Chronic Bile Duct Injury Associated with Fibrotic Matrix Microenvironment Provokes Cholangiocarcinoma in p53-Deficient Mice

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Abstract

Intrahepatic cholangiocarcinoma (CCA) is a lethal malignancy of the biliary epithelium associated with p53 mutations, bile duct injury, inflammation, and fibrosis. Here, to validate these processes in CCA, we developed a liver cirrhosis model driven by chronic intermittent toxin exposure, which provokes bile duct injury/necrosis and proliferation, fibroblast recruitment, and progressive extracellular matrix (ECM) changes. Fibrotic changes in the matrix microenvironment, typified by increased type I and III collagens and fibroblast recruitment, were shown to stimulate biliary epithelium hyperplasia with subsequent progression to malignant intrahepatic CCA only in mice harboring a p53 mutant allele. These murine CCAs bear histologic and genetic features of human intrahepatic CCA, including dense peritumoral fibrosis, increased inducible nitric oxide synthase, nitrotyrosine, and cyclooxygenase-2 expression, c-Met activation, cErbB2 overexpression, down-regulation of membrane-associated E-cadherin, and p53 codon 248 mutation. Thus, p53 deficiency, chronic bile duct injury/proliferation, and the fibrotic matrix microenvironment cooperate to induce intrahepatic CCA, highlighting the key role of the ECM microenvironment in this common liver cancer. (Cancer Res 2006; 66(13): 6622-7)

Introduction

Cholangiocarcinoma (CCA) is an aggressive malignancy of the intrahepatic and extrahepatic bile ducts that is notable for limited therapeutic responsiveness and poor prognosis (1). Intrahepatic CCA ranks as the second most common liver cancer in the world and accounts for up to 33% of CCA cases (1, 2). Despite its prominence and devastating clinical course, there is a rudimentary understanding of the genetic, cell biological, and environmental mechanisms driving the genesis and progression of CCA and its subtypes (3).

Clinical conditions associated with an increased risk of intrahepatic CCA include liver fluke infection, ingestion of salted fish infected with dimethylnitrosamine-producing bacterial strains, hepatolithiasis, primary sclerosing cholangitis, hepatitis C viral infection, and liver cirrhosis secondary to other nonbiliary causes (1). There is increasing evidence that liver cirrhosis is a risk factor for intrahepatic CCA development in humans (4–8). Notably, fibrosis is common to several risk factors of intrahepatic CCA, such as hepatolithiasis (9) and primary sclerosing cholangitis (10).

Collectively, these observations have fueled speculation that chronic cycles of epithelial destruction and renewal, coupled with inflammation, fibrosis, and exposure to mutagenic agents, are possible factors integral to human cholangiocarcinogenesis (1). In support of the contribution of inflammation to this cancer, human intrahepatic CCAs show inducible nitric oxide (NO) synthase (iNOS) overexpression and presence of nitrotyrosine (proteins are subjected to nitrosylation by the increased production of NO by iNOS; ref. 11). The fibrosis associated with chronic inflammation and injury may also contribute to CCA pathogenesis, particularly through an increase in extracellular matrix (ECM) components, which are known to participate in the regulation of bile duct differentiation during development (12). In considering the hallmarks of cancer (13), there exists strong and growing evidence that the microenvironment can influence carcinogenesis (14, 15).

Cancer-relevant signaling pathways seem to be targeted in human intrahepatic CCA, including the tumor suppressor p53. Comparative genomic hybridization, loss of heterozygosity analysis, and spectral karyotyping have shown that 17p deletion, specifically the region encompassing the p53 tumor suppressor gene, is a frequent genomic alteration in human intrahepatic CCA (16, 17). Cancer-relevant mutations involving p53 have been reported at different frequencies ranging from 10% to 50% of all intrahepatic CCA cases (18–20). Abnormal nuclear accumulation of p53 has also been reported in human intrahepatic CCA (21–25) and attributed to either p53 mutation or ill-defined epigenetic regulation possibly involving MDM2 (18, 19). Mutations in p53 have been reported in advanced disease; however, increased p53 expression in dysplastic lesions and adjacent nonneoplastic bile duct epithelium has suggested a possible role for p53 dysregulation during the formative stages of intrahepatic CCA development (25).

The study of mechanisms driving intrahepatic CCA would be facilitated by faithful mouse models of the human disease. To date, intrahepatic CCA lesions occur occasionally in aflatoxin B1–treated p53+/− mice transgenic for hepatitis B virus surface antigen (26), and an intrahepatic CCA-prone condition has been observed in mice harboring an ErbB2 transgene expressed in the biliary tract epithelium (27). Exposure of mice to CCl4 and alcohol results in an increase in bile duct proliferation in association with cirrhosis,
fibrosis, and fibrillar ECM deposition (28), and, in humans, occupational CCl4 exposure seems to increase the risk of intrahepatic CCA (29), perhaps due to cholangiocyte destruction and proliferation (30). Exposure of rats to CCl4 induces such cellular effects in cholangiocytes (31).

In the present study, we examined the effect of liver microenvironmental changes (ECM and inflammation) and p53 germline mutation in the pathogenesis of intrahepatic CCA. The experimental system used in this work provided evidence for the contribution of continuous cycles of cholangiocyte destruction and regeneration, coupled with bile duct fibrosis and accumulation of fibroblasts as permissive microenvironmental factors favoring the emergence of a highly penetrant intrahepatic CCA-prone phenotype in the context of germline p53 mutations.

Materials and Methods

Mice. p53+/− (C57Bl6, The Jackson Laboratory, Bar Harbor, ME) and mTERT+/−, mTERT+/-, and p53+/−, and p53−/− mice that were also either mTERT+/+ or mTERT−/− (Supplementary Materials and Methods). Mice were subjected to CCl4 administration (10 μL/g body weight of a 10% solution in olive oil) by i.p. injection three times weekly (Monday, Wednesday, and Friday) for 4 months beginning at an age of 6 weeks.

Study design. During the course of the CCl4 treatment, 11 p53+/+, 14 p53+/−, and 7 p53−/− mice were sacrificed and examined to assess the development of liver disease/cirrhosis and cancer-associated changes and to examine the effects of CCl4 on the biliary epithelium. A separate cohort of 27 p53+/+, 28 p53+/−, and 13 p53−/− mice was followed after the end of the CCl4 treatment to assess cancer development.

Histologic analysis. Mice were treated and sacrificed in accord with approved protocols as per institutional guidelines. The livers were removed, inspected for the appearance of surface nodules, and subsequently fixed in formalin and embedded in paraffin. Tissue sections from the paraffin-embedded livers were cut and stained with H&E to enable histologic characterization of the observed liver nodules. Sections were examined for the presence of tumors, which were classified as either hepatocellular carcinoma or CCA; CCA was defined as a solid proliferation of atypical epithelial cells arranged in ducts or tubules, which formed a discrete mass. The nonlesional liver tissue was examined for inflammation, bile ductal or epithelial cells arranged in ducts or tubules, which formed a discrete mass.

Results

Role of Bile Duct Injury, Cholangiocyte Proliferation, Fibrosis, Fibroblast Accumulation, and p53 Status in the Emergence of Bile Duct Hyperplasia and Early Carcinomas

During the course of CCl4 treatment (from 1-16 weeks after initiation of treatment), several mice (ages 7-22 weeks) were examined for CCl4 toxicity and possible early neoplastic changes, including (a) the degree of bile duct injury/necrosis (loss of bile ducts from portal tract; necrosis or dropout), apoptosis (by terminal deoxyuridylate transferase–mediated dUTP nick end labeling staining), and proliferation (increased number of bile duct profiles in portal tract or perportal tissue) in H&E- and CK-19-stained sections and (b) the level of fibrosis as assessed by Masson-Trichrome–stained sections (detects fibrillar collagens) and immunoreactivity to type III collagen, FSP1 (a fibroblast marker), and α-SMA (marker for activated stellate cells, which acquire a myofibroblast-like phenotype). Bile duct injury/necrosis and proliferation were observed in CCl4-treated mice, and the extent of pathology was similar regardless of the p53 genotype (Fig. 1A; Supplementary Fig. S1A and B; data not shown; a total of 11 p53+/+, 14 p53+/−, and 7 p53−/− mice were examined). Apoptosis was observed in 7 of 25 cases examined and was only observed in p53+/+ and p53+/− mice but not in p53−/− mice, consistent with the prominent role of p53 as an inducer of apoptosis (35).

The importance of the ECM in normal bile duct development prompted us to assess a possible correlation between the degree of fibrosis (Supplementary Materials and Methods) in the livers and the presence of bile duct injury/necrosis, apoptosis, or proliferation. Although bile duct injury/necrosis and apoptosis per se did not correlate with the degree of fibrosis (Supplementary Fig. S2B; P = 0.2638 and Supplementary Fig. S2C; P = 0.3544, respectively), the extent of bile duct proliferation was most prominently associated with increasing fibrosis and the emergence of cirrhosis irrespective of the p53 genotype (Fig. 1A and B; Supplementary Table S1; P = 0.0216). Fibrosis was further confirmed by evaluating the expression for type III collagen, FSP1, and α-SMA, all of which exhibited higher levels of expression in the cirrhotic livers when compared with livers with minimal fibrosis (Fig. 1C).
These results are in line with previous observations of increased bile duct proliferation in cirrhotic mouse livers and mirror similar correlative studies reporting bile duct proliferation and cirrhosis in humans (28, 36). In our studies, CCl₄ exerts a uniform toxic effect on the bile duct epithelium; however, the induction of extensive bile duct proliferation coincides significantly with increasing fibrillar collagen deposition and fibrosis. As bile duct proliferation has been reported as a precursor lesion to human CCA (37), our findings implicate the cirrhotic microenvironment in the initiation of intrahepatic CCA.

Next, bile duct proliferation and neoplastic transformation was assessed during and following the full course of CCl₄ treatment. Foci of more florid bile duct proliferation with crowding, early infiltrative growth, and some cytologic atypia [increased nuclear-cytoplasmic ratio, nuclear pleomorphism, and coarse chromatin (i.e., with a histologic appearance suggestive of early carcinomas)] appeared toward the end and shortly following treatment in 6 of 15 p53+/- mice. In contrast, the 14 p53+/+ and 19 p53+/- mice examined within the same timeframe did not develop early carcinomas (Fig. 2A and B). Only 6 to 7 months later did 3 of 17 p53+/- and 1 of 15 p53+/- mice develop early carcinoma foci. These results suggest that p53 loss and bile duct cell hyperplasia cooperate in the transition to early carcinomas, a view further supported by the increased tumor multiplicity in p53+/- mice compared with p53+/- mice (see below).

**Impact of p53 Status on Intrahepatic CCA Incidence and Histologic Presentation**

Following full treatment, the various p53 cohorts were surveyed, revealing progression to more advanced intrahepatic CCA lesions only in p53+/- and p53+/- mice. The intrahepatic CCAs were characterized histologically by a mass-forming proliferation of infiltrating tubules and glands (Fig. 2C), positive staining for the biliary epithelial cell marker, cytokeratin-19 (Supplementary Fig. S5A), and were commonly associated with a collagenous stroma as assessed by the Masson-Trichrome stain, elevated expression of type III collagen, and accumulation of activated fibroblasts (Supplementary Fig. S5B; data not shown). Metastatic CCA was observed in 1 of 5 p53+/- mice (lung metastasis) and 1 of 7 p53+/- mice (lymph node metastasis; Supplementary Fig. S3C and D). p53 deficiency was associated with increased incidence of intrahepatic CCA (p53+/-; 54%; p53+/-; 18%; P = 0.0288) and a significant reduction in tumor latency (Fig. 2D; p53+/-; 29.3 weeks median survival; p53+/-; 52 weeks median survival; P < 0.0001). Furthermore, p53+/- mice more frequently developed multiple CCAs compared with p53+/- mice (average of 7.4 and 1.2 CCAs, respectively; P = 0.0177). Collectively, these results support a role for p53 loss in initiation and progression of intrahepatic CCA in the setting of bile duct proliferation and associated fibrosis.

**Molecular Profile of Mouse CCAs Mirrors the Human Disease**

p53. Validation of p53 loss in CCA pathogenesis was documented further by robust anti-p53 nuclear immunoreactivity in the cancer cells (Supplementary Fig. S4A and B) and by the presence of single point mutations resulting in amino acid substitutions that targeted the DNA-binding domain (Gly242Asp, Arg245Cys, and Ser258Ala; Supplementary Fig. S4C). The codon 242 and 245 mutations correspond precisely to those altered in human intrahepatic CCA. Moreover, Arg245 (human codon 248) mutation is the most frequent p53 lesion in human cancers and would impair p53 DNA binding (38). Additional alterations included an exon 8 mutation (Ala273Val in the DNA-binding domain) and a nonsynonymous exon 7 mutation (in the Ile273 codon). Notably, all mutations detected were G to A or C to T substitutions, for p53 loss in initiation of CCl₄ treatment). The mice with cirrhosis were 11 to 22 weeks old. With levels 2 to 3 were 12 to 22 weeks old, and 7 to 20 weeks old, the mice with fibrosis with no evidence of fibrosis and cirrhosis (labeled with SMA) in sections from livers (labeled with collagen 3), type I collagen accumulation. Magnification, ×100. B, percentage mice that showed bile duct proliferation (assessed on H&E-stained sections) concurrently with various levels of fibrosis (assessed on Masson-Trichrome–stained sections). Bile duct proliferation was observed in mice that were 14 to 22 weeks old (8-16 weeks after initiation of CCl₄ treatment). The mice examined with fibrosis levels of 0 to 1 were 7 to 20 weeks old, the mice with fibrosis levels 2 to 3 were 12 to 22 weeks old, and the mice with collagen levels 11 to 22 weeks old. C, percentage cells staining positive for p53 loss in initiation and progression of intrahepatic CCA in the setting of bile duct proliferation and associated fibrosis.

**Figure 1.** Effects of CCl₄ on biliary epithelial cells and fibrosis. A, left, white arrow, H&E-stained section showing bile duct injury/necrosis and bile duct degeneration. Magnification, ×200. Middle, inset, white arrows, H&E-stained section showing bile duct hyperplasia. Magnification, ×200. Right, inset, white arrows, blue, Masson-Trichrome–stained section showing bile duct proliferation (hyperplasia) in areas with type I collagen accumulation. Magnification, ×100. B, percentage mice that showed bile duct proliferation (assessed on H&E-stained sections) concurrently with various levels of fibrosis (assessed on Masson-Trichrome–stained sections). Bile duct proliferation was observed in mice that were 14 to 22 weeks old (8-16 weeks after initiation of CCl₄ treatment). The mice examined with fibrosis levels of 0 to 1 were 7 to 20 weeks old, the mice with fibrosis levels 2 to 3 were 12 to 22 weeks old, and the mice with collagen levels 11 to 22 weeks old. C, percentage cells staining positive for p53 loss in initiation and progression of intrahepatic CCA in the setting of bile duct proliferation and associated fibrosis.
iNOS, Nitrotyrosine, and COX-2 Expression. The p53 transversions fueled speculation that they may relate to inflammation and associated induction of NO by iNOS (NO can then react with oxygen to generate N₂O₃), which reacts with amines of DNA bases, resulting in their deamination and G:C to A:T transitions (41). In our mouse model, 7 of 10 CCAs showed overexpression of iNOS, and these same tumors contained increased nitrotyrosine (which reflects nitrosylation of proteins by a product released from the reaction of NO with superoxide), consistent with NO production and associated induction of NO by iNOS (NO can then react with amines of DNA bases, resulting in their deamination and G:C to A:T transitions (41)). Adjacent normal bile ducts stained negative for both iNOS and nitrotyrosine (Fig. 3A).

COX-2 expression is common in human intrahepatic CCAs, is induced by iNOS, and promotes cell growth in mouse cholangiocytes (42, 43). Correspondingly, robust expression of COX-2 was detected in 6 of 10 (60%) mouse CCAs (Fig. 3A), and 5 of 6 COX-2-positive tumors showed concurrent iNOS overexpression. Adjacent normal bile ducts stained negative for COX-2 (Fig. 3A).

c-Met, cErbB2, and E-Cadherin Expression. Activated c-Met, cErbB2 overexpression, and down-regulation of E-cadherin are common alterations in human intrahepatic CCA (3). Analysis of baseline c-Met expression revealed that ~80% of tumors possessed a robust c-Met signal and c-Met activation (expression of phosphorylated c-Met; Fig. 3B). c-Met has been suggested to play a role early in human cholangiocarcinogenesis based on robust expression in hyperplastic bile ducts, although the activation status of the receptor was not assayed (42, 44). In our model, although analysis of c-Met activation status revealed no staining in six examples of bile duct hyperplasia (Fig. 3B), all of the four early carcinomas stained positive for phosphorylated Met (Fig. 3B; P = 0.0048). The metastatic lesions also stained positive for phosphorylated Met (data not shown). Therefore, the onset of c-Met activation coincided with the transition from bile duct proliferation to early carcinomas and remained robust in more advanced CCA tumors.

As shown in Fig. 3B, strong cErbB2 expression was detected in ~70% of CCAs. Staining for cErbB2 was weak or undetectable in adjacent normal liver or bile ducts and in six lesions of bile duct hyperplasia, but strong staining was observed in two of five early carcinomas (Fig. 3B; P = 0.1818). Furthermore, strong cErbB2 expression persisted in the two metastatic lesions (data not shown). Such expression patterns suggest a role for cErbB2 in CCA initiation and progression and are consistent with previous transgenic mouse studies establishing a role for cErbB2 overexpression in intrahepatic CCA development (27). Analysis of E-cadherin revealed strong membranous expression in normal bile ducts (Fig. 3B), whereas ~75% of advanced CCA lesions showed altered subcellular localization with only weak cytoplasmic signal for E-cadherin (Fig. 3B). Examination of lesions with bile duct hyperplasia and early carcinomas revealed membrane-associated E-cadherin expression in four of five hyperplasias and five of five cases of early carcinomas (Fig. 3B). Thus, loss of membrane-associated E-cadherin coincides with progression to advanced CCA.

Discussion

This study provides insights into the pathogenesis of intrahepatic CCA, underscoring the combined effect of toxin-mediated bile duct injury, liver fibrosis, and p53 mutation in biliary carcinogenesis. This CCA model shares features prominent in the human disease, including the presence of intrahepatic fibrosis and increased inflammation (as evidenced by overexpression of iNOS and nitrotyrosine) and a molecular profile, including overexpression of COX-2, c-Met activation, cErbB2 overexpression, E-cadherin suppression, and specific p53 mutations. In exploring the evolution of CCA, CCl4 proved particularly effective inducing bile duct injury and proliferation, gradual onset of fibrosis in association with accumulation of fibroblasts, and fibrillar collagen matrix mirroring the common histopathologic correlates of human CCA. This study provides the first experimental confirmation that cholangiocyte injury and proliferation in association with inflammation and fibrosis promote the emergence of CCA in the context of p53 germline mutations. Furthermore, this study stresses the importance of the ECM microenvironment in cancer development.
As p53 transversions are frequent in human intrahepatic CCA (20), it is interesting to note that G to A and C to T transitions in p53 are encountered in cancers associated with chronic inflammation, such as ulcerative colitis. These cancers are also characterized by increased level of iNOS, an enzyme responsible for the generation of NO (45). Correspondingly, CCl₄ has been shown to induce NO, an array of cytokines, along with oxidative stress and free radicals (40), which may explain the specific G to A and C to T mutations observed in our studies with mouse CCA. In support of this theory, we have observed overexpression of iNOS and nitrotyrosine in our mouse CCAs, suggesting that there is ongoing inflammation. In this regard, chronic inflammation and NO production have also been linked to human intrahepatic CCA (11). Taken together, our model provides support for the carcinogenic effects of chronic inflammation and injury to the bile duct epithelium and emergence of intrahepatic CCA in the context of germline p53 mutations.

Intrahepatic accumulation of ECM components, particularly type I collagen, is observed during the formative as well as advanced stages of human intrahepatic CCA, and liver cirrhosis has been suggested to be an etiologic factor in facilitating the emergence of human intrahepatic CCA. During the course of the CCl₄ protocol, there is a gradual increase in type I collagen deposition, culminating in the development of fibrosis, characterized by increased deposition of type III collagen, and accumulation of activated fibroblasts, which under the influence of various cytokines can produce diverse ECM components permissive for cell proliferation and migration. The fibrotic matrix microenvironment is composed of molecules, such as types I, III, and IV collagens, tenacin and fibrin. These molecules when organized in a fibrillar matrix mesh can influence the proliferation and migration of inflamed cholangiocytes and potentially aid in recruitment of activated fibroblasts and endothelial cells to organize a “reactive stroma” around early CCA lesions. Collectively, our results suggest that chronic inflammation in conjunction with fibrotic matrix microenvironment could provide permissive molecular cues to cholangiocytes and other resident cellular components, such as fibroblasts, to facilitate progression of intrahepatic CCA in the context of germline p53 mutations. This model also provided evidence for c-Met activation and cErbB2 overexpression in both early and late stages of cholangiocarcinogenesis and loss of

Figure 3. Molecular profile of mouse CCAs mirrors human CCA. A, overexpression of iNOS, expression of nitrotyrosine, and overexpression of COX-2 in CCA. Black arrows, absence of iNOS, nitrotyrosine, and COX-2 staining in adjacent normal bile ducts. B, activation of c-Met and overexpression of cErbB2 in early carcinoma and CCA and loss of E-cadherin in CCA. Top column, different lesion; left row, different immunohistochemical stain. For phosphorylated Met-stained sections, the magnifications are ×200 (normal liver), ×100 (CCA), ×100 (early carcinoma), and ×200 (hyperplasia). Phosphorylated Met-stained section of a normal liver. Arrow, normal bile duct, which shows no staining. For cErbB2-stained sections, the magnifications are ×100 (normal liver), ×100 (CCA), ×200 (early carcinoma), and ×100 (hyperplasia). cErbB2-stained section of a normal liver: Arrow, normal bile duct, which shows only weak staining. cErbB2-stained section of a CCA: Arrow, strong staining within the tumor. For E-cadherin-stained sections, the magnifications are ×200 (normal liver), ×100 (CCA), ×100 (early carcinoma), and ×200 (hyperplasia). E-cadherin-stained section of a normal liver: Arrow, positively stained bile duct. E-cadherin-stained section of a CCA: Arrow, normal bile tract within the tumor, which shows positive membrane staining in contrast to the rest of the tumor (where there is loss of membrane expression of E-cadherin and the appearance of weaker cytoplasmic staining). E-cadherin-stained section of a bile duct proliferation: Arrow, positively stained lesion. C, cancer-relevant events in the different stages of cholangiocarcinogenesis. Histopathologic progression to intrahepatic CCA observed in this mouse model (which recapitulates the events in human CCA) and the sequence of molecular events in the progression to CCA.
membrane-associated E-cadherin in intrahepatic CCA progression, thus providing a molecular framework of intrahepatic CCA genesis and progression and a model to identify additional mutational events (Fig. 3C). Because cholangiocarcinogenesis is preceded by an evolving process of bile duct injury/inflammation/fibrosis, our toxin-based model provides an essential microenvironmental dimension that is not readily captured in models built solely on germline engineering of relevant mutations.

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