Bile acids promote diethylnitrosamine-induced hepatocellular carcinoma via increased inflammatory signaling

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Bile acids promote diethylnitrosamine-induced hepatocellular carcinoma via increased inflammatory signaling. Am J Physiol Gastrointest Liver Physiol 311: G91–G104, 2016. First published May 5, 2016; doi:10.1152/ajpgi.00027.2015.—Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and seventh in women (9, 12). Each year an estimated 20,000 new cases of HCC are diagnosed in the US and the trend is rising. The treatment options for HCC remain limited partly because the mechanisms of pathogenesis of HCC are not completely known (27). Previous studies have implicated increased bile acids in pathogenesis of HCC but the mechanisms are not fully elucidated (3, 34, 35).

Bile acids, initially known for their role in lipid and vitamin metabolism, are now considered critical signaling molecules involved in cross tissue signaling, especially in the liver-gut axis (15). Both protective and pathogenic roles of bile acids in variety of diseases including drug-induced liver injury, steatohepatitis, and cancers of colon and liver have been identified (10). Whereas several studies have linked bile acids to hepatocellular carcinoma (HCC) (21, 22, 25, 34, 35), the exact mechanisms by which bile acids promote HCC are not known. Previous studies indicate that mouse models with increase in total circulating bile acids including FXR (NR1H4) knockout (KO), BSEP (Abcb11) KO, and MDR2 (Abcb4) KO mice exhibit spontaneous HCC formation. Decreasing bile acids in FXR KO mice results in reduction of spontaneous HCC (35). Although these studies implicate increase in bile acids to HCC promotion, the mechanisms remain elusive.

We studied the mechanisms of bile acid-mediated tumor promotion using a classic initiation-promotion protocol of diethylnitrosamine (DEN)-initiated liver tumors in C57BL/6 mice. Using a global gene expression analysis and traditional methods, we have identified NF-κB-mediated inflammatory signaling pathways as a mechanism behind the tumor promotion by bile acids. Furthermore, we show that increased bile acids may promote stemness-related genes in hepatocytes. Finally, our studies have also identified increased bile acid as a component of human HCC pathogenesis. These studies provide evidence that increased bile acids can promote liver cancer with diverse mechanisms.

METHODS

Animals, treatments, and tissue collection. Animal studies were conducted as described before (32). The C57BL/6 mice were purchased from Jackson laboratories (Bar Harbor, Me) and housed in the University of Kansas Medical Center Laboratory Animal Resources Vivarium. All studied were performed in male mice only. The role of cholic acid in promoting DEN-initiated hepatic tumors was evaluated using a classic initiation-promotion protocol (Fig. 1A). Male mice pups were injected with either vehicle or DEN (15 μg/kg dissolved in saline) at postnatal day 15. All mice (n = 5 to 8 per group) were weaned on normal rodent chow and maintained on rodent chow till 8 mo of age. At 8 mo, two groups of mice, one DEN treated and other vehicle treated were fed diet containing 0.2% cholic acid (CA) till 10 mo of age. All mice were killed at 10 mo of age. Livers and serum were collected and processed as described before (8, 32). All animal
Fig. 1. Cholic acid (CA) promotes diethylnitrosamine (DEN)-induced hepatic carcinogenesis. A: scheme showing the experimental design. B: representative photographs of mouse liver obtained from DEN + normal diet (ND) and DEN + 0.2% CA treated mice. Arrows point to liver tumors. C: bar graph showing number of liver tumors per mouse. D: percent survival in various treatment groups. Data are expressed as mean ± SE. *P < 0.05, significant difference from all other groups. E: representative photomicrographs of hemtoxylin-eosin (H&E)-stained liver sections from ND (i), 0.2% CA (ii), DEN + ND (iii), and DEN + 0.2% CA (iv) taken at ×400 magnification. Arrowheads demark the tumor boundary and arrows point to inflammatory cell infiltrate.
studies were approved by and performed in accordance with the Institutional Animal Care and Use Committee at University of Kansas Medical Center.

Mouse hepatocyte isolation and treatments. Primary mouse hepatocytes were isolated from 2- to 3-mo-old male C57BL/6 mice using standard two-step collagenase perfusion method as previously described (1, 2). Hepatocytes were cultured on collagen-coated plates, allowed to adhere for 3 h, washed, and then treated with either vehicle or bile acids as described before (1). The bile acids used in these study include taurocholic acid (TCA), deoxycholic acid (DCA, also called desoxycholic acid), and chenodeoxycholic acid (CDCA) at either 100 or 200 μM. Cells were harvested 24 h after bile acid treatment, and mRNA was isolated and used for real-time PCR studies.

Histology and immunohistochemistry. Paraffin-embedded liver sections (4-μm thick) were used for hematoxylin-eosin (H&E) staining and immunohistochemical detection of proliferating cell nuclear antigen (PCNA), CD34, CD3, CD20, CD45, and reticulin as described before (8, 16, 32). H&E-stained slides were used for necrosis scoring and PCNA-stained slides were used for PCNA scoring as described before (7). Antibodies used include DAKO rabbit polyclonal antibodies against α-1-antitrypsin, α-feto protein (AFP), and CD3, CD20, and CD45 (Carpentaria, CA) as well as Cell Marque/Epitomics rabbit monoclonal anti-CD34 (Burlingame, CA).

Biochemical analysis of proteins and mRNA. Western blotting was used to detect protein levels in RIPA extracts and real-time PCR was used to quantify mRNA as described before (32). Primary and secondary antibodies were purchased from Cell Signaling Technologies (Danvers, MA).

Bile acid analysis. Serum and liver total bile acids were measured enzymatically using Total bile acid assay kit (BQ Kits, San Diego, CA), as per the manufacturer’s protocol described previously (6). Specific serum and hepatic bile acids were analyzed by UPLC-MS (Waters, Milford, MA) using methods similar to described previously (6) at the NIH sponsored West Coast Metabolomics Center in University of California, Davis.

Microarray analysis. Global gene expression analysis was performed using Affymetrix Mouse 430_2.0 Chip and pooled mRNA isolated from four to five separate mice livers per group. Microarrays and Ingenuity Pathway Analysis (IPA) analysis were performed as described before (33). The raw data have been submitted to GEO database (GSE75445).

Human serum bile acid analysis. All human studies were approved by and were performed in accordance to Ethics Committee and the Clinical Trials office of the Third Affiliated Hospital of Sun Yat-sen University. Total serum bile acids were quantified in de-identified serum samples from normal, cirrhotic, and HCC patients using Automated Biochemical Analyzer Hitachi 7180 (Hitachi). Global gene expression analysis was performed using the criteria noted in Table 1. Tumors were identified as either hepatic adenoma, dysplastic nodules, or well-differentiated HCC. The tumors in the DEN + 0.2% CA group were accompanied with significant increase in inflammatory cell infiltration with visible inflammatory cell foci adjacent to and within the liver tumors (Fig. 1, Eii and Eiv). Hepatic adenomas were characterized by liver cells resembling normal hepatocytes, which formed slightly thickened liver cell plates that do not exceed two cells thick. Adenomas were enclosed in a well-developed reticulin framework, lacked internal nodularity, portal tracts, or fibrosis. Dysplastic nodules showed a uniform population of hepatocytes, often with in-
creased nuclear to cytoplasmic ratios. Focally, the hepatic plates were expanded greater than or equal to three cells thick, and there was focal disruption of reticulin framework. Few mitoses were visible. Well-differentiated hepatocellular carcinoma exhibited increased nuclear:cytoplasmic ratios, absence of portal tracts, expansion of hepatic plates greater than three cells thick, and disruption and loss of normal reticulin framework and focal pseudoglandular formation. Mitoses, including atypical mitoses, were frequent (Fig. 1, Eii-Eiv).

Some but not all tumors in the DEN + 0.2% CA group exhibited intracellular and intranuclear inclusions within the neoplastic hepatocytes (Fig. 2Ai). These globules were not observed in the background benign hepatocytes. These globules were brightly eosinophilic, varied in size and number per cell, and were concentrated within the tumor nodules. The globules were negative for both periodic acid-Schiff (PAS), PAS/diastase, α-1-antitrypsin, AFP, or lipids (Oil Red O staining). Further immunohistochemical analysis revealed that these globules were brightly eosinophilic, varied in size and number per cell, and were concentrated within the tumor nodules. The globules were negative for both periodic acid-Schiff (PAS), PAS/diastase, α-1-antitrypsin, AFP, or lipids (Oil Red O staining). Further immunohistochemical analysis revealed that these globules were brightly eosinophilic, varied in size and number per cell, and were concentrated within the tumor nodules. The globules were negative for both periodic acid-Schiff (PAS), PAS/diastase, α-1-antitrypsin, AFP, or lipids (Oil Red O staining). Further immunohistochemical analysis revealed that these globules were brightly eosinophilic, varied in size and number per cell, and were concentrated within the tumor nodules. The globules were negative for both periodic acid-Schiff (PAS), PAS/diastase, α-1-antitrypsin, AFP, or lipids (Oil Red O staining). Further immunohistochemical analysis revealed that these

Fig. 2. Histological characterization of hepatocellular carcinoma (HCC). A: representative photomicrographs of H&E-stained sections from DEN + 0.2% CA treated mouse showing globular inclusions (i, arrowheads) and immunohistochemical detection of ubiquitin (ii) and CK8 (iii). B: representative photomicrographs of CD34 immunohistochemistry on liver sections from ND (i) and DEN + 0.2% CA (ii) treated mice. Liver section from DEN + 0.2% CA treated mouse showing vascular invasion of an HCC. iii: H&E staining (×200). iv: CD34 immunohistochemistry (×200). *Vascular invasion. C: representative photomicrographs of reticulin staining in ND (i) and DEN + 0.2% CA treated mice (ii: ×400. iii: ×200). *HCC within the section and arrowheads show tumor boundary. D: representative photomicrographs of CD3 (i), CD45 (ii), and CD20 (iii) staining in DEN + 0.2% CA treated mouse. All photographs at ×400 magnification unless mentioned otherwise.
granules were positive for ubiquitin and CK8 (Fig. 2, Aii-Aiii), which has been demonstrated in HCC associated with cholestasis (14).

We performed several additional special staining to further characterize the HCC in the DEN + 0.2% CA group. CD34 staining highlighted the capillarization of the sinusoids in neoplastic vs. nonneoplastic tissue (Fig. 2, Bi-Bii). The endothelial cells wrapping the nests and trabeculae phenotypically resembled capillary endothelium (CD34 positive) rather than normal hepatic sinusoidal endothelium (CD34 negative). Interestingly, we observed vascular invasion characterized by CD44-positive capillarization within the vessel in only the DEN + 0.2% CA treated mice (Fig. 2, Biit and Biv). Reticulin staining confirmed the presence of hepatocellular carcinoma and showed widened hepatic plates, disruption of normal hepatic plate architecture, and decreased number/amount of reticulin fibers compared with background nonlesional liver (Fig. 2, Ci-Ciii). The reticulin framework was found to be absent or markedly decreased or distorted, with irregular or absent staining of the borders of the hepatic plates in the HCC. Finally, we characterized the inflammatory infiltrates associated with HCC in the DEN + 0.2% CA group by staining for CD3, CD20, and CD45 (Fig. 2, Di-Diii). The data indicate that the inflammatory infiltrate in and around HCC observed in DEN + 0.2% CA group consisted mainly of CD45-positive lymphocytes with a mixed population of CD3-positive T cells and CD20-positive B cells (Fig. 2, Di and Dii).

Increased proliferation and decreased apoptosis in cholic acid-promoted liver tumors. To determine changes in cell proliferation and cell death, we performed immunohistochemical analysis for PCNA and terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay on paraffin embedded liver sections from all four groups (Fig. 3). PCNA staining indicated a moderate increase in hepatocyte proliferation after 0.2% CA feeding, which was not statistically significant (Fig. 3, A and B). A significant increase in cell proliferation was observed in both DEN + ND and DEN + 0.2% CA treated mice compared with ND and 0.2% CA treated mice. Although number of PCNA-positive cells were moderately higher in DEN + 0.2% CA treated mice, the values were not statistically significant than the DEN + ND group. Western blot analysis corroborated the PCNA immunohistochemistry. A significant increase in cyclin D1 and PCNA protein was observed in the 0.2% CA, DEN + ND, and DEN + 0.2% CA groups (Fig. 3C). Interestingly, levels of p21, the main cell cycle inhibitor, decreased in the 0.2% CA, DEN + ND, and DEN + 0.2% CA groups and were lowest in the DEN + 0.2% CA group.

TUNEL assay results indicated a significant increase in apoptosis in the 0.2% CA, DEN + ND, and DEN + 0.2% CA groups compared with ND control mice (Fig. 3, D and E). However, DEN + 0.2% CA treated mice exhibited 50% lower apoptosis compared with the DEN + ND group. Interestingly, 0.2% CA treatment by itself and in combination with DEN induced significant scattered single cell necrosis as demonstrated by cytoplasmic TUNEL staining, a characteristic of necrotic cell death. TUNEL data were further corroborated by Western blot analysis of total and activated caspase 3 (Fig. 3F). A significant increase in activated caspase 3 was observed in both DEN + ND and DEN + 0.2% CA treated mouse livers but the levels of active caspase 3 protein were significantly lower in the DEN + 0.2% CA group compared with the DEN + ND treated group.

Global gene expression changes in cholic acid-promoted liver tumors. To determine the mechanisms behind promotion of DEN-induced liver tumors by 0.2% CA, we studied global gene expression changes using Affymetrix microarrays. The extensive data generated in these studies have been submitted to GEO database. We used threefold change cut off for significance. With the use of this cut off, the 0.2% CA-induced expression of 20 genes and decreased expression of 20 genes compared with ND (Table 2). The DEN + ND group had 335 genes upregulated and 28 genes downregulated as compared ND. When gene expression changes in 0.2% CA were compared with those in the DEN + 0.2% CA group, 318 genes were significantly upregulated and 59 genes were downregulated. Finally, in the DEN + 0.2% CA group 18 genes were upregulated and 17 genes were downregulated compared with the DEN + ND group.

To further gain insights into specific signaling pathways changed during promotion of DEN-induced liver tumors by cholic acid, we analyzed the data using IPA. The upstream regulator analysis is used to identify possible activation of transcription factors and nuclear receptors predicted based on the gene expression data. Upstream regulator analysis on the gene array data indicated NF-κB and NANO signaling pathways were predicted to be upregulated in the DEN + 0.2% CA group (Table 3). IPA also predicted downregulation of other transcription regulators including RBPI (regulator in Notch signaling pathway), estrogen receptor, glucocorticoid receptor, and SREBP1. Furthermore, we performed upstream regulator analysis on cytokines and growth factors, which revealed several inflammatory cytokines including IL-1β and TNF-α to be activated (Table 3).

Increased inflammatory signaling in cholic acid-promoted liver tumors. Histological analysis, gene array data and upstream regulator analysis of gene expression data together indicated increase in inflammatory signaling in DEN + 0.2% CA treated livers compared with all other groups. The main difference between the DEN + ND and DEN + 0.2% CA groups was the increased inflammation in the DEN + 0.2% CA group. Therefore, we further investigated whether TNF-α-IL-1β-NF-κB activation is involved in increased inflammation and tumor promotion following cholic acid feeding. Real-time PCR analysis revealed a twofold increase in TNF-α and IL-1β transcripts following 0.2% CA treatment compared with ND (Fig. 4, A and B). The DEN alone did not cause increase in TNF-α mRNA but resulted in twofold increase in IL-1β mRNA. However, a sixfold increase in TNF-α and IL-1β mRNA was observed in the DEN + 0.2% CA compared with the ND group. Western blot analysis indicated a significant increase in total p65 levels in the DEN + ND and DEN + 0.2% CA groups but the DEN + 0.2% CA group had substantially higher p65 protein compared with any other the group (Fig. 4C). The total IkBα protein was unchanged in any treatment, but the phosphorylated form of IkBα (targeted for degradation) was significantly higher in the DEN + 0.2% CA group consistent with increased p65 protein levels. Finally, Western blot analysis indicated a moderate increase in IKKα/β, the primary regulator of IkB/NF-κB interaction in DEN + ND treated mice. However, phosphorylated (active) IKKα/β protein was higher in the DEN + 0.2% CA group.
Fig. 3. Analysis of cell proliferation and apoptosis in CA-promoted DEN-induced HCC. 

A: representative photomicrographs of PCNA immunohistochemistry performed on paraffin embedded liver sections from various treatment groups. Arrows indicate cells in S phase of cell cycle. 

B: quantification of PCNA staining. Bar graph shows numbers of PCNA-positive cells in high power (×400) fields in various treatment groups. Data are expressed as mean ± SE. *P < 0.05, significant difference from ND. 

C: Western blot analysis of cyclin D1, PCNA, and p21 conducted using total liver cell extracts. 

D: representative photomicrographs of terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay immunofluorescent staining on liver sections from ND (i), 0.2% CA (ii), DEN + ND (iii), and DEN + 0.2% CA (iv). White arrowheads point to cells in apoptosis and yellow arrows point to necrotic cells. 

E: bar graph showing quantification of TUNEL assay performed using paraffin embedded liver sections from various treatment groups. Data are expressed as mean ± SE. *P < 0.05, significant difference from DEN+ND. !P < 0.5, significantly different from ND group. 

F: Western blot analysis of total and activated caspase-3 conducted using total liver extracts from various treatment groups.
DEN-induced liver tumors by 0.2% cholic acid

Table 2. Global gene expression changes in promotion of DEN-induced liver tumors by 0.2% cholic acid

| Group Comparison       | Upregulated genes | Downregulated Genes |
|-----------------------|-------------------|---------------------|
| 0.2% CA vs. ND        | 20                | 20                  |
| DEN + ND vs. ND       | 335               | 28                  |
| DEN + 0.2% CA vs. ND  | 569               | 130                 |
| DEN + 0.2% CA vs. 0.2% CA | 310            | 59                  |
| DEN + 0.2% CA vs. DEN + ND | 18        | 17                  |

Global gene expression changes are number of genes that changed (up or down) more than 3-fold. CA, cholic acid; ND, normal diet; DEN, diethylnitrosamine.

(Fig. 4D). Taken together, these data indicate higher activation of IKKα/β in the DEN + 0.2% CA group compared with all other groups.

Changes in MAPK signaling in cholic acid-promoted liver tumors. Previous studies have indicated that bile acids are able to induce MAPKs, which further exacerbate inflammatory signals. To determine MAPK activation during promotion of DEN-induced HCC by cholic acid, we investigated levels of total and phosphorylated AKT, ERK, JNK, and p38 MAPK by Western blot (Fig. 5). The results indicate that 0.2% CA and DEN + ND treated mice showed a moderate increase in JNK activation (ratio of phosphorylated protein to total protein) but DEN + 0.2% CA mice had significant activation (4-fold higher than ND) of JNK (Fig. 5, A and C). AKT activation was induced twofold by 0.2% CA treatment but was surprisingly decreased in both the DEN + ND and DEN + 0.2% CA groups compared with ND treated mice (Fig. 5, A and D). A very mild increase in ERK activity was observed in both the 0.2% CA and DEN + 0.2% CA groups (Fig. 5, B and E). Finally, p38 activity decreased significantly in the DEN + ND and DEN + 0.2% CA treated groups with more than threefold decrease in the DEN + 0.2% CA group compared with the ND group (Fig. 5, B and F). Taken together, these data indicate a significant increase in JNK activity and significant decrease in p38 MAPK activity accompanied higher tumor incidence in DEN + 0.2% CA treated mice.

Increased bile acids during HCC pathogenesis in mice. To determine whether development of HCC is accompanied with changes in bile acids, we quantified total and specific bile acids in serum and livers from all groups of mice. We analyzed a total of 20 specific bile acids in liver and serum out of which eight bile acids contributed to over 97% of the total bile acids. These data were further analyzed to determine the percentage each bile acid contributed to total bile acids measured. The data indicate significant increase in serum bile acids in 0.2% CA and DEN + 0.2% CA treated mice (Fig. 6A). Interestingly, the mice in the DEN + ND group also showed an overall increase in total serum bile acids but there was significant variation. Further analysis revealed substantial changes in the bile acid composition (Fig. 6, B–E). Cholic acid feeding alone substantially decreased amounts of muricholic acid and tauro-β-muricholic acid and increased levels of cholic acid, taurocholic acid, chenodeoxycholic acid, deoxycholic acid. Intriguingly, DEN treatment alone (in the DEN + ND treated group) increased levels of cholic acid, taurocholic acid, and tauro-β-muricholic acid and decreased the levels of muricholic acid. Similarly, a substantial increase in deoxycholic acid and cholic acid along with decrease in tauro-β-muricholic acid was observed in serum of DEN + 0.2% CA treated mice.

Total liver bile acid analysis showed substantial increase in total hepatic bile acid content in the 0.2% CA and DEN + 0.2% CA treated groups (Fig. 6F). The DEN + ND treated mice also had increase in total liver bile acids with a lesser degree of variation compared with serum values but it was not statistically significant than the ND group. Specific bile acid analysis (Fig. 6, G–J) revealed decrease in tauro-β-muricholic acid and increase in taurocholic acid and taurodeoxycholic acid in mice fed 0.2% CA diet. Treatment with DEN alone (in DEN + ND treated group) did not show significant change in bile acid composition except moderate increase in taurocholic acid and slight decrease in tauro-β-muricholic acid. However, DEN + 0.2% CA treated mice showed significant decrease in tauro-β-muricholic acid and substantial increase in cholic acid, taurocholic acid, and deoxycholic acid. Taurodeoxycholic acid levels increased compared with the ND and DEN + ND group but remained the same compared with 0.2% CA group.

Bile acid treatment induces genes associated with stemness. Our gene array studies indicate extensive activation of

Table 3. Upstream regulators activated and inhibited in DEN +0.2% CA group

| Upstream Regulator | Predicted Activation State | Activation z-Score | P Value of Overlap |
|--------------------|---------------------------|--------------------|--------------------|
| **Transcription Factors** |                           |                    |                    |
| NFκB               | Activated                 | 2.792              | 1.38E-04           |
| NUPR1              | Activated                 | 2.558              | 7.10E-05           |
| NANOG              | Activated                 | 2.236              | 4.73E-02           |
| RBPJ               | Inhibited                 | −2.000             | 2.49E-02           |
| Estrogen receptor  | Inhibited                 | −2.111             | 1.31E-01           |
| NR3C1              | Inhibited                 | −2.219             | 5.26E-02           |
| SREBF1             | Inhibited                 | −2.592             | 5.81E-06           |
| **Cytokines and Growth Factors** |                       |                    |                    |
| IFNγ               | Activated                 | 3.240              | 9.10E-05           |
| IL1β               | Activated                 | 3.068              | 3.54E-06           |
| TNFα               | Activated                 | 2.846              | 7.98E-06           |
| IFNα2              | Activated                 | 2.570              | 1.09E-05           |
| IL1α               | Activated                 | 2.447              | 2.73E-03           |
| CSF2               | Activated                 | 2.236              | 1.94E-01           |
| IFNL1              | Activated                 | 2.138              | 8.11E-04           |
| IL1RN              | Inhibited                 | −2.345             | 5.82E-04           |
Nanog, a factor associated with stemness. To determine whether bile acids can regulate factors associated with stemness in hepatocytes, we treated primary mouse hepatocytes with bile acids including taurocholic acid, deoxycholic acid, and chenodeoxycholic acid. Our data indicate that taurocholic acid and deoxycholic acid, but not chenodeoxycholic acid, could induce stemness genes including Myc, KLF4, Nanog, Oct4, and Sox2 between 2- and 15-fold (Fig. 7). These data indicate that high concentrations of bile acids may induce factors associated with hepatic dedifferentiation and stemness.

Increased bile acids during HCC pathogenesis in humans. To determine whether increase in bile acid is associated with HCC pathogenesis in humans, we determined serum bile acids in a total of 32 normal, 30 cirrhotic without HCC, and 36 HCC patients (Fig. 8). The clinical characteristics of patients are provided in Table 4. The data indicate an overall increase in serum bile acids in both cirrhotic without HCC and with HCC compared with normal patients with no difference between the two cirrhotic groups.

DISCUSSION

Role of bile acids in regulation of metabolism, cell growth, and cancer promotion apart from their classic role in digestion has been well recognized (10, 15). In the liver, bile acids play a critical role in liver homeostasis, liver regeneration, and development of liver cancers (4). Several animal models with increase in total bile acids exhibit spontaneous liver cancer development including the whole body FXR−/− mice and the BSEP-KO mice (21, 22, 35). Interestingly, sequestration of bile acids using cholestyramine can decrease liver cancer incidence in FXR−/− mice (35). Whereas these studies clearly indicate that increased bile acids are a risk factor in liver cancer pathogenesis, the mechanisms remain unknown. Furthermore, whether increased bile acids are associated with HCC development in humans is also not known. The data presented here demonstrate that the increase in total bile acids is associated with human HCC development and show that increased inflammation as the possible mechanisms of bile acid-mediated tumor promotion.

We used a classic initiation-promotion model of DEN-induced liver carcinogenesis to study the mechanisms of bile acid-induced HCC promotion. We choose cholic acid mainly because it is one of the major bile acids in humans and previous studies with cholic acid feeding in diet provided background information necessary for dose selection (6, 36). Our results obtained from a DEN-induced initiation-promotion model clearly demonstrate that bile acids can act as tumor promoters in liver carcinogenesis. Additionally, our data taken together with previous studies confirms that bile acids alone are not complete carcinogens, as bile acid feeding, albeit for short duration, did not result in liver cancers. Previous studies have demonstrated that bile acids can stimulate cell proliferation during liver regeneration (6, 19). We investigated whether the higher tumor promotion by bile acids is simply due to increases cell proliferation in the liver. However, our data indicate that cholic acid-promoted tumors exhibited only a moderate increase in cell proliferation but a much significant decrease in apoptosis as compared all other treatments. Further, cholic acid feeding resulted in scattered single cell necrotic cell death in the liver both in the 0.2% CA and DEN + 0.2% CA groups, which was absent in the ND and DEN + ND groups. It is plausible that the necrotic cell death observed may be a trigger for inflammation and in the DEN + 0.2% CA group; the inflammatory signaling further helps in selecting the mutated cells towards tumorigenesis. The molecular mechanisms by
Fig. 5. Changes in MAPK signaling in CA-promoted DEN-induced HCC. Western blot analysis of total and phosphorylated JNK and AKT (A) and total and phosphorylated ERK and p38 MAPK (B) conducted using total liver extracts from various groups. C-F: densitometric analysis of Western blots of JNK, AKT, ERK, and p38, respectively, conducted using blots shown in A and B.
Fig. 6. Changes in bile acids in serum and liver during CA-induced promotion of DEN-initiated tumors. A: quantification of total bile acids in serum in mice from various treatment groups. B–E: bar graphs showing contribution of each bile acid to total bile acids in serum of ND, 0.2% CA, DEN + ND, and DEN + 0.2% CA groups. F: quantification of total bile acids in livers of mice from various treatment groups. G–J: bar graphs showing contribution of each bile acid to total bile acids in serum of ND, 0.2% CA, DEN + ND, and DEN + 0.2% CA groups. Data are expressed as mean ± SE. In B–E and G–J, all comparisons are made to respective bile acid concentration in ND group. *P < 0.05, significant difference. **P < 0.01, significant difference.
which increased bile acids induce cell death, increase inflammation, and promote cell proliferation have been investigated in the past. Bile acids can signal via a number of FXR-dependent and -independent mechanisms to induce cell death including EGFR-dependent Egr1 activation, TGR5-mediated signaling in nonparenchymal cells in the liver, and S1P2 receptor-mediated signaling (1, 24, 29). These data suggest that bile acids may promote tumorigenesis by disturbing the natural balance between cell proliferation and compensatory cell death in the liver.

Fig. 7. Bile acids induce stemness genes in mouse hepatocytes. Bar graphs showing mRNA levels of Myc, KLF4, Nanog, Oct4, and Sox2 as measured by Real-Time PCR using total RNA extracted from primary mouse hepatocytes treated with various bile acids for 24 h. Data are expressed as mean ± SE. *P < 0.05, significant difference. **P < 0.01, significant difference. ***P < 0.001, significant difference from vehicle-treated group.
and DEN + 0.2% CA groups, it is possible that an underlying Nanog-Yap signaling axis may be involved in bile acid-induced promotion of liver cancers, which require additional investigations.

Our upstream analysis on transcription factors and cytokines revealed NF-κB, TNF-α, and IL-1β to be highly upregulated in the DEN + 0.2% CA group, which was corroborated by detailed analysis of these factors in the liver tissues. These data suggest that enhanced inflammatory signaling is involved in tumor promotion by bile acids. These data are consistent with previous observation that bile acids are proinflammatory molecules (1, 2, 10, 15, 26). Previous studies have also shown increase in IL-1β in whole body FXR-KO mice, which have spontaneous liver tumors (21). These data also raise a question regarding the primary receptor involved in this proinflammatory and protumorigenic effect of bile acids. Previous observations indicate that FXR and TGR5 activation by bile acids is anti-inflammatory whereas EGFR activation by bile acids leads to proinflammatory signaling (2, 26, 29). Further studies are needed to determine the role of these various receptors, which are expressed in different cell types in the liver, in bile acid-induced tumor promotion.

One of the important contributions of this study is the determination of changes in bile acid composition during HCC pathogenesis. Although our data, not surprisingly, revealed increased bile acids in cholic acid fed groups, they also indicated an increase in total serum and liver bile acids in the DEN + ND treated group. These mice were only treated with DEN and experienced a slow progressive tumor growth. The significant increase in total and specific bile acids in DEN treated mice indicates that loss of bile acid regulation is an integral part of HCC pathogenesis. These data were further supported by highest increase in bile acids in the DEN + 0.2% CA treated mice above all other groups, indicating an additive effect of DEN and cholic acid treatment. The common theme in bile acids changes associated with HCC formation was a decrease in tauro-β-muricholic acid and an increase in cholic acid, muricholic acid, and deoxycholic acid. Out of these, the significant increase in deoxycholic acid (DCA, also called desoxycholic acid) known to induce inflammatory signaling in the liver (17, 30) is consistent with our other observations. It is plausible that increased DCA may trigger proinflammatory signaling resulting in tumor promotion in the DEN + 0.2% CA group. Finally, our analysis of serum bile acids in normal, cirrhotic, and HCC patients indicated an increase in circulating bile acids in cirrhotic and HCC patients further supporting the role of bile acids in tumor promotion.

Previous observations indicate that HCC development is relative rare in adult cholestatic disease but is more common in

Table 4. Gender and age distribution of clinical cases

| Gender | Number of Cases | Median Age | Standard Deviation |
|--------|----------------|------------|--------------------|
| Normal |                |            |                    |
| Male   | 16             | 40.5       | 14.93              |
| Female | 16             | 34         | 14.36              |
| Male   | 27             | 48         | 10.8               |
| Female | 9              | 51         | 10.61              |
| Cirrhosis | 24          | 42.5       | 8.72               |
| Female | 6              | 55.5       | 3.72               |

Fig. 8. Serum total bile acids in normal, cirrhotic and HCC patient samples. Data are expressed as mean ± SE. ***p < 0.001, significant difference from normal patients.
children with cholestasis, as seen in children with mutations in BSEP (22). Given the fact that HCC is an adult disease, formation of HCC in children with cholestasis further indicates that increased bile acids may result in faster promotion of an adult disease in the children. HCC may be rare in adult cholestatic patients but deregulation of bile acid homeostasis secondary to loss of FXR has been observed in both liver cirrhosis and nonalcoholic steatohepatitis (NASH), two conditions closely associated with HCC development (13, 34). Thus our findings have revealed a previously unrecognized role of bile acids in promotion of HCC of multiple etiologies.

In summary, our data indicate that bile acids can promote liver tumors via increasing inflammatory signaling, endoplasmic reticulum stress, and possibly selective survival of tumor-initiating stem cells. Whereas additional studies are needed to fully understand the mechanisms behind bile acid-mediated tumor growth, our data suggest that reducing bile acids may be a plausible treatment option in inhibiting tumor formation in early cirrhotic patients without HCC and reducing tumor growth in HCC patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L.S., K.B., P.B., G.E., B.B., C.W., N.R., M.W.M.J., S.G., M.O., H.L., and U.A. performed experiments; L.S., K.B., P.B., B.B., C.W., N.R., S.G., M.O., H.L., and U.A. analyzed data; L.S., P.B., G.E., B.B., C.W., M.W.M.J., S.G., H.L., and U.A. approved final version of manuscript; P.B. and U.A. edited and revised manuscript; S.G., M.O., and U.A. interpreted results of experiments; M.O. and U.A. prepared figures; U.A. conception and design of research; U.A. revised manuscript; S.G., M.O., and U.A. performed experiments; L.S., K.B., P.B., B.B., C.W., N.R., S.G., M.O., and U.A. interpreted results of experiments; U.A. conceived and designed the research; U.A. performed experiments; L.S., K.B., P.B., B.B., C.W., N.R., S.G., M.O., and U.A. wrote the manuscript; S.G., M.O., and U.A. revised the manuscript; L.S., K.B., P.B., B.B., C.W., N.R., S.G., M.O., and U.A. revised the manuscript; and U.A. edited and revised manuscript.

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