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

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Abstract

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.

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Author Contribution: AV, CA and KY designed and executed the experiments, collected, analyzed and interpreted the data, and wrote the manuscript. YH performed and oversaw microarray gene expression data analysis. YZ participated in animal experiments. ST conducted siRNA studies and sequence predictions. ST, MS and LRC interpreted histological preparations and immunohistochemistry. BZS and JML supervised the whole project and participated in data interpretation and writing the manuscript.

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Methods—The intracellular domain of Notch was conditionally expressed in hepatoblasts and their progeny (hepatocytes and cholangiocytes) in mice, through Cre expression, under the control of an albumin and α -fetoprotein enhancer and promoter (AFP-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 (*Igf2*); 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 IGF 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 MAML 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

INTRODUCTION

Primary liver cancer is the third leading cause of cancer death worldwide¹, and its incidence in the United States has tripled between 1975 and 2005². The most frequent subtype is hepatocellular carcinoma (HCC), which has a complex pathogenesis related to its diverse etiological factors including cirrhosis due to 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 ablation³. 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 year⁴. While 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 states⁵. In the liver, Notch acts in a temporal- and dose-dependent manner to coordinate biliary fate and morphogenesis⁶. 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 frequency⁷. 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 anti-tumoral effects following its inhibition^{10–12}. Of note, none of the previously reported studies on Notch and HCC included genetic engineered models for pathway de-regulation. We previously developed a series of gain-of-function and loss-of-function reagents to characterize the role of Notch during liver development⁶. Herein, we report that long-term exposure 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

See Supplementary Information for details.

Animal model

Mice used in the study were previously described⁶. A schematic representation of the bigenic AFP-NICD mice model is depicted in Figure 1a. Briefly, the Rosa^{NICD} strain harbors a constitutively active form of Notch1 inserted in the Rosa26 locus, downstream of loxP-flanked transcriptional stop sequences¹³. 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 promoter¹⁴. 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.

mRNA 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). For newborn data, liver samples were obtained from newborn bigenic mice and monogenics from six and five pups respectively; and profiled with the Whole Mouse Genome Oligo Microarray G4122A (Agilent Technologies). 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 datasets: 1) HCV-associated dataset (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) Multi-etiology HCC (n=144, formalin-fixed paraffin-embedded samples (FFPE)): HCC from different etiologies (GSE19977). Samples from these two datasets were obtained from the HCC Genomic Consortium (Mount Sinai School of Medicine, New York; Hospital Clínic, Barcelona; Istituto Nazionale dei Tumori, Milan). 3) Publicly available datasets (n=407): five HCC datasets including different etiologies (Supplementary Table 1).

Expression data of liver cancer cell lines was obtained from the Broad-Novartis Cancer Cell Line Encyclopedia¹⁹, publicly available at <http://www.broadinstitute.org/ccle>. Expression from 318 cancer cell lines (Affymetrix U133 Plus 2.0 arrays) was obtained from the cancer Biomedical Informatics Grid (caBIG®), hosted by the National Cancer Institute. We acknowledge Glaxo-Smith-Kline for generating and providing these data (<https://array.nci.nih.gov/caarray/project/woost-00041>).

Immunohistochemistry and reverse transcriptase PCR

Four µm FFPE tissue sections from mice and human tissues were used for immunohistochemistry (IHC) studies²⁰. Primary antibodies: CK19 (1:1000, courtesy from Prof Douglas Melton, Harvard University), SOX9 (1:100, Millipore), CD31 (1:50, Abcam), Vimentin (1:100, Cell Signaling), and pan-Cytokeratin (1:100, Santa Cruz Biotechnology).

One µg of RNA was reverse-transcribed into cDNA using the SuperScript® III Reverse Transcriptase (Invitrogen) and analyzed through semi-quantitative PCR amplification.

Double-strand Sanger sequencing (*NOTCH1* mutations)

Mutation analysis included re-sequencing of 50 genomic DNA human HCC samples for the coding sequences of exons 26, 27, and 34 (covered by 6 amplicons) from the *NOTCH1* gene

(gene ID: 4851, CCDS: 43905.1). 300 ng purified genomic DNA per experimental sample (at least 20 ng/ μ L) were used for this analysis. After PCR amplification, NTPs and oligos were removed by enzymatic treatment, and the PCR products directly sequenced on each strand using the PCR primers (Supplementary Table 2). Analysis was conducted by GENEWIZ, Inc.

Cell viability assay

Proliferation assays were conducted on 96-well plates. Approximately 2000 cells/well were seeded in media containing 10% FBS and DMSO or the γ -secretase inhibitor (GSI) Compound E (Calbiochem). Media (with DMSO or treatment) was replaced every 48h. Five days after seeding, cell proliferation was assessed using CellTiter-Glo Luminescent Viability assay (Promega). HepG2, SNU423 and SNU475 were obtained from the American Type Culture Collection (ATCC) cell bank and Huh7 from the Japanese Collection of Research Bioresources (JCRB).

Transfection and immunofluorescence

Cells were plated (MatTek) (40,000/35 mm bottom plate) and infected 24h later with GFP-only control viruses or a dominant negative DN-MAML-GFP fusion construct. This dominant negative protein results in strong and selective inhibition of transcriptional output from all Notch receptors²¹. For immunofluorescence studies, 48h post-infection, cells were fixed in 2% PFA, permeabilized with 70% ethanol and blocked in 0.5% BSA. Samples were incubated with phospho-histone H3 (pHH3) antibody (Cell Signaling), Alexa 594-conjugated secondary antibody (Invitrogen) and DAPI counterstaining. Proliferation was quantified as the ratio of pHH3-stained nuclei among GFP-positive cells in 5 to 7 low magnification fields.

Data analysis

Mann-Whitney U-test, Spearman's coefficient and Fisher-exact test were used for variable comparisons. Microarray data were normalized and log2-transformed using the RMA algorithm, genes were filtered for variability using PreprocessDataset module in Gene Pattern²². Differential gene expression in mice was evaluated with the Significance Analysis of Microarrays (SAM)²³, using FDR²⁴ and q-value for multiple testing correction. Differentially expressed genes (q-value<0.05) with a fold-change >2 constituted the Notch signatures (tumor and newborn). Predictions of these signatures in human samples and cell lines were performed using the Nearest Template Prediction (NTP) method²⁵. Overlap of the Notch tumor signature with other HCC signatures was conducted using Cramer's V coefficient¹⁷.

The Notch Gene List (n=96) was generated by merging previously reported lists of Notch genes^{26,27} (Supplementary Table 3), excluding duplicates according to HGNC²⁸. Differential expression of these genes between HCC with the Notch signature and normal controls were conducted using SAM analysis and validated with the ComparativeMarkerSelection module from Gene Pattern²².

RESULTS

Liver-specific activation of Notch pathway promotes oncogenesis

We sought to evaluate long-term effects of Notch signaling *in vivo* by using bigenic AFP-Notch Intracellular Domain (AFP-NICD) mice, in which Cre-mediated recombination in embryonic hepatoblasts results in the expression of a constitutively active form of Notch1 in >95% of hepatoblasts and cholangiocytes postnatally⁶ (Fig. 1a). At 6-months of age, AFP-NICD mice developed liver tumors at an estimated frequency of 33% (1/3) and 50% (2/4) at

9 months. Beyond 12 months, all AFP-NICD mice showed macroscopic liver tumors (12/12, Fig. 1b), while none of the control monogenic mice (Rosa^{NICD}) had tumors. Bigenic animals had significant worse survival than monogenics (Fig. 1c).

H&E staining of tumors revealed HCC with varying degrees of differentiation in the majority of analyzed tumors (7/10, Fig. 1d). Accompanying these HCCs, we also identified areas formed by proliferative non-malignant hepatocytes that tended to group together and replace normally shaped hepatocytes, resembling dysplastic foci found in HCC patients (Fig. 1d, “Dysplasia”). All livers displayed various degrees of ductular proliferation, highlighted by CK19 staining (Fig. 1d), a feature present in livers of bigenic mice at birth⁶. In the remaining tumors (3/10), CK19+ cells infiltrated the malignant nodules and provoked a significant distortion of liver architecture (Fig. 1d, “Atypical pattern”). In addition to hepatocytes (normal, dysplastic or malignant) and CK19+ cells, there was an additional cell population formed by high-proliferative small cells, with reduced cytoplasm and oval nuclei. These cells showed histological features of so-called progenitor cells, exhibiting scattered staining for pan-cytokeratin and progressive loss of CK19 staining (Supplementary Fig. 1). These cells lacked expression of either CD31 or vimentin, excluding a mesenchymal phenotype. They were located either forming nests within dysplastic/tumor nodules, or dispersed among normal hepatocytes. Overall, these data indicate that long-term activation of Notch in the mouse liver recapitulates different stages of human hepatocarcinogenesis and results in full-blown HCC, including histological features associated with progenitor cell expansion.

Notch-induced tumors exhibit *Igf2* promoter reactivation

To examine the possible molecular alterations governing Notch-induced tumorigenesis, we performed genomic profiling of five tumors derived from AFP-NICD mice using whole-genome transcriptome arrays, and compared them with four monogenic livers. Analysis of differentially expressed genes (Supplementary Table 4) revealed significant (q-value<0.05) up-regulation of Notch target genes such as *Spp1* (37.4 fold), *Sox9* (4.4 fold), *Nrarp* (2.98 fold), *Hes1* (2.9 fold) and *Hey2* (2.8 fold) in tumors. In addition, genes involved in cell cycle regulation were significantly up-regulated, including *Dnase1*, *Cdk1*, *Ccna2*, *Ccnd1*, *Ccnb2* (all >2.5 fold up-regulated). Tumors showed significant down-regulation of members of the cytochrome P450 superfamily, which are involved in hepatic detoxification and homeostasis (e.g., *Cyp2a22*, *Cyp2b13* and *Cyp2b9*, all >15 fold down-regulated). As expected, Gene Set Enrichment Analysis identified gene sets defining oncogenic phenotypes in tumors, whereas control livers were markedly enriched in gene sets associated with metabolic biosynthesis (Supplementary Table 5). Analysis of Gene Ontology terms found concordant results (Supplementary Table 6).

The mitogen *Igf2* was markedly induced in AFP-NICD tumors (16.3 fold up-regulated). *H19*, an imprinted gene for a long non-coding RNA that is commonly co-regulated with *Igf2*, was also over-expressed (39.5 fold). Typically, fetal liver express *Igf2* transcribed from three different promoters that can be detected using exon specific PCR (Supplementary Fig. 2a). Since previous reports have documented *IGF2* overexpression and reactivation of fetal *IGF2* promoters in human HCC^{29,30}, we tested whether this was also occurring in Notch-induced tumors in mice. *Igf2* promoters P1–P3 were strongly induced in tumors (4/5) when compared to control liver, mirroring the *Igf2* mRNA array levels (Supplementary Fig. 2b).

We also sought to identify candidate oncogenic partners during Notch-induced transformation. Hence, we profiled livers from AFP-NICD mice (n=6) at birth (postnatal days 0–2) -prior to the development of HCC or dysplasia- and compared them to monogenic livers (n=5). As predicted, Notch target genes were significantly up-regulated in bigenic

newborn livers (Supplementary Table 7). Consistent with these results, the Notch pathway appeared among top canonical pathways enriched upon Ingenuity Pathway analysis (Supplementary Table 8). In contrast to the tumors, the imprinted genes *Igf2* and *H19* were not significantly deregulated in newborn AFP-NICD mice. This finding suggests that *Igf2* may cooperate with activated Notch during malignant progression.

Activation of the Notch pathway occurs frequently in human HCC

We then used comparative functional genomics to determine whether Notch pathway activation occurs frequently in human HCC samples. For that purpose, we generated a 384-gene signature using differentially expressed genes between Notch-induced tumors in AFP-NICD mice and control liver from monogenics (Supplementary Table 9). The Notch tumor signature was first tested in our cohort of human samples recapitulating hepatitis C (HCV)-associated hepatocarcinogenesis (normal liver [n=10], cirrhosis [n=13], dysplastic nodules [n=18] and full-blown HCC [n=91]^{15,16}) using NTP²⁵. Interestingly, a subset of cancerous tissues held a significant prediction for the signature but none of non-cancerous tissue, which suggested that the signature was mostly capturing Notch activation in transformed tissues. When NTP was performed in HCCs, the Notch signature predicted activation of Notch in 29 out of 91 (31.8%) human HCC (Fig. 2). To rule out whether the signature was capturing unspecific genomic signals of malignant transformation unrelated to Notch activation, we defined a separate signature -consisting of 276 genes- using liver tissue from newborn bigenic animals (before tumor onset). When applied to human HCC, even though fewer samples were predicted with this Notch newborn signature (18%, 17/91), they were still significantly enriched in the Notch tumor signature ($P<0.001$; Supplementary Fig. 3). Interestingly, both signatures were enriched in a robust HCC subclass recently identified (Proliferation class¹⁵, $P<0.001$; Supplementary Fig. 3). Thus, the 386-gene Notch tumor signature identifies a subgroup of nearly a third of human HCCs associated with hepatitis C virus.

Having captured a subset of Notch-associated human HCCs based on the signature generated in murine tumors, we next determined the status of potential downstream effectors in these samples. To do so, we first crafted a Notch gene list (n=96) by merging previously reported lists of Notch pathway genes^{26,27} (Supplementary Table 3). When evaluating their differential expression between Notch-activated HCC (n=29) and normal liver (n=10, Fig. 2), we found a significant up-regulation of several Notch activators such as ligands (e.g., *JAG1*), α -secretases (e.g., *ADAM17*), effectors (e.g., *MAML1*, *NOV*) and target genes (e.g., *SOX9*, *SPPI*, *HEY1*). Pathway inhibitor *ITCH* was significantly down-regulated. In accordance with data from murine tumors, Notch-activated human HCCs were significantly enriched for high levels of *IGF2* expression (Fisher exact test $P=0.003$, Fig. 2), an alteration we previously found to be associated with fetal promoter reactivation²⁹. In addition, these tumors exhibited activation of the IGF, AKT and MTOR pathways as determined by phospho(p)-IGF-1R, p-Akt and p-RPS6 IHC staining (Fig. 2). To address possible mechanisms underlying Notch activation in humans, we integrated gene expression data with DNA copy number changes in the same cohort for Notch pathway genes. We failed to find high-level amplifications affecting any putative Notch gene, but there were DNA gains affecting the *HEY1* locus (chromosome 8). *HEY1* is a Notch target gene significantly up-regulated in HCCs with the Notch signature compared to normal tissue and tumors without the signature, mostly in those samples with DNA gains (Fig. 2 and Supplementary Fig. 4).

We further tested the performance of the signature on an additional set of 144 HCCs from different etiologies including also hepatitis B infection and alcohol-induced HCC¹⁷. Consistent with the results obtained using the HCV set, 29.2% (42/144) of these tumors exhibited the Notch signature. To assess the validity of this signature in biological samples, we performed IHC for the Notch target SOX9 in a subset of HCC with (n=27) and without

(n=24) the Notch signature. Nuclear staining of SOX9 (Fig. 3a) was more prevalent in HCCs with the Notch signature than without (19/27 vs 6/24, $P=0.001$), and mRNA levels of SOX9 were associated with both SOX9 nuclear staining and the Notch signature prediction ($P<0.001$, Fig. 3b).

SOX9 has been shown to maintain pancreatic progenitors and it has been proposed as a marker of progenitor cells with bi-potentiality in the adult liver^{31,32}. An emerging role of SOX9 in cancer promotion and metastasis-seeding has also been suggested³³. *SOX9* was highly up-regulated in Notch-activated HCC (10.6 fold compared to normal liver, Fig. 2). Additionally, we observed increased *SOX9* levels in an aggressive miRNA-based molecular subclass of HCC (C3 subclass³⁴), and identified miR-30 as a candidate regulator of *SOX9* (Supplementary Fig. 5).

Previous studies have identified activating *NOTCH1* mutations in solid tumors⁸. Hence, we sought to estimate their prevalence in our dataset and searched for the presence of mutations at hotspot exons 26, 27 and 34 of the *NOTCH1* gene using direct sequencing in 50 human HCCs from our HCV-related dataset. Two samples showed non-synonymous mutations (4%), indicating that activating mutations in hotspots of the *NOTCH1* gene do not account for pathway deregulation in most HCC cases.

The Notch signature co-clusters within the Proliferation class and predicts response to selective Notch inhibition

To better understand the predictive performance of the Notch 384-gene signature in human HCC, we further evaluated five publicly available HCC datasets, including a total of 407 samples using the NTP method (Supplementary Table 1). We also integrated data of Notch predictions with our previously described molecular classification of HCC¹⁵. As a whole, between 20–37% of samples in each HCC dataset showed the presence of the Notch signature (Fig. 4a). Of note, when we focused on those datasets with higher number of significant predictions based on a false discovery rate (FDR) <0.05 , the prediction rate for Notch ranged between 32–37%, very close to that of our own cohorts (~30%). In most datasets, there was a significant correlation between the Notch signature and our previously defined Proliferation class¹⁵, a class characterized by genomic signals related to cell cycle progression and proliferation. Thereafter, we tested the similarity between the Notch signature and signatures previously reported to confer aggressive clinical behavior in HCC using Cramer's V coefficient¹⁷. The Notch signature co-clustered with a group including the Proliferation class¹⁵, a CK19+ progenitor-derived HCC signature generated in rats³⁵, a cholangiocarcinoma-like gene expression trait described in HCC³⁶, and a prognostic TGF- β signature generated in mouse hepatocytes³⁷ (Fig. 4b). The Notch signature was not correlated with clinical outcome in these patients; what points towards its limited role as a prognostic biomarker in HCC¹⁸.

To further evaluate the Notch 384-gene signature and determine whether it predicts sensitivity to Notch pathway inhibitors, we applied this signature to a large panel of cancer cell lines (n=318) whose expression data is available online (caBIG®). We found that the signature captured Notch activation mainly in cells derived from solid tumors, as opposed to hematological malignancies, and liver cancer cell lines showed varied profiles (Fig. 5a). Then, we focused on the Notch signature prediction in a panel of 21 liver cancer cell lines (CCLE¹⁹, Fig. 5b) and selected three lines with the Notch signature (SNU423, SNU475 and SNU449) and two without (HepG2 and Huh7) for further analysis. When incubated with 2 μ M Compound E, a GSI that inhibits ligand-dependent Notch signaling, SNU423 and SNU475 showed significant decrease in cell viability, whereas HepG2 and Huh7 remained irresponsive ($P=0.002$, Fig. 5c). To obtain a more specific inhibition of the pathway, we transfected SNU449, SNU475 and HepG2 cells with a construct containing a dominant

negative form of the transcriptional co-activator of Notch MAML1 (DN-MAML-GFP). SNU449 and SNU475 (Notch signature-positive) showed decreased proliferation upon DN-MAML-GFP infection in comparison to GFP-only cells ($P<0.03$, Fig. 5d). In contrast, HepG2 (Notch signature-negative) showed an increase in proliferation ($P=0.036$, Fig. 5d).

DISCUSSION

In this study, we have undertaken a comprehensive and integrative approach to explore the role of Notch signaling in liver cancer pathogenesis. We have found that Notch signaling promotes liver carcinogenesis in a genetically engineered mouse model, and that this pathway is activated in one third of human HCCs. Specifically, our studies demonstrate that (i) Liver-specific Notch activation in mice recapitulates features of human hepatocarcinogenesis, including dysplasia and HCC; (ii) Several genes involved in the Notch signaling cascade are de-regulated in HCC, particularly *SOX9*; (iii) *Notch1* is a *bona fide* oncogene in experimental liver cancer; (iv) Notch and IGF signaling are frequently co-activated in experimental and human HCCs, and *Igf2* induction occurs via reactivation of silenced *Igf2* promoters; (v) A 384-gene signature obtained from Notch-induced tumors is able to recognize pathway activation in approximately 30% of human HCCs from different etiologies; (vi) Inhibiting Notch signaling in liver cancer cells lines that harbored the Notch signature, but not those that lacked it, resulted in growth inhibition. Taken together, these data suggest that activation of Notch signaling is pivotal in liver oncogenesis.

The role of the Notch cascade in solid tumors is controversial⁷, and despite strong data indicating that Notch activation facilitates tumor progression in the liver^{38,39}, some reports suggest the opposite^{10,11,40}, a discrepancy that may be due to the high context-dependency of the Notch cascade⁷. Ours is the first study to use a genetically engineered mouse model to test the tumorigenicity impact of liver-specific Notch activation *in vivo*. In our model, HCCs arose with a latency of greater than 6 months, suggesting that additional hits may be needed in addition to Notch deregulation to achieve cell transformation. In this regard, we found strong up-regulation of *Igf2* in Notch-derived tumors, raising the possibility that *Igf2* acts as an oncogenic partner of Notch in this setting. Reactivation of *Igf2* fetal promoters and their tight correlation with mRNA levels lends credence to such a cooperative pathogenic role, as both promoter reactivation and *IGF2* up-regulation are well recognized features of human HCC^{29,30}. Whether there is a functional crosstalk between both cascades in HCC needs further evidence. Previous reports suggested a cooperative oncogenic role between Notch and other pathways such as RAS⁴¹.

We undertook a comparative functional genomics⁴² approach to connect molecular pathogenic features of human cancer to the mouse model by integrating high-density genomic data. This approach has previously been used in HCC⁴², which resulted in the identification of best-fit mouse models to model human HCC. In line with this concept, we aimed to translate detection of aberrant Notch activation in rodent HCC to humans by identifying common transcriptome deregulation patterns in murine Notch-activated tumors and human HCC. To this end, we generated a 384-gene signature of Notch-activated HCC from mice, and determined its predictive performance in 642 human HCC samples. Overall, 30–35% of HCCs were confidently predicted to harbor the signature, regardless of etiology and disease stage. Strikingly, tumors with the signature were significantly enriched in the “Proliferation class” of our previously described molecular classification¹⁵, and showed de-regulation of several Notch pathway genes such as *HEY1*, which has been recently proposed as an oncogene in HCC⁴³. Recent data suggest that this class is heterogeneous, capturing different genomic signals such as activation of TGF β ¹⁸, MET⁴⁴, IGF^{15,29} and progenitor-derived HCC⁴⁵.

Detailed pathological examination of Notch-induced HCC identified 3 major cell populations: (1) “proliferative” duct-like cells which were positive for CK19; (2) hepatocyte-like cells which were negative for CK19, including normal, dysplastic and malignant hepatocytes; and (3) atypical small oval-shaped cells which formed nests within tumors that were CK19 negative but positive for scattered pan-Cytokeratin staining. All Notch-induced tumors showed various degrees of nuclear staining for the Notch target gene *Sox9*, and *SOX9* overexpression was frequently observed in human HCCs, particularly those tumors bearing the Notch signature. A recent study³² suggests that Sox9 marks a pluripotent population within the liver, and thus it is possible that Notch acts during hepatocarcinogenesis by expanding a pre-existing progenitor-like cell or conferring progenitor-like properties to differentiated cells during hepatocarcinogenesis.

The success of targeted therapies that block oncogene addiction loops, such as vemurafenib⁴⁶ in mutant BRAF melanomas or crizotinib in lung cancer patients with ALK rearrangements⁴⁷, underscores the importance of identifying molecular drivers of cancer. At present, HCC lacks any known oncogene addiction loop amenable for selective targeting. Recent failures in phase 3 trials with systemic agents (e.g., sunitinib negative in first line and brivanib negative in first and second line) highlight the need for new therapeutic targets and a change in trial design to routinely include enrichment strategies based on molecular markers of response, and the Notch signature fulfills both criteria. Similarly to other molecular biomarkers of response in oncology (e.g., ALK rearrangements in lung cancer), lack of prognosis performance of the Notch signature does not diminish its potential role as a therapeutic biomarker. We provide evidence of differential response to selective Notch inhibition depending upon the presence or absence of a Notch signature, both with a pharmacological inhibitor (GSI) and a specific molecular inhibitor (dominant negative MAML) of Notch signaling. This finding raises the possibility that the estimated 30% of HCCs exhibiting Notch pathway activation could be responsive to Notch inhibition. Prospective trials, ideally including populations enriched with biomarkers of Notch activation, will be necessary to address this issue. Of note, interrogation of a cohort of 318 cancer cell lines for the presence of the 384-gene signature predicted Notch activation only in cells derived from solid tumors. The presence of differential genomic traits between Notch-induced solid tumors and hematological malignancies might be expected with such a context-dependent pleiotropic pathway.

In summary, this study provides evidence that the Notch pathway is involved in the pathogenesis of HCC, with 30–35% of tumors exhibiting pathway activation according to a novel gene signature across different datasets. Overall, our findings indicate that Notch may be an appealing target for new drug development initiatives in HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FDR	false discovery rate
FFPE	formalin-fixed paraffin-embedded
GSI	γ -secretase inhibitor
HCC	hepatocellular carcinoma
IHC	immunohistochemistry
NICD	Notch intracellular domain
NTP	nearest template prediction

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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 (i.e., infiltration of duct-like cells with marked architectural distortion), and intense ductular proliferation (as observed in CK19 staining image).

Figure 2. Notch activation and de-regulation in human HCC

Prediction of the Notch signature (red in the first row) in HCCs of the HCV-related dataset (n=91). Each square represents data of each individual sample. One third of the samples (31.8%) had activation of Notch based on a significant prediction for the presence of the signature (FDR<0.05, Nearest Template Prediction method). Tumors with the Notch signature were significantly enriched in the Proliferation class of our molecular classification of HCC¹⁵ (Proliferation=green). In addition, HCCs with activated Notch were significantly enriched in different markers of IGF pathway activation including phosphorylated(p)-Akt, p-IGF1R, p-RPS6 (immunohistochemistry), as well as high expression levels of *IGF2*²⁹. The bottom panel shows Notch Pathway Gene List (see methods for details) genes found significantly deregulated between Notch-activated HCC and those HCC without the signature as well as with normal liver (FDR<0.05, red and blue bar in the first row, respectively). Deregulation magnitude is graded based on a red (over-expression) and green gradient (down-regulation) normalized to the median expression value in normal liver.

Figure 3. De-regulation of the Notch target gene *SOX9* in HCC

(a) Representative images of nuclear SOX9 staining. Top panels show SOX9 staining in mice: bile duct staining in livers from control monogenic mice (upper left), hepatocytes and cholangiocytes in murine tumors (upper right). Bottom panels show human HCC samples with negative (bottom left) and positive (bottom right) nuclear staining. (b) Boxplots of *SOX9* mRNA levels from HCC samples (n=51) with and without the Notch signature (left) or SOX9 nuclear staining (right).

Figure 4. Signature-based activation of Notch across HCC datasets

(a) Performance of the Notch signature across HCC datasets. Top rows in each dataset represent Notch predictions, bottom rows show our molecular classification of HCC¹⁵ (Proliferation=green) for each sample. In all except one dataset (HCC-Izuka), tumors with Notch activation are enriched in patients of the Proliferation class. **(b)** Heatmap of Cramer's V coefficient showing correlation between the Notch signature and other previously reported signatures that predict clinical aggressive behavior^{17,48}. Briefly, Cramer's V statistic values range from 0 to 1, being 0.36–0.49 substantially correlated, >0.5 strongly correlated and 1 identical.

Figure 5. Notch signature predicts response to Notch inhibition *in vitro*

(a) Prediction of the Notch signature in 318 cancer cell lines from the Cancer Biomedical Informatics Grid, caBIG® (dark green: solid tumors' cell lines, light green: hematological malignancies' cell lines). (b) Notch signature prediction in 21 liver cancer cell lines (Broad-Novartis Cancer Cell Line Encyclopedia)¹⁹. Cell lines used in further analysis are highlighted in bold. (c) Cell viability upon 5-day incubation with a γ -secretase inhibitor. Decrease in viability was restricted to cells harboring the Notch signature; standard deviation is shown. (d) Representative images of pH3 staining in DN-MAML-GFP and GFP-only (control) transfected cells, white arrowheads highlight transfected proliferating cells (GFP+ and pH3+). Quantification of cell proliferation was evaluated by enumerating pH3+ GFP+ cells as a percentage of total GFP+ cells in liver cancer cell lines 48h post-infection; standard deviation is shown.