**Abstract**

Intrahepatic cholangiocarcinoma (IHCC) is a primary cancer of the liver with an increasing incidence and poor prognosis. Preclinical studies of the etiology and treatment of this disease are hampered by the relatively small number of available IHCC cell lines or genetically faithful animal models. Here we report the development of a genetically engineered mouse model of IHCC that incorporates two of the most common mutations in human IHCC, activating mutations of Kras (Kras<sup>G12D</sup>) and deletion of p53. Tissue-specific activation of Kras<sup>G12D</sup> alone resulted in the development of invasive IHCC with low penetrance and long latency. Latency was shortened by combining Kras<sup>G12D</sup> activation with heterozygous or homozygous deletion of p53 (mean survival of 56 weeks vs. 19 weeks, respectively), which also resulted in widespread local and distant metastasis. Serial analysis showed that the murine models closely recapitulated the multistage histopathologic progression of the human disease, including the development of stroma-rich tumors and the premalignant biliary lesions, intraductal papillary biliary neoplasms (IPBN), and Von Meyenburg complexes (VMC; also known as biliary hamartomas). These findings establish a new genetically and histopathologically faithful model of IHCC and lend experimental support to the hypothesis that IPBN and VMC are precursors to invasive cancers. *Cancer Res;* **72**(6); 1557–67. © 2012 AACR.

**Introduction**

Intrahepatic cholangiocarcinoma (IHCC) is thought to arise from the intrahepatic biliary system based on common histologic and molecular properties (1). These tumors are characterized by an aggressive course and early metastasis. The incidence of IHCC is on the rise and although effective palliative chemotherapy has recently been defined, treatment options for the majority of patients remain limited (2, 3). Many aspects of its biology and genetics remain incompletely defined including key questions relating to (i) the cell(s)-of-origin, (ii) the precursor lesions in the liver, their molecular profiles, and their relationship to fully established IHCC, and (iii) the functional impact of oncogenes and tumor suppressor genes on malignant progression.

Although the genetic basis for IHCC has not been fully elucidated, mutations in a number of established oncogenes and tumor suppressors are well described. Recurrent mutations have been observed in the KRAS oncogene, which is activated in 20% to 50% of tumors (4–6). Mutations in BRAF were described in 2 European cohorts but have not been reported in the U.S. studies (1). p53 inactivation is the most common tumor suppressor lesion, observed in 37% of IHCC (7). In addition, subsets of IHCC show mutations or deletions of SMAD4 and p16<sup>INK4A</sup> (4, 8).

Pathologic studies of the biliary system in diseased livers and of biliary lesions adjacent to IHCC have led to a proposed multistage progression model for the development of invasive cancers from the normal hepatic epithelium (9). In particular, lesions known as biliary intraepithelial neoplasia (BilIN) and intraductal papillary biliary neoplasms (IPBN) are thought to be precursors of IHCC and have been graded according to the degrees of architectural distortion and cellular atypia (10, 11). Additional lesions of the biliary tract include malformations of the ductal systems referred to as biliary hamartomas or Von Meyenburg complexes (VMC), although the relationship of these lesions to IHCC is less clear (12, 13). Importantly, despite these detailed pathologic descriptions, the genetic features of the different biliary ductal lesions and the capacity of these lesions to give rise to IHCC remain undefined.

Experimental model systems have been central to providing basic and preclinical insights into many cancer types. Although a number of advances have been made in this regard in IHCC,
there is currently an incomplete array of systems for the study of this malignancy. For example, only a small number of IHCC cell lines are reported in the literature, with most published experimental studies employing no more than 2 or 3 lines. Alternatively, studies often employ a combination of IHCC, EHCC, and gallbladder cell lines, although these different types of biliary cancer carry distinct mutational profiles. A number of carcinogen-induced models of primary liver tumors in mammalian systems have been described (14–17) and the transduction of viral oncoproteins has also enabled transformation of carcinogen-treated hepatic epithelium both in vitro and in vivo (18, 19). Genetically engineered mouse (GEM) models have also been developed to model tumors with similarities to IHCC. p53 mutant mice develop cholangiocarcinomas upon repeated carcinogen exposure, although there is long latency in this model (20). Liver-targeted delivery of mouse poloma virus middle T antigen (PyMT) using a transgenic avian retroviral system induces focal regions of IHCC as well as more prominent hepatocellular carcinoma (HCC) lesions in Trp53 and Ink4a/Arf knockout mice (21). Mixed HCC/IHCC histology is also seen in mice with liver-specific inactivation of the NF2, Savi, and Mst1/Mst2 tumor suppressor genes, although HCC is the predominant component in each case (reviewed in ref. 22). Combined homozygous deletion of conditional Smad4 and Pten alleles in the liver via crosses to the Albumin-Cre strain causes tumors histologically similar to IHCC (23). Although providing important systems to study malignant transformation of liver cells, these models have not been reported to exhibit progressive precursor lesions of the biliary tract nor do they accurately incorporate the most common genetic lesions seen in the human disease.

GEM models designed to mimic both genetic and pathologic aspects of cancer have proven critical to drug development efforts, biomarker identification, and the study of early disease (24, 25). To create a model of IHCC based on oncogenic mutations commonly observed in the human disease, we generated compound mutant mice with Albumin-Cre–mediated somatic activation of KrasG12D and deletion of p53 in the hepatic parenchyma. We report that cooperation between these 2 relevant genetic alterations in the hepatic epithelium leads to a model of IHCC that recapitulates the histologic and molecular features of multistage progression of human IHCC. We employ this model to study the role of preinvasive lesions as an indicator of disease in the presence of fresh complete media rinsed 2× with PBS and scraped off the plate in the presence of ice-cold lysis buffer. Cell Signaling lyse buffer (catalog no. 9803) including Sigma Protease Inhibitor Cocktail (catalog no. P8340), Sigma Phosphatase Inhibitor Cocktail 2 (catalog no. P5726), and Sigma Phosphatase Inhibitor Cocktail 3 (catalog no. P0044) were used for all experiments. Protein concentration was determined using the Bradford assay.

**Materials and Methods**

**Mice: mutant mouse strains**

All animal studies were conducted in accordance with the AAALAC accredited University Committee on Animal Resources (UCAR). All mouse strains used in these studies have been previously described and characterized (26–28). Specifically KrasG12D, p53−/−, and Alb-Cre mutants stained were intercrossed to achieve the desired cohorts as outlined above. The genetic background was mixed. Individual mice within experimental cohorts were followed until signs of illness including poor grooming, abdominal bloating, diminished activity, or weight loss, at which point a full necropsy was done followed by histologic analysis.

**Histology**

Two board-certified pathologists with a specialization in hepatic histopathology independently reviewed and classified tumors. In all cases there was agreement about the histologic diagnosis. Lymph nodes, lungs, and spleen were included in a survey for metastasis in all individuals with tumors.

**Tumor cell lines**

After sampling of tumors for histology and molecular profiling, 3- to 5-mm samples of tumor derived from mutant mouse strains described above were cut adjacent to sample evaluated histologically and subjected to collagenase/trypsin digestion. After washing, cells were placed in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum and fed until a confluent monolayer was formed. Cells were passaged 3 to 4 times before molecular characterization that included IF staining with CK-19 to establish ductal origin and repeated genotyping for Kras and p53 to establish hepatic origin.

**Immunohistochemistry**

Formalin-fixed paraffin sections were hydrated and heated antigen retrieval was carried out when necessary. Sections were then incubated with primary antibody overnight at 4°C. Species- and isotype-matched IgG were used in place of the primary antibodies as a negative control.

**Western bloting**

Whole tissues (liver and whole tumor) were snap frozen in liquid nitrogen and crushed immediately using an electronic pestle into ice-cold lysis buffer. Cells used were grown to 60% to 80% confluence in the presence of fresh complete media rinsed 2× with PBS and scraped off the plate in the presence of ice-cold lysis buffer. Cell Signaling lyse buffer (catalog no. 9803) including Sigma Protease Inhibitor Cocktail (catalog no. P8340), Sigma Phosphatase Inhibitor Cocktail 2 (catalog no. P5726), and Sigma Phosphatase Inhibitor Cocktail 3 (catalog no. P0044) were used for all experiments. Protein concentration was determined using the Bradford assay.

**Antibodies**

Cell Signaling: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; H:C10) #2118, P-Akt Ser473 #9271, Akt #9272, p44/42 mitogen-activated protein kinase (MAPK; Erk1/2, 137F5) #4695, P-p44/42 MAPK (Erk1/2) #9201/Tyr204; D13.14.4E) #4695, p53 (1C12) #2524, LC3A (D5068) #4599, Santa Cruz: alpha-Tubulin (TU-02) sc-8035, p21 (H-164) sc-756, p16 (M-156) sc-1207, Smad4 (B-8) sc-7966, cytokeratin 19 (M-17): sc-3311, Abcam: p19ARF ab80. Progen: p62. Dako: pan-CK #Z0622 1:1000, AFP (4A0008) 1:400.
Kras and p53 Cooperate to Cause Intrahepatic Cholangiocarcinoma

**Autophagy assays**

Culture conditions: cells were grown in DMEM with 10% FBS, l-glutamine, and no antibiotics, and media was changed every 1 to 2 days or the morning before analysis. pBabe-LC3-GFP (Addgene, 11546) was used to produce virus using NIH3T3 cells. Standard infection protocol with polybrene was followed to establish low-passage cell explants with the GFP-LC3 autophagy reporter. To determine GFP-LC3 foci, cells were plated onto cover slips in the presence and absence of chloroquine (CQ) at the indicated dosage and fixed with 4% paraformaldehyde. Confocal images were taken with FV1000 Olympus laser scanning microscope. At least 50 cells were counted for each cell line under different treatment conditions. Cells containing more than 5 puncta were considered positive for autophagy. For the cell proliferation assays, cells were seeded in 24-well plates at 10,000 cells per well. Treatment with CQ at the indicated dose was done the day after plating. At the indicated time points, cells were fixed with 10% formalin and stained with 0.1% crystal violet. Dye was extracted with 10% acetic acid and optical density at 595 nm was determined as a measure of relative cell density.

**Statistical analysis**

All statistical analysis was done using Prism statistical software version 4.0a May 11, 2003. Survival was determined by the absence of liver pathology on autopsy were considered positive for autophagy. Animals that displayed signs of illness and were found to have advanced cancers on necropsy were included as events for survival analysis. Animals that died at autopsy. Animals that died for reasons other than advanced cancer, as determined by the absence of liver pathology on autopsy, were censored.

**Results**

Mutations in Kras and p53 are among the most commonly described genetic changes in IHCC. To model these genetic features in the mouse, we used a conditionally activated allele for Kras$^{G12D}$ (LSL-Kras$^{G12D}$) and a conditional knockout allele for p53 (p53$^{lox/lox}$, refs. 26, 27). The LSL-Kras$^{G12D}$ allele is expressed at endogenous levels after Cre-mediated excision of a transcriptional stopper element and the p53$^{lox/}$ allele is engineered to sustain Cre-mediated excision of exons 2 to 10 rendering the gene functionally inactive. Although the cell of origin of IHCC is not established, both the differentiated biliary epithelial cells as well as resident progenitor cells within the liver, commonly referred to as "oval cells," have been implicated (29). Further complicating the understanding of the ontogeny of IHCC is the finding that human IHCC harbors regions of abnormal intermediate hepatocytes and that HCC expresses markers typically seen in IHCC (30). Given the undefined cellular origins of these tumors, we sought an approach that would target mutations throughout the adult. To accomplish this, we used the Albumin-Cre (Alb-Cre) transgene that is initially active in liver progenitors during late embryogenesis and has been shown to produce effective recombination of floxed alleles in both the adult hepatocytes and cholangiocytes (Fig. 1A; ref. 28). Cohorts of compound mutant mice were created with the experimental genotypes Alb-Cre;Kras$^{G12D}$ (n = 8), Alb-Cre;Kras$^{G12D}$/p53$^{fl}$ (n = 20), and Alb-Cre;Kras$^{G12D}$/p53$^{+/}$ (n = 22)—from here on designated Kras, Kras-p53$^{1/1}$, and Kras-p53$^{3/3}$, respectively.

**Kras$^{G12D}$ cooperates with p53 inactivation to cause hepatic transformation**

Kras, Kras-p53$^{3/3}$, and Kras-p53$^{1/1}$ cohorts were produced at the expected ratios and showed no evidence of early developmental abnormalities. These animals were monitored until they developed signs of illness (abdominal bloating, ascites, etc.). All statistical analysis was done using Prism statistical software version 4.0a May 11, 2003. Survival was determined by the absence of liver pathology on autopsy were considered positive for autophagy. Animals that displayed signs of illness and were found to have advanced cancers on necropsy were included as events for survival analysis. Animals that died at autopsy. Animals that died for reasons other than advanced cancer, as determined by the absence of liver pathology on autopsy, were censored.

**Figure 1.** Kras$^{G12D}$ and p53 mutation in the hepatic epithelium cooperate to cause cholangiocarcinoma. A, modeling strategy; compound mutant Mice harboring albumin-Cre transgene, LSL-Kras$^{G12D}$, and p53$^{lox/lox}$ were created to conditionally activate Kras$^{G12D}$ and delete p53 in the hepatic epithelium. Lox P sites are indicated with red triangles. B, Kaplan–Meier survival analysis of cohorts of compound mutant animals. The p53 status is indicated; all of the mice have the LSL-Kras$^{G12D}$ allele. C, gross tumor nodules in the Kras-p53 model. Arrowheads indicate tumor nodules seen in an animal with 3 distinct tumors. The arrow in image (ii) indicates enlarged lymph nodes. The tumor in the lower aspect of image (i) is cystic and fluid filled and was found to be an IPBN.
diminished activity, and cachexia). Most of the Kras mice were healthy up to an age of 75 weeks, although 1 of 8 developed a lethal liver mass at 36 weeks of age. p53 deficiency greatly increased the penetrance and accelerated disease onset as the Kras-p53L/+ and Kras-p53L/L mice developed tumors as early as 32 and 9 weeks, respectively (mean survival 52 weeks and 19 weeks; Fig. 1B). Upon necropsy these animals were found to have solid liver tumors ranging in size from 2 to 20 mm and presented as isolated nodules (n = 14 mice) or as multiple independent lesions (n = 6 mice; Table 1). In many animals, cystic fluid-filled lesions were found adjacent to the solid masses (Fig. 1C). Hepatic lesions frequently showed hemorrhage into the peritoneal cavity and had evidence of tumor necrosis. Tumors were highly metastatic, with 75% of tumor-bearing animals displaying gross evidence of invasion into adjacent organs (diaphragm, bowel, pancreas, and stomach) or distant metastasis (with dissemination to the lymph nodes, spleen, lungs, and peritoneal cavity; Fig 2B and Table 1). Mice harboring Alb-Cre;p53L/L (n = 10) were followed to 1 year of age without evidence of illness, and subsequent necropsy and histologic survey did not reveal abnormal hepatic pathology (data not shown). Thus, Kras\textsuperscript{G12D} promotes metastatic liver tumorigenesis that is significantly accelerated by the heterozygous and homozygous inactivation of p53.

**Table 1. Tumor histology, number of nodules, precursor lesions found, sites of metastasis, and age**

<table>
<thead>
<tr>
<th>ID</th>
<th>Age (wk)</th>
<th>Tumor type</th>
<th>Size (cm)</th>
<th>Cancer precursor</th>
<th>Gross metastasis/invasion</th>
</tr>
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<tr>
<td>Kras\textsuperscript{G12D} P53\textsuperscript{L/L}</td>
<td>100</td>
<td>9.0</td>
<td>HCC</td>
<td>1.5</td>
<td>None</td>
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<tr>
<td>460</td>
<td>11.7</td>
<td>Mixed</td>
<td>0.5</td>
<td>IPBN</td>
<td>Lung, spleen, under liver diaphragm, stomach, pancreas</td>
</tr>
<tr>
<td>199</td>
<td>13.1</td>
<td>CC</td>
<td>1.5</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>444</td>
<td>15.7</td>
<td>CC</td>
<td>2.0</td>
<td>Unknown</td>
<td>Diaphragm, intestine, pancreas, lung</td>
</tr>
<tr>
<td>537</td>
<td>18.1</td>
<td>CC</td>
<td>1.0</td>
<td>VMC</td>
<td>Lung, diaphragm, lymph nodes</td>
</tr>
<tr>
<td>518</td>
<td>18.3</td>
<td>CC</td>
<td>0.4</td>
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<tr>
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<td>VMC</td>
<td>Lower lymph nodes</td>
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<tr>
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<td>19.4</td>
<td>HCC</td>
<td>3.0</td>
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<td>None</td>
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<td>0.5</td>
<td>VMC</td>
<td>Diaphragm</td>
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<tr>
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<td>23.3</td>
<td>CC</td>
<td>1.0</td>
<td>None</td>
<td>Lung</td>
</tr>
<tr>
<td>476</td>
<td>32.0</td>
<td>CC</td>
<td>0.5</td>
<td>None</td>
<td>Lungs, lymph nodes</td>
</tr>
<tr>
<td>719</td>
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<td>CC</td>
<td>1.0</td>
<td>IPBN</td>
<td>Lung, diaphragm, lymph nodes</td>
</tr>
<tr>
<td>232</td>
<td>31.9</td>
<td>HCC</td>
<td>2.0</td>
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<td>None</td>
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<tr>
<td>339</td>
<td>37.6</td>
<td>CC</td>
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<td>None</td>
</tr>
<tr>
<td>231</td>
<td>39.1</td>
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<td>Intestine, pancreas</td>
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<tr>
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<tr>
<td>338</td>
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<td>344</td>
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<tr>
<td>408</td>
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<td>CC</td>
<td>1.6</td>
<td>None</td>
<td>Lungs, lymph nodes, intestine</td>
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<tr>
<td>Kras\textsuperscript{G12D} P53\textsuperscript{L/+}</td>
<td>495</td>
<td>35.7</td>
<td>CC</td>
<td>2.0</td>
<td>Unknown</td>
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</table>

**Albumin-Cre: Kras GEM models recapitulate the histopathologic features of human IHCC**

Microscopic analysis of the liver tumors revealed that 83% showed IHCC histology, with 66% (n = 19) containing exclusively IHCC and 17% (n = 5) showing a mixed phenotype of IHCC and HCC elements. A smaller number had exclusively HCC histology (17% n = 5; Table 1, and Fig. 2A). The IHCC showed a range of cellular differentiation, including well-differentiated tumors with clear glandular architecture and areas of mucin production, as well as poorly differentiated tumors with minimal gland formation and large pleomorphic cells showing nuclear atypia and frequent mitosis (Fig. 2A). Often different grades of differentiation were seen in the same tumor. The histologic spectrum of IHCC was similar among the different mouse cohorts.
To further characterize the tumors, we stained tissue sections for cytokeratins (pan-CK) that mark the biliary epithelium and have been used clinically to distinguish IHCC from HCC. Correspondingly, immunochemistry for pan-CK revealed robust staining of the regions with the biliary ductal morphology, consistent with IHCC histopathology (Fig. 2C). In mixed tumors, the areas of glandular epithelium stained positive for pan-CK (Fig. 2Aiii arrowhead and Fig. 2C arrowhead), whereas the regions morphologically consistent with HCC were pan-CK negative (Fig. 2Aiii arrow). We also stained tissue sections for alpha-fetoprotein (AFP) a marker of HCC. Immunochemistry for AFP showed staining of all HCC and HCC components of mixed tumors, whereas IHCC and IHCC components of mixed tumors did not stain (Fig. 2Ciii). Early metastasis is a hallmark of human IHCC and similarly microscopic IHCC metastases were found in nearly all animals examined, including those without evident gross metastasis. These lesions were found in the lung, lymphatic system and the spleen, peritoneal cavity, and veins (Fig. 2B). Trichrome staining revealed collagen deposition throughout the IHCC but not in most HCC, a pattern reminiscent of the corresponding human tumors, in which IHCCs, but not HCC, are characterized by a dense stroma (desmoplasia; Fig 2D). Overall, the histopathologic appearance of the murine IHCC displayed a striking similarity to the human disease, exhibiting varying degrees of differentiation and early metastasis.

**IHCC is associated with IPBN and Von Meyenburg complexes, or biliary hamartomas**

Histologic survey of hepatic parenchyma adjacent to IHCC, as well as isolated regions of grossly normal liver, revealed the presence of premalignant lesions within the bile ducts. These lesions resembled intraductal papillary neoplasms of the bile ducts (IPBN) and biliary hamartomas (also known as VMC) that have been described in humans (11, 12). In several cases, there was evidence that the IHCC arose directly from IPBN (Fig. 1C and Fig. 3B). The presence of additional lesions in regions distal to the primary tumors suggests that there was multifocal initiation of these precursor lesions. IPBN were grossly evident (Fig. 1Cii—lower cystic lesion with arrowhead), characterized by dilated bile ducts (Fig. 3Ai and iii) and were found connected to the biliary tree. These structures were lined by pancreaticobiliary and intestinal epithelium and exhibited varying degrees of dysplasia, with a notable frequent association of the largest lesions with contiguous areas of invasive carcinoma (Fig. 3B). Although a papillary architecture was not universally appreciated, the presence of low-grade dysplasia in some areas and high-grade dysplasia/carcinoma in situ in others mimicked...
the typical presentation seen in the human disease (Fig 3Ai–iv). These observations support the IPBN-to-IHCC progression model for this cancer type.

In addition to the IPBN, another type of structural abnormality was seen in focal regions of the biliary ducts, consisting of dilated, haphazardly arranged ductal structures embedded in a fibrous stroma adjacent to portal areas. Such structures, found in 36% of animals harboring IHCC ($n = 5/14$; Fig. 4i–v), showed resemblance to biliary hamartomas or VMCs described in humans. Whereas these lesions were typically small and focused, in 2 animals we also observed more extensive lesions that extended through an entire lobe of the liver (Fig. 4Aii). Whereas VMCs are generally regarded as benign, recent case reports of cholangiocarcinoma arising from a VMC have raised the question of its potential role as an IHCC precursor lesion (12, 31). Consistent with this notion, we found IHCC
apparently arising directly from adjacent VMC in 4 animals (Fig. 4ii). VMC showed strong staining for pan-CK consistent with their ductal morphology (Fig. 4ii and 4bii). The findings of both IPBN and VMC, both independent of and in association with IHCC, among mutant Kras-p53 animals provided experimental evidence for a progression model of IHCC that includes both IPBN as well as VMC.

**Molecular features of IHCC**

To characterize the molecular features of IHCC arising in the Kras-p53 mice, we used both early-passage cell lines derived from the primary tumors and whole tumor lysates. Tumor cells were used to evaluate other established tumor suppressor genes and provide a pure tumor-derived material without stromal contamination. CK-19, a common marker of IHCC, confirmed the origins of early-passage tumor explants with all derivative cells showing positive immunostaining (data not shown). As expected p53 and its downstream target, p21, were not expressed in the IHCC (Fig. 5A) consistent with somatic deletion (data not shown). Among tumors arising in Kras-p53L/+, mice, we found loss of the wild-type allele in 4 of 6 animals consistent with spontaneous deletion during the course of tumorigenesis (Fig. 5B). Next, we examined Smad4 and p16Ink4a, which are additional tumor suppressors known to be inactivated in human IHCC (4, 32, 33). Western blot analysis showed that Smad4 expression was maintained in all 6 IHCC examined, whereas p16Ink4a expression was absent in 2 lines (335 and 460). The p19Arf tumor suppressor, expressed from a common locus with p16Ink4a, was also absent in line #335, suggesting somatic deletion of the Ink4a/Arf (CDKN2A) locus. All other lines retained various levels of p19Arf expression in these p53-deficient tumors, consistent with the overlapping functions of p53 and p19Arf in constraining tumor progression (34). The spontaneous loss of wild-type p53 in tumors arising in the Kras-p53L/+ cohort and the spurious loss of p16Ink4a mirrors the molecular profile of the human disease and is in keeping with key functions of the p53/p19Arf and Rb/p16Ink4a pathways in suppressing KRAS-driven tumorigenesis.

Next we carried out a molecular analysis of IHCC to determine the status of pathways that are commonly altered in human IHCC. Whole IHCC lysates were compared with both normal liver as well as with normal appearing liver from Kras-p53 animals. Both MAPK/mitogen-activated protein kinase extracellular signal–regulated kinase (MEK) and phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathways have been shown to be active and important for growth and survival in tumors characterized by KRAS mutation, and strategies to target these downstream effectors of KRAS are in development in IHCC (35, 36). Consistent with KRAS activation and signaling through MAPK/MEK signaling pathways, we found total ERK1/2 levels to be elevated in tumors compared with both normal liver as well as with normal appearing liver from Kras-p53 animals (Fig. 5C) that latter of which exhibited no increased activity of the pathway despite the presence of a Kras mutation in the majority of cells. Phosphorylation of residues Thr 202 and Tyr 204 indicative of Erk1/2 activity was present in IHCC, but not found in the untransformed hepatic epithelium (Fig. 5C). Similarly PI3K/AKT pathway activity was seen as

**Figure 5.** IHCC harbors molecular derangements characteristic of the human disease and activation of downstream RAS effector pathways. A. early-passage (P1-3) IHCC cell lysates were tested for the expression of tumor suppressor genes. Twenty-five micrograms of protein were loaded in each lane and murine pancreatic cell lines with previously defined genetic profiles (p53 or Ink4a/Arf mutant vs. wild type) were used as controls (49). p53 expression is absent in all IHCC, as is p21 expression. Whereas the majority of tumors retained expression of p19Arf, line #335 lost expression. Lines #460 and #335 both showed absent p16Ink4a expression. SMAD4 expression was maintained across all lines. B. PCR reactions to detect the p53 WT (+) and p53lox alleles (top) and p53-null (−) allele (bottom) in normal tissue (tail clip) from p53lox/lox (lane 10), p53−/− (lane 9), and p53 lox/− (lane 8) mice and in tumor cell lines (lanes 1-7). Primers and conditions used are previously described in detail (27). A total of 4/6 tumor cell lines from Kras-p53−/− animals (232, 338, 344, and 408) showed no WT allele, indicating loss of heterozygosity at the p53 locus. Tumor line 449 shows retention of the p53 null allele with longer exposure (data not shown). Tumor cell line #449 from a Kras-p53−/− animal shows retention of the p53+ allele. C, Western blot analysis of whole liver lysates from 10 weeks old wild-type and Kras-p53+/− animals compared with IHCC tumor lysates evaluating the relative activity of RAS effectors ERK and AKT showing activity of ERK and AKT. Total LC3 levels were increased in IHCC as compared with normal and mutant liver (LC3 is lower band, upper band is nonspecific).
evidenced by phosphorylation of AKT Ser 473 in IHCC. Enhanced expression and activation of components of both MAPK/MEK and PI3K pathways is consistent with the observations made among human tumors as well as with other tumors driven by KRAS and points toward the potential utility of inhibitors under development targeting these pathways.

**IHCC are characterized by elevated levels of autophagy required for growth**

The panel of cell lines from our GEM model provides a genetically defined experimental system to begin to test the role of key pathways in the pathogenesis of the KRAS-p53 mutant subset of IHCC. Notably, tumors driven by mutated \textit{RAS} have been difficult to target as compared with cancers which harbor more "drugable" mutated or amplified oncoproteins (37). Combinational approaches focused on key downstream pathways, such as combined PI3K and MEK inhibition as discussed above, may offer strategies toward effective treatments. At the same time insights into the unique metabolic requirements of \textit{RAS}-driven tumors and, in particular, a dependence on autophagy have also led to new therapeutic inroads (38–40). Autophagy (or macroautophagy) is the process of autodigestion (or "self-eating") that both maintains cellular homeostasis and enables cells to meet basic energy requirements in times of stress. The induction of autophagy has been proposed to play a dual role in cancer, functioning to meet the metabolic requirements of \textit{RAS}-driven tumors and, in particular, a dependence on autophagy have also led to new therapeutic inroads (38–40). Autophagy (or macroautophagy) is the pro-

Discussion

We have generated a GEM model in which KRAS activation combined with \textit{p53} inactivation results in the reproducible development of IHCC that mimics the human disease on a pathologic and molecular level. Importantly, the tumors in this model arise from the malignant progression of precursor lesions in the bile ducts, enabling us to provide novel insight into histopathogenesis of the disease, showing in particular that both IPBN and VMC can give rise to IHCC. In terms of the molecular features, the tumors in this model show spontaneous loss of \textit{p16\(^{INK4a}\)} and show activation of the MAPK/MEK and PI3K/AKT pathways, recapitulating observations from the human cancer. This model provides a relevant foundation for further understanding of the earliest precancerous stages of IHCC, the influence of additional genetic lesions on tumor biology, and for evaluating therapeutics.

Although IHCC was the dominant histologic tumor type, we also found a small proportion of mixed IHCC/HCC and HCC. Mixed IHCC/HCC are well recognized clinically and, although sharing some histologic characteristics with HCC, behave more similarly to IHCC in terms of the rapidity of disease progression, elevation of tumor markers, and responsiveness to chemotherapy (43). The relationships between dominant histologic phenotype (HCC vs. IHCC), molecular underpinning, and clinical behavior of primary liver tumors are not straightforward (30). Recent expression analysis of primary tumors, including HCC, IHCC, and mixed tumors, revealed a subset with a histologic diagnosis of HCC but an IHCC-like expression profile which correlated with a more aggressive clinical course (44). Thus, a molecular overlap exists among the human primary liver tumors, despite clear histologic differences among subtypes. The development of the mixed tumors and occasional evidence of HCC may reflect the timing of Alb-Cre-mediated recombination that is first evident during late embryogenesis, and thus targets hepatic progenitors and mature hepatocytes, as well as biliary tract epithelium. \textit{Kras} mutations are found in rare subsets of HCC induced by mutagen exposure and associated with \textit{p53} mutations in this setting (45). Because the entire liver was targeted for mutation and as biliary epithelial cells are only a small proportion of the liver parenchyma, it is notable that the dominant phenotype was biliary cancer. This suggest that either biliary cells have particular susceptibility to \textit{Kras} transformation or that \textit{Kras} and \textit{p53} mutations shift cellular differentiation toward a biliary phenotype. Future studies using promoters to target Cre

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expression to subpopulations of cells within the liver (biliary, hepatocyte, or other intermediaries) could clarify this issue.

Both IPBN and VMCs were found as isolated lesions as well as in association with IHCC in our GEM model. IPBN are an established precursor to human IHCC, whereas the relationship of VMC to malignant tumors in humans has not been defined. VMCs are found in approximately 5% of adults in autopsies series and are associated with hepatic cysts (13). More recently reports of VMC showing progressive cytologic atypia in association with IHCC have raised the question of their role in association with IHCC in our GEM model. IPBN are an established precursor to human IHCC, whereas the relationship of VMC to malignant tumors in humans has not been defined. VMCs are found in approximately 5% of adults in autopsies series and are associated with hepatic cysts (13). More recently reports of VMC showing progressive cytologic atypia in association with IHCC have raised the question of their importance in a progression model of the disease (12). In our model, we found instances of clear histologic progression of IPBN, as well as VMC, to contiguous IHCC, thereby providing direct evidence that both of these precursors can be initiated by KRAS and can progress to invasive cancer. There was no overlap in regions harboring IPBN and VMC, suggesting that they arise independently of each other. It is also notable that a group of tumor-bearing animals showed no evidence for such structures or of any other precursor lesions. One possible explanation for both the independent ontogeny of the IPBN and VMC, and the absence of such lesions in some mice, may be that each scenario is associated with a different cell of origin. For example, Kras-p53 mediated transformation of early liver progenitors (hepatoblasts), adult progenitors (e.g., oval cells), and more mature forms of the biliary epithelium or hepatocytes may be associated with a different cellular differentiation program during early tumorigenesis. Alternatively, such differences in precursor lesions may reflect the differential impact of additional acquired molecular alterations driving IHCC progression, as has been reported in pancreatic cancer GEM models (46).

Autophagy is active in derivative cell lines and blockade of autophagy with CQ inhibits growth of derivative cell lines consistent with findings in other Kras-driven tumors (38, 39). Although most RAS-driven cancer cell lines have evidence of active autophagy, the effect of blocking this process has variable effects on cell proliferation, with certain tumor types—including pancreatic cancer—exhibiting...
marked sensitivity to CQ, whereas growth of lung cancer cell lines was effected to a lesser extent. We find that cell lines from our model are significantly growth inhibited with a corresponding block in autophagy by CQ. This fits well with evidence from human tumors and cell lines suggesting the importance of autophagy in the human disease and provides impetus to further evaluate CQ and other drugs that may impact this process in IHCC (47, 48).

In summary, we have created a tractable IHCC GEM model on the basis of the genetics of the human disease. The multistage evolution of the tumors provides a context to explore questions about the role of different cells of origin and the molecular and histologic progression of precancerous stages of IHCC. In addition, the highly reproducible development of IHCC in this model provides a foundation for testing the influence of additional genetic lesions on tumor biology and a platform for the preclinical testing of promising new therapies.

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Kras$^{G12D}$ and p53 Mutation Cause Primary Intrahepatic Cholangiocarcinoma

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