Cell Lineage Tracing Reveals a Biliary Origin of Intrahepatic Cholangiocarcinoma

Rachel V. Guest1, Luke Boulter1,2, Timothy J. Kendall2, Sarah E. Minnis-Lyons3, Robert Walker1, Stephen J. Wigmore6, Owen J. Sansom6, and Stuart J. Forbes1

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.

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-CreER T2) or biliary (CK19-CreER T2) 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-CreER T2 but not CK19-CreER T2 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.

Authors' Affiliations: 1MRC Centre for Regenerative Medicine; 2Human Genetics Unit, University of Edinburgh; 3Department of Surgery and Transplantation Medicine, Royal Infirmary of Edinburgh, Edinburgh; and 4Beatson Institute for Cancer Research, Glasgow, Scotland, United Kingdom

Corresponding Author: Stuart J Forbes, MRC Centre for Regenerative Medicine, 5 Little France Drive, Edinburgh bioQuarter, Edinburgh EH16 4UJ, Scotland, United Kingdom. Phone: 44-0131-6519-569; Fax: 44-0131-6519-501; E-mail: stuart.forbes@ed.ac.uk

doi: 10.1158/0008-5472.CAN-13-1911

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Materials and Methods

Mice
CK19CreERT26ReYFP mice on a mixed genetic background (a kind gift from Guoqiang Gu, Vanderbilt University Medical Center, Nashville, TN) and Trp53tm1Brn (The Jackson Laboratory) were used in this study.

Experimental protocol
For induction of Cre activity, 6-week-old CK19CreERT26ReYFP 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) in drinking water for 26 weeks to induce tumor formation. To assess noncancerogenic injury models, mice received 1 µL/g carbon tetrachloride (Sigma) or olive oil (Sigma) intraperitoneally for 16 weeks or 3,5-diethoxycarbonyl-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 H2O2 and Protein Block (Invitrogen). Sections were incubated overnight with the following primary antibodies: GFP, CK19, Cyp2D6, Sox9 and Notch1 (Abcam), Nanog (eBiosciences), or Oct4 (Santa Cruz following primary antibodies: GFP, CK19, Cyp2D6, Sox9 and Notch1 (Abcam), Nanog (eBiosciences), or Oct4 (Santa Cruz). Sections were incubated overnight with the directly conjugated secondary antibodies (Invitrogen). After washing in PBS, the sections were counterstained with diamidino-2-phenylindole) (DAPI; Sigma) and Fluoromount (SouthernBiotech).

Study approval
All animal experiments were approved by the University of Edinburgh animal ethics committee and conducted with the local ethical approval 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 TRPS1 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-CreERT26ReYFP;Trp53loxP), in which Cre recombination induces excision of exons 2 to 10 of the Trp53 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 CK19CreERT26ReYFP (80%) but not CK19CreERT26ReYFP;Trp53lox/lox (0%) or CK19CreERT26ReYFP;Trp53lox/lox (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 CK19CreERT26ReYFP 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+ 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 Oct4 (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 CK19CreERTR26RYFP mouse has not 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

Figure 1. Transgenic system of tamoxifen-inducible, Cre-mediated cell tracking with Trp53 deletion in CK19CreERT eYFPTR26p53f/f mice. A, transgenic construct of fluorescent labeling and tumor suppressor deletion in CK19 cells in response to tamoxifen in 6-week-old mice. B, in the presence of Cre and tamoxifen (TM), eYFP activity is seen within small ductules as well as large bile ducts. The eYFP+ population expands following 14 days of dietary DDC; scale bars, 50 μm. Quantitative analysis of Cre efficiency 72 hours post injection in Cre+ mice exposed to tamoxifen (n = 5), Cre- mice without tamoxifen (n = 3), and Cre+ mice exposed to tamoxifen (n = 8). C, following tamoxifen injection, eYFP positivity is seen only in CK19-expressing cells. These are cholangiocytes that also express the biliary markers Sox9. No colocalization is seen with the mature hepatocyte marker Cyp2D6; scale bars, 50 μm.
labeled tumors were not observed after 30 weeks of TAA administration in the CK19CreER 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 cholangiocytes. A, experimental strategy of tamoxifen induction in CK19CreER<sup>T2R26RYFP</sup>p53<sup>+/−</sup> mice followed by oral administration of 600 mg/mL TAA for 26 weeks. B, multifocal tumors developed only in CK19CreER<sup>T2R26RYFP</sup>p53<sup>−/−</sup> (homozygous for p53 deletion; n = 5) and not CK19CreER<sup>T2R26RYFP</sup>p53<sup>+/−</sup> (n = 14) or CK19CreER<sup>T2R26RYFP</sup>p53<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.

Authors’ Contributions

Conception and design: R.V. Guest, L. Boulter, S.E. Minnis-Lyons, S.J. Wigmore, O.J. Sansom, S.J. Forbes

Development of methodology: R.V. Guest

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

Writing, review, and/or revision of the manuscript: R.V. Guest, L. Boulter, S.E. Minnis-Lyons, S.J. Wigmore, S.J. Forbes

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Walker, S.J. Wigmore

Study supervision: S.J. Wigmore, S.J. Forbes

Acknowledgments

The authors thank Guoqiang Gu, Vanderbilt University Medical Center, Nashville, TN for the provision of mice.

Grant Support

This work was supported by the MRC Centre for Regenerative Medicine, University of Edinburgh. Funding was provided by the Wellcome Trust, the Medical Research Council, and Cancer Research UK. R.V. Guest is supported by Wellcome Trust Clinical Research Training Fellowship; L. Boulter is supported by a Cancer Research UK project grant and an MRC research grant; T.J. Kendall is supported by a Wellcome Trust Intermediate Clinical Fellowship; S.E. Minnis-Lyons is supported by an MRC Scottish Clinical Pathology Fellowship; R. Walker is supported by a Cancer Research UK project grant; S.J. Wigmore is supported by the Scottish Higher Education Funding Council; O.J. Sansom is supported by Cancer Research UK and the European Research Council; and S.J. Forbes is supported by the MRC and a Cancer Research UK project grant.

Received July 9, 2013; revised October 31, 2013; accepted November 20, 2013; published OnlineFirst December 5, 2013.
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doi:10.1158/0008-5472.CAN-13-1911

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