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[12,5-7]. 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\(^{[11-14]}\), whereas the age-adjusted incidence and mortality rates for extrahepatic cholangiocarcinoma have been reported to be declining over a comparable time period\(^{[14,12]}\). 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\(^{[12,14]}\).

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\(^{[11,18-20]}\). 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. 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. 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\(^{[21,23]}\). 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
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].

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-ERK and the phosphatidylinositol 3-kinase (PI3K)-Akt pathways [23]. Activation of the ras-raf-MEK-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
Moreover, preferential binding of phospholipase Cγ 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 ≥ 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 a sampling size of
intrahepatic cholangiocarcinoma specimens in the study to be meaningful\textsuperscript{[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\textsuperscript{[44,46-50,52]}, but not all of the published studies. Moreover, in some of these studies, mouse monoclonal ErbB2 antibody preparations were used\textsuperscript{[41,44,48,49]} whereas others were performed with rabbit polyclonal anti-ErbB2 antibodies\textsuperscript{[48,49,50-52]}. Furthermore, while in some studies no distinctions were made between plasma membrane and cytoplasmic immunostaining when scoring tumors ostensibly positive for ErbB2 overexpression\textsuperscript{[49-57]}, others more appropriately relied largely on evidence of positive membranous immunostaining when classifying positive cases\textsuperscript{[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 (≥ 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\textsuperscript{[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\textsuperscript{[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%\textsuperscript{[51]}, 21.1%\textsuperscript{[46]}, 21.6%\textsuperscript{[54]}, 47%\textsuperscript{[53]}, and 81%\textsuperscript{[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\textsuperscript{[40]} 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 1 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\textsuperscript{[50,51]}. 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\textsuperscript{[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\textsuperscript{[50]} 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\textsuperscript{[50]} 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...
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. Shiraiishi 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[61] 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[61] 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, Altimari et al[62] 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[63] 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[63] 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
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
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[51] 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[60], 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[51] 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[60] 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[64,76,89]. 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[80] 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 CHOLANGIOCARCINOGNESIS

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[61,62]. 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[60]. 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[60].

Hepatic cirrhosis and early formed cholangiophibic precursor lesions precede the development of intrahepatic cholangiocarcinoma in both furan- and thioacetamide-treated rats. Here it is interesting that Neu
overexpression has also been observed in metaplastic/dysplastic glands within these early cholangiobiliary 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 genetically engineered 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[66]. 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 BDEneu 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 BDEneco cells that were derived by stably transfecting BDE1 cells to only express the gene for neomycin resistance[68]. BDEneu 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, BDEneu 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 on 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 BDEneu 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

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

| Model                     | Tumor Classification                                      | ErbB2/Neu | Tyrosine phosphorylation | Ref.  |
|---------------------------|-----------------------------------------------------------|------------|--------------------------|-------|
| Furan rat model           | Intestinal-type cholangiocarcinoma                        | Wild-type mRNA Increased Increased Increased [63,64] |
| Thioacetamide rat model   | Intestinal-type cholangiocarcinoma                        | NA NA Increased NA [65] |
| P53 deficiency/CCl₄ mouse model | Intestinal-type cholangiocarcinoma                        | NA NA Increased NA [66] |
| Rat BDEneu orthotopic cell transplantation model | Ductal cholangiocarcinoma                                | Mutated Increased Increased Increased [68,69] |

NA: Not assessed.
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 4 Rat BDEneu 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 x 10⁶ tumorigenic rat BDEneu cholangiocytes overexpressing mutationally-activated Neu, together with associated peritoneal metastases (arrows); B: Expt 1. mean tumor size ± 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 x 10⁶ BDEneu cells (BDEneu-BD), compared with no tumors formed in rats comparably inoculated with control non-tumorigenic BDEneo cells (BDEneo-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 ± SD of hepatic tumors (relative to that of corresponding non-tumorous liver) formed at 3 wk after bile duct inoculation of 4 x 10⁶ BDEneu cells (BDEneu-BD) versus the mean wet weight of hepatic tumors that formed over the same time period following inoculation of the BDEneu cells directly into liver (BDEneu-L). P < 0.001 (BDEneu-BD tumor versus BDEneu-L tumor), n = 4; D: Representative low power photomicrograph demonstrating neoplastic cholangiocytes within a rapidly growing BDEneu-BD intrahepatic cholangiocarcinoma to be strongly immunoreactive for plasma membrane Neu; E: Photomicrograph showing malignant cholangiocytes within a BDEneu-BD intrahepatic cholangiocarcinoma to exhibit strong uniformity of positive immunoreactivity for activated Neu phosphorylated at tyrosine 1248; F: Photomicrograph of a BDEneu-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 BDEneu model). (D × 66, E, F × 132).
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\[^8^3\], may be acting to promote cholangiocarcinogenesis in the biliary tract\[^8^0,^8^4,^8^5\] 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\[^8^6\] 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\[^8^0\] 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\[^8^7\]. Comparable mechanisms have been reported for bile-acid mediated induction of COX-2 and Mcl-1 in human and rat hepatic stellate cells\[^8^8\], 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

| Expected size (bp) | Actin (p43) | Cyclin D1 | MAPK (p42/44) | Neu (p185) | Phospho-Akt | Phospho-Neu | Phospho-Akt |
|-------------------|------------|-----------|---------------|------------|-------------|-------------|------------|
| 1500              | 206        | 234       | 260           | 264        | 260         | 264         | 264        |
| 1000              | 191        | 206       | 227           | 253        | 253         | 253         | 253        |
| 600               | 206        | 234       | 260           | 264        | 260         | 264         | 264        |
| 100               | 206        | 234       | 260           | 264        | 260         | 264         | 264        |

*ego* gene

EGFR and/or ErbB2 have been shown to be prominently detected in C611B cholangiocarcinoma cells. C611B and BDEneu cholangiocarcinoma cell lines. C611B overexpress the wild-type c-neu gene\[^6^4\]^, whereas BDEneu cells overexpress mutational-activated neu oncogene\[^6^8,^6^9\]. 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.

Western blotting and RT-PCR 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\[^6^4\]^, whereas BDEneu cells overexpress mutational-activated neu oncogene\[^6^8,^6^9\]. 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.

**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\[^6^4\]^, whereas BDEneu cells overexpress mutational-activated neu oncogene\[^6^8,^6^9\]. 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 transactivation and subsequent activation of p42/44 MAPK and MAPK, are each shown to prominently detected in C611B cholangiocarcinoma.
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-1α[80].

**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[61,62,74] 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[90-93]. 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[96,98,94].

Vadlamudi et al[85] 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[86]. Benoit et al[87] 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 carcinoma cells[109], 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[89]. 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[80] 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[80], Lai et al[88] 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 (BDE Neu cells) were also found to be positively immunoreactive for cytoplasmic COX-2[89] (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[101,102]. 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[106,108]. Isomoto et al[109] 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[80] 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 MUC4 has been frequently observed in human intrahepatic cholangiocarcinomas, and both MUC1 and MUC4 have been reported as being independent prognostic factors for predicting poor outcomes in patients with mass-forming intrahepatic cholangiocarcinoma. MUC1 overexpression has also been detected in rat intrahepatic cholangiocarcinoma, and more recently, MUC1 mRNA was demonstrated to be significantly increased in the highly tumorigenic rat BDEneu cholangiocarcinoma cell line 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. 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.

The transmembrane mucins MUC1 and MUC4 interact by different mechanisms with ErbB family receptors and each can function to potentiate ErbB-dependent signal transduction. 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. Notably, the MUC1 cytoplasmic tail has been shown to interact with all four members of the ErbB family of receptor tyrosine kinases. 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. 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. 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. More recently, MUC1 has also been reported to modulate TGF-α-dependent cancer progression, as well as to regulate EGFR stability upon activation, 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 c-Src. This interaction, in turn, stimulates binding of the MUC1 cytoplasmic domain to the molecular chaperone HSP90, thus forming a complex to transport MUC1 oncopeptide to the mitochondrial outer membrane, where it may then act to facilitate neoplastic development by blocking activation of the intrinsic apoptotic pathway.

In contrast to MUC1, MUC4 has been shown to function as a novel intramembrane ligand and modulator for ErbB2 receptor tyrosine kinase. Specifically, it has been proposed that EGF-like domains on MUC4 interact with the cognate receptor portion of ErbB2. However, the actual mechanism whereby MUC4 can signal through ErbB2 is complex and still not completely understood. Carraway and his associates first reported that MUC4 alone was capable of inducing tyrosine phosphorylation of ErbB2 and up-regulation of p27Kip1 (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 p27Kip1 repression. More recently, it was reported by Funes et al. 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 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.

MUC 4 has also been reported to suppress apoptosis in cells in the presence or absence of NRG treatment. Indeed, based on the cumulative data for MUC4 interaction with ErbB, Carraway et al. 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.

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. Positive immunoreactivity for Met was determined to be most prominent in well differentiated human cholangiocarcinomas and to be low in poorly differentiated tumors. 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 in liver of p53-/- mice following CCl\(_4\) 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\(^{66}\) 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-\(\alpha\) 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\(^{111}\). PGE\(_2\) 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 EGF\(^{12,13}\). Moreover, TGF-\(\alpha\) and PGE\(_2\), 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\(^{116,139}\) 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\(\alpha\) (HIF-1\(\alpha\)), 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\(^ {148}\). PGE\(_2\) has also been reported to up-regulate VEGF in cancer cells via transactivation of EGFR\(^ {49}\). 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\(^ {88}\). 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\(^ {139}\). 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, 2C4) | Recombinant humanized mAb        | Dimerization domain of ErbB2        | Intravenous infusion    | Phase II / II                          | [151,153,154] |
| Cetuximab (Erbitux, 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                | Recombinant humanized bispecific mAb | Extracellular domain of EGFR       | Intravenous infusion    | Phase I / II                           | [157,161] |
| Gefitinib (Iressa)     | Anilinoquinazoline/Thiazolylquinazoline | EGFR tyrosine kinase              | Oral                    | Limited approval (NSCLC)                | [157,162-164] |
| Erlotinib (Tarceva)    | Anilinoquinazoline/Thiazolylquinazoline | 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 (Peltinib)     | Cyaanoquinoline/Reversible TKI   | EGFR tyrosine kinase               | Oral                    | Phase I / II                           | [157,166,173] |
| BIBW-2992              | Anilinoquinazoline/Reversible TKI | EGFR and ErbB2 tyrosine kinases    | Oral                    | Phase I / II                           | [168,174] |
| CI-1033 (Canertinib)   | Anilinoquinazoline/Reversible TKI | Pan-ErbB tyrosine kinases          | Oral                    | Phase I / II                           | [153,157,164,168] |
| HKI-272                | Cyaanoquinoline/Reversible 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.

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. 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[169,180] 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[79], or the MEK inhibitor CI-1040[182], 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[183]. 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 in vitro 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 biliary duct carcinoma cell line | Gefitinib induced increase in radiosensitivity of HuCCT1 and TFK-1 cells                                                                                                                                                | [177]|
| Cetuximab + erlotinib | EGFR + MEK Erk1/2 | 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 in vitro and HuCCT1 tumor growth arrest in vivo | [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 in vitro and in vivo 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 in vitro 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 in vitro cell growth; EG1-1 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 in vivo 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 in vitro 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/ ErbB2 | BK5.erbB2 transgenic mouse constitutively expressing wild-type rat ErbB2 | Gallbladder adenocarcinoma                                                                         | Both agents produce significant chemorepresentative 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. This TKI also failed to block p42/44 MAP kinase phosphorylation and tumor growth in vitro 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. 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. 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.

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. Moreover, in this study, cetuximab was shown to be equally effective when administered alone or in combination with erlotinib in partially suppressing...
of further note, these respective cultured cholangiocarcinoma cell lines (data not shown).

cyclin D1 protein expression and with a prominent apoptosis being induced in
of C611B and HuCCT1 growth (Neu signaling (≥ 5 μmol/L) and those associated with marked inhibition of in vitro cancer cell growth (≥ 6 μmol/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 BK5erbB2 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 BK5erbB2 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[178]. 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
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 \textit{in vitro} 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 \textit{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 \textit{in vitro} as well as \textit{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” \textit{in vitro} 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 \textit{neu}-transformed rat cholangiocytes (BDE\textit{neu} cells), this treatment produced a discernible decrease in 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 \textit{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 BDE\textit{neu} cholangiocytes\[^{188,189}\] 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 kinase).
phosphorylated p185

co, 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, 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-1α and tumor extracellular matrix components, like laminin 5, 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. 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. 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. The efficacy of EGFR-TKIs, such as gefitinib or erlotinib, has been linked to activating
mutations in lung cancer cell EGFR\cite{199,201}, whereas secondary mutations, such as EGFR T790M mutation, can also lead to resistance to EGFR-TKI therapy\cite{197,201} and to lapatinib-mediated inhibition of receptor autophosphorylation\cite{202}. Kras mutation has also been demonstrated to be an important predictor of resistance to therapy with ErbB-TKIs in some solid tumors\cite{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\cite{198,199}.

In addition to secondary mutations, various other mechanisms of acquired resistance to ErbB-targeted therapies have also been described, including EGFR ubiquitination\cite{203}, signaling interplay between the EGFR and IGF-1 receptors\cite{204}, steric hindrance of ErbB2 by MUC4\cite{205}, and flexibility of the ErbB receptor family signaling system in the face of inhibition of a single member, such as EGFR\cite{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\cite{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\cite{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\cite{209}. Elevated TGF-α also appeared to predict for a partial response to lapatinib in patients with advanced EGFR and/or ErbB2 solid malignancies\cite{210}. However, Ishikawa et al\cite{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\cite{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\cite{213}, although it is now well established that herceptin when administered in combination with anthracyclines, can induce significant cardiotoxicity\cite{213,214}. 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\cite{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\cite{213,214}. Intestinal lung disease, elevated hepatic transaminases, and nephritic syndrome have also been rarely reported to occur with small drug EGFR-TKIs\cite{214}, and a high prevalence of hypersensitivity reactions to cetuximab, including anaphylaxis, has been reported in some areas of the United States\cite{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 to 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 therapies 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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REFERENCES

1. Sirica AE. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. Hepatology 2005; 41: 5-15
2. Malhi H, Gores GJ. Cholangiocarcinoma: molecular advances in understanding a deadly old disease. J Hepatol 2006; 45: 856-867
3. Shimoda M, Kubota K. Multi-disciplinary treatment for cholangiocellular carcinoma. World J Gastroenterol 2007; 13: 1500-1504
4. Shaib Y, El-Serag HB. The epidemiology of cholangiocarcinoma. Semin Liver Dis 2004; 24: 115-125
5. Nakajima T, Kondo Y, Miyazaki M, Okui K. A histopathologic study of 102 cases of intrahepatic cholangiocarcinoma: histologic classification and modes of spreading. Hum Pathol 1988; 19: 1228-1234
6. Bae JY, Park YN, Nakanuma Y, Lee WJ, Kim JY, Park C. Intestinal type cholangiocarcinoma of intrahepatic large bile duct associated with hepatitis B virus--a new histologic subtype for further investigation. Hepatogastroenterology 2002; 49: 626-629
7. Nakanuma Y, Sasaki M, Ishikawa A, Tsui W, Chen TC, Huang SF. Biliary papillary neoplasm of the liver. Histol Histopathol 2002; 17: 851-861
8. Suh KS, Chang SH, Lee HJ, Roh HR, Kim SH, Lee KU. Clinical outcomes and apomucin expression of intrahepatic cholangiocarcinoma according to gross morphology. J Am Coll Surg 2002; 195: 782-789
9. Yamasaki S. Intrahepatic cholangiocarcinoma: macroscopic type and stage classification. J Hepatobiliary Pancreat Surg 2003; 10: 288-291
10. Sasaki A, Kawano K, Aramaki M, Ohno T, Tahara K, Kitano S. Correlation between tumor size and mode of spread in mass-forming intrahepatic cholangiocarcinoma. Hepatogastroenterology 2004; 51: 224-228
11. Patel T. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. Hepatology 2001; 33: 1353-1357
12. Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD. Intestinal type cholangiocarcinoma of intrahepatic large bile duct. A nationwide case-control study. Cancer Epidemiol Biomarkers Prev 2006; 15: 1198-1203
13. Shaib YH, El-Serag HB, Davila JA, Morgan R, McGlynn KA. Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. Gastroenterology 2005; 128: 620-626
14. McGlynn KA, Tarone RE, El-Serag HB. A comparison of trends in the incidence of hepatocellular carcinoma and intrahepatic cholangiocarcinoma in the United States. Cancer Epidemiol Biomarkers Prev 2006; 15: 1198-1203
15. Ben-Menachem T. Risk factors for cholangiocarcinoma. Eur J Gastroenterol Hepatol 2007; 19: 615-617
16. Welzel TM, Mellekkjaer L, Gloria G, Sakoda LC, Hsing AW, El Ghormli L, Olsen JH, McGlynn KA. Risk factors for intrahepatic cholangiocarcinoma in a low-risk population: a nationwide case-control study. Int J Cancer 2007; 120: 638-641
17. Patel T, Singh P. Cholangiocarcinoma: emerging approaches to a challenging cancer. Curr Opin Gastroenterol 2007; 23: 317-323
18. Gores GJ. Cholangiocarcinoma: current concepts and insights. Hepatology 2003; 37: 961-969
19. Lazaridis KN, Gores GJ. Cholangiocarcinoma. Gastroenterology 2005; 128: 1655-1667
20. Fava G, Marzioni M, Benedetti A, Glaser S, DeMorrow S, Francis H, Alpini G. Molecular pathology of biliary tract cancers. Cancer Lett 2007; 250: 155-167
21. Roskoski R Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun 2004; 319: 1-11
22. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005; 5: 341-354
23. Normanno N, Bianco C, Strizzi L, Mancino M, Maislo MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: an overview. Curr Drug Targets 2005; 6: 243-257
24. Linghi B, Carpenter G. ErbB receptors: new insights on mechanisms and biology. Trends Cell Biol 2006; 16: 649-656
25. Hobbie SS, Coffing SL, Le AT, Cameron EM, Williams EE, Andrew M, Blommel EN, Hammer RP, Chang H, Rice DJ. 2nd. Neuregulin isoforms exhibit distinct patterns of ErbB family receptor activation. Oncogene 2002; 21: 8442-8452
26. Werneburg NW, Yoon JH, Higuchi H, Gores GJ. Bile acids activate EGF receptor via a TGF-alpha-dependent mechanism in human cholangiocyte cell lines. Am J Physiol Gastrointest Liver Physiol 2003; 285: G31-G36
27. Il M, Yamamoto H, Adachi Y, Maruyama Y, Shimomura Y. Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp Biol Med (Maywood) 2006; 231: 20-27
28. Fuller SJ, Sivarajah K, Sugden PH. ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. J Mol Cell Cardiol 2008; 44: 831-854
29. Wolf-Yadlin A, Kumar N, Zhang Y, Hautaniemi S, Zaman M, Kim HD, Grantcharova V, Laufenburger DA, White FM. Effects of HER2 overexpression on cell signaling networks governing proliferation and migration. Mol Syst Biol 2006; 2: 54
30. Harari D, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. Oncogene 2000; 19: 6102-6114
31. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem 2000; 275: 8806-8811
32. Pai R, Nakamura T, Moon WS, Tarnawski AS. Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. FASEB J 2000; 17: 1640-1647
33. Han C, Michalopoulos GK, Wu T. Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. J Cell Physiol 2006; 207: 261-270
34. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW, Ward CW. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol Cell 2003; 11: 495-505
35. Michalopoulos GK, Khan Z. Liver regeneration, growth factors, and amphiregulin. Gastroenterology 2005; 128: 503-506
36. Kikuchi K, Carbalaj S, Chan K, Beltran L, Rufino L, Shen J, Matsumoto T, Yoshimi N, DiGiovanni J. Constitutive expression of ErbB-2 in gallbladder epithelium results in development of adenocarcinoma. Cancer Res 2001; 61: 6971-6976
37. Zhou BP, Hung MC. Dysregulation of cellular signaling by HER2/neu in breast cancer. Semin Oncol 2003; 30: 38-48
38. Bhat-Nakshatri P, Sweeney CJ, Nakshatri H. Identification of signal transduction pathways involved in constitutive NF-kappaB activation in breast cancer cells. Oncogene 2002; 21: 2066-2078
39. Makino K, Day CP, Wang SC, Li YM, Hung MC. Upregulation of I KKalpha/ I KKbeta by integrin-linked kinase is required for HER2/neu-induced NF-kappaB antiapoptotic pathway. Oncogene 2004; 23: 3883-3887
Voravud N, Foster CS, Gilbertson JA, Sikora K, Waxman J. Oncogene expression in cholangiocarcinoma and in normal hepatic development. *Hepat Pathol* 1989; 20: 1163-1168

Collier JD, Guo K, Mathew J, May FE, Bennett MK, Corbett IP, Basset MF, Burt AD. c-erbB-2 oncogene expression in hepatocellular carcinoma and cholangiocarcinoma. *J Hepatol* 1992; 14: 377-380

Brunt EM, Swanson PE. Immunoreactivity for c-erbB-2 oncoprotein in benign and malignant diseases of the liver. *Am J Clin Pathol* 1992; 97: 583-581

Chow NH, Huang SM, Chan SH, Mo LR, Hwang MH, Su WC. Significance of c-erbB-2 expression in normal and neoplastic bile duct tissue of biliary tract. *Anticancer Res* 1995; 15: 1055-1059

Terada T, Ashida K, Endo K, Horie S, Maeta H, Matsunaga Y, Takashima K, Ohta T, Kitamura Y. c-erbB-2 protein is expressed in hepatolithiasis and cholangiocarcinoma. *Histopathology* 1998; 33: 325-331

Suzuki H, Isaji S, Pairojkul C, Uttaravichien T. Comparative clinicopathological study of resected intrahepatic cholangiocarcinoma in northeast Thailand and Japan. *J Hepatobiliary Pancreat Surg* 2000; 7: 206-211

Itô Y, Takeda S, Sasaki Y, Sakon M, Yamada T, Ishiguro S, Imaoka S, Tsujimoto M, Higashiyama S, Monden M, Matsuura N. Expression and clinical significance of the erbB family in intrahepatic cholangiocellular carcinoma. *Pathol Res Pract* 2001; 197: 95-100

Aishima SI, Taguchi KI, Sugimachi K, Shimada M, Sugimachi K, Tsuneyoshi M. c-erbB-2 protein overexpression relates to cholangiocarcinogenesis and progression of intrahepatic cholangiocarcinoma. *Histopathology* 2002; 40: 269-278

Ukita Y, Kato M, Terada T. Gene amplification and mRNA and protein overexpression of c-erbB-2 (HER-2/neu) in human intrahepatic cholangiocarcinoma as detected by fluorescence in situ hybridization, in situ hybridization, and immunohistochemistry. *J Hepatol* 2002; 36: 780-785

Endo K, Yoon BJ, Pairojkul C, Demetris AJ, Sirica AE. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology* 2002; 36: 439-450

Atlimari A, Fiorentino M, Gabusi E, Grupponi E, Corti B, D’Errico A, Grigioni WF. Investigation of ErbB1 and ErbB2 expression for therapeutic targeting in primary liver tumours. *Dig Liver Dis* 2003; 35: 322-328

Nakazawa K, Dobashi Y, Suzuki S, Fuji H, Takeda Y, Ooi A. Amplification and overexpression of c-erbB-2, epidermal growth factor receptor, and c-met in biliary tract cancers. *J Pathol* 2005; 206: 356-365

Srettakorn J, Kaewpila N, Burns GF, Leong AS. FAT, E-cadherin, beta catenin, HER 2/neu, Ki67 immunooexpression, and histological grade in intrahepatic cholangiocarcinoma. *J Clin Pathol* 2005; 58: 1249-1254

Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hansa WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF. American Society of Clinical Oncology / College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007; 25: 118-145

Nonomura A, Ohta G, Nakamura Y, Izumi R, Mizukami Y, Matsubara F, Hayashi M, Watanabe K, Takayanagi N. Simultaneous detection of epidermal growth factor receptor (EGF-R), epidermal growth factor (EGF) and ras p21 in cholangiocarcinoma by an immunocytotoxic method. *Lever 1988; 8: 157-166

Jan YH, Yeh TS, Yeh JN, Yang HR, Chen MF. Expression of epidermal growth factor receptor, apomucins, matrix metalloproteinases, and p53 in rat and human cholangiocarcinoma: appraisal of an animal model of cholangiocarcinoma. *Ann Surg* 2004; 239: 89-94

Butitta F, Barassi F, Fresu G, Felicioni L, Chella A, Paoluzzi D, Lattanzio G, Salvatore S, Campele PP, Rosini S, Iarussi T, Mucilli F, Sacco R, Mazzotti A, Marchetti A. Mutational analysis of the HER2 gene in lung tumors from Caucasian patients: mutations are mainly present in adenocarcinomas with bronchioalveolar features. *Int J Cancer* 2006; 119: 2586-2591

Bekaii-Saab T, Williams N, Plass C, Calero MV, Eng C. A novel mutation in the tyrosine kinase domain of ERBB2 in hepatocellular carcinoma. *BMC Cancer* 2006; 6: 278

Leone F, Cavalloni G, Pignochino Y, Sarotto I, Ferraris R, Picciobello W, Venesio T, Capusotti L, Risio M, Aglietta M. Somatic mutations of epidermal growth factor receptor in bile duct and gallbladder carcinoma. *Clin Cancer Res* 2006; 12: 1680-1685

Gwak GY, Yoon JH, Shin CM, Ahn YJ, Chung JK, Kim YA, Kim TY, Lee HS. Detection of response-predicting mutations in the kinase domain of the epidermal growth factor receptor gene in cholangiocarcinomas. *J Cancer Res Clin Oncol* 2005; 131: 649-652

Shiraishi K, Kusano N, Okita S, Oga A, Okita K, Sasaki K. Genetic aberrations detected by comparative genomic hybridization in biliary tract cancers. *Oncoology* 1999; 37: 42-49

Obama K, Ura K, Li M, Katagiri T, Tsunoda T, Nomura A, Satoh S, Nakamura Y, Furukawa Y. Genome-wide analysis of gene expression in human intrahepatic cholangiocarcinoma. *Hepatology* 2005; 41: 1339-1348

Su WC, Shiech SC, Liu HS, Chen CY, Chow NH, Lin NZ. Expression of oncogene products HER2/Neu and Ras and fibrosis-related growth factors bFGF, TGF-beta, and PDGF in bile from biliary malignancies and inflammatory disorders. *Dig Dis Sci* 2001; 46: 1387-1392

Sirica AE, Radaeva S, Caran N. NEU overexpression in the furer rat model of cholangiocarcinogenesis compared with biliary ductal cell hyperplasia. *Am J Pathol* 1997; 151: 1685-1694

Radaeva S, Ferreira-Gonzalez A, Sirica AE. Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: A link between biliary intestinal metaplasia and mucin-producing cholangiocarcinoma. *Hepatology* 1999; 29: 1453-1462

Yeh CN, Maître A, Lee KE, Jan YY, Chen MF. Thiocacetamide-induced intestinal-type cholangiocarcinoma in rat: an animal model recapitulating the multi-stage progression of human cholangiocarcinoma. *Carcinogenesis* 2004; 25: 631-636

Farazi PA, Zeisberg M, Glickman J, Zhang Y, Kalluri R, DePinho RA. Chronic bile duct injury associated with fibrotic matrix microenvironment provokes cholangiocarcinoma in p53-deficient mice. *Cancer Res* 2006; 66: 6622-6627

Yang L, Faris RA, Hixson DC. Long-term culture and characteristics of normal rat liver bile duct epithelial cells. *Gastroenterology* 1993; 104: 840-852

Lai GH, Zhang Z, Shen XN, Ward DJ, Dewitt JL, Holt SE, Rozich RA, Hixson DC, Sirica AE. erbB-2/neu transformed rat cholangiocytes recapitulate key cellular and molecular features of human bile duct cancer. *Gastroenterology* 2005; 129: 2047-2057

Sirica AE, Zhang Z, Lai GH, Asano T, Shen XN, Ward DJ, Mahatme A, Dewitt JL. A novel ‘patient-like’ model of cholangiocarcinoma progression based on bile duct inoculation of tumorigenic rat cholangiocyte cell lines. *Hepatology* 2008; 47: 1178-1190

Kwon YK, Bhattacharyya A, Albert JA, Giannobile WV, Cheon K, Stiles CD, Pomeroy SL. Activation of ErbB2 during wallerian degeneration of sciatic nerve. *J Neurosci* 1997; 17: 8293-8299

Cicenas J, Urban P, Küng W, Vuaroqueaux V, Labuhn M, Wight E, Eppenberger U, Eppenberger-Castori S. Phosphorylation of tyrosine 1248-ERBB2 measured by...
chemiluminescence-linked immunoassay is an independent predictor of poor prognosis in primary breast cancer patients. *Eur J Cancer* 2006; 42: 636-645

72 Lai GH, Sirica AE. Establishment of a novel rat cholangiocarcinoma cell culture model. *Carcinogenesis* 1999; 20: 2335-2340

73 Sirica AE, Lai GH, Zhang Z. Biliary cancer growth factor pathways, cyclo-oxygenase-2 and potential therapeutic strategies. *J Gastroenterol Hepatol* 2001; 16: 363-372

74 Sirica AE, Lai GH, Endo K, Zhang Z, Yoon BI. Cyclooxygenase-2 and ERBB-2 in cholangiocarcinoma: potential therapeutic targets. *Semin Liver Dis* 2002; 22: 305-313

75 Lai GH, Zhang Z, Sirica AE. Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with epidem to suppress rat cholangiocarcinoma growth in vitro through a mechanism involving enhanced Akt inactivation and increased activation of caspases-9 and -3. *Mol Cancer Ther* 2003; 2: 265-271

76 Carver RS, Stevenson MC, Scheving LA, Russell WE. Diverse expression of ErbB receptor proteins during rat liver development and regeneration. *Gastroenterology* 2002; 123: 2017-2027

77 Yoon JH, Gwak GY, Lee HS, Bronk SF, Werneburg NW, Gores GJ. Enhanced epidermal growth factor receptor activation in human cholangiocarcinoma cells. *J Hepatol* 2004; 41: 808-814

78 Lee JH, Rim HJ, Bak UB. Effect of Clonorchis sinensis infection and dimethylaminoazobenzene administration on the induction of cholangiocarcinoma in Syrian golden hamsters. *Korean J Parasitol* 1993; 31: 21-30

79 Thamavit W, Pairojkul C, Tiwawech D, Shirai T, Ito N. Strong promoting effect of Opisthorchis viverrini infection on dimethylaminoazobenzene-initiated hamster liver. *Cancer Lett* 1994; 78: 121-125

80 Lee JH, Rim HJ, Sell S. Heterogeneity of the "oval-cell" response in the hamster liver during cholangiocarcinogenesis following Clonorchis sinensis infection and dimethylaminoazobenzene treatment. *J Hepatol* 1997; 26: 1313-1322

81 Chaimuangraj S, Thamavit W, Tsuda H, Moore MA. Experimental investigation of opisthorchiasis-associated cholangiocarcinoma induction in the Syrian hamster - pointers for control of the human disease. *Asian Pac J Cancer Prev* 2003; 4: 8-95

82 Leilooi W, Yongvanit P, Wongkham C, Top sirni N, Sripa B, Sithithaworn P, Hanai S, Miwa M. Altered gene expression in Opisthorchis viverrini-associated cholangiocarcinoma in hamster model. *Mol Carcinogen* 2006; 45: 279-287

83 Changbumrun S, Tungtrongchit R, Migasena P, Chamroonengan S. Serum unconjugated primary and secondary bile acids in patients with cholangiocarcinoma and hepatocellular carcinoma. *J Med Assoc Thai* 1990; 73: 81-90

84 Kinami Y, Ashida Y, Gotoda H, Seto K, Kojima Y, Takashima S. Promoting effects of bile acid load on the occurrence of cholangiocarcinoma induced by disopropanolminosamine in hamsters. *Oncology* 1993; 50: 46-51

85 Kinami Y, Miyakoshi M, Fujikawa K. Bile acid load on the DNA distribution pattern of bile ductules and cholangiocarcinoma induced by disopropanolminosamine in hamsters. *Oncology* 1998; 55: 77-86

86 Yoon JH, Higuchi H, Werneburg NW, Kaufmann SH, Gores GJ. Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. *Gastroenterology* 2002; 122: 985-993

87 Yoon JH, Werneburg NW, Higuchi H, Canbay AE, Kaufmann SH, Akgul C, Edwards SW, Gores GJ. Bile acids inhibit Mcl-1 protein turnover via an epidermal growth factor receptor/RAF-1-dependent mechanism. *Cancer Res* 2002; 62: 6500-6505

88 Kim KM, Yoon JH, Gwak GY, Kim W, Lee SH, Jang JJ, Lee HS. Bile acid-mediated induction of cyclooxygenase-2 and McI-1 in hepatic stellate cells. *Biochem Biophys Res Commun* 2006; 342: 1108-1113

89 Mariette C, Perrais M, Leteurtre E, Jonckheere N, Hémon B, Pigny P, Batra S, Aubert JP, Triboulet JP, Van Seuningen I. Transcriptional regulation of human mucin MUC4 by bile acids in oesophageal cancer cells is promoter-dependent and involves activation of the phosphatidylinositol 3-kinase signalling pathway. *Biochem* 2004; 377: 701-708

90 Pierssen G, Jonckheere N, Vincent A, Hémon B, Ducouroiu Mf, Copin MC, Mariette C, Van Seuningen I. Regulation of the human mucin MUC4 by tumor-enhanced cyclooxygenase and taurodeoxycholic bile acids in oesophageal cancer cells is mediated by hepatocyte nuclear factor 1alpha. *Biochem* 2007; 40: 81-91

91 Charlyaleratsk S, Sirikulchayonanta V, Mayer D, Kopp-Schneider A, Förstenberger G, Marks F, Möller-Decker K. Aberrant cyclooxygenase isozyme expression in human intrahepatic cholangiocarcinoma. *Gut* 2001; 48: 80-90

92 Hayashi N, Yamamoto H, Hiraoka N, Dono K, Ito Y, Okami J, Kondo M, Nagano H, Umeshiba K, Sakon M, Matsuura N, Nakamori S, Monden M. Differential expression of cyclooxygenase-2 (COX-2) in human bile duct epithelial cells and bile duct neoplasm. *Hepatology* 2001; 34: 638-650

93 Wu T. Cyclooxygenase-2 and prostaglandin signaling in cholangiocarcinoma. *Biochim Biophys Acta* 2005; 1755; 135-150

94 Zhi YH, Liu RS, Song MM, Tian Y, Long J, Tu W, Guo RX. Cyclooxygenase-2 promotes angiogenesis by increasing vascular endothelial growth factor and predicts prognosis in gallbladder carcinoma. *World J Gastroenterol* 2005; 11: 3724-3728

95 Vadlamudi R, Mandal M, Adam L, Steinbach G, Mendelsohn J, Kumar R. Regulation of cyclooxygenase-2 pathway by HER2 receptor. *Oncogene* 1999; 18: 305-314

96 Wang SC, Lien HC, Xia W, Chen IF, Lo HW, Wang Z, Ali-Seyed M, Lee DF, Bartholomeusz G, Ou-Yang F, Giri DK, Hung MC. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. *Cancer Cell* 2004; 6: 251-261

97 Benoit V, Relic B, Leval XD, Chariot A, Merville MP, Bours V. Regulation of HER-2 oncogene expression by cyclooxygenase-2 and prostaglandin E2. *Oncogene* 2004; 23: 1631-1635

98 Han C, Wu T. Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. *J Biol Chem* 2005; 280: 24053-24063

99 Zhang L, Jiang L, Sun Q, Peng T, Lou K, Liu N, Leng J. Prostaglandin E2 enhances mitogen-activated protein kinase/Erk pathway in human cholangiocarcinoma cells: involvement of EP1 receptor, calcium and EGF receptors signaling, *Mol Cell Biochem* 2007; 305: 19-26

100 Sugawara H, Yasoshima M, Katayangaki K, Kono N, Watanabe Y, Harada K, Nakamura Y. Relationship between interleukin-6 and proliferation and differentiation in cholangiocarcinoma. *Histopathology* 1998; 33: 145-153

101 Goydos JS, Brunfield AM, Frezza E, Booth A, Lotze MT, Carle SE. Marked elevation of serum interleukin-6 in patients with cholangiocarcinoma: validation of utility as a clinical marker. *Am Surg* 1998; 227: 398-404

102 Cheon YK, Cho YD, Moon JH, Jang JY, Kim YS, Kim YS, Lee MS, Lee JS, Shim CS. Diagnostic utility of interleukin-6 (IL-6) for primary bile duct cancer and changes in serum IL-6 levels following photodynamic therapy. *Ann J Gastroenterol* 2007; 102: 2164-2170

103 Rosen HR, Winkle PJ, Kendall BJ, Diehl DL. Biliary interleukin-6 and tumor necrosis factor-alpha in patients undergoing endoscopic retrograde cholangiopancreatography. *Dig Dis Sci* 1997; 42: 1290-1294
Okada K, Shimizu Y, Nambu S, Higuchi K, Watanabe A. Interleukin-6 functions as an autocrine growth factor in a cholangiocarcinoma cell line. J Gastroenterol Hepatol 1994; 9: 462-467

Yokomuro S, Tsuji H, Lunz JG 3rd, Sakamoto T, Ezure T, Murase N, Demetris AJ. Growth control of human biliary epithelial cells by interleukin 6, hepatocyte growth factor, transforming growth factor beta1, and activin A: comparison of a cholangiocarcinoma cell line with primary cultures of non-neoplastic biliary epithelial cells. Hepatology 2000; 32: 26-35

Isomoto H, Kobayashi S, Wernberg NW, Bronk SF, Guicciardi ME, Frank DA, Gores GJ. Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. Hepatology 2005; 42: 1329-1338

Meng F, Yamagishi Y, Ueno Y, Patel T. Over-expression of interleukin-6 enhances cell survival and transformed cell growth in human malignant cholangiocarcinomas. J Hepatol 2006; 44: 1055-1065

Isomoto H, Mott JL, Kobayashi S, Wernberg NW, Bronk SF, Haan S, Gores GJ. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. Gastroenterology 2007; 132: 384-396

Han C, Demetris AJ, Stolz DB, Xu L, Lim K, Wu T. Modulation of Stat3 activation by the cytosolic phospholipase A2alpha and cyclooxygenase-2-controlled prostaglandin E2 signaling pathway. J Biol Chem 2006; 281: 24833-24846

Qiu Y, Ravi L, Kung HJ. Requirement of ErbB2 for signalling by interleukin-6 in prostate cancer cells. Nature 1998; 393: 83-85

Grant SL, Hammacher A, Douglas AM, Goss GA, Mansfield RK, Heath JK, Begley CG. An unexpected biochemical and functional interaction between gp130 and the EGF receptor family in breast cancer cells. Oncogene 2002; 21: 460-474

Webbe H, Henson R, Meng F, Mize-Berge J, Patel T. Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. Cancer Res 2006; 66: 10517-10524

Higashi M, Yonezawa S, Ho JI, Tanaka S, Irimura T, Kim YS, Sato E. Expression of MUC1 and MUC2 mucin antigens in intrahepatic bile duct tumors: its relationship with a new morphological classification of cholangiocarcinoma. Hepatology 1999; 30: 1347-1355

Matsunuma N, Yamamoto M, Aruga A, Takasaki K, Nakano M. Correlation between expression of MUC1 core protein and outcome after surgery in mass-forming intrahepatic cholangiocarcinoma. Cancer 2002; 94: 1770-1776

Shibahara H, Tamada S, Higashi M, Goto M, Batra SK, Hollingsworth MA, Imai K, Yonezawa S. MUC4 is a novel prognostic factor of intrahepatic cholangiocarcinoma-mass forming type. Hepatology 2004; 39: 220-229

Sasaki M, Ikeda H, Nakamura Y. Expression profiles of MUC mucus and trefoil factor family (TFF) peptides in the intrahepatic biliary system: physiological distribution and pathological significance. Prog Histochem Cytochem 2007; 42: 61-110

Thomas MB. Biological characteristics of cancers in the gallbladder and biliary tract and targeted therapy. Crit Rev Oncol Hematol 2007; 61: 44-51

Carraway KL, Ramauer VP, Haq B, Carothers Carraway CA. Cell signaling through membrane mucins. Bioessays 2003; 25: 66-71

Singh PK, Hollingsworth MA. Cell surface-associated mucins in signal transduction. Trends Cell Biol 2006; 16: 467-476

Singh AP, Chaturvedi P, Batra SK. Emerging roles of MUC4 in cancer: a novel target for diagnosis and therapy. Cancer Res 2007; 67: 433-436

Li Y, Yu WH, Ren J, Chen W, Huang L, Kharbanda S, Loda M, Kufe D. Hergulin targets gamma-catenin to the nucleolus by a mechanism dependent on the DF3/MUC1 oncoprotein. Mol Cancer Res 2003; 1: 765-775

Pochampalli MR, Bitler BG, Schroeder JA. Transforming growth factor alpha dependent cancer progression is modulated by Mucl. Cancer Res 2007; 67: 6591-6598

Pochampalli MR, el Bejani RM, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. Oncogene 2007; 26: 1693-1701

Ren J, Bharai A, Raina D, Chen W, Ahmad R, Kufe D. MUC1 oncoprotein is targeted to mitochondria by heregulin-induced activation of c-Src and the molecular chaperone HSP90. Oncogene 2006; 25: 20-31

Jepson S, Komatsu M, Hau B, Arango ME, Huang D, Carraway CA, Carraway KL. Muc4/sialomucin complex, the intramembrane ErbB2 ligand, induces specific phosphorylation of ErbB2 and enhances expression of p27kip1, but does not activate mitogen-activated kinase or protein kinase B/akt pathways. Oncogene 2001; 21: 7524-7532

Funes M, Miller JK, Lai C, Carraway KL 3rd, Sweeney C. The mucin Muc4 potentiates neutregulin signaling by increasing the cell-surface populations of ErbB2 and ErbB3. J Biol Chem 2006; 281: 19310-19319

Terao T, Nakamura Y, Sirica AE. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and in hepatolithiasis. Hum Pathol 2007; 38: 175-180

Lai GH, Radaeva S, Nakamura T, Sirica AE. Unique epithelial cell production of hepatocyte growth factor/ scatter factor by putative precancerous intestinal metaplasias and associated intestinal-type biliary cancer chemically induced in rat liver. Hepatology 2000; 31: 1257-1265

Schieving LA, Stevenson MC, Taylormoore JM, Traxler P, Russell WE. Integral role of the EGFR receptor in HGF-mediated hepatocyte proliferation. Biochem Biophys Res Commun 2002; 290: 197-203

Khoury H, Naujokas MA, Zuo D, Sangwan V, Frigault MM, Petkiewicz S, Dankort DL, Muller WJ, Park M. HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion. Mol Biol Cell 2005; 16: 550-561

Fischer OM, Giordano S, Comoglio PM, Ullrich A. Reactive oxygen species mediate Met receptor transactivation by G protein-coupled receptors and the epidermal growth factor receptor in human carcinoma cells. J Biol Chem 2004; 279: 35333-35341

Elmore LW, Sirica AE. "Intestinal-type" of adenocarcinoma preferentially induced in right/caudate liver lobes of rats treated with furan. Cancer Res 1993; 53: 254-259

Zhang Z, Lai GH, Sirica AE. Colecistokind-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. Hepatology 2004; 39: 1028-1037

Matsumoto K, Nakamura T. Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. Int J Cancer 2006; 119: 477-483

Benckert C, Jonas S, Cramer T, von Marschall Z, Schäfer G, Peters M, Wagner K, Radke C, Wiedenmann B, Neuhaus P, Höcker M, Rosewicz S. Transforming growth factor beta 1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells. Cancer Res 2003; 63: 1083-1092

Tang D, Nagano H, Yamamoto H, Wada H, Nakamura M, Kondo M, Ota H, Yoshioka S, Kato H, Damdinsuren B, Marubashi S, Miyamoto A, Takeda Y, Umeshiba K, Dono K, Wakasa K, Mondon M. Angiogenesis in cholangiocellular carcinoma: expression of vascular endothelial growth factor, angiopoietin-1/2, thrombospondin-1 and clinicopathological significance. Oncol Rep 2006; 15: 525-532

Yoshikawa D, Ojima H, Iwasaki M, Hiroaki N, Kosuge T, Kasai S, Hirohashi S, Shibata T. Clinopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma. Br J Cancer 2008; 99: 7055
138 Park BK, Paik YH, Park JY, Park KH, Bang S, Park SW, Chung JH, Park YN, Song SY. The clinicopathologic significance of the expression of vascular endothelial growth factor-C in intrahepatic cholangiocarcinoma. *Am J Clin Oncol* 2006; 29: 138-142

139 Aishima S, Nishihara Y, Iwai T, Taguchi K, Taketomi A, Maehara Y, Tsuneyoshi M. Lymphatic spread is related to VEGF-C expression and D2-40-positive myofibroblasts in intrahepatic cholangiocarcinoma. *Mod Pathol* 2008; 21: 256-264

140 Ogasa S, Yano H, Higaki K, Takayama A, Akiba J, Shiotani K, Kojiro M. Expression of angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in human biliary tract carcinoma cells. *Hepatol Res* 2001; 20: 97-113

141 Kawahara N, Ono M, Taguchi K, Okamoto M, Shimada M, Takenaka K, Hayashi K, Mosher DF, Sugimachi K, Tsuneyoshi M, Kuwano M. Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma. *Hepatology* 1998; 28: 1512-1517

142 Aishima S, Taguchi K, Sugimachi K, Asayama Y, Nishi H, Shimada M, Sugimachi K, Tsuneyoshi M. The role of thymidine phosphorylase and thrombospondin-1 in angiogenesis and progression of intrahepatic cholangiocarcinoma. *Int J Surg Pathol* 2002; 10: 47-56

143 Yun I, Benlimame N, Nie ZR, Xiao D, Wang T, Al Moustafa AE, Esumi H, Milani J, Hynes NE, Pages G, Aloiou-Jamali MA. Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol Cell Biol* 2002; 13: 4029-4044

144 Aloiou-Jamali MA, Song DJ, Benlimame N, Yen L, Deng X, Hernandez-Perez M, Wang T. Regulation of multiple tumor microenvironment markers by overexpression of single or paired combinations of ErbB receptors. *Cancer Res* 2003; 63: 3764-3774

145 Kloss KS, Wyszomierski SL, Sun M, Tan M, Zhou X, Li P, Yang W, Yin G, Hilttman WN, Yu D. ErbB2 increases vascular endothelial growth factor protein synthesis via activation of mammalian target of rapamycin/p70S6K leading to increased angiogenesis and spontaneous metastasis of human breast cancer cells. *Cancer Res* 2006; 66: 2028-2037

146 Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1a (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol* 2001; 21: 3995-4004

147 Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, Cheng QJ, Jove R, Yu H. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *OncoGene* 2005; 24: 5592-5596

148 Pore N, Jiang Z, Gupta A, Cervera G, Kao GD, Maity A. EGFR tyrosine kinase inhibitors decrease VEGF expression by both hypoxia-inducible factor (HIF)-1-dependent and HIF-1-independent mechanisms. *Cancer Res* 2006; 66: 3197-3204

149 Ding YB, Shi RH, Tong JD, Li XY, Zhang GX, Xiao WM, Yang JG, Bao Y, Wu J, Yan ZG, Wang XH. PGE2 up-regulates vascular endothelial growth factor expression in MKN28 gastric cancer cells via epidermal growth factor receptor signaling system. *Exp Oncol* 2005; 27: 108-113

150 Vogel CL, Franco SX. Clinical experience with trastuzumab (herceptin). *Breast J* 2003; 9: 452-462

151 Esteva FJ. Monoclonal antibodies, small molecules, and vaccines in the treatment of breast cancer. *Oncologist* 2004; 9 Suppl 3: 4-9

152 Zhang H, Benezov A, Wang Q, Zhang G, Drebin J, Murall R, Greene M. ErbB receptors: from oncogenes to targeted cancer therapies. *Cancer Invest* 2007; 117: 2051-2058

153 Rabindran SK. Antitumor activity of HER-2 inhibitors.
kinase inhibitor of epidermal growth factor receptor 1 (EGFR) and 2 (HER2) in a 2 week on 2 week off schedule. J Clin Oncol (meeting abstract) 2006; 24 Suppl: 3025

175 Rabindran SK, Discafani CM, Rosfjord EC, Baxter M, Floyd M, Golas J, Hallett BD, Nilakantran R, Overbeek E, Reich MF, Shen R, Shi X, Tsou HR, Wang YF, Wissner A. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. Cancer Res 2004; 64: 3958-3965

176 Ariyama H, Qin B, Baha E, Tanaka R, Mitsugi K, Harada M, Nakano S. Gefitinib, a selective EGFR tyrosine kinase inhibitor, induces apoptosis through activation of Bax in human gallbladder adenocarcinoma cells. J Cell Biochem 2006; 97: 724-734

177 Miyata H, Sasaki T, Kuwahara K, Serikawa M, Chayama K. The effects of ZD1839 (Iressa), a highly selective EGFR tyrosine kinase inhibitor, as a radiosensitiser in bile duct carcinoma cell lines. Int J Oncol 2006; 28: 915-921

178 Jimeno A, Rubio-Viqueira B, Amador ML, Oppenheimer D, Bouraud N, Kulesza P, Sebastiani V, Maia T, Hidalgo M. Epidermal growth factor receptor kinase dynamics influences response to epidermal growth factor receptor targeted agents. Cancer Res 2005; 65: 3003-3010

179 Hidalgo M, Amador ML, Jimeno A, Mezzadra H, Patel P, Chan A, Nielsen ME, Maia T, Altio K. Assessment of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor signaling in HuCCT-1 human cholangiocarcinoma by serial fine needle aspiration. Mol Cancer Ther 2006; 5: 1895-1903

180 Zhang Z, Sirica AE. Simultaneous inhibition of ErbB1 and ErbB2 signaling significantly enhances the growth suppression of rat and human cholangiocarcinoma cell lines. FASEB J 2007; 21: A71-A72

181 Wiedmann M, Fiesthettel J, Blüthner T, Tannapfel A, Kamenz T, Kluge A, Mössner J, Caca K. Novel targeted approaches to treating biliary tract cancer: the dual epidermal growth factor receptor and ErbB-2 tyrosine kinase inhibitor NVP-AEE788 is more efficient than the epidermal growth factor receptor inhibitors gefitinib and erlotinib. Anticancer Drugs 2006; 17: 783-795

182 Kiguchi K, Ruffino L, Kawamoto T, Ajiki T, Digiovanni J. Qin B, Baba E, Tanaka R, Mitsugi K, Harada M, Nakano S. Gefitinib, a selective EGFR tyrosine kinase inhibitor, induces apoptosis through activation of Bax in human gallbladder adenocarcinoma cells. J Cell Biochem 2006; 97: 724-734

183 Pak P, Alligood KJ, Spector NL. Anti-tumor activity of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance in patients (pts) with advanced biliary tree cancer (BTC) or hepatocellular cancer (HCC). A California Consortium (CCC-P) Trial. J Clin Oncol (meeting abstract) 2006; 24 Suppl: 4010

184 Siegel-Lakhai WS, Beijnen JH, Vervenne WL, Boot H, Keessen M, Versola M, Koch KM, Smith DA, Pandite L, Michel DJ, Schellens JH. Phase I pharmacokinetic study of the safety and tolerability of lapatinib (GW572016) in combination with oxaliplatin/fluorouracil/leucovorin (FOLFOX4) in patients with solid tumors. Clin Cancer Res 2007; 13: 4495-4502

185 Miura F, Okazumi S, Takayama W, Asano T, Makino H, Shibut K, Ochiai T. Hemodynamics of intrahepatic cholangiocarcinoma: evaluation with single-level dynamic CT during hepatic arteriography. Abdom Imaging 2004; 29: 467-471

186 Lu Y, Liang K, Li X, Fan Z. Responses of cancer cells with wild-type or tyrosine kinase domain-mutated epidermal growth factor receptor (EGFR) to EGFR-targeted therapy are linked to downregulation of hypoxia-inducible factor-1alpha. Mol Cancer 2007; 6: 63

187 Fravonic A, Gunaratnam L, Smith K, Robert I, Patten D, Lee S. Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. Proc Natl Acad Sci USA 2007; 104: 13092-13097

188 Giannelli G, Azarri A, Fransvea E, Porcelli L, Antonaci S, Paradiso A. Laminin-5 offsets the efficacy of gefitinib (Iressa) in hepatocellular carcinoma cells. Br J Cancer 2004; 91: 1964-1969

189 Kuwai T, Nakamura T, Kim SJ, Sasaki T, Kitadai Y, Langleby RR, Fan D, Hamilton SR, Fidler IJ. Intratumoral heterogeneity for expression of tyrosine kinase growth factor receptors in human colon cancer surgical specimens and orthotopic tumors. Ann J Pathol 2008; 172: 358-366

190 Ono M, Kuwano M. Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. Clin Cancer Res 2006; 12: 7242-7251

191 Pao W. Defining clinically relevant molecular subsets of lung cancer. Cancer Chemother Pharmacol 2006; 58 Suppl 1: s11-s15

192 Wang SE, Narasanna A, Perez-Torres M, Xiang B, Wu FY, Yang S, Carpenter G, Gazzard AF, Muthuswamy SK, Arteaga CL. HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer Cell 2006; 10: 25-38

193 Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. Cancer Sci 2007; 98: 1817-1824

194 Massarelli E, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, Bekele BN, Herbst RS, Wistuba II. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res 2007; 13: 2890-2896

195 Yu Z, Boggon TJ, Kobayashi S, Jin C, Ma PC, Dowlati A, Kern JA, Tenen DG, Halmos B. Resistance to an irreversible epidermal growth factor receptor (EGFR) inhibitor in EGFR-mutant lung cancer reveals novel treatment strategies. Cancer Res 2007; 67: 10417-10427

196 Gilmer TM, Cable L, Alligood K, Rusnak D, Spehar G, Gallagher KT, Woldu E, Carter HL, Truesdale AT, Shewchuk L, Wood ER. Impact of common epidermal growth factor receptor and HER2 variants on receptor activity and inhibition by lapatinib. Cancer Res 2008; 68: 571-579

197 Lu Y, Li X, Liang K, Luwor R, Siddik ZH, Mills GB, Mendelsohn J, Fan Z. Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance in patients with advanced biliary tree cancer (BTC) or hepatocellular cancer (HCC). A California Consortium (CCC-P) Trial. J Clin Oncol (meeting abstract) 2006; 24 Suppl: 4010

198 www.wjgnet.com
resistance escaping treatment by the anti-EGFR monoclonal antibody cetuximab. *Cancer Res* 2007; 67: 8240-8247

204 **Jones HE**, Gee JM, Hutcheson IR, Knowlden JM, Barrow D, Nicholson RI. Growth factor receptor interplay and resistance in cancer. *Endocr Relat Cancer* 2006; 13 Suppl 1: S45-S51

205 **Nahta R**, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* 2006; 3: 269-280

206 **Rajput A**, Koterba AP, Kreisberg JI, Foster JM, Willson JK, Brattain MG. A novel mechanism of resistance to epidermal growth factor receptor antagonism in vivo. *Cancer Res* 2007; 67: 665-673

207 **Stommel JM**, Kimmelman AC, Ying H, Nabioullin R, Ponugoti AH, Wiedemeyer R, Stogh AH, Bradner JE, Ligon KL, Brennan C, Chin L, DePinho RA. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science* 2007; 318: 287-290

208 **Mueller KL**, Hunter LA, Ethier SP, Boerner JL. Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. *Cancer Res* 2008; 68: 3314-3322

209 **Pino MS**, Shrader M, Baker CH, Cognetti F, Xiong HQ, Abbruzzese JL, McConkey DJ. Transforming growth factor alpha expression drives constitutive epidermal growth factor receptor pathway activation and sensitivity to gefitinib (Iressa) in human pancreatic cancer cell lines. *Cancer Res* 2006; 66: 3802-3812

210 **Spector NL**, Xia W, Burris H 3rd, Hurwitz H, Dees EC, Dowlati A, O’Neil B, Overmoyer B, Marcom PK, Blackwell KL, Smith DA, Koch KM, Stead A, Mangum S, Ellis MJ, Liu L, Man AK, Bremer TM, Harris J, Bacus S. Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. *J Clin Oncol* 2005; 23: 2502-2512

211 **Ishikawa N**, Daigo Y, Takano A, Taniwaki M, Kato T, Hayama S, Murakami H, Takeshima Y, Inai K, Nishimura H, Tsuchiya E, Kohno N, Nakamura Y. Increases of amphiregulin and transforming growth factor-alpha in serum as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancers. *Cancer Res* 2005; 65: 9176-9184

212 **Jimeno A**, Tan AC, Colfa J, Rajeshkumar NV, Kulesza P, Rubio-Viqueira B, Wheelhouse J, Diosdado B, Messersmith WA, Iacobuzio-Donahue C, Maitra A, Varella-Garcia M, Hirsch FR, Meijer GA, Hidalgo M. Coordinated epidermal growth factor receptor pathway gene overexpression predicts epidermal growth factor receptor inhibitor sensitivity in pancreatic cancer. *Cancer Res* 2008; 68: 2841-2849

213 **Force T**, Krause DS, Van Etten RA. Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition. *Nat Rev Cancer* 2007; 7: 332-344

214 **Johnston JB**, Navaratnam S, Pitz MW, Maniate JM, Wiehech E, Baust H, Gingerich J, Skiris GP, Murphy LC, Los M. Targeting the EGFR pathway for cancer therapy. *Curr Med Chem* 2006; 13: 3483-3492

215 **Chung CH**, Mirakhrur B, Chan E, Le QT, Berlin J, Morse M, Murphy BA, Satinover SM, Hosen J, Mauro D, Siebos RJ, Zhou Q, Gold D, Hatley T, Hicklin DJ, Platts-Mills TA. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N Engl J Med* 2008; 358: 1109-1117

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