**Priority Report**

**Cell Lineage Tracing Reveals a Biliary Origin of Intrahepatic Cholangiocarcinoma**

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**Abstract**

Intrahepatic cholangiocarcinoma is a treatment refractory malignancy with a high mortality and an increasing incidence worldwide. Recent studies have observed that activation of Notch and AKT signaling within mature hepatocytes is able to induce the formation of tumors displaying biliary lineage markers, thereby raising the suggestion that it is hepatocytes, rather than cholangiocytes or hepatic progenitor cells that represent the cell of origin of this tumor. Here, we use a cholangiocyte-lineage tracing system to target p53 loss to biliary epithelia and observe the appearance of labeled biliary lineage tumors in response to chronic injury. Consequent to this, upregulation of native functional Notch signaling is observed to occur spontaneously within cholangiocytes and hepatocytes in this model as well as in human intrahepatic cholangiocarcinoma. These data prove that in the context of chronic inflammation and p53 loss, frequent occurrences in human disease, biliary epithelia are a target of transformation and an origin of intrahepatic cholangiocarcinoma. *Cancer Res; 74(4); 1005–10. ©2013 AACR.*

**Introduction**

The unexplained increase in incidence of intrahepatic cholangiocarcinoma (1, 2), coupled with its poor response to chemotherapeutics and high mortality, necessitates a greater understanding of the biology of this aggressive malignancy, in which the cell of origin remains unclear. The historic assumption that these tumors arise from the oncogenic transformation of mature biliary epithelia has been based on a glandular histologic morphology, location within and adjacent to the biliary network, and expression of cholangiocyte-specific proteins, including mucin and biliary cytokeratins 7 and 19 (3). Substantive evidence for this origin, however, has been lacking. Patients with primary sclerosing cholangitis and liver fluke infection, diseases characterized by chronic biliary inflammation and epithelial proliferation, are up to 161 and 27 times more likely to develop biliary tract cancers compared with the general population (4, 5). Bipotential hepatic progenitor cells (HPC) have also been considered as a cellular source of intrahepatic cholangiocarcinoma in light of the existence of combined hepatocellular cholangiocarcinoma (6), tumors with features of both cholangiocarcinoma and hepatocellular carcinoma, as well as cholangiolocellular carcinoma, characterized by ductular reaction and cords resembling the Canals of Hering (7).

Interestingly, the incidence of intrahepatic cholangiocarcinoma is increased in chronic hepatocellular injury such as hepatitis C virus and hepatitis B virus (8) infection, indicating a more complex cellular origin of these cancers. Recent work has demonstrated that mature hepatocytes possess potential for transdifferentiation into intrahepatic cholangiocarcinoma, a phenomenon dependent on intracellular Notch signaling (9, 10). This concurs with the known role of Notch in the specification of hepatoblasts during ontogeny as well as observations that Notch is able to reprogram postnatal and terminally differentiated hepatocytes into biliary epithelia with the capacity to form ductular structures (11–13).

In recent fate-tracing experiments, chemically induced tumors were established in transgenic mice carrying an inducible heritable label for either hepatocyte (Alb-CreERT²) or biliary (CK19-CreERT²) lineages. Unexpectedly, in the absence of transgenic Notch overexpression, labeled neoplastic nodules positive for epithelial cell adhesion molecule were observed in tumors arising in Alb-CreERT² but not CK19-CreERT² animals, which suggested that intrahepatic cholangiocarcinoma arose from hepatocytes rather than cholangiocytes in that model (10). Given the unexpected nature of these findings within the clinical context of this disease and their implications for the development of future therapy, we set out to assess whether targeted loss of tumor suppressor function within cholangiocytes can precipitate intrahepatic cholangiocarcinoma formation using an independent transgenic strategy and, hence, whether biliary epithelia should still be considered a cell of origin of intrahepatic cholangiocarcinoma.
Materials and Methods

Mice

CK19CreERT26ReYFP mice on a mixed genetic background (a kind gift from Guoqiang Gu, Vanderbilt University Medical Center, Nashville, TN) and Trp53<sup>mut<sub>lox</sub></sup> (The Jackson Laboratory) were used in this study.

Experimental protocol

For induction of Cre activity, 6-week-old CK19CreERT<sup>T</sup>eYFPp53<sup>+/−</sup> mice were administered three intraperitoneal injections of 4 mg tamoxifen (Sigma) reconstituted in olive oil (Sigma) at a concentration of 30 mg/mL on alternate days. Mice were harvested 72 hours following tamoxifen administration to assess efficiency of Cre recombination. A separate cohort went on to receive 600 mg/mL thioacetamide (TAA; Sigma) intraperitoneally for 26 weeks to induce tumor formation. To assess noncarcinogenic injury models, mice received 1 μL/g carbon tetrachloride (Sigma) or olive oil (Sigma) intraperitoneally for 16 weeks or 3.5-dioxyxycarbonyl-1,4-dihydro-collidine (DDC; Sigma) diet (0.1% Purina 5015 Mouse chow) for 14 days.

Immunohistochemistry

Livers were fixed overnight in 4% aqueous buffered formalin, embedded in paraffin, and cut into 5-μm sections. The tissue underwent microwave antigen retrieval using Tris-EDTA with 0.1% Tween20 (Sigma) and blocked with H<sub>2</sub>O<sub>2</sub> and Protein Block (Invitrogen). Sections were incubated overnight with the following primary antibodies: GFP, CK19, Cyp2D6, Sox9, and Oct4 (Abcam), Nanog (eBiosciences), or Oct4 (Santa Cruz Biotechnology). After washing in PBS, the directly conjugated Notch1 (Abcam), Nanog (eBiosciences), or Oct4 (Santa Cruz Biotechnology) were used according to species with DAPI (4’,6-diamidino-2-phenylindole)-Fluoromount (SouthernBiotech). Immunohistochemistry.

Study approval

All animal experiments were approved by the University of Edinburgh animal ethics committee and conducted with the U.K. Home Office approval. Human specimens were collected prospectively from patients undergoing hepatic resection at the Royal Infirmary of Edinburgh with local ethical approval and informed patient consent.

Results and Discussion

Up to 26% of patients with intrahepatic cholangiocarcinoma carry mutations in the <i>TRPS3</i> gene [up to 44.4% in fluke-associated intrahepatic cholangiocarcinoma (14)], primarily single-base substitutions at CpG sites, resulting in loss of tumor suppressor function (15). In our tamoxifen inducible experimental system, we have, therefore, targeted functional loss of p53 in CK19-expressing cells that are synchronously labeled with a Cre inducible eYFP reporter (CK19-CreERT; R26ReYFP;Trp53<sup>loxP</sup>), in which Cre recombination induces excision of exons 2 to 10 of the <i>Trp53</i> gene, and also the stop locus upstream of eYFP (Fig. 1A). In the healthy and injured mouse liver, CK19 expression is found on cholangiocytes lining medium and large-sized bile ducts as well as terminal ductules in the Canals of Hering, but not in hepatocytes (Fig. 1B). In the absence of Cre or tamoxifen, no eYFP expression was seen in either healthy or injured liver, but following induction with tamoxifen at 6 weeks old, eYFP positivity was observed in 14% of all cholangiocytes (Fig. 1C). No cell types other than biliary epithelia were labeled.

One week following Cre induction, mice were initiated on TAA to induce tumor formation (Fig. 2A; ref. 16). After 26 weeks, multifocal tumors were observed in the livers of CK19CreERT<sup>T</sup>eYFPp53<sup>+/−</sup> (80%) but not CK19CreERT<sup>T</sup>eYFPp53<sup>−/−</sup> (0%) or CK19CreERT<sup>T</sup>eYFPp53<sup>−/+</sup> (0%) animals (Fig. 2B). eYFP positivity was observed in all histologically identified neoplastic nodules, and this colocalized with expression of the ductular markers CK19 and Sox9. No cells were dually positive for eYFP and the mature hepatocyte marker Cyp2D6 (Fig. 2C).

In light of the emerging role for Notch in driving cholangiocarcinogenesis, we then looked to identify the cellular expression of the Notch 1 receptor within this model. Membranous and nuclear positivity of activated Notch1 was observed widely in the epithelium of the malignant ducts and frequently colocalized with eYFP staining (Fig. 3A). Interestingly, positivity was also seen to occur within nuclei of hepatocytes, particularly those located adjacent to the cancerous stroma (Fig. 3B). We went on to assess whether Notch1 was also expressed in nonmalignant models of liver injury and observed strong ductular positivity in the context of the DDC biliary injury dietary model, but none during chronic hepatocyte regeneration with carbon tetrachloride or in the uninjured mouse liver (Fig. 3C). Furthermore, this pattern of Notch activity is recapitulated in human resected intrahepatic cholangiocarcinoma specimens, in which the strongest positivity is observed within malignant ducts, as well as in hepatocytes adjacent to the invasive front of the tumors (Fig. 3D). Hepatic lineage-tracing experiments have proved problematic; indeed the CK19CreERT<sup>T</sup>R26ReYFP mouse has hitherto not been widely adopted for cell-specific gene deletion experiments due to poor efficiency. p53 deletion at the point of tamoxifen administration does not result in increased labeling efficiency, but is likely to cause a preferential expansion of the eYFP<sup>+</sup> compartment in response to TAA-induced injury, making it more probable that a transforming event will occur in this population of cells compared with labeled cells in a similar fate-tracing system without p53 deletion. We believe this to be a robust and representative model of biliary carcinogenesis, given the frequent combination of p53 loss and chronic biliary inflammation observed in human disease. eYFP positivity was observed in all animals in which tumors arose as well as in each and every focus of malignancy. We observed colocalization between eYFP and the M3 acetylcholine receptor, a marker of mature cholangiocytes, occasional colocalization with CD4 and no colocalization with the stem cell markers Nanog and Oct 4 (17; Supplementary Fig. S1). A likely cell of origin is, therefore, the mature cholangiocyte, although we cannot eliminate the possibility of stem cells, progenitors or
intermediates as targets of transformation. Interestingly, given the lineage-tracing system used here, these would be CK19\(^{+}\) cells. Given the CK19CreER\(^T\) eYFP\(^{+}\)Trp53\(^{+/+}\) mouse has hitherto exhibited lineage labeling of hepatocytes, we can conclude that the eYFP\(^{+}\) tumor cells here, arise from cholangiocytes rather than hepatocytes. It is unclear why...
labeled tumors were not observed after 30 weeks of TAA administration in the CK19CreER<sup>T</sup>T2R26RYFP system published by Sekiya and colleagues; however, our data clearly and definitively attest that biliary epithelia can be a cell of origin of intrahepatic cholangiocarcinoma in an independent CK19-based transgenic system.

Figure 2. Intrahepatic cholangiocarcinoma is derived from CK19<sup>+</sup> cholangiocytes. A, experimental strategy of tamoxifen induction in CK19CreER<sup>T</sup>eYFP<sup>p53<sup>−/−</sup></sup> mice followed by oral administration of 600 mg/mL TAA for 26 weeks. B, multifocal tumors developed only in CK19CreER<sup>T</sup>eYFP<sup>p53<sup>−/−</sup></sup> (homozygous for p53 deletion; n = 5) and not CK19CreER<sup>T</sup>eYFP<sup>p53<sup>+/−</sup></sup> (n = 14) or CK19CreER<sup>T</sup>eYFP<sup>p53<sup>−/−</sup></sup> (n = 5) animals, and only following TAA administration. C, coimmunofluorescent staining of eYFP with the biliary lineage markers CK19 and Sox9. All eYFP<sup>+</sup> cells were seen to be CK19<sup>+</sup>. eYFP positivity did not overlap with the mature hepatocyte marker Cyp2D6. Nuclei are stained with DAPI; scale bars, 50 μm.
Primary liver cancers are a phenotypically and molecularly heterogeneous group of malignancies without a stereotypical mutational signature. It has been suggested that such heterogeneity reflects in part the diversity of the underlying cells of origin (17), although this remains unproved. What is evident, however, is the plasticity of hepatic lineages. Following oncogenic transduction, mature hepatocytes, HPCs, and hepato blasts all have potential for reprogramming into tumor-initiating cells with acquisition of CD133+ expression, side population fractions as well as tumor-forming and metastatic capacity (18). Cellular differentiation seems to trigger distinct transcriptional programs in response to the same oncogenic stimulus; however, all transduced cells independent of origin, are able to form tumors of multiple lineages.

Our data support the published evidence for Notch as driver of biliary oncogenesis (19) by demonstrating active signaling within the ductular epithelium in intrahepatic cholangiocarcinoma in both human and mouse. The observation of strong Notch1 intracellular domain expression within hepatocyte nuclei adjacent to the desmoplastic stroma substantiates previous experiments that have shown Notch1 activation within these cells acts as a transdifferentiating factor (9, 10). Moreover, this model of reprogramming is further strengthened by the capacity of constitutively activated Notch2 in albumin-expressing cells to induce intrahepatic cholangiocarcinoma formation and accelerate DEN-induced hepatocellular carcinoma, which is less differentiated than wild-type controls (20). This Notch high state, able to prime the peritumoral parenchyma for transdifferentiation, has significant therapeutic implications for the many patients who develop intrahepatic cholangiocarcinoma on a background of chronic hepatocellular injury (8). We believe that these findings unify and
clarify previous reports and explain how chronic biliary damage can lead to cholangiocarcinoma arising from biliary epithelium. In conclusion, we have definitively shown that even in the absence of transgenic Notch activation, intrahepatic cholangiocarcinoma can arise from the biliary epithelia. Future therapeutic strategies should target the Notch pathway as a driver of tumorigenesis in this aggressive malignancy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.V. Guest, T.J. Kendall, R. Walker, S.J. Wigmore
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.V. Guest, L. Boulter, T.J. Kendall, S.J. Wigmore, S.J. Forbes
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References


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