Chemopreventive and Therapeutic Efficacy of Orally Active Tyrosine Kinase Inhibitors in a Transgenic Mouse Model of Gallbladder Carcinoma

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Abstract

Biliary tract cancer (BTC) is the second most common primary hepatobiliary cancer after hepatocellular cancer. At the time of diagnosis, most BTC are at an advanced stage and are unresectable. There is presently no effective curative treatment of the advanced disease nor is there any effective clinical therapy that will prevent the development of BTC. All of these factors render gallbladder cancer nearly incurable with a poor survival rate. The aim of our study was to provide a better understanding of the mechanisms involved in the development of gallbladder carcinoma as the advancement of more effective treatment options would significantly improve prognosis. In the present study, we examined the effect of gefitinib, a selective epidermal growth factor receptor/tyrosine kinase inhibitor (EGFR/TKI), on the development of gallbladder carcinoma in BK5.erbB2 mice. In addition, we examined the effect of another quinazoline derivative, GW2974, which is able to block the activation of both the EGFR and erbB2, in this model. Animals were treated with either 400 ppm gefitinib or 200 ppm GW2974 as a supplement in the diet using either a chemopreventive or therapeutic protocol. The results show that both compounds were potent chemopreventive and therapeutic agents in this mouse model of human BTC. The results also suggest that activation of the EGFR plays an important role in development of BTC in this model and that targeting both the EGFR and erbB2 may be an effective strategy for treatment of this disease.

Approximately 8,000 cases of biliary tract cancer (BTC) are reported annually in the United States (1). Nearly two thirds of these tumors arise in the gallbladder, making it the most common BTC and fifth most common gastrointestinal tract cancer. Cholangiocarcinoma, which originates in the bile duct and periampullary region, comprises the remainder of BTC. The incidence of BTC has considerable geographic variation. High standardized mortality ratios of BTC are found in cancer registries for South American countries such as Chile, Peru, and Columbia and for Asian countries such as Japan and Thailand (2). Japan has one of the world’s highest age-adjusted cancer death rates related to BTC and it seems steadily increasing (2.5% and 11.5% in 1980 and 1998, respectively; ref. 3). There are indications that the incidence of gallbladder carcinoma may also be increasing in Western countries (4).

The early stages of BTC are usually asymptomatic. To date, very few studies have attempted to decipher the molecular and cellular mechanism(s) involved in the development of BTC; thus, very little is known regarding the sequence of events that lead to this disease. A limiting factor has been the lack of relevant animal models for the study of early events in BTC. Presently available animal models are based on exposure to chemical carcinogens. In most of these models, the latency between the treatment and tumor development is long and the tumor incidence is relatively low; however, the furan rat model described by Sirica et al. gives rise to a very high incidence of cholangiocarcinoma in liver (5, 6).

Recently, we reported development of transgenic mice where expression of a rat erbB2 cDNA is targeted to the basal layer of multiple epithelial tissues, including the biliary tract epithelium, by the bovine keratin 5 (BK5) promoter (BK5.erbB2 mice; refs. 7, 8). Tumors arise at various sites of the biliary tract as a consequence of elevated erbB2 expression. In particular, adenocarcinoma of the gallbladder develops in ~70% of homozygous BK5.erbB2 transgenic mice by 3 months of age. In the biliary tract epithelium of these mice, overexpression of erbB2 leads to an increase in epidermal growth factor receptor (EGFR) protein, erbB2/EGFR heterodimer formation and activation of the EGFR (i.e., tyrosine phosphorylation). ErbB2 overexpression has also been reported in a significant percentage of human BTC (9–15). In one study, 30 of 43 cases (69.6%) and 14 of 43 cases (32.6%) of gallbladder adenocarcinoma had amplification of erbB2 DNA or overexpression of erbB2 protein, respectively (9). In another study, 7 of 11 cases...
(63.6%) of gallbladder adenocarcinoma had overexpression of erbB2 protein (10). In addition, protein levels of both EGFR and its ligand, transforming growth factor-erbB2 protein (10). In addition, protein levels of both EGFR (63.6%) of gallbladder adenocarcinoma had overexpression of erbB2 and EGFR protein, respectively (18).

In the present study, we examined the effect of gefitinib, a selective EGFR/tyrosine kinase inhibitor (TKI), on the development of gallbladder carcinoma in BK5.erbB2 mice. In addition, we have examined the effect of another quinazoline derivative, GW2974, which is able to block the activation of erbB2 and EGFR protein, respectively (18).

The experimental design allowed evaluation of both the chemopreventive and therapeutic efficacy of these TKIs against gallbladder carcinoma in BK5.erbB2 mice. The results indicate that targeting the EGFR alone or in combination with erbB2 was effective at both prevention and treatment of gallbladder carcinoma in BK5.erbB2 transgenic mice.

Materials and Methods

Histologic analysis of gallbladder. Mice were sacrificed by cervical dislocation at 3 months of age and immediately autopsied. Gallbladders were fixed in formalin and embedded in paraffin before sagittal sectioning. Sections of 5 μm were cut and stained with H&E. Mice were injected i.p. with bromodeoxyuridine (Sigma, St. Louis, MO) in PBS (100 μg/g body weight) 30 minutes before sacrifice. For the analysis of labeling index, paraffin sections were stained using anti-bromodeoxyuridine antibody (7).

Treatment. Gefitinib and GW2974 were supplied in lyophilized form from AstraZeneca (Cheshire, United Kingdom) and GlaxoSmithKline (Research Triangle, NC), respectively. For the chemopreventive protocol, treatment with either 400 ppm gefitinib or 200 ppm GW2974 was initiated in utero due to the rapid development of gallbladder carcinoma (50-60% incidence of gallbladder carcinoma at 2 weeks of age) in the BK5.erbB2 mice. Mice received gefitinib or GW2974 as a supplement in the semisynthetic AIN76 diet 3 weeks before mating and throughout the study. Homozygous and wild-type offspring were sacrificed and examined at 3 months of age. For the therapeutic protocol, 8-week-old animals began treatment with either the gefitinib diet or the GW2974 diet (200 ppm) for 4 weeks. Prior studies have established that the incidence of gallbladder carcinoma reaches a plateau at 8 weeks of age. Thirty minutes before sacrifice, mice were injected with bromodeoxyuridine (100 μg/g of body weight) in both protocols. To evaluate systemic toxicity, body weight, feed consumption, and neurologic function were monitored biweekly. All experiments were carried out with strict adherence to institutional guidelines for minimizing distress in experimental animals.

Ultrasound biomicroscopy. Ultrasound images of gallbladders were generated by a high-frequency ultrasound imaging system (Model Vevo 60, VisualSonics, Toronto, Canada).

Immunofluorescence staining. The expression and localization of erbB2, phospho-erbB2 (p-erbB2), EGFR, phospho-EGFR (p-EGFR), and cyclooxygenase-2 (COX-2) were determined using immunofluorescence on sections of gallbladders as described previously (7, 8). Rabbit polyclonal antibody against erbB-2, p-erbB2, EGFR, p-EGFR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and COX-2 (IBI, Fujikoa, Japan) were used as the primary antibodies. FITC-conjugated, affinity-purified F(ab)2 fragment of antimouse IgG (Jackson ImmunoResearch Lab, West Grove, PA) was used as the secondary antibody. The sections were analyzed using an Olympus laser confocal microscope and an Olympus BX560 microscope.

Toluidine blue staining. Toluidine blue staining was done as previously described (20).

Terminal deoxynucleotidyl transferase–mediated nick-end labeling assay. Apoptosis-induced DNA strand breaks were detected using the protocols outlined in the Roche In situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN).

Western blot analysis. For Western blot analysis, epithelial cell lysates were prepared from 50 gallbladders from nontransgenic mice, five gallbladder tumors from BK5.erbB2 mice, and five gallbladders from BK5.erbB2 mice treated with either gefitinib or GW2974 were pooled. We were not able to discern macroscopically in the gallbladders from mice treated with either gefitinib or GW2974. Gallbladder epithelial cell lysates were electrophoresed through 7% SDS/polyacrylamide gels as previously described (8). Separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking with Odyssey blocking reagent for 1 hour at room temperature in TBS [0.5 mol/L NaCl, 20 mmol/L Tris (pH 7.5)], protein levels of erbB2, p-erbB2 (1:500, Santa Cruz Biotechnology), phospho-MAPK (1:500, Cell Signaling Technology, Beverly, MA), and COX-2 (1:1,000, Cayman Chemical, Ann Arbor, MI) were detected by incubating the membrane with the corresponding antibodies. Protein bands were visualized using the Odyssey Image after the incubation with secondary antibodies labeled with IRDye800 or Alexa680 (Cy5.5) in 0.1% Tween Odyssey blocker for 30 minutes at room temperature. The relative differences between control and treated samples were quantitated using the Odyssey Image system.

Mitogen-activated protein kinase activity assay. Approximately 0.25 to 2 mg of protein from whole cell lysates of gallbladder were immunoprecipitated with 4 μg of anti-phospho-MAPK antibody. The kit for the kinase assays was purchased from Upstate Biotechnology (Charlottesville, VA), and assays were done according to the manufacturer’s instruction.

Statistical analysis. All of the data are expressed as the mean ± SD. Significant differences were determined using the Kendall tau-b test. P < 0.05 was considered significant. All of the statistical analyses were done using StatView software (Abacus Concept Inc., Berkeley, CA).

Results

Chemoprevention of gallbladder carcinoma by orally active tyrosine kinase inhibitors. Initially, we examined whether oral administration of TKIs would prevent the development of gallbladder carcinoma in BK5.erbB2 mice. In this chemopreventive protocol, the treatment of TKI was initiated in utero due to the rapid development of gallbladder carcinoma in this mouse model. Female mice, 8 weeks of age, received 400 ppm of gefitinib or 200 ppm GW2974 as a supplement in a semisynthetic diet, AIN76, 3 weeks before mating. Offspring received this diet until they reached 3 months of age and then were sacrificed for analyses. Administration of TKIs via this protocol resulted in a significant decrease in the incidence of gallbladder carcinoma in BK5.erbB2 mice (Fig. 1). Detailed histologic examination showed the incidence of gallbladder carcinoma in the gefitinib- and GW2974-treated group was 18% and 8%, respectively, at the end of the study. This corresponded to a 75% and 89% decrease in tumor incidence compared with the incidence in BK5.erbB2 mice receiving the control diet. Examination by ultrasound of a subset (7–9) of animals from each group revealed that no tumors had developed in BK5.erbB2 mice treated with either gefitinib or GW2974 by 20 to 27 days of age. In contrast, four of nine BK5.erbB2 mice on the control diet developed tumors by 19 to 28 days of age.

Therapeutic efficacy of tyrosine kinase inhibitors. We also examined whether oral administration of either gefitinib or GW2974 would be effective as therapeutic agents against...
gallbladder carcinoma in BK5.erbB2 mice. For these experiments, 2-month-old mice were selected for treatment as the incidence of gallbladder carcinoma reaches a plateau of ~70% by this age. BK5.erbB2 mice received either 400 ppm gefitinib or 200 ppm GW2974 in the diet for 1 month. These treatments resulted in a significant decrease in the incidence of gallbladder carcinoma to 17% and 3% for gefitinib and GW2974, respectively (Fig. 1). These reductions corresponded to a 77% and 95% decrease in tumor incidence compared with BK5.erbB2 mice receiving the control diet.

During the course of these experiments, the status of the gallbladders in each group was monitored weekly by high-frequency ultrasound biomicroscopy (n = 7 and 10 for gefitinib and GW2974, respectively). Figure 2A, left shows a representative image of a gallbladder from a 2-month-old BK5.erbB2 mouse before GW2974 treatment. Figure 2A, middle shows the image of the gallbladder from the same mouse after GW2974 treatment for 23 days. Note the dramatic regression of the tumor and only hyperplasia is evident. Figure 2A, right shows the H&E staining of the hyperplastic lesion in this gallbladder after 30 days of GW2974 treatment.

No mortality or neurologic deficiencies were observed in any nontransgenic or BK5.erbB2 mice treated with either gefitinib or GW2974 (either protocol). After weaning, there was no statistically significant difference in the average consumption of the various diets (Table 1). There was a slight (nonstatistically significant) increase in the average body weight of BK5.erbB2 mice treated with TKIs under both the chemopreventive and therapeutic protocols, which may be attributed to the slight increase in feed consumption observed in these groups (Table 1).

### Histopathologic examination of gallbladders from mice treated with gefitinib and GW2974

Adenocarcinoma of the gallbladder was observed in 12 of 17 (71%) and 21 of 29 (72%) mice on the control diet in the chemopreventive and therapeutic protocols, respectively. In the majority of these lesions, tumor cells filled the lumen, occupying at least one third of the distal portion of the gallbladder. All of the tumors were diagnosed as well-differentiated adenocarcinomas (Fig. 2B, top left). We evaluated 22 (chemoprevention protocol) and 18 (therapeutic protocol) gallbladders from mice treated with gefitinib and found that 4 of the 22 (18.2%) and 3 of the 18 (16.7%), in the respective protocols, were diagnosed as adenocarcinoma (Fig. 2B, top middle). Of the gallbladders from mice treated with GW2974, only 1 of 12 (8.3%) and 1 of 34 (2.9%) from the chemoprevention and therapeutic protocols, respectively, were diagnosed as adenocarcinoma. These latter cases of adenocarcinoma were apparently refractory to the treatment and both were diagnosed as carcinoma in situ (Fig. 2B, bottom middle). In these cases, the lumen was partially filled by tumor cells, particularly at the distal portion of the gallbladder. In most of the gallbladder adenocarcinomas that responded to either gefitinib or GW2974, we observed low-grade epithelial hyperplasia, particularly in the distal region. Thickened connective tissue, decreased vascularization (Fig. 2B, top right and bottom right), a significantly lower labeling index as determined by bromodeoxyuridine incorporation, and an increase in the number of apoptotic cells as determined by terminal deoxynucleotidyl transferase–mediated nick-end labeling assay (Fig. 2C) were also observed following treatment with either TKI. In addition, mast cells, which stained positive with toluidine blue (Fig. 2D), were observed in the stromal area of gallbladders from BK5.erbB2 mice treated with either gefitinib or GW2974.

Histologic evaluation of the gallbladder did not reveal any significant differences between nontransgenic mice treated with these TKIs compared with the nontransgenic mice on the control diet.

### Status of epidermal growth factor receptor and erbB2 in gallbladders of tyrosine kinase inhibitor–treated mice.

To investigate the mechanistic basis of the inhibitory and indeed regressive effects of gefitinib and GW2974 on gallbladder adenocarcinoma in BK5.erbB2 mice, protein lysates of gallbladders obtained from the therapeutic protocol were
Fig. 2. Effects of gefitinib and GW2974 detected by histologic and ultrasound analyses. A. Regression of gallbladder carcinoma by GW2974 treatment as detected by ultrasound biomicroscopy. All images are from a single animal depicting the response representative of the treatment group. Left, ultrasound image of gallbladder carcinoma (maximum size: 1.35 mm) before GW2974 treatment. Middle, ultrasound image of gallbladder on the 23rd day of treatment; this image indicates regression of the carcinoma (observed size: 0.48 mm). Right, the lesion that remained was confirmed by H&E staining as hyperplasia. B. Histologic evaluation of gallbladders from BK5.erbB2 mice treated with gefitinib and GW2974 (therapeutic protocol). Top left, gallbladder of BK5.erbB2 mouse receiving AIN76 control diet. Top middle, gallbladder adenocarcinoma that was refractory to gefitinib treatment. Top right, typical histologic features of the gallbladder from BK5.erbB2 mice treated with AIN76 diet containing 400 ppm gefitinib. Bottom middle, gallbladder carcinoma in situ that was refractory to the GW2974 treatment. Bottom right, typical histologic features of the gallbladder from BK5.erbB2 mice treated with AIN76 diet containing 200 ppm GW2974. C. Reduction of the labeling index (top) and induction of apoptosis (bottom) in the gallbladder of mice receiving either control diet (left), gefitinib diet (middle), or GW2974 diet (right). The labeling index was determined by staining for bromodeoxyuridine (BrdU) and apoptotic cells were detected by the terminal deoxynucleotidyl transferase – mediated nick-end labeling (TUNEL) method. D. Mast cells (arrow) detected by toluidine blue staining in the gallbladder of a BK5.erbB2 mouse treated with gefitinib diet (left) or GW2974 diet (right).
analyzed for levels of erbB2, p-erbB2, EGFR, and p-EGFR by both Western blot analysis and immunofluorescence staining. ErbB2, p-erbB2, EGFR, and p-EGFR levels were constitutively elevated in lysates of gallbladders from BK5.erbB2 mice on the control diet compared with nontransgenic mice (Fig. 3).

Treatment with gefitinib and GW2974 resulted in decreased levels of both erbB2 and EGFR. Furthermore, levels of p-erbB2 and p-EGFR were markedly reduced (Fig. 3). In this regard, there were 1.6- and 1.9-fold decreases in EGFR and erbB2 levels, respectively, in the gallbladders of BK5.erbB2 mice treated with gefitinib. The alterations in EGFR and erbB2 protein levels were even more striking following GW2974 treatment, with respective decreases of 4.3- and 4.5-fold observed (Fig. 3C). These changes are relative to the levels of erbB2 and p-erbB2, were 12.5- and 5.6-fold, respectively, in the gallbladders of BK5.erbB2 mice that received only the control diet after normalization to the level of β-actin. Decreases in the ratios of phosphorylated forms of EGFR and erbB2, normalized to total protein levels of EGFR and erbB2, were 1.6- and 1.9-fold, respectively, in the gallbladders of BK5.erbB2 mice treated with gefitinib. This same pattern was observed following treatment with GW2974, although the relative decrease in phosphorylated EGFR and erbB2 was not as marked (Fig. 3).

The findings from the Western blot analyses shown in Fig. 3 were supported by results from immunofluorescence staining of tissue sections from treated and untreated mice (Fig. 4). The levels of erbB2, p-erbB2, EGFR, and p-EGFR were significantly elevated in the gallbladder carcinomas of the BK5.erbB2 mice. The levels of erbB2 and EGFR in the gallbladders from both gefitinib- and GW2974-treated mice were decreased to levels seen in normal gallbladders from nontransgenic mice. p-EGFR and p-erbB2 levels were essentially undetectable by immunostaining in either treatment group. The expression of these proteins shown in nontransgenic gallbladder was either very low or not detectable.

Analysis of the adenocarcinoma in situ that was refractory to treatment with GW2974 showed no detectable levels of p-erbB2 and p-EGFR and also lower levels of expression of erbB2, EGFR, and COX-2 proteins compared with gallbladders from the BK5.erbB2 control group (data not shown).

**Analysis of cyclooxygenase-2 protein level following treatment with tyrosine kinase inhibitors.** Previously, we reported that COX-2 protein levels were up-regulated in the gallbladders of BK5.erbB2 mice (8). In this study, we also examined the effects of gefitinib and GW2974 on the levels of COX-2 in gallbladder tissue. Analysis by Western blot (Fig. 3) and immunofluorescence (Fig. 4) showed that COX-2 levels were decreased in gallbladders from BK5.erbB2 mice treated with either TKI under the therapeutic protocol. COX-2 protein levels were also decreased in gallbladders from gefitinib- and GW2974-treated mice on the chemoprevention protocol (data not shown).

**Status of mitogen-activated protein kinase activity in gallbladder of tyrosine kinase inhibitor–treated mice.** We also reported that mitogen-activated protein kinase (MAPK) was activated in the gallbladder of BK5.erbB2 mice (8). In this study, activity of MAPK, as assessed by Western blot analysis for phospho-MAPK and direct assay of MAPK activity, was decreased in gallbladders from mice treated with both TKIs under the therapeutic protocol (Fig. 5).

**Discussion**

Overexpression and/or amplification of erbB2 has been widely reported in a number of human cancers including gallbladder (9–11) and cholangiocarcinoma (10, 12, 13, 15, 21). The fact that constitutive overexpression of erbB2 in gallbladder epithelium of mice leads to a high incidence of adenocarcinoma (8) suggests that constitutive expression and/or activation of this erbB family member may be involved in the

<table>
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<th>Protocol</th>
<th>Mouse (n)</th>
<th>Diet</th>
<th>Body weight (g) ± SD</th>
<th>Daily intake (g/d)*</th>
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<tr>
<td>Chemopreventive</td>
<td>Nontransgenic</td>
<td>8 (4 M, 4 F)</td>
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<td>34.0 ± 4.1</td>
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<td></td>
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<td>Gefitinib</td>
<td>31.8 ± 4.6</td>
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<td></td>
<td></td>
<td>15 (8 M, 7 F)</td>
<td>GW 2974</td>
<td>32.4 ± 4.9</td>
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<td></td>
<td>BK5.erbB2</td>
<td>17 (8 M, 9 F)</td>
<td>Control AIN76</td>
<td>27.1 ± 3.8</td>
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<tr>
<td></td>
<td></td>
<td>22 (10 M, 12 F)</td>
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<td>12 (7 M, 5 F)</td>
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<td>30.5 ± 2.9</td>
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<td>Control AIN76</td>
<td>33.8 ± 4.9</td>
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<td></td>
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<td>26.0 ± 3.7</td>
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<td>29 (16 M, 13 F)</td>
<td>GW 2974</td>
<td>31.4 ± 4.2</td>
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Abbreviations: M, male; F, female.

* Daily intake was calculated based on the total feed consumption of five mice over 5 days at 2 to 3 months of age.
development of human BTC. In our previous work, we found that the EGFR was up-regulated (protein level) and activated (tyrosine phosphorylation) in gallbladder tissue from these mice (8). ErbB2 has been shown to lack a specific ligand and affects erbB signaling through the formation of heterodimers with other erbB family members (22, 23). Molecular conformational energy calculations of erbB2 and EGFR tyrosine kinase domains indicate a preference for heterodimer formation (24, 25) and erbB2 is the preferred interacting partner for EGFR (26, 27). This interaction also reduces the rate of EGFR degradation (22). Thus, elevated EGFR protein in gallbladder epithelium of BK5.erbB2 transgenic mice may be due to reduced receptor degradation through forced heterodimer formation as a result of transgene expression. These observations also suggested that activation of EGFR may play an important role in the development of biliary tract tumors in BK5.erbB2 mice and in human tumors with elevated expression of erbB2.

To further explore the role of EGFR signaling in development of BTC in BK5.erbB2 mice, we used gefitinib, a selective EGFR/TKI, and GW2974, a dual EGFR/TKI and erbB2/TKI to examine the effects of these compounds on the development of gallbladder carcinoma in BK5.erbB2 mice. Gefitinib is an orally active, selective EGFR/TKI that blocks signal transduction pathways implicated in proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth (28, 29). A generally cytostatic growth inhibiting activity of gefitinib has been shown in a wide range of human cancer cell lines that express functional EGFR, including prostate, breast, ovarian, colon, epidermoid, and small cell and non–small cell lung cancers (30–33). As shown in Fig. 1, gefitinib possessed significant activity as both a chemopreventive agent and a therapeutic agent for gallbladder carcinoma in BK5.erbB2 mice. Interestingly, a small number of tumors were refractory to gefitinib in both protocols (n = 4 and 3 adenocarcinomas, respectively). In these tumors, there still appeared to be partial chemopreventive and therapeutic effects based on tumor size, labeling index, and induction of apoptosis. Mast cells were often observed in the stromal area of gallbladders from BK5.erbB2 mice treated with either gefitinib or GW2974. Marked infiltration of mast cells has been observed around fibrotic bile ducts in heptolithiasis (34), which may play an important role in fibrogenesis around the damaged bile ducts (35). Therefore, increased fibrogenesis in the stromal area and infiltration of mast cells in the gefitinib- and GW2974-treated gallbladders may

![Fig. 3. Western blot analysis of EGFR and erbB2 status following treatment with gefitinib and GW2974. A-B, Western blot analysis of the levels of EGFR, p-EGFR, erbB2, p-erbB2, and COX-2 in the gallbladders of nontransgenic mice, BK5.erbB2 mice, and BK5.erbB2 mice treated with either gefitinib or GW2974 (therapeutic protocol), respectively. C, ratios of the levels of total EGFR (white columns in left) and erbB2 (white columns in right), protein and phosphorylated EGFR (black columns in left) and erbB2 (black columns in right) in the gallbladders normalized to the levels found in mice that received the control diet. BK5.erbB2 mice were treated with either AIN76 control diet, AIN76 diet containing 400 ppm gefitinib, or AIN76 diet containing 200 ppm GW2974 under the therapeutic protocol. For Western blot analysis, epithelial cells lysates were prepared from 50 gallbladders from nontransgenic mice, gallbladder tumors from five BK5.erbB2 mice, and gallbladders from five BK5.erbB2 mice treated with gefitinib or GW2974 were pooled. Tumors were not visible macroscopically in the gallbladders from either gefitinib- or GW2974-treated groups.](www.aacjournals.org)
indicate a reactive regeneration process after the regression of tumor cells.

Many studies using selective EGFR/TKIs have shown evidence for EGFR as a critical molecule in the development of a tumor in which both erbB2 and EGFR are overexpressed. For example, tumor cells expressing high levels of erbB2 are highly sensitive to the selective EGFR/TKI, gefitinib (36–38). In our study, gefitinib inhibited phosphorylation of both EGFR and erbB2 in gallbladders of BK5.erbB2 mice (Figs. 3-4). Significant reduction in phosphorylation of both EGFR and erbB2 also has been reported in tumor cell lines treated with gefitinib (36, 37). Anderson et al. reported that gefitinib does not prevent formation of EGFR/erbB2 heterodimers but rather blocks the ability of the EGFR to phosphorylate its heterodimeric partner (38). These studies suggest that the activity of gefitinib on erbB2 phosphorylation in vivo is not attributable to a direct competition for ATP binding to erbB2 but rather is mediated through its effects on the EGFR. These results also suggest that the synergistic cooperation between erbB2 and EGFR may play a critical role in the development of gallbladder carcinoma. Interruption of EGFR function with selective EGFR/TKIs may disrupt EGFR-erbB2 cross-talk, leading to inhibition of both EGFR and erbB2 tyrosine kinases.

GW2974 is one of the most potent quinazoline dual EGFR and erbB2 inhibitors and it exerts its effect by targeting the conserved ATP binding site of these molecules. This compound shows potent in vitro inhibition of both EGFR and erbB2 kinase domains with an IC_{50} < 80 nmol/L. Growth of erbB2 and EGFR expressing tumor cell lines is inhibited by GW2974 at concentrations of <0.4 μmol/L and it was a potent inhibitor of tumor growth in EGFR and erbB2 overexpressing cells using a tumor xenograft model (19). In the current study, almost complete inhibition of tumor growth was observed in both chemopreventive and therapeutic protocols in which BK5.erbB2 mice were treated orally with GW2974 via the diet. Only one case of carcinoma in situ was found in each treatment protocol, whereas all other gallbladders exhibited low-grade epithelial hyperplasia. Notably, GW2974 showed a greater inhibitory effect than gefitinib on the development of gallbladder carcinoma in BK5.erbB2 mice. GW2974 also showed a strong reduction of the phosphorylated forms of both EGFR and erbB2 in the gallbladders of BK5.erbB2 mice (Fig. 3). These results suggest that the greater inhibition of gallbladder carcinoma may be attributable to the direct inhibitory effect of GW2974 against both the EGFR and erbB2 tyrosine kinases. Because gallbladder carcinoma development in BK5.erbB2 mice is driven by elevated expression of erbB2 using the bovine K5 promoter, we cannot state conclusively that GW2974 or other dual specific TKIs would be more effective in treating human BTC. Nevertheless, our data suggest that this could be possible especially in tumors where both erbB2 and EGFR are overexpressed and activated.

An interesting finding in the current study was that treatment with both TKIs reduced not only phosphorylation levels but also total protein levels of EGFR and erbB2 in the gallbladders of BK5.erbB2 mice. The mechanism(s) for the reduction of EGFR and erbB2 protein levels in TKI-treated gallbladders is not known. However, neither TKI had any effect on expression of the transgene as assessed by semiquantitative reverse transcription-PCR (data not shown). It has been reported that EGFR activation
TKIs Inhibit Gallbladder Cancer

Fig. 5. MAPK activity in gallbladder tissue lysates prepared from mice treated with TKIs. Following immunoprecipitation with a MAPK antibody, one half of the immunoprecipitates were subjected to Western blot analysis for phospho-MAPK (p-MAPK) (top) and the other half analyzed for MAPK activity (bottom). *, P < 0.05; †*, P < 0.001. Immunoprecipitates were subjected to Western blot analysis for phospho-MAPK (p-MAPK) (top) and the other half analyzed for MAPK activity (bottom). *, P < 0.05; †*, P < 0.001.

may signal an autoregulatory loop (39, 40). Therefore, inhibition of EGFR phosphorylation by these TKIs may reduce this feedback loop resulting in decreased levels of EGFR and possibly erbB2 proteins. Further experiments will be necessary to determine the exact mechanism(s) for the reduced levels of EGFR and erbB2 proteins.

Previously, we reported that COX-2 (mRNA and protein) levels were up-regulated in gallbladder carcinomas from BK5.erbB2 mice (8) and considerable evidence indicates that COX-2 is elevated in many human epithelial tumors (41) including BTC (42–45). Accumulating evidence suggests a strong positive correlation between erbB2 and COX-2 expression in BTC (5, 8, 45) though COX-2 overexpression alone may not be sufficient to induce gallbladder carcinoma. ErbB2 may play a key role in regulating COX-2 expression in neoplastic and precancerous biliary tract epithelial cells. A link between erbB2 signaling and COX-2 expression has been established by several studies including our recent report (5, 8, 45–47). A strong positive correlation between erbB2 and COX-2 protein expression has been observed in cholangiocarcinomas from both humans and rats (5, 45). Additionally, activation of the erbB2/erbB3 pathway induced COX-2 in colorectal cancer cells (46). Increased levels of COX-2 mRNA and protein and prostaglandin E2 synthesis were detected in erbB2 transformed human mammary epithelial cells (47). Several reports have also suggested that the EGFR may be involved in regulation of COX-2 expression. Matsuura et al. have shown that activation of the EGFR signaling pathway due to enhanced transforming growth factor-α expression plays an important role in the induction of COX-2 by IFN-γ in human epidermal keratinocytes (48). Recently, it has been reported that EGFR is activated by chenodeoxycholate, the primary hydroxyphobic bile acid, and functions to induce COX-2 expression by a MAPK cascade in a human cholangiocarcinoma cell line (49).

In conclusion, we have shown that the novel TKIs, gefitinib and GW2974, are potent chemopreventive and therapeutic agents against gallbladder adenocarcinoma that develop in BK5.erbB2 mice. This is the first demonstration of the anticancer effects of TKIs against primary tumors overexpressing EGFR and erbB2 using a genetically engineered mouse model. Interestingly, activation of erbB2 and EGFR was still observed in those adenocarcinomas that were refractive to treatment with gefitinib or GW2974 (Fig. 5). These data on COX-2 and MAPK indicate that EGFR/erbB2 downstream signaling events were also blocked by treatment with the TKIs.

In conclusion, we have shown that the novel TKIs, gefitinib and GW2974, are potent chemopreventive and therapeutic agents against gallbladder adenocarcinoma that develop in BK5.erbB2 mice. This is the first demonstration of the anticancer effects of TKIs against primary tumors overexpressing EGFR and erbB2 using a genetically engineered mouse model. Interestingly, activation of erbB2 and EGFR was still observed in those adenocarcinomas that were refractive to treatment with gefitinib (data not shown). Further investigation is definitely warranted regarding the mechanism and/or series of events that allowed sustained phosphorylization and activation of erbB2/EGFR thus mediating resistance to treatment via tyrosine kinase inhibition. Nevertheless, based on our results, targeting the EGFR and possibly erbB2 could provide a potentially new and effective therapy for patients with BTC.

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Chemopreventive and Therapeutic Efficacy of Orally Active Tyrosine Kinase Inhibitors in a Transgenic Mouse Model of Gallbladder Carcinoma

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