Dysregulation of the Hedgehog pathway in human hepatocarcinogenesis

Jason K. Sicklick 1,6, Yin-Xiong Li 4,6, Aruna Jayaraman 2, Rajesh Kannangai 1, Yi Qi 6, Perumal Vivekanandan 1, John W. Ludlow 1, Kourosh Owzar 5, Wei Chen 6, Michael S. Torbenson 3 and Anna Mae Diehl 1,6

1 Department of Surgery and Division of Surgical Oncology, 2 Division of Gastroenterology and 3 Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA; 4 Department of Cell Biology and Pediatrics, 5 Department of Biostatistics and Bioinformatics and 6 Division of Gastroenterology and Duke Liver Center, Duke University Medical Center, Durham, NC, USA and 7 Vesta Therapeutics, Durham, NC, USA

Abstract

Introduction

Dysregulation of the Hedgehog (Hh) pathway has been implicated in the genesis of cancers that are derived from multiple tissue types (1). Along with studies of other developmentally regulated signaling pathways, such as Wnt, these findings have added to a growing body of evidence for the stem cell theory of cancer, which holds that tumors, like normal tissues, are generated by a small number of self-renewing stem cells (1). From embryogenesis to adulthood, skin and gastrointestinal progenitors are regulated by Hh signaling (2–4). This pathway is activated when Sonic hedgehog (SHH) or Indian hedgehog (IHH) ligand bind to their receptor, Patched (PTC). When unoccupied by ligand, PTC is a tumor suppressor that binds and represses Smoothened (SMO), a proto-oncoprotein, from activating downstream components and transcription of target genes. Here we show that in HCCs, overexpression of the SMO proto-oncogene, as well as an increase in the stoichiometric ratio of SMO to Ptc mRNA levels, correlated with tumor size, a prognostic indicator in HCC biology.

Hedgehog (Hh) pathway activation promotes tumors in several endodermally derived tissues, but its role in the pathogenesis of hepatocellular carcinoma (HCC) is unknown. Although normal hepatocytes lack Hh signaling, activation of the Hh pathway in endodermal progenitors is required for liver development. Thus, we hypothesized that hepatocarcinogenesis may involve regulation of Hh signaling. This pathway is activated when Hh ligand binds to its receptor, Patched (PTC). In an unoccupied state, PTC normally functions as a tumor suppressor that inhibits Smoothened (SMO), a proto-oncogene, from activating downstream components and transcription of target genes. Here we show that in HCCs, overexpression of the SMO proto-oncogene, as well as an increase in the stoichiometric ratio of SMO to Ptc mRNA levels, correlated with tumor size, a prognostic indicator in HCC biology. In one tumor we identified a novel SMO mutation in an evolutionarily conserved residue. We also demonstrated that HCC cell lines (HepG2 and Hep3B) expressed Hh pathway components and activated Hh transcriptional targets. In Hep3B cells, cyclopamine, an inhibitor of wild-type SMO, had no effect, but KAAD-cyclopamine, a blocker of oncogenic SMO, inhibited Hh signaling activity by 50%, decreased expression of the hepatocarcinogenic oncogene, c-myc, by 8-fold, and inhibited the growth rate of Hep3B cells by 94%. These data support our hypothesis that Hh signaling is dysregulated in human hepatocarcinogenesis. We demonstrate that overexpression of and/or tumorigenic activation of the SMO proto-oncogene mediates c-myc overexpression, which plays a critical role in hepatocarcinogenesis and suggests that SMO is a prognostic factor in HCC tumorigenesis.

Abbreviations: ρ, Spearman’s rank correlation; Afp, alpha-fetoprotein; Cyc, Cyclopamine; GUS, β-glucuronidase; HCC, hepatocellular carcinoma; Hh, Hedgehog; Ihh, Indian hedgehog; KAAD-cyclopamine or KAAD-Cyc, 3-Keto-N-(aminoethylamino)phosphinylcinnamoyl)-cyclopamine; mRNA, messenger RNA; PTC, Patched; RT–PCR, reverse transcription polymerase chain reaction; SMO, Smoothened; Shh, Sonic hedgehog; Tom, Tomatidine.
Hedgehog pathway in human hepatocarcinogenesis

despite the liver’s requirement for Hh signaling during embryogenesis (17). The pivotal role of Hh in liver development is proven by evidence that induction of Shh promotes hepatogenesis, whereas pancreatic differentiation ensues in the absence of Shh (17). The latter finding suggests that the liver and pancreas are derived from a common, Hh-responsive endodermal progenitor. If Hh regulates progenitors in postnatal livers, the Hh pathway may play an overlooked role in the formation of liver cancers. This possibility is further suggested by our recent finding that hepatic stellate cells, which reside in the mesenchyme of adult livers, produce Hh ligands (18). Herein, we evaluate our hypothesis that Hh signaling regulates hepatocarcinogenesis by examining Hh pathway expression and function in cultured hepatocellular carcinoma (HCC) cell lines, comparing Hh pathway expression in non-neoplastic and malignant human livers, and correlating the expression of Hh pathway components with human HCC biology.

Materials and methods

Animal care

Adult, male Ptc-lacZ reporter mice were obtained from Dr P.A. Beachy (Johns Hopkins University, Baltimore, MD). Animal experiments fulfilled NIH, Johns Hopkins University and Duke University requirements for humane animal care.

Ptc-lacZ staining and reporter assay

We studied mice in which one allele of Ptc is replaced in-frame with the β-galactosidase gene by homologous recombination in order to evaluate Hh signaling in the liver. As Ptc is a transcriptional target of the GLI proteins, expression of β-galactosidase indicates activation of the Hh pathway (19,20). Staining and quantification of reporter expression were performed as described previously using the β-galactosidase Detection Kit (Promega, Madison, WI) (21).

Culture of cell lines

HepG2, Hep3B and C3H10T½ cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured according to their instructions. The HCT116 cell line was purchased from the Duke University Cancer Center Tissue Culture Facility (Durham, NC) and cultured according to supplier instructions.

Isolation of hepatocytes

Donated human livers, not suitable for orthotopic liver transplantation, were obtained from federally designated organ procurement organizations. Informed consent was obtained from next of kin for use of the livers for research purposes. The portal vein and/or the hepatic artery were cannulated and the organ perfused with EGTA-containing buffer for 15 min followed by digestion with 125 mg/l Liberase (Roche, Nutley, NJ), a highly purified preparation used for this purpose. The liver was cut into 1 cm pieces, and the tissue was dispersed by using a combination of collagenase, elastase, and DNase. Enzymatic digestion of the liver was performed according to the manufacturer's recommendations. After a 3.5 h incubation, the liver was incubated in a solution of DMEM containing 10% FCS, 1% glutamine, and 10% heparin in the absence of antibiotics. The liver was then suspended in 1000 μl PBS and digested with 1 mg/ml DNase I (Roche). The digested tissue was filtered through a 70 μm filter, and the resulting cell suspension was centrifuged at 400 x g for 10 min. The isolated hepatocytes were then cultured on silicone-coated tissue culture dishes at a density of 10⁶ cells/ml in DMEM containing 10% FCS, 1% glutamine, 50 μg/ml Penicillin/streptomycin, 100 U/ml penicillin and 100 μg/ml streptomycin. The culture medium was changed every 2 days.

Pharmacological regulation of Hh signaling

The Hep3B cell line was treated with regulators of the Hh signaling pathway in a dose- and time-dependent fashion. Cultures were treated with mouse IgG1 isotype control antibody reconstituted in sterile phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) as per manufacturer instructions (Roche). Specificity of the experiments was confirmed by Western blot analysis. The Hep3B cell line was plated at a density of 5000 cells per well, cultured for 24 h, and then treated with reagent medium or appropriate control medium for up to 96 h. Cells were then incubated with tetrazolium reagent (2-(4-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyltetrazolium chloride)). Absorbance was read at 490 nm using a microplate reader.

Statistical analysis

Descriptive measures were calculated as the mean ± SD, median, or percent of total if relevant. Statistical differences between the distributions of two continuous variables were assessed using the Wilcoxon–Mann–Whitney test (49). In two sample problems, the employment of non-parametric tests over their parametric counterparts is generally more powerful than the underlying distributions are a priori not known. In the
situation of small sample sizes, the utility of non-parametric tests may be limited due to low power to detect stochastic discrepancies. Therefore, we quantitatively assessed these discrepancies using Welch’s version of the t-test (50). We note that the control of the Type I error is not guaranteed as the underlying distributions are not necessarily normal and that the observations within each sample are not mutually independent by virtue of the normalization method. The pairwise associations between target gene expression levels and continuous clinical outcomes [e.g. tumor size and serum alpha-fetoprotein (AFP) level] were estimated using Spearman’s rank correlation (ρ) (49). Given the sample size and the presence of ties in the data, the null distribution was approximated using 50 000 permutation replicates rather than using asymptotics. To explore gene expression relationships, exploratory cluster was approximated using 50 000 permutation replicates rather than using asymptotics. To explore gene expression relationships, exploratory cluster analyses using Spearman’s correlation coefficient, as the distance measure, were employed. P-values were not adjusted for multiple testing.

Results

Normal hepatocytes lack Hh pathway activity

Given that Hh pathway activation is obligatory for liver bud formation, it is conceivable that cells in adult livers might have residual Hh pathway activity. To address this issue, we studied Ptc-lacZ mice where Hh-responsive elements in Ptc, a known downstream gene target of the Hh pathway, drive β-galactosidase expression to report Hh activity. We examined three healthy Ptc-lacZ mice (10–14 weeks old) to determine if mature hepatocytes exhibited Hh activity. LacZ-expressing hepatocytes were not detected at ×20 (Figure 1A) or ×100 magnifications (Figure 1B), although there were numerous β-galactosidase-positive cells in the wall of the gallbladder (×40 magnification) (Figure 1C), consistent with the role of Hh signaling in gallbladder cancer (15). Our finding that mature hepatocytes lacked Hh activity was consistent with results from other groups (7,15), and further verified by our subsequent studies of primary hepatocytes isolated from the livers of two additional Ptc-lacZ mice. Protein extracted from the freshly isolated hepatocyte fraction did not exhibit β-galactosidase activity (data not shown).

Malignant human HCC lines express Hh pathway components

In order to determine if malignant hepatocytes express components of the Hh signaling pathway, we studied two well-characterized in vitro models of liver cancer, the HepG2 and Hep3B cell lines (51). Using two-step RT–PCR we found that both lines expressed the Hh ligands, Shh and Ihh, the tumor-suppressor gene Ptc, the proto-oncogene Smo, as well as the downstream transcription factor, Gli1 (Figure 2A). Quantitative real-time RT–PCR was done to compare gene expression in the two cancer cell lines and Percoll-isolated primary human hepatocytes (Hep). In each assay, expression levels were normalized to that of the housekeeping gene, β-glucuronidase (Gus), in the same RNA samples. As expected, both malignant and non-malignant hepatocytes expressed Albumin (Figure 2B). However, when compared to Albumin gene expression in Hep, the HepG2 cells expressed 2.1-fold more Albumin (P < 0.0004) and the Hep3B cells expressed 40% less Albumin (P < 0.0007). Consistent with the routine use of the immature hepatocyte marker, AFP, as a serologic marker for HCC, both cancer cell lines strongly expressed this gene, while expression was barely detected in Hep. The HepG2 and Hep3B cancer lines expressed 208 064-fold (P < 0.0001) and 602-fold (P < 0.0001) more Afp than Hep, respectively (Figure 2D). Expression of Hh ligands and Hh pathway signaling components was detected in both non-malignant and malignant hepatocytes (Figure 2D). However, compared to Hep, the two HCC lines had 3- to 50-fold higher expression of Ihh (HepG2, P < 0.069; Hep3B, P < 0.055), Ptc (HepG2, P < 0.0011; Hep3B, P < 0.026) and Smo (HepG2, P < 0.012; Hep3B, P < 0.05). Interestingly, the relative expression levels of Ptc, a tumor-suppressor gene, and Smo, a proto-oncogene, differed between the two HCC lines. HepG2 cells expressed relatively more Ptc than Smo, whereas Hep3B expressed higher levels of Smo relative to Ptc. These findings suggested that the activation of GLI1, a downstream target of SMO, may inherently differ between the two cancer cell lines.

Hep3B cells have Hh signaling activity

To further evaluate the relationship between Hh pathway expression and function, we assessed transcriptional activity of Gli, a downstream target of Hh signaling, in the Hep3B line, which had high expression of Smo relative to Ptc. Results were compared to an Hh-responsive, positive control cell line (C3H10T½) that was co-transfected with plasmids for a Gli-luciferase reporter and constitutively active Smo (Figure 3A). As expected, C3H10T½ cells had endogenous Gli reporter activity, consistent with basal Hh pathway activity. Transfection of Smo further increased Gli-luciferase activity in these cells (P < 0.0024). Although not statistically significant, basal Gli activity in Hep3B cells was slightly higher than that of the positive control cell line (C3H10T½). Smo transfection of Hep3B cells also significantly enhanced their Hh reporter activity (P < 0.0002). This 4.4-fold increase in reporter activity was also slightly higher than the 3.5-fold increase which Smo induced in the positive control cell line. The specificity of our assay was confirmed using an Hh-unresponsive, colon cancer cell line (HCT116) (15) as a negative control. These cells demonstrated a lack of luciferase

Fig. 1. Normal adult hepatocytes lack Hh pathway activity. Liver sections of transgenic Ptc-lacZ mice in which β-galactosidase reports cellular Hh activity (blue) at (A) ×20 and (B) ×100 magnifications. (C) In the same sections, the gallbladder wall was a positive control (×40 magnification).
reporter activity upregulation in the presence of Smo over-expression as compared to the C3H10T½ cells (P < 0.0002) or Hep3B cells (P < 0.0001) (Figure 3A).

**Hep3B cell viability is reduced by an inhibitor of the Hh pathway**

As mentioned earlier, Hh signaling promotes the viability and growth of various foregut tumors. We evaluated the influence of Hh pathway activity on Hep3B viability by culturing the line with neutralizing antibody to Hh (5E1) or the pharmacological SMO blocker, Cyc, for up to 72 h in a dose-dependent fashion. Neither treatment reduced the viability of Hep3B cells as compared to controls treated with either isotype control antibody or Tom, an inactive Cyc analog (Figure 3B). However, treatment with KAAD-Cyc, an agent that can inhibit oncogenically mutated SMO (26), inhibited Hep3B viability.
in a dose-related fashion, with significant decreases in viability noted at the 1000 nM dose ($P < 0.013$, Figure 3C). This dose of KAAD-Cyc reduced the Hep3B growth rate from 48 to 96 h by 94% (Figure 3D). These findings suggested that Hh activity promoted the viability of the Hep3B cell line.

Hh pathway inhibition regulates gene expression and pathway activity in Hep3B cells

Other groups have shown that induction of the c-myc proto-oncogene is critical for human hepatocarcinogenesis (52,53) and that its expression is regulated by Hh signaling (54). Therefore, it is important to determine if inhibiting Hh activity affects c-myc in Hep3B cells. We found that a 5 day treatment with KAAD-Cyc decreased Hep3B mRNA expression of c-myc by 7.7-fold as compared to Tom-treated controls (Figure 3E; $P < 0.046$). Similarly, KAAD-Cyc inhibition of SMO reduced Smo expression by 4.2-fold ($P < 0.0008$), consistent with reports that SMO may regulate Smo expression (55). These changes in gene expression are relatively selective because KAAD-Cyc had no effect upon the expression of cyclin B1, D1, D2 or E1 mRNA (data not shown). This contrasts with what others have observed when Hh signaling is blocked with Cyc in medulloblastoma, another Hh-responsive cancer (54).

To establish whether blocking SMO influenced Hh-regulated transcriptional activity, we treated replicate Hep3B cultures for 1–2 days with 1000 nM Tom or KAAD-Cyc, and then analyzed the Hh reporter activity of the cells. KAAD-Cyc treatment reduced Hh-responsivity by 50% at one day ($P < 0.029$) and 38% ($P < 0.005$) at two days when compared...
to the Tom-treated controls (Figure 3F). These reductions in reporter activity were particularly notable because the inherent inducibility of reporters allows for a greater dynamic range for activation than for repression (56). Therefore, these findings confirmed that Hep3B cells have Hh signaling activity and demonstrated that Hh activity was reduced by treatment with KAAD-Cyc.

Oncogenic SMO inhibitor effects are independent of M1 and M2 Smo mutations

In order to determine if oncogenic Smo gene mutations could underlie the differential sensitivity of Hep3B cells to KAAD-Cyc and Cyc, we amplified DNA from Hep3B and HepG2 cell lines and performed direct sequencing analysis for previously described point mutations in the Smo gene. Point mutations in exons 9 (M2) and exons 10 (M1) hot spots are known to cause sporadic basal cell carcinomas (14). Our sequencing analysis did not indicate a mutation at these loci in either cell line (data not shown). Thus, Hep3B resistance to Cyc and sensitivity to KAAD-Cyc could not be explained by an activating Smo mutation at these sites, but suggested the potential for point mutations at other positions in the gene that have not been described as being oncogenic.

Expression of Smo correlates with tumor size in human HCC

Given that two human HCC cell lines overexpress Hh components and that one of the lines (Hep3B) exhibited constitutive Hh signaling activity, we evaluated the Hh pathway in 14 patients with HCC who underwent resection or liver transplantation. The mean tumor size in these individuals was 4.06 ± 2.48 cm (range 1–11). Patient demographics are noted in Table I.

Total RNA was extracted from the paired non-neoplastic livers and HCCs. Using two-step real-time RT–PCR we compared Hh pathway expression in each patient’s HCC with that in the respective non-neoplastic liver tissue at the resection margin. Cluster analysis using Spearman’s rank correlation as the distance measure demonstrated that tumors that expressed more Shh than their adjacent non-neoplastic livers also tended to overexpress Ihh (p = 0.68, P < 0.01). Half of the 14 tumors also had an increase in Gli1 expression, ranging from 1.5- to 131-fold higher than the non-neoplastic tissue. HCCs that had relative overexpression of Smo tended to have higher Gli1 expression (p = 0.47, P < 0.091). This suggested that Smo overexpression in some of the tumors was associated with increased Hh activity.

Gene expression patterns were then analyzed for their relationship to patient and tumor characteristics. Overall, the 14 tumors averaged a 2.5-fold increase in Smo proto-oncogene expression. In 6 out of 14 HCCs (42.9%), expression of Smo was upregulated more than 3-fold. No tumors had significantly decreased Smo expression (Figure 4A). Moreover, expression of the Smo proto-oncogene positively correlated with HCC tumor size (p = 0.54, P < 0.051).

In 10 tumors (71.4%), expression of the Ptc tumor-suppressor gene was significantly different than the respective patients’ non-neoplastic liver specimens. Ptc expression was decreased 3- to 4-fold in seven tumors (50%), while three tumors (21.4%) had 8- to 9-fold increases in Ptc expression. Interestingly, tumor size inversely correlated with Ptc tumor-suppressor gene expression although the relationship was not statistically significant (p = −0.23, P = 0.43).

Like the Hep3B line, 100% of the HCCs had higher expression levels of Smo than Ptc. The expression of Smo relative to Ptc, ranged from 1.4- to 758-fold higher in the tumors (111.6 ± 198.1) as compared with 3.7- to 97.0-fold higher in the non-neoplastic livers (23.8 ± 28.1). And like overexpression of Smo alone, the ratio of Smo to Ptc expression in the tumors directly correlated with HCC tumor size (Figure 4B; ρ = 0.57, P < 0.04).

Recent work in other foregut tumors has demonstrated that growth of these tumors can be driven by endogenous overexpression of the Shh and Ihh ligands (15). Gli1 overexpression with concomitant Shh overexpression is also necessary for xenograft growth of prostate cancers (57). However, Shh, Ihh and Gli1 mRNA transcript levels did not correlate with the size of resected liver tumors (P > 0.10).

Clinically, serum levels of the HCC tumor marker, AFP, are used in the diagnosis and follow-up of patients with malignant liver tumors. Therefore, we examined the relationships among preoperative serum AFP levels, tumor size, as well as expression of the Ptc tumor-suppressor gene and the Smo proto-oncogene. In our study cohort, serum AFP levels were slightly elevated in 11 tumors (91.7%). However, there was no empirical evidence to suggest that tumor size correlated with the preoperative serum AFP level (p = 0.10, P = 0.75). Moreover, in our cohort, serum AFP levels did not correlate with the expression of Ptc (ρ = 0.342, P = 0.27), Smo (ρ = 0.48, P = 0.12) or the ratio of Smo:Ptc (ρ = −0.13, P = 0.70) in the tumors.

Novel mutation of Smo identified in human HCC

In order to determine if Smo activation in HCCs was accompanied by Smo gene mutations, we amplified DNA from the tumors and from the non-neoplastic liver of 12 of the patients studied above in whom samples were available for analysis. Direct sequencing of these tissues did not reveal a point mutation at the M2 or M1 Smo oncogene hot spots in the cohort

Table I. Demographics, underlying diseases and tumor related factors for the cohort

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<th>Factor</th>
<th>Number (N = 14)</th>
<th>Percentage</th>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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exons 9 and 10, respectively (data not shown) (14). However, a 67-year-old female with a necrotic tumor and hepatitis C-induced cirrhosis was found to have a heterozygous point mutation in her tumor, as well as in her non-neoplastic but cirrhotic liver (Figure 5A and B). This mutation was not observed in the other livers that were analyzed (Figure 5C). This mutation, an A to T transversion at position 1723, resulted in a missense mutation causing a lysine to become a methionine at amino acid 575. Given the location of this residue in the carboxy-terminal cytoplasmic tail of SMO, it is conceivable that binding of interacting factors, such as PTC, may be altered (14,47). Moreover, this lysine residue is highly conserved amongst 10 vertebrate species. Given our findings that the relative expression of Smo to Ptc correlated with tumor size, the potential biological activity of this Smo mutation is supported by our finding that this patient’s non-neoplastic liver had the highest ratio of Smo to Ptc expression (97.0) of all 14 patients analyzed. Furthermore, comparison of the non-neoplastic and HCC tissues from this patient demonstrated increased tumor expression of Smo ($P < 0.0016$), as well as the two transcriptionally regulated Hh targets, Ptc ($P < 0.049$) and Gli1 ($P < 0.021$), by 1.8- to 6.8-fold, respectively (Figure 5D).

Discussion

While the worldwide incidence of liver cancer is expected to rise over the next decade, HCC already has one of the highest cancer-related mortalities and prevalences (58). Unfortunately, many of the mechanisms for initiation and progression of HCC remain elusive. Our results identify one potential mechanism for hepatocarcinogenesis, namely dysregulation of the Hh signaling pathway. Although this pathway is known to play a key role in hepatic specification of endodermal progenitors during embryogenesis (17), it has not been considered as a growth regulator in adult livers because mature hepatocytes lack Hh pathway activity. Despite this, we demonstrated that 100% of the 14 human HCCs and 2 human HCC cell lines that we examined express mRNAs that encode components of the Hh signaling pathway. Moreover, expression of the Hh pathway component and proto-oncogene, Smo, significantly correlated with tumor size and this may be mediated by mechanisms that regulate c-myc expression. The role of Smo in hepatocarcinogenesis was further supported by our identification of the first point mutation of Smo in a liver tumor. This novel mutation is located in an evolutionarily conserved domain in the Smo gene. Although we were unable to identify a similar point mutation in two HCC cell lines, Hep3B’s sensitivity only to the oncogenic SMO inhibitor, KAAD-Cyc, suggested that it also may harbor unidentified Smo mutations outside the recognized genomic hot spots.

Various factors that drive Hh activation promote non-liver cancers in adults. For example, some basal cell carcinomas of the skin, rhabdomyosarcomas and medulloblastomas are attributed to Ptc mutations that prevent the PTC–SMO interaction that normally represses SMO, thereby permitting persistent SMO activity (5). Other basal cell carcinomas result from activating mutations in Smo itself (14). Several types of gastrointestinal tract malignancies, including adenocarcinomas of the esophagus, stomach, duodenum, pancreas and gallbladder, may overexpress Shh and Ihh ligands that activate SMO (15). Cyc generally inhibits the growth of such
had an A to T transversion in both her non-neoplastic (but cirrhotic) liver and in her tumor. The non-neoplastic liver also had overexpression of Smo relative to Ptc, attesting to the cumulative nature of events leading to HCC formation and suggesting that Hh signaling may play a previously unsuspected role in the progression from cirrhosis to liver cancer. Further studies to clone this mutated Smo gene are required to fully define its tumorigenic capacity.

In general, it is accepted that enhanced Hh pathway activation leads to downstream expression of target genes including Ptc and Gli1, and hence, the levels of these transcripts are often used as surrogate markers of Hh pathway activity (59). However, current evidence suggests that other, less understood, mechanisms also influence the cellular content of Ptc and Gli1 transcripts. For instance, transient overexpression of Ptc in neural tubes leads to inhibition of GLI1 transcriptional activity in cells with high levels of Ptc mRNA (60). This observation is consistent with other evidence that PTC can downregulate GLI1 activation of gene transcription independent of the canonical cascade of Hh signaling (61). In addition, HCCs often develop in cirrhotic livers (62–64), which contain increased numbers of activated, myofibroblastic hepatic stellate cells (HSC) (65). We have shown previously that activated HSC express Ptc (18) and others have demonstrated Ptc transcripts in some cirrhotic patients (66,67). More than two-thirds of the patients with HCC in our study had underlying cirrhosis, and the accompanying accumulation of HSC probably increased Ptc expression in the non-neoplastic liver tissue, inter-individual differences in Ptc expression in non-neoplastic livers also influenced our results. Nevertheless, compared to adjacent non-neoplastic liver tissues, 21.4% of the HCC exhibited increased Ptc expression and 50% had increased expression of Gli1. Also, HCCs with higher levels of Smo tended to have higher Gli1 expression. Thus, we suggest that dysregulation of Hh signaling occurs during hepatocarcinogenesis and this appears to result from increases in Smo that may occur without necessitating the striking increases in Ptc expression that have been typical of other gastrointestinal tumors (15).

Our studies also demonstrate interactions between c-myc and the Hh pathway during hepatocarcinogenesis, because treatment of Hep3B cells with KAAD-Cyc reduced expression of c-myc, a key oncogenic factor in hepatocarcinogenesis (52). The potential importance of Hh pathway interactions with c-myc during hepatic neoplasia is consistent with a report that C-MYC enhances growth of SHH-induced medulloblastomas (68). Others have demonstrated that c-myc expression directly correlates with HCC tumor size (69) and increased HCC size is associated with worse 5 and 10-year survival (70). Herein, we demonstrate that HCC size positively correlated with Ptc expression, and that large tumors (>5 cm) had higher ratios of Smo to Ptc expression than small tumors (<5 cm). In our studies of the Hep3B line, Smo inhibition decreased Hh activity and also reduced the expression of c-myc. Taken together, these results suggest that SMO-mediated increases in c-myc might enhance HCC growth, implicating Smo as a poor prognostic factor in HCC.

Evidence that Hh pathway dysregulation is associated with liver cancer in adults has intriguing implications. Neither we nor others have been able to demonstrate Hh signaling in mature hepatocytes, but the Hh pathway must regulate...
primitive liver progenitors because it is required for hepatic specification of endodermal cells during embryogenesis (17). The genesis of cancers in several other endodermally derived tissues, including the lung, proximal gastrointestinal tract and pancreas, has recently been attributed to the over-activation of Hh signaling (1). It is conceivable that carcinogenesis in various adult endodermally derived tissues, including the liver, sometimes involves malignant transformation of residual, Hh-responsive progenitors (1). Hh-responsive progenitors are also implicated in the formation of basal cell carcinomas of the skin and certain types of central nervous system cancers (14,54). Our new evidence for Hh pathway expression and function in a human HCC line demonstrates that some malignant hepatocytes are Hh-responsive. Along with the acknowledged decline in liver cell sensitivity to Hh during liver maturation, this finding suggests that some liver cancers might arise from populations of relatively immature liver cells that retain Hh sensitivity. These results complement and extend findings in other types of cancer and suggest that, as in these malignancies, progenitors play a role in the genesis of HCC.

Although the expression of such Hh-regulated progenitors in adult livers remains unproven, a large hepatic mesenchymal tumor was recently reported in a child with Gorlin’s syndrome (71). Since individuals with Gorlin’s syndrome have germ cell tumors recently reported in a child with Gorlin’s syndrome adult livers remains unproven, a large hepatic mesenchymal progenitors in post-natal liver might retain Hh sensitivity. This possibility is supported by some of our own recent work which shows that HSC, major components of the adult liver mesenchyme, produce Hh ligands (18). That myofibroblastic HSC produce Hh ligands is notable because these cells accumulate in cirrhosis, a major risk factor for HCC in all systems cancers (14,54). Our new evidence for Hh pathway expression and function in a human HCC line demonstrates that some malignant hepatocytes are Hh-responsive. Along with the acknowledged decline in liver cell sensitivity to Hh during liver maturation, this finding suggests that some liver cancers might arise from populations of relatively immature liver cells that retain Hh sensitivity. These results complement and extend findings in other types of cancer and suggest that, as in these malignancies, progenitors play a role in the genesis of HCC.

Supplementary material

Supplementary material is available at http://www.carcin.oupjournals.org/

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References

Hedgehog pathway in human hepatocarcinogenesis


(PMID: 16003737; PubMed in progress).


