Role of ErbB family receptor tyrosine kinases in intrahepatic cholangiocarcinoma

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Abstract

Aberrant expression and signaling of epidermal growth factor receptor (ErbB) family receptor tyrosine kinases, most notably that of ErbB2 and ErbB1, have been implicated in the molecular pathogenesis of intrahepatic cholangiocarcinoma. Constitutive overexpression of ErbB2 and/or ErbB1 in malignant cholangiocytes has raised interest in the possibility that agents which selectively target these receptors could potentially be effective in cholangiocarcinoma therapy. However, current experience with such ErbB-directed therapies have at best produced only modest responses in patients with biliary tract cancers. This review provides a comprehensive and critical analysis of both preclinical and clinical studies aimed at assessing the role of altered ErbB2 and/or ErbB1 expression, genetic modifications, and dysregulated signaling on cholangiocarcinoma development and progression. Specific limitations in experimental approaches that have been used to assess human cholangiocarcinoma specimens for ErbB2 and/or ErbB1 overexpression and gene amplification are discussed. In addition, current rodent models of intrahepatic cholangiocarcinogenesis associated with constitutive ErbB2 overexpression are reviewed. Select interactive relationships between ErbB2 or ErbB1 with other relevant molecular signaling pathways associated with intrahepatic cholangiocarcinoma development and progression are also detailed, including those linking ErbB receptors to bile acid, cyclooxygenase-2,

interleukin-6/gp130, transmembrane mucins, hepatocyte growth factor/Met, and vascular endothelial growth factor signaling. Lastly, various factors that can limit therapeutic efficacy of ErbB-targeted agents against cholangiocarcinoma are considered.

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INTRODUCTION

Intrahepatic cholangiocarcinoma, also known as peripheral cholangiocarcinoma, is a primary epithelial cancer that arises within liver and which exhibits differentiation markers of biliary epithelial cells or cholangiocytes^[1-3]. This rare, but highly malignant hepatobiliary cancer accounts for approximately 10%-15% of all primary liver cancer^[3,4]. More than 90% of intrahepatic cholangiocarcinomas are classified histologically as well-to-moderately differentiated tubular adenocarcinomas, although other rare histological variants, including papillary, adenosquamous, and intestinal-type carcinoma also occur^[1,2,5-7]. Typically, a desmoplastic reaction of variable degrees is a common histological feature and in some cases may be the most prominent characteristic of the tumor^[1,2].

Morphologically, intrahepatic cholangiocarcinomas have been further classified as either having a mass-forming, periductular infiltrating, or intraductal growth pattern^[1,2,8-10]. Of these morphological types, the intraductal growing cholangiocarcinoma is the least common, but has a more favorable prognosis than either

the mass-forming or periductular infiltrating types. Some tumors may also manifest a combination of growth patterns (i.e. mass-forming and periductular infiltrating), thereby precluding an absolute morphology-based classification system based solely on a single type of growth pattern.

Intrahepatic cholangiocarcinoma typically carries a very poor prognosis and the challenges posed by this cancer are formidable. Most notably, early diagnosis of intrahepatic cholangiocarcinoma is problematic, with a vast majority of patients being diagnosed at first presentation with advanced malignant disease. Thus, treatment options are limited and prospects for long-term survival are for the most part dismal. Current epidemiological data have further indicated a global increase over the past two to three decades in the age-adjusted incidence and mortality rates of intrahepatic cholangiocarcinoma^[4,11-14], whereas the age-adjusted incidence and mortality rates for extrahepatic cholangiocarcinoma have been reported to be declining over a comparable time period^[4,12]. It is of further interest that the most significant rise in intrahepatic cholangiocarcinoma incidence was noted among the older (≥ 65 years of age) rather than younger age groups analyzed^[4,12].

It has recently been reported that only about 10% of patients with intrahepatic cholangiocarcinoma have been found to have a known established risk factor, such as primary sclerosing cholangitis, hepatolithiasis, infestation with the liver flukes *Opisthorchis viverrini* or *Clonorchis sinensis*, and choledochal cysts^[15]. Thus, a vast majority of patients presenting with intrahepatic cholangiocarcinoma do not have a history of these well-recognized risk factors and the cause for the rising incidence, particularly among older age groups, of this often fatal hepatobiliary malignancy remains unclear. However, in addition to the more well-established risk factors listed above, a number of chronic liver diseases, including alcoholic liver disease, hepatitis C and B, human immunodeficiency virus infection, unspecified cirrhosis, and diabetes have also been recently reported to be associated with the development of intrahepatic cholangiocarcinoma^[12-14,16,17].

Common features of the few well-established risk factors and the more recently analyzed pre-existing chronic liver conditions seemingly predisposing for intrahepatic cholangiocarcinoma include chronic inflammation and bile duct cell injury often combined with cholestasis and altered bile composition. Molecular perturbations brought about by the milieu of cholangitis and cholestasis have been linked to the initiation, promotion and/or progression stages of cholangiocarcinogenesis^[1,18-20]. Among the pathways affected and demonstrated to be playing a role in the molecular pathogenesis of intrahepatic cholangiocarcinogenesis are those mediated by the ErbB family of receptor tyrosine kinases, most notably involving the dysregulation of ErbB2 (HER2/neu) and/or epidermal growth factor receptor (EGFR) signaling. This review will critically evaluate the role

played by the ErbB family receptor tyrosine kinases in the development and progression of intrahepatic cholangiocarcinoma. Specifically, the significance of aberrant ErbB2 and EGFR expression and genetic alterations in relation to the pathogenesis of human intrahepatic cholangiocarcinoma will be assessed. Experimental models linking constitutive overexpression of activated ErbB2 to intrahepatic cholangiocarcinoma development will also be described. In addition, relevant interactive relationships between ErbB2, as well as EGFR, with other key molecular pathways associated with intrahepatic cholangiocarcinoma development and/or progression and the effects of bile acids on ErbB receptor signaling in cholangiocarcinoma cells will be discussed. Lastly, the potential value of EGFR and/or ErbB2 as molecular targets in intrahepatic cholangiocarcinoma therapy will be assessed.

THE ERBB FAMILY OF RECEPTOR TYROSINE KINASES AND THEIR SPECIFIC LIGANDS

The ErbB family of class I receptor tyrosine kinases is comprised of four distinct receptors: EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. Each of these plasma membrane receptors, in turn, is composed of an extracellular ligand-binding domain, a transmembrane lipophilic domain, and a conserved cytoplasmic tyrosine kinase domain^[21-24]. All of these receptors, with the exception of ErbB2, bind receptor specific ligands belonging to the EGF-family of growth factors. These EGF-related growth factors have been divided into three groups^[21,23]. The first group, which includes epidermal growth factor (EGF), transforming growth factor- α (TGF- α), and amphiregulin, binds specifically to EGFR. A second group, which shows dual specificity by binding to both EGFR and ErbB4, includes heparin-binding EGF, epiregulin, and betacellulin. Neuregulins (NRGs), compose the third group, with NRG1 and NRG2 (heregulins) being ligands for ErbB3 and ErbB4, and NRG3 and NRG4 binding only to ErbB4^[21,23,25]. EGF-family growth factors are produced in cells as transmembrane precursors, which can then be shed as soluble active ligands through proteolysis catalyzed by cell surface acting proteases, most notably through the activity of matrix metalloproteinases (MMPs)^[22,26,27]. This mechanism of cell surface growth factor shedding plays an important role in regulating ligand availability and receptor activation^[22,27].

The binding of EGF-family ligands to the extracellular domain of ErbB receptors induces homo- or heterodimerization of the receptor proteins and activation of the intrinsic tyrosine kinase domain, resulting in the trans-phosphorylation of specific tyrosine residues within the receptor's cytoplasmic tail^[21-23]. The phosphorylated residues then serve as docking or recruitment sites for a variety of signaling proteins^[22,24,28,29], which in turn, initiate downstream signaling cascades and other molecular activities that

Mechanisms of ErbB family receptor activation and main downstream signaling pathways

Figure 1 Simplified scheme depicting ErbB receptor tyrosine kinase activation and downstream signaling pathways relevant to intrahepatic cholangiocarcinogenesis.

regulate such fundamental biological responses as cell differentiation, cell proliferation, cell survival, cell migration, and angiogenesis (Figure 1). The type and amplitude of the downstream signaling pathways that are activated and their resulting biological effects are governed by the complexity and diversity of the ErbB network regulating these responses, and as such, are dictated in large part by which ErbB receptors are being expressed, the number of receptors being expressed and their dimerization and tyrosine phosphorylation profiles, the type and concentration of stimulating ligand, receptor stability at the cell surface, and the potential for receptor transactivation and/or cross-talk with other receptor-mediated signaling pathways^[22,23,30-33].

ErbB2, because of the unique conformation of its ectodomain compared with the ectodomains of EGFR and ErbB3, is the only member of the ErbB receptor family that does not allow for the binding of any of the known soluble EGF-like ligands^[21-23]. The crystal structure of a truncated ErbB2 ectodomain has further revealed a conformation that resembles that of an activated state poised to interact with other ErbB receptors^[21,34]. This unique structure helps to explain why ErbB2 has an enhanced capacity for heterodimerization and is the preferred dimerization partner for all other ErbB receptors^[23]. Moreover, by having a fixed conformation that resembles the ligand-activated state, it is not surprising that ErbB2 exhibits a constitutively high basal kinase activity and is closely linked to human oncogenesis^[23,35]. It is also relevant that ErbB2 overexpression can result in ligand-independent homodimerization and that EGFR levels may be increased in tumors induced in association with the

constitutive overexpression of ErbB2^[23,36].

ErbB2-containing heterodimers (i.e. ErbB2/EGFR or ErbB2/ErbB3) are associated with a more robust signaling than that generated by homodimers^[23]. In this context, it is notable that ErbB2/ErbB3 heterodimers are most potent in terms of stimulating cell mitogenesis and neoplastic transformation^[21,23]. The dominant signaling capacity of ErbB2/ErbB3 heterodimers appears to be paradoxical since ErbB2 is a ligandless receptor for EGF family growth factors, while ErbB3 has an impaired kinase activity^[21]. However, an allosteric mechanism for the activation of the kinase domain of ErbB3 by its interaction with ErbB2 has been predicted^[24]. Furthermore, the potent signaling exhibited by ErbB2/ErbB3 heterodimers relates to their capacity to strongly activate both the *ras-raf*-MEK-p42/44 mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)-Akt pathways^[23]. Activation of the *ras-raf*-MEK-MAPK (ERK) pathway involves the recruitment of select adaptor proteins (Grb-2 or Shc) to the receptor, and is a key pathway for driving cellular proliferation^[22,23]. The PI3K-Akt pathway, which regulates cell survival and anti-apoptotic signals is activated by recruitment of the p85 adaptor subunit of PI3K to the receptor^[22,23]. Specifically, ErbB3 (and ErbB4) activates PI3K through select p85 docking sites on the cytoplasmic tyrosine kinase domain. Additional factors contributing to potent signaling capacity of the ErbB2/ErbB3 heterodimers is their increased stability at the cell surface and their ability to evade downregulation mechanisms, thereby leading to prolonged signaling^[23]. ErbB2 also acts to decrease the rate of ligand dissociation from ErbB2-containing

Figure 2 ErbB2 expression in human intrahepatic cholangiocarcinoma. A: Differences in overall reported frequencies of archival human intrahepatic cholangiocarcinoma specimens scored as being immunohistochemically-positive for ErbB2 expression using non-uniform experimental methods and criteria; B: Range of reported frequencies of archival human intrahepatic cholangiocarcinomas scored as being moderately-to-strongly positive (+2 to +3) for ErbB2 immunoreactivity following antigen unmasking by heating of tissue specimens in citric acid buffer, pH 6.0. Number in () = number of cases analyzed; number in [] = reference number; In A, ¹refers to Japanese cases analyzed; ²refers to Thai cases analyzed.

heterodimeric complexes^[23].

In addition to activating the intracellular *ras-raf*-MEK-MAPK and PI3K-Akt cascades, signaling through ErbB family receptors also activates other key cell regulatory molecules affecting cell differentiation, cell cycle progression, and malignant transformation and/or progression, including transcription factors such as *c-myc*, *c-Jun*, *c-fos*, Ets family members, signal transduction and activator of transcription (STAT) proteins, and nuclear factor- κ B (NF- κ B)^[21-23,37-39]. Cyclin D1 is an important cell cycle regulator downstream of ErbB receptor signaling, which promotes G1/S phase cell cycle progression^[23,30]. Moreover, preferential binding

of phospholipase C γ to the tyrosine kinase domain of the EGFR receptor mediates the generation of lipid second messengers diacylglycerol and inositol 1, 4, 5-triphosphate^[21].

ErbB2 and/or EGFR together with EGF-like peptides are frequently found to be overexpressed in various epithelial cancers of both the human and experimental animal models. Mechanisms associated with the aberrant constitutive activation of ErbB2 or EGFR in malignant tumors include not only those associated with a persistent paracrine or autocrine production of growth factor ligands within the tumor, but also may occur as a result of receptor gene amplification and/or transcription-mediated protein overexpression, or by mutational activation. As ErbB2, and to a lesser extent EGFR, have been the most studied of the ErbB family receptor members in intrahepatic cholangiocarcinoma, much of the remainder of this review will focus on establishing their relevance to the molecular pathogenesis of intrahepatic cholangiocarcinoma and their potential as molecular targets for therapy against this lethal cancer.

ABERRANT ERBB2 EXPRESSION IN HUMAN INTRAHEPATIC CHOLANGIOCARCINOMA

The human ErbB2 receptor tyrosine kinase is a 185 kDa transmembrane glycoprotein encoded by the *c-erbB-2* proto-oncogene localized to chromosome 17q. Since 1989, several independent studies have been published describing the results of immunohistochemical analyses of ErbB2 oncoprotein expression in cancerous epithelium of human intrahepatic cholangiocarcinomas relative to that of normal bile ducts in adult liver^[40-52]. In each of these studies, ErbB2 immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue samples from human intrahepatic cholangiocarcinomas of either Eastern and/or Western origin, which were mostly obtained from surgical files, but in some studies, also included specimens from autopsy files^[44,48]. ErbB2 immunostaining was either not detected or only weakly detected in normal intrahepatic bile duct epithelium in adult and in fetal human livers^[40,43,44,47,49]. On the other hand, reported incidences of intrahepatic cholangiocarcinoma cases overexpressing immunoreactive ErbB2 in their cancerous epithelium were found to vary considerably. Figure 2A highlights differences in the published frequencies (%) of analyzed cases of human intrahepatic cholangiocarcinomas that showed positive 1+ (weak) to $\geq 2+$ (moderate to strong) immunoreactivity for ErbB2. The obvious disparities among these published values are likely due, at least in part, to one or more of the following possibilities: (1) differences in tissue processing procedures used to optimize antigen availability, (2) use of different ErbB2 antibody preparations, (3) variations in the criteria or methods used to score and quantify positive cases of ErbB2 overexpression, and (4) in some studies, having too small of a sampling size of

intrahepatic cholangiocarcinoma specimens in the study to be meaningful^[41,42].

Notable differences among the experimental approaches used in these various independent studies included the fact that antigen retrieval procedures involving heating tissue specimens in citrate buffer, pH 6.0, were employed in only some^[44,46-50,52], but not all of the published studies. Moreover, in some of these studies, mouse monoclonal ErbB2 antibody preparations were used^[41-46,48,49] whereas others were performed with rabbit polyclonal anti-ErbB2 antibodies^[40,47,50-52]. Furthermore, while in some studies no distinctions were made between plasma membrane and cytoplasmic immunostaining when scoring tumors ostensibly positive for ErbB2 overexpression^[40,47], others more appropriately relied largely on evidence of positive membranous immunostaining when classifying positive cases^[43,44,48-52]. Also, the methods used in these studies to assess human intrahepatic cholangiocarcinomas for ErbB2 overexpression have been for the most part semi-quantitative, with no standard or uniform guidelines having been adopted to date for quantifying or validating ErbB2 overexpression in human cholangiocarcinogenesis. In this context, the results shown in Figure 2B are somewhat revealing, since they demonstrate that a greater degree of conformity among the reported frequency of expression values shown in Figure 2A can be achieved when the data are reevaluated in terms of (1) being derived only from those studies in which antigen unmasking was accomplished through heating of the tissue specimens in citric acid buffer, pH 6.0, and (2) when only those tumors scored as being moderately-to-strongly-positive ($\geq 2+$) in their immunostaining reactions are used in calculating the overall frequencies of ErbB2-overexpressing tumors. It also follows that before further progress can be made in using immunohistochemistry to reliably and reproducibly assess cases of human intrahepatic cholangiocarcinoma for ErbB2 overexpression, there is a real need to establish rigorous clinical practice guidelines specific for this cancer, as were recently recommended by the American Society of Clinical Oncology and the College of American Pathologists for ErbB2 testing and validation in human breast cancer^[53]. Less subjective and more analytical methods, such as the use of computer-assisted image analysis for quantifying immunostaining reactions are also needed to permit more objective measurements of ErbB2 immunoreactivity (as well as that of other marker proteins) in fixed tissue sections from human intrahepatic cholangiocarcinomas and related risk conditions. In this regard, utilizing microdensitometry measurements of immunostaining reactions, Endo *et al*^[49] had demonstrated a strong positive correlation between levels of plasma membrane ErbB2 immunoreactivity and that of cytoplasmic cyclooxygenase-2 (COX-2) in human intrahepatic cholangiocarcinogenesis. The data generated from this quantitative immunohistochemical study are consistent with a growing body of experimental evidence that supports a strong positive relationship between ErbB2

receptor expression and signaling and COX-2 protein up-regulation and increased activity in the pathogenesis of intrahepatic cholangiocarcinoma (see below).

Not surprisingly, and likely for the same reasons described above for ErbB2, comparable immunohistochemical studies aimed at assessing EGFR overexpression in archival specimens of human intrahepatic cholangiocarcinomas from surgical files also yielded disparate results. For example, in five different published studies, the frequencies of intrahepatic cholangiocarcinomas exhibiting 2+ to 3+ immunostaining for EGFR were reported to be 10.7%^[51], 21.1%^[46], 21.6%^[54], 47%^[55], and 81%^[50], respectively. In the positive tumors, EGFR immunostaining, with varying degrees of heterogeneity, was largely localized to the plasma membranes of the cancerous epithelial cells. Ito *et al*^[46] further observed that 39.5% of their analyzed cases ($n = 38$) of human intrahepatic cholangiocarcinoma showed 2+-positive cytoplasmic immunostaining for ErbB3, with 10.5% of the total tumors analyzed also found to be 2+-positive for cytoplasmic ErbB4 immunostaining. In addition, these investigators reported that 21.1% of the cases of intrahepatic cholangiocarcinomas included in their study exhibited positive immunostaining for all four ErbB family members, while 36.8% of the tumors analyzed were found to exhibit positive immunostaining reactions for three of the four type I receptors. However, the purely descriptive nature of this study makes it impossible to predict specific receptor dimerization preferences that may be operative in these analyzed tumors.

While uncommon, activating mutations in the tyrosine kinase domain of ErbB2 have been recently described for a subset of lung cancer patients^[56]. Likewise, a novel H878Y missense mutation in the tyrosine kinase domain of ErbB2 has also been recently demonstrated in 11% (2/18) of examined cases of human hepatocellular carcinoma^[57]. However, in this same study, none of 22 analyzed cases of biliary cancer were found to harbor the *erbB2* H878Y mutation. All hepatocellular carcinoma and biliary cancer samples analyzed in this particular study were further observed to be negative for gain-of-function somatic mutations affecting the catalytic domain of the EGFR gene. In contrast, Leone *et al*^[58] recently described somatic mutations in the tyrosine kinase domain of EGFR in a subgroup of patients with either cholangiocarcinoma or gallbladder carcinoma. Gwak *et al*^[59] also recently reported that 13.6% (3/22) of examined cases of human cholangiocarcinoma exhibited EGFR mutations in the kinase domain. While still limited in scope, these collective findings suggest that mutations in the tyrosine kinase domain may be playing a role (albeit limited) in sustaining the activation of ErbB receptors, most particularly EGFR, in a subset of human biliary tract cancers, including some intrahepatic cholangiocarcinomas. Thus, identifying such activating mutations could conceivably contribute towards predicting a positive response of a few select biliary tract

Figure 3 Representative photomicrographs demonstrating *c-erbB2* gene amplification and a corresponding strong positive immunoreactivity for activated ErbB2 oncoprotein in the neoplastic cholangiocytes of a human intrahepatic cholangiocarcinoma. A and B: Sections of two different intrahepatic cholangiocarcinomas with neoplastic cholangiocytes exhibiting FISH nuclear signals for *c-erbB2* (orange dots) and for CEP17 (centromeric region of chromosome 17; green dots). Orange/green values indicate *c-erbB2*/CEP17 signal ratio for each cholangiocarcinoma. An orange/green ratio of > 2.0 = a tumor with *c-erbB2* amplification (B), whereas a ratio value at or approximating 1.0 = a tumor without *c-erbB2* amplification (A). The neoplastic cholangiocytes in the intrahepatic cholangiocarcinomas depicted in A (immunohistochemical staining not shown) and in B (with corresponding immunohistochemical staining shown in C) were each found to exhibit strong positive immunoreactivity at the plasma membrane for activated ErbB2 oncoprotein, using an antibody directed against phosphotyrosine 1248 of ErbB2. Tyrosine 1248 is the main autophosphorylation site in the carboxy terminal domain of ErbB2 linked to downstream signaling through the *ras-raf*-MEK-p42/44 MAPK signal transduction pathway. Thus, activation of ErbB2 via autophosphorylation at tyrosine 1248 in neoplastic cholangiocytes of intrahepatic cholangiocarcinomas can be demonstrated either in the presence or absence of *c-erbB2* amplification. (A, B $\times 330$, C $\times 132$).

cancers to ErbB targeted therapies (see below).

Presently, there have only been limited efforts to investigate *c-erbB2* gene amplification in human intrahepatic cholangiocarcinomas. Moreover, as has been

the case with immunohistochemistry, current studies that utilized either fluorescence *in situ* hybridization (FISH), exemplified in Figure 3, or chromogenic *in situ* hybridization (CISH) analysis to evaluate *c-erbB2* amplification in cases of human cholangiocarcinoma have also produced variable and conflicting results. Shiraishi *et al*^[60], employing two-color FISH using probes specific for *c-erbB2* and chromosome 17 centromere, did not detect evidence of *c-erbB2* amplification in six analyzed cases of human biliary tract cancers, including two peripheral and three hilar cholangiocarcinomas that showed a gain in 17q. In contrast, Ukita *et al*^[48] employing FISH to evaluate gene amplification using only a *c-erbB2* probe reported increased *c-erbB2* signals in all 22 of their analyzed cases of intrahepatic cholangiocarcinoma. In this study, putative *c-erbB2* amplification was detected in the form of cluster signals in most of the tumors. On the other hand, there was a notable discordance between the immunohistochemical findings for ErbB2 overexpression and the FISH data for *c-erbB2* amplification. Furthermore, since Ukita *et al*^[48] did not assess for changes in chromosome 17 copy number, it is not possible to determine from their results the extent to which aneuploidy or chromosome 17 polysomy alone may have contributed to their findings. More recently, Altamari *et al*^[50] using CISH detected putative *c-erbB2* amplification in 4% of cases of human intrahepatic cholangiocarcinoma that were also found to be moderately to strongly immunoreactive for ErbB2 oncoprotein. However, this study also did not include an internal control for chromosome 17 copy number. On the other hand, Nakazawa *et al*^[51] using two color FISH with probes for *c-erbB2* and chromosome 17 centromere reported no evidence of *c-erbB2* amplification in 28 analyzed cases of human intrahepatic cholangiocarcinomas. Contrary to the findings of others (see Figure 2) Nakazawa *et al*^[51] also reported that none of their analyzed cases of intrahepatic cholangiocarcinoma were immunohistochemically positive for ErbB2 overexpression.

The limited scope of these reported findings together with the fact that experimental variations and non-standardized criteria were used in assessing *c-erbB2* amplification in human intrahepatic cholangiocarcinoma clearly indicates the need for further studies in order to more definitively assess the extent to which gene amplification may be contributing to ErbB2 overexpression in these tumors. Universal and reliable standards need to be adopted for assessing *c-erbB2* amplification in biliary tract malignancies, including intrahepatic cholangiocarcinoma, leading to a more rigorously controlled assessment of *c-erbB2* gene amplification in relation to protein overexpression. It is also important to be able to more definitively assess if *c-erbB2* amplification might provide a more reliable and less variable indicator than immunohistochemistry for predicting favorable therapeutic responses of patients with intrahepatic cholangiocarcinomas to target-based treatments with ErbB2 inhibitors.

Another important deficiency in our current

understanding of ErbB2 and EGFR overexpression in human intrahepatic cholangiocarcinomas is the noticeable lack of mRNA data. *In situ* hybridization (ISH) has been used to investigate *c-erbB2* mRNA expression in the cancerous epithelium of archival formalin-fixed, paraffin-embedded specimens of human cholangiocarcinoma^[48], but the staining pattern that was observed was seen to be diffusely intranuclear and to a much lesser extent cytoplasmic, suggesting some possibility of artifact. Regardless, these reported ISH results are descriptive and not quantitative, and have not been validated by more definitive analytical methods, such as real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR). Utilizing laser microbeam microdissection and cDNA microarray analysis, Obama *et al*^[61] demonstrated a more than five-fold increase in *c-erbB2* expression in 3 (14%) and more than a two-fold increase in 6 (28%) of 21 cases of human intrahepatic cholangiocarcinomas analyzed. However, it was not evident if the microarray data for *c-erbB2* was validated by real-time RT-PCR. Here it may also be relevant that Endo *et al*^[49], utilizing computer-assisted image analysis to evaluate plasma membrane ErbB2 immunostaining, have reported strongly-positive immunoreactivity for plasma membrane ErbB2 to be most prominent in the cancerous epithelium of well differentiated intrahepatic cholangiocarcinomas, whereas most of the tumors analyzed by Obama *et al*^[61] were classified as being moderately to poorly differentiated. Settakorn *et al*^[52], on the other hand, observed a low level of ErbB2 immunoreactivity in all ($n = 9$) of their analyzed archival cases of low grade human intrahepatic cholangiocarcinoma and strongly-positive ErbB2 immunoreactivity in 10 of 22 cases of the higher grade tumors. The fact that different commercial antibodies (monoclonal *versus* polyclonal) were used by Endo *et al*^[49] and Settakorn *et al*^[52], respectively, may account, at least in part, for the discrepancies in their reported immunohistochemical findings. Nevertheless, it is evident from the limited and conflicting results described above that further studies are needed to more clearly define the association between tumor grade and *c-erbB2* mRNA and protein expression in human intrahepatic cholangiocarcinoma.

A number of immunohistochemical studies have demonstrated increased expression of ErbB2 oncoprotein in variable percentages of noncancerous biliary proliferative disorders associated with human cholangiocarcinogenesis, including hepatolithiasis and PSC^[44,47,49]. These findings suggest that ErbB2 overexpression associated with risk conditions characterized by cholangitis and partial biliary obstruction may represent a relatively early event linked to human intrahepatic cholangiocarcinogenesis. Interestingly, Su *et al*^[62] also measured a higher mean level of ErbB2 oncoprotein in bile collected from patients with cholangiocarcinoma than in bile collected from patients with biliary tract infection, biliary stone disease, or normal controls. This study further supports ErbB2 as playing a role in human cholangiocarcinogenesis

and suggests that elevated ErbB2 in bile might have some use in identifying individuals with high risk for developing biliary tract cancers. But again, these findings are limited in scope and need to be significantly expanded and substantiated before ErbB2 in bile can be accepted as a possible diagnostic test for predicting cholangiocarcinoma risk. Moreover, in none of the studies indicated in Figure 2, was the activation status (tyrosine phosphorylation) of the ErbB2 or EGFR receptors determined. In this regard, there is a further need to rigorously establish the tyrosine phosphorylation status of ErbB2 expressed in cholangiocarcinoma cells (i.e. see Figure 3C) compared with non-malignant cholangiocytes in liver, together with that of other ErbB receptor family members. Moreover, this should be done in conjunction with determining associated changes in receptor mRNA and protein expression levels. Such a comprehensive analysis of ErbB receptor family expression and activation would be more informative than that reflected in currently published studies, particularly when viewed in terms of contributing towards devising and tailoring specific strategies aimed at targeting ErbB signaling pathways for intrahepatic cholangiocarcinoma therapy.

ERBB2 AND RODENT MODELS OF CHOLANGIOCARCINOGENESIS

Neu is the rat homologue of human ErbB2. Table 1 lists experimental rodent models in which Neu overexpression has been demonstrated to be associated with the molecular pathogenesis of intrahepatic cholangiocarcinoma. Western and Northern blotting, together with immunohistochemistry and ISH have been used to demonstrate Neu protein and mRNA to both be significantly overexpressed in the cancerous epithelium of furan-induced intrahepatic intestinal-type cholangiocarcinomas when compared with hyperplastic bile ducts/ductules in the liver of bile duct-ligated rats and with normal adult rat liver^[63,64]. In these tumors, *c-neu* was further determined not to be amplified nor to exhibit evidence of a key activating point mutation within its transmembrane domain^[64]. In addition, Neu oncoprotein overexpressed in furan-induced rat cholangiocarcinomas was tyrosine phosphorylated, indicative of a constitutively activated signaling state. Here, it would seem that constitutive overexpression of activated Neu in furan-induced rat intrahepatic cholangiocarcinomas is the result of an altered transcriptional event rather than to mechanisms involving gene amplification or mutation. Similarly, Neu protein was further demonstrated by immunohistochemistry to be overexpressed in cancerous epithelium of intestinal-type cholangiocarcinoma induced in the livers of rats treated with thioacetamide when compared with normal cholangiocytes^[65].

Hepatic cirrhosis and early formed cholangiofibrotic precursor lesions precede the development of intrahepatic cholangiocarcinoma in both furan- and thioacetamide-treated rats. Here it is interesting that Neu

Table 1 Rodent models of intrahepatic cholangiocarcinoma constitutively overexpressing ErbB2/Neu in cancerous epithelium

Model	Tumor			ErbB2/Neu			Tyrosine phosphorylation	Ref.
	Development time	Incidence (%)	Classification	c-erbB-2/neu	mRNA	Protein		
Furan rat model	> 1 yr	70-100	Intestinal-type cholangiocarcinoma	Wild-type/non-amplified	Increased	Increased	Increased	[63,64]
Thioacetamide rat model	16-22 wk	100	Intestinal-type cholangiocarcinoma	NA	NA	Increased	NA	[65]
P53 deficiency/CCl ₄ mouse model	≥ 16 wk	40	Ductal cholangiocarcinoma	NA	NA	Increased	NA	[66]
Rat BD Eneu orthotopic cell transplantation model	4 wk	100	Ductal cholangiocarcinoma	Mutated	Increased	Increased	Increased	[68,69]

NA: Not assessed.

overexpression has also been observed in metaplastic/dysplastic glands within these early cholangiofibrotic lesions, as well as in the later developed invasive cholangiocarcinomas, compared with negative or marginal levels of Neu detected in normal or hyperplastic bile ductular epithelium^[64,65]. More recently, CCl₄-induced hepatic fibrosis was shown to be associated with the promotion of intrahepatic cholangiocarcinoma in p53-deficient mice^[66], with strong Neu immunostaining having also been seen in the cancerous epithelium of a majority of the mass-forming liver tumors, as well as in some metastatic lesions. Here too, Neu expression was detected in early intrahepatic cholangiocarcinomas as well, with only weak or undetected Neu immunostaining being observed in normal bile ducts and in small bile duct hyperplasia. Overall, these experimental results are consistent with previously described epidemiological and immunohistochemical findings for human intrahepatic cholangiocarcinogenesis, which support a role for hepatic cirrhosis as a potential risk factor in the pathogenesis of intrahepatic cholangiocarcinoma development^[13,16]. They further suggest Neu overexpression to be a factor in both the early and later stages of intrahepatic cholangiocarcinogenesis.

More direct evidence for the involvement of Neu in the development of biliary tract malignancies, including intrahepatic cholangiocarcinoma comes from genetic-based animal and cell models. Transgenic mice (*BK5. erbB2* mice) generated to constitutively overexpress wild-type rat *c-neu* developed gallbladder adenocarcinoma at a 100% incidence and intrahepatic cholangiocarcinomas at 30% incidence by 8 mo of age^[36]. No mutations in either *K-ras* or *p53* were detected in 16 of the murine gallbladder adenocarcinomas analyzed, with *p53* being found to be elevated in only one of these tumors. On the other hand, Neu and EGFR protein (but not ErbB3 or ErbB4) were found to be increased and hyperphosphorylated on tyrosine residues in gallbladder tissue from the *BK5.erbB2* mice. Increased heterodimer formation between Neu and EGFR and increased p42/44 MAPK activation were also observed in gallbladder from these transgenic mice when compared with that from control mice not expressing the rat *c-neu* transgene, further implicating increased Neu signaling in development of biliary tract cancers. Consistent

with these transgenic mouse data, it has recently been demonstrated that when non-tumorigenic immortalized adult rat cholangiocytes (BDE1 cells)^[67] were stably transfected with a constitutively expressed mutant rat *neu* gene, they became highly tumorigenic^[68,69]. These malignant transformants, designated as BD Eneu cells, overexpressed activated Neu^[68,69], as well as EGFR (Sirica, A.E., unpublished data), and exhibited elevated levels of phospho-p42/44 MAPK and phospho-Akt when compared with both non-tumorigenic parent BDE1 cells and non-tumorigenic control BD Eneo cells that were derived by stably transfecting BDE1 cells to only express the gene for neomycin resistance^[68]. BD Eneu cells transplanted *via* bile duct inoculation into the livers of isogenic rats rapidly formed large moderately differentiated mass-forming intrahepatic cholangiocarcinomas that recapitulated clinical, cellular, and molecular features of advanced human disease^[68,69] (Figure 4). Notably, BD Eneu tumors were found to be highly invasive and metastatic, to produce bile duct obstruction at the hepatic hilus, and to retain strong immunoreactivity in their cancerous epithelium for Neu constitutively phosphorylated at tyrosine 1248. Tyrosine 1248 is known to be a major autophosphorylation site at the cytoplasmic tyrosine kinase domain of Neu oncoprotein that reflects the activation status of the receptor and couples Neu to the *ras-raf*-MEK-p42/44 MAPK signal transduction pathway^[70,71].

The C611B cell line is another novel rat cholangiocarcinoma cell line that constitutively overexpresses Neu when compared with hyperplastic rat intrahepatic bile ducts/ductules induced in bile duct-ligated rats^[72-75]. This cell line was established from a transplantable mucin-producing cholangiocarcinoma expressing wild-type *neu* that was derived from the furan rat cholangiocarcinogenesis model^[72]. Like BD Eneu cells, C611B cells express Neu protein phosphorylated at tyrosine 1248, as well as activated Akt and p42/44 MAPK (Figure 5A). Inoculation of C611B cells into the inguinal fat pads or livers of isogenic rats also yields a 100% incidence of mass-forming adenocarcinomas that closely resemble in their histopathology well differentiated mucin-producing tubular intrahepatic cholangiocarcinomas of the human^[72]. As exemplified by the RT-PCR data shown in Figure 5B, C611B

Figure 4 Rat BD Eneu cell transplantation model of intrahepatic cholangiocarcinoma progression. A: Gross pathology of a bisected invasive hepatic tumor (T) formed in the liver of an isogenic Fischer 344 rat at 3 wk after bile duct inoculation of 4×10^6 tumorigenic rat BD Eneu cholangiocytes overexpressing mutationally-activated Neu, together with associated peritoneal metastases (arrows); B: Expt. 1. mean tumor size $\pm$ SD (as a % of total liver weight) of hepatic tumors that formed at a 100% incidence in isogenic rat livers at 3 wk after bile duct inoculation of 4×10^6 BD Eneu cells (BD Eneu-BD), compared with no tumors formed in rats comparably inoculated with control non-tumorigenic BD Eneo cells (BD Eneo-BD) transfected to stably express the neomycin resistance gene only, in the absence of oncogenic *neu* $n = 6$; C: Expt. 2. Differences in mean wet weight $\pm$ SD of hepatic tumors (relative to that of corresponding non-tumorous liver) formed at 3 wk after bile duct inoculation of 4×10^6 BD Eneu cells (BD Eneu-BD) versus the mean wet weight of hepatic tumors that formed over the same time period following inoculation of the BD Eneu cells directly into liver (BD Eneu-L). ^b $P \leq 0.001$ (BD Eneu-BD tumor versus BD Eneu-L tumor). $n = 4$; D: Representative low power photomicrograph demonstrating neoplastic cholangiocytes within a rapidly growing BD Eneu-BD intrahepatic cholangiocarcinoma to be strongly immunoreactive for plasma membrane Neu; E: Photomicrograph showing malignant cholangiocytes within a BD Eneu-BD intrahepatic cholangiocarcinoma to exhibit strong uniformly positive immunoreactivity for activated Neu phosphorylated at tyrosine 1248; F: Photomicrograph of a BD Eneu-BD tumor demonstrating positive cytoplasmic immunostaining for COX-2 in the cholangiocytes of the neoplastic ducts. Positive immunostaining in D-F is indicated by brown reaction product. (See References 68 and 69 for a more complete description of rat BD Eneu model). (D $\times 66$, E $\times 132$).

cholangiocarcinomas simultaneously express mRNA for ErbB growth factor ligands (i.e. TGF- α , heregulin) and for three of the four ErbB family receptors, with the exception of ErbB4, which is also not detected in normal rat liver^[76]. These data, although limited, suggest that ligand-dependent activation of ErbB family receptors are likely operative in these tumors, and that cognate ligands, such as TGF- α , may be playing an important role in this process^[26]. EGFR immunoreactivity has also been detected in the cancerous epithelium of 100% of

analyzed specimens of rat cholangiocarcinomas induced by thioacetamide^[55], further suggesting that signaling through EGFR may also be a relevant contributing factor to the genesis and progression of intrahepatic cholangiocarcinoma^[77]. However, additional mechanism-based studies are needed to more fully substantiate this likely possibility.

It is surprising that there is a noticeable lack of published data on the use of well established hamster models of cholangiocarcinogenesis, such as those

Figure 5 Western blot (A) and RT-PCR (B) analyses. A: Relevant protein and phosphorylation profiles associated with aberrant Neu expression in rat C611B and BDEneu cholangiocarcinoma cell lines. C611B overexpress the wild-type *c-neu* gene^[64], whereas BDEneu cells overexpress mutationally-activated *neu* oncogene^[68,69]. Note that both cell lines are positive for phospho-Neu, phospho-Akt, and phospho-p42/44 MAPK, as well as show prominent expression of cyclin D1; B: RT-PCR analysis of *c-erbB* family receptor together with heregulin, TGF- α , and COX-2 mRNAs detected in malignant neoplastic ducts obtained by laser capture microdissection from a hepatic tumor formed in an isogenic Fischer 344 male rat following cell transplantation of rat C611B cholangiocarcinoma cells directly into liver. Note that as expected, among the ErbB family receptors, only ErbB4 mRNA is not detected in the tumor ducts. Moreover, it is also significant that mRNA signals for both the EGFR ligand TGF- α and for COX-2, which is known to be up-regulated in response to EGFR and/or Neu signaling, are each shown to be prominently detected in C611B cholangiocarcinoma.

which combine tumor initiating doses of carcinogenic nitrosamines with *Opisthorchis viverrini* or *Clonorchis sinensis* infection^[78-82] as a means to investigate the role of aberrant ErbB family receptor signaling in intrahepatic cholangiocarcinoma development and progression. This may be due to the fact that only very limited hamster-specific immunochemical and molecular reagents are currently available for such studies. Nevertheless, the establishment of novel rodent models as those described above and in Table 1 have proven to be valuable resources for investigating the role of ErbB-family receptors in intrahepatic cholangiocarcinogenesis. Clearly more studies are needed in this regard, but from the standpoint of clinical relevance, it is also apparent that

some recently developed rodent models of intrahepatic cholangiocarcinoma development and progression have the potential of serving as powerful preclinical platforms for rapidly testing novel ErbB targeted treatment strategies for cholangiocarcinoma prevention and therapy.

INTERACTIVE RELATIONSHIPS BETWEEN ERBB FAMILY RECEPTORS AND OTHER MOLECULAR PATHWAYS

The interplay between the EGFR and/or ErbB2 receptor tyrosine kinases with other diverse signaling pathways provides multiple functional links by which intrahepatic cholangiocarcinoma growth and/or progression may be promoted or potentiated. Furthermore, such interactive molecular relationships can have important implications with respect to devising novel molecular targeting strategies for cholangiocarcinoma therapy or chemoprevention^[1]. EGFR and/or ErbB2 have been linked to other molecular pathways implicated in intrahepatic cholangiocarcinoma growth, apoptosis resistance, angiogenesis and/or invasion and metastasis. Each of these depicted interactions will be discussed separately, although it is important to keep in mind that they are likely functioning in concert.

Bile acids

Secondary bile acids, such as deoxycholic acid and its conjugated forms, which have been reported to be increased in the serum of cholangiocarcinoma patients^[83], may be acting to promote cholangiocarcinogenesis in the biliary tract^[20,84,85] through a mechanism involving bile acid-mediated activation of EGFR, leading to up-regulation of COX-2 and protein turnover inhibition of the potent antiapoptotic protein, myeloid cell leukemia 1 (Mcl-1). Werneburg *et al*^[26] have demonstrated that bile acids like deoxycholic acid can act to activate EGFR in cultured human H-69 cholangiocyte and KMBC cholangiocarcinoma cell lines by a TGF- α -dependent mechanism mediated by bile acid stimulation of matrix metalloproteinase activity catalyzing TGF- α membrane release. Yoon *et al*^[86] have further shown deoxycholic acid and conjugated forms, such as taurochenodeoxycholate, to significantly induce COX-2 expression in cultured human KMBC cholangiocarcinoma cells *via* EGFR transactivation and subsequent activation of p42/44 MAPK, p38 MAPK, and c-jun-N-terminal kinase (JNK). In addition, deoxycholic acid was found to prolong Mcl-1 protein turnover in cultured KMBC cholangiocarcinoma cells by an EGFR/Raf-1-dependent mechanism^[87]. Comparable mechanisms have been reported for bile-acid mediated induction of COX-2 and Mcl-1 in human and rat hepatic stellate cells^[88], suggesting that bile acids might also be playing a role in enhancing the survival and proliferation of myofibroblastic tumor stromal cells associated with the desmoplastic reaction typically characteristic of intrahepatic cholangiocarcinoma. It may also be of interest that deoxycholic acid, as well as

taurodeoxycholic, and taurochenodeoxycholic bile acids have recently been reported to regulate the expression of MUC4, a modulator of ErbB2 signaling (see below), in human esophageal carcinoma cells by a promoter-dependent mechanism involving activation of the PI3K pathway^[89], and in the case of taurodeoxycholic and taurochenodeoxycholic bile acids, have also been found to be mediated by hepatocyte nuclear factor-1 α ^[90].

Cox-2

Cox-2, the inducible form of prostaglandin endoperoxidase, has been reported to be commonly up-regulated in the malignant neoplastic epithelium of intrahepatic cholangiocarcinomas of both the human^[49,91,92] and of experimental rodent models^[68,69,73-75] (Figures 4F and 5B). Accumulating evidence over the past several years has further supported COX-2-derived prostaglandin signaling as playing an important role in cholangiocarcinogenesis as a mediator of mitogenesis, anti-apoptosis, and angiogenesis^[93,94]. Moreover, a close relationship between COX-2-derived prostaglandin signaling and that of EGFR or ErbB2 overexpression and activation has been established in animal and cell models of biliary tract carcinogenesis^[36,68,74].

Vadlamudi *et al*^[95] were the first to demonstrate the regulation of COX-2 expression by heterodimeric ErbB2 signaling. Furthermore, the presence of a nuclear ErbB2 has been identified, which appears to act as a transcriptional regulator of COX-2^[96]. Benoit *et al*^[97] have further demonstrated that COX-2, through the production of its major enzymatic product prostaglandin E₂ (PGE₂), positively regulates ErbB2 expression, supporting a model in which ErbB2 and COX-2 regulate each other in a positive loop. Cross-talk between COX-2-derived PGE₂ and EGFR has also been demonstrated in human cholangio- and hepatocellular carcinoma cells^[33,98], and EGFR kinase inhibitors were found to decrease COX-2 expression levels in cultured human cholangiocarcinoma cells^[77], suggesting a similar positive regulatory loop for EGFR and COX-2. In addition, the recent findings of Zhang *et al*^[99] have suggested that in human cholangiocarcinoma cells, PGE₂-enhanced phosphorylation of MAPK/ERK is, at least in part, mediated through EP1 receptors and EGFR phosphorylation that is associated with increased intracellular calcium concentration.

As previously noted in this review, a positive correlation between immunohistochemically-determined ErbB2 expression and COX-2 up-regulation has been described in human cholangiocarcinogenesis^[49]. COX-2 up-regulation has also been determined to closely parallel constitutive *c-neu* protein and mRNA overexpression in cholangiocarcinoma cells and tumors derived from the furan rat model of intrahepatic cholangiocarcinogenesis^[73,74]. Of particular interest are the findings of Kiguchi *et al*^[36] who observed a prominent up-regulation of COX-2 protein and mRNA in gallbladder tissues and tumors from *BK5. erbB2* transgenic mice constitutively overexpressing wild-type rat *c-neu*, thereby adding more convincing

support for the role of Neu (ErbB2) in COX-2 expression in biliary tract carcinogenesis. Consistent with the findings of Kiguchi *et al*^[36], Lai *et al*^[68] more recently reported COX-2 protein and mRNA, together with a concomitant overproduction of PGE₂, to be significantly induced in rat BDE1 cholangiocytes upon *in vitro* malignant neoplastic transformation of these cells with mutationally-activated rat *neu* oncogene. Neoplastic epithelium of tumors overexpressing mutationally-activated Neu formed from orthotopically transplanted *neu*-transformed rat cholangiocytes (BDEneu cells) were also found to be positively immunoreactive for cytoplasmic COX-2^[68] (Figure 4F).

Interleukin-6 (IL-6)/gp130

The inflammatory cytokine IL-6 has been demonstrated to be frequently overexpressed in malignant neoplastic cells of human well differentiated intrahepatic cholangiocarcinomas^[100] and to be increased in the serum and bile of cholangiocarcinoma patients^[101-103]. IL-6 has been further shown to function as an autocrine growth factor for human cholangiocarcinoma cell lines^[100,104,105], and to up-regulate Mcl-1 in malignant human cholangiocyte cell lines^[106,107].

Gp130 is the signal transducing element of the IL-6 receptor. Current data support a mechanism whereby sustained IL-6-induced phosphorylation (activation) of gp130, leading to enhanced activation of STAT-3, contributes to cholangiocarcinoma cell mitogenesis and enhanced Mcl-1 up-regulation and resistance to apoptosis^[106-109]. Isomoto *et al*^[108] have further reported that sustained IL-6/STAT-3 signaling in human cholangiocarcinoma cells is due to epigenetic silencing of suppressor of cytokine signaling 3 (SOCS-3). Here, it is also noteworthy that COX-2-derived PGE₂ was recently found by Han *et al*^[109] to induce IL-6 production through activation of the EP4 receptor and subsequent phosphorylation of gp130/STAT-3 in SG231 human cholangiocarcinoma cells, demonstrating the potential for cross-talk between the COX-2/PGE₂ and IL-6/gp130 pathways in cholangiocarcinoma. In this same study, it was also found that PGE₂ signaling in human CCLP1 human cholangiocarcinoma cells could activate STAT-3 through EP1 receptor-mediated intracellular activation of *c-Src* and EGFR^[109].

Qiu *et al*^[110], utilizing human prostate carcinoma cells, were the first to demonstrate that ErbB2 forms a complex with the gp130 subunit of the IL-6 receptor, thereby serving as a functional component of IL-6 receptor signaling activating the p42/44 MAPK pathway. Grant *et al*^[111] subsequently reported gp130 to be constitutively associated with ErbB2 and ErbB3 in human breast cancer cells and found EGF to induce tyrosine phosphorylation of gp130 in these cells, further supporting a functional interaction between gp130 and the ErbB receptor family. Interestingly, IL-6 overexpression was recently reported to also decrease EGFR promoter methylation in human cholangiocarcinoma cells, leading to enhanced EGFR protein expression^[112].

MUC1 and MUC4

Aberrant overexpression of the transmembrane mucins MUC1 and MUC 4 has been frequently observed in human intrahepatic cholangiocarcinomas^[55,113-116], and both MUC1 and MUC4 have been reported as being independent prognostic factors for predicting poor outcomes in patients with mass-forming intrahepatic cholangiocarcinoma^[113-115]. MUC1 overexpression has also been detected in rat intrahepatic cholangiocarcinoma^[55], and more recently, MUC1 mRNA was demonstrated to be significantly increased in the highly tumorigenic rat BDEneu cholangiocarcinoma cell line^[68] over that of non-tumorigenic parent BDE1 cholangiocytes, as well as of that expressed in a spontaneously transformed rat cholangiocyte cell line, designated as BDEsp, which was less tumorigenic than the BDEneu cell line^[69]. MUC4 has also been cited as being overexpressed, as well as co-localized with Neu protein, in gallbladder adenocarcinoma formed in *BK5. erbB2* transgenic mice overexpressing the wild-type rat *c-neu* transgene^[117].

The transmembrane mucins MUC1 and MUC4 interact by different mechanisms with ErbB family receptors and each can function to potentiate ErbB-dependent signal transduction^[118-120]. The cytoplasmic tail of MUC1 harbors several tyrosine residues that when phosphorylated may provide critical docking sites for initiating downstream cytoplasmic signaling pathways relevant to cancer development and progression^[118]. Notably, the MUC1 cytoplasmic tail has been shown to interact with all four members of the ErbB family of receptor tyrosine kinases^[118,119]. EGFR-mediated phosphorylation of the MUC1 cytoplasmic tail at its YEKV motif has further been demonstrated to enhance its affinity for β -catenin, potentially favoring decreased cell adhesion and in the case of tumor cells, increased invasiveness^[118,119]. Heregulin was also reported to enhance the formation of MUC1-ErbB2 complexes in tumor cells and to induce the binding of the MUC1 cytoplasmic domain with γ -catenin and targeting of the MUC1 cytoplasmic tail- γ -catenin complex to the nucleolus^[121]. This latter finding also supports cross-talk between the ErbB2 receptor tyrosine kinase and MUC1. However, the role of a MUC1 cytoplasmic tail- γ -catenin complex in the nucleolus still needs to be elucidated, particularly in relation to malignant neoplastic cell transformation and progression.

MUC1's cytoplasmic tail, through its interaction with ErbB family receptor tyrosine kinases, has been demonstrated to potentiate ErbB signaling mediated through Grb2-Sos-activation of the Ras-p42/44 MAPK signaling cascade^[118,119]. More recently, MUC1 has also been reported to modulate TGF- α -dependent cancer progression^[122], as well as to regulate EGFR stability upon activation^[123], thereby suggesting a possible alternative pathway whereby cancer development and progression may be promoted *via* a mechanism based on MUC1-mediated inhibition of EGFR degradation. MUC1's C-terminal subunit has further been reported to be translocated to mitochondria by a mechanism in

which heregulin-induced tyrosine phosphorylation of ErbB receptors results in the activation of α -Src^[124]. This interaction, in turn, stimulates binding of the MUC1 cytoplasmic domain to the molecular chaperone HSP90, thus forming a complex to transport MUC1 oncoprotein to the mitochondrial outer membrane, where it may then act to facilitate neoplastic development by blocking activation of the intrinsic apoptotic pathway^[124].

In contrast to MUC1, MUC4 has been shown to function as a novel intramembrane ligand and modulator for ErbB2 receptor tyrosine kinase^[118-120]. Specifically, it has been proposed that EGF-like domains on MUC4 interact with the cognate receptor portion of ErbB2^[119]. However, the actual mechanism whereby MUC4 can signal through ErbB2 is complex and still not completely understood. Caraway and his associates^[118,125] first reported that MUC4 alone was capable of inducing tyrosine phosphorylation of ErbB2 and up-regulation of p27^{kip} (a cyclin-dependent kinase inhibitor), but did not produce activation of MAPKs or PI3K-Akt. On the other hand, these researchers found that in the presence of NRG1, MUC4 potentiated ErbB2/ErbB3 phosphorylation, as well as enhanced p42/44 MAPK and Akt activation and p27^{kip} repression^[118,125]. More recently, it was reported by Funes *et al*^[126] that MUC4 potentiates NRG1 signaling by making more ErbB2/ErbB3 receptors available at the plasma membrane for interaction with the NRG1 ligand, without affecting the total quantity of receptors expressed by the cell. Interestingly, in this study, without NRG1, no evidence of MUC4-stimulated ErbB2 receptor tyrosine phosphorylation was detected. The basis for this difference with earlier reported results^[118,125] is unclear, but it seems that MUC4 may be acting to stabilize the ErbB2/ErbB3-NRG1 complex at the plasma membrane by suppressing its internalization^[119,126].

MUC 4 has also been reported to suppress apoptosis in cells in the presence or absence of NRG treatment^[118]. Indeed, based on the cumulative data for MUC4 interaction with ErbB2, Caraway *et al*^[118] have proposed that in polarized untransformed epithelium, the MUC4-ErbB2 complex in the absence of NRG1 receptor signaling would promote cell differentiation. In contrast, in unpolarized cancer cells, MUC4 and NRG1 could function simultaneously to augment ErbB2/ErbB3 signaling, leading to downstream activation of both the Ras-p42/44 MAPK and PI3K-Akt signaling pathways and transcriptional regulation of cyclin D1, promoting cancer cell growth, survival, and/or progression^[119].

HGF/Met

Met, the heterodimeric tyrosine kinase receptor for hepatocyte growth factor (HGF), its cognate ligand, is commonly overexpressed in the neoplastic epithelium of intrahepatic cholangiocarcinomas of both human and rodent origin^[47,64-66,72,127]. Positive immunoreactivity for Met was determined to be most prominent in well differentiated human cholangiocarcinomas and to be low in poorly differentiated tumors^[47,127]. Rat models of intrahepatic cholangiocarcinoma overexpressing

Neu were further found to concomitantly overexpress Met^[64,65,72]. In this context, it is noteworthy that like Neu, constitutive overexpression of tyrosine phosphorylated Met in the cancerous epithelium of furan-induced rat intrahepatic cholangiocarcinomas was determined not to be the result of gene amplification, but to correlate with increased Met mRNA expression^[64]. Constitutively-activated Met has also been detected in intrahepatic cholangiocarcinomas formed in liver of p53^{-/-} mice following CCl₄ treatment^[66].

Typically, in epithelial cancers, Met activation occurs mainly through a paracrine mechanism with stromal cell-secreted HGF, although evidence supporting the existence of a HGF/Met autocrine growth control circuit in cholangiocarcinoma cell lines of both human^[105] and rat^[73,128] origin has been described. However, cross-talk between EGFR and Met has been demonstrated to also occur in malignant epithelial cell lines in the absence of HGF, but in the presence of TGF- α or EGF^[31]. EGFR signaling has also been implicated in HGF-induced hepatocyte proliferation^[129], and downstream signaling from HGF/Met has been demonstrated to synergize with ErbB2 to enhance malignant progression^[130]. Furthermore, evidence has been provided indicating that EGFR can transactivate the Met receptor tyrosine kinase in human hepatocellular and pancreatic carcinoma cells^[131]. PGE₂ generated by COX-2 has also been shown in human colon and hepatocellular carcinoma cells to transactivate the Met receptor in a manner dependent on functional EGFR^[32,33]. Moreover, TGF- α and PGE₂, factors which are overexpressed in cholangiocarcinoma cells^[74,132,133], may function as inducers of HGF production by tumor stromal fibroblasts^[134]. Overall, these findings support interactive functional relationships between ErbB family receptors, HGF/Met, and COX-2 that are likely contributing in a major way towards regulating intrahepatic cholangiocarcinoma growth and progression.

VEGF

Moderate to strong expression of the angiogenic factor, vascular endothelial growth factor (VEGF or VEGF-A), as well as of the lymphangiogenic factor VEGF-C, has been detected in the cancerous epithelium of a significant percentage of human intrahepatic cholangiocarcinomas^[135-139] and in human cholangiocarcinoma cell lines^[135,140]. Increased VEGF expression has been reported to be associated with a significant vascularization of human intrahepatic cholangiocarcinomas, as assessed by microvessel density^[135,136]. In comparison, hypovascularity of cholangiocarcinoma may be related to a down-regulation of VEGF together with an up-regulation of the angiogenesis inhibitor thrombospondin-1^[136,141,142], although further studies are needed to validate the likelihood of this being a primary mechanism underlying the limited angiogenesis observed in cases of intrahepatic cholangiocarcinoma.

Cultured rat BDE1 cholangiocytes stably transfected

with constitutively expressed rat neu oncogene overexpress VEGF^[68]. Moreover, ErbB homo- and heterodimers have been demonstrated to increase VEGF expression in other tumorigenic cell types, with EGFR/ErbB2 and ErbB2/ErbB3 heterodimers determined to be the most potent inducers of VEGF mRNA expression when compared with various other paired combinations, such as EGFR/ErbB3 or ErbB2/ErbB4^[143]. Interestingly, in both mouse and human cell models, thrombospondin-1 mRNA and protein were down-regulated *in vitro* and in tumors overexpressing specific paired combinations of ErbB receptors, compared with cells stably transfected to overexpress only single ErbB receptors^[144].

ErbB2-mediated up-regulation of VEGF has been shown to involve activation of PI3K-Akt, mTOR and p70S6 kinase^[145], as well as to be linked to increased expression of hypoxia inducible factor-1 α (HIF-1 α), an activator of VEGF transcription, *via* a PI3K-Akt-dependent mechanism^[146]. In addition, targeting STAT3 was found to block HIF-1 and VEGF expression induced by ErbB2 and other oncogenic growth signaling pathways^[147]. Moreover, EGFR tyrosine kinase inhibitors have been shown to decrease VEGF expression in cancerous epithelial cells by both HIF-1-dependent and HIF-1-independent mechanisms^[148]. PGE₂ has also been reported to up-regulate VEGF in cancer cells *via* transactivation of EGFR^[149]. Thus, aberrant ErbB receptor signaling in malignant cells, including cholangiocarcinoma cells, has important implications not only for promoting cancer cell growth and progression, but also for regulation of the tumor microenvironment.

THERAPEUTIC TARGETING OF ERBB RECEPTOR TYROSINE KINASES IN CHOLANGIOCARCINOMA CELLS

Conventional chemotherapy, which does not distinguish between cancer cells and normal healthy cells is of limited benefit in the treatment of unresectable or metastatic intrahepatic cholangiocarcinoma^[183]. However, in recent years, the emergence of target-based cancer therapies has provided the option of developing and testing novel treatment strategies that have the potential of improving therapeutic efficacy against cancers like intrahepatic cholangiocarcinoma, which are typically refractory to conventional cancer chemotherapeutic modalities. Since ErbB receptor tyrosine kinases, most notably EGFR and/or ErbB2, are frequently overexpressed and/or constitutively activated in human cancers, including intrahepatic cholangiocarcinoma, agents that selectively target ErbB receptor family members have generated considerable interest among clinicians and researchers alike, with monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKIs) having advanced the furthest in clinical development^[155]. Table 2 lists mAbs and TKIs targeting ErbB family receptors that are currently approved and/or in phase clinical trials for the treatment of various

Table 2 Selected anti-cancer agents targeting ErbB family receptors

Agent	Class/Type	Target	Route of administration	Development stage	Ref.
Trastuzumab (Herceptin)	Recombinant humanized mAb	Extracellular domain of ErbB2	Intravenous infusion	Approved (ErbB2-positive breast cancer)	[150-152]
Pertuzumab (Omnitarg, 2C ₄)	Recombinant humanized mAb	Dimerization domain of ErbB2	Intravenous infusion	Phase II / III	[151,153,154]
Cetuximab (Erbix, C225)	Recombinant human/mouse chimeric mAb	Extracellular domain of EGFR	Intravenous infusion	Approved (EGFR-positive metastatic colorectal cancer and squamous cell carcinoma head and neck cancer)	[151,155-157]
Panitumumab (ABX-EGF, Vectibix)	Fully human mAb	Extracellular domain of EGFR	Intravenous infusion	Approved (EGFR-positive metastatic colorectal cancer)	[157-159]
Matuzumab (EMI-72000)	Recombinant humanized mAb	Extracellular domain of EGFR	Intravenous infusion	Phase I / II	[157,159,160]
MDX-447	Humanized bispecific mAb	Extracellular domain of EGFR and high affinity IgG receptor CD64	Intravenous infusion	Phase I / II	[157,161]
Gefitinib (Iressa)	Anilinoquinazoline/Reversible TKI	EGFR tyrosine kinase	Oral	Limited approval (NSCLC)	[157,162-164]
Erlotinib (Tarceva)	Anilinoquinazoline/Reversible TKI	EGFR tyrosine kinase	Oral	Approved (NSCLC and pancreatic cancer)	[153,155,157,165,166]
Lapatinib (Tykerb, GW572016)	Thiazolylquinazoline/Reversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Approved (ErbB2-positive advanced metastatic breast cancer)	[157,166-169]
PKI-166	Pyrrolopyrimidine/Reversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Phase I	[153,155,157,166,170]
BMS-599626	Pyrrolotriazine/Reversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Phase I	[171,172]
EKB-569 (Pelitinib)	Cyanoquinoline/Irreversible TKI	EGFR tyrosine kinase	Oral	Phase I / II	[157,166,173]
BIBW-2992	Anilinoquinazoline/Irreversible TKI	EGFR and ErbB2 tyrosine kinases	Oral	Phase I / II	[168,174]
CI-1033 (Canertinib)	Anilinoquinazoline/Irreversible TKI	Pan-ErbB tyrosine kinases	Oral	Phase I / II	[153,157,164,168]
HKI-272	Cyanoquinoline/Irreversible TKI	Pan-ErbB tyrosine kinases	Oral	Phase I / II	[153,164,168,175]

mAb: Monoclonal antibody; TKI: Tyrosine kinase inhibitor; EGFR: Epidermal growth factor receptor; NSCLC: Non-small cell lung cancer.

human solid cancers.

Recent preclinical studies, summarized in Table 3, have demonstrated that select TKIs targeting either EGFR or ErbB2, as well as those producing dual inhibition of EGFR and of ErbB2, are capable of effectively suppressing cellular growth and inducing significant apoptosis in human and rodent biliary cancer cell lines *in vitro*, and in some studies were also found to significantly inhibit tumor growth in athymic nude mice that had been subcutaneously xenografted with select human biliary tract carcinoma cell lines. Orally active TKIs were also shown to exhibit both chemopreventive and therapeutic effects in the *BK5.erbB2* transgenic mouse model of gallbladder carcinoma^[182]. These cumulative preclinical results provide a “proof of principal” for EGFR and/or ErbB2 targeting as being a promising strategy for the chemoprevention and/or adjuvant therapy of biliary tract cancers. Most importantly, select preclinical studies further indicated that dual targeting of EGFR and ErbB2 with TKIs, such as lapatinib^[180] or NVP-AEE788^[181], as well as treatments with EGFR or ErbB2 inhibitors administered in combination with other small drug inhibitors, such as the COX-2 inhibitor celecoxib^[75], or the MEK inhibitor

CI-1040^[179], were found to be significantly more potent than corresponding treatments with the single targeting agents alone in suppressing cholangiocarcinoma or gallbladder carcinoma cell growth *in vitro* and/or the tumorigenic growth of biliary tract cancer xenografts *in vivo*. Consistent with previously described results obtained with other carcinoma cell types^[169,184], growth suppression and induced apoptosis of cultured rat and human cholangiocarcinoma cell lines produced by *in vitro* treatments with the dual EGFR/ErbB2-TKI lapatinib or by single agent TKIs combined to simultaneously inhibit EGFR and ErbB2 tyrosine phosphorylation were also observed to be correlated with prominent dose-dependent inhibition of both p42/44 MAPK and Akt activation^[180] (Figure 6). On the other hand, while the dual EGFR/ErbB2 inhibitor NVP-AEE788 was reported to suppress tumorigenic growth in nude mice subcutaneously implanted with human biliary tract cancer cell lines by mechanisms involving downstream blocking of p42/44 MAPK signaling, induction of apoptosis, and inhibition of angiogenesis, treatment with this agent had no apparent effect on reducing Akt activation in the tumor xenografts^[181]. In contrast, the EGFR-TKI gefitinib, was determined to effectively

Table 3 Preclinical biological effects of ErbB RTK inhibitors alone or combined with other target-based treatments for biliary tract cancer cells

Agent	Target	Experimental condition	Biliary cancer cell line/tumor	Biological effects	Ref.
Gefitinib	EGFR	Cell culture	HAG-1 human gallbladder adenocarcinoma cell line	Dose-dependent <i>in vitro</i> cell growth inhibition by arresting cells in G0/G1, followed by progressive cell apoptosis; inhibition of EGFR phosphorylation and of Erk1/2 and Akt activation; decreased cyclin D1 mRNA and induced accumulation of p27 protein, a negative cell cycle regulator	[176]
Gefitinib + Ionizing radiation	EGFR	Cell culture	HuCCT1 human intrahepatic cholangiocarcinoma cell line; TFK-1 human bile duct carcinoma cell line	Gefitinib induced increase in radiosensitivity of HuCCT1 and TFK-1 cells	[177]
Cetuximab + erlotinib	EGFR	Cell culture and subcutaneous tumor xenografts in athymic nude mice	HuCCT1 cell line	Combined treatment with cetuximab blunted erlotinib-induced EGFR up-regulation and regulated in HuCCT1 growth inhibition and apoptosis <i>in vitro</i> and HuCCT1 tumor growth arrest <i>in vivo</i>	[178]
Gefitinib + CI-1040	EGFR + MEK-Erk1/2	Cell culture and subcutaneous tumor xenografts in athymic nude mice	HuCCT1 cell line	Drug combination significantly more effective than single agent treatments in suppressing both <i>in vitro</i> and <i>in vivo</i> tumor cell growth; combination treatment dramatically decreased phosphorylation levels of EGFR and Erk1/2 in cultured cells and in xenografted tumors, whereas HuCCT1 cells were found to be resistant to treatments with gefitinib or CI-1040 alone	[179]
Lapatinib	EGFR/ ErbB2	Cell culture	Rat C611B and human HuCCT1 cholangiocarcinoma cell lines	Lapatinib was demonstrated to be a potent inhibitor of C611B and HuCCT1 cholangiocarcinoma cell growth <i>in vitro</i> by a mechanism involving inhibition of EGFR and ErbB2 activation, suppression of p42/44 MAPK and Akt phosphorylation, and induction of apoptosis	[180]
NVP-AEE788	EGFR/ ErbB2 and VEGFR-2	Cell culture and subcutaneous tumor xenografts in athymic nude mice	EGI-1, TFK-1, CC-SW-1, CC-LP-1 and SK-ChA-1 human extrahepatic bile duct cancer cell lines; MZ-ChA-1 and MZ-CA-2 human gallbladder adenocarcinoma cell lines	NVP-AEE788 more efficacious than the EGFR RTK inhibitors gefitinib and erlotinib in suppressing <i>in vitro</i> cell growth; EGI-1 tumors in mice treated with NVP-AEE788 had significantly reduced volume and mass compared with those in placebo-treated mice, while erlotinib was without effect in inhibiting <i>in vivo</i> tumor growth; main mechanisms of NVP-AEE788 drug action were suppression of Erk1/2 phosphorylation, induced apoptosis, and inhibition of tumor angiogenesis	[181]
Emodin + Celecoxib	ErbB2 + COX-2	Cell culture	C611B rat intrahepatic cholangiocarcinoma cell line	Emodin and celecoxib combined to synergistically suppress anchorage-dependent and anchorage-independent cell growth <i>in vitro</i> through a mechanism involving enhanced inhibition of ErbB2 activation, decreased phospho-Akt, and enhanced caspase-9 and -3 activation, resulting in significantly increased apoptosis	[75]
Gefitinib or GW2974	EGFR EGFR/ ErbB2	BK5.erbB2 transgenic mice constitutively expressing wild-type rat ErbB2	Gallbladder adenocarcinoma	Both agents produce significant chemopreventative and therapeutic effects in reducing gallbladder adenocarcinoma incidence, which was associated with prominent decreases in both the phosphorylation and protein levels of EGFR and ErbB2, with significantly decreased Erk1/2 activity and with a reduction in COX-2 protein levels in BK5.erbB2 mouse gallbladders	[182]

RTK: Receptor tyrosine kinase; EGFR: Epidermal growth factor receptor; VEGFR-2: Vascular endothelial growth factor receptor-2; COX-2: Cyclooxygenase-2.

block EGFR tyrosine phosphorylation and associated downstream activation of Akt, but had a minimal effect on inhibiting p42/44 MAP kinase activation in cultured human HuCCT1 cholangiocarcinoma cells^[179]. This TKI also failed to block p42/44 MAP kinase phosphorylation and tumor growth *in vivo* in athymic nude mice subcutaneously transplanted with HuCCT1 cells. The fact that the HuCCT1 cholangiocarcinoma cell line expresses a mutant *Kras* may explain why upstream inhibition of EGFR by gefitinib was ineffective in blocking p42/44 MAPK activation in these cells^[179]. However, gefitinib given in combination with the MEK inhibitor CI-1040 was associated with a profound inhibition of HuCCT1 tumor growth that appeared to be linked to either a greater p42/44 MAP kinase

inhibition or to simultaneous inhibition of Akt and p42/44 MAPK signaling^[179]. In comparison, gefitinib alone suppressed both the autophosphorylation of p42/44 MAPK and to a lesser degree that of Akt in the HAG1 human gallbladder carcinoma cell line^[176].

Interestingly, the EGFR-TKI erlotinib was found to up-regulate EGFR in HuCCT1 cholangiocarcinoma cells, but combined treatment with the anti-EGFR antibody cetuximab or blockage of erlotinib-induced EGFR synthesis by a small interfering RNA abrogated this TKI effect, and resulted in an inhibition of cell proliferation and increased apoptosis in the HuCCT1 cells^[178]. Moreover, in this study, cetuximab was shown to be equally effective when administered alone or in combination with erlotinib in partially suppressing

Figure 6 Dose-dependent inhibition test. A: Western blots demonstrating dose-dependent suppression by the dual ErbB1 (EGFR)/ErbB2 (Neu) inhibitor lapatinib of phospho-EGFR^{Tyr 1173} and/or of phospho-ErbB2/Neu^{Tyr 1248}, together with concomitant downstream inhibition of phospho-Akt and of phospho-p42/44 MAPK expressed in cultured human HuCCT1 and in rat C611B cholangiocarcinoma cells, respectively. Note that the lapatinib treatment *in vitro* did not affect corresponding total protein levels, but in both cell lines induced a dose-dependent activation of the apoptotic enzyme, caspase-3; B: Dose response curves for lapatinib on suppressing anchorage-dependent growth of cultured rat C611B and human HuCCT1 cholangiocarcinoma cells. Each value represents the mean $\pm$ SD ($n = 3$). Note the close relationship between the lapatinib concentrations required to induce prominent suppression of EGFR and/or ErbB-2/Neu signaling ($\geq 5 \mu\text{mol/L}$) and those associated with marked inhibition of *in vitro* cholangiocarcinoma cell growth ($\geq 6 \mu\text{mol/L}$). Lapatinib-induced suppression of C611B and HuCCT1 growth *in vitro* correlated with both a down-regulation of cyclin D1 protein expression and with a prominent apoptosis being induced in these respective cultured cholangiocarcinoma cell lines (data not shown).

tumorigenic growth in HuCCT1 bearing mice, suggesting that antibody-induced down-regulation of EGFR may provide an effective strategy for circumventing resistance

to specific EGFR-TKI inhibitors in cholangiocarcinoma and other EGFR-expressing cancer cell types.

In the *BK5.erbB2* transgenic mouse model, decreased gallbladder carcinoma incidence produced by chemopreventive or therapeutic treatments involving single targeting of EGFR with gefitinib or dual targeting of EGFR/ErbB2 with GW2974 were each demonstrated to not only reduce tyrosine phosphorylation levels, but also total protein levels of EGFR and ErbB2 in the gallbladders of responsive mice^[182]. Of further note, these targeted treatments were also associated with decreases in COX-2 protein level and of MAPK activity in gallbladders from both the gefitinib and GW2974-treated *BK5.erbB2* mice, with GW2974 showing a greater inhibitory effect than gefitinib on the development of gallbladder carcinoma and also a strong reduction in the tyrosine phosphorylated forms of both EGFR and ErbB2. Interestingly, gallbladder adenocarcinomas refractory to the gefitinib treatment were reported to exhibit sustained activation of EGFR and ErbB2.

ErbB2-TKI emodin in combination with the COX-2 inhibitor celecoxib was further demonstrated to act synergistically to suppress anchorage-dependent and-independent growth of rat C611B cholangiocarcinoma cells overexpressing activated wild-type ErbB2 through a mechanism involving enhanced Akt inactivation leading to increased apoptosis^[75]. From a clinical standpoint, targeted treatments, including the use of select EGFR- and/or ErbB2-TKIs combined with other agents that act to suppress the phosphorylation of Akt are likely to prove to be more effective than single agent treatments for the therapy of biliary tract cancers. In this context, it is noteworthy that both the EGFR-TKI gefitinib and the Akt inhibitor LY294002 were each determined in a preclinical study to increase the radiosensitivity of human HuCCT1 and TFK1 biliary carcinoma cell lines by suppressing Akt activation^[177]. Thus, preclinical testing of various paradigms utilizing select targeting of aberrant EGFR and/or ErbB2 signaling affecting MAPK and/or Akt activation, in combination with other classes of agents that act to suppress Akt activation, would seem to be warranted as a potential strategy for developing new adjuvant therapies for biliary tract carcinomas, including cholangiocarcinoma.

As reflected in Table 4, there have been to date only a limited number of published clinical studies on EGFR and/or ErbB2 targeting in human biliary tract cancers. Unfortunately, while the various treatments described in these studies were determined in most cases to be well tolerated, the anti-tumor effects produced by such treatments in which ErbB receptor targeting was included as part of the therapeutic regimen, was at best modest, and mostly without effect in the vast majority of biliary tract cancer patients who presented with unresected or metastatic disease. Moreover, the extent of EGFR expression was reported not to be significantly associated with outcome^[185]. In one case report, a patient with metastatic cholangiocarcinoma was described as having a response to cetuximab-based therapies; however, the patient's tumors were determined

Table 4 Outcomes of ErbB-targeted therapies in patients with advanced biliary cancer

Assessable patients	Tumor	ErbB status	ErbB inhibitor	Target	Administration	Response	Ref.
40	Unresected or metastatic biliary tract cancers (gallbladder, intra- and extrahepatic bile duct)	29/36 (81%) assessable tumor samples positive for EGFR expression	Erlotinib	EGFR	Single agent	PR-3 patients; SD-17 patients; median time to disease-progression of 2.6 mo	[185]
1	Metastatic cholangiocarcinoma	Negative EGFR expression in tumor	Cetuximab	EGFR	In combination with 5-fluorouracil, folic acid, and radiotherapy	PR in intra-chemotherapeutic state	[186]
1	Unresected cholangiocarcinoma with peritoneal carcinomatosis	Positive EGFR expression in tumor	Cetuximab	EGFR	In combination with gemcitabine	PR with 30% reduction in hepatic mass and disappearance of peritoneal carcinomatosis as shown by computed tomography	[187]
9	Unresected cholangiocarcinoma with disease progression after at least 3 cycles of gemcitabine-oxaliplatin	9/9 (100%) tumor samples positive for EGFR expression, with all being negative for membranous ErbB2	Cetuximab	EGFR	In combination with gemcitabine-oxaliplatin	After 6 mo, CR-1 patient; PR-1 patient; SD-1 patient; progressive disease-6 patients; all patients relapsed, with a median time to disease progression of 4 mo	[188]
17	Unresected advanced biliary tract cancers (gallbladder, bile duct)	Not reported	Lapatinib	EGFR/ErbB2	Single agent	No observed responses; 5 patients with SD; cohort closed due to no noted lapatinib activity	[189]
6 with biliary tract cancers out of a total of 34 with various types of solid tumors	Advanced cholangiocarcinoma (5) or gallbladder cancer (1)	Not investigated	Lapatinib	EGFR/ErbB2	In combination with oxaliplatin/5-fluorouracil/leucovorin	PR-1 patient with cholangiocarcinoma and 1 patient with gallbladder cancer	[190]

CR: Complete response; PR: Partial response; SD: Stable disease.

by immunohistochemistry not to express EGFR^[186]. Also, while immunohistochemistry was used in some of these clinical therapeutic studies to confirm a positive EGFR status^[185,187,188] of the tumors prior to treatment, unlike some of the preclinical *in vivo* studies described above^[179,181,182] (Table 3), no effort was made in any of the studies listed in Table 4 to investigate the effects of the ErbB-target agents being tested on markers of biological activity (i.e. suppression of EGFR tyrosine phosphorylation; inhibition of p42/44 MAPK and/or Akt activation, suppression of cyclin D1) in tumors of responsive *versus* non-responsive patients.

In view of the promising preclinical results obtained with ErbB-targeting agents against human and rodent biliary tract cancer cells, including cholangiocarcinoma cells *in vitro* as well as *in vivo* in tumor xenograft and transgenic models, it is disappointing that the current clinical experience with ErbB-directed therapies have resulted in only very limited anti-tumor activity having been demonstrated in select patients with biliary tract cancers. However, these (albeit limited) clinical findings are also quite consistent with an increasing body of data from different clinical trials demonstrating at best, only modest therapeutic responses of various other epithelial tumor types to single-drug treatments with ErbB inhibitors. In addition, the discrepancies between current preclinical and clinical data point out a real need to establish “patient-like” *in vivo* models of cholangiocarcinoma progression that would more

closely mimic the advanced human disease, and which could better serve to more accurately predict the clinical efficacy of ErbB-directed therapies. In this context, it is noteworthy that very recent preliminary studies in the author’s laboratory have demonstrated that when the dual EGFR/ErbB2-TKI lapatinib was administered to rats by gavage over a three week period, beginning at 2 d after bile duct inoculation of oncogenic *neu*-transformed rat cholangiocytes (BD_Eneu cells), this treatment produced an approximately 70% inhibition in mean intrahepatic tumor wet weight compared with that of tumors formed in the livers of vehicle control-treated rats. In contrast, when the lapatinib treatment was delayed and given to rats with more advanced tumors that resulted in bile duct obstruction, it was without any anti-tumor effect (Sirica AE, Zhang Z, and Campbell DJ, unpublished data). As already indicated above, a similar lack of clinical response was reported by Ramanathan *et al*^[189] in a Phase II study evaluating the anti-tumor efficacy of lapatinib as a single agent treatment in patients with advanced biliary tree cancer. Thus, the rat model of intrahepatic cholangiocarcinoma progression based on bile duct inoculation of highly tumorigenic BD_Eneu cholangiocytes^[68,69] would appear to closely reproduce certain relevant clinical (i.e. bile duct obstruction, icteric liver, elevated serum bilirubin), pathologic (moderately differentiated invasive ductal cholangiocarcinoma with desmoplasia, peritoneal carcinomatosis), and key molecular features (i.e. overexpression of tyrosine

Table 5 Factors affecting ErbB-targeted therapies for intrahepatic cholangiocarcinoma and other biliary tract cancers**Factors**

Patient selection and sampling size
Suboptimal drug dosing and/or scheduling
Tumor microenvironment and bioavailability
Intratumoral heterogeneity in receptor expression and activation
Receptor dynamic effects
Mutational effects
Different mechanisms of acquired resistance
Constitutive overexpression of ErbB family ligands
Co-activation of multiple receptor tyrosine kinases resulting in signaling redundancy and interplay
Lack of uniform biomarkers to effectively predict therapeutic response
Co-morbid disease and toxicity

phosphorylated p185^{neu}, COX-2, and MUC1) of the progressive human disease, as well as permit for a rapid preclinical assessment within a three-to-four week period of innovative molecular targeting strategies that include specific targeting of ErbB2 (and EGFR) regulated signaling pathways. Validating such target-based ErbB-targeting strategies in this “patient-like” model may prove to be most useful in developing new paradigms for adjuvant therapies for intrahepatic cholangiocarcinoma. However, more definitive basic and translational studies are now required to determine the significance of this preclinical rat model of intrahepatic cholangiocarcinoma progression for predicting the clinical efficacy of ErbB-directed therapies against the early *versus* advanced human malignant disease.

FACTORS AFFECTING THERAPEUTIC RESPONSES TO ERBB-TARGET AGENTS

Table 5 lists various factors that should be considered when devising strategies for ErbB-target-based therapies against intrahepatic cholangiocarcinoma. Obviously, patient selection is important and there is a real need now for clinical trials aimed at identifying biological markers that would better predict which of those with this malignant disease would best benefit from such ErbB-directed therapies. It also seems apparent from the limited clinical data that is currently available that single agent testing in cholangiocarcinoma patients with advanced disease that is refractory to conventional therapeutic modalities may not be the best way to assess the therapeutic value of agents that target ErbB receptor signaling pathways. Here again, it can be restated that preclinical models of intrahepatic cholangiocarcinoma that closely recapitulate clinical and molecular features of human disease progression are needed to test novel therapeutic paradigms that combine ErbB-targeted therapies with other target-based therapies in an effort to maximize the inhibition of redundant signaling pathways that aberrantly regulate malignant tumor growth and survival. In addition, preclinical testing of paradigms that combine ErbB target-based therapies

with current conventional modalities could further lead to more rational approaches to developing new strategies for adjuvant management of tumor cell resistance, recurrence or progression. Because this cancer is relatively rare, it is also apparent that for the testing of ErbB-targeted therapies against intrahepatic cholangiocarcinoma to be statistically meaningful and rigorously controlled, multi-center trials set in well established treatment centers for hepatobiliary cancer located in both Eastern and Western countries would be needed to achieve appropriate patient selection. Furthermore, well controlled preclinical and multi-center clinical trials should also be directed towards evaluating the chemopreventative effects of ErbB-directed targeted treatments alone and/or in combination with other therapies in select patient populations with known high risk premalignant conditions for intrahepatic cholangiocarcinoma.

Data on optimum dosing and scheduling of ErbB-targeted agents alone and in combination with other target-based or conventional treatments against intrahepatic cholangiocarcinoma are severely lacking and need to be developed, both preclinically and clinically. Tumor microenvironment and bioavailability is also an important factor in determining the potential therapeutic effectiveness of ErbB-targeted therapies against intrahepatic cholangiocarcinoma. For example, intrahepatic cholangiocarcinomas are often hypovascularized^[136,141,142,191], thereby limiting the possibility of ErbB-targeted agents of reaching the malignant cells in sufficient concentrations to be therapeutically effective. In addition, hypoxic tumor microenvironments inducing HIF-1 α ^[192,193] and tumor extracellular matrix components, like laminin 5^[194], may alter the effectiveness of ErbB directed antineoplastic therapies. Moreover, intratumoral heterogeneity of expression for tyrosine kinase growth factor receptors (i.e. EGFR and ErbB2) indicate that targeting of single ErbB family receptor tyrosine kinase receptors in adenocarcinoma types, including intrahepatic cholangiocarcinoma, may not in itself be sufficient to elicit a strong therapeutic response^[195]. ErbB receptor dynamics have also been shown to influence response to ErbB-targeted agents. For example, as described above, exposure of HuCCT1 human cholangiocarcinoma cells to erlotinib was shown to induce EGFR protein and mRNA expression in these cells, leading to resistance to EGFR tyrosine kinase inhibition^[178]. However, cetuximab or blockage of EGFR synthesis in HuCCT1 cells by small interfering RNA (siRNA) was found to abrogate erlotinib-induced EGFR up-regulation in these cells, suggesting that resistance and sensitivity to EGFR-targeted agents are dynamic events, and that combinations of more than one type of ErbB-directed therapy may be needed to elicit a positive therapeutic outcome.

Mutational events play a key role in mediating resistance or sensitivity to cell-based ErbB-targeted therapies^[196-202]. The efficacy of EGFR-TKIs, such as gefitinib or erlotinib, has been linked to activating

mutations in lung cancer cell EGFR^[199,201], whereas secondary mutations, such as EGFR T790M mutation, can also lead to resistance to EGFR-TKI therapy^[197,201] and to lapatinib-mediated inhibition of receptor autophosphorylation^[202]. *Kras* mutation has also been demonstrated to be an important predictor of resistance to therapy with ErbB-TKIs in some solid tumors^[197,199,200]. In addition, tumor cells with ErbB2 mutations were reported to be resistant to EGFR-TKI treatment, but sensitive to ErbB2 inhibitors, both *in vitro* and *in vivo*^[198,199].

In addition to secondary mutations, various other mechanisms of acquired resistance to ErbB-targeted therapies have also been described, including EGFR ubiquitination^[203], signaling interplay between the EGFR and IGF-1 receptors^[204], steric hindrance of ErbB2 by MUC4^[205], and flexibility of the ErbB receptor family signaling system in the face of inhibition of a single member, such as EGFR^[206]. Moreover, it has been hypothesized that co-activation of multiple receptor tyrosine kinases, (i.e. EGFR and/or ErbB2, Met, PDGFR, IL-6R) in solid malignant tumors results in redundant inputs that drive downstream signaling, therefore limiting the effectiveness of therapies that target just a single receptor tyrosine kinase^[207]. For example, Met/*c-Src* signaling has been recently shown to mediate EGFR tyrosine phosphorylation and cell growth in cultured SUM229 breast cancer cells in the presence of EGFR-TKIs^[208].

Presently, there is no uniform set of biomarkers that can be used to accurately predict a therapeutic response in solid tumors, including intrahepatic cholangiocarcinoma, to ErbB-directed therapies. TGF- α expression has recently been reported to drive constitutive EGFR pathway activation and sensitivity to gefitinib in human pancreatic cancer cell lines^[209]. Elevated TGF- α also appeared to predict for a partial response to lapatinib in patients with advanced EGFR and/or ErbB2 solid malignancies^[210]. However, Ishikawa *et al*^[211] have also presented data to suggest that increased levels of TGF- α and of amphiregulin in serum may serve as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancer. More recently, Jimeno *et al*^[212], utilizing global gene expression profiling together with gene set enrichment analysis, defined an EGFR pathway-based signature that was predictive of a therapeutic response to erlotinib and cetuximab in a subset of xenografted human pancreatic cancers. Further studies are needed, however, to determine if this approach can be effective in a clinical setting as a means of defining biomarkers of ErbB-directed therapies.

Impaired liver function and cirrhosis, together with older age, genetic background, co-morbidities, such as cardiovascular disease, and immune-status all need to also be considered as factors that may affect the potential use and/or effectiveness of ErbB target-based treatments for intrahepatic cholangiocarcinoma therapy. Presently, only a very few clinical trials have investigated cardiotoxicities of the various ErbB target agents,

using defined cardiac endpoints, such as left ventricular function^[213], although it is now well established that herceptin when administered in combination with anthracyclines, can induce significant cardiotoxicity^[153,213]. On the otherhand, treatments with small drug TKI, like lapatinib, erlotinib, and gefitinib, or with the anti-EGFR mAbs cetuximab and panitumumab, have been reported to be associated with low cardiotoxicity^[213]. Skin rash is a classic adverse effect of ErbB-targeted therapy and diarrhea is relatively common in patients treated with EGFR antibodies, or with TKIs, including lapatinib, erlotinib, and gefitinib^[153,213,214]. Interstitial lung disease, elevated hepatic transaminases, and nephritic syndrome have also been rarely reported to occur with small drug EGFR-TKIs^[214], and a high prevalence of hypersensitivity reactions to cetuximab, including anaphylaxis, has been reported in some areas of the United States^[215]. Thus, ErbB target-based therapies are not without risk, although the current clinical experience has indicated that such therapies are generally well tolerated.

FINAL REMARKS

While it now seems apparent that aberrant EGFR and/or ErbB2 expression and signaling is associated with the molecular pathogenesis of intrahepatic cholangiocarcinoma, there is still a significant gap in our knowledge as how to best exploit such alterations in terms of targeted therapies that can then be successfully translated into positive clinical outcomes. More definitive approaches, including cDNA microarray analysis, quantitative immunohistochemistry, and possibly proteomics, need to be evaluated in terms of their potential for profiling individual patient samples (cholangiocarcinoma tumor tissue and cytological specimens and corresponding serum and/or bile samples) for select molecular biomarkers that would be predictive of a therapeutic response to ErbB targeted therapies. In addition, there is a need to develop more effective drug delivery systems for these agents, in order to maximize their bioavailability. Preclinical animal platforms for rapidly testing target-based treatments of intrahepatic cholangiocarcinoma that closely mimic key clinicopathological and molecular features of the human disease also need to be more fully developed and assessed for their ability to more effectively predict therapeutic responses in human clinical trials. Furthermore, based on the complex interactive growth factor receptor signaling and microenvironment properties of solid tumors like intrahepatic cholangiocarcinoma, it seems evident that novel therapeutic strategies involving multiple targeted therapeutics aimed at both cancer and tumor stromal cell targets should be rigorously explored as a means of achieving more effective molecular therapies for this devastating cancer.

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