

# Role of Growth Factor Signaling Pathways in Biliary Tract Cancer

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## Abstract

Biliary-tract carcinomas (BTCs) are relatively infrequent but highly lethal malignancies. Novel targets for therapeutic or chemopreventive approaches are urgently needed. However, the knowledge of genomic mutations in BTC is less extensive than that of other gastrointestinal cancers. In this chapter, we will discuss the role of growth factors and their receptors (receptor tyrosine kinases, RTKs), downstream signaling pathways of these RTKs and inflammatory mediators during gallbladder carcinogenesis based on our study using a mouse model for human BTC as well as additional information in the literature.

## 1 Introduction

Biliary tract carcinomas (BTCs), which include cancers of the gallbladder (GBCs) and the intra- and extra-hepatic biliary tree, are relatively infrequent but highly lethal malignancies [1]. Although there have been advances in the diagnosis and management of BTCs, these cancers still prove challenging to treat due to their insensitivity to conventional therapies and the inability to prevent or detect early tumor formation. These factors render gallbladder cancer nearly incurable with a five-year survival rate of only 5–21 % [2–6]. Novel targets for therapeutic or chemopreventive approaches are urgently needed. We previously generated transgenic mice that overexpress wild-type rat erbB2 in epithelial tissues under the control of the bovine keratin 5 (BK5) promoter (BK5.erbB2 mice) [7]. Overexpression of erbB2 in basal epithelial cells of the gallbladder led to the development of adenocarcinoma of the gallbladder and cystic duct in 90 % of these transgenic mice by 2–3 months of age. This was the first direct demonstration that erbB2 overexpression could lead to the development of BTC [7]. We have shown that BK5.erbB2 transgenic mice are a valid model for investigating mechanisms underlying the development of GBCs and other BTCs. We have found

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that protein levels of erbB2 as well as protein levels of epidermal growth factor receptor (EGFR) are elevated in the gallbladder in BK5.erbB2 transgenic mice. In addition, we have found elevated levels of COX-2/PGE<sub>2</sub> and elevated activity of Akt, MAPK (mitogen-activated protein kinase), and mTOR (mammalian target of rapamycin) in the GBC from these mice. These molecular alterations are similar to those reported in human GBC or BTC.

In this chapter, we will discuss the role of growth factors and their receptors (receptor tyrosine kinases, RTKs), downstream signaling pathways of these RTKs, and inflammatory mediators during gallbladder carcinogenesis. Understanding the growth factor signaling pathways upregulated in GBC will provide critical clues for novel therapeutic and chemopreventive strategies using drugs and/or agents that selectively target these specific pathways.

## 2 Molecular Aspect of BTC

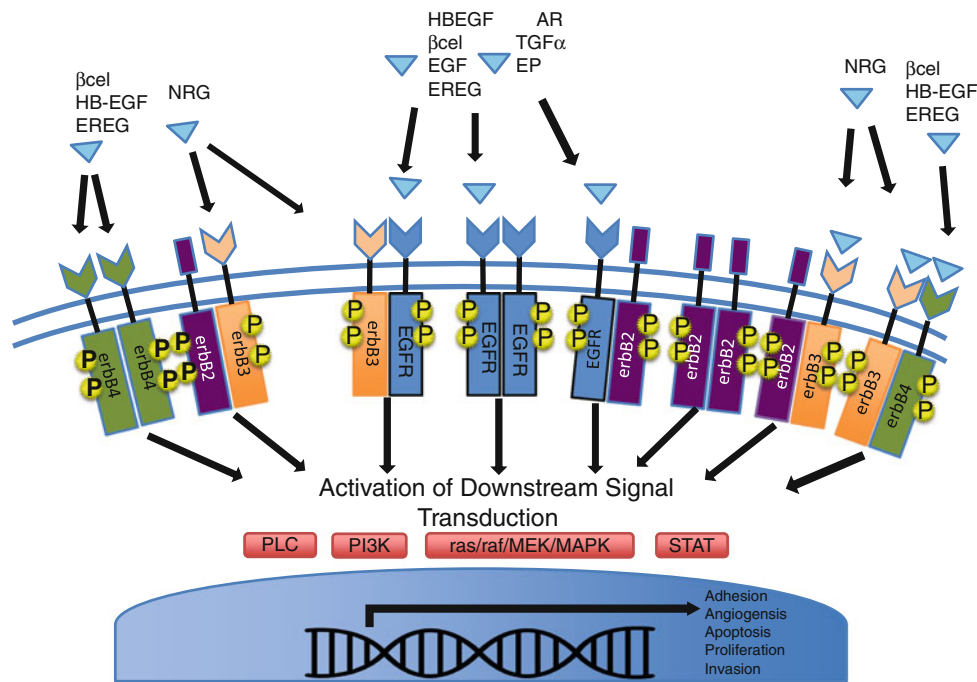
The knowledge of genomic mutations in BTC is less extensive than that of other gastrointestinal cancers. Although the molecular aspects of BTC remain poorly understood, several genetic abnormalities have been described in specimens of human GBC. Genetic alterations in p53 or K-ras may contribute to the development of certain types of GBC [8–13]. In this regard, Hanada et al. [11] found that the incidence of p53 mutations and protein expression was significantly less in the polypoid type (adenoma–carcinoma sequence) of GBC compared with the flat type (de novo development). Li et al. [14] have reported that p53 overexpression was detected in 43 % of adenomas, 60 % of dysplasias, and 57 % of GBCs in addition to frequent observation of reduced p21<sup>WAF1/CIP1</sup> expression. Mutations in codon 12 of K-ras are seen infrequently in GBC, except in those cases where the carcinoma is associated with an anomalous junction of the pancreaticobiliary duct (APDJ) [8–13]. Recent studies describe the presence of p53 mutation in 92 % of invasive GBC [13]. ErbB2 overexpression has been reported in a significant percentage of GBCs [14–16] and cholangiocarcinomas [16–21]. The protein levels of both EGFR and its ligand, transforming growth factor- $\alpha$  (TGF $\alpha$ ), assessed by immunostaining, are elevated in human BTC including GBC [22, 23]. Accumulating evidence suggests that COX-2, an inducible enzyme responsible for conversion of arachidonic acid to prostaglandins, may play a variety of roles in the gastrointestinal tract including pathogenic processes such as neoplasia [24]. A recent study demonstrated a relationship between erbB2 overexpression and COX-2 upregulation in human colorectal cancer cells [25]. Elevated COX-2 expression has been demonstrated in well-differentiated human hepatocellular carcinoma [26, 27] and GBC [28] compared with low or non-detectable COX-2

expression in poorly differentiated tumors. Very recently, Sirica's group reported a strong positive correlation between the immunostaining intensities of erbB2 and COX-2 in BTC. COX-2 was observed not only in the furan rat cholangiocarcinoma model, but also in human cholangiocarcinomas [29], supporting the possibility that erbB2 plays a key role in regulating COX-2 expression in neoplastic and precancerous biliary tract epithelial cells. Grossman et al. [30] reported a specific COX-2 inhibitor, but not COX-1 inhibitor, decreased mitogenesis, and increased human gallbladder cell apoptosis associated with decreased prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). This suggests that the COX enzymes and the prostanooids may play a role in the development of gallbladder cancer and that COX-2 inhibitors may have a therapeutic role in gallbladder neoplasms [30].

## 3 Role of ErbB RTKs and Their Downstream Signaling Pathways in the Development of BTC

### 3.1 ErbB2 and EGFR in Human BTC

To date, very few studies have addressed the molecular and cellular mechanisms underlying the development of BTC as described above; however, several lines of evidence suggest a role for the erbB receptor family. Overexpression and activation of erbB2 have been reported in a significant percentage of human BTC [15, 16, 31, 32]. In one study, 30 of 43 cases (69.6 %) and 14 of 43 cases (32.6 %) of GBCs had amplification of erbB2 DNA or overexpression of erbB2 protein, respectively [15]. In another study, 7 of 11 cases (63.6 %) of GBCs showed overexpression of erbB2 protein [16]. Yukawa et al. [17] reported erbB2 protein expression in 9 of 13 cases (69 %) of GBCs considered to be relatively early-stage tumors (all 13 cases were histologically diagnosed as well-differentiated tubular adenocarcinoma), yet erbB2 protein expression was undetectable in tumors that were more advanced. Furthermore, ErbB2 has been shown to be overexpressed in the neoplastic glandular epithelium of furan- and thioacetamide-induced intestinal-type cholangiocarcinomas in rat liver [33, 34]. It has also been reported that erbB2-transformed rat cholangiocytes, which overexpressed activated erbB2, obtained a tumorigenic feature when transplanted into isogenic rats, yielding a 100 % incidence of BTCs [34]. Overexpression and activation of epidermal growth factor receptor (EGFR) have also been reported in 30–60 % of BTC samples [31, 32, 35] and were shown to be correlated with negative clinical and pathologic features, such as distant metastasis and poor dedifferentiation [22, 36–38]. These data suggest that altered expression and activity of erbB2 and EGFR are major mechanisms underlying human BTC carcinogenesis [39].



**Fig. 1** ErbB family signaling system. Cross talk between erbB2/EGFR and other erbB receptor tyrosine kinase members and downstream signaling which leads to cell proliferation, survival, and migration (see text in detail)

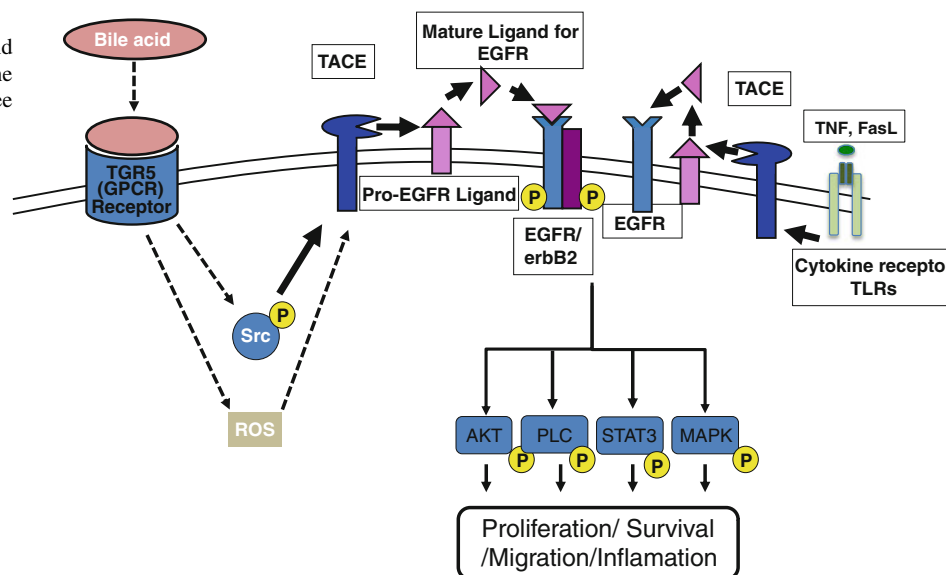
### 3.2 erbB RTK Family

Several lines of evidence suggest a role for the erbB receptor family as described above. A number of RTKs have been described [40–42]. Among them is the erbB family of RTKs consisting of the epidermal growth factor receptor (EGFR/erbB1), erbB2 (neu), erbB3, and erbB4 [43]. ErbB family RTKs have been shown to be important for normal development as well as in neoplasia [40, 44] (Fig. 1). Although all of the erbB family members share similarities in primary structure, receptor activation mechanism, and signal transduction patterns, they bind to different ligands. EGFR binds to and can be activated by a number of different ligands of the EGF family, including EGF, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin [45, 46], epigen (EP), and epiregulin (EREG). The neuregulin subfamily consists of various isoforms referred to as 1–4. These ligands bind to erbB4 and/or erbB3. Betacellulin, HB-EGF, and epiregulin have also been shown to bind to erbB4. Ligand-dependent activation of erbB family receptors can lead to heterodimerization, particularly of EGFR, erbB3 and erbB4 with erbB2. To date, no ligand has been identified for erbB2. ErbB3 cannot generate signals in isolation because the kinase function of this receptor is impaired, thus relying on interaction with erbB2 for signaling.

Post-receptor signaling by activated erbB family members includes signaling through Ras/MEK/MAPK/Erk (extracellular signal-regulated kinase), phospholipase C $\gamma$ , signal transducer and activation of transcription (STATs), and phosphatidylinositol 3-kinase (PI3K) pathways that are common to nearly all RTKs (Fig. 1). Although the membrane-anchored peptide can be biologically active through juxtacrine signaling, in most cases, the extracellular domain is proteolytically cleaved by a metalloprotease activity present in the cell membrane. This process is known as “ectodomain shedding” and leads to the release of the soluble growth factor, which may act in an endocrine, paracrine, or autocrine fashion [47].

To allow paracrine or autocrine interaction of the EGFR ligands with the receptor, the membrane-tethered ligand precursors need to be released by a proteolytic reaction. This important step is mediated mainly by membrane-anchored metalloproteases of the ADAM (a disintegrin and metalloprotease) family [48]. ADAM17, which is also known as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-converting enzyme, or TACE, together with ADAM10, is thought to play a central role. ADAM17 can cleave the AR, EREG, TGF- $\alpha$ , and HB-EGF membrane-anchored precursors, while ADAM 10 is a key sheddase for EGF and BTC, and can also cleave the HB-HGF transmembrane precursor [45, 46, 49]. Transactivation of the EGFR by ligands of G-protein-coupled receptors (GPCRs) is perhaps the best characterized example of EGFR

**Fig. 2** ErbB2/EGFR transactivation through TLRs, GPCR, and TACE and cross talk between src which leads to the development of biliary tract cancer (see text in detail)



activation by heterologous ligands [48]. These include angiotensin II (ANG II), lysophosphatidic acid (LPA), endothelin-I, thrombin, IL-8, and prostaglandins such as PGE2 [48]. Different mechanisms have been proposed to mediate ADAM activation by GPCRs. Elevation of the intracellular levels of  $\text{Ca}^{2+}$  or reactive oxygen species (ROS) is likely to be involved as well as phosphorylation reactions involving protein kinase C (PKC), ERK, or c-Src [48]. As previously indicated, transactivation of the EGFR is not exclusive of GPCR-triggered signaling. Studies carried out in keratinocytes have established that the expression and release of EGFR ligands can be elicited by the cytokines  $\text{TNF-}\alpha$  and interferon- $\gamma$  (INF- $\gamma$ ) [50]. This has been recently observed also for the proapoptotic factor Fas ligand (FasL). Interestingly, it was shown that transactivation of the EGFR through the secretion of ligands such as AR contributed to mediate part of the inflammatory responses to FasL in human epidermis [51] (Fig. 2).

### 3.3 Animal Models for Human BTC

#### 3.3.1 Background

As mentioned, 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, and 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 BTC, intrahepatic

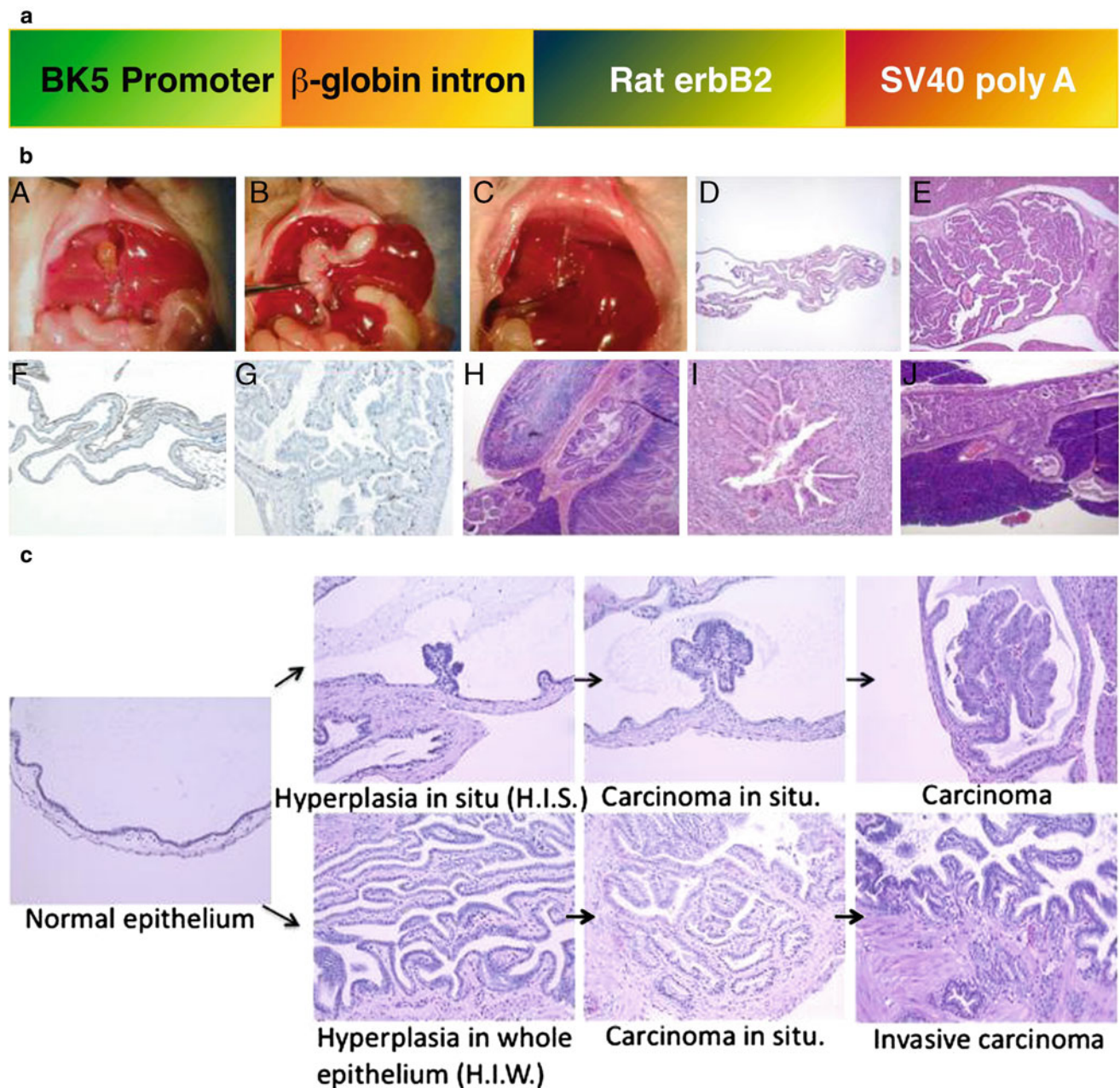
cholangiocarcinoma [52, 53]. In this model, treatment of rats with furan rapidly induced intestinal metaplasia and associated cholangiofibrosis in the right/caudate liver of rats [54]. Long-term treatment with furan (daily dose of 30 mg/kg of body weight, five times weekly by gavage for 9–13 weeks) resulted in the preferential development of cholangiocarcinoma [53]. The incidence of cholangiocarcinoma was 70–90 % in rats treated with furan by 16 months. The furan-induced cholangiocarcinoma in this rat model characteristically overexpressed erbB2, COX-2, and c-Met [54]. In addition to this model, combined treatment of Syrian golden hamster with dihydroxy-di-n-propyl nitrosamine and liver fluke infestation was shown to be associated with the enhancement of cholangiocarcinomas and preneoplastic lesions in the gallbladder [55].

Recently, we developed BK5.erbB2 transgenic mice, where expression of the rat erbB2 cDNA is targeted to the basal layer of multiple epithelial tissues, including the biliary tract epithelium [7, 56] (Fig. 3a). Adenocarcinoma of the gallbladder develops in 90 % of the homozygous BK5.erbB2 transgenic mice by 2–3 months of age [7]. The BK5.erbB2 transgenic mouse line represents the first genetically engineered mouse model for investigating the mechanism(s) underlying the development of GBCs and other BTCs. The remainder of this section will be devoted to a summary of this model and its initial utilization for preclinical therapeutic studies.

#### 3.3.2 BK5.erbB2 Mouse Model of Gallbladder Cancer

Necropsy of adult BK5.erbB2 mice revealed that the gallbladder was dramatically enlarged and had a white, opaque appearance (Fig. 3bB). Enlarged gallbladders were often associated with a significantly dilated common bile duct



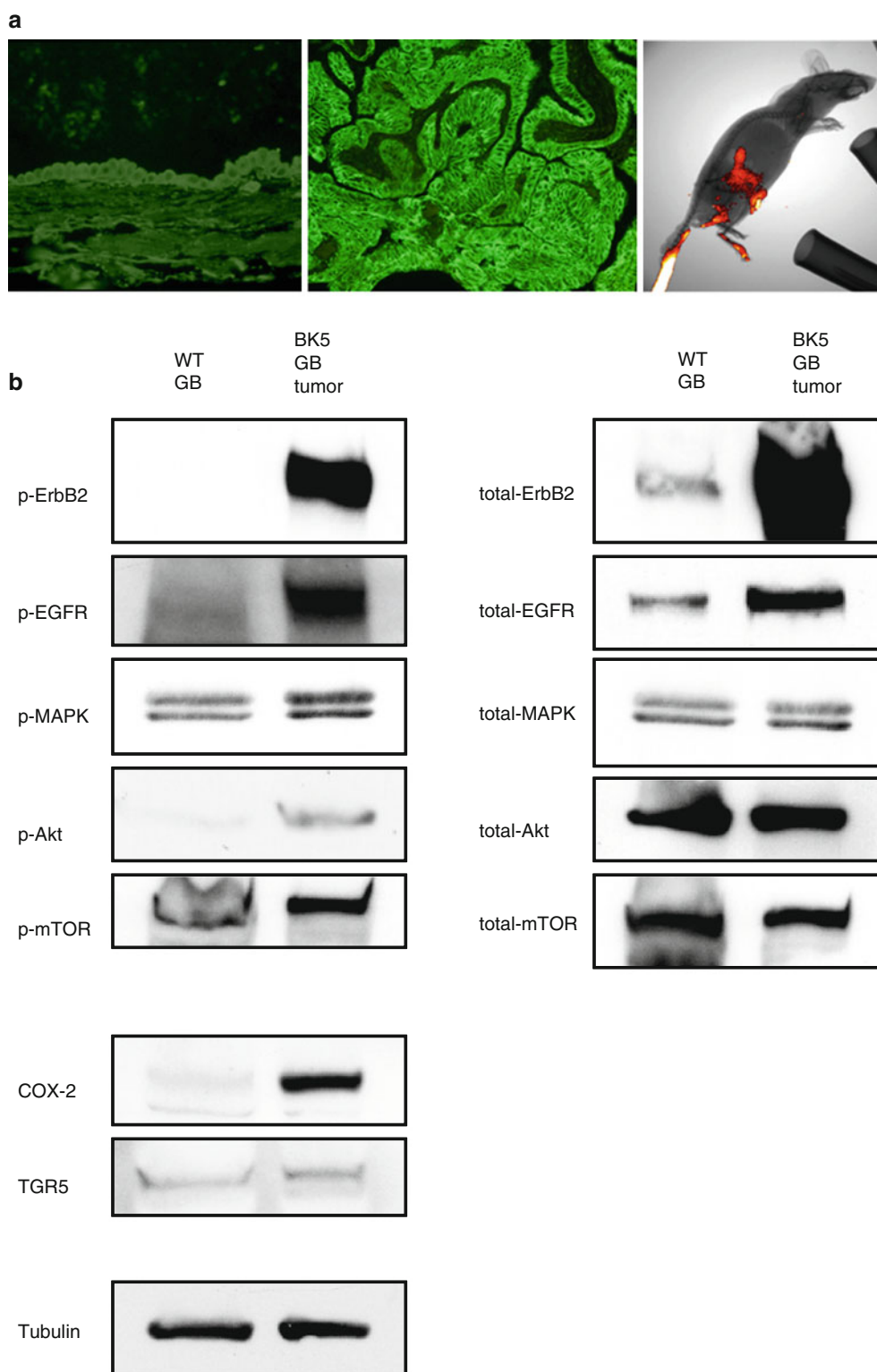


**Fig. 3** **a** The DNA construct used to generate BK5.erbB2 mice. **b** Gross appearance and histological evaluations of BTC in BK5.erbB2 mice. **A** Gallbladder of wild-type mouse, **B** BK5.erbB2 mouse at 3 months of age, **C** anomalous fasciculus form of gallbladder in the early stage of gallbladder development (2 weeks of age) in BK5.erbB2 mouse, **D** H and **E** staining of gallbladder in wild-type mouse and **E** BK5.erbB2 mouse, **F** BrdU staining of gallbladder in wild-type mouse and **G** BK5.erbB2 mouse, **H** H and **E** staining of the ampulla of

Vater, **I** intrahepatic cholangiocarcinoma, and **J** the junction of the pancreaticobiliary duct (JPBD) in a 3-month-old BK5.erbB2 mouse. **c** Two different pathways of development of GBC in BK5.erbB2 mice. (*Upper figures*) Carcinoma arising from hyperplasia in situ shown in an adenoma/hyperplasia/carcinoma sequence. (*Lower figures*) Carcinoma arising from hyperplasia shown in a de novo sequence. (*Figure on left*) Normal gallbladder from wild-type control mouse. Some of figures are adopted from Kiguchi K et al. [7]

(Fig. 3bB). This enlarged hepatic duct from the liver and the cystic duct from the gallbladder unite to form the enlarged common bile duct, which extends posteriorly through the pancreas and intestinal wall, where it opens to the mucosal surface of the duodenum as the ampulla of

Vater (Fig. 3B and H). Most of the gallbladders in young BK5.erbB2 mice (<3 weeks) possess an anomalous fasciculus structure (Fig 3bC). The majority of the GBCs completely filled the lumen (Fig. 3E), although some showed focal lesions.



**Fig. 4 a** Expression of ErbB2: immunostaining for erbB2 in gallbladder from wild-type mouse (*left*) and BK5.erbB2 mouse (*middle*) of 3-month-old mouse. Image of GBC detected by Kodak in-vivo Imaging System FX-Pro, 48 h after EGF-labeled probe injection (*right, arrow*). High-intensity area in tail is injection site

(*arrow head*). **b** Analysis of protein statuses and kinase activation in gallbladder from wild-type and BK5.erbB2 mice. Whole tissue lysates from wild-type and BK5.erbB2 mice were analyzed via Western blot with antibodies against indicated molecules. Tubulin was used as an internal control

Analysis of the mucosa adjacent to the GBC observed in the mice allowed segregation into two categories based on etiology: carcinoma arising from hyperplasia in situ (HIS, 14 cases out of 34 GBCs from BK5.erbB2 mice, 41 %) or hyperplasia in whole mucosa (HIW, 59 %) (Fig. 3c). GBC tumors arising from HIW were more likely to be invasive (70 %,  $p < 0.01$ ) compared to those arising from HIS (14 %). Tumors were characterized by branching structures with finger-like projections covered with high columnar epithelium and hyperchromatic nuclei. Most of the tumors were diagnosed as well-differentiated adenocarcinomas. Carcinoma cells frequently invaded into the surrounding connective tissues. In addition, hypervascularization was a characteristic feature of these tumors. Staining with CD31, a marker for endothelial cells, revealed extensive vascularization in the adenocarcinomas from BK5.erbB2 mice [7]. Adenocarcinomas from BK5.erbB2 mice exhibited a significantly elevated labeling index (a marker of proliferation) compared to normal gallbladder epithelium as determined by staining with antibromodeoxyuridine (BrdU) antibody (Fig. 3bG). Tumor cells of the common bile duct often invaded into the pancreatic duct (Fig. 3bJ). The ampulla of Vater was dilated, and hyperplasia of the epithelium was observed in transgenic mice. Pronounced congestion of bile, inflammation, necrosis, hyperplasia of biliary duct cells, and/or tumor development was also frequently observed in intrahepatic biliary ducts of transgenic mice (Fig. 3bI).

### 3.3.3 Status of EGFR and ErbB2 in GBC of BK5.erbB2 Mice

Persistent expression of the erbB2 transgene was observed in the epithelia of both gallbladder and intrahepatic biliary duct as well as in gallbladder adenocarcinoma (Fig. 4a) and cholangiocarcinomas [7]. Endogenous erbB2 expression was only weakly detectable in both the intrahepatic biliary duct and gallbladder from wild-type mice (Fig. 4a). Western blot analysis of gallbladder tissue lysates showed that the level of erbB2 protein was significantly elevated in BK5.erbB2 mice compared to that of wild-type mice, as expected (Fig. 4b). ErbB2 was also hyperphosphorylated after adjustment for total erbB2 protein level (Fig. 4b). Interestingly, the level of EGFR protein (but not erbB3 or erbB4 protein) was elevated and hyperphosphorylated on tyrosine residues in gallbladder tissue from BK5.erbB2 mice (Fig. 4b). Additional analyses by immunoprecipitation of EGFR and erbB2 followed by Western blot analysis for erbB2 and EGFR, respectively, confirmed elevated heterodimer formation between erbB2 and EGFR in gallbladder tissue of BK5.erbB2 mice [7]. Furthermore, to detect gallbladder tumors in BK5.erbB2 mice in vivo, we utilized a molecular imaging system. BK5.erbB2 mice were injected via tail vein with either EGF-labeled NHS ester conjugate with infrared dye 800CW (IRDye 800CW EGF

probe) or IRDye 800CW Carbonate as control. The distribution of the IRDye 800CW EGF was visualized by the Kodak in vivo Imaging System FX-Pro (Carestream Health Inc., Rochester, NY). 48 h after the injection, the EGF probe accumulated in the gallbladder (Fig. 4a). The background signal in the gallbladders of mice injected with IRD 800CW Carbonate as well as the gallbladder of wild-type mice injected with the EGF probe was undetectable (data not shown). This preliminary experiment indicates that the level of EGFR and/or erbB2 is significantly high in the gallbladder of BK5.erbB2 mice and that this bioimaging technique can be a useful tool for tracking tumor size in longitudinal in vivo experiments.

### 3.3.4 MAPK, Akt, and mTOR in Gallbladder Tissue of BK5.erbB2 Mice

The activation status of signaling molecules downstream of erbB2/EGFR and the status of other proteins were also examined. Although total protein levels of MAPK were not changed (Fig. 4b), the level of phosphorylation of MAPK was increased in the gallbladder of transgenic mice. Furthermore, phospho-Akt, but not total Akt level, was elevated in the gallbladder of BK5.erbB2 mice as assessed by Western blot analysis (Fig. 4b). We have reported that mTOR and other signaling molecules both immediately upstream (Akt, MAPK) and downstream (p70S6 K) of mTOR are hyperphosphorylated in gallbladder tissues from BK5.erbB2 mice compared to corresponding tissue from wild-type mice [39]. We also found that cyclin D1, bcl-2, c-Met, E-cadherin, and b-catenin were upregulated in the gallbladder tissue of BK5.erbB2 compared to wild-type mouse by Western blot analysis [7].

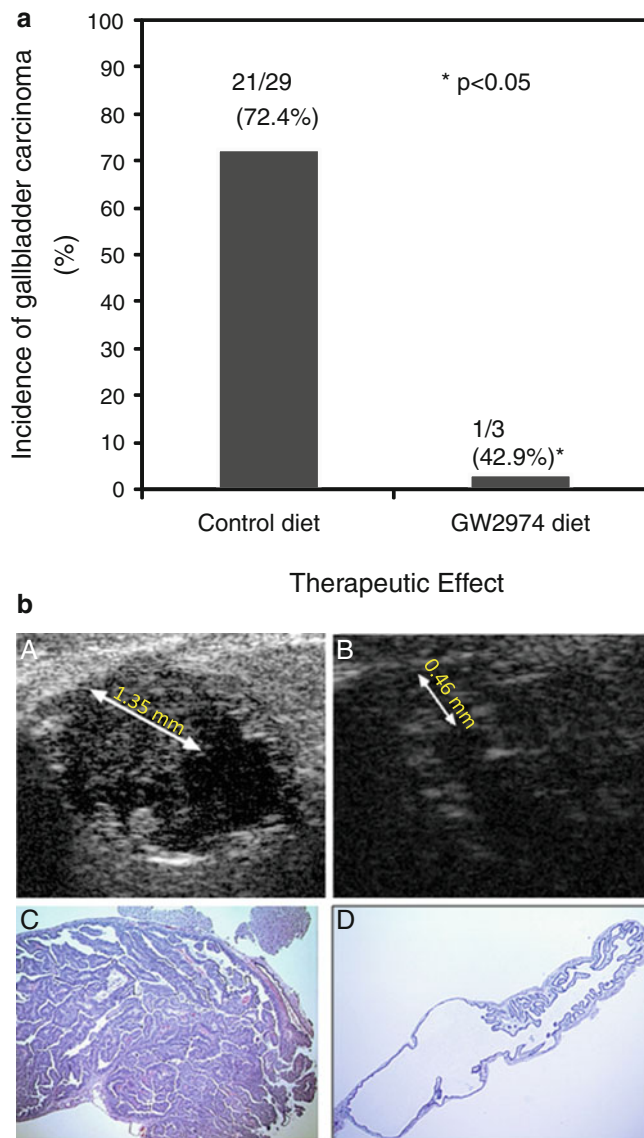
### 3.3.5 Increased COX-2 Protein and mRNA Expression, PGE2 Synthesis, and Phosphorylation of PLA2 in GBC of BK5.erbB2 Mice

The protein level (determined by immunohistochemistry and Western blot) and mRNA expression (determined by RT-PCR) of COX-2 were significantly elevated in the gallbladder tissue of BK5.erbB2 mice compared to wild-type mice (Fig. 4b) and [7]. The level of PGE2 was also found to be elevated in the tissue [47]. These results suggest that elevated prostaglandins, particularly PGE2, may play an important role in the development of GBC in BK5.erbB2 mice. Phosphorylated form of phospholipase A2 (PLA2), but not total PLA2, was also elevated in the gallbladder tissue of BK5.erbB2 mice (not shown).

### 3.3.6 Therapeutic Studies with Specific Molecular Targeting Agents Using BK5.erbB2 Mice

Similarities in molecular alterations, such as overexpression and/or activation of erbB2, EGFR, Akt, and COX-2 between BTCs in BK5.erbB2 mice and humans, make





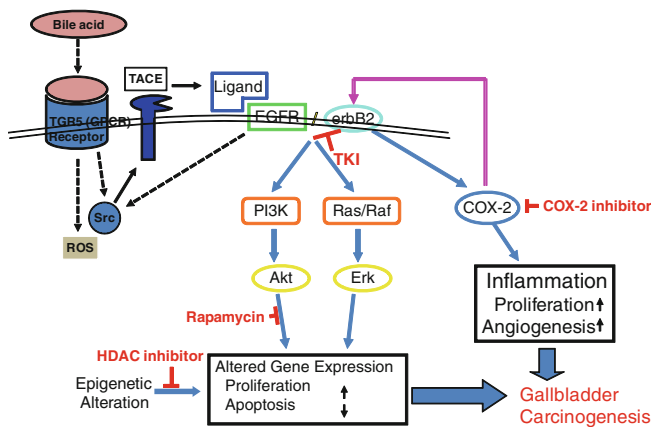
**Fig. 5** Therapeutic effect of tyrosine kinase inhibitor, GW2974, and HDAC inhibitor, PCI-24781, on the incidence of GBC in BK5.erbB2 mice. **a** Incidence of GBC in BK5.erbB2 mice. Two month-old BK5.erbB2 mice were treated with AIN76 control diet or AIN76 diet containing 200 ppm GW2974 for one month. \*,  $P < 0.01$ . **b** Effects of GW2974 detected by ultrasound and **b** histological analyses. Regression of GBC by GW2974 treatment as detected by ultrasound biomicroscopy. All images are from a single animal depicting the response representative of the treatment group. **A** Ultrasound image of GBC (maximum size: 1.35 mm) before GW2974 treatment. **B** Ultrasound image of gallbladder on the 23rd day of treatment; this image indicates regression of the carcinoma (observed size: 0.46 mm). **C** Gallbladder of BK5.erbB2 mouse receiving AIN76 control diet. **D** Typical histological features of the gallbladder from BK5.erbB2 mice treated with AIN76 diet containing 200 ppm GW2974. Figures are adopted from Kiguchi K et al. [56]

BK5.erbB2 transgenic mice a unique animal model for further mechanistic studies regarding the role of erbB2/EGFR and their downstream signaling in the development and growth of BTC, as well as a promising tool for the development of new treatment and/or prevention modalities. We have used this model successfully in several pre-clinical therapeutic studies using tyrosine kinase inhibitors [56], a COX-2 inhibitor [57], an mTOR inhibitor [58], and histone deacetylase (HDAC) inhibitor [59]. Figure 5 shows the therapeutic effect of GW2974, a dual specific erbB2/EGFR inhibitor [60]. In this experiment, BK5.erbB2 mice received 200 ppm GW2974 in the diet for 1 month. Treatment with GW2974 resulted in a significant decrease in the incidence of GBC to 3 % (Fig. 5a). These reductions corresponded to a 95 % decrease in tumor incidence compared with BK5.erbB2 mice receiving the control diet, which had a GBC incidence of 72 % as determined by histopathological examination. The impact of treatment is very clearly seen in the ultrasound images in Fig. 5b. H and E staining in the right panels clearly shows that the dramatic regression of the tumor with only hyperplasia is still evident (Fig 5b). The labeling index determined by BrdU staining was also reduced in the gallbladder of mice receiving GW2974.

Treatment with GW2974 resulted in decreased levels of both erbB2 and EGFR. Furthermore, levels of p-erbB2 and p-EGFR were markedly reduced [56]. Nearly complete inhibition of tumor development by GW2974 suggests a level of erbB2 dependency during gallbladder tumor development in BK5.erbB2 mice. Treatment of BK5.erbB2 mice with the HDAC inhibitor, PCI-24781, for 1 month prevented 79 % of GBCs cases from progression and showed a clinical effect in 47 % of cases. This effect was associated with downregulation of erbB2 mRNA, ErbB2 protein/activity, and EGFR activity and upregulation of acetylated histone and acetylated tubulin [58]. These results indicate that the significant therapeutic/inhibitory effect that this HDAC has on the development of gallbladder tumors is due to its ability to block the activation of both erbB2 and EGFR.

We also examined the effects of a COX-2 inhibitor, CS-706, on the development of GBCs using the BK5.erbB2 mouse model. Ultrasound image analysis as well as histological evaluation revealed a significant therapeutic effect of CS-706 on the GBCs, either as reversion to a milder phenotype or as inhibition of tumor progression. The antitumor effect was associated with inhibition of prostaglandin E2 synthesis. CS-706 treatment also downregulated the activation of erbB2 and EGFR, resulting in decreased levels of phosphorylated Akt and COX-2 in GBCs of BK5.erbB2





**Fig. 6** Proposed pathway in which erbB2/EGFR, COX-2, bile acid, and src may play a role during the BTC carcinogenesis. ErbB2 overexpression/activation may accelerate the transactivation cascade of during the development of BTC. Figure is adopted from Kiguchi et al. [75]

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 BTC [57].

In addition, BK5.erbB2 mice were treated with rapamycin by i.p. injection (5 mg/kg BW, once daily for 14 days). Rapamycin significantly reduced the incidence and severity of GBCs in BK5.erbB2 mice in a dose-dependent manner. Tumors responsive to treatment exhibited a higher number of apoptotic cells. Furthermore, rapamycin treatment led to decreased levels of phosphorylated p70 S6 kinase (Thr389) in gallbladder tissue as assessed by both Western blot and immunofluorescence analyses. Immunofluorescence staining revealed elevated phosphorylated Akt (Ser473) and phosphorylated mammalian target of rapamycin (mTOR; Ser2448) in human GBC compared with normal gallbladder tissue. Based on the fact that the Akt/mTOR pathway is activated in human GBC, rapamycin and related drugs may be effective therapeutic agents for the treatment of human GBC with activated Akt/mTOR pathway [58]. Proposed inhibitory effect of each therapeutic compound on the signaling pathways in GBC of BK5.erbB2 mice is shown in Fig. 6.

#### 4 The Role of Bile Acid During BTC Carcinogenesis

Exposure to high levels or abnormal composition of bile acid is associated with an increased incidence of cancer of the laryngopharyngeal tract, esophagus, stomach, pancreas, small intestine, and colon [61]. Bile acids, which are synthesized from cholesterol, have long been recognized as essential for dietary lipid absorption; however, an important role for bile acids as signaling molecules has emerged in recent years [62–64]. Bile acids activate EGFR, MAPK, and

PI3-K/Akt signaling pathways in hepatocytes [65, 66]. More recent evidence suggests that bile acids may activate RTKs and downstream signaling molecules, indirectly, in a G-protein-coupled receptor (GPCR)-dependent manner [67] mediated by ADAM family peptidases [68, 69]. The role of cell signaling by these organic acids in the development of human biliary tract cancer remains unknown. A recent study from our laboratory [70] demonstrated that the secondary conjugated bile acid, taurochenodeoxycholic acid (TCDC), increased proliferation of primary cultured gallbladder epithelial cells from BK5.erbB2 mice and human BTC cells. TCDC treatment activated erbB2/EGFR and downstream signaling molecules in both primary cultured cells and human BTC cells. TCDC also increased the expression of EGFR ligands and TACE activity in human BTC cells. These results suggest that during the development of BTC, bile acid may act as a promoter when erbB2 is activated in gallbladder epithelial cells.

Previous lines of evidence have suggested that the non-receptor tyrosine kinase, c-Src, elicits cross talk between TGR5 (a GPCR) and EGFR to transduce bile acid signaling for activation of EGFR [71–74]. Figure 6 shows the proposed role of erbB2/EGFR and other key molecules as well as possible cross talk in the development of BTC in BK5.erbB2 mice.

#### 5 Conclusion and Future Direction

The dismal outcomes that generally result from gallbladder carcinoma and other BTCs explain the pessimism that surrounds treatment for these cancers. Nevertheless, more aggressive surgical technique, advanced oncologic, and radiation therapy have led many institutions to report an increase in long-term survival rates. Although these treatments are progressive, the efforts directed toward early detection and novel treatment derived from basic research to determine the mechanisms involved in BTC development may play a key role in the improvement of patients' survival. New drugs that selectively target specific augmented molecule(s) such as erbB2 and COX-2 in BTC and associated risk conditions may serve as potentially effective adjunct therapeutic strategies for this cancer, for which there is currently no effective medical treatment. In addition, identification of novel candidate gene(s) or protein(s), which regulate these mechanisms, may provide not only potential therapeutic targets, but also novel tumor markers for this lethal disease. The BK5.erbB2 transgenic mouse model provides a unique opportunity to study the mechanisms involved in the development of this cancer.

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## References

- Wistuba II, Gazdar AF (2004) Gallbladder cancer: lessons from a rare tumour. *Nat Rev Cancer* 4(9):695–706
- Carriaga MT, Henson DE (1995) Liver, gallbladder, extrahepatic bile ducts, and pancreas. *Cancer* 75(1 Suppl):171–190
- Cuberta-fond P, Gainant A, Cucchiario G (1994) Surgical treatment of 724 carcinomas of the gallbladder. Results of the French Surgical Association Survey. *Ann Surg* 219(3):275–280.
- Ruckert JC, Ruckert RI, Gellert K, Hecker K, Muller JM (1996) Surgery for carcinoma of the gallbladder. *Hepato-gastroenterology* 43(9):527–533
- Oertli D, Herzog U, Tondelli P (1993) Primary carcinoma of the gallbladder: operative experience during a 16 year period. *Eur J Surg* 159(8):415–420
- Ogura Y, Mizumoto R, Isaji S, Kusuda T, Matsuda S, Tabata M (1991) Radical operations for carcinoma of the gallbladder: present status in Japan. *World J Surg* 15(3):337–343
- Kiguchi K, Carbajal S, Chan K, Beltran L, Ruffino L, Shen J, Matsumoto T, Yoshimi N, DiGiovanni J (2001) Constitutive expression of ErbB-2 in gallbladder epithelium results in development of adenocarcinoma. *Cancer Res* 61(19):6971–6976
- Itoi T, Watanabe H, Ajioka Y, Oohashi Y, Takel K, Nishikura K, Nakamura Y, Horil A, Saito T (1996) APC, K-ras codon 12 mutations and p53 gene expression in carcinoma and adenoma of the gall-bladder suggest two genetic pathways in gall-bladder carcinogenesis. *Pathol Int* 46(5):333–340
- Watanabe H, Date K, Itoi T, Matsubayashi H, Yokoyama N, Yamano M, Ajioka Y, Nishikura K (1999) Histological and genetic changes in malignant transformation of gallbladder adenoma. *Ann Oncol Official J Eur Soc Med Oncol/ESMO* 10(Suppl 4):136–139
- Hanada K, Itoh M, Fujii K, Tsuchida A, Ooishi H, Kajiyama G (1997) K-ras and p53 mutations in stage I gallbladder carcinoma with special attention to growth patterns. *Europ J Cancer* 33:1136–1140
- Hanada K, Tsuchida A, Iwao T, Eguchi N, Sasaki T, Morinaka K, Matsubara K, Kawasaki Y, Yamamoto S, Kajiyama G (1999) Gene mutations of K-ras in gallbladder mucosae and gallbladder carcinoma with an anomalous junction of the pancreaticobiliary duct. *Am J Gastroenterol* 94(6):1638–1642
- Hidaka E, Yanagisawa A, Seki M, Takano K, Setoguchi T, Kato Y (2000) High frequency of K-ras mutations in biliary duct carcinomas of cases with a long common channel in the papilla of Vater. *Cancer Res* 60(3):522–524
- Wee A, Teh M, Raju GC (1994) Clinical importance of p53 protein in gall bladder carcinoma and its precursor lesions. *J Clin Pathol* 47(5):453–456
- Li X, Hui AM, Shi YZ, Takayama T, Makuuchi M (2001) Reduced p21(WAF1/CIP1) expression is an early event in gallbladder carcinogenesis and is of prognostic significance for patients with carcinomas of the gallbladder. *Hum Pathol* 32(8):771–777
- Suzuki T, Takano Y, Kakita A, Okudaira M (1993) An immunohistochemical and molecular biological study of c-erbB-2 amplification and prognostic relevance in gallbladder cancer. *Pathol Res Pract* 189(3):283–292
- Chow NH, Huang SM, Chan SH, Mo LR, Hwang MH, Su WC (1995) Significance of c-erbB-2 expression in normal and neoplastic epithelium of biliary tract. *Anticancer Res* 15(3):1055–1059
- Yukawa M, Fujimori T, Hirayama D, Idei Y, Ajiki T, Kawai K, Sugiura R, Maeda S, Nagasako K (1993) Expression of oncogene products and growth factors in early gallbladder cancer, advanced gallbladder cancer, and chronic cholecystitis. *Hum Pathol* 24(1):37–40
- Suzuki H, Isaji S, Pairojkul C, Uttaravichien T (2000) Comparative clinicopathological study of resected intrahepatic cholangiocarcinoma in northeast Thailand and Japan. *J Hepatobiliary Pancreat Surg* 7(2):206–211
- Ito Y, Takeda T, Sasaki Y, Sakon M, Yamada T, Ishiguro S, Imaoka S, Tsujimoto M, Higashiyama S, Monden M, Matsuura N (2001) Expression and clinical significance of the erbB family in intrahepatic cholangiocellular carcinoma. *Pathol Res Pract* 197(2):95–100
- Aishima SI, Taguchi KI, Sugimachi K, Shimada M, Sugimachi K, Tsuneyoshi M (2002) c-erbB-2 and c-Met expression relates to cholangiocarcinogenesis and progression of intrahepatic cholangiocarcinoma. *Histopathology* 40(3):269–278
- Ukita Y, Kato M, Terada T (2002) Gene amplification and mRNA and protein overexpression of c-erbB-2 (HER-2/neu) in human intrahepatic cholangiocarcinoma as detected by fluorescence in situ hybridization, in situ hybridization, and immunohistochemistry. *J Hepatol* 36(6):780–785
- Lee CS, Pirdas A (1995) Epidermal growth factor receptor immunoreactivity in gallbladder and extrahepatic biliary tract tumours. *Pathol Res Pract* 191(11):1087–1091
- Lee CS (1998) Transforming growth factor alpha immunoreactivity in human gallbladder and extrahepatic biliary tract tumours. *Eur J Surg Oncol* 24(1):38–42
- DuBois RN, Eberhart CF, Williams CS (1996) Introduction to eicosanoids and the gastroenteric tract. *Gastroenterol Clin N Am* 25(2):267–277
- Vadlamudi R, Mandal M, Adam L, Steinbach G, Mendelsohn J, Kumar R (1999) Regulation of cyclooxygenase-2 pathway by HER2 receptor. *Oncogene* 18(2):305–314
- Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T, Sata M (1999) Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. *Hepatology* 29(3):688–696
- Kondo M, Yamamoto H, Nagano H, Okami J, Ito Y, Shimizu J, Eguchi H, Miyamoto A, Dono K, Umeshita K, Matsuura N, Wakasa K, Nakamori S, Sakon M, Monden M (1999) Increased expression of COX-2 in nontumor liver tissue is associated with shorter disease-free survival in patients with hepatocellular carcinoma. *Clin Cancer Res* 5(12):4005–4012
- Ghosh M, Kawamoto T, Koike N, Fukao K, Yoshida S, Kashiwagi H, Kapoor VK, Agarwal S, Krishnani N, Uchida K, Miwa M, Todoroki T (2000) Cyclooxygenase expression in the gallbladder. *Int J Mol Med* 6(5):527–532
- Endo K, Yoon BI, Pairojkul C, Demetris AJ, Sirica AE (2002) ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology* 36(2):439–450
- Grossman EM, Longo WE, Panesar N, Mazuski JE, Kaminski DL (2000) The role of cyclooxygenase enzymes in the growth of human gall bladder cancer cells. *Carcinogenesis* 21(7):1403–1409
- Kim YW, Huh SH, Park YK, Yoon TY, Lee SM, Hong SH (2001) Expression of the c-erb-B2 and p53 protein in gallbladder carcinomas. *Oncol Rep* 8(5):1127–1132
- Kawamoto T, Krishnamurthy S, Tarco E, Trivedi S, Wistuba II, Li D, Roa I, Roa JC, Thomas MB (2007) HER receptor family: novel candidate for targeted therapy for gallbladder and extrahepatic bile duct cancer. *Gastrointest Cancer Res* 1(6):221–227
- Radaeva S, Ferreira-Gonzalez A, Sirica AE (1999) Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: a link between biliary intestinal metaplasia and mucin-producing cholangiocarcinoma. *Hepatology* 29(5):1453–1462
- Yeh CN, Maitra A, Lee KF, Jan YY, Chen MF (2004) Thioacetamide-induced intestinal-type cholangiocarcinoma in rat: an animal model recapitulating the multi-stage progression of human cholangiocarcinoma. *Carcinogenesis* 25(4):631–636

35. Leone F, Cavalloni G, Pignochino Y, Sarotto I, Ferraris R, Piacibello W, Venesio T, Capussotti L, Risio M, Aglietta M (2006) Somatic mutations of epidermal growth factor receptor in bile duct and gallbladder carcinoma. *Clin Cancer Res* 12(6):1680–1685
36. Kaufman M, Mehrotra B, Limaye S, White S, Fuchs A, Lebowicz Y, Nissel-Horowitz S, Thomas A (2008) EGFR expression in gallbladder carcinoma in North America. *Int J Med Sci* 5:285–291
37. Sasaki T, Hiroki K, Yamashita Y (2013) The role of epidermal growth factor receptor in cancer metastasis and microenvironment. *Biomed Res Int* 546318–546325
38. Ogo Y, Nio Y, Yano S, Toga T, Koike M, Hashimoto K, Itakura M, Maruyama R (2006) Immunohistochemical expression of HER-1 and HER-2 in extrahepatic biliary carcinoma. *Anticancer Res* 26(1B):763–770
39. Kiguchi K, DiGiovanni J (2009) Role of growth factors and signal transduction pathways in biliary tract cancer. In: Clifton Fuller M, Charles Thomas M (eds) *Biliary tract and gallbladder cancer: diagnosis and therapy*. Demos Medical Publishing
40. Aaronson SA (1991) Growth factors and cancer. *Science* 254:1146–1153
41. Schlessinger J, Ullrich A (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron* 9:389–391
42. Ullrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203–212
43. Dougall W, Qian X, Peterson N, Miller M, Samanta A, Greene M (1994) The neu-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene* 9:2109–2123
44. Gullick WJ (1991) Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. *Br Med Bull* 47:87–98
45. Wieduwilt MJ, Moasser MM (2008) The epidermal growth factor receptor family: biology driving targeted therapeutics. *Cell Mol Life Sci (CMLS)* 65(10):1566–1584
46. Yarden Y, Shilo BZ (2007) SnapShot: EGFR signaling pathway. *Cell* 131(5):1018
47. Schneider MR, Wolf E (2009) The epidermal growth factor receptor ligands at a glance. *J Cell Physiol* 218(3):460–466
48. Ohtsu H, Dempsey PJ, Eguchi S (2006) ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *Am J Physiol Cell Physiol* 291(1):C1–10
49. Berasain C, Nicou A, Garcia-Irigoyen O, Latasa MU, Urtasun R, Elizalde M, Salis F, Perugorria MJ, Prieto J, Recio JA, Corrales FJ, Avila MA (2012) Epidermal growth factor receptor signaling in hepatocellular carcinoma: inflammatory activation and a new intracellular regulatory mechanism. *Dig Dis* 30(5):524–531
50. Pastore S, Mascia F, Mariani V, Girolomoni G (2008) The epidermal growth factor receptor system in skin repair and inflammation. *J Invest Dermatol* 128(6):1365–1374
51. Farley SM, Purdy DE, Ryabinina OP, Schneider P, Magun BE, Jordanov MS (2008) Fas ligand-induced proinflammatory transcriptional responses in reconstructed human epidermis. Recruitment of the epidermal growth factor receptor and activation of MAP kinases. *J Biol Chem* 283(2):919–928
52. Sirica AE, Lai GH, Zhang Z (2001) Biliary cancer growth factor pathways, cyclo-oxygenase-2 and potential therapeutic strategies. *J Gastroenterol Hepatol* 16(4):363–372
53. Elmore LW, Sirica AE (1993) “Intestinal-type” of adenocarcinoma preferentially induced in right/caudate liver lobes of rats treated with furan. *Cancer Res* 53(2):254–259
54. Elmore LW, Sirica AE (1991) Phenotypic characterization of metaplastic intestinal glands and ductular hepatocytes in cholangiofibrotic lesions rapidly induced in the caudate liver lobe of rats treated with furan. *Cancer Res* 51(20):5752–5759
55. Thamavit W, Moore MA, Hiasa Y, Ito N (1988) Enhancement of DHPN induced hepatocellular, cholangiocellular and pancreatic carcinogenesis by *Opisthorchis viverrini* infestation in Syrian golden hamsters. *Carcinogenesis* 9(6):1095–1098
56. Kiguchi K, Ruffino L, Kawamoto T, Ajiki T, DiGiovanni J (2005) Chemopreventive and therapeutic efficacy of orally active tyrosine kinase inhibitors in a transgenic mouse model of gallbladder carcinoma. *Clin Cancer Res* 11(15):5572–5580
57. Kiguchi K, Ruffino L, Kawamoto T, Franco E, Kurakata S, Fujiwara K, Hanai M, Rumi M, DiGiovanni J (2007) Therapeutic effect of CS-706, a specific cyclooxygenase-2 inhibitor, on gallbladder carcinoma in BK5.ErbB-2 mice. *Mol Cancer Ther* 6(6):1709–1717
58. Wu Q, Kiguchi K, Kawamoto T, Ajiki T, Traag J, Carbajal S, Ruffino L, Thames H, Wistuba I, Thomas M, Vasquez KM, DiGiovanni J (2007) Therapeutic effect of rapamycin on gallbladder cancer in a transgenic mouse model. *Cancer Res* 67(8):3794–3800
59. Kitamura T, Connolly K, Ruffino L, Ajiki T, Lueckgen A, DiGiovanni J, Kiguchi K (2012) The therapeutic effect of histone deacetylase inhibitor PCI-24781 on gallbladder carcinoma in BK5.erbb2 mice. *J Hepatol* 57(1):84–91
60. Rusnak DW, Affleck K, Cockerill SG, Stubberfield C, Harris R, Page M, Smith KJ, Guntrip SB, Carter MC, Shaw RJ, Jowett A, Stables J, Topley P, Wood ER, Brignola PS, Kadwell SH, Reep BR, Mullin RJ, Alligood KJ, Keith BR, Crosby RM, Murray DM, Knight WB, Gilmer TM, Lackey K (2001) The characterization of novel, dual ErbB-2/EGFR, tyrosine kinase inhibitors: potential therapy for cancer. *Cancer Res* 61(19):7196–7203
61. Bernstein H, Bernstein C, Payne CM, Dvorakova K, Garewal H (2005) Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* 589(1):47–65
62. Raufman JP, Zimniak P, Bartoszko-Malik A (1998) Lithocholyltaurine interacts with cholinergic receptors on dispersed chief cells from guinea pig stomach. *Am J Physiol* 274(6 Pt 1):G997–1004
63. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science* 284(5418):1365–1368
64. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B (1999) Identification of a nuclear receptor for bile acids. *Science* 284(5418):1362–1365
65. Qiao L, Studer E, Leach K, McKinsty R, Gupta S, Decker R, Kukreja R, Valerie K, Nagarkatti P, El Deiry W, Molkentin J, Schmidt-Ullrich R, Fisher PB, Grant S, Hylemon PB, Dent P (2001) Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. *Mol Biol Cell* 12(9):2629–2645
66. Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P, Hylemon PB (2002) Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. *Hepatology* 35(2):307–314
67. Hylemon PB, Zhou H, Pandak WM, Ren S, Gil G, Dent P (2009) Bile acids as regulatory molecules. *J Lipid Res* 50(8):1509–1520
68. Mifune M, Ohtsu H, Suzuki H, Nakashima H, Brailoiu E, Dun NJ, Frank GD, Inagami T, Higashiyama S, Thomas WG, Eckhart AD, Dempsey PJ, Eguchi S (2005) G protein coupling and second messenger generation are indispensable for metalloprotease-dependent, heparin-binding epidermal growth factor shedding through angiotensin II type-1 receptor. *J Biol Chem* 280(28):26592–26599
69. Higashiyama S, Nanba D (2005) ADAM-mediated ectodomain shedding of HB-EGF in receptor cross-talk. *Biochim Biophys Acta* 1751(1):110–117

70. Kitamura T, Srivastava J, DiGiovanni J, Kiguchi K (2013) Bile Acid Accelerates ErbB2-Induced Pro-Tumorigenic Activities in Biliary Tract Cancer. *Mol Carcinog* (on line)
71. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K (2002) Identification of membrane-type receptor for bile acids (M-BAR). *Biochem Biophys Res Commun* 298(5):714–719
72. Dent P, Fang Y, Gupta S, Studer E, Mitchell C, Spiegel S, Hylemon PB (2005) Conjugated bile acids promote ERK1/2 and AKT activation via a pertussis toxin-sensitive mechanism in murine and human hepatocytes. *Hepatology* 42(6):1291–1299
73. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M (2003) A G protein-coupled receptor responsive to bile acids. *J Biol Chem* 278(11):9435–9440
74. Vassileva G, Golovko A, Markowitz L, Abbondanzo SJ, Zeng M, Yang S, Hoos L, Tetzloff G, Levitan D, Murgolo NJ, Keane K, Davis HR, Jr., Hedrick J, Gustafson EL (2006) Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation. *Biochem J* 398(3):423–430
75. Kiguchi K (2014) Molecular aspects of cholangiocarcinoma. *J Hepatobiliary Pancreat Sci* (In Press)



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