Notch Signaling Is Activated in Human Hepatocellular Carcinoma and Induces Tumor Formation in Mice

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BACKGROUND & AIMS: The Notch signaling pathway is activated in leukemia and solid tumors (such as lung cancer), but little is known about its role in liver cancer. **METHODS:** The intracellular domain of Notch was conditionally expressed in hepatoblasts and their progeny (hepatocytes and cholangiocytes) in mice. This was achieved through Cre expression under the control of an albumin and α-fetoprotein (AFP) enhancer and promoter (AFP-Notch intracellular domain [NICD]). We used comparative functional genomics to integrate transcriptome data from AFP-NICD mice and human hepatocellular carcinoma (HCC) samples (n = 683). A Notch gene signature was generated using the nearest template prediction method. **RESULTS:** AFP-NICD mice developed HCC with 100% penetrance when they were 12 months old. Activation of Notch signaling correlated with activation of 3 promoters of insulin-like growth factor 2; these processes appeared to contribute to hepatocarcinogenesis. Comparative functional genomic analysis identified a signature of Notch activation in 30% of HCC samples from patients. These samples had altered expression in Notch pathway genes and activation of insulin-like growth factor signaling, despite a low frequency of mutations in regions of *NOTCH1* associated with cancer. Blocking Notch signaling in liver cancer cells with the Notch activation signature using γ-secretase inhibitors or by expressing a dominant negative form of mastermind-like 1 reduced their proliferation in vitro. **CONCLUSIONS: Notch signaling is activated in human HCC samples and promotes formation of liver tumors in mice. The Notch signature is a biomarker of response to Notch inhibition in vitro.**

Keywords: Genetically Engineered Mouse Model; Notch Activation; Gene Expression Profiling.

BASIC AND TRANSLATIONAL LIVER

Primary liver cancer is the third leading cause of cancer death worldwide,[1] and its incidence in the United States has tripled between 1975 and 2005.[2] The most frequent subtype is hepatocellular carcinoma (HCC), which has a complex pathogenesis related to its diverse etiologic factors including cirrhosis because of viral hepatitis (B and C) and/or alcohol abuse. Overall, less than 30% of newly diagnosed HCC patients are eligible for curative therapies such as resection, liver transplantation, or local ablation.[3] Patients diagnosed at advanced stages have a bleak prognosis, although the recent identification of sorafenib as an effective molecular therapy has extended their survival to a median of approximately 1 year.[4] Although this therapy is not curative, it has changed the landscape of translational research in the field, underscoring the importance of dissecting the molecular drivers of HCC.

The Notch pathway is an evolutionarily conserved signaling module that participates in embryonic cell fate decisions and regulates stem/progenitor cell states.[5] In the liver, Notch acts in a temporal- and dose-dependent manner to coordinate biliary fate and morphogenesis.[6] A causative role for Notch signaling is well established in T-cell acute lymphoblastic leukemia, where mutations or chromosomal aberrations affecting the *NOTCH1* gene are found with high frequency.[7] In addition, activation of the Notch pathway has been described in several other solid tumors.[8,9]

Data regarding Notch involvement in HCC are limited and ambiguous in terms of antitumoral effects following its inhibition.[10–12] Of note, none of the previously reported studies on Notch and HCC included genetic engineered models for pathway deregulation. We previously developed a series of gain-of-function and loss-of-function reagents to characterize the role of Notch during liver development.[6] Herein, we report that long-term exposure

Abbreviations used in this paper: **AFP, α-fetoprotein; GFP, green fluorescent protein; GSI, γ-secretase inhibitor; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Igf2, insulin-like growth factor 2; IHC, immunohistochemistry; mRNA, messenger RNA; NICD, Notch intracellular domain; NTP, nearest template prediction; PCR, polymerase chain reaction.**

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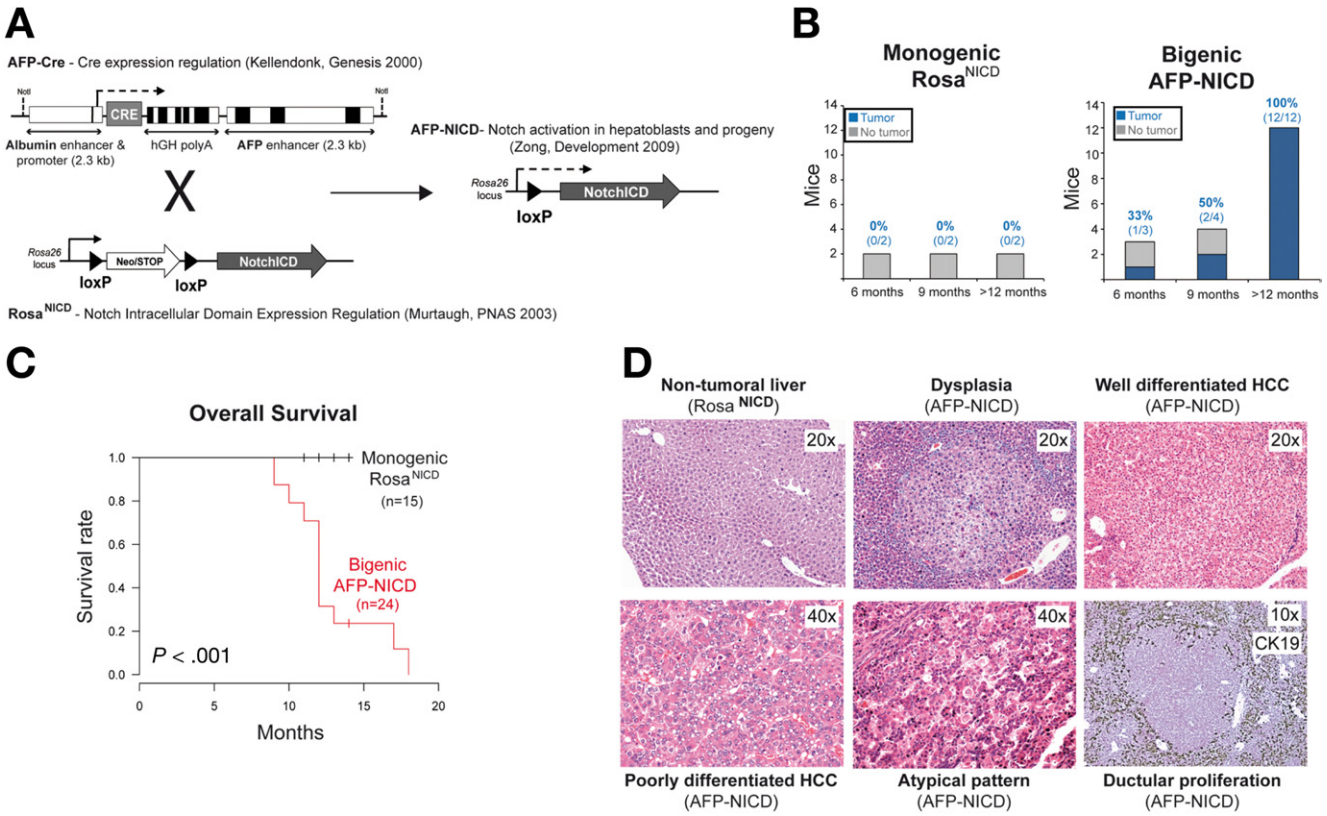

Figure 1. Activated Notch induces liver oncogenesis in vivo. (*A*) Schematic representation of the generation of bigenic mice over-expressing a constitutively active form of Notch specifically in the liver (*AFP-NICD*). (*B*) Tumor incidence in AFP-NICD bigenic and control NICD monogenic mice. (*C*) Kaplan–Meier curves of mice survival. (*D*) Representative H&E images of control liver, a dysplastic nodule, HCC with different degrees of differentiation, HCC with atypical pattern (ie, infiltration of duct-like cells with marked architectural distortion), and intense ductular proliferation (as observed in CK19 staining image).

to constitutive Notch signaling in the liver induces HCC in mice with high penetrance and that Notch pathway activation occurs in roughly one-third of human HCCs.

Materials and Methods

For additional information, please see Supplementary Materials and Methods.

Transcript profiling: Submitted microarray data accession numbers are GSE33486 and GSE33560 (GEO Omnibus: http://www.ncbi.nlm.nih.gov/geo/).

Animal Model

Mice used in the study were previously described.[6] A schematic representation of the bigenic α-fetoprotein (AFP)-Notch intracellular domain (NICD) (AFP-NICD) mice model is depicted in Figure 1*A*. Briefly, the Rosa^{NICD} strain harbors a constitutively active form of Notch1 inserted in the Rosa26 locus, downstream of loxP-flanked transcriptional stop sequences.[13] Liver specificity was provided by the AFP-Cre mice strain, in which Cre recombinase is expressed under the regulatory control of the AFP enhancer and albumin promoter.[14] Liver tissues from Rosa^{NICD} mice were used as controls. Studies were performed in compliance with guidelines and procedures for the humane use of animals established by the University of Pennsylvania.

Messenger RNA Profiling

For Notch-induced HCC analyses, we used 5 liver tumors of AFP-NICD mice and 4 liver control samples from Rosa^{NICD} mice. Expression profiling was conducted with the GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). For newborn data, liver samples were obtained from newborn bigenic mice and monogenics from 6 and 5 pups, respectively, and profiled with the Whole Mouse Genome Oligo Microarray G4122A (Agilent Technologies, Santa Clara, CA). Raw data are deposited at GEO Omnibus (GSE33486 and GSE33560, http://www.ncbi.nlm.nih.gov/geo/). We analyzed expression data from 683 samples (642 HCCs, 18 dysplastic nodules, 13 cirrhotic tissues, and 10 normal livers)[15–18] from 3 different data sets: (1) HCV-associated data set (n = 132, fresh-frozen tissue): different stages of hepatitis C-related hepatocarcinogenesis (normal liver [n = 10], cirrhosis [n = 13], dysplastic nodules [n = 18], and HCC [n = 91]), including both gene expression and DNA copy number data (GSE9843 and GSE20594). (2) Multietiology HCC (n = 144, formalin-fixed paraffin-embedded samples): HCC from different etiologies (GSE19977). Samples from these 2 data sets were obtained from the HCC Genomic Consortium (Mount Sinai School of Medicine, New York, NY; Hospital Clínic, Barcelona, Spain; Istituto Nazionale dei Tumori, Milan, Italy). (3) Publicly available data sets (n = 407): 5 HCC data sets including different etiologies (Supplementary Table 1).

Expression data of liver cancer cell lines was obtained from the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE),[19] publicly available at http://www.broadinstitute.org/ccle. Expression from 318 cancer cell lines (Affymetrix U133 Plus 2.0 arrays; Affymetrix) was obtained from the cancer Biomedical Informatics Grid (caBIG), hosted by the National Cancer Institute (Bethesda, MD). We acknowledge Glaxo-Smith-Kline (Brentford,