	19	5	(26.3)	14	(73.7)	0.001*
	Moderately or poorly	35	26	(74.3)	9	(25.7)	
Vascular invasion	Negative	41	21	(51.2)	20	(48.8)	0.093
	Positive	13	10	(76.9)	3	(23.1)	
Lymphatic invasion	Negative	34	17	(50.0)	17	(50.0)	0.125
	Positive	20	14	(70.0)	6	(30.0)	

*Statistically significant according to Fisher's exact test.

Table 4. Multivariate analysis of risk factors affecting neck metastasis

Step	Variable	OR	95% CI	P value
Step 1				
	Sex	0.72	0.024-21.629	0.853
	T classification	0.17	0.010-2.902	0.221
	Differentiation	2.76	0.363-20.974	0.327
	Vascular invasion	5.68	0.373-86.479	0.211
	Cox-2	155.98	5.785-4205.749	0.003*
	E-cadherin	0.05	0.002-1.411	0.078
Step 2				
	T classification	0.18	0.011-2.862	0.224
	Differentiation	2.66	0.368-3412.194	0.332
	Vascular invasion	5.26	0.398-1.264	0.208
	Cox-2	144.04	6.080-19.220	0.002*
	E-cadherin	0.05	0.002-69.631	0.068
Step 3				
	T classification	0.13	0.009-1.984	0.143
	Vascular invasion	6.34	0.504-79.851	0.153
	Cox-2	188.89	8.030-4443.022	0.001*
	E-cadherin	0.02	0.001-0.570	0.021*
Step 4				
	T classification	0.24	0.024-2.414	0.227
	Cox-2	111.10	7.002-1762.637	< 0.001*
	E-cadherin	0.02	0.001-0.424	0.012*
Step 5				
	Cox-2	53.49	5.706-501.489	< 0.001*
	E-cadherin	0.06	0.005-0.589	0.016*

OR, odds ratio; CI, confidence interval. *Statistically significant according to multiple logistic regression analysis.

the chemopreventive benefit of celecoxib was found only for familial adenoma polyposis [63], but was not observed for others including Barrett's esophagus [64], oral premalignant lesions [65], and cervical intraepithelial neoplasia [66]. Furthermore, although a number of trials have been conducted to examine the benefit of celecoxib in combination with chemotherapy in the treatment of advanced cancers of the pancreas, lung, and colorectum, no additional therapeutic effect has been demonstrated [67-72]. These discouraging outcomes of Cox-2 inhibition also have led to a notion that PGE₂ inhibition by targeting downstream EP receptors might provide superior therapeutic effects and specificity compared to simply shutting down the entire Cox cascade.

Our *in vitro* study demonstrated that selective pharmacological inhibition of Cox-2 using celecoxib, as well as that of EP2 using the receptor-specific antagonist PF-044-18948, repressed their migration ability in HPSCC cells through suppression of EMT by restoring E-cadherin expression with its membra-

nous localization and reducing vimentin expression via downregulating the transcriptional repressors of E-cadherin. These results suggest that targeting EP2 is an attractive strategy for novel therapeutics aimed at preventing the development and progression of malignant tumors including HPSCC. Because of the comparable effects of PF-04418948 at much lower dose than that of celecoxib without inhibiting other prostanoids signaling pathways downstream of Cox-2, the EP2 antagonist can be a promising alternative to the selective Cox-2 inhibitor with a much less risk of adverse effects, although further extensive evaluation is indispensable to elucidate its clinical feasibility.

Notably, the degree of the effect of Cox-2/EP2 inhibition on the expression of EMT-related molecules was shown to depend on the baseline expression levels of both Cox-2 and E-cadherin (CDH-1) in each cell; i.e., tumor cells expressing higher Cox-2 and lower E-cadherin appear to be more susceptible to Cox-2/EP2 inhibition in terms of reversing EMT. Such a finding regarding Cox-2 inhibition is in accordance with previous studies that examined cancer cells of bladder [29, 31], colon [30], and tongue [33], whereas that regarding EP2 inhibition has not yet been reported. Accordingly, it is indicated that the anti-EMT effects of EP2 antagonist, as well as Cox-2 inhibitor, could be predictable in advance by evaluating the baseline expression level of Cox-2, E-cadherin, and other certain downstream molecules individually.

However, in our migration assay, the suppressive effects of celecoxib and PF-04418948 on cell migration were not necessarily consistent with those on the expression of EMT-related molecules; the suppressive effect of celecoxib was much greater in BICR6 than in FaDu, whereas that of PF-04418948 was almost even between the cells. From a different point of view, the strength of the suppressive effect of both reagents on migration was in proportion with that on the expression of EMT-related molecules in FaDu cells, but this was not the case in BICR6 cells wherein celecoxib showed markedly strong suppression of cell migration. Such discordance found within these results is presumably attributed to the fact that these effects of the reagents are not uniform in a dose-

and/or time-dependent manner as observed in our preliminary experiments, as well as to the possible Cox-2-independent anti-cancer effect of celecoxib that may also affect migratory ability [73, 74]. In terms of the EP2-dependent regulation of cell migration involved in EMT, our finding is in line with a previous study in which EP2 activation, via either PGE₂ or the EP2 agonist butaprost, enhanced cell migration and invasion by upregulating snail expression in hepatocellular carcinoma (HCC) cells, although neither the expression of typical EMT-related markers including E-cadherin nor the effect of EP2 inhibition were presented [75].

Aside from EMT-derived functions, the inhibitory effects of the reagents on cell proliferation in our study showed a tendency rather opposite to that shown in the assays of migration and EMT-related molecules expression, i.e., the inhibitory effects on proliferation were observed clearly in FaDu cells but not in BICR6 cells, with a consistent dominance of celecoxib in both cells. However, such inconsistency was not necessarily unexpected, because it could be partly ascribed to certain disparities in the molecular mechanisms between cell migration and proliferation, and also to a relatively low association between proliferation activity and the expression level of EMT-related molecules.

Regarding the transcriptional repressors of E-cadherin regulated downstream of the Cox-2/PGE₂/EP2 pathway, we found that celecoxib suppressed the expression of snail predominantly compared to other repressors in BICR6 cells and quite exclusively in FaDu cells, whereas PF-04418948 attenuated that of snail and ZEB1 in the both cells and of twist only in FaDu cells. These results suggest a relatively important role of snail and ZEB1 in reversing EMT by inhibiting this pathway in HPSCC cells, particularly in those expressing higher Cox-2 and lower E-cadherin. This responsibility of snail and ZEB1 was first demonstrated in a study using genetic manipulation of Cox-2 expression and PGE₂-treatment in NSCLC cells [22], and was later confirmed in a study using three Cox-2 inhibitors in bladder cancer cells [31]. Although EP2 antagonist-induced downregulation of the transcriptional repressors of E-cadherin has not been reported so far, EP2 activation-upregulated snail was further confirmed in HCC cells [75]. We previously found that expression of SIP1, another key transcrip-

tional repressor of E-cadherin [76, 77], was also correlated with Cox-2 expression, and was downregulated consistently similar to snail and twist by all three Cox-2 inhibitors examined in OSCC cells [33], however, this was not the case in the present study (data for SIP1 are not shown). Such inconsistent results appeared to be attributable to disparities in relative dominance among these transcriptional repressors, which may depend on each origin of cancer cells.

Despite its theoretically appealing strategy, the anti-cancer effect of targeting EP2 using selective EP2-antagonists has not been investigated extensively except for the few following studies. Another small molecular EP2-selective antagonist, TG4-155, was demonstrated to suppress PGE₂-induced prostate cancer cell proliferation and invasion, accompanied by downregulation of the inflammatory cytokines IL-1 β and IL-6 [78]. More recently, TG6-10-1, a novel brain-permeable EP2 antagonist, was reported to reduce Cox-2 activity-driven malignant glioma cell proliferation, invasion, and migration, and to cause cell cycle arrest and apoptosis [79]. Regarding PF-04418948, although its direct effect on cancer cells has not been reported previously, its administration in a colitis-associated colon cancer model of mice was shown to suppress tumor formation by inhibiting inflammatory responses in the tumor microenvironment where EP2 is expressed in infiltrating neutrophils and tumor-associated fibroblasts. This suppression is exerted via repressing the infiltration of these inflammatory cells through downregulation of their own chemoattractant CXCL1 [80]. Our findings with PF-04418948 in HPSCC are in agreement with these studies regarding the promising position of EP2 antagonists as an alternative to Cox-2 inhibitors.

An increasing number of studies have investigated the molecular mechanism by which PGE₂/EP2 signaling serves the acquisition of a malignant phenotype as reviewed recently [13, 48, 55]. EP2 activation by PGE₂ can activate adenylate cyclase that increases the cytoplasmic levels of cAMP, leading to activation of protein kinase A (PKA) that directly phosphorylates and activates its downstream transcription factors such as T-cell factor (TCF), lymphoid enhancer factor (LEF) [81], and cAMP response

element-binding protein (CREB) [82], thereby regulating a wide range of biological functions. In addition, PGE₂-stimulated EP2 can activate and dissociate its coupled G protein subunits, which then act in two ways: the G α s subunit binds to Axin to release GSK-3 β from its complex with Axin, whereas the G β / γ subunits activate PI3K/Akt to promote the phosphorylation and inactivation of released GSK-3 β . These processes lead to nuclear translocation of β -catenin and subsequent activation of its target genes [83]. Intriguingly, the PGE₂/EP2-stimulated PI3K/Akt/GSK-3 β / β -catenin pathway is also shown to activate the cofactors TCF-4 and LEF-1, which results in upregulation of Cox-2 [84], suggesting a positive feedback regulation of Cox-2 expression through this axis. Moreover, activated EP2 also forms a complex with β -arrestin-1 and Src, leading to activation of Src/EGFR that subsequently activate H-Ras, ERK1/2, and AKT, thereby stimulating multiple signaling pathways/effectors via G protein-independent mechanisms [85]. These various molecular mechanisms activated by PGE₂/EP2 are thought to serve multifarious functions involved in cancer development and progression including proliferation, migration, invasiveness, survival, and therapeutic resistance of cancer cells, as well as angiogenesis, immunosuppression, and chronic inflammation in the tumor microenvironment. Considering such versatile roles of EP2, as well as Cox-2, it is conceivable that, besides suppressing EMT, a variety of mechanisms are implicated in the anti-cancer effects of selective EP2 antagonists and Cox-2 inhibitors, and that these mechanisms contribute cooperatively to their various effects.

In the clinical specimens of HPSCC examined using immunohistochemistry, the expression of Cox-2 and E-cadherin was inversely correlated, not only regarding the incidence of immunopositivity statistically, but also concerning the cellular localization that displays their reciprocal expression pattern *in situ*. A significant inverse correlation between these molecules was also found in bladder cancer [86], while a similar inverse expression pattern *in situ* in individual cases was also observed in NSCLC, OSCC, and colon cancer [22, 23, 87]. These findings corroborate the close involvement of Cox-2 with EMT in cancer tissues and suggest that the degrees of Cox-2 upregulation and

E-cadherin downregulation vary depending on microscopically specific sites, e.g., from the invasive front to the center of cancer nests.

As for correlations with clinicopathological variables in HPSCC, while the univariate analysis showed that enhanced expression of Cox-2 and reduced expression of E-cadherin, as well as less differentiation, were significantly correlated with neck lymph node metastasis, the multivariate analysis revealed that both Cox-2 overexpression and E-cadherin downregulation were independent predictors of neck metastasis in this cohort. The result regarding Cox-2 is in accordance with the several previous immunohistochemical studies that also found a positive correlation of Cox-2 with neck metastasis in HNSCC [88-90] including HPSCC [91], though all results but one [90] were confined to univariate analysis. On the other hand, the result concerning E-cadherin is also consistent with recent studies that examined laryngeal [92] and tongue SCC [93] using multivariate analysis, as well as with our previous studies that examined the above-mentioned correlation with delayed neck metastasis [77] or at mRNA expression level [33] in tongue SCC. The previous and the present findings support the notion that Cox-2-promoted pleiotropic function including enhanced migration ability via EMT induction can be responsible for developing lymphatic metastasis in HPSCC. Accordingly, suppression of EMT by inhibiting Cox-2/PGE₂/EP2 signaling using selective EP2 antagonists and/or Cox-2 inhibitors is expected to prevent the initiation and progression of lymph node metastasis, which may contribute to improving the treatment outcomes of various malignant tumors including HPSCC. Further studies remain to be conducted in preclinical and clinical settings to examine whether EP2 antagonists exhibit sufficient anti-cancer effect while minimizing the risk of adverse reactions in comparison with Cox-2 inhibitors.

Conclusions

The present study demonstrated that a selective EP2 antagonist, as well as a Cox-2 inhibitor, suppressed migration ability of HPSCC cells by reversing EMT through restoring E-cadherin expression and reducing vimentin expression. In addition, Cox-2 overexpression and E-cadherin downregulation were shown to be

independently predictive of neck metastasis, suggesting a critical implication of these molecules in lymphatic metastasis. Taken together, targeting Cox-2/EP2 signaling, especially using EP2 antagonists, can be a promising therapeutic strategy by exerting an anti-metastatic effect via EMT reversal for the treatment of patients with various cancers including HPSCC.

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Disclosure of conflict of interest

None.

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