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Advances in Brief

Constitutive Expression of ErbB-2 in Gallbladder Epithelium Results in Development of Adenocarcinoma[1]

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Abstract

Overexpression of ErbB-2 in the basal layer of biliary tract epithelium led to the development of gallbladder adenocarcinoma in 100% of transgenic mice by 3 months of age. In addition, tumors developed in other parts of the biliary tree (*e.g.*, cholangiocarcinoma). Adenocarcinoma of the gallbladder appeared to arise via a stepwise process involving hyperplasia, adenoma formation, and then adenocarcinoma formation. Increased ErbB-2/epidermal growth factor receptor heterodimer formation, activation of mitogen-activated protein kinase, and up-regulation of cyclooxygenase-2 levels (mRNA and protein) were observed in gallbladder epithelium of these mice. These mice represent a unique new animal model for studying biliary tract carcinogenesis.

Introduction

The biliary tract transports, stores, and releases bile into the duodenum for digestion and consists of the gallbladder and bile ducts (including the cystic, intrahepatic, common, and pancreatico-biliary ducts). In humans, the incidence of BTC[4] has considerable geographic variations. High standardized mortality ratios of BTC are found in cancer registries for South American countries such as Chile, Peru, and Colombia and for Asian countries such as Japan and Thailand (1). Japan has one of the world's highest age-adjusted cancer death rates related to BTC, and it appears to be steadily increasing (5.7 and 11.5 in 1980 and 1998, respectively; Ref. 2). In the United States, ~7500 new cases of BTC are diagnosed per year, and ~5000 of those cases are diagnosed as gallbladder cancer (3). Certain predisposing factors have been linked to gallbladder cancer, such as chronic cholelithiasis, obesity, and the presence of an anatomically anomalous pancreatico-biliary junction (4). Genetic alterations in *p53* or K-*ras* may contribute to the development of certain types of gallbladder cancer (5–9). In addition, ErbB-2 overexpression has been reported in a significant percentage of gallbladder adenocarcinomas (10–12). Gallbladder carcinoma is a relatively aggressive and frequently lethal cancer. Unfortunately, many gallbladder carcinomas are in an advanced stage at the time of diagnosis, and metastases to the liver and regional lymph nodes are common. These factors contribute to the relatively poor prognosis for this cancer (5-year survival rate of <5%; Ref. 13). To improve the prognosis of patients with gallbladder carcinoma, more effective techniques for early diagnosis and a better understanding of the mechanisms involved in the development of gallbladder cancer are essential.

Spontaneous gallbladder cancer is rare in mice and rats. Only eight gallbladder tumors in seven B6C3F1 mice and one Swiss Webster mouse were reported in a database of nearly 80,000 mice (14). Six of these eight tumors were benign, and the two adenocarcinomas were of low-grade malignancy. There have been several reports of chemically induced neoplastic lesions of the mouse gallbladder. A low incidence of gallbladder adenocarcinomas can be induced by continuous administration of either *N,n*-propyl-*N*-formylhydrazine (15) or 2-acetamidofluorene (16) to mice. Although spontaneous gallbladder tumors are extremely rare in hamsters (17), neoplasia can be induced by chronic exposure to 3-methylcholanthrene via slow release capsules implanted in the gallbladder (18) or a combination of exposure to nitrosodimethylamine (in drinking water) and cholesterol pellets (implanted in gallbladder; Ref. 19). These two treatment protocols reportedly yield incidences of 61 and 68% of gallbladder carcinoma, respectively, at ~6 months after implantation (19). In contrast, Hoch-Legeti *et al.* (20) reported a high incidence of spontaneous gallbladder adenocarcinoma in two inbred strains of guinea pigs that was further increased by whole body radiation. With respect to the cholangiocarcinoma model, the furan rat model described by Sirica and co-workers (21, 22) gives rise to a very high incidence of cholangiocarcinoma in liver. In addition, in the Syrian golden hamster combined treatment with dihydroxy-di-*n*-propyl nitrosamine and liver fluke infestation was associated with enhancement of cholangiocarcinomas and preneoplastic lesions in the gallbladder (23).

Recently, our laboratory generated transgenic mice that overexpress wild-type rat *ErbB-2* under the control of the *bovine keratin 5* (*BK5*) promoter (24). This report describes the development and characteristics of biliary tract cancers in *BK5.ErbB-2* transgenic mice and provides evidence that ErbB-2 signaling plays a role in this process.

Materials and Methods

Generation and Identification of Transgenic Mice. *BK5.ErbB-2A* transgenic mice were generated as described previously (24). Transgenic animals were identified by PCR of DNA isolated from the tails of weanlings using oligonucleotides specific for the rabbit β-globin cDNA as described previously (24).

Histological Analysis. Biliary tract tissues were fixed in formalin and embedded in paraffin prior to sectioning. Sections of 7 μm were cut and stained with H&E. Serial sectioning (25 μm between each section) was also performed to analyze the pancreatico-biliary duct junction and the ampulla of Vater. Mice received i.p. injections of BrdUrd in PBS (100 μg/g body weight) 30 min before sacrifice.

Analysis of Transgene Expression. The expression and localization of the transgene as well as the expression of endogenous mouse ErbB-2 were determined using immunofluorescence on sections of bile duct cancers as described previously (24). Rabbit polyclonal antibody against the epitope corresponding to amino acids 1169–1186 mapping to the COOH terminus of the precursor

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[4] The abbreviations used are: BTC, biliary tract cancer; BrdUrd, bromodeoxyuridine; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3′-kinase; RT-PCR, reverse transcription-PCR; EGFR, epidermal growth factor receptor; COX, cyclooxygenase.

forms of human neu gp 185 (C-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as the primary antibody.

Immunohistochemical Staining for p53, CD31, and COX-2 Expression. The anti-p53 polyclonal antibody CM-1 (Signet Labs, Inc., Dedham, MA) was used for the immunohistochemical analysis of p53. Immunolocalization was performed by the streptavidin-biotin immunoperoxidase method, using formalin-fixed, paraffin-embedded sections. Frozen sections (10 μm) were used to determine CD31 expression. Sections were incubated with rat antimouse CD31 monoclonal antibody (BD PharMingen, San Diego, CA) overnight at 4°C. After three washes with BSA/PBS, the sections were incubated with the secondary FITC-conjugated, affinity-purified F(ab′)2 fragment of goat antirat IgG (Jackson Immuno Research Lab, West Grove, PA). The sections were analyzed using a Fluoroview Laser Confocal microscope (Olympus America, Melville, NY). The expression and localization of COX-2 was determined by goat antirabbit COX-2 polyclonal antibody (Cayman Chemical, Ann Arbor, MI) using the same method for the detection of ErbB-2.

Western Analysis. Whole cell lysates and the immunoprecipitates, which were prepared as described previously (24), were electrophoresed through 7–10% SDS/polyacrylamide gels, transferred to polyvinylidene difluoride membranes and analyzed using the following: anti-ErbB antibodies (Santa Cruz Biotechnology, Inc.); the anti-phosphotyrosine antibody PY20 (BD Transduction Laboratories, San Diego, CA); anti-MAPK (Erk1 and Erk2; Santa Cruz Biotechnology, Inc.); the phospho-MAPK antibody (Cell Signaling Technology, Beverly, MA); and the rabbit COX-2 antibody (Cayman Chemical, Ann Arbor, MI), as described previously (24). The relative differences between control and treated samples were quantitated by densitometry using a Visage 60 analyzer (BioImage, Millipore Corp., Bedford, MA).

Analysis of K-*ras* and *p53* Mutations. Genomic DNA was isolated from 15 gallbladder carcinomas from *Bk5.ErbB-2A* mice and used to amplify regions containing K-*ras* codons 12, 13, and 61 and *p53* exons 5–8 using the following primer sets: K-*ras* codons 12 and 13, sense 5′-GTAAGGCCTGCTGAAAATG-3′ and antisense 5′-GGGTCGTACTCATCCACAA-3′; K-*ras* codon 61, sense 5′-TTCTCAGGACTCCTACAGGAAACAA-3′ and antisense 5′-TTAAACCCACCTATAATGGTGAATA-3′; *p53* exon 5, sense 5′-ACACCTGATCGTTACTCGGCTTGTC-3′ and antisense 5′-GTCCTAACCCCACAGGCGGTGTTGA-3′; *p53* exon 6, sense 5′-CGGCTTCTGACTTATTCTTGCTCTT-3′ and antisense 5′-CTAGGCTGGAGTCAACTGTCTCTAA-3′; *p53* exon 7, sense 5′-CTGTAGTGAGGTAGGGAGCGACTTC-3′ and antisense 5′-GGCGGGACTCGTGGAACAGAAAC-3′; and *p53* exon 8, sense 5′-TACTGCCTTGTGCTGGTCCTTTTCT-3′ and antisense 5′-GTGACTTTGGGGTGAAGCTCAACAG-3′. The PCR products were sequenced for mutations in all three codons of the K-*ras* gene and four exons of the *p53* gene.

MAPK, PI3K, and src Kinase Assay. Approximately 0.25–2 mg of protein from whole lysates of gallbladder were immunoprecipitated with 4 μg of either anti-phosphospecific MAPK kinase rabbit polyclonal antibody or anti-PI3K antibody (Upstate Biotechnology, Lake Placid, NY) or 300 ng of anti-src monoclonal antibody (Oncogene, Boston, MA). Kits for these kinase assays were purchased from Upstate Biotechnology, and assays were performed according to the manufacturer's instructions.

RT-PCR Analysis. Upon removal, gallbladders were snap frozen, and total RNA was isolated using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. RT-PCR analysis for *COX-2* was performed as described by Shattuck-Brandt *et al.* (25). H-*ras* was used as an internal amplification control as described previously (26).

Results

Gross Appearance of the Biliary Tract in *BK5.ErbB-2A* Mice. We obtained three founders from the *BK5.ErbB-2* construct (Fig. 1*a*) as described previously (24). Homozygous mice from the line with the strongest phenotype, designated *BK5.ErbB-2A*, were used for this study. Necropsy of adult *BK5.ErbB-2A* mice revealed that the gallbladder was dramatically enlarged and had a white, opaque appearance (Fig. 1*b*). Enlarged gallbladders were often associated with a significantly dilated common bile duct (Fig. 1*c*). The hepatic duct from the liver and the cystic duct from the gallbladder unite to form the 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. 1*c*). The short common channel formed from the merging of the bile duct and the pancreatic duct was anatomically normal in most of the older transgenic and nontransgenic mice, as was the ampulla of Vater.

Histological Analysis of the Biliary Tract in *BK5.ErbB-2A* Mice. We evaluated 20 female and 32 male homozygous *BK5.ErbB-2A* mice ranging from 2 weeks to 8 months of age. Adenocarcinoma of the gallbladder was observed in 44 of 52 of these mice (Table 1). All of the 3–8-month-old mice presented with these tumors, which along with the papillary adenomas, were seen as early as 2–3 weeks of age. The incidence of adenocarcinomas of the adjacent biliary tract in mice $\geq$4 months of age was 100, 87, and 30% for the cystic duct, common bile duct, and intrahepatic biliary duct (cholangiocarcinoma), respectively (Table 1). The majority of the gallbladder tumors completely filled the lumen (Fig. 1, *e* and *g*). In younger mice ($\leq$2 months), foci of adenocarcinoma cells were detected within adenomas, and in older mice ($\geq$3 months), carcinoma cells predominated in all lesions. The tumors were characterized by branching structures with finger-like projections covered with high columnar epithelium and hyperchromatic nuclei (Fig. 1, *e* and *g*). Most of the tumors were diagnosed as well-differentiated adenocarcinomas. Carcinoma cells frequently invaded into the surrounding connective tissues (Fig. 1*g*). In addition, hypervascularization was a characteristic feature of these tumors. Staining with CD31, a marker for endothelial cells, revealed extensive vascularization in an adenocarcinoma from a *BK5.ErbB-2A* mouse (Fig. 2*b*). Adenocarcinomas exhibited a significantly elevated labeling index, as determined by staining with anti-BrdUrd antibody compared with normal gallbladder epithelium (Fig. 2, *c* and *d*).

Although gross examination did not reveal any significant abnormalities of the junction of the pancreatico-biliary duct or the ampulla of Vater in *BK5.ErbB-2A* mice, microscopic analysis of H&E-stained serial sections showed that tumor cells of the common bile duct had invaded into the pancreatic duct (Fig. 1*h*). In addition, the ampulla of Vater was dilated, and hyperplasia of the epithelium was observed in transgenic mice (data not shown). Pronounced congestion of bile, inflammation, necrosis, hyperplasia of biliary duct cells, and/or tumor development (cholangiocarcinoma) was also observed frequently in intrahepatic biliary ducts of transgenic mice (Fig. 2*e*). Hyperplasia of the intrahepatic biliary duct and cholangiocarcinoma were observed as early as 1 and 3 months of age, respectively.

Detection of Transgene Expression. The localization of transgene expression in the biliary tract was determined by indirect immunofluorescence staining. Persistent expression of the transgene was observed in the epithelium of both gallbladder (Fig. 2*g*) and intrahepatic biliary ducts (data not shown) of *BK5.ErbB-2A* mice. Transgene expression was also observed in the epithelial component of both gallbladder adenocarcinomas (Fig. 2*g*) and cholangiocarcinomas (Fig. 2*f*). Endogenous ErbB-2 expression was only weakly detectable in both the intrahepatic biliary duct (data not shown) and gall bladder from nontransgenic mice (Fig. 2*h*).

Status of ErbB Family Members in Gallbladder. Direct Western blot analyses of gallbladder tissue lysates showed that the level of ErbB-2 protein was significantly elevated in *BK5.ErbB-2A* mice compared with that of nontransgenic mice, as expected (Fig. 3*a*). Interestingly, the level of EGFR protein was also elevated in gallbladder tissue of transgenic mice (Fig. 3*a*). Quantitation showed elevations of epidermal growth factor (EGFR/ErbB-1) and ErbB-2 of 5.2 $\pm$ 0.6- and 5.6 $\pm$ 0.9-fold, respectively, after normalization of these levels from four separate experiments (3 mice/group for each experiment) to β-actin. The level of ErbB-3 protein was similar between nontransgenic and transgenic mice, and the level of ErbB-4 protein appeared to be decreased in the gallbladder of transgenic mice (Fig. 3*a*).

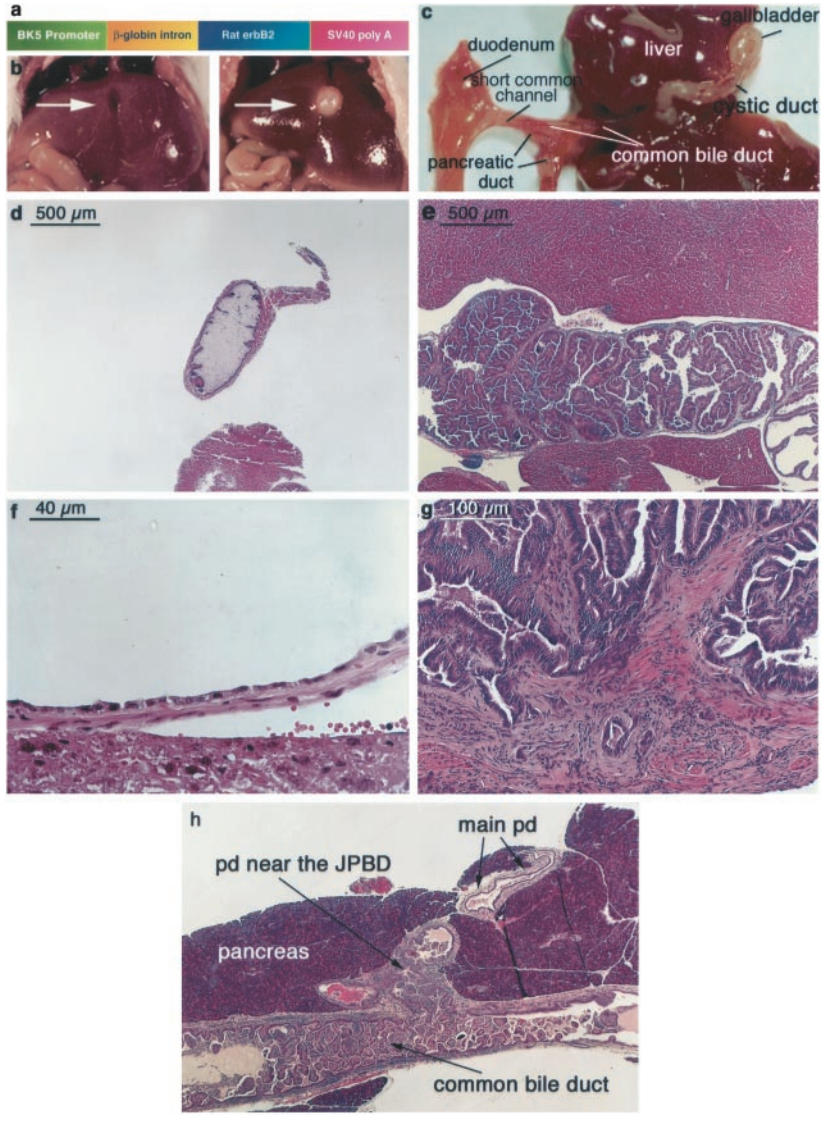

Fig. 1. Transgenic construct and comparison of the gallbladder from a *BK5.ErB-2A* transgenic mouse and a nontransgenic littermate. *a*, the DNA construct used to generate *BK5.ErbB-2A* mice. *b*, gallbladder of nontransgenic littermate (*left*) and *BK5.ErbB-2A* mouse (*right*) at 3 months of age. *c*, gross appearance of gallbladder and adjacent biliary tract of *BK5.ErbB-2A* mouse. H&E-stained sections of gallbladder in nontransgenic littermates (*d* and *f*) and *BK5.ErbB-2A* mice (*e* and *g*) at 0.5 months (*d* and *e*) and 3 months of age (*f* and *g*). *h*, H&E-stained section of the junction of the pancreatico-biliary duct (*JPBD*) in a 3-month-old *BK5.ErbB-2A* mouse.

Gallbladder tissue lysates were further analyzed by immunoprecipitation with antibodies to specific ErbB family members and then Western blotted with PY20 antibody to determine the phosphorylation status. Both ErbB-2 and EGFR (but not ErbB-3 or ErbB-4) were hyperphosphorylated on tyrosine residues in gallbladder tissue from *BK5.ErbB-2A* mice (Fig. 3*b*). There were approximately 4.8 ± 0.8- and 3.6 ± 0.6-fold increases in phosphotyrosine content for EGFR and ErbB-2, respectively, in gallbladder of transgenic mice *versus* nontransgenic mice after normalization of these activities from three separate experiments (2–3 mice/group for each experiment) for relative protein levels (Fig. 3*b*).

The increase in tyrosine phosphorylation of the EGFR and ErbB-2 suggested an increase in heterodimer formation between ErbB-2 and EGFR. This was confirmed by examination of the microsomal fraction of gallbladder. Immunoprecipitation of ErbB-2 followed by Western blot analysis for the EGFR revealed that coimmunoprecipitation of EGFR with ErbB-2 was significantly elevated (>20-fold) in transgenic mice (Fig. 3*c*).

Table 1 *Incidence of the pathological lesions in gallbladder and intrahepatic biliary duct in BK5.ErbB-2A transgenic mice*[a]

	Age (mo)				
Lesion	0.5	1	2	3	4–8
Gallbladder					
Adenoma	50% (2/4)	43% (3/7)	33% (2/6)	0% (0/12)	0% (0/23)
Adenocarcinoma	50% (2/4)	43% (3/7)	67% (4/6)	100% (12/12)	100% (23/23)
Liver					
Cholangiocarcinoma	0% (0/4)	0% (0/7)	0% (0/6)	25% (3/12)	30% (7/23)
Cystic duct adenocarcinoma	50% (2/4)	43% (3/7)	67% (4/6)	100% (12/12)	100% (23/23)
Common bile duct					
Adenocarcinoma	0% (0/4)	28% (2/7)	50% (3/6)	84% (10/12)	87% (20/23)

[a] Numbers in parentheses are positive cases of total examined for that age group.

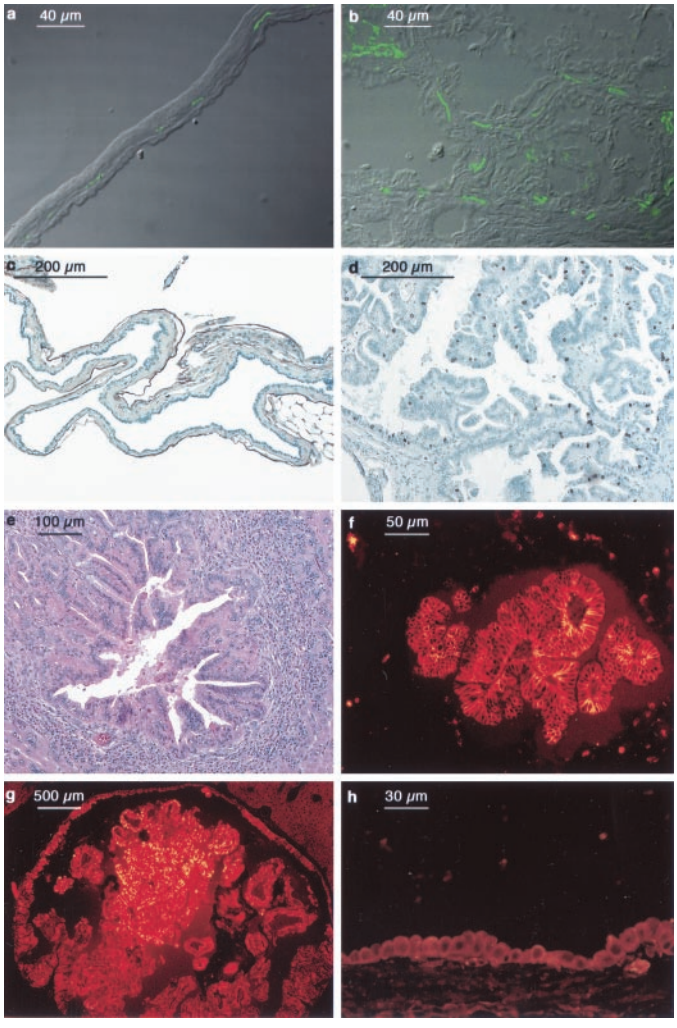

Fig. 2. Pattern of expression and localization of CD31 in gallbladder sections from a 3-month-old nontransgenic (*a*) and *BK5. ErbB-2A* (*b*) mouse. Note the hypervascularization with FITC-positive lesions for vascular endothelial cells in the gallbladder carcinoma of the transgenic mouse. BrdUrd-stained sections of gallbladder from a nontransgenic (*c*) and a transgenic (*d*) mouse are shown. Note the high level of BrdUrd-positive cells in the gallbladder carcinoma of the transgenic mouse. *e*, H&E-stained section of intrahepatic biliary lesion from a 3-month-old *BK5.erbB-2A* mouse. *f*, immunostaining for ErbB-2 in the same lesion. Immunostaining for ErbB-2 in gallbladder from a 3-month-old transgenic mouse (*g*) and a nontransgenic mouse of the same age (*h*).

Analysis of MAPK, src, and PI3K Activities. We analyzed the activity of MAPK, src kinase, and PI3K in gallbladder tissue lysates of *BK5.ErbB-2A* mice and nontransgenic littermates. MAPK activity was significantly elevated in tissue lysates of transgenic mice (1.6-fold; $P < 0.05$; Fig. 3*d*), and this increase in activity correlated with a higher level of phosphorylation of MAPK proteins (Fig. 3*d*). The protein levels of MAPK (Erk1 and Erk2) were similar in gallbladder lysates of transgenic mice and nontransgenic littermates (Fig. 3*d*). There was no difference in either src kinase or PI3K activity in gallbladder lysates of transgenic mice *versus* nontransgenic littermates.

Analysis of Mutations in K-*ras* or *p53*. Mutation analyses were conducted to determine whether genetic alterations were the underlying cause of tumor development in the gallbladder of *BK5.ErbB-2A* mice. Direct sequencing revealed no mutations in either K-*ras* (codons 12, 13, and 61) or *p53* (exons 5–8) in 16 gallbladder adenocarcinomas from *BK5.ErbB-2A* mice. Elevated expression of p53 protein was detected in only 1 of the 16 (8%) gallbladder adenocarcinomas examined (data not shown).

Analysis of COX-2 Expression. RT-PCR analysis using primers specific for murine COX-2 revealed elevated levels of mRNA expression in all five gallbladder RNA samples isolated from transgenic mice (Fig. 3*e*). Immunofluorescence analysis substantiated that COX-2 protein was also elevated in gallbladder epithelium in all of the *BK5.ErbB-2A* transgenic mice examined (8 of 8; Fig. 3*f*).

Discussion

During the course of our studies to explore the role of ErbB family members in epithelial tumorigenesis, we generated a transgenic mouse that overexpresses wild-type rat ErbB-2 protein under the control of the *BK5* promoter. It has been established that the *BK5* promoter directs transgene expression to the basal layer of many epithelia (27). Our results show for the first time that this promoter also directs expression of transgenes to the biliary tract epithelium. As a result of ErbB-2 overexpression in biliary tract epithelium, tumors arose at various sites. In particular, papillary adenocarcinoma of the gallbladder developed in all homozygous *BK5.ErbB-2A* mice between 3 and 8 months of age. Currently, an animal model for gallbladder cancer does not exist, and there is very little data on this type of cancer in experimental animals. Therefore, the availability of a relevant mouse model of gallbladder cancer would be useful for studying this devastating form of human cancer.

Although alterations in ErbB-2 signaling have been implicated in neoplastic transformation *in vitro* (28) and in neoplasia in both experimental animals (24) and humans [reviewed by Klapper *et al.* (29)], only a few studies have investigated the role of ErbB-2 in human gallbladder cancer (10–12). In one study, 30 of 43 cases (69.6%) and 14 of 43 cases (32.6%) of gallbladder adenocarcinoma had amplification of *ErbB-2* DNA or overexpression of ErbB-2 protein, respectively (10). In another study, 7 of 11 cases (63.6%) of gallbladder adenocarcinomas showed overexpression of ErbB-2 protein (11). Yukawa *et al.* (12) reported ErbB-2 protein expression in 9 of 13 cases (69%) of gallbladder cancer considered to be relatively early stage tumors (all 13 cases were histologically diagnosed as well-differentiated tubular adenocarcinoma), yet ErbB-2 protein expression was undetectable in tumors that were more advanced. With respect to cholangiocarcinoma, hyperphosphorylation of ErbB-2 has been demonstrated to be a characteristic feature of intrahepatic cholangiocarcinomas induced in the livers of furan-treated rats (30, 31). These data suggest that *ErbB-2* amplification and/or overexpression may be involved in the development of human gallbladder cancer and cholangiocarcinoma.

Western blot analyses demonstrated that all four ErbB family members (*i.e.*, EGFR, ErbB-2, ErbB-3, and ErbB-4) were expressed in gallbladder from both nontransgenic mice and *BK5.ErbB-2A* mice. Immunoprecipitation followed by Western blotting showed that *BK5.ErbB-2A* mice exhibited: (*a*) as expected, elevated ErbB-2 protein levels; (*b*) elevated EGFR protein levels; and (*c*) elevated phosphotyrosine levels of EGFR and ErbB-2 but not ErbB-3 or ErbB-4. Additional analyses confirmed elevated heterodimer formation between ErbB-2 and EGFR in gallbladder tissue of transgenic mice. Thus, gallbladder carcinoma in *BK5.ErbB-2A* mice may arise via elevated signaling through ErbB-2:ErbB-2 homodimers and/or ErbB-2:EGFR heterodimers.

Examination of downstream signaling pathways revealed no change in src activity or PI3K activity, but MAPK activity was elevated in gallbladder tissue isolated from *BK5.ErbB-2A* mice. These data suggest that MAPK signaling pathways may play a role in producing the gallbladder phenotype in these mice. It is worth noting that MAPK activity is also elevated in the epidermis of *BK5.ErbB-2A* mice, where spontaneous tumors also develop (24).

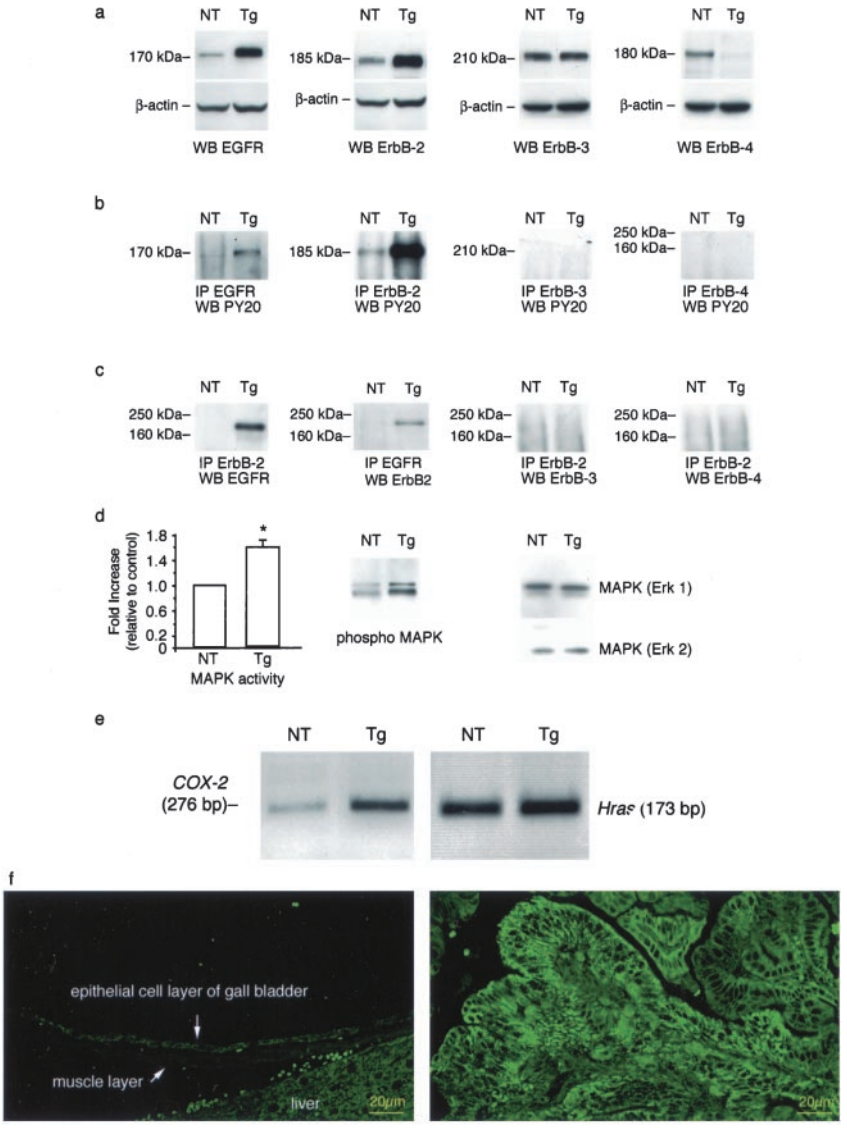

Fig. 3. Western blot (*WB*) analysis of ErbB family members and analysis of MAPK activity and expression of COX-2 mRNA and protein in gallbladder of nontransgenic (*NT*) mice and *BK5.ErbB-2A* transgenic (*Tg*) mice (3 months old). *a*, the whole cell lysate was analyzed by WB with antibodies to EGFR, ErbB-2, ErbB-3, and ErbB-4. Protein was normalized to β-actin. *b*, whole cell lysates were also immunoprecipitated (*IP*) with antibodies to these receptors, followed by WB analysis with the phosphotyrosine-specific antibody PY20. *c*, WB analysis of heterodimer formation of ErbB family members to ErbB-2 in the gallbladder of *BK5.ErbB-2A* transgenic mice. Lysates were immunoprecipitated with an antibody to one receptor and then analyzed by WB with an antibody to a different receptor. *d*, MAPK activity of gallbladder. Whole cell lysates of gallbladder were immunoprecipitated with polyclonal MAPK antibody. One-half of the immunoprecipitates were subjected to WB analysis for phospho-MAPK (*middle*) and MAPK (*Erk1* and *Erk2*; *right*), and the other half were analyzed for MAPK activity (*left*). There was a significant increase in MAPK activity in the transgenic mice ($P < 0.05$; Mann-Whitney *U* test). *e*, RT-PCR analysis of *COX-2* mRNA expression in gallbladder from nontransgenic (*NT*) and *BK5.ErbB-2A* (Tg) mice at 3 months of age. H-*ras* was used as an internal amplification control. *f*, immunostaining for COX-2 in gallbladder from nontransgenic (*left*) and *BK5.ErbB-2A* (*right*) mice.

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 (32). A recent study demonstrated a relationship between ErbB-2 overexpression and COX-2 deregulation in human colorectal cancer cells (33). In addition, evidence was presented supporting a role for ErbB-2:ErbB-3 heterodimers in the constitutive up-regulation of the COX-2 pathway (33, 34). Fig. 3 of the current study shows that the levels of COX-2 mRNA and protein were significantly elevated in gallbladder tissue and tumors from *BK5.ErbB-2A* transgenic mice. These observations implicate the involvement of COX-2 in the development of gallbladder tumors in *BK5.ErbB-2* mice. Furthermore, these data implicate signaling from ErbB-2:ErbB-2 and/or ErbB-2:EGFR dimers in the up-regulation of COX-2 in the gallbladder epithelium of *BK5.ErbB-2A* mice.

It has been proposed that there are two primary morphological pathways for development of gallbladder carcinoma in humans; one involves the sequential development of carcinoma from adenoma, and in the other, carcinomas develop via a process lacking preneoplastic conditions (*de novo* development; Ref. 5). All of the gallbladder adenocarcinomas seen in *BK5.ErbB-2A* mice were well-differentiated lesions, although some tumors in older animals showed more advanced features. Because hyperplasia preceded tumor formation and adenocarcinoma cells could be seen within adenomas, we suggest that tumor development in *BK5.ErbB-2A* mice may best represent an adenoma-carcinoma model of gallbladder cancer, although further work will be necessary to substantiate this hypothesis.

Several studies have reported *p53* mutations and protein overexpression in human gallbladder carcinomas (5–7). Hanada *et al.* (7) found that the incidence of *p53* mutations and protein expression was significantly less in the polypoid type (adenoma-carcinoma sequence) of gallbladder carcinoma compared with the flat type (*de novo* development). Mutations in codon 12 of K-*ras* are seen infrequently in gallbladder carcinoma, except in those cases where the carcinoma is associated with an anomalous junction of the pancreatico-biliary duct (5–9). We did not detect any mutations in either K-*ras* or *p53* in 16 gallbladder carcinomas, and p53 protein overexpression was detected in only one of the samples. The similarities between gallbladder cancer in *BK5.ErbB-2A* mice and human gallbladder cancer occurring via the adenoma-carcinoma sequence in terms of *p53* and K-*ras* alterations further support the relevance of this animal model.

In conclusion, overexpression of wild-type ErbB-2 in basal epithelial cells of gallbladder leads to the development of adenocarcinoma of the gallbladder and cystic duct in 100% of homozygous *BK5.ErbB-2A* transgenic mice and also the development of cholangiocarcinoma at an incidence of 30%. *BK5.ErbB-2A* transgenic mice

appear to represent a unique new animal model for further mechanistic studies involving the role of ErbB-2 in the development and growth of BTCs, as well as a promising tool for the development of new treatment and/or prevention modalities.

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