Hedgehog Signaling Blockade Delays Hepatocarcinogenesis Induced by Hepatitis B Virus X Protein

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Abstract

The hepatitis B virus (HBV) encoded X protein (HBx) contributes centrally to the pathogenesis of hepatocellular carcinoma (HCC). Aberrant activation of the Hedgehog (Hh) pathway has been linked to many tumor types including HCC. Thus, experiments were designed to test the hypothesis that HBx promotes HCC via activation of Hh signaling. HBx expression correlated with an upregulation of Hh markers in human liver cancer cell lines, in liver samples from HBV infected patients with HCC, and in the livers of HBx transgenic mice (HBxTg) that develop hepatitis, steatosis, and dysplasia, culminating in the appearance of HCC. The findings in human samples provide clinical validation for the in vitro results and those in the HBxTg. Blockade of Hh signaling inhibited HBx stimulation of cell migration, anchorage-independent growth, tumor development in HBxTg, and xenograft growth in nude mice. Results suggest that the ability of HBx to promote cancer is at least partially dependent upon the activation of the Hh pathway. This study provides biologic evidence for the role of Hh signaling in the pathogenesis of HBV-mediated HCC and suggests cause and effect for the first time. The observation that inhibition of Hh signaling partially blocked the ability of HBx to promote growth and migration in vitro and tumorigenesis in two animal models implies that Hh signaling may represent an “oncogene addiction” pathway for HBV-associated HCC. This work could be central to designing specific treatments that target early development and progression of HBx-mediated HCC. Cancer Res; 72(22):1–9. ©2012 AACR.

Introduction

The hepatitis B virus (HBV) “oncoprotein,” HBx, is a transactivating protein that contributes to hepatocellular carcinoma (HCC) by affecting cell-cycle regulation, DNA repair, multiple signaling pathways as well as cellular genes that are important for cell proliferation, inflammation, angiogenesis, immune responses, and epigenetics (1–4). Aberrant Hedgehog (Hh) pathway activation is seen in many tumor types where it accounts for about one-third of all cancer deaths (5). In the canonical pathway, Hh signaling is initiated by the binding of Hh ligands Sonic (Shh), Indian (Ihh), or Desert (Dhh) to the Patched (PTCH) receptor, which becomes internalized, leading to the activation of Smoothened (SMO) via release from PTCH-dependent suppression. SMO activates the Gli transcription factors that regulate the expression of Hh target genes (6). Altered Hh signaling contributes to tumor progression and invasion (7, 8). HBx has been shown to stabilize Gli1 and Gli2 in vitro (9), but the biologic implications of these findings are not clear. Thus, experiments were designed to test whether HBx promotes HCC, in part, through the activation of Hh signaling.

Recent work showed that HBV and HCV increased hepatocyte production of ligands that activate Hh signaling, thereby expanding the pool of Hh-responsive cells that promote liver fibrosis and cancer (10). Hh activation occurs in response to liver injury (e.g., growth of hepatic progenitors, inflammation, vascular remodeling, and liver fibrosis) in chronic liver disease (CLD; 11, 12). Inhibition of Hh signaling in HCC cell lines decreased expression of Hh target genes and resulted in apoptosis (13). Gli2 and Gli1 were shown to be primary and secondary mediators of Hh signaling, respectively (14, 15). Specifically, Gli2 upregulates Gli1 by direct interaction with the Gli1 promoter (16). Gli2 also plays a predominant role in the proliferation of HCC cells (17). Thus, Gli2 was further investigated here in HBx-mediated HCC.

Prior work has shown elevated Hh signaling markers in HCC (18), but their relationship to HBx, and whether they contributed to the cause or outcome of HCC, is not known. HBx correlated with the upregulated expression of Hh markers in vitro (8), but the biologic and pathologic consequences of this upregulation was not explored. In this work, these questions were addressed both in vitro and in vivo using 2 animal models.
The first consisted of HBxTg that develop progressive pathology in the liver very similar to that observed among HBV carriers, culminating in the appearance of HCC (19, 20). In these mice, HBx expression is not seen until after birth, meaning that the mice are not tolerant to HBx. As HBx expression increases with age, so does the severity of CLD. This model permits evaluation of the relationships between HBx, upregulation of Hh markers, and the pathogenesis of HCC. The second model consisted of HBx positive human HCC xenografts growing as subcutaneous tumor in nude mice. In this model, elevated Hh signaling was evaluated in tumor growth. The combined results support the hypothesis that HBx contributes to HCC by stimulating Hh signaling.

Materials and Methods

Cell lines

HepG2 cells were stably transfectd with HBx (HepG2X) or the control bacterial chloramphenicol acetyltransferase (CAT; HepG2CAT) genes by recombinant retroviruses and cultured without the selection of individual clones as previously described (21). Huh7X and Huh7CAT cells were prepared and cultured in the same way. These cell lines have been used in numerous studies that have been published (21).

Patient samples

Formalin-fixed, paraffin-embedded tumor (HCC)/nontumor (adjacent liver) tissues were obtained from Chinese patients who underwent surgery at the Third Military Medical University, Chongqing, China. All patients were hepatitis B surface antigen-positive in blood; 21 were males, 1 was female, and the age range was from 35 to 60 years (average: 47 years). Samples were used for diagnostic purposes and then for this study. Ten uninfected human liver tissue slides (Abcam) were used as controls. The use of these samples was approved by the Institutional Review Boards at all participating universities.

Quantitative RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen). Quantitative real-time PCR (qRT-PCR) was conducted using SensiFAST SYBR kit (Bionline). Primer Sequences are shown in Supplementary Table S1. Threshold cycles (Ct) were calculated by the StepOnePlus Detection System (Applied Biosystems). Target gene levels in the treated cells are presented as a ratio to levels detected in control cells according to the Ct method (22).

Western blot analyses

Liver tissues were rinsed in ice-cold PBS and homogenized in lysis buffer (Cell Signaling) with protease inhibitor cocktail (Sigma). Cell debris was removed by double centrifugation at 14,000 × g for 15 minutes. Protein extracts from cells were prepared using same lysis buffer. For Western blot analysis, 150 μg of protein extracts from liver tissues, 60 μg from Huh7CAT and Huh7X cells, and 100 μg from HepG2CAT and HepG2X cells were separated on SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were incubated with antibodies against Gli2, PTCH1 (Santa Cruz Biotechnology) or β-actin (Sigma). The blots were developed using the ECL plus kit (Amersham).

Immunohistochemistry

HBx transgenic and control mice, 3, 6, 9, 12 months old, were euthanized, their livers removed, fixed in formalin, and embedded in paraffin (23). Liver morphology was evaluated by hematoxylin and eosin staining. Mouse and human tissue sections were deparaffinized, dehydrated, treated with Universal REVE antigent retrieval (Innovex), and stained using the UltraVision Detection System (Thermo Scientific). For human tissues, antibodies used were anti-HBx (anti-99; 24), anti-Shh (Epitomics), anti-Gli2 (GenWay), anti-PTCH1, and anti-Ihh (Abcam). For mouse tissues, anti-HBx (anti-99), anti-Shh, anti-Ihh, anti-PTCH1 (Millipore), and anti-Gli2 (Abcam) were used. Normal mouse or rabbit immunoglobulin G (IgG; Vector Labs) were used to rule out false-positive responses. Preabsorption of primary antibodies with corresponding antigens was conducted to insure specificity. Scoring was based upon colorimetric evaluation.

Hh signaling inhibition

For in vitro experiments, the Smo inhibitor GDC-0449 (Vismodegib; Selleck Chemicals) was reconstituted in dimethyl sulfoxide (DMSO; Sigma) and used at a final concentration of 1 μmol/L for 24 hours. The ligand inhibitor, Shh neutralizing antibody (5E1), was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and used at 10 μg/mL for 24 hours. For in vivo experiments, GDC-0449 was reconstituted in 2-hydroxypropyl-β-cyclodextrin (Sigma) in water 45% (w/v) and used at 25 mg/kg for HBxTg and at 30 mg/kg for nude mice.

Phenotypic assays

Cell migration, with or without GDC-0449, was evaluated using 24-well BD BioCoat Matrigel Invasion Chambers (BD Biosciences). GDC-0449 was added for the duration of the assay. To assess anchorage-independent growth (soft agar assay) cells were seeded (25) with or without GDC-0449. Colonies were counted after 22 days. Medium in all wells was changed twice a week.

Treatment of mice

HBxTg used herein have been previously described (19, 20). Twelve-month-old mice were treated daily with GDC-0449 or control vehicle by intraperitoneal injection (6 mice per group, total 19 injections). For xenograft experiments, male nude mice (Hsd: Athetic Nude-Fox1tm1, Harlan Labs) 3 to 8 weeks old, were injected subcutaneously in the flank with 0.2 mL containing 1 × 107 viable cells in PBS (5 mice for each cell line). Treatment with GDC-0449 or vehicle (19 injections) was started after tumor volumes reached about 0.6 cm3. Tumor volumes were estimated by caliper measurements as described (26). At the end of the experiment, tumors were removed. Volumes were also measured by water displacement, and their wet weights were determined.

Mice were housed in a pathogen-free room under controlled temperature and humidity. All animal protocols have been
approved by the Temple University Institutional Animal Care and Use Committee.

Statistics

The relationship between HBx and Hh markers by immunohistochemistry (IHC) was determined using 2 × 2 comparisons in the \( \chi^2 \) test. Statistical significance was considered when \( P < 0.05 \). The Student \( t \) test was used to calculate the significance of mean difference in all other measurements. Significant relationships were identified when \( P < 0.05 \).

Results

HBx stimulates Hh signaling

Lysates from HBx-expressing (HepG2X, Huh7X) and from HBx-negative (HepG2CAT, Huh7CAT) cells were analyzed for Hh components by qRT-PCR and Western blot analysis. qRT-PCR showed increased levels of Shh (2-fold; \( P < 0.01 \)), Gli2 (4-fold; \( P < 0.05 \)), and PTCH1 (2.4-fold; \( P < 0.05 \)) in HepG2X compared with HepG2CAT cells (Fig. 1). Upregulation of these mRNAs in Huh7X compared with Huh7CAT cells was 4.7-fold for Shh (\( P < 0.005 \)), 4.4-fold for Gli2 (\( P < 0.005 \)), and 2.7-fold for PTCH1 (\( P < 0.01 \); Fig. 1). The SMO antagonist, GDC-0449, now in Phase II trials for several cancers (27), was used to inhibit Hh signaling. GDC-0449 treatment of HepG2X cells decreased mRNA levels of Shh by 2.6-fold (61%, \( P < 0.005 \)), PTCH1 by 6.8-fold (85%, \( P < 0.05 \)), and Gli2 by 2.4-fold (58%, \( P < 0.05 \)). In Huh7X cells, mRNA levels were reduced by 6.1-fold for Shh (84%, \( P < 0.005 \)), by 4.1-fold for PTCH1 (76%, \( P < 0.005 \)), and by 4.9-fold for Gli2 (80%, \( P < 0.005 \)). In control cell cultures, there was no significant difference in these Hh markers with or without drug.

Hh signaling was also inhibited by the neutralizing antibody to Shh (5E1), which prevents Shh from binding to PTCH. In HepG2X cells, this resulted in decreased Shh mRNA (5.9-fold; 83%, \( P < 0.005 \)), PTCH1 mRNA (2.8-fold; 64%, \( P < 0.05 \)), and Gli2 mRNA (2.3-fold; 57%, \( P < 0.05 \)). In Huh7X cells, reduction was 9.1-fold for Shh (89%, \( P < 0.05 \)), 2.7-fold for PTCH1 (63%, \( P < 0.01 \)), and 7.7-fold for Gli2 (87%, \( P < 0.01 \); Supplementary Fig. S1). In control cells, 5E1 had no effect, implying that HBx transactivates Hh signaling.

When Gli2 and PTCH1 levels were evaluated by Western blot analysis, both were elevated in HepG2X compared with HepG2CAT cells (1.8-fold; \( P < 0.02 \) for Gli2 and 2-fold; \( P < 0.02 \) for PTCH1; Fig. 1). They were also elevated in Huh7X compared with Huh7CAT cells (2.5-fold; \( P < 0.01 \) for Gli2 and 1.9-fold; \( P < 0.03 \) for PTCH1; Fig. 1). It was not possible to conduct accurate Western blot analysis for Shh and Ihh, as they are mostly extracellular. GDC-0449 reduced Gli2 in HepG2X cells (2.2-fold; 55%, \( P < 0.02 \)) and in Huh7X cells (3.8-fold; 74%, \( P < 0.02 \)), but not in treated compared with untreated control cells (Fig. 1). GDC-0449 also reduced PTCH1 in HepG2X (1.8-fold; 44%, \( P < 0.02 \)) and Huh7X cells (2.6-fold; 61%, \( P < 0.01 \)), but not in the HBx negative cultures. Hence, HBx stimulates expression of Hh components in human liver cancer cells.

HBx promotion of cell migration and growth in soft agar is largely Hh dependent

Both Hh signaling (28) and HBx (29) promote cell migration. To determine whether HBx-stimulated migration was Hh dependent, cells were treated with GDC-0449. Migration of Huh7X cells was blocked an average of 4.4-fold (\( P < 0.01 \)) and HepG2X cells by 2.2-fold (\( P < 0.02 \)), but there was no effect of
treatment upon HBx negative cells (Fig. 2). Hence, HBx stimulation of cell migration correlated with upregulation of Hh markers, although the migration of HBx negative cells was largely independent of Hh pathway activity.

Prior work has shown that HBx promotes anchorage-independent growth (21). To determine whether this depends upon Hh signaling, HBx positive and negative cells were seeded into soft agar with or without GDC-0449. GDC-0449 decreased the clonability of Huh7X cells by 2.2-fold compared with untreated cells (\( P < 0.01 \)) and of HepG2X cells by 1.8-fold compared with untreated cells (\( P < 0.03 \); Fig. 2). Growth of HBx negative cells was not significantly different under identical conditions, suggesting that Hh signaling contributes to HBx-associated, anchorage-independent growth.

**Hh markers in human liver and HCC samples**
Paraffin-embedded tissues from 22 deidentified patients were used to evaluate HBx and Hh markers by IHC. Among these, 17 patients had tumor and adjacent nontumor liver, 3 only had tumor, and 2 had nontumor liver. HBx staining, mostly cytoplasmic (Fig. 3), was seen in 15 of 20 tumors (75%) and in all 19 nontumor livers (100%; Supplementary Table S3). Cytoplasmic Shh staining (Fig. 3) was observed in 12 of 19 nontumor samples (63%) and in 12 of 20 tumors (60%). Five patients had Shh staining in tumor and nontumor (Supplementary Table S3). In HCC, strong Shh staining was seen at the growing margin of tumors (Fig. 4). Cytoplasmic Ihh was seen in 14 of 19 nontumor cases (74%), in 6 of 14 cases of HCC (43%), and in both compartments of 4 patients (Supplementary Table S3, Figs. 3 and 4). Nuclear Gli2 was observed in 11 of 19 nontumor samples (58%), in 11 of 20 tumors (55%), and in 6 patients in both compartments (Figs. 3 and 4). Membranous PTCH1 was observed in 10 of 19 nontumor samples (53%), in 9 of 20 tumors (45%), and in both compartments in 4 patients.

![Figure 2](image2.png)

Figure 2. Phenotypic changes associated with Hh signaling in HBx positive and negative cells with or without GDC-0449. A, representative images of HBx expressing cells that migrated through Matrigel basement membrane (×200); B, quantification of the results in A (mean expression ± SD of 3 assays). Cells were treated with DMSO (dark bars) or with GDC-0449 (light bars). \(* P < 0.01; \)**, \( P < 0.02 \). C, anchorage-independent growth of Huh7X and HepG2X with or without GDC-0449. D, quantification of the results in C (mean expression ± SD of 3 assays). Cells were treated with DMSO (dark bars) or with GDC-0449 (light bars). \(*, P < 0.01; \)**, \( P < 0.03 \).

![Figure 3](image3.png)

Figure 3. Costaining of HBx with Gli2 (×400; A), with Ihh (×200; B), and with Shh (×400; C) in nontumor sections of clinical samples from HBV-infected patients.
Ten commercially available liver sections from uninfected individuals were negative for HBx, Gli2, Shh, PTCH1, and Ihh (data not shown). Staining with normal IgG proved specificity of staining (Fig. 4). Thus, Hh signaling is activated in nontumor and tumor of HBV patients with HCC.

When these relationships were evaluated by χ² analysis (Supplementary Table S2), there was a strong correlation between HBx and all Hh markers in nontumor liver, where HBx staining was stronger and widespread, as previously reported (30), but not in adjacent tumor, where HBx expression was often among scattered cells. This suggests a tight correlation between HBx expression and activated Hh signaling in nontumor liver. It appears that once triggered by HBx, Hh signaling remains activated in HCC even in cells without detectable HBx expression.

Hh markers in HBx transgenic mice

The centrality of HBx to the development of HCC is recapitulated in HBxTg that develop progressive lesions in the liver as in human carriers (19, 20). These mice develop hepatitis and steatosis by 5 to 6 months of age, dysplastic nodules by 8 to 9 months, and visible HCC by 12 months in 100% of mice. This is accompanied by increased HBx expression with age in the liver. Nontransgenic littersmates had no lesions in their livers at any age (Supplementary Fig. S2). Staining for HBx, Gli2, and Shh in livers from 3, 6, 9, and 12 months old transgenic mice showed an increase in all these markers with age (Fig. 5). As in human livers, HBx and Shh staining was mostly cytoplasmic, although membranous Shh was also seen. Gli2 staining was nuclear, with some cytoplasmic localization (Fig. 5). PTCH1 was membranous, although Ihh was cytoplasmic, membranous, and within some liver sinusoids (Supplementary Fig. S3A). Livers from nontransgenic littersmates were negative for all Hh markers (Supplementary Fig. S3B). Thus, Hh signaling is increasingly activated in HBxTg with age and the severity of CLD.

When χ² analysis was conducted on these markers in mice of different ages, there were statistically significant relationships between HBx and each of the Hh markers in the liver. The most striking relationship in the livers of 3-month-old mice was between HBx and Gli2 (Supplementary Table S4). In tumors from 12-month-old mice, the correlation between HBx and Hh markers no longer existed.

Hh signaling in the pathogenesis of HBx associated HCC

To determine whether HBx promotes hepatocarcinogenesis via Hh signaling, the effect of GDC-0449 on tumor development in HBxTg and tumor growth in xenograft-bearing nude mice was evaluated. Untreated 12-month-old HBxTg had multiple tumors on the surface of their livers (Fig. 6A) although most GDC-0449-treated mice had fewer tumors. These differences were statistically significant (Fig. 6B). Excised tumors showed lower levels of Gli2 in GDC-0449-treated mice compared with controls (Fig. 6C). The latter was confirmed by staining, where no Gli2 was observed in treated compared with untreated mice (Fig. 6D). Shh staining was much weaker and dispersed compared with untreated mice (Fig. 6D). Thus, inhibition of Hh signaling resulted in decreased number of tumors.

In the xenograft experiments, nude mice bearing HBx positive or negative xenografts were treated with GDC-0449 or vehicle (Fig. 7 and Supplementary Fig. S4). The contribution of HBx only to cell growth via Hh signaling was assessed as described in the legend of Fig. 7. GDC-0449 inhibited the growth of HepG2X compared with HepG2CAT tumors by approximately 2-fold (P < 0.01; Fig. 7A). Identical experiments with Hu7X cells showed differences of 5.6-fold (P < 0.005; Fig. 7B). There was little difference in the tumor size of HepG2CAT and Hu7CAT cells whether or not they were treated (data not shown). Tumor volumes determined by water displacement showed similar to those determined by caliper measurements.
be “addicted” to an activated Hh pathway in chronic HBV infection. Thus, the ability of HBx to promote HCC appears to depend upon the activation of Hh signaling, suggesting that HCC may be "addicted" to an activated Hh pathway in chronic HBV infection.

### Discussion

This is the first report showing that activated Hh signaling is linked to the expression of HBx in the pathogenesis of HCC. Elevated Hh markers in HBx positive HepG2 and Huh7 cells correlated with the ability of HBx to promote cell migration and growth in soft agar. GDC-0449 and Shh neutralizing antibody reduced the expression of Hh markers in these cells, cell migration and growth (Figs. 1 and 2, Supplementary Fig. S1). Importantly, elevated Hh marker expression was seen in HBxTg (but not in nontransgenic littermates; Fig. 5). These results were validated in tissue samples from patients with HBV-associated HCC, where costaining between HBx and Hh markers was seen in the livers but not in HCC nodules (Fig. 3, Supplementary Table S2), suggesting that once Hh markers were upregulated by HBx, they remained elevated even when HBx was no longer detectable. Treatment of HBxTg with GDC-0449 yielded significantly fewer tumors as well as depressed Gli2 expression (Fig. 6), suggesting that Hh signaling contributed to tumor development. GDC-0449 also inhibited the growth of HBx expressing xenografts in nude mice (Fig. 7). Thus, the ability of HBx to promote HCC appears to depend upon the activation of Hh signaling, suggesting that HCC may be "addicted" to an activated Hh pathway in chronic HBV infection.

The results from Fig. 1 show upregulated expression of Shh, PTCH1, and Gli2 in the presence of HBx, although the underlying mechanism(s) remain to be defined. Although these results suggest that these Hh components are transcriptionally activated by HBx, other work (9) has shown that HBx does not upregulate Gli transcription factors, but posttranslationally stabilizes them. Although both studies used HepG2 and Huh7 cells, the experimental designs were different. In particular, prior work used transient transfection, whereas the work here was carried out with stably transfected cell lines. The latter is more representative of the host-virus relationship in the chronically infected liver and also reflects the relationship between HBx and Hh markers in cell culture and in HBxTg. Gli2 can be posttranslationally stabilized by deacetylation (31), which may occur by the recruitment of mSin3A-HDAC1 deacetylase complex by HBx (4, 32). Gli2 transcriptional activation by HBx is also possible. The Gli2 promoter has SMAD and T-cell factor/lymphoid enhancer factor binding sites, making it responsive to TGF-β and β-catenin (25, 33), both of which are transactivated by HBx (34, 35). Because Gli2 is expressed in the absence of Hh signaling (36), it may be activated by HBx through TGF-β1. Although the HBx activation of Hh signaling may upregulate Hh target genes (such as PTCH1), the elevated expression of Shh, which is not an Hh target gene, must occur by other mechanisms. The upregulation of Shh in HBx expressing cells (Fig. 1) could be mediated through HBx activation of NF-κB (37), which binds to the Shh promoter and induces Shh expression (38). HBx activation of canonical Hh signaling is also suggested by the correlation between HBx and Hh markers in chronically infected human liver (Fig. 3) and in HBxTg livers with age (Fig. 5, Supplementary Table S4). TGF-β1 may also

### Figure 6. Relationship between Hh signaling and HCC in HBxTg.

A, HCC nodules (circled) on the surface of the liver. B, the number of visible nodules observed on livers (n = 6 HBxTg per group) after injections of vehicle (dark bars) or GDC-0449 (light bars). Tumor numbers for individual mice are shown above each bar. The average tumor number is shown above each group. C, Western blot analysis for Gli2 in livers from transgenic mice treated with vehicle (−) or GDC-0449 (+). D, staining for Gli2 and Shh on serial sections of tumors (T) and nontumor (NT) livers from HBxTg treated with vehicle (top) or GDC-0449 (bottom). Magnification is ×100 for each panel and ×400 for each insert.
promote canonical Hh signaling, as TGF-β1 upregulates Shh mRNA and protein (39). The finding that treatment of HBx expressing cells with Shh neutralizing antibody 5E1 resulted in decreased levels of Gli2 and PTCH1 mRNAs (Supplementary Fig. S1) also supports a role for canonical signaling in HBx-mediated Hh activation. Thus, HBx may promote Shh expression by multiple pathways, and may underlie differences in the presence, frequency, and distribution of some of the Hh markers evaluated by staining.

The importance of HBx promoting tumorigenesis through the activation of Hh signaling is underscored by experiments using GDC-0449, which blocked the ability of HBx to stimulate cell migration and anchorage-independent growth (Fig. 2). These findings correlated with suppressed levels of Gli2, PTCH1, and Shh (Fig. 1). Stimulation of migration is a part of epithelial-to-mesenchymal transition that results in the remodeling of liver during CLD and promotion of metastasis during cancer progression. The role of Hh signaling in HBx-mediated tumor progression was confirmed in xenograft experiments and in HBxTg, where GDC-0449 inhibited tumor growth (Figs. 6 and 7). Among transgenic mice, GDC-0449 treatment also correlated with decreased expression of Gli2 and Shh (Fig. 6). Although it is not clear how Shh is suppressed after GDC-0449 treatment (Fig. 1), this has been shown elsewhere (40, 41), implying an unidentified feedback loop in Hh signaling. Thus, Hh signaling may be important in the early stages of hepatocarcinogenesis. This is further indicated by the strong correlation between HBx staining and the appearance of Hh components before the detection of HCC in HBxTg (Fig. 5) and in infected human liver and HCC (Fig. 3). If HBx-mediated activation of Hh signaling results in the altered expression of Hh target genes, it may contribute to the pleiotrophic properties of HBx. Thus, HBx may constitutively activate Hh signaling in the pathogenesis of HCC, suggesting that Hh signaling may be a therapeutic target in this tumor type.

Although aberrant Hh activation associated with mutations has been documented in several tumor types, such mutations are rare in HCC (18). Aberrant Hh activation also occurs in nontumor liver (Figs. 3 and 5), suggesting that HBx may trigger
Hh signaling before tumor development. The oxidative environment in CLD appears to trigger Hh signaling and promotes HBx expression, which contributes to tumor development (42). The fact that normal human hepatocytes are resistant to Hh ligand-mediated signaling, that Hh responsive cells often consist of immature hepatocytes and/or tissue progenitors (17), and that HBx promotes the development of "stemness" in the liver (25), also suggests that HBx activates Hh signaling before the development of tumor.

HBx-mediated activation of Hh signaling might also be involved in the "oncogene addiction" of HCC. Two pathways, Raf/MEK/MAPK and PI3K/Akt, are known to be oncogene "addicted" in HCC (43). These pathways potentiate Hh signaling through noncanonical pathways (7, 44, 45) that are activated by HBx (46, 47). If so, this would provide strong rationale for the development of combination therapies that focus upon Gli2. Because there are some 50 drug candidates being tested in roughly 200 clinical trials (48), a major problem contributing to the development of resistance and failure of so many trials may be the lack of combination therapies targeting pathways associated with "oncogene addiction." Perhaps the linkage of HBx to activated Hh signaling in the pathogenesis of HCC will result in therapies that are better targeted to prevent tumor appearance and/or block the growth and relapse of established tumors.

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