Adjuvant Gemcitabine Therapy Improves Survival in a Locally Induced, R0-Resectable Model of Metastatic Intrahepatic Cholangiocarcinoma

Engin Gürlevik,1* Bettina Fleischmann-Mundt,1* Nina Armbricht,1 Thomas Longerich,2 Norman Woller,1 Arnold Kloos,1 Dirk Hoffmann,3 Axel Schambach,3 Thomas C. Wirth,1 Michael P. Manns,1 Lars Zender,4 Stefan Kubicka,1,5* and Florian Kühnel1*

Complete surgical tumor resection (R0) for treatment of intrahepatic cholangiocarcinoma (ICC) is potentially curative, but the prognosis remains dismal due to frequent tumor recurrence and metastasis after surgery. Adjuvant therapies may improve the outcome, but clinical studies for an adjuvant approach are difficult and time-consuming for rare tumor entities. Therefore, animal models reflecting the clinical situation are urgently needed to investigate novel adjuvant therapies. To establish a mouse model of resectable cholangiocarcinoma including the most frequent genetic alterations of human ICC, we electroporated Sleeping Beauty-based oncogenic transposon plasmids into the left liver lobe of mice. KRas-activation in combination with p53-knockout in hepatocytes resulted in formation of a single ICC nodule within 3-5 weeks. Lineage tracing analyses confirmed the development of ICC by transdifferentiation of hepatocytes. Histologic examination demonstrated that no extrahepatic metastases were detectable during primary tumor progression. However, formation of tumor satellites close to the primary tumor and vascular invasion were observed, indicating early invasion into normal tissue adjacent to the tumor. After R0-resection of the primary tumor, we were able to prolong median survival, thereby observing tumor stage-dependent local recurrence, peritoneal carcinomatosis, and lung metastasis. Adjuvant gemcitabine chemotherapy after R0-resection significantly improved median survival of treated animals. Conclusion: We have developed a murine model of single, R0-resectable ICC with favorable characteristics for the study of recurrence patterns and mechanisms of metastasis after resection. This model holds great promise for preclinical evaluation of novel multimodal or adjuvant therapies to prevent recurrence and metastasis after R0-resection. (HEPATOLOGY 2013;58:1031-1041)

Biliary tract cancers (BTC) are characterized by aggressive adenocarcinomas that are clinically classified into gallbladder carcinomas as well as distal, perihilar, and intrahepatic cholangiocarcinomas (ICC). Within the liver, ICC is the second most common primary hepatic malignancy worldwide, with a rapidly increasing incidence.1-3 Intrahepatic and extrahepatic cholangiocarcinomas (ECC) are characterized by specific clinical challenges and disease-related risk factors.4,5 Furthermore, there is growing evidence that the frequency of characteristic genetic alterations significantly varies between ICC and ECC.6 While KRas-mutations are only observed in ~15% of ECC,7 it is the most frequent genetic alteration in ICC with an incidence of up to 54%, suggesting a central role of aberrant KRas-activation in ICC formation.8 It is also

**Abbreviations:** BTC, biliary tract cancers; CAF, cancer-associated fibroblast; ECC, extrahepatic cholangiocarcinomas; ICC, intrahepatic cholangiocarcinomas; MAPK, mitogen-activated protein kinase; R0, surgical resection

From the 1Clinic for Gastroenterology, Hepatology, and Endocrinology, Hannover Medical School, Hannover, Germany; 2Institute for Pathology, University of Heidelberg, Heidelberg, Germany; 3Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany; 4Department of Internal Medicine I, University Hospital Tübingen, Tübingen, Germany; 5Cancer Center Reutlingen, District Hospital Reutlingen, Germany.

Supported by the Deutsche Forschungsgemeinschaft (SFB-TRR77), Deutsche Krebshilfe and Wilhelm-Sander-Stiftung.

Received December 14, 2012; accepted April 15, 2013.

*Stefan Kubicka and Florian Kühnel contributed equally to the paper.
known that p53-deficient mice are prone to develop cholangiocarcinomas upon exposure to carcinogens. Recent observations in a germline genetically engineered mouse model with albumin-Cre-mediated activation of oncogenic KRas-G12D together with p53 inactivation further confirm the significant role of these molecular alterations in ICC development. It has been a long-term paradigm that ICC development is initiated by malignant transformation of intrahepatic biliary epithelial cells or liver progenitor stem cells. But most recently, two independent studies demonstrated that ICC can also arise from differentiated hepatocytes by Notch-mediated conversion into biliary lineage cells. Although the latter molecular mechanism may also sufficiently explain the observation that hepatocyte-specific clinical risk factors such as viral hepatitis and alcohol consumption can contribute to development of ICC, until now it is not known whether differentiated intrahepatic cholangiocarcinomas can also arise from adult hepatocytes by Notch-independent molecular alterations.

Complete surgical resection (R0) of the primary tumor is the preferred treatment of ICC. Along with the development of novel medical imaging technologies and refined surgical methods, increasing numbers of ICC patients will be available for resection. However, despite advances in clinical diagnosis and liver resection techniques, the prognosis of patients with R0-resected ICC is still dismal. Early tumor spreading and outgrowth of metastasis result in disease recurrence and 5-year survival of patients who underwent resection range from 15% to 40%. Retrospective analyses identified several parameters, such as small tumor size, well-differentiated tumor grade, absence of multifocal tumors, regional lymph node involvement, or vascular invasion, as independent favorable prognostic factors. The role of adjuvant therapies for patients of R0-resected ICC is under intense investigation. In a recently published meta-analysis comprising 20 studies of adjuvant therapy of BTC, the outcome of patients undergoing surgical resection with those receiving additional adjuvant therapy has been compared and a clear clinical benefit can be achieved by adjuvant therapy for high-risk patients. However, among the studies included in this meta-analysis there was only one randomized investigation and only one retrospective study on patients with ICC.

With respect to the difficulties of conducting extended clinical phase III trials for rare tumor entities, such as ICC, preclinical animal models are required for investigation of new adjuvant treatment strategies and molecular mechanisms. Reflecting clinical challenges, an ideal preclinical animal model for ICC should include the resection of the primary tumor. Therefore, we established a model for locally restricted tumor formation. Using a Sleeping Beauty-based transposon system and in vivo plasmid electroporation technique we were able to locally transduce the hepatic parenchyma. Recapitulating the most frequent molecular alterations in human ICC, oncogenic KRas-insertion combined with p53 inactivation transform adult hepatocytes in vivo into cholangiocarcinoma. Potentially curative resection of the developed single ICC nodules prolonged survival of the animals with the subsequent observation of tumor stage-dependent local recurrence and distant metastases. Since these recurrence patterns reflect the clinical situation in humans, we were able to establish for the first time a clinically relevant and reliable animal model for investigations of novel adjuvant therapies after R0-resection of ICC.

Materials and Methods

Animal Experiments. Six to eight-week-old p53fl/fl mice (Strain B6.129P2-Trp53tm1Bmr/J) were used for the experiments. Mice were anesthetized with ketamine (100 mg/kg intraperitoneally, Albrecht, Germany) and xylazin (10 mg/kg intraperitoneally, Bayer, Germany) for 60-90 minutes and the left liver lobe or tumor was prepared for electroporation or resection, respectively.

Ethics Approval. All in vivo experiments were conducted according to the German guidelines for animal care and use of laboratory animals (TierSchG) with the approval of the Hannover Medical School animal facility.

Address reprint requests to: Stefan Kubicka, M.D., or Florian Kuehnel, Ph.D., Clinic for Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Carl Neuberg Str. 1, 30625 Hannover, FR Germany. E-mail: Kubicka.stefan@mh-hannover.de, or kuehnel.florian@mh-hannover.de

Copyright © 2013 by the American Association for the Study of Liver Diseases. View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.26468

Potential conflict of interest: Dr. Kubicka consults, advises, and is on the speakers’ bureau for Roche, Amgen, and Sanofi.

Additional Supporting Information may be found in the online version of this article.
For electroporation of the liver, the Square Wave Electroporator (CUY21SC, Nepa Gene, Japan) was used. Electric pulses for plasmid transfer into the liver tissue were generated with a tweezers-type electrode (CUY650P5, 5 mm diameter). The large lobe of the liver was used for subcapsular DNA injection (50 μL of 0.5 μg/μL DNA) with a 27G needle and the injected region was then placed between the electrode disks. Two electric pulses were administered twice with 75 msec duration at a voltage of 75 mV and an interval of 500 msec.

For the resection, a laparotomy was performed and the tumor-bearing liver lobe was prepared by cutting the connective tissue between liver and diaphragm. Without touching the tumor, a very fine bipolar forceps (0.4 mm, ERBE, Germany) was positioned proximal to the porta hepatitis followed by coagulation at 15 W. The coagulation line was cut with a scissors and the affected lobe was extracted. To facilitate reproducibility, resection margin and size of remaining tissue was controlled to confirm almost complete removal of the lobe. The extension of resection (removal of the tumor-bearing liver lobe) was identical for all tumors. For sham-operation the tumor-bearing livers were left untreated after laparotomy. The abdominal wound was closed by suturing. During the surgical procedure, mice were kept under infrared light until awakening. Mice received metamizol (0.8 mg/mL, Ratiopharm, Germany) with drinking water as postoperative analgesia. For adjuvant therapy, gemcitabine (100 mg/kg bodyweight) was injected intraperitoneally once weekly for 4 weeks.

Plasmids. For Sleeping Beauty-mediated integration, we used the hyperactive transposase construct pPGK-SB13 as described (kindly provided by David A. Largaespada, Univ. of Minnesota). As transposon plasmid for subsequent cloning procedures, we used the pT3/EF1α plasmid as backbone containing duplicated inverted repeats and EF1α promoter (Xin Chen, UCSF, Addgene plasmid 31789). All cloning procedures are described in the Supporting Materials. For expressing Cre-recombinase the plasmid pPGK-Cre-bpA was used (Klaus Rajewsky, MDC, Berlin, Addgene plasmid 11543).

Histologic Analyses and Immunohistochemistry. Tissue specimens were fixed in 4% buffered formalin and embedded in paraffin. For histopathological analysis, samples were sectioned (2 μm) and stained with hematoxylin and eosin (H&E). For native green fluorescent protein (GFP) detection, sections were covered with citifluor (Citiflour, London, UK) and investigated by fluorescence microscopy. For immunohistochemical studies the following antibodies were used: anti-GFP/Egfp (ab290-50, Abcam), anti-HNF4α (ab41898, Abcam), anti-CK19 (14-9898-82, eBioscience), and anti-vimentin (ab92547, Abcam) with Alexa-Fluor488 or Alexa-Fluor555 (Invitrogen) coupled secondary antibody. Nuclei were counterstained with DAPI (Sigma). Phospho-ERK1/2 was visualized by DAB-staining. Sections were treated with 3% H2O2 and incubated with the primary pERK1/2 (p44/42)-antibody (4376, Cell Signaling), secondary biotin-anti-rabbit-antibody (Invitrogen), streptavidin-HRP (Invitrogen), and DAB (Zytomed). Nuclei were counterstained with hematoxylin.

Statistics. To determine statistical significance, survival curves were analyzed by log-rank test. P < 0.05 was considered statistically significant.

Additional materials and methods are provided in the Supporting Materials.

Results

Induction of a Locally Restricted Tumor Nodule. To initiate a locally restricted, single tumor nodule in the liver, which is accessible to complete removal by surgical resection, we established an orthotopic gene transfer model using in situ electroporation of oncogenic plasmids. After subcapsular injection of plasmids for Sleeping-Beauty transposase-mediated genomic transgene integration, the injected liver region was placed between the electrode disks and electric pulses were applied for local tissue transfection (Fig. 1A). Since KRas-activation and p53-alteration can be frequently found in BTC, predominantly in the ICC subtype, we investigated this genetic setup in mice. For this purpose, a transposon plasmid encoding for mutant KRas-G12V was used for SB13 transposase-mediated insertion into the genome of electroporated cells (Fig. 1B). p53-knockout was realized by using p53fl/fl mice and co-delivery of a plasmid for Cre-recombinase, thus keeping the restriction to electroporated cells. After injection of plasmids into the liver followed by immediate electroporation, we observed reliable formation of a single tumor nodule in all animals within 3-5 weeks, indicating potent oncogenic activity of KRas-G12V on the background of genetic p53-knockout in the adult liver of mice (Fig. 1C).

Investigations of the Origin of Transduced Cells After Electroporation. Since the cellular origin of ICC is a matter of current debate, we addressed the nature of transduced cells. Successfully electroporated cells could be easily identified with a plasmid expressing EGFP under control of the constitutively active...
EF1α-promoter. For cell lineage determination we performed costainings for HNF4α as a classical marker of hepatocytes and for CK19 to detect cholangiocytes. We observed that all electroporated cells expressing EGFP were also positive for HNF4α, indicating that the electroporation technique resulted in successful transfection of hepatocytes (Fig. 2A, upper lane). In contrast, EGFP expression could not be observed in any cell which was positive for the biliary cell marker CK19 (Fig. 2A, lower lane). Separate quantitation of EGFP-positive hepatocytes and cholangiocytes in a set of tissue-sections confirmed that electroporation failed to transduce cholangiocytes (Fig. 2B). To verify these observations by lineage tracking experiments, we designed three reporter plasmids for expression of EGFP under the control of the EF1α-promoter (upper lane) or the albumin-promoter (middle lane). In contrast, CK19-promoter dependent, cholangiocyte-specific EGFP expression was completely absent (Fig. 2C, lower lane). For a quantitative confirmation of these results, messenger RNA (mRNA) from the electroporated tissue area was isolated (Fig. 2D). The figure shows significant EGFP expression under transcriptional control of the albumin or EF1α-promoter. EGFP expression was again absent if a construct under control of the CK19-promoter was applied. These data confirm that hepatocytes, but not cholangiocytes, are the primary target of transduction by the electroporation procedure.

**Oncogenic KRas-G12V and p53-Inactivation in Liver Lead to Tumor Formation With Histopathologic Features of ICC.** Based on genetic analysis, KRas-activation in combination with p53-knockout seems to promote ICC. As a result of electroporation of the left liver lobe, reliable formation of a single tumor nodule was observed in mice that received KRas-G12V-insertion and p53-knockout triggered by transient coexpression of the Cre-recombinase (Fig. 3A). In contrast, KRas-G12V-activation or p53-knockout alone did not induce tumor growth, at least within the time investigated. The chosen oncogenic setup limited the life span of mice to 5-8 weeks following electroporation due to primary tumor progression. Analysis of tumor growth after electroporation by measuring the tumor area in histologic sections showed that growth was slightly decelerated after rapid growth between days 14 and 21 (Fig. 3B). When we performed histopathologic examinations in tissue of primary tumor and the tumor margin, we could clearly identify a mass-forming, intrahepatic cholangiocarcinoma reflecting the bile-ductular type, which is characterized by glandular and ductular structures intersected by extended regions of tumor stroma (Fig. 3C, upper lane). It is well known that activated KRas leads to downstream induction of the mitogen-activated protein kinase (MAPK)-pathway by phosphorylation of Erk. Staining tumor tissue slices for phospho-Erk revealed that ductular cells are highly positive for this MAPK-pathway activation marker (Fig. 3C, lower lane). Transformed cells should also be deleted of p53 by the cotransfected Cre-recombinase. To analyze KRas expression and p53-knockout, single tumor cells were isolated from tumor tissue and then analyzed by quantitative polymerase chain reaction (qPCR). Using this approach, tumor cells can be completely separated from tumor stroma and healthy liver cells, thus allowing accurate
determination of tumor-specific p53 and KRas levels. Complementary DNA (cDNA) quantification of tumor cells showed high expression of KRas, whereas p53 was absent in comparison with nontransduced liver of p53fl/fl mice or the murine tumor cell line CMT64 (p53wt/KRas-G12V) as controls (Fig. 3D). To further confirm the complete p53-knockout, genotyping of isolated tumor cells was performed showing Cre-mediated recombination of intron 1 and intron 10 (Fig. 3E, bottom lane). For comparison, the non-recombined loxP-sites within the introns were amplified from liver tissue of nontransduced mice as well as wild-type alleles from the cell line CMT64, where the corresponding PCR products are shorter (Fig. 3E, middle lanes). As internal control, an exon 11-specific PCR-fragment was detected in all analyzed samples (Fig. 3E, upper lane).

At the timepoint of death due to primary tumor growth, no metastases could be detected in any other organs. However, we observed satellites that were located close to the primary tumor. Importantly, these satellites seemed to already affect the vascular system, as they could be found to infiltrate vessels located in the periportal field (Fig. 3F, left). Staining phospho-ERK in these satellite structures also showed MAPK-pathway activation (Fig. 3F, right). Linking expression of KRas-G12V to GFP by way of an IRES-motif allowed us to identify those cells within the tumor that are direct progeny of initially transduced cells after electroporation. Microscopical analyses of tumor

Fig. 2. Electroporation resulted in transduction of hepatocytes. (A) Livers of p53fl/fl mice were electroporated using a transposon-based plasmid expressing EGFP under control of the constitutively active EF1α-promoter. Three days after electroporation, livers were harvested and the electroporation scar was investigated by paraffin sections. The upper lane shows a coimmunofluorescence after staining of EGFP (green) and HNF4α (red), whereas a communostaining of EGFP (green) and CK19 (red) is shown in the bottom lane. Nuclei were counterstained with DAPI (400× magnification). (B) By microscopic inspection of a set of tissue-sections, EGFP-fluorescence was separately assessed in HNF4α-positive hepatocytes and CK-19-positive cholangiocytes (mean + SD, n = 10 sections). (C) Livers were electroporated by transposon-based plasmids containing an EGFP expression cassette under the control of different tissue-specific/constitutive promoters including the constitutively active EF1α-promoter, the hepatocyte-specific mouse albumin-promoter (prAlb), or the cholangiocyte-specific mouse CK19-promoter, respectively. Three days after electroporation, livers were investigated for native EGFP-fluorescence in unstained paraffin sections (C, left row) and aligned with the corresponding serial H&E-stained sections (C, right row; 200× magnification). (D) Total RNA was isolated from the electroporated region of the liver tissue and EGFP mRNA levels were determined by RT-qPCR (mean ± SD, n = 3 mice per group).
Fig. 3. KRas-G12V expression in combination with p53-knockout promotes development of ICC. Livers of p53fl/fl mice were electroporated with a transposon-based plasmid for expression of the KRas-G12V oncogene, or with the Cre-recombinase expression plasmid, or with a combination of both. (A) Survival of mice after electroporation was monitored (n = 19 per group). (B) After electroporation of the plasmids pT/KRas-G12V and PGK-Cre, mice were harvested at the indicated timepoints and tumor size was determined (mean ± SD, n = 4 mice per timepoint). (C) Tumor-bearing livers were harvested and investigated by histological sections. The figure shows an H&E (upper lane) and phopho-Erk staining (lower lane) of tumor tissue (left) and the tumor margin (right; 100× magnification). (D) Cell cultures from tumors were obtained and single-cell-derived colonies were investigated for mRNA levels of KRas (left) and p53 (right) by RT-qPCR. The results were compared with healthy liver of p53fl/fl mice or the tumor cell line CMT64 (mean ± SD, n = 3 cultured single cell colonies from n = 2 individual tumors). (E) p53flox genotyping of DNA from liver of p53fl/fl mice (3′ and 5′ LoxP-site alleles), cultured single cell tumor colonies (Trp53D2-10 allele, representative example of n = 3 cultured colonies) and mouse tumor cell line CMT64 (Trp53 wildtype alleles). (F) H&E-stained section from liver tissue adjacent to the tumor showing a tumor satellite with vascular invasive character (left). The bile duct is indicated by black arrow, the artery by a white arrow, and the central vein with infiltrating tumor cells is marked by an asterisk (200× magnification). The phospho-Erk staining of a satellite is shown on the right side (200× magnification).
sections for GFP-fluorescence showed ICC-characteristic glandular structures, which were formed by cells carrying the KRas-G12V oncogene (Fig. 4A). To confirm the classification of these tumors by cell lineage-specific markers, coimmunostainings were performed (Fig. 4B). In addition to the GFP-fluorescence indicating KRas-G12V-expressing tumor cells, sections were additionally stained for HNF4α to visualize tumor cells that may have retained a hepatocellular character (Fig. 4B, upper lane). In GFP-positive tumor cells, we could not detect any costaining of HNF4α. HNF4α expression was restricted to the adjacent nonmalignant liver parenchyma. To investigate the biliary cell character of the tumor, CK19 costaining was performed (Fig. 4B, middle lane). The figure shows CK19-costaining in oncogene-positive glandular structures, which represent the malignant backbone of ICC. With regard to our above-described findings that ICCs derived from electroporated hepatocytes, this result suggests that tumor cells acquired biliary lineage characteristics during the process of transformation and tumor progression. In ICC, glandular and ductal tumor structures are frequently found embedded in stromal cancer-associated fibroblasts (CAFs). CAFs can be visualized by staining of vimentin. Corresponding costaining on tumor sections showed a high number of vimentin-positive cells surrounding the glandular structures (Fig. 4B, lower lane). Since these cells do not express KRas-G12V/GFP and are therefore not progeny of tumor cells, they are most likely CAFs as part of a desmoplastic stroma reaction to the tumor.

Resection of Neoplasms at the Early Stage Significantly Improves Survival of Tumor-Bearing Mice. The established tumor model of ICC has the striking advantage of resectability due to locally restricted growth within one liver lobe. Next, survival of
mice after resection of liver tumors was monitored and tumor size at the timepoint of resection was determined to investigate a possible correlation between prognosis and tumor size. Therefore, tumors were classified in three groups with regard to their size. After resection, histopathologic analysis revealed pathologic formations that were characteristically associated with the tumor size (Fig. 5A, left). In particular, satellites could be observed in tumors achieving a primary tumor size of more than 5 mm in diameter (Fig. 5A, right). Tumors exceeding 10 mm in diameter develop high-grade, poorly differentiated cancer cells with the loss of glandular and ductal structures (Fig. 5A, bottom). Survival monitoring clearly showed that the outcome is directly correlated with the tumor size (Fig. 5B). We observed that resection at early stages was curative (tumor size ≤3 mm). However, if tumors reached a size of more than 5 mm, resection was no longer curative, but was at least life-prolonging when compared to large tumors with a diameter exceeding

Fig. 5. Tumor size at the time of surgical resection is a major determinant of survival. Livers of p53fl/fl mice were electroporated with PGK-Cre and pT/Kras-G12V. After tumor development, tumor diameter was determined, neoplastic tissue was resected, and inspected by histology. (A) Tumors were classified by tumor size and representative histologies are shown for each size class (left, 100× magnification). Satellite formation was investigated at 200× magnification (right). (B) Survival was monitored from the timepoint of resection. Mice were classified with regard to tumor size at time of resection (n = 8 mice each group). (C) Histologies of local tumor recurrence/intrahepatic metastasis (100× magnification, left) and distant lung metastasis (200× magnification, right) as examples of disease relapse after resection. (D) Resected specimens were orthogonally sectioned to the resection margin and stained with H&E. A representative example of the microscopic analysis is shown. In all samples, the resection margin (left; 40× magnification) was found with a minimum distance of 9 mm to the most proximal satellite structure. Satellites (indicated by black arrow) were detectable with a maximum distance of 700 μm to the margin of the primary tumor (right; 100× magnification).
10 mm. Histopathologic analysis of mice to examine possible causes of death showed disease relapse with strong local tumor recurrence/intrahepatic metastases (Fig. 5C, left) accompanied by peritoneal carcinomatosis and small lung metastases (Fig. 5C, right lane). Since metastases could not be found in nonresected animals, the prolonged life span gained by resection obviously allows for the outgrowth of circulating micrometastases. As an important hallmark of R0-resection, the resection margin should be absolutely tumor-free. In orthogonally sectioned samples of tumors following resection, in all samples we achieved a minimum distance of at least 9 mm between tumor tissue and resection margin (Fig. 5D, left). Since satellites were detectable with a maximum visible distance of around 700 μm to the tumor margin, the performed resections were actually R0 (Fig. 5D, right).

**Adjuvant Therapy With Gemcitabine Prolongs Survival.** It is common sense in clinical oncology that satellite formation with vascular invasion during tumor progression can be regarded as a strong indication for local tumor recurrence after resection. Concepts for adjuvant therapies are recommended and deserve more detailed investigations to overcome life-limiting disease relapse. Therefore, we tested in our model the therapeutic efficacy of adjuvant gemcitabine treatment as used in recent clinical cholangiocarcinoma trials. At first, gemcitabine treatment as a palliative approach in sham-operated mice showed no survival benefit compared to the sham-operated control group (Fig. 6), whereas tumor resection itself resulted in prolonged survival compared to both sham-operated groups. Compared to the resection group, we observed a significant survival benefit by an adjuvant gemcitabine approach. Therefore, our data indicate a life-prolonging outcome if gemcitabine is used in the adjuvant setting after surgical R0-resection of ICC. Together, our results show that our established model reflects both genetic and clinical characteristics of human ICC and thus represents an excellent tool to investigate novel adjuvant ICC therapies in an animal model of highly predictive value.

**Discussion**

Intrahepatic cholangiocarcinoma is representative of tumor entities where resection of the primary tumor is the only option for cure, but frequent disease recurrence limits survival. In general, clinical development of novel cancer therapies is expensive and time-consuming, since a large number of promising agents or strategies fail in late-stage clinical trials. Those disappointing results emphasize that preclinical animal models of reliable predictive value are urgently required, particularly for investigations of adjuvant systemic therapies.

To meet these predictive requirements genetically engineered mouse models have been developed and several new treatment strategies could be developed on the basis of these models. However, limitations of germline models, such as time-consuming generation, unpredictable tumor formation, and low genetic flexibility lead to the establishment of nongermline models such as the mosaic tumor model using intrahepatic transfer of oncogenic transposons by retrograde liver transduction. This transposon-based model provides exceptional genetic flexibility and a short induction time. However, due to the simultaneous development of multiple tumor foci, the mosaic model cannot be used for investigations of novel therapies in the adjuvant setting, where potentially curative R0-resection is mandatory. We investigated in this study an approach to induce an autochthonously grown and resectable tumor by local transfection of the liver parenchyma using electroporation techniques. To this end, we used transposase-mediated oncogenic KRas-G12V-insertion combined with Cre-mediated p53-knockout, which resulted in locally restricted formation of an intrahepatic tumor. Histopathologic analyses and coimmunostainings demonstrated development of ICC characterized by expression of the biliary marker CK19 within the tumor cells. CK19 expression was clearly connected to cellular insertion of the oncogenic KRas-G12V transposon. Additionally, the desmoplastic
stroma surrounding the characteristic glandular tumor structures was visualized by vimentin staining of CAFs. Primary tumor growth analysis and survival showed that the oncogenic effect of the KRas-G12V mutant was only exerted in combination with p53-knockout. Within the investigated time, KRas-G12V insertion or p53-knockout alone did not result in any tumor formation. Since we anticipated the predominant transduction of hepatocytes but not cholangiocytes, the exclusive development of liver tumors with characteristic histologic features of ICC was remarkable. By lineage-tracing experiments we indeed identified hepatocytes as primary target cells of electroporation-mediated gene transfer but not cholangiocytes, which have been expected as a source of ICC. However, the originating cell type of ICC is currently a matter of debate since an in vitro study has provided initial evidence for transdifferentiation of mature hepatocytes into bile duct cells. Further evidence for the Notch-driven conversion of hepatocytes was confirmed with an elaborate transgenic mouse model of ICC by Sekiya and Suzuki. In contrast, we investigated the role of the most abundant genetic alterations of ICC and demonstrated that genetic engineering of adult hepatocytes in vivo by oncogenic KRas-activation and p53-inactivation also resulted in ICC formation. Our results suggest the existence of Notch-independent mechanisms enabling hepatobiliary transdifferentiation. Our model of KRas/p53-induced ICC is consistent with a recent study using a genetic albumin-Cre mouse model for activation of KRas-G12D and p53-knockout wherein the majority of tumors could be classified as ICC. However, in contrast to our study, where ICC was exclusively detectable, some tumors in this germline model were identified as hepatocellular carcinoma (HCC) or showed a mixed phenotype. These differences may be explainable by the fact that we transformed adult cells, whereas the albumin-Cre-mediated KRas/p53-induced ICC is initially activated in liver progenitors during late embryogenesis.

Outgrowth of metastases and disease recurrence is life-limiting in ICC patients after surgical resection of the tumor. In our model, we could not detect any manifest metastasis when the primary tumor was not removed by resection. However, primary tumor formation was frequently accompanied by formation of satellites and vascular invasion, which can be interpreted as a premetastatic stage. Because of single tumor formation, we could use this model to investigate the pattern of tumor recurrence after curative R0-resection and the impact of adjuvant systemic therapy in ICC. After surgical resection of the primary tumor, we observed a clear correlation between survival outcomes and tumor stages at the time of surgery, which were classified by tumor size and histopathologic grading. While R0-resection was curative in early ICC, we observed a high incidence of local recurrence/ intrahepatic metastases, peritoneal carcinomatosis, and frequent manifestation of lung metastases in advanced ICC. These findings reflect the clinical situation in humans, where the liver is the most common site of recurrence after resection of the primary tumor in ICC patients. Therefore, adjuvant systemic therapy is the favorable treatment of biliary tract cancers. With adjuvant gemcitabine chemotherapy we could show a significantly improved survival of animals after R0-resection. In contrast to the ineffective palliative gemcitabine treatment in our model, our results strongly recommend adjuvant gemcitabine chemotherapy for advanced ICC.

We established for the first time a murine tumor R0-resection model of an autochthonously developed ICC. Our animal model overcomes several important drawbacks of germline genetically engineered mouse models. Therefore, it holds great promise for preclinical evaluation of novel targeted therapies. Most important, this model facilitates investigations of how tumor genetics influence the outcome of adjuvant and neoadjuvant therapies. In addition, it appears likely that the locoregional electroporation method of oncogenic transposons can be easily adapted to genetic and morphologic modeling of further tumor entities to allow for predictive preclinical studies.

References

1. Patel T. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. Hepatology 2001;33:1353-1357.
2. 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.
3. Hammill CW, Wong LL. Intrahepatic cholangiocarcinoma: a malignancy of increasing importance. J Am Coll Surg 2008;207:594-603.
4. Gatto M, Braguzzi MC, Semeraro R, Napoli C, Gentile R, Torrice A, et al. Cholangiocarcinoma: update and future perspectives. Dig Liver Dis 2010;42:253-260.
5. Patel T. Cholangiocarcinoma. Nat Clin Pract Gastroenterol Hepatol 2006;3:33-42.
6. Hezel AF, Deshpande V, Zhu AX. Genetics of biliary tract cancers and emerging targeted therapies. J Clin Oncol 2010;28:3531-3540.
7. Rashid A, Ueki T, Gao YF, Houllihan PS, Wallace C, Wang BS, et al. K-ras mutation, p53 overexpression, and microsatellite instability in biliary tract cancers: a population-based study in China. Clin Cancer Res 2002;8:3156-3163.

8. Tannapfel A, Benicke M, Katalinic A, Uhlmann D, Koeckler F, Haus J, et al. Frequency of p16(INK4A) alterations and K-ras mutations in intrahepatic cholangiocarcinoma of the liver. Gut 2000;47:721-727.

9. 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.

10. O'Dell MR, Huang JL, Whitney-Miller CL, Deshpande V, Rothberg P, Grose V, et al. Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma. Cancer Res 2012;72:1557-1567.

11. Sempoux C, Fan C, Singh P, Obeidat K, Roayaie S, Schwartz M, et al. Cholangiolocellular carcinoma: an innocent-looking malignant tumor mimicking ductular reaction. Semin Liver Dis 2011;31:104-110.

12. Jiang W, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. A prognostic scoring system based on clinical features of intrahepatic cholangiocarcinoma. Ann Oncol 2011;22:1644-1652.

13. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. J Clin Invest 2012;122:3914-3918.

14. Tyson GL, El-Serag HB. Risk factors for cholangiocarcinoma. HEPATOLOGY 2011;54:173-184.

15. Puhalla H, Schuell B, Pokorny H, Kornek GV, Scheithauer W, Gruenwald C. Cholangiolocellular carcinoma: an innocent-looking malignant liver tumor mimicking ductular reaction. Semin Liver Dis 2011;31:104-110.

16. Weber SM, Jarnagin WR, Klimstra D, DeMatteo RP, Fong Y, Blumgart LH. Intrahepatic cholangiocarcinoma: resectability, recurrence pattern, and predictive factors. HPB (Oxford) 2011;13:59-63.

17. Lieser MJ, Barry MK, Rowland C, Ilstrup DM, Nagorney DM. Surgical management of intrahepatic cholangiocarcinoma: a 31-year experience. J Hepatobiliary Pancreat Surg 1998;5:41-47.

18. Endo I, Gonen M, Yopp AC, Dalal KM, Zhou Q, Klimstra D, et al. Intrahepatic cholangiocarcinoma: rising frequency, improved survival, and determinants of outcome after resection. Ann Surg 2008;248:84-96.

19. Jarnagin WR, Shoup M. Surgical management of cholangiocarcinoma. Semin Liver Dis 2004;24:189-199.

20. Jiang W, Zeng ZC, Tang ZY, Fan J, Sun HC, Zhou J, et al. A prognostic scoring system based on clinical features of intrahepatic cholangiocarcinoma: the Fudan score. Ann Oncol 2011;22:1644-1652.

21. Guglielmi A, Ruzzenente A, Campagnaro T, Pachera S, Valdegamberi A, Nicoli P, et al. Intrahepatic cholangiocarcinoma: prognostic factors after surgical resection. J Clin Oncol 2012;30:1934-1940.

22. Horgan AM, Amir E, Walter T, Knox JJ. Adjuvant therapy in the treatment of biliary tract cancer: a systematic review and meta-analysis. J Clin Oncol 2012;30:1934-1940.

23. Suzuki T, Shin BC, Fujikura K, Matuzuaki T, Takata K. Direct gene transfer into rat liver cells by in vivo electroporation. FEBS Lett 1998;425:436-440.

24. Carlson CM, Frandsen JL, Kirchhof N, Melvor RS, Largespada DA. Somatic integration of an oncogene-harboring Sleeping Beauty transposon models liver tumor development in the mouse. Proc Natl Acad Sci U S A 2005;102:17059-17064.

25. Tward AD, Jones KD, Yant S, Cheung ST, Fan ST, Chen X, et al. DISTINCT pathways of genomic progression to benign and malignant tumors of the liver. Proc Natl Acad Sci U S A 2007;104:14771-14776.

26. Rijken AM, van Gulik TM, Polak MM, Gouma DJ. Diagnostic and prognostic value of incidence of K-ras codon 12 mutations in resected distal bile duct carcinoma. J Surg Oncol 1998;68:187-192.

27. Nault JC, Zucman-Rossi J. Genetics of hepatobiliary carcinogenesis. Semin Liver Dis 2011;31:173-187.

28. Ozaki M, Nakajima Y, Kakehi K, Kumagai K, Takéuchi M, Nakajima M, et al. Ki-ras mutations and p53 protein expressions in intrahepatic cholangiocarcinomas: relation to gross tumor morphology. Gastroenterology 1995;109:1612-1617.

29. Isi T, Tomita S, Nakachi A, Miyazato H, Shimoji H, Kusano T, et al. Analysis of microsatellite instability, K-ras gene mutation and p53 protein overexpression in intrahepatic cholangiocarcinoma. Hepatogastroenterology 2002;49:604-608.

30. Nakamura Y, Sato Y, Harada K, Sasaki M, Xu J, Ikeda H. Pathological classification of intrahepatic cholangiocarcinoma based on a new concept. World J Hepatol 2010;2:419-427.

31. Sirica AE. The role of cancer-associated myofibroblasts in intrahepatic cholangiocarcinoma. Nat Rev Gastroenterol Hepatol 2012;9:44-54.

32. Nathan H, Aloia TA, Vauthey JN, Abdalla EK, Zhu AX, Schulick RD, et al. A proposed staging system for intrahepatic cholangiocarcinoma. Ann Surg Oncol 2009;16:14-22.

33. Sala M, Fuster J, Llovet JM, Navasa M, Sole M, Varela M, et al. High pathological risk of recurrence after surgical resection for hepatocellular carcinoma: an indication for salvage liver transplantation. Liver Transpl 2004;10:1294-1300.

34. Murakami Y, Uemura K, Hayasidani Y, Sudo T, Hashimoto Y, Ohge H, et al. Indication for postoperative adjuvant therapy in biliary carcinoma based on analysis of recurrence and survival after surgical resection. Dig Dis Sci 2009;54:1360-1364.

35. Ellis MC, Cassera MA, Verto JT, Orloff SL, Hansen PD, Billing KG. Surgical treatment of intrahepatic cholangiocarcinoma: outcomes and predictive factors. HPB (Oxford) 2011;13:59-63.

36. Heyer J, Kwong LN, Lowe SW, Chin L. Non-germline genetically engineered mouse models for translational cancer research. Nat Rev Cancer 2010;10:470-480.

37. Singh M, Lima A, Molina R, Hamilton P, Clermont AC, Devasthali V, et al. Assessing therapeutic responses in Kras mutant cancers using genetically engineered mouse models. Nat Biotechnol 2010;28:585-593.

38. Zuber J, Radtke I, Pardee TS, Zhao Z, Rappaport AR, Luo W, et al. Genetic and epigenetic mechanisms of therapeutic resistance in Kras(G12D) tumor models liver tumor development in the mouse. Proc Natl Acad Sci U S A 2005;102:17059-17064.

39. Zuber J, Radtke I, Pardee TS, Zhao Z, Rappaport AR, Luo W, et al. Mouse models of human AML accurately predict chemotherapy response. Genes Dev 2009;23:877-889.

40. Nishikawa Y, Ushio Y, Watanabe H, Tokiati T, Omori Y, Su M, et al. Transdifferentiation of mature rat hepatocytes into bile duct-like cells in vitro. Am J Pathol 2005;166:1077-1088.