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TOPIC HIGHLIGHT

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AKT and ERK1/2 signaling in intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinomas (ICC) are neoplasms that originate from cholangiocytes and can occur at any level of the biliary tree. Surgical resection is the current therapy of choice for this highly aggressive cancer. However, the 5-year survival still is poor, with high recurrence rates. Due to the intrahepatic growth a significant proportion of patients present with advanced disease and are not candidates for curative surgery or transplantation. The existing palliative options are of limited benefit and there is a great necessity for novel therapeutic options. In this article we review the role of the phosphoinositide 3-kinase(PI3K)/ AKT and extracellular regulated kinase (ERK) signaling pathways in ICC and present new data on the prognostic value of these protein kinases. Finally, we discuss future upcoming therapeutic options based on targeting these signaling pathways.

Key words: Extra cellular regulated kinases; Cholangiocarcinoma; Prognosis; Oncology; Immunohistochemistry

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INTRODUCTION

Cholangiocarcinomas (CCC) are rare malignant tumors arising from the biliary tract. According to their anatomic location they can be categorized as either intrahepatic or extrahepatic. Although intrahepatic cholangiocarcinoma (ICC) is less frequent than extrahepatic carcinoma, within malignant liver tumors it ranks second after hepatocellular carcinoma. The incidence of cholangiocarcinoma differs considerably in different geographic regions, with the incidence highest in Southeast Asia[1]. In western countries ICC accounts for about 10% of primary liver malignancies with increasing incidence. Established risk factors for development of cholangiocarcinoma are liver fluke infestation especially with opisthorchis viverini, primary sclerosing cholangitis, hepatolithiasis, anomalous biliary-pancreatic malformation, choledochal cysts, thorotrast exposure, liver cirrhosis and hepatitis C infection[2]. However, most cholangiocarcinomas arise in the absence of known underlying risk factors.

The prognosis of ICC is very poor. Due to its intrahepatic localization, symptoms occur late in the course of the disease and patients often present with an advanced tumor. Most patients exhibit a median survival of less than 9 mo after diagnosis. To date, complete resection has been the only curative therapy. Recent trends are to advocate accurate preoperative staging with an aggressive surgical approach to achieve complete tumor resection[2,3]. In the majority of patients ICC presents at an advanced stage or patients have associated co-morbidity that preclude surgery. This patient group needs adequate palliation such as chemotherapy. Biliary decompression, which can be achieved by surgery, radiology or endoscopy, is an additional palliative treatment option. However, since ICC rarely cause biliary congestion, these additional palliative options are rarely used.

The importance of an optimal preoperative assessment of resectability and the lack of potent optional (adjuvant) therapeutic approaches, emphasize the necessity of novel prognostic and predictive parameters. This review focuses on the relevance of two central biological signaling pathways - namely the AKT and ERK1/2 pathway - in ICC. The development of phospho-specific antibodies for immunohistochemistry allows the evaluation of signaling activity of these pathways in pathological specimens. Phosphorylation of proteins either on specific tyrosine or serine residues is a posttranslational event modulating the activity of many key signaling molecules in the cell including AKT and ERK1/2[4]. We recently demonstrated AKT and ERK phosphorylation as an independent prognostic parameter in breast cancer and colorectal cancer, respectively[5,6]. Others found an association of AKT

and ERK1/2 with decreased survival in various human malignomas including breast cancer, colorectal cancer, pancreatic cancer, malignant melanoma, leukemia and mucoepidermoid cancer[5,7-10]. This review describes the biological background of AKT and ERK in ICC, presents our own new data, analyses the potential prognostic relevance and discusses upcoming therapeutic options.

GENERAL BIOLOGY OF THE AKT AND ERK SIGNALING PATHWAYS

AKT/PKB (Protein kinase B)

AKT is a central player in the regulation of metabolism, cell survival, motility, cell cycle progression and transcription. AKT is a serine/threonine kinase and its activation is induced by phosphorylation mediated by Phosphoinositide (PI) 3-kinase (PI3K) in association with tyrosine kinase receptors. The AKT family comprises three mammalian isoforms, PKBα, PKBβ and PKBγ (AKT1, AKT2 and AKT3, respectively), which are products from different genes and share a conserved structure. AKT includes three functional domains: an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal regulatory domain. PI3K is localized upstream of the AKT kinase and is essential for the activation of AKT. PI3K and thereby AKT are activated upon (1) autophosphorylation of receptor tyrosine kinases induced by ligands (such as growth factors), (2) activation of cytokine receptors, (3) stimulation of G-Protein coupled receptors, or (4) activation of integrin signaling[11,12]. Upon PI3K mediated generation of the second messenger PtdIns (3,4,5) P_3 AKT is translocated from the cytoplasm to the plasma membrane. Once recruited to the membrane, AKT is activated by a phosphoinositide-dependent kinase 1 and 2 (PDK1, PDK2) dependent multistep process that results in the phosphorylation of both Threonine 308 and Serine 473 residues necessary for full AKT activation. Due to the great number of downstream substrates AKT kinase modulates a variety of central cellular processes as summarized in Figure 1.

ERK (extracellular regulated kinases)

ERK1/2 - also referred to as p44 and p42 MAP kinases - are ubiquitously expressed kinases and represent one component of the three mitogen activated protein kinases (MAPK) cascades that are activated by an enormous array of stimuli. The MAPK pathways phosphorylate and activate numerous proteins, including transcription factors, cytoskeletal proteins, kinases and other enzymes. Each of the three MAPK pathways contains a three-tiered kinase cascade comprising a MAP kinase kinase kinase (synonyms: MAPKKK, MAP3K, MEKK or MKKK), a MAP kinase kinase (synonyms: MPKK, MAP2K, MEK or MKK) and the MAPK. This review focuses on ERK1 and ERK2 that form a part of the MAPK module containing Raf MAPKKKs (A-Raf- B-Raf, C-Raf/Raf1) and the MEK1/MEK2 MAPKKs. All MAPKs are activated by dual phosphorylation of the conserved threonine and tyrosine residues. ERK1 and ERK2 are induced by stimuli such as tyrosine receptor kinases or G-protein coupled receptors. This leads to activation of Ras, which then triggers a complex network including the activation of Raf isoforms. ERK1/2 exhibit a variety of substrates including several key transcription factors (Figure 1). Depending upon the intensity and duration of stimulation ERK1/2 can result in proliferation of differentiation[13].

TARGETS OF THE AKT AND ERK PATHWAY

Both AKT and ERK mediate their effect *via* several substrates, which may be localized in the nuclei or in the cytoplasm. AKT can potentially phosphorylate over 9000 substrates in mammalian cells including typical cytoplasmic as well as nuclear proteins. Thus, AKT activity not surprisingly can be detected in both the nucleus and the cytoplasm[14]. When interpreting immunohistochemically stained tissue slides, it is important to keep the subcellular localization of activated AKT or ERK in mind. Moreover it seems as if both kinases show different subcellular localizations in various human carcinomas. In a colon cancer study, we showed pAKT immuno-localization in the nucleus and cytoplasm[6], in contrast to our results in ICC with a restricted immunoreactivity in the cytoplasm.

Cytosolic substrates for ERK include several pathway components involved in ERK negative feedback regulation. Multiple residues on SOS (son of sevenless homolog) are phosphorylated by ERK following growth factor stimulation. SOS phosphorylation destabilizes the SOS-Grb2 complex, eliminating SOS recruitment to the plasma membrane and interfering with Ras activation of the ERK pathway. It has also been proposed that negative feedback by ERK occurs through direct phosphorylation of the epidermal growth factor EGF receptor at Thr669[15].

ERK1 and ERK2 regulate transcription indirectly by phosphorylating the 90 kDa ribosomal protein S6 kinases (RSKs), a family of broadly expressed Ser/Thr kinases activated in response to mitogenic stimuli, including growth factors and tumor-promoting phorbol esters[16]. Active RSKs appear to play a major role in transcriptional regulation, translocating to the nucleus and phosphorylating such factors as the product of proto-oncogene *c-fos* at Ser362, serum response factor (SRF) at Ser103, and cyclic AMP response element-binding protein (CREB) at Ser133[17,18]. Recent studies revealed a spatial control of ERK signaling by Sef (similar expression of FGF), a recently identified inhibitor, whose action mechanisms are not fully defined. Sef acts as a molecular switch for ERK signaling by specifically inhibiting nuclear translocation of ERK without inhibiting its activity in the cytoplasm[19]. Further studies are necessary to elucidate the regulatory mechanisms of Sef.

Upon phosphorylation, nuclear translocation of ERK1 and ERK2 is critical for both gene expression and DNA replication induced by growth factors. Probably the best-characterized transcription factor substrates of ERKs are ternary complex factors (TCFs) including Elk-1, which is directly phosphorylated by ERK1 and ERK2 at multiple sites[20]. Upon complex formation with SRF, phosphorylated TCFs transcriptionally activate the numerous mitogen-inducible genes regulated by serum response elements

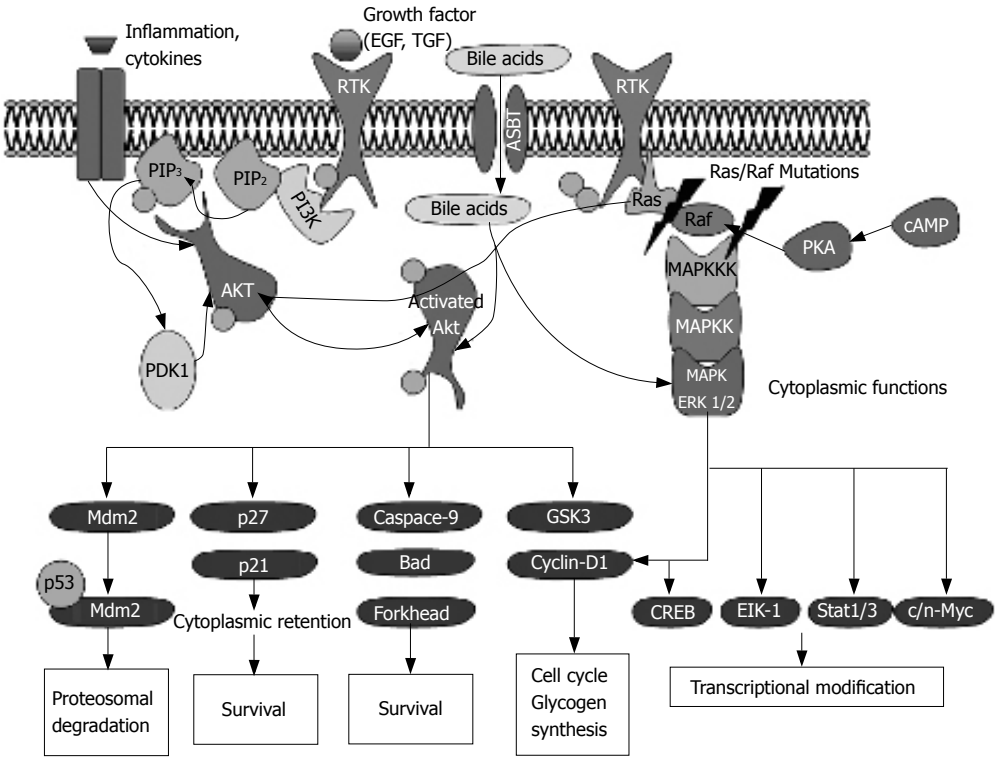

Figure 1 Overview of the AKT and ERK signaling pathway.

(SREs)[21]. Another direct target of ERK, at least *in vitro*, is the product of proto-oncogene c-*myc*, a short-lived transcription factor involved in multiple aspects of growth control[22].

AKT may mediate its functions both in the cytoplasm and in the nucleus. AKT phosphorylates a great variety of substrates involved in the regulation of key cellular functions including cell growth and survival, glucose metabolism and protein translation. Most of the well-known targets are located in the cytoplasm but it is noteworthy that many of the substrates of AKT are proteins that function in the nucleus. Some relevant cytoplasmic targets of AKT are GSK3 (glycogen synthase kinase), IRS-1 (insulin receptor substrate-1), PDE-3B (phosphodiesterase-3B), BAD, human caspase 9, Forkhead and NF-B transcription factors, BRCA1, MDM2 (murine double minute), mTOR (mammalian target of rapamycin), eNOS (endothelial nitric oxide synthase), Raf protein kinase and $p21^{Cip/Waf1}$. Several of these targets such as MDM2 translocate to the nucleus upon activation *via* AKT [23-28]. Similar to ERK1/2, immunohistochemical analysis of pAKT in various human cancers shows both cytoplasmic and nuclear immunoreactivity. *In vitro* studies showed that AKT is activated by membrane localization and later translocated to the cytosol and nucleus[29]. The function of nuclear activated AKT is not yet fully understood. Among the nuclear substrates there are fatty acid synthase[30], estrogen and androgen hormone receptors[31,32] and transcriptional factors of the FOXO family[33]. All these factors are supposed to increase cell survival.

SPECIAL ASPECTS OF AKT AND ERK IN CHOLANGIOCARCINOMA

Both the AKT and ERK pathways can be activated *via* several growth factors/survival factors, stimulation of G-protein-coupled receptors, or activation of integrin signaling. Due to the great array of activators relevant to different human cancers, we will focus on those activators that are likely to be specific for ICC.

Activation by growth factors

Both the AKT and the ERK pathway are inducible *via* growth factor stimulation. It is known that a subset of ICC exhibit overexpression of the EGF and HER-2 receptor[34]. Indeed, in our study group 21.3% (13/61) of ICC showed strong EGFR overexpression, which was confirmed by others[35]. Due to the relatively large subset of ICC with EGF or HER-2 expression activation of these ligands might be relevant for the progression of ICC.

Activation by cytokine receptors

A well-known risk factor for the development of cholangiocarcinoma is chronic inflammation. Chronically inflamed biliary epithelium is exposed to cytokines and chemokines. Indeed, cholangiocarcinoma cells constitutively secrete Interleukin 6 (IL-6), which is supposed to be a pivotal cytokine for cholangiocarcinogenesis[36-38]. A recent paper found ERK1/2 and AKT in cholangiocarcinoma cell lines to be activated *via* the cytokine receptor C-X-C motif chemokine receptor 4 (CXCR4) *via* the CXC chemokine ligand 12 (CXCL12). Thus, inflammation signals mediated by cytokine receptors are capable of inducing the ERK and AKT pathway[39].

Activation by oncogenes

It is crucial to know that AKT and ERK can be induced by mutation of oncogenes such as *Ras* and *Braf*. *Ras* is frequently mutated in many tumors, and is associated with constitutive activation of the ERK1/2 MAPKs. A

recent study showed that in contrast to hepatocellular carcinoma (HCC), *K-ras* and *Braf* mutations are a frequent event in cholangiocarcinoma[40]. In fact 22% of the cholangiocarcinoma analysed exhibited a *K-ras* mutation and 45% a *B-Raf* mutation, respectively.

Since Ras and Braf proteins are members of the Ras/Raf/MAPKKK/MAPKK/MAPK pathway, activating mutations of *K-ras* and *B-raf* should result in increased activation of MAPK. Indeed it was shown, that tumors harboring a *B-raf* mutation exhibited stronger ERK1/2 immunostaining[40]. *K-ras* mutations are a frequent event in colorectal cancer and we recently demonstrated a consecutive activation of the ERK pathway in cancers with mutated *K-ras*[6]. These data indicate that frequent disruption of alterations in the Ras/Raf/MAPKKK/MAPKK/MAPK pathway, either by *Ras* or *Braf* mutations, may play a central role in cholangiocellular carcinogenesis.

Activation by bile acids

Cholangiocytes are exposed to high concentrations of bile acids. It is known that bile acids after cellular uptake by apical sodium bile acid cotransporter (ASBT), are capable of altering the AKT and ERK signaling pathways[41]. Moreover, the EGFR-pathway appears to be activated by extracellular bile acids *via* its ligand transforming growth factor TGF-alpha in cholangiocytes[42] and the human cholangiocarcinoma cell line[43]. The EGFR pathway, once activated initiates several signaling cascades, including the AKT and ERK pathways.

ERK AND AKT PATHWAYS AND PROGNOSIS

Little is known about the prognostic relevance of the ERK and AKT pathways in intrahepatic cholangiocarcinoma. In fact, there is only one study that was based on a small cohort of 24 samples that does not discriminate between extra- and intrahepatic cholangiocarcinoma[44]. Thus, we aimed to elucidate the clinical impact of activated AKT and ERK pathways in a large and homogenous cohort of solely intrahepatic cholangiocarcinoma.

We would like to present new data derived from a large study of 62 intrahepatic cholangiocarcinomas. Between 1998 and 2006 a total of 62 patients with a mean age of 58 ± 11.5 years were available for this study. The study comprised consecutive patients who underwent surgery for liver resection. Patients solely undergoing an explorative laparatomy without subsequent resection or with hilar cholangiocarcinoma, gallbladder carcinoma or mixed hepato/-CCC were excluded from the study. The diagnosis of ICC was based on histology by examination of the resected liver specimen.

IMMUNOHISTOCHEMISTRY (IHC)

Phospho-AKT and Phospho-ERK

Immunostaining with pAKT (1/2/3) serine 473 was carried out with a monoclonal anti-phospho-AKT antibody (Cell Signaling Technology, Beverly, Massachusetts, USA). Subsequent to antibody retrieval, the primary antibodies were incubated for 30 min at 1:20 dilution. Antibody detection was performed with peroxidase-conjugated streptavidin and diaminobenzidine as chromogen. Tumors were classified according to their cytoplasmic staining intensity: negative (0), moderate (1) and strong (2).

Monoclonal phospho-p44/42 MAPK antibody (threonine 202/ tyrosine 204; Cell Signaling Technology, Beverly, Massachusetts, USA) was used at a 1:100 dilution. Tumor cells with strong specific immunostaining, independent of the amount of stained cells, were scored as strongly positive (2+). Tumors exhibiting a detectable but faint immunostaining were scored as weak (1+), whereas tumors with a minimal, hardly detectable or missing staining pattern were classified as negative (0).

EGFR

Immunostaining with EGFR was carried out with a monoclonal anti-EGFR antibody (Zymed Laboratories Inc, CA, USA). The primary antibodies were incubated for 30 min at 1:100 dilution. Antibody demonstration was achieved using the commercially available anti-mouse IgG detection kit (EnVision, DakoCytomation, Carpenteria, CA, USA). Classification was performed following the guidelines of PharmDx^{Tm} (DakoCytomation). Tumor samples lacking immunostaining were classified as negative (0), whereas the remaining were classified into 1+, 2+ or 3+ depending on the level of immunoreactivity.

Ki67 Immunostaining, TUNEL

The growth fraction was determined as previously described[45]. *In situ* DNA fragmentation was established using the terminal desoxyribonucleotide transferase (TdT)-mediated dUTP nick end labelling technique (TUNEL) as previously described[45].

Statistical analysis

Immunostainings were assessed by two of the authors in a blind-trial fashion without knowledge of the clinical outcome. Interobserver agreement[46] of pERK and pAKT was substantial (kappa = 0.78 and 0.63). Relationships between ordinal parameters were investigated using two tailed χ^2 analysis (or Fisher's exact test where patient numbers were small). Overall survival (OS) curves were estimated using the Kaplan-Meier method, and any differences in the survival curves were compared by the log-rank test.

IHC of pAKT, pERK and EGFR

Immunostaining for pAKT was localized to the cytoplasm of tumor cells. Twenty two patients (37.3%) were classified as negative, 24 (40.7%) as moderately positive and 13 (22%) as strongly positive. Immunostaining of pERK exhibited a specific nuclear and cytoplasmatic staining pattern. Representative pAKT and pERK immunostainings are shown in Figure 2. In all, 26 (41.9%) tumors lacked pERK immunostaining, 19 (30.6%) tumors exhibited a moderate staining intensity and 17 (27.4%) tumors were classified as strongly positive. Immunostaining of EGFR was localized to the membrane of the tumor cells. Due to the lack of paraffin material, two cases were excluded from EGFR-

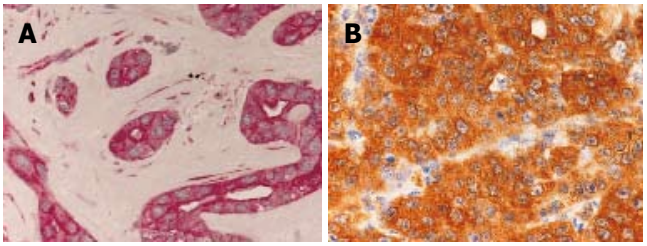

Figure 2 Light micrograph displaying strong phospho-ERK1/2 (**A**) and strong phospho-AKT (**B**) expression as analyzed by immunohistochemistry in intrahepatic cholangiocarcinoma (x 400).

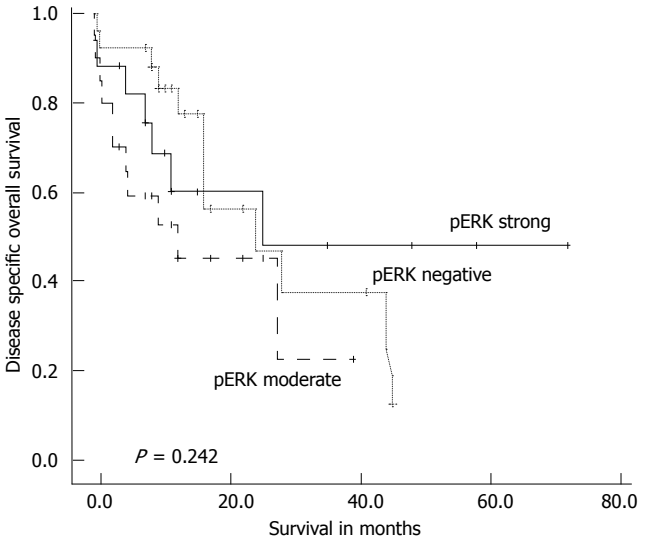

Figure 3 Kaplan-Meier survival plot for disease specific overall survival in the complete series of 62 intrahepatic cholangiocarcinoma in relation to pERK immunostaining intensity. Log-rank test: $P = 0.242$.

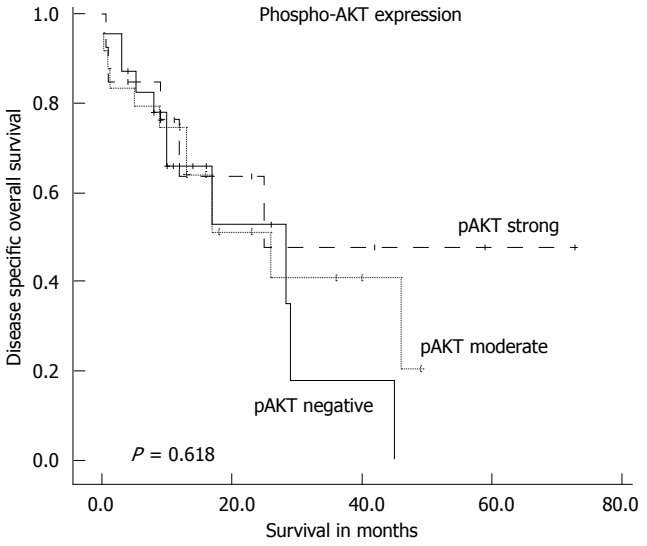

Figure 4 Kaplan-Meier survival plot for disease specific overall survival in the complete series of 59 intrahepatic cholangiocarcinoma in relation to pAKT immunostaining intensity. Log-rank test: $P = 0.618$.

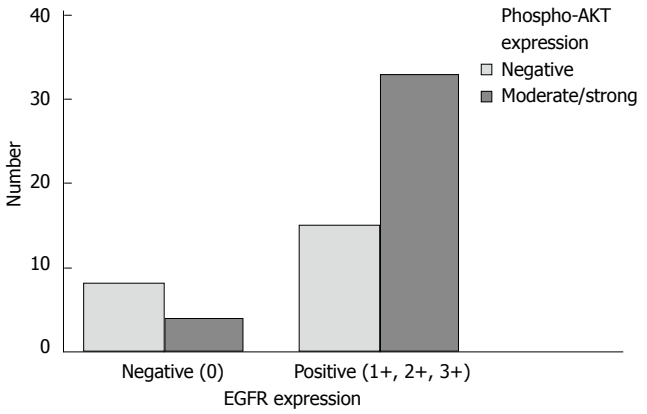

Figure 5 Intrahepatic cholangiocarcinoma with positive EGFR expression exhibit significantly more frequent AKT activation (χ^2 analysis: $P = 0.028$).

IHC. In all, 12 (19.7 %) tumors were classified as negative, 19 (31.1 %) as 1+, 17 (28.9%) as 2+ and 13 (21.3%) as 3+. χ^2 analysis revealed that EGFR-positive tumors (1+, 2+, 3+) had a statistically significant association with AKT activation ($P = 0.028$; Figure 5) but not with ERK activation. Kaplan-Meier survival analysis did not reveal a prognostic relevance of pAKT, pERK (Figures 3 and 4) or EGFR expression nor were any of the parameters associated with apoptosis or growth fraction (data not shown).

CONCLUSION

The ERK and AKT pathways are of central relevance and can induce growth factor independent growth, insensitivity to apoptotic signals, ability to invade and alter response to chemotherapeutic drugs. The fact that mutations of the Ras/Raf/MAPKKK/MAPKK/MAPK pathway occur in more than 60% of cholangiocarcinomas indicates the importance of these pathways for the carcinogenesis of cholangiocarcinomas and highlights new strategies in the treatment of this disease[47]. Moreover, the finding of a relatively large subset of ICC presenting overexpression of growth factors such as EGFR and HER-2/neu support the notion, that these growth factors play a relevant role in the progression of ICC. Our results support the notion that the AKT but not the ERK1/2 pathway is induced by EGFR overexpression, since we found a coexpression of pAKT and EGFR in ICC. However, neither ERK1/2 nor AKT activation influenced patient survival in this large series of ICC. Thus, both kinases may not serve as potential prognostic markers in this highly aggressive disease. Our results are in contrast to a previous study suggesting AKT as a favourable prognostic parameter in cholangiocarcinoma[44]. This study by Javle *et al* did not discriminate between intrahepatic and extrahepatic cholangiocarcinoma and is based on a small cohort with 24 patients; thus, the data derived should be interpreted with caution. The main advantage of the present study is the large number of patients ($n = 62$) in combination with a high homogeneity of this series composed of consecutively resected ICC.

Both the AKT and ERK pathways are topics of preclinical and clinical trials. A promising candidate is the multikinase inhibitor Sorafenib, which inhibits several

kinases including the Raf kinase, an upstream activator of the ERK pathway[48,49]. A recent study published the results of a phase Ⅱ study of sorafenib in patients with advanced hepatocellular carcinoma. It was demonstrated that HCC patients with higher pERK baseline levels had a longer time to progression following treatment with sorafenib thus pointing towards the possible relevance of activated pERK as an useful biomarker in HCC[48,49]. With regard to carcinomas of the biliary system the results of a phase Ⅱ study of sorafenib in patients with unresectable or metastastic gallbladder carcinoma or cholangiocarcinoma have been presented in Abstract form[50]. Sorafenib as a single agent did not result in a clinically significant objective response rate in patients with gallbladder and cholangiocarcinoma, but demonstrated an impact on survival that may be comparable to commonly used chemotherapy regimens. These promising data point towards novel therapeutic options utilizing multikinase inhibitors such as Sorafenib. In addition, the inhibition of upstream activators of the AKT and ERK pathways such as EGFR and HER-2 might also constitute novel therapeutic approaches. The results in a small number of patients showed that cetuximab, a monoclonal antibody against EGFR, is well tolerated and provides good palliative effects in advanced cholangiocarcinoma[51].

Despite the widely acknowledged potential of AKT inhibitors as anticancer therapy, few have made it to clinical trials. Wortmannin and LY294002 may have limited clinical utility owing to their lack of specificity, associated adverse effects, poor pharmacology, and poor solubility[52,53]. *In vivo* use of LY294002 in mice has been associated with many adverse effects, including death[54]. Furthermore, in mice, Wortmannin induces liver and bone marrow toxicity, and LY294002 can induce dermatitis and inhibit growth. Therefore, although Wortmannin and LY294002 inhibit the PI-3K/AKT pathway, their drawbacks raise doubts about their suitability as leading candidates for additional drug development.

Currently studies aimed at testing the clinical relevance of AKT inhibitors such as VQD-002, [Triciribine (TCN-P)] which is a tricyclic nucleoside that inhibits activated AKT, are being conducted. For example, the MD Anderson Cancer Center, Houston, TX (USA), and the H. Lee Moffitt Cancer Center, Tampa, FL (USA) have an ongoing Phase Ⅰ/Ⅱa clinical trial with VQD-002 in ovarian, pancreatic, breast, and colorectal cancer patients with tumors that over-express AKT (http://www.vioquestpharm.com/content/VQ0A002_about.html). Regarding the MAPK pathway, oral MEK inhibitors such as CI-1040 are currently being tested in a multicenter phase Ⅱ study in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer[55].

In addition to the above-mentioned kinases, another potential therapeutic target is worth mentioning. Against the background of NSAIDs, recent studies have evaluated selective COX-inhibitors for their effect on cell growth and invasion of cholangiocarcinoma cells *in vitro* and in nude mice[56-60]. Treatment with COX-2 inhibitors resulted in induced apoptosis and inhibited proliferation. In a recent study we demonstrated the independent prognostic value of immunohistochemical COX-2 protein expression in resected ICC[45], thereby offering a further potential additional adjuvant therapeutic approach with COX-2 inhibitors facilitating an optimised therapeutic strategy. Moreover COX-2 may serve as a target for chemoprevention in high-risk patients.

Significant progress has been made in understanding this disease, and patients are being diagnosed earlier at specialized centers. Moreover, an optimized preoperative assessment of resectability as well as an aggressive intraoperative approach to achieve complete tumor resection might increase long-term survival[3]. However, a significant proportion of patients present with advanced disease and are not candidates for curative surgery. The palliative options, mainly consisting of chemotherapy, are of limited benefit, as cholangiocarcinomas respond poorly to existing therapies. Therefore, further clinical and preclinical trials are necessary in order to develop novel therapeutic options based on new tumor targets such as AKT, ERK and EGFR. Although the activation of these pathways did not show an impact on survival in ICC - at least in this study - in contrast to many other human carcinomas, an interruption of these pathways or associated signaling molecules by specific inhibitors might, nevertheless, have favorable effects on long-term survival for this highly aggressive cancer.

REFERENCES

1 **Shaib Y**, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 115-125

2 **Patel T**. Cholangiocarcinoma. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 33-42

3 **Lang H**, Sotiropoulos GC, Frühauf NR, Dömland M, Paul A, Kind EM, Malagó M, Broelsch CE. Extended hepatectomy for intrahepatic cholangiocellular carcinoma (ICC): when is it worthwhile? Single center experience with 27 resections in 50 patients over a 5-year period. *Ann Surg* 2005; **241**: 134-143

4 **Hunter T**. A thousand and one protein kinases. *Cell* 1987; **50**: 823-829

5 **Schmitz KJ**, Otterbach F, Callies R, Levkau B, Hölscher M, Hoffmann O, Grabellus F, Kimmig R, Schmid KW, Baba HA. Prognostic relevance of activated Akt kinase in node-negative breast cancer: a clinicopathological study of 99 cases. *Mod Pathol* 2004; **17**: 15-21

6 **Schmitz KJ**, Wohlschlaeger J, Alakus H, Bohr J, Stauder MA, Worm K, Winde G, Schmid KW, Baba HA. Activation of extracellular regulated kinases (ERK1/2) but not AKT predicts poor prognosis in colorectal carcinoma and is associated with k-ras mutations. *Virchows Arch* 2007; **450**: 151-159

7 **Dai DL**, Martinka M, Li G. Prognostic significance of activated Akt expression in melanoma: a clinicopathologic study of 292 cases. *J Clin Oncol* 2005; **23**: 1473-1482

8 **Min YH**, Eom JI, Cheong JW, Maeng HO, Kim JY, Jeung HK, Lee ST, Lee MH, Hahn JS, Ko YW. Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable. *Leukemia* 2003; **17**: 995-997

9 **Xu X**, Sakon M, Nagano H, Hiraoka N, Yamamoto H, Hayashi N, Dono K, Nakamori S, Umeshita K, Ito Y, Matsuura N, Monden M. Akt2 expression correlates with prognosis of human hepatocellular carcinoma. *Oncol Rep* 2004; **11**: 25-32

10 **Yamamoto S**, Tomita Y, Hoshida Y, Morooka T, Nagano H, Dono K, Umeshita K, Sakon M, Ishikawa O, Ohigashi H, Nakamori S, Monden M, Aozasa K. Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2004; **10**: 2846-2850

11 **Wymann MP**, Zvelebil M, Laffargue M. Phosphoinositide

3-kinase signalling--which way to target? *Trends Pharmacol Sci* 2003; **24**: 366-376
12 **Foster FM**, Traer CJ, Abraham SM, Fry MJ. The phosphoinositide (PI) 3-kinase family. *J Cell Sci* 2003; **116**: 3037-3040
13 **Marshall CJ**. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 1995; **80**: 179-185
14 **Rosner M**, Hanneder M, Freilinger A, Hengstschläger M. Nuclear/cytoplasmic localization of Akt activity in the cell cycle. *Amino Acids* 2007; **32**: 341-345
15 **Northwood IC**, Gonzalez FA, Wartmann M, Raden DL, Davis RJ. Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J Biol Chem* 1991; **266**: 15266-15276
16 **Chen RH**, Chung J, Blenis J. Regulation of pp90rsk phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor-, phorbol ester-, and cyclic AMP-mediated signal transduction. *Mol Cell Biol* 1991; **11**: 1861-1867
17 **Xing J**, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* 1996; **273**: 959-963
18 **Chen RH**, Abate C, Blenis J. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc Natl Acad Sci USA* 1993; **90**: 10952-10956
19 **Torii S**, Kusakabe M, Yamamoto T, Maekawa M, Nishida E. Sef is a spatial regulator for Ras/MAP kinase signaling. *Dev Cell* 2004; **7**: 33-44
20 **Marais R**, Wynne J, Treisman R. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* 1993; **73**: 381-393
21 **Gille H**, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH, Shaw PE. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J* 1995; **14**: 951-962
22 **Gupta S**, Seth A, Davis RJ. Transactivation of gene expression by Myc is inhibited by mutation at the phosphorylation sites Thr-58 and Ser-62. *Proc Natl Acad Sci USA* 1993; **90**: 3216-3220
23 **Altiok S**, Batt D, Altiok N, Papautsky A, Downward J, Roberts TM, Avraham H. Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-Kinase/AKT in breast cancer cells. *J Biol Chem* 1999; **274**: 32274-32278
24 **Datta SR**, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999; **13**: 2905-2927
25 **Zhou BP**, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol* 2001; **3**: 245-252
26 **Zimmermann S**, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* 1999; **286**: 1741-1744
27 **Schmitz KJ**, Grabellus F, Callies R, Wohlschlaeger J, Otterbach F, Kimmig R, Levkau B, Schmid KW, Baba HA. Relationship and prognostic significance of phospho-(serine 166)-murine double minute 2 and Akt activation in node-negative breast cancer with regard to p53 expression. *Virchows Arch* 2006; **448**: 16-23
28 **Chen WS**, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev* 2001; **15**: 2203-2208
29 **Franke TF**, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. *Cell* 1997; **88**: 435-437
30 **Van de Sande T**, Roskams T, Lerut E, Joniau S, Van Poppel H, Verhoeven G, Swinnen JV. High-level expression of fatty acid synthase in human prostate cancer tissues is linked to activation and nuclear localization of Akt/PKB. *J Pathol* 2005; **206**: 214-219
31 **Vilgelm A**, Lian Z, Wang H, Beauparlant SL, Klein-Szanto A, Ellenson LH, Di Cristofano A. Akt-mediated phosphorylation and activation of estrogen receptor alpha is required for endometrial neoplastic transformation in Pten+/- mice. *Cancer Res* 2006; **66**: 3375-3380
32 **Lin HK**, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proc Natl Acad Sci USA* 2001; **98**: 7200-7205
33 **Vogt PK**, Jiang H, Aoki M. Triple layer control: phosphorylation, acetylation and ubiquitination of FOXO proteins. *Cell Cycle* 2005; **4**: 908-913
34 **Settakorn J**, Kaewpila N, Burns GF, Leong AS. FAT, E-cadherin, beta catenin, HER 2/neu, Ki67 immuno-expression, and histological grade in intrahepatic cholangiocarcinoma. *J Clin Pathol* 2005; **58**: 1249-1254
35 **Nonomura A**, Ohta G, Nakanuma 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 immunocytochemical method. *Liver* 1988; **8**: 157-166
36 **Park J**, Tadlock L, Gores GJ, Patel T. Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. *Hepatology* 1999; **30**: 1128-1133
37 **Isomoto H**, Kobayashi S, Werneburg 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
38 **Moss SF**, Blaser MJ. Mechanisms of disease: Inflammation and the origins of cancer. *Nat Clin Pract Oncol* 2005; **2**: 90-97; quiz 1 p following 113
39 **Kobayashi S**, Werneburg NW, Bronk SF, Kaufmann SH, Gores GJ. Interleukin-6 contributes to Mcl-1 up-regulation and TRAIL resistance via an Akt-signaling pathway in cholangiocarcinoma cells. *Gastroenterology* 2005; **128**: 2054-2065
40 **Tannapfel A**, Sommerer F, Benicke M, Katalinic A, Uhlmann D, Witzigmann H, Hauss J, Wittekind C. Mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. *Gut* 2003; **52**: 706-712
41 **Xia X**, Francis H, Glaser S, Alpini G, LeSage G. Bile acid interactions with cholangiocytes. *World J Gastroenterol* 2006; **12**: 3553-3563
42 **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
43 **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
44 **Javle MM**, Yu J, Khoury T, Chadha KS, Iyer RV, Foster J, Kuvshinoff BW, Gibbs JF, Geradts J, Black JD, Brattain MG. Akt expression may predict favorable prognosis in cholangiocarcinoma. *J Gastroenterol Hepatol* 2006; **21**: 1744-1751
45 **Schmitz KJ**, Lang H, Wohlschlaeger J, Reis H, Sotiropoulos GC, Schmid KW, Baba HA. Elevated expression of cyclooxygenase-2 is a negative prognostic factor for overall survival in intrahepatic cholangiocarcinoma. *Virchows Arch* 2007; **450**: 135-141
46 **Landis JR**, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**: 159-174
47 **Peyssonnaux C**, Eychène A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* 2001; **93**: 53-62
48 **Liu L**, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, Wilhelm S, Lynch M, Carter C. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 2006; **66**: 11851-11858
49 **Abou-Alfa GK**, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, De Greve J, Douillard JY, Lathia C, Schwartz B, Taylor I, Moscovici M, Saltz LB. Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2006; **24**: 4293-4300
50 **El-Khoueiry AB**, Rankin C, Lenz HJ. SWOG 0514: a phase II study of sorafenib (BAY 43-9006) as single agent in patients

(pts) with unresectable or metastatic gallbladder cancer or cholangiocarcinomas. *J Clin Oncol* 2007; **25** Suppl 18: A4639
51 **Paule B**, Bralet M, Herelle M, Rage E, Ducreux M, Guettier C, Adam R. Cetuximab plus gemcitabine/oxaliplatin (GEMOX) for patients with unresectable/recurrent intrahepatic cholangiocarcinoma refractory to GEMOX. *J Clin Oncol* 2007; **24** Suppl 18: 14084
52 **West KA**, Castillo SS, Dennis PA. Activation of the PI3K/Akt pathway and chemotherapeutic resistance. *Drug Resist Updat* 2002; **5**: 234-248
53 **Guo H**, Gao C, Mi Z, Zhang J, Kuo PC. Characterization of the PC4 binding domain and its interactions with HNF4alpha. *J Biochem* 2007; **141**: 635-640
54 **Hu L**, Zaloudek C, Mills GB, Gray J, Jaffe RB. In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). *Clin Cancer Res* 2000; **6**: 880-886
55 **Rinehart J**, Adjei AA, Lorusso PM, Waterhouse D, Hecht JR, Natale RB, Hamid O, Varterasian M, Asbury P, Kaldjian EP, Gulyas S, Mitchell DY, Herrera R, Sebolt-Leopold JS, Meyer MB. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. *J Clin Oncol* 2004; **22**: 4456-4462
56 **Zhang Z**, Lai GH, Sirica AE. Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. *Hepatology* 2004; **39**: 1028-1037
57 **Wu T**, Leng J, Han C, Demetris AJ. The cyclooxygenase-2 inhibitor celecoxib blocks phosphorylation of Akt and induces apoptosis in human cholangiocarcinoma cells. *Mol Cancer Ther* 2004; **3**: 299-307
58 **Han C**, Leng J, Demetris AJ, Wu T. Cyclooxygenase-2 promotes human cholangiocarcinoma growth: evidence for cyclooxygenase-2-independent mechanism in celecoxib-mediated induction of p21waf1/cip1 and p27kip1 and cell cycle arrest. *Cancer Res* 2004; **64**: 1369-1376
59 **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**: 303-313
60 **Lai GH**, Zhang Z, Sirica AE. Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with emodin 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

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