Therapeutic effect of CS-706, a specific cyclooxygenase-2 inhibitor, on gallbladder carcinoma in BK5.ErbB-2 mice

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Abstract

Biliary tract cancer is still challenging to treat and manage due to its poor sensitivity to conventional therapies and the inability to prevent or detect the early tumor formation. The most well known risk factor for gallbladder cancer is the presence of chronic inflammation, usually related to gallstones. It has been suggested that cyclooxygenase-2 (COX-2) plays a variety of roles in the gastrointestinal tract, including pathogenic processes such as neoplasia. Recently, we have generated transgenic mice that over-express rat ErbB-2 under the control of bovine keratin 5 promoter (BK5.ErbB-2 mice). Homozygous BK5.ErbB-2 mice develop adenocarcinoma of gallbladder with an ~90% incidence. In addition to the activation of ErbB-2 and epidermal growth factor receptor, mRNA and protein levels of COX-2 were up-regulated in the gallbladder carcinomas that developed in these transgenic mice. The aim of this study was to examine the effects of a COX-2 inhibitor, CS-706, on the development of gallbladder carcinomas using the BK5.ErbB-2 mouse model. Ultrasound image analysis as well as histologic evaluation revealed a significant therapeutic effect of CS-706 on the gallbladder tumors, either as reversion to a milder phenotype or inhibition of tumor progression. The anti-tumor effect was associated with inhibition of prostaglandin E2 synthesis. CS-706 treatment also down-regulated the activation of ErbB-2 and epidermal growth factor receptor, resulting in decreased levels of phosphorylated Akt and COX-2 in gallbladder cancers of BK5.ErbB-2 mice. Based on our results, targeting COX-2 could provide a potentially new and effective therapy alone or in combination with other therapeutic agents for patients with biliary tract cancer. [Mol Cancer Ther 2007;6(6):1709–17]

Introduction

Although there have been advances in diagnosis and management of biliary tract cancer (BTC), it is still proving challenging to treat due to its poor sensitivity to conventional therapies and the inability to prevent or detect early tumor formation. All of these factors render gallbladder cancer nearly incurable, with a 5-year survival rate of only 5% to 21% (1–5). An estimated 20,000 new cases of liver and BTC are diagnosed annually in the United States, and each year, 4,000 patients will die of BTC, accounting for ~1% of all deaths from cancer (6). Nearly two thirds of these arise in the gallbladder, making it the most common BTC and fifth most common gastrointestinal tract cancer, whereas the remainder originates in the intrahepatic bile ducts and periampullary region (7).

Presence of a chronic inflammatory state, usually related to gallstones, has been found to be the most significant risk factor for gallbladder cancer. There is a known association between gallstones and gallbladder carcinoma, with gallstones present in 74% to 92% of patients with gallbladder carcinoma (8, 9). Pathogenic bacteria are cultured from the gallbladders of patients with gallbladder carcinoma at a significantly greater frequency than patients with simple cholelithiasis (10). Typhoid carriers may also suffer chronic inflammation of the gallbladder and have been described to have a significantly higher risk of gallbladder carcinoma than the rest of the population (11, 12). The presence of anomalous pancreaticobiliary ductal junction, which causes a long-term inflammation, has also been suggested in numerous reports as a risk factor for gallbladder carcinoma (13).

Accumulating evidence suggests that cyclooxygenase (COX)-2, an inducible enzyme responsible for conversion of arachidonic acid to prostaglandins, plays a variety of roles in the gastrointestinal tract, including pathogenic processes such as neoplasia (14). Elevated COX-2 expression has been shown in well-differentiated human hepatocellular carcinoma (15, 16) and gallbladder carcinoma (17). Grossman et al. (18) reported that a specific COX-2 inhibitor (SC58125), but not COX-1 inhibitor (valeryl salicylic acid), decreased mitogenesis and increased human gallbladder cell apoptosis associated with decreased prostaglandin E2 (PGE2). This suggests that prostaglandins may play a role in the development of gallbladder cancer and that COX-2 inhibitors may have a therapeutic role in gallbladder neoplasms. A recent study showed a relationship between ErbB-2 overexpression and COX-2
up-regulation in human colorectal cancer cells (19). Sirica et al. suggested a strong positive correlation between the immunostaining intensities of ErbB-2 and COX-2. In this regard, COX-2 was observed not only in the human cholangiocarcinoma model (20) but also in human cholangiocarcinoma (21), supporting the possibility that ErbB-2 plays a key role in regulating COX-2 expression in neoplastic and precancerous biliary tract epithelial cells.

Several years ago, our laboratory generated transgenic mice that overexpress wild-type rat ErbB-2 under the control of the BK5 promoter (22). Overexpression of wild-type ErbB-2 in basal epithelial cells of gallbladder led to the development of adenocarcinoma of the gallbladder and cystic duct in ~90% of these transgenic mice. In addition, these mice also developed cholangiocarcinoma at a lower incidence of ~30%. Several similarities between gallbladder carcinoma in BK5.ErbB-2 mice and human gallbladder carcinoma include histopathologic observation, overexpression of proteins, such as ErbB-2 and COX-2, and alterations of p53 and K-ras (23). BK5.ErbB-2 transgenic mice seem to represent a unique new animal model for further mechanistic studies involving the role of ErbB-2 and COX-2 in the development and growth of BTC as well as a promising tool for the development of new treatment and/or prevention modalities.

In our current study, we examined the effects of a newly engineered COX-2 inhibitor, CS-706 (Sankyo Co. Ltd.), on the development of gallbladder carcinoma in the BK5.ErbB-2 mouse model. The long-term goal is to develop new, more effective treatment options that will lead to improved prognosis for this devastating type of cancer.

Materials and Methods

Breeding and Identification of Transgenic Mice

BK5.ErbB-2 transgenic mice are bred in the Griffin Animal Resource Facility at the M. D. Anderson Cancer Center, Science Park-Research Division, campus. For the purposes of these experiments, hemizygous BK5.ErbB-2 transgenic mice were mated together to generate an F2 population consisting of wild-type (25%), hemizygous (50%), and homozygous (25%) transgenic mice. Transgenic mice were identified by PCR of DNA isolated from the tails of weanlings using oligonucleotides specific for the rabbit β-globin cDNA as described previously (22). BK5.ErbB-2 mice homozygous for the transgene exhibit a gross phenotype characterized by alopecia and wrinkled skin (22). Hemizygous BK5.ErbB-2 mice are distinguished by their altered hair coat characterized by a ruffled and shaggy appearance, which is relatively easy to distinguish these from nontransgenic or homozygous BK5.ErbB-2 littermates. To confirm the difference of transgene expression between homozygous and hemizygous transgenic mice, fluorescence immunostaining for ErbB-2 protein in tail skin was done. The intensity of fluorescence for rat ErbB-2 has been shown to correlate with obvious differences of physical characteristics in nontransgenic, hemizygous, and homozygous transgenic mice (22).

Animals and Treatment Protocols

Homozygous BK5.ErbB-2 mice and nontransgenic littermates received a control AIN76A diet and an experimental diet containing 60, 100, or 160 ppm of CS-706 (Sankyo Co. Ltd.). Due to the toxicity, treatment with CS-706 at 160 ppm to these mice was discontinued, and the mice were sacrificed for histologic evaluation. Most of the mice treated with this dose had diarrhea; however, body weight changes between control diet group and 160 ppm CS-706--treated group were not statistically significant in both nontransgenic and BK5.ErbB-2 mice (data not shown). Although most of the mice treated with 160 ppm of CS-706 had edematous intestines and colons, histologic analysis did not reveal any particular abnormal lesions in tissues, including intestines, colon, bone marrow, liver, kidney, and spleen, with the exception of one BK5.ErbB-2 mouse in which fatty liver was observed. These diets were stored at 4°C and fresh diet was supplied every other day. The animals were monitored biweekly to evaluate systemic toxicity, body weight, and feed consumption. Feed consumption was measured by the average changes in body weight. All experiments were carried out with strict adherence to institutional guidelines for minimizing distress in experimental animals.

Histologic Analysis

Biliary tract tissues were fixed in formalin and embedded in paraffin before sectioning. Serial sections (28 μm between each section) of 7 μm were cut and stained with H&E.

Proliferating Cell Nuclear Antigen Staining

The expression of proliferating cell nuclear antigen (PCNA) was detected by a PCNA staining kit (Zymed Laboratories). This procedure was done following the protocol outlined in the staining kit.

Immunofluorescence Staining

The expression and localization of ErbB-2, phosphorylated ErbB-2 (p-ErbB-2), epidermal growth factor receptor (EGFR), phosphorylated EGFR (p-EGFR), and COX-2 were determined using immunofluorescence on sections of gallbladders as described previously (22, 23). Rabbit polyclonal antibody against ErbB-2, p-ErbB-2 (Santa Cruz Biotechnology, Inc.), EGFR, p-EGFR (Cell Signaling Technology), and COX-2 (IBL) was used as the primary antibodies. FITC-conjugated, affinity-purified F(ab′)2 fragment of anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used as the secondary antibody. The sections were analyzed using an Olympus laser confocal microscope and an Olympus BX60 microscope.

Terminal Deoxynucleotidyl Transferase – Mediated dUTP Nick End Labeling Assay

Apoptosis-induced DNA strand break was detected by a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay kit. This assay was done by the procedures outlined in the Roche In situ Cell Death Detection kit (Roche Molecular Biochemicals).

Western Blot Analysis

For Western blot analysis, epithelial cell lysates were prepared from 50 gallbladders from nontransgenic mice on
control AIN76A diet, 5 gallbladders from BK5.ErbB-2 mice on control AIN76A diet, and 5 gallbladders from BK5.ErbB-2 mice treated with CS-706 and pooled. Gallbladder epithelial cell lysates were electrophoresed through 7% SDS-polyacrylamide gels as described previously (24). Separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking the membranes with 5% nonfat milk in TBS [20 mmol/L Tris, 150 mmol/L NaCl (pH 7.5)] for 1 h at room temperature, protein levels of ErbB-2, p-ErbB-2 (1:500; Santa Cruz Biotechnology), phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (1:500; Cell Signaling Technology), and COX-2 (1:1,000; IBL) were detected by incubating with corresponding antibodies as described previously (24). Signals were detected by the enhanced chemiluminescence (25) substrates (Pierce). Then, densitometry was done on detected signals normalizing the values to β-actin levels in corresponding samples. All experiments were done in triplicate and experiments were repeated at least twice.

Detection of Gallbladder Images Using a High-Frequency Ultrasound Biomicroscopy

The images of gallbladders from BK5.ErbB-2 mice were monitored every 2 weeks during the treatment by ultrasound biomicroscopy (model VS40, Visual Sonics).

PGE2 Production Assay

The concentration of PGE2 in serum and gallbladder tissues was determined by enzyme-linked immunoassay according to the manufacturer’s manual (Cayman Chemical Co.). Total protein concentration of tissue was determined with the Bradford colorimetric assay. Determinations were done thrice using three independent samples. PGE2 values were expressed as nanograms of PGE2 per milligrams of tissue protein and picograms of PGE2 per microliter of serum volume.

Results

Evaluation of Therapeutic Efficacy of CS-706

We examined whether oral administration of CS-706 had a therapeutic effect on the gallbladder carcinoma that developed in BK5.ErbB-2 mice. Two-month-old mice, in which the incidence of gallbladder carcinoma reaches a plateau, received CS-706 in the diet for 1 month. During the course of experiments to evaluate the therapeutic efficacy of this COX-2 inhibitor for gallbladder carcinoma in this mouse model, images of gallbladders were monitored every 2 weeks by ultrasound biomicroscopy. The therapeutic effect of CS-706 was evaluated by ultrasound images and histologic analyses as follows: complete response, achieved in the case of the disappearance of an assessable tumor by ultrasound biomicroscopy; partial response (PR), required a >30% reduction of all the measurable lesions in the original tumor; minimum change (MC), <30% of change in the measurable size compared with the original tumor; and progressive disease (PG), required an increase of 30% of the measurable lesions or the appearance of new lesion(s). Serial sections of all cases stained by H&E were subjected to histologic analysis to verify the therapeutic evaluation.

Evaluation of the therapeutic effects of CS-706 was as follows: in the control diet group (n = 9), seven (77.8%) and two (22.2%) cases were diagnosed as PG and MC, respectively (Fig. 1). In the group treated with 60 ppm of CS-706 (n = 10), two (20%), three (30%), and five (50%) were diagnosed as PG, MC, and PR, respectively (Fig. 1); whereas in the group treated with 100 ppm of CS-706 (n = 19), four (21.0%), six (31.6%), and nine (47.4%) were diagnosed as PG, MC, and PR, respectively (Fig. 1). Seventy-eight percent of gallbladder tumors had PG in the control diet group; however, only 20% and 21% of tumors displayed a progressive phenotype in 60 and 100 ppm of CS-706–treated groups, respectively. Furthermore, ~80% of gallbladders (80% and 79% for 60 and 100 ppm of CS-706 treatment, respectively) showed either therapeutic efficacy (PR) or prevention from progression (MC) when treated with CS-706. These effects of both 60 and 100 ppm of CS-706 are statistically significant (Fisher’s exact test) compared with the control diet (Fig. 1). Figure 2A shows a typical ultrasound image of gallbladder from a 2-month-old BK5.ErbB-2 mouse (before treatment) and the same mouse at 3 months old with control diet without CS-706 (after treatment). The images show that the tumor dramatically progressed during 2 to 3 months of age in BK5.ErbB-2 mouse on control diet, which was diagnosed as PG. Figure 2B shows a case of MC from a mouse treated with CS-706 from 2 to 3 months of age.

None of the nontransgenic or BK5.ErbB-2 mice treated with 60 or 100 ppm of CS-706 showed any signs of toxicity or neurologic abnormalities. Average body weight gain showed a smaller increase only in nontransgenic mice treated with 100 ppm of CS-706 compared with control diet group. In contrast, a larger increase in BK5.ErbB-2 mice.
treated with both 60 and 100 ppm of CS-706 was observed compared with control diet group ($P < 0.05$). Efficacy of CS-706 against gallbladder tumors may contribute to an improvement of cachexia in BK5.ErbB-2 mice. There was no significant difference of the average diet consumption in both nontransgenic and BK5.ErbB-2 mice treated with CS-706 (60 and 100 ppm) compared with control diet.

**Figure 2.** Therapeutic effects of CS-706 detected by ultrasound analysis. A, typical ultrasound images of gallbladder carcinomas diagnosed with PG from a BK5.ErbB-2 mouse receiving AIN76A control diet. B, typical ultrasound images of gallbladder carcinomas diagnosed as PR. C, MC from BK5.ErbB-2 mice receiving AIN76A diet containing 60 ppm (B-1 and C-1) and 100 ppm (B-2 and C-2) of CS-706. Left, ultrasound image of gallbladder carcinoma before CS-706 treatment at 2 mo of age; middle, ultrasound image of gallbladder after treatment with CS-706 for 2 wks; right, ultrasound image of gallbladder after treatment with CS-706 for 1 mo.
Histopathologic Examination of Gallbladders from Mice Treated with CS-706

In the majority of adenocarcinomas observed in gallbladders diagnosed as PG from BK5.ErbB-2 treated with both control and CS-706 diet, tumor cells occupied at least one third of the gallbladder lumen (Fig. 3A). All of these tumors were diagnosed as well-differentiated adenocarcinomas. Of the gallbladders from mice exposed to 60 and 100 ppm of CS-706, 5 of 10 and 9 of 19, respectively, were diagnosed as PR. Figure 3B shows typical H&E staining of gallbladders diagnosed as PR from BK5.ErbB-2 mice treated with AIN76A diet containing 100 ppm CS-706. The epithelium of the gallbladder contains mixed lesions of hyperplasia and adenocarcinoma. In one case from BK5.ErbB-2 mice treated with 100 ppm of CS-706, not only was the original tumor almost abolished but the resulting H&E revealed only hyperplastic epithelium remaining (Fig. 3C). In cases of PR, the lumen was partially filled by tumor cells mixed with hyperplastic epithelial lesions (Fig. 3C), with a lower labeling index as determined by staining with anti-PCNA antibody (percentage of positive cells was 3.8 ± 0.6%; n = 5) and a higher induction of apoptosis determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (percentage of positive cells was 3.8 ± 0.6%; n = 5) assay compared with that of PG (38.6 ± 6.5% and 1.9 ± 0.3% for PCNA and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining, respectively; n = 2; Fig. 4). Notably, histologic evaluation of the gallbladders did not reveal any significant differences between nontransgenic mice treated with CS-706 compared with the nontransgenic mice on the control diet.

Analyses of COX-2 Protein Levels following Treatment with CS-706

We previously reported that COX-2 protein was up-regulated in the gallbladders of BK5.ErbB-2 mice (23). We examined the effects of CS-706 on the levels of COX-2 in gallbladder tissue. Both immunohistochemical (Fig. 5A and B) and Western blot analysis (Fig. 5C) showed that the elevated COX-2 level was markedly decreased in gallbladders from BK5.ErbB-2 mice treated with CS-706 compared with that from BK5.ErbB-2 mice on the control diet.

CS-706 Strongly Inhibited PGE2 Production in Both Serum and Gallbladders of BK5.ErbB-2 Mice

PGE2 is the major product of COX-2–catalyzed reaction. To examine the effect of CS-706 on PGE2 levels in BK5.ErbB-2 mice, we measured PGE2 levels in both serum and gallbladders by enzyme immunoassay. PGE2 levels in both serum and gallbladders from BK5.ErbB-2 mice treated

Table 1. Average body weight changes and the diet intake in nontransgenic and BK5.ErbB-2 mice treated with control diet or CS-706

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Treatment</th>
<th>Diet consumption (g)</th>
<th>Body weight (end of treatment), g</th>
<th>Average body weight change (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTg</td>
<td>Control diet</td>
<td>4.2 ± 1.3</td>
<td>33.9 ± 3.9</td>
<td>6.85 ± 1.59</td>
</tr>
<tr>
<td>NTg</td>
<td>CS-706 (60 ppm)</td>
<td>4.5 ± 1.2</td>
<td>40.6 ± 3.0</td>
<td>6.43 ± 1.06</td>
</tr>
<tr>
<td>NTg</td>
<td>CS-706 (100 ppm)</td>
<td>4.6 ± 1.5</td>
<td>33.5 ± 4.0</td>
<td>4.77 ± 1.45*</td>
</tr>
<tr>
<td>Tg</td>
<td>Control diet</td>
<td>3.6 ± 0.9</td>
<td>29.9 ± 3.3</td>
<td>3.55 ± 1.09</td>
</tr>
<tr>
<td>Tg</td>
<td>CS-706 (60 ppm)</td>
<td>3.8 ± 0.9</td>
<td>35.0 ± 4.7</td>
<td>5.18 ± 1.06*</td>
</tr>
<tr>
<td>Tg</td>
<td>CS-706 (100 ppm)</td>
<td>3.9 ± 1.1</td>
<td>32.4 ± 3.9</td>
<td>6.36 ± 2.21*</td>
</tr>
</tbody>
</table>

NOTE: Significant differences were determined using an independent two-sample t test. A value of P < 0.05 was considered to be significant.

Abbreviations: NTg, nontransgenic; Tg, transgenic.

*Significantly different from control diet group of nontransgenic mice.

Significantly different from control diet group of BK5.ErbB-2 mice.

Figure 3. Therapeutic effects of CS-706 detected by histologic analysis (H&E staining). A, gallbladder from BK5.ErbB-2 mouse receiving AIN76A control diet. B, typical histologic features of gallbladder diagnosed as PR from BK5.ErbB-2 mice treated with AIN76A diet containing 100 ppm CS-706. C, gallbladder from BK5.ErbB-2 mice treated with AIN76A diet containing 100 ppm CS-706, in which the original tumor was almost abolished. Total number of H&E-stained gallbladder from mice treated with control diet and 100 ppm CS-706 was three and five, respectively.
with control diet were significantly higher than those from nontransgenic mice (*, $P < 0.05$ by two-way ANOVA test; Fig. 6). Treatment of CS-706 strongly inhibited PGE$_2$ production in both serum and gallbladders from BK5.ErbB-2 mice treated with 100 ppm of CS-706 compared with those from BK5.ErbB-2 mice treated with control diet (**, $P < 0.05$ by two-way ANOVA test; Fig. 6).

**Status of ErbB-2 and EGFR in Gallbladders of CS-706–Treated Mice**

To investigate potential mechanisms for the effects of CS-706 on gallbladder adenocarcinoma development in BK5.ErbB-2 mice, protein levels of ErbB-2, activated ErbB-2 (p-ErbB-2), EGFR, and activated EGFR (p-EGFR) were examined in gallbladder lysates by Western blot analysis. In CS-706–treated BK5.ErbB-2 mice, gallbladder tissue proteins showed a 1.4-fold decrease in p-EGFR and 1.5-fold decrease in p-ErbB-2 levels (Fig. 7). Furthermore, treatment with CS-706 resulted in both a decreased level of total ErbB-2 and EGFR. These changes are relative to levels of EGFR, ErbB-2, and their phosphorylated forms in gallbladders from BK5.ErbB-2 mice (data not shown). In this study, activity of Akt was assessed by Western blot analysis for phosphorylated Akt, which decreased in gallbladders from mice treated with CS-706 (Fig. 7).

**Discussion**

Overexpression and/or amplification of COX-2 has been widely reported in several human cancers, including BTC (14–17, 26, 27). In our previous studies, we found that COX-2 was up-regulated (both protein and mRNA) in gallbladder carcinoma from BK5.ErbB-2 mice (23) and the elevated level of COX-2 protein was significantly down-regulated by the treatment of tyrosine kinase inhibitors (TKI) in vivo, which correlated with a significant decrease in incidence of gallbladder carcinoma. In this regard, gefitinib and GW2974, specific EGFR and EGFR/ErbB-2 inhibitors, respectively, resulted in a substantial reduction of incidence of gallbladder tumors (only 16.6% and 2.9% incidence, respectively, compared with 72.4% of incidence for control diet group; ref. 28). These results indicated that COX-2 up-regulation may be critical in the development of BTC in BK5.ErbB-2 mice, in which ErbB-2 is overexpressed and EGFR/ErbB-2 is activated, suggesting COX-2 as a potential target for the treatment of BTC. Thus, we examined the therapeutic effects of an orally active COX-2 inhibitor, CS-706, on gallbladder carcinoma in BK5.ErbB-2 mice. CS-706 (2-[ethoxyphenyl]-4-methyl-1-[4-sulfamoylphenyl]-1H-pyrrole) is a novel COX-2–selective inhibitor with an in vitro selectivity ratio similar to that for celecoxib (29). The structure of CS-706 was recently published (30). Nonclinical data indicate that CS-706 has potent anti-inflammatory, analgesic, and anticaner effects as well as anticahesive effects in tumor-bearing mice (31). Previous studies have shown that CS-706, a novel COX-2 inhibitor, markedly reduced the number of polyps in APC$^{Min}$ mice, a model of familial adenomatous carcinoma (31). In addition, this compound dose dependently reduced incidence and number of malignant colorectal tumors by 50% to 84% in azoxymethane-treated rats (31). Furthermore, elevated PGE$_2$ production and total COX activity in azoxymethane-induced tumors were suppressed by CS-706 (31). This drug is currently in phase 2 studies in the United States, and its efficacy and safety have been shown (32). CS-706, due to its similar structure and COX-2 selectivity compared with celecoxib, may have a lower potential for
cardiovascular risk. CS-706 has an \textit{in vitro} selectivity ratio (IC$_{50}$ ratio for COX-2 versus COX-1) of $\sim 7:1$, similar to that for celecoxib, which shows a more favorable cardiovascular risk-benefit profile compared with rofecoxib. In nonclinical distribution studies, radiolabeled rofecoxib, but not CS-706 or celecoxib, showed retention and accumulation in the thoracic aorta, suggesting that CS-706 may differ from rofecoxib in the binding to aortic proteins (30). The cardiovascular safety of CS-706 remains to be established in controlled clinical trials.

In this study, 100 ppm of CS-706 prevented 84.2\% of cases from progression and showed a clinical effect (PR) in 52.6\% of cases. These inhibitory effects are significant when compared with the control diet group in which 75\% of cases showed progression of the original tumor. These results indicate that this COX-2 inhibitor may delay tumor progression and have a role in cancer therapy, especially in combination with other anticancer agents. Other agents showing therapeutic efficacy in this model of human gallbladder cancer include rapamycin, an inhibitor of mammalian target of rapamycin (33), and TKIs (28). Selective COX-2 inhibitors are now being evaluated in conjunction with chemotherapy, surgery, and radiotherapy in patients with cancers of the lung, colon, breast, esophagus, pancreas, liver, cervix, and brain (34). Several reports have shown the mutual inhibitory effect of TKI and COX-2 inhibitors on the development and growth of tumors. A combination of an EGFR TKI and a COX-2 inhibitor significantly reduced intestinal polyps in APC$^{min/+}$ mice (35). This combination regimen also inhibited the growth of squamous cell carcinoma of the head and neck both \textit{in vitro} and \textit{in vivo} (36). These results, including our current and previous studies, suggest that the combination of TKIs and COX-2 inhibitors may be explored as a potential treatment for human BTC.

Significant correlation of COX-2 and ErbB-2 expression has also been reported in several human cancer types, such as cholangiocarcinoma, colon cancer, and breast cancer (19, 37–39). Recently, Wang et al. (40) reported that ErbB-2 forms a complex at a specific nucleotide sequence of the COX-2 promoter and is able to stimulate its transcription. In our study, we showed that COX-2 inhibitor treatment resulted in the decreased levels of p-ErbB-2 and p-EGFR, which correlated with the reduction of COX-2 protein level. ErbB-2, the phosphorylated forms of both ErbB-2 and EGFR, and COX-2 were constitutively up-regulated in the gallbladder of BK5.ErbB-2 mice compared with that of nontransgenic mice (23). These results suggest that there may be direct interaction between ErbB-2 (and/or EGFR) and COX-2 in the gallbladder carcinoma of BK5.ErbB-2 mice. In addition, the results from our previous study,
which showed that the levels of ErbB-2, p-ErbB-2, p-EGFR, Akt, and phosphorylated Akt (p-Akt) were all decreased in TKI-treated mice (28), together with the current results provide further evidence for a direct interaction between ErbB-2 and COX-2 in the gallbladder carcinoma in BK5.ErbB-2 mice. Western blot analysis also showed that phosphorylated Akt, but not total Akt, level was decreased in the gallbladders from BK5.ErbB-2 mice treated with CS-706 (Fig. 7). It has been reported that ErbB-2 signals through Akt to induce COX-2 expression in breast cancer cells (41) and that COX-2 and PGE	extsubscript{2} lead to an enhanced ErbB-2 expression (42). Furthermore, a COX-2 inhibitor blocked phosphorylation of Akt in human cholangiocarcinoma cells (43) and inhibition of Akt reduced COX-2 expression in the ErbB-2–overexpressing SKBR-3 cells (41). Sheng et al. (44) reported that treatment with PGE	extsubscript{2} increases Akt activity, which is required for the growth stimulation of PGE	extsubscript{2} in human colon cancer cells. p-EGFR/p-ErbB-2 offers docking sites for the SH2 and SH3 domains of various signaling molecules, including the phosphatidylinositol-3-OH kinase (45), which activates downstream targets, including Akt. Akt has been implicated in the control of cell survival and metabolism (46). Akt is constitutively activated in human cancer as a result of mutation/deletion of the tumor suppressor PTEN, a phospholipid phosphatase that negatively regulates Akt activation (47). Akt has also been shown to negatively regulate, directly or indirectly, the activity of several proapoptotic members of the bcl-2 family (48). Recently, Zhou et al. (49) have reported that the dual EGFR/ErbB-2 inhibitor GW2974 potentially inhibits constitutive and/or ligand-induced EGFR or ErbB-2 tyrosine phosphorylation and ERK and Akt phosphorylation in all five breast cancer cell lines, except SUM 185 cells, which correlated with the antiproliferative response. The authors have also suggested that the potential mechanism of resistance in SUM 185 cells is the failure of GW2974 to inhibit downstream ERK and Akt activation despite inhibition of ErbB-2 phosphorylation (49). Furthermore, positive correlation determined by immunohistochemistry between COX-2 and phosphorylated Akt in human BTC has been reported (43, 50), and overexpression of COX-2 induces phosphorylation of Akt in hepatocellular carcinoma cells (51). Although our Western blot analysis did not show a change in the levels of either total ERK or phosphorylated ERK (data not shown), the phosphorylated Akt level was decreased in the gallbladders from BK5.ErbB-2 mice treated with CS-706. These studies, including our results, support a link between EGFR/ErbB-2 and COX-2 expression mediated by Akt activity (41, 42, 50, 52, 53). Based on these findings, we postulate that reduced Akt activation results directly from inhibition of COX-2 and reduced signaling through EGFR/ErbB-2. We are currently crossing COX-2 null mice with BK5.ErbB-2 transgenic mice to address this hypothesis more directly.

In conclusion, we have shown that the novel COX-2 inhibitor CS-706 has therapeutic efficacy against gallbladder adenocarcinoma that develops in BK5.ErbB-2 mice. Based on our results, targeting the COX-2 could provide a potentially new and effective therapy both alone and in combination with other chemotherapeutic agents for patients with BTC.

Acknowledgments

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References


Figure 7. Western blot analysis of ErbB-2, p-ErbB-2, EGFR, p-EGFR, Akt, and phosphorylated Akt (p-Akt) levels in the gallbladders of BK5.ErbB-2 mice on control diet and BK5.ErbB-2 mice treated with 100 ppm CS-706. Epithelial cell lysates were prepared from gallbladder tumors from five BK5.ErbB-2 mice, and gallbladders from BK5.ErbB-2 mice treated with CS-706 were pooled.
Molecular Cancer Therapeutics

Therapeutic effect of CS-706, a specific cyclooxygenase-2 inhibitor, on gallbladder carcinoma in BK5.ErbB-2 mice

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