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Bile Salt Export Pump is Dysregulated with Altered Farnesoid X Receptor Isoform Expression in Patients with Hepatocellular Carcinoma

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Abstract

As a canalicular bile acid effluxer, bile salt export pump (BSEP) plays a vital role in maintaining bile acid homeostasis. BSEP deficiency leads to severe cholestasis and hepatocellular carcinoma (HCC) in young children. Regardless of the etiology, chronic inflammation is the common pathological process for HCC development. Clinical studies showed that bile acid homeostasis is disrupted in HCC patients with elevated serum bile acid level as a proposed marker for HCC. However, the underlying mechanisms remain largely unknown. In this study, we found that BSEP expression was severely diminished in HCC tissues and markedly reduced in adjacent non-tumor tissues. In contrast to mouse, human BSEP was regulated by farnesoid x receptor (FXR) in an isoform-dependent manner. FXR α 2 exhibited a much more potent activity than FXR α 1 in transactivating human BSEP *in vitro* and *in vivo*. The decreased BSEP expression in HCC was associated with altered relative expression of FXR α 1 and FXR α 2. The FXR α 1/FXR α 2 ratios were significantly increased with undetectable FXR α 2 expression in one third of the HCC tumor samples. Similar correlation between BSEP and FXR isoform expression was confirmed in hepatoma Huh 7 and HepG2 cells. Further studies showed that intrahepatic proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) were significantly elevated in HCC tissues. Treatment of Huh 7 cells with IL-6 and TNF- α resulted in a marked increase in the FXR α 1/FXR α 2 ratio concurrent with a significant decrease in BSEP expression. In conclusion, BSEP expression was severely diminished in HCC patients associated with alteration of FXR isoform expression induced by inflammation, and the restoration of BSEP expression through suppressing inflammation in the liver may re-establish the bile acid homeostasis.

Keywords

BSEP; FXR; HCC; Bile acids; Gene regulation

INTRODUCTION

Bile acid homeostasis is achieved through a tightly regulated enterohepatic circulation, the rate-limiting step of which is canalicular secretion of bile acids through bile salt export pump (BSEP, ABCB11) (1, 2). Modulation of BSEP expression or function by inherited or acquired factors has a profound impact on bile acid homeostasis. The expression of BSEP is coordinately regulated by multiple transactivation pathways (3–7), notably the bile acid/farnesoid x receptor (FXR, NR1H4) signaling pathway (3, 4). Activation of FXR by bile acids strongly induces BSEP expression *in vitro* and *in vivo* (3, 4). Such feed-forward regulation of BSEP by FXR is considered a major mechanism for preventing excessive accumulation of toxic bile acids in hepatocytes. Two FXR genes, FXR α and FXR β , have been identified (8–10). FXR α is functional in all species tested while FXR β is a pseudogene in humans (10). Alternative promoter and splicing result in four isoforms of FXR α (FXR α 1–4) (11, 12) with predominant expression of FXR α 1 and FXR α 2 in human liver (11). Currently, the pathophysiological significance of FXR isoform-specific regulation remains unknown.

Maintenance of bile acid homeostasis is vital for health and disruption of bile acid balance is associated with various diseases. Many pieces of evidence support a role of excessive intrahepatic bile acids in the development of hepatocellular carcinoma (HCC). Children with a deficiency in BSEP develop severe cholestasis and HCC at early ages (13, 14). Certain genetic variations in BSEP are associated with susceptibility to develop HCC (15). FXR knockout mice (FXR $^{-/-}$) with dysregulation of BSEP spontaneously developed HCC as they aged (16, 17). It is generally accepted that chronic exposure of hepatocytes to high levels of bile acids contributes to liver tumor development. Indeed, feeding FXR $^{-/-}$ mice with a diet containing bile acid strongly promoted N-nitrosodiethylamine-initiated liver tumorigenesis, whereas lowering bile acid pool with a bile acid sequestrant considerably reduced the malignant lesions (16). Thus disruption of bile acid homeostasis due to impairments in BSEP expression may contribute to the pathogenesis of HCC.

HCC is the most common primary liver cancer and one of the leading causes for cancer-related deaths globally. The etiology of HCC mainly includes viral hepatitis (18, 19), alcoholic and nonalcoholic fatty liver disease (20–22), and metabolic syndromes (23, 24). Regardless of the etiology, the common pathological process for HCC development is chronic liver injury and inflammation (25, 26). Clinical studies showed that bile acid levels in serum and urine were significantly elevated with a concurrent decrease in fecal bile acids in HCC patients (27–31), indicating disruption of bile acid homeostasis. Elevated serum bile acid level has been proposed as a clinical marker for HCC (27–30). Currently, the underlying mechanisms for bile acid imbalance in HCC patients are largely unknown.

In this study, we demonstrated that BSEP expression was dysregulated with altered FXR isoform expression in HCC tissues and hepatoma cell lines Huh 7 and HepG2. Transactivation studies *in vitro* and *in vivo* established that in contrast to mouse, human BSEP was isoform-specifically regulated by FXR with FXR α 2 being the predominant regulator. Additional studies revealed that proinflammatory cytokines IL-6 and TNF- α significantly elevated in HCC tissues and altered the FXR α 1/FXR α 2 ratio with concurrent decreases in BSEP expression in Huh 7 cells. A potential link from inflammation to disruption of bile acid homeostasis through alteration in the relative expression of FXR isoforms and subsequent BSEP dysregulation was proposed in patients with HCC.

MATERIALS AND METHODS

Reagents and suppliers

Chemicals and reagents for polymerase-chain reaction (PCR), cell culture, transfection, and luciferase assays were described previously (32). Recombinant human FXR α 2, IL-1 β , IL-6 and TNF- α were purchased from Pierce.

Liver samples

Fourteen healthy human liver samples and 22 HCC tumor (HCC-T) samples with 11 paired adjacent non-tumor (HCC-NT) tissues were obtained from the University of Virginia, University of Pennsylvania and Ohio State University through the Cooperative Human Tissue Network (CHTN). The detailed information on HCC patients was provided in Supplement Table 1 and 2. The protocol for using human tissues was approved by the Institutional Review Board (IRB) at the University of Rhode Island (URI).

Plasmid constructs

Human and mouse BSEP promoter reporters, phBSEP(-2.6kb) and pmBSEP(-2.6kb), were prepared as described previously (6, 32). Expression plasmids for human FXR α 2 and FXR α 1 were provided by Dr. Matthew Stoner and Dr. David Mangelsdorf (University of Texas Southwestern Medical Center).

Reporter luciferase assay

The reporter luciferase activity assays were carried out in Huh 7 cells in a 24-well plate format and the luciferase activities were detected with a Dual-Luciferase Reporter Assay as described (33).

Living imaging with *in vivo* imaging system (IVIS)

Eighteen CD-1 female mice were obtained from Charles River Laboratory and randomly divided into three groups. Mice were injected with 5 μ g phBSEP(-2.6kb) and either 5 μ g FXR α 1, FXR α 2 or pcDNA5 vector, respectively and subjected to IVIS 100 Living Imaging detection with a exposure time of 45 seconds. The study was approved by the Institutional Animal Care and Use Committees (IACUC) at URI.

Quantitative real-time PCR

Total RNA preparation from liver samples, Huh 7 and HepG2 cells, cDNA synthesis and TaqMan real-time PCR were carried out as described (33). Transcript levels of FXR, BSEP, IL-1 β , IL-6 and TNF α were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

Semi-quantification of FXR α 1 and FXR α 2 expression

A 600bp fragment encompassing the 12- nucleotides inserted in FXR α 1 was amplified with a sense primer 5'-TGTGGAGACAGAGCCTCTGGATACCACTATAATGC-3' and an antisense primer 5'-GAACATAGCTTCAACCGCAGACCCTTTTCAGCAAG-3'. The PCR products were equally divided into three tubes and completely digested with BstZ 17I, Bsm I or buffer only, followed by separating the DNA fragments on 1.5% agarose gel and quantified.

Cytokine treatment

Huh 7 cells were treated with 30ng/ml of IL-1 β , IL-6 or TNF α in a medium containing 1% dislipidated FBS (Invitrogen) for 48h. Total cellular RNA was extracted and cDNA was

synthesized, followed by quantification of total FXR, FXR α 1, FXR α 2 and BSEP expression.

Western blotting

Crude membrane fractions from liver tissues were prepared as described (34). Membrane fractions containing 25 μ g of total proteins were loaded into each well. After transfer, the membrane was split with the top portion blotted for BSEP (~175kD) with an anti-human BSEP mAb (sc- 74500, Santa Cruz Biotechnologies) and the bottom portion blotted for GAPDH (35kD) with the rabbit anti-human GAPDH polyclonal antibodies (G9545, Sigma-Aldrich). FXR detection in FXR α 1 or FXR α 2-transfected cells or mouse livers was performed using mouse anti-human FXR mAb (H00009971-M02, Abnova). The expression levels of GAPDH were used to normalize BSEP or FXR expression.

Two-dimension electrophoresis followed by Western blot (2DWB)

Nuclear extracts from human liver tissues and HepG2 cells were used to detect FXR α 1 and FXR α 2 according to the protocol provided in Supplement Fig. 1.

Mass spectrometry (MS)

Nuclear extracts from human liver tissues and HepG2 cells were applied to MS to detect FXR α 1 and FXR α 2 protein. The detailed protocol was provided in the Supplement Fig. 2.

Statistic analysis

Student t-test was applied to pair-wise comparison for normally distributed data. Non-parametric Mann-Whitney test was used for pair-wise comparison for non-normally distributed data. P value of 0.05 or lower was considered statistically significant.

RESULTS

BSEP expression was dramatically decreased in HCC tissues

Previous studies showed that bile acid levels increased in the serum and urine and decreased in fecal excretion in HCC patients (27–31). We therefore hypothesized that BSEP transcription was dysregulated in patients with HCC. To test the hypothesis, BSEP mRNA levels were quantitatively determined in liver samples from HCC patients and healthy subjects. As shown in Fig. 1A, when compared with healthy controls, the level of BSEP mRNA was significantly reduced in both HCC tumor (HCC-T) and adjacent non-tumor (HCC-NT) tissues. The median level of BSEP mRNA decreased by 72% and 97% in HCC-NT ($p=0.029$) and HCC-T ($p<0.001$) samples, respectively. No statistical significance was detected between HCC-NT and HCC-T ($p=0.053$). The data demonstrated that BSEP transcription was severely dysregulated in HCC patients.

To determine whether the transcriptional dysregulation of BSEP in HCC patients correlates with BSEP protein expression, Western blotting was performed to quantify BSEP protein in all liver samples. As shown in Fig. 1B and 1C, compared with healthy subjects, BSEP protein levels dramatically decreased in HCC-NT ($p=0.001$) and HCC-T ($p<0.001$) samples. The median levels of expression decreased by 97% and 99% in HCC-NT and HCC-T samples, respectively. Almost no BSEP protein was detected in any HCC-T samples. It was thus concluded that BSEP expression severely diminished in HCC patients. It should be noted that such diminished expression of BSEP did not result from decreased FXR expression as no statistically significant changes in total FXR expression were detected among the groups (Fig. 1D), suggesting other underlying mechanisms.

Isoform-dependent transactivation of human BSEP by FXR *in vitro*

FXR α 1 and FXR α 2 are predominant isoforms expressed in human liver (11). A previous study showed that mouse ileal bile acid-binding protein was regulated differently by FXR α 1 and FXR α 2, while regulation of mouse BSEP by FXR α 1 and FXR α 2 displayed little disparities (12). To determine whether human BSEP is transcriptionally regulated by FXR in an isoform-specific manner, human BSEP promoter reporter, phBSEP(-2.6kb), was cotransfected into Huh 7 cells with FXR α 1 or FXR α 2. Mouse BSEP promoter reporter, pmBSEP(-2.6kb), was included in the experiments as a control. As shown in Fig. 2A, FXR α 2 exhibited over 20 fold higher activity than FXR α 1 in transactivating human BSEP. In contrast, FXR α 1 and FXR α 2 exhibited comparable activity with mouse BSEP (Fig. 2B). It should be noted that the observed difference in human BSEP transactivation was not due to the different levels of protein expression from the transfected FXR isoforms (Fig. 2C). The data demonstrated that human BSEP was predominantly regulated by FXR α 2. Therefore, in addition to total FXR levels, the relative expression levels of FXR α 1 and FXR α 2 should play an important role in determining human BSEP expression.

To demonstrate the effect of FXR α 1/FXR α 2 ratios on BSEP transactivation, a series of FXR α 1 and FXR α 2 plasmid DNA in various ratios with a constant total amount of FXR were co-transfected with human BSEP promoter reporter in Huh 7 cells. As shown in Fig. 2D, BSEP transactivation levels gradually decreased as FXR α 1/FXR α 2 ratios increased. It was thus established that the FXR α 1/FXR α 2 ratio is a determinant in regulating human BSEP expression.

Isoform-dependent transactivation of BSEP by FXR *in vivo* in mice

To further confirm the isoform-dependent regulation of human BSEP, we performed an *in vivo* study in mice using IVIS 100 Living Imaging System. No signal was detected in mice receiving pcDNA5 vector and phBSEP(-2.6kb), indicating that human BSEP transactivation by endogenous mouse FXR is minimal (Fig. 3A). Detectable signals were readily captured in mice co-injected with human FXR α 1 and phBSEP(-2.6kb). Much more robust signals were detected in mice co-injected with human FXR α 2 and phBSEP(-2.6kb). Quantification of the signals showed that FXR α 2-induced human BSEP transactivation was 4.5 times higher than FXR α 1 on average (Fig. 3B) with similar levels of FXR protein expression in mice injected with FXR α 1 or FXR α 2 (Fig. 3C). The data above concluded that human BSEP was regulated by FXR in an isoform-dependent manner both *in vitro* and *in vivo* with FXR α 2 being the dominant regulator.

FXR α 1/FXR α 2 ratios were significantly altered in HCC tissues

After demonstrating the isoform-dependent regulation of human BSEP, we investigated whether alteration in relative expression of FXR α 1 and FXR α 2 is a potential mechanism for BSEP dysregulation in patients with HCC.

Digestion with restriction enzymes BstZ 17I and Bsm I differentiates the two FXR isoforms (Fig. 4A). The PCR products derived from FXR α 1 plasmid templates were completely digested by BstZ 17I, whereas no digestion was observed with Bsm I (Fig. 4B). On the other hand, Bsm I could completely digest the PCR products derived from the FXR α 2 plasmid templates, while no digestion was detected with BstZ 17I. In liver samples, the PCR products resistant to either Bsm I or BstZ 17I digestion represent the relative amount of FXR α 1 and FXR α 2 (Fig. 4B). In healthy subjects, FXR α 1 was expressed more abundantly than FXR α 2 with a median FXR α 1/FXR α 2 ratio of 1.4 (Fig. 4C), and such ratio significantly increased to 2.1 ($p=0.001$) in HCC-NT and 2.8 ($p=0.002$) in HCC-T samples. More strikingly, the expression of FXR α 2 was undetectable in 7 of the 22 HCC-T samples and was therefore not included in Fig. 4C and statistical analysis. The data established that

the FXR α 1/FXR α 2 ratios varied greatly in patients with HCC and that such alteration occurred in both tumor and adjacent non-tumor tissues.

BSEP expression correlated with FXR isoform expression in hepatoma Huh 7 and HepG2 cells

To investigate whether BSEP expression in hepatoma cell lines correlates with FXR isoform expression as revealed in HCC tissues, BSEP and FXR isoform expression levels were quantified in Huh 7 and HepG2 cells. As shown in Fig. 5A, BSEP transcripts were readily detected in Huh7 cells although at a decreased level when compared with healthy liver samples. However, BSEP mRNA was undetectable in HepG2 cells. It should be noted that such differences in BSEP transcription was not caused by the variation of total FXR expression because there was no statistical difference in total FXR expression between the two cell lines (Fig. 5B). Detection of FXR isoforms revealed that both FXR α 1 and FXR α 2 were expressed with an FXR α 1/FXR α 2 ratio of 1.72 in Huh 7 cells. In contrast, no FXR α 2 expression was detected in HepG2 cells (Fig. 5C). The results indicated that Huh 7 cells behaved similarly to most of the HCC tissues with increased FXR α 1/FXR α 2 ratio and decreased BSEP expression while HepG2 cells behaved as the 7 HCC-T samples with no detectable FXR α 2 and completely diminished BSEP expression. The data thus confirmed the phenomenon observed in HCC tissues.

To further solidify the findings and determine whether supplying exogenous FXR α 2 to HepG2 cells can restore the expression of endogenous BSEP, HepG2 cells were transiently transfected with increasing amounts of FXR α 2, followed by quantification of BSEP mRNA. As shown in Fig. 5D, transfection of exogenous FXR α 2 indeed resulted in endogenous BSEP expression in an FXR α 2 dose-dependent manner. The data strongly supported the notion that undetectable BSEP expression in HepG2 cells was due to the absence of FXR α 2.

Alteration of FXR isoform protein expression in HCC and HepG2 cells

To determine whether the data on FXR isoform expression at mRNA level correlate with their expression at protein levels, FXR α 1 and FXR α 2-specific proteins were detected by 2DWB and MS in representative liver tissues and HepG2 cells. Since HepG2 cells only expressed FXR α 1 at mRNA level, the respective FXR α 1 and FXR α 2 proteins were detected by 2DWB with nuclear extracts from untransfected and FXR α 2-transfected HepG2 cells (Supplement Fig. 1). In the healthy tissue, FXR α 2 was dominantly expressed while a new form of FXR protein started to appear in the HCC-NT sample. More significantly, FXR α 1 and the new form FXR protein were significantly increased in HCC-T samples (Supplement Fig. 1). The relative expression of FXR α 1 was increased from 11% in healthy tissue to 59% of total FXR in HCC-T samples (Table 1). In the detection of FXR α 1 and FXR α 2 protein by MS, the fingerprints of two signature peptides (FXR α 1: EMGMLAECMYTGLLTEIQCK and FXR α 2: EMGMLAECLLTEIQCK) were shown in Supplement Fig. 2. The relevant mass spectral abundance of the precursor ions of the two signature peptides provided relative levels of the two proteins in the samples. Consistent with the data from 2DWB, relative FXR α 1 expression was markedly increased from 9% in healthy tissue to 85% of total FXR in HCC-T samples (Table 1). Taken together, the data demonstrated that the relative expression of the two FXR isoforms was significantly altered in HCC. Such alteration may represent a mechanism for BSEP dysregulation in HCC patients.

Proinflammatory cytokines IL-6 and TNF- α increased FXR α 1/FXR α 2 ratios with concurrent decreases in BSEP expression

It is well established that chronic liver injury and inflammation are the common pathological processes leading to HCC development (25, 26). To determine whether inflammation plays

a role in altering relative expression of FXR α 1 and FXR α 2, we treated Huh 7 cells with IL-1 β , IL-6 and TNF- α , followed by determination of FXR α 1/FXR α 2 ratios and BSEP expression. As shown in Fig. 6A, treatment with IL-6 and TNF- α significantly shifted the relative expression of FXR α 1 and FXR α 2. The FXR α 1/FXR α 2 ratios increased as much as 2.5 fold in cells treated with IL-6 (p=0.015) or TNF- α (p=0.003), while IL-1 β had no detectable effects (p=0.61). As expected, alteration in FXR α 1/FXR α 2 ratios corresponded with BSEP expression (Fig. 6B), which decreased by 59% (p<0.001) and 31% (p=0.036) in cells treated by IL-6 and TNF- α , respectively. On the contrary, treatment with IL-1 β resulted in a 30% increase in BSEP expression with no statistical significance (p=0.15). Treatment with IL-6 (p=0.6), TNF- α (p=0.83), or IL-1 β (p=0.17) induced unnoticeable alteration in total FXR expression (Fig. 6C). In summary, IL-6 and TNF- α , but not IL-1 β , substantially increased FXR α 1/FXR α 2 ratio and decreased BSEP expression.

Previous studies have shown that serum IL-6 and TNF- α concentrations were increased in HCC patients (35, 36). Based on our finding, we propose that the levels of proinflammatory cytokines IL-6 and TNF- α would be elevated in HCC tissues. Indeed, as shown in Fig. 7, IL-6, TNF- α and IL-1 β mRNA levels were greatly elevated in HCC-NT and HCC-T samples when compared with healthy controls. The median IL-6 mRNA levels increased by 13.2 fold in HCC-NT (p<0.001) and 24.2 fold in HCC-T (p<0.001) samples, while the median TNF- α mRNA level increased by 4.5 fold in HCC-NT (p=0.017) and 4.8 fold in HCC-T (p<0.001) samples. Significant elevation of IL-1 β mRNA levels were also detected in HCC-NT (p=0.019) and HCC-T (p<0.001). The data revealed that the expression levels of proinflammatory cytokines IL-6, TNF- α and IL-1 β significantly elevated in HCC tissues.

DISCUSSION

As the canalicular effluxer of bile acids, BSEP has a direct impact on the enterohepatic circulation of bile acids. In this study, we showed that BSEP expression was severely diminished in both HCC and tumor-surrounding tissues, suggesting a reduced biliary secretion of bile acids and accumulation of bile acids in the liver and body. The finding was in agreement with the clinical measurements of bile acids in HCC patients (27–30). In one study, total serum bile acid levels increased 5.7 fold in HCC patients (30). In another study, serum glycochenodeoxycholic acid and taurocholic acid elevated 6.7 and 25.4 fold, respectively while glycocholic acid levels increased 6.4 fold in serum and 45 fold in urine in HCC patients (28). On the other hand, fecal metabolome profiling revealed that bile acids dramatically decreased in HCC patients (31). Therefore, it was concluded that dysregulation of BSEP was at least one of the mechanisms leading to the disruption of bile acid homeostasis in HCC patients.

FXR α 1 and FXR α 2 are the two predominant FXR isoforms in the human liver (11) with isoforms FXR α 3 and FXR α 4 being undetectable (data not shown). A previous study showed that mouse BSEP was comparably transactivated by FXR α 1 and FXR α 2 (12). In contrast, we found that human BSEP was differentially regulated by FXR α 1 and FXR α 2 with FXR α 2 being the predominant regulator *in vitro* and *in vivo*. Such species-specific regulation of BSEP is not uncommon (6). Currently, the mechanisms for isoform-dependent and species-specific regulation of BSEP by FXR are under investigation.

Similar to human BSEP, other FXR target genes including ileal bile acid-binding protein (12), syndecan-1 (37) and fibrinogens (38) also exhibited isoform-specific regulation by FXR. However, at present, its physiological and/or pathological significance are unknown. In this study, we demonstrated that FXR α 1 and FXR α 2 expression was significantly altered in HCC tissues in association with a marked reduction of BSEP expression. Such observation in HCC tissues was fully in agreement with the data from hepatoma cell lines

Huh 7 and HepG2 cells. We thus concluded that alteration in relative expression of FXR isoforms may represent an underlying mechanism for BSEP dysregulation in HCC patients. However, it remains to be determined whether other mechanisms such as epigenetic modifications and alteration in coregulator recruitment, which occur commonly in tumorigenesis, also indirectly contribute to BSEP dysregulation in HCC.

In the 2DWB, a new form of FXR protein appeared (Supplement Fig. 1) in HCC-NT and HCC-T samples but absent in healthy tissue. It remains to be determined whether it is a modified FXR α 1, FXR α 2 or a new isoform of FXR. Comparison of the data at mRNA and protein levels, it was noted that FXR α 1 was more abundantly expressed at the mRNA levels while FXR α 2 expression was predominantly expressed at the protein level in the same healthy tissue. Similarly, FXR α 2 mRNA levels were undetectable while FXR α 2 proteins were detected by both 2DWB and MS in the representative HCC-T samples. Such discrepancy may indicate different post-transcriptional regulations of the two isoforms. Additional studies are required to provide a full explanation for the phenomenon.

Chronic inflammation is the hallmark for the development of HCC (25, 26). Consistently, serum IL-6 and TNF- α concentrations significantly increased in HCC patients (35, 36) and mouse model (39). There are also studies demonstrating that IL-6 and TNF- α play an etiological role in the development of HCC (39, 40). Alternative splicing from a single promoter results in FXR α 1 and FXR α 2 (11, 12). In this study, we found that IL-6 and TNF- α but not IL-1 β markedly altered relative expression of FXR α 1 and FXR α 2 with a concurrent decrease in BSEP expression (Fig. 6A and 6B), indicating a possible crosstalk between the FXR alternative splicing process and IL-6 and TNF- α signaling pathways. Further studies are required to establish and reveal the mechanistic insights of such crosstalk. The fact that in contrast to IL-6 and TNF- α , IL-1 β had no significant effects on FXR isoforms and BSEP expression (Fig. 6A and 6B) indicates that not general but cytokine-specific chronic inflammation leads to alteration of FXR isoform and subsequently BSEP expression.

In comparison with healthy liver samples, adjacent HCC-NT tissues exhibited a significant decrease in BSEP expression although at the levels not as severe as in HCC-T (Fig. 1), indicating that BSEP dysregulation is not limited to the tumor but extended to adjacent liver tissues. Similarly, the expression levels of proinflammatory cytokines IL-6, TNF- α and IL-1 β were markedly elevated in HCC-NT tissues at comparable levels to that in HCC-T (Fig. 7). The data are in agreement with the notion that chronic inflammation leads to decreased BSEP expression and tumorigenesis may further worsen BSEP dysregulation. However, it remains to be determined whether such elevation in proinflammatory cytokine levels and BSEP dysregulation extends to the entire liver.

Bile acids have been recognized as carcinogens and/or tumor promoters (41). Currently, whether disruption of bile acid homeostasis is a trigger for initiating HCC development remains to be established. However, a large body of evidence indicates that excessive bile acids in the liver contribute to the pathogenesis of HCC (13, 14, 16, 17, 41–43). Indeed, chronic exposure of hepatocytes to high levels of bile acids due to genetic defects in BSEP in human (13, 14) or dysregulation of BSEP in FXR knockout mice (16, 17) do spontaneously develop HCC. Bile acids are also implicated as etiological agents in colon and esophagus cancer development (41–43). Based on our finding that BSEP expression was severely diminished due to inflammation-mediated alteration of FXR isoform expression, restoration of BSEP expression through controlling chronic inflammation in the liver may re-establish the bile acid homeostasis, providing a possible approach for treatment and/or prevention of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BSEP	bile salt export pump
HCC	hepatocellular carcinoma
FXR	farnesoid X receptor
HCC-T	HCC tumor
HCC-NT	HCC non-tumor
IL-1β	interleukin-1beta
IL-6	interleukin-6
TNF-α	tumor necrosis factor-alpha
CDCA	chenodeoxycholic acid
DMSO	dimethyl sulfoxide
PCR, polymerase-chain reaction. GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IVIS	<i>in vivo</i> imaging system
CHTN	cooperative human tissue network. 2DWB, two-dimension electrophoresis followed by Western blot
MS	Mass spectrometry

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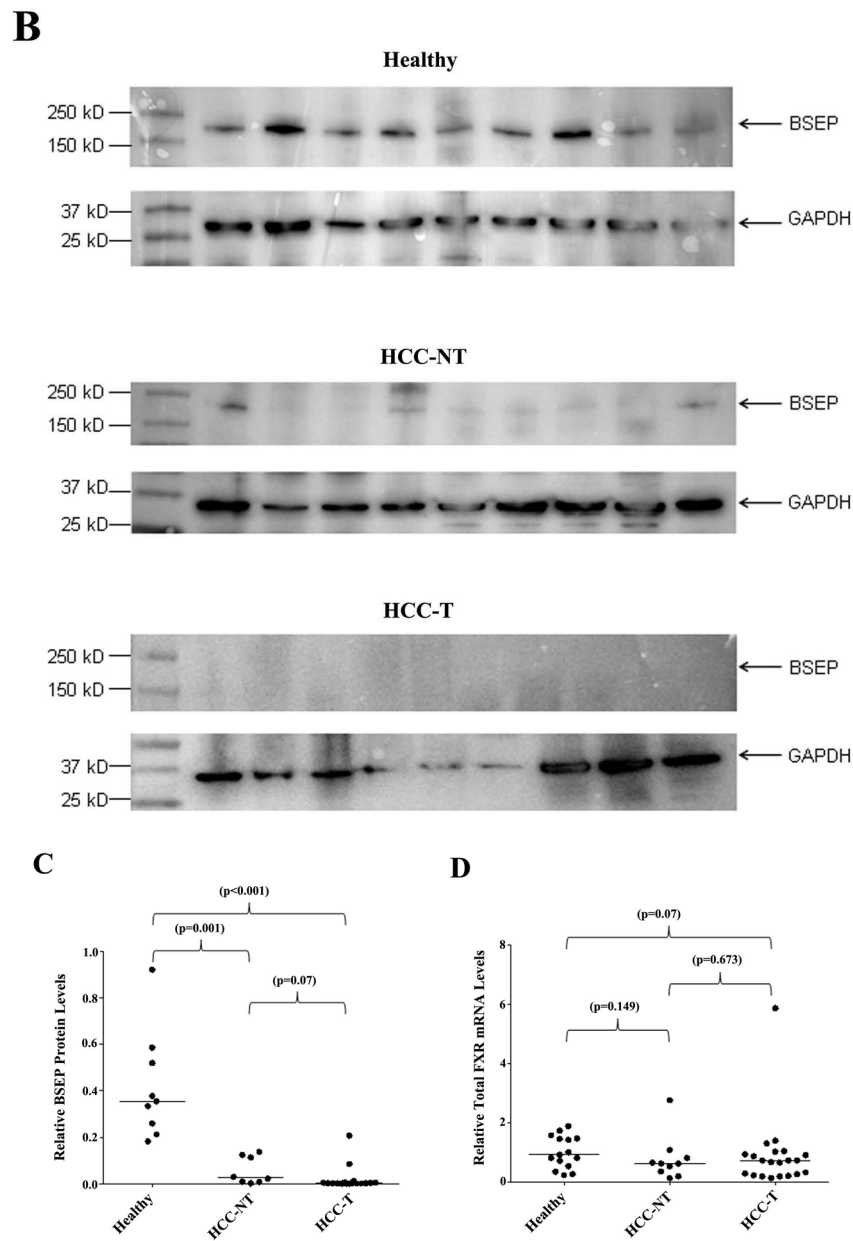
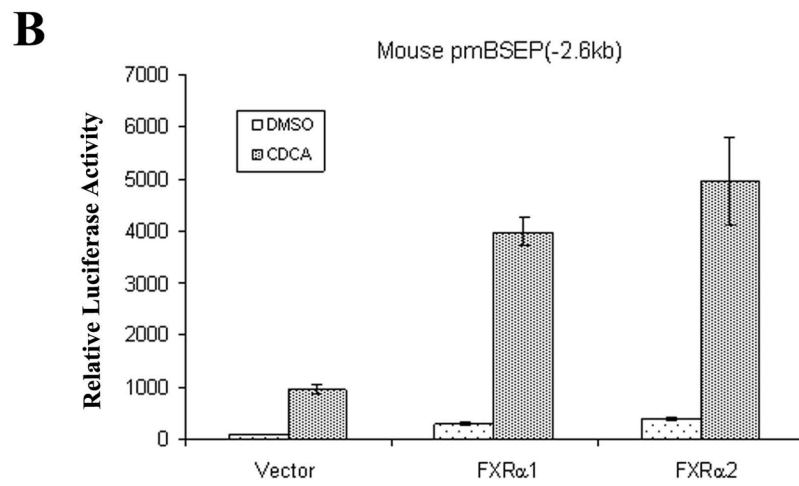
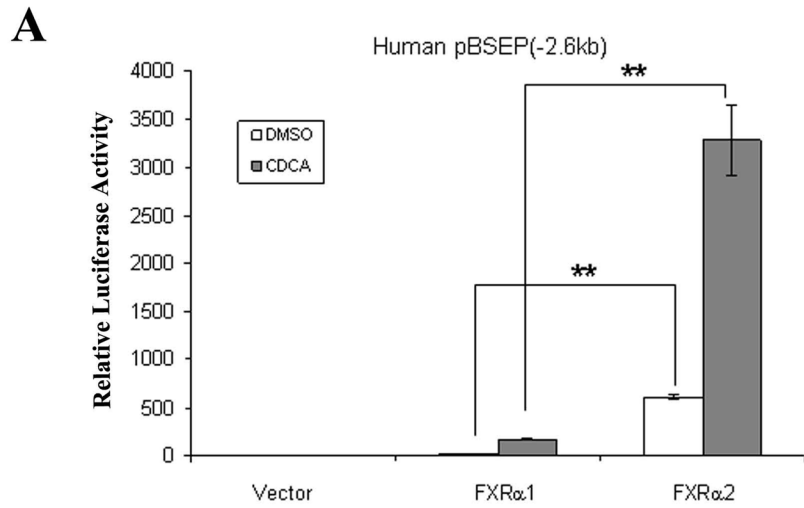


Fig. 1. BSEP expression was markedly decreased in HCC tissues

(A) Expression levels of BSEP mRNA, (B, C) BSEP protein, and (D) total FXR mRNA in healthy, HCC tumors (HCC-T) and paired adjacent non-tumor (HCC-NT) tissues. BSEP and total FXR mRNA levels were quantified by TaqMan real-time PCR with GAPDH levels as internal standards. BSEP protein levels were detected by Western blot and normalized with GAPDH levels on the same blot. The median value of each group was indicated by a short line. Non-parametric Mann-Whitney test was used for pair-wise comparison.



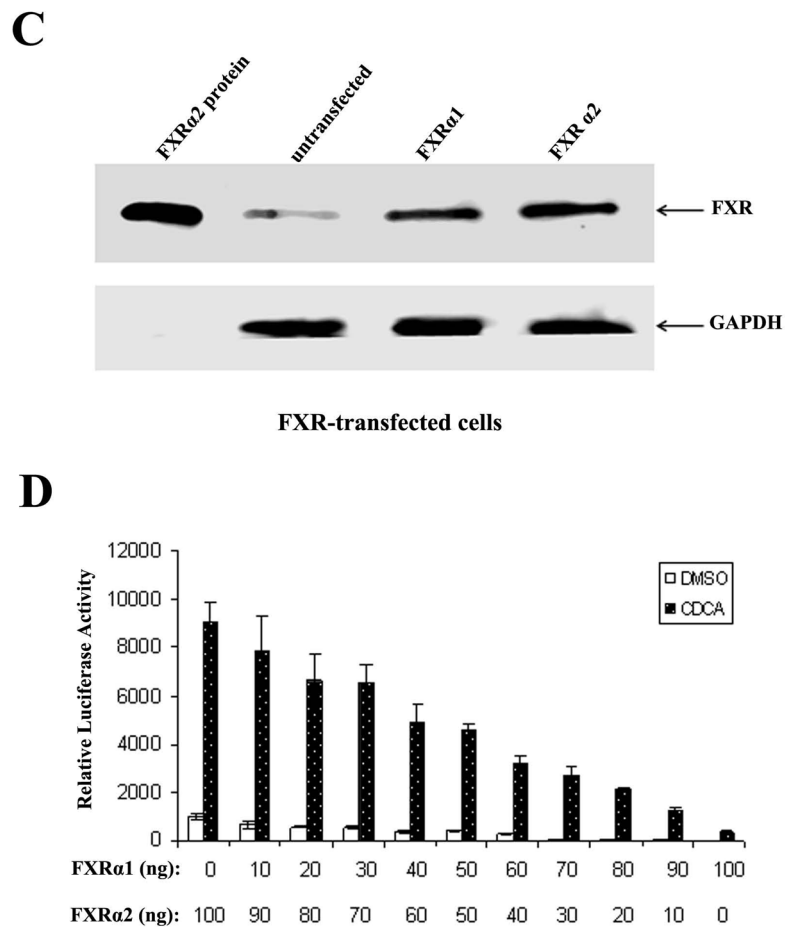


Fig. 2. Isoform-dependent transactivation of human BSEP by FXR *in vitro*

(A) FXR α 1 and FXR α 2-mediated transactivation of human BSEP promoter reporter phBSEP(-2.6kb) and (B) mouse BSEP promoter reporter pmBSEP(-2.6kb) in the presence of vehicle DMSO (0.1%) or CDCA (10 μ M). The data were presented as mean \pm SD of at least three separate experiments. Student's t-test was applied to pairwise comparison. A double asterisk (**) indicates $p < 0.001$. (C). FXR protein levels were detected with Western blot in FXR α 1 or FXR α 2-transfected cells. (D) The effects of FXR α 1/FXR α 2 ratios on human BSEP transactivation.

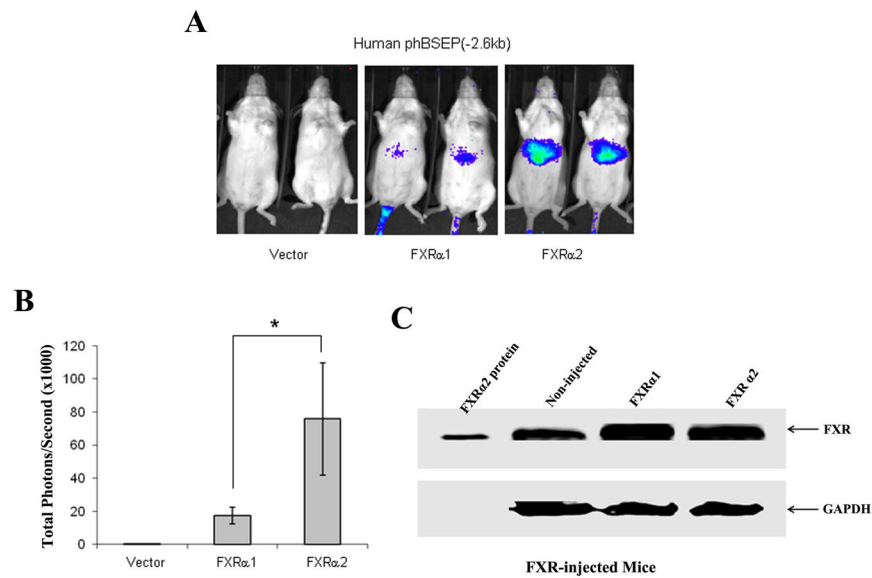


Fig. 3. Isoform-dependent transactivation of human BSEP by FXR *in vivo* in mice
 (A) Three groups of CD-1 female mice received human promoter reporter phBSEP(-2.6kb) and either pcDNA5 vector, FXR α 1 or FXR α 2 expression plasmid DNA through hydrodynamic tail vein injection. The transactivation levels were detected with IVIS Living Imaging system. Two representative mice from each group were presented. (B) Quantification of total signals (photons) in a fixed area covering the entire liver surface was carried out with Living Imaging Software and presented as mean \pm SD. The asterisk (*) indicates $p < 0.05$ by the Student's t-test. (C) FXR protein levels in the liver of FXR α 1 or FXR α 2-injected mice were detected with Western blot.

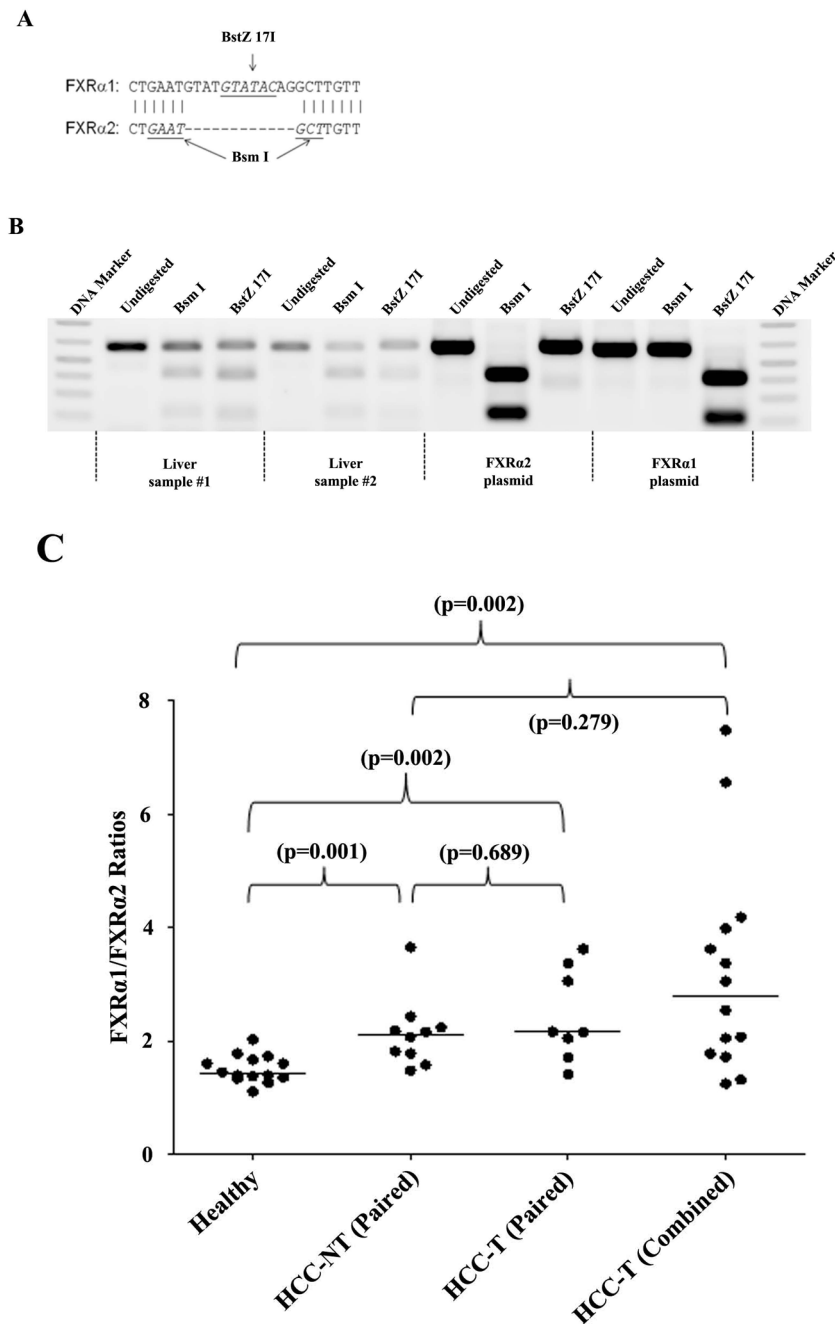


Fig. 4. FXRα1/FXRα2 ratios were significantly increased in HCC tissues

FXRα1 and FXRα2 differ by an insertion of 12 nucleotides in the hinge region in FXRα1 which is absent in FXRα2. A restriction enzyme site for BstZ 171 is located within the inserted 12 nucleotides. Another restriction enzyme site for Bsm I is present in the junction of the insertion in FXRα2 but destroyed by the insertion in FXRα1. Therefore, digestion with BstZ 171 or Bsm I differentiates FXRα1 from FXRα2. (A) Sequence alignment of FXRα1 and FXRα2 in the insertion junction. (B) Results from two representative healthy liver samples and FXRα1 and FXRα2 plasmid controls were presented. PCR fragments (600bp) containing the insertion junction were completely digested with BstZ 171 or Bsm I generating two fragments with sizes of 400bp and 200bp. The intensity of the remaining

600bp bands after digestion with BstZ 17I or Bsm I represented the expression levels of FXR α 2 or FXR α 1. (C) FXR α 1/FXR α 2 ratios significantly increased in HCC-NT and HCC-NT samples. The median value of each group was indicated by a short line. Non-parametric Mann-Whitney test was used for pair-wise comparison.

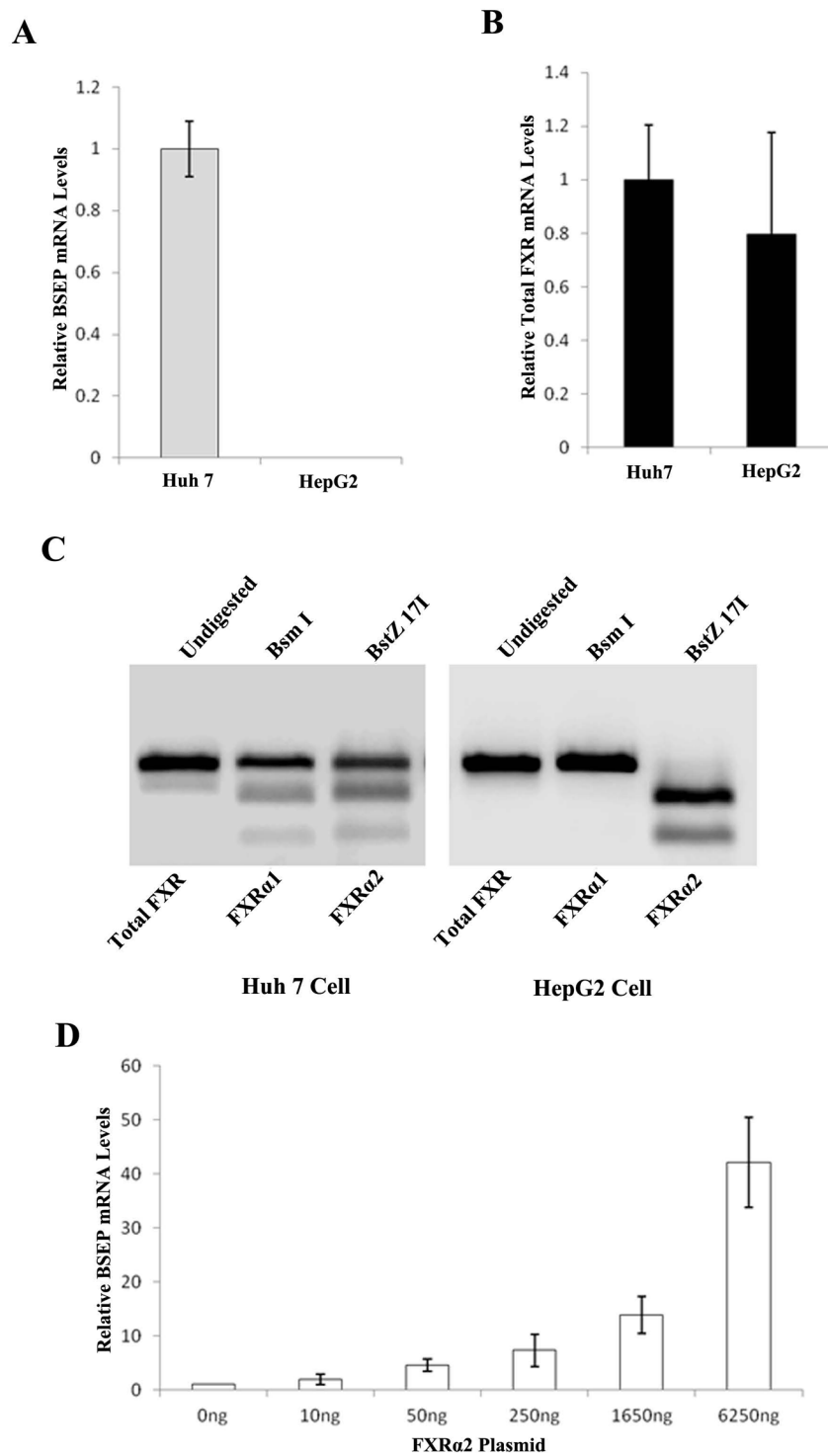
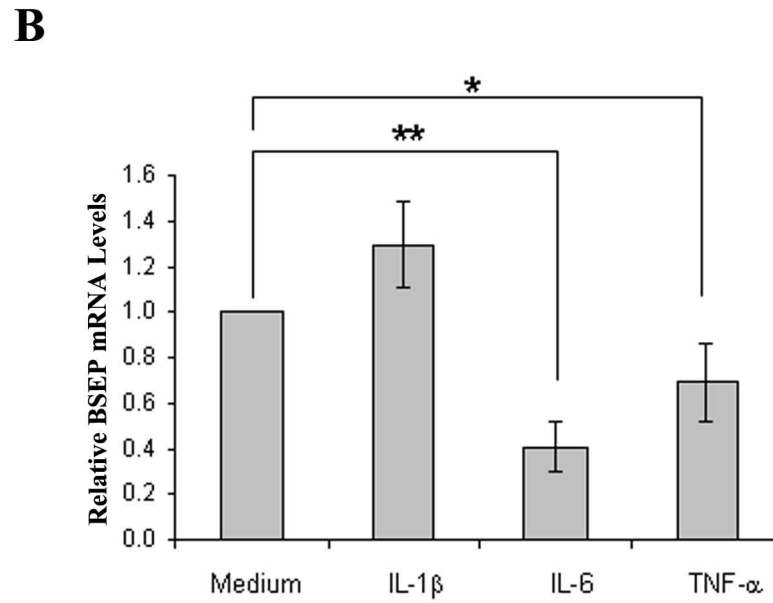
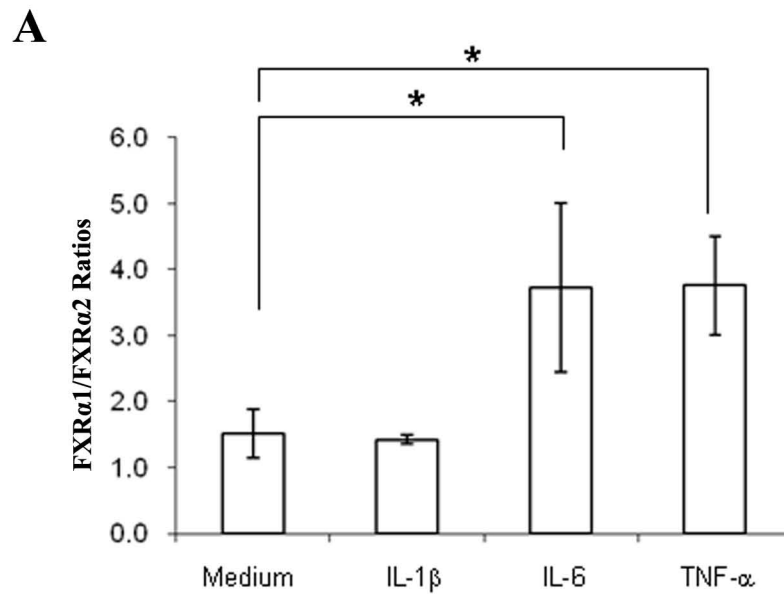


Fig. 5. BSEP expression correlated with FXR isoform expression in Huh 7 and HepG2 cells (A) Expression levels of BSEP mRNA and (B) total FXR mRNA were quantified by TaqMan real-time PCR with GAPDH levels as internal standards. No BSEP mRNA expression was detected in HepG2. (C) Expression of FXR α 1 and FXR α 2 in Huh 7 and HepG2 cells. No FXR α 2 expression was detected in HepG2 cells. (D) Exogenously

transfected FXR α 2 restored endogenous BSEP expression in a dose dependent manner in HepG2 cells.



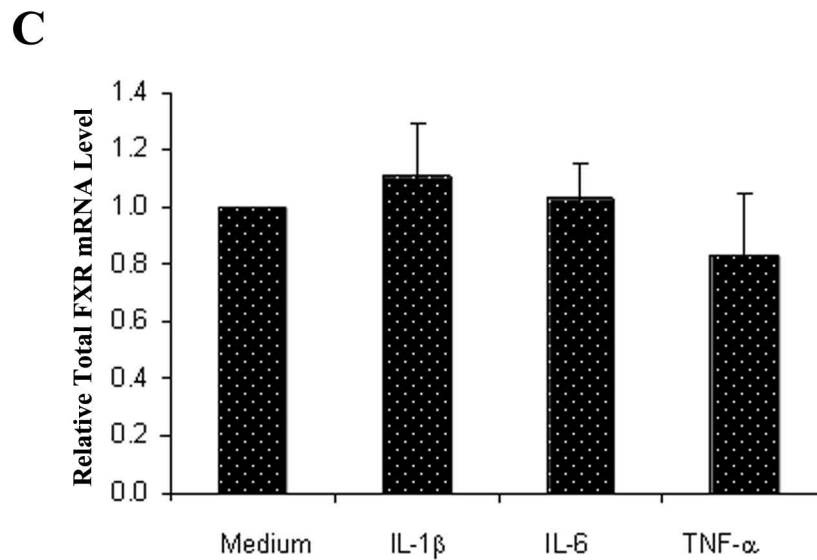


Fig. 6. Proinflammatory cytokines IL-6 and TNF- α altered FXR α 1/FXR α 2 ratio and BSEP expression

(A) Huh 7 cells were treated with proinflammatory cytokines IL-1 β , IL-6 and TNF- α at a concentration of 30ng/ml for 48h, followed by determination of FXR α 1/FXR α 2 ratios, (B) BSEP and (C) total FXR mRNA levels. Student's t-test was applied to pair-wise comparison. A single asterisk (*) indicates $p < 0.05$ and a double asterisk (**) indicates $p < 0.001$.

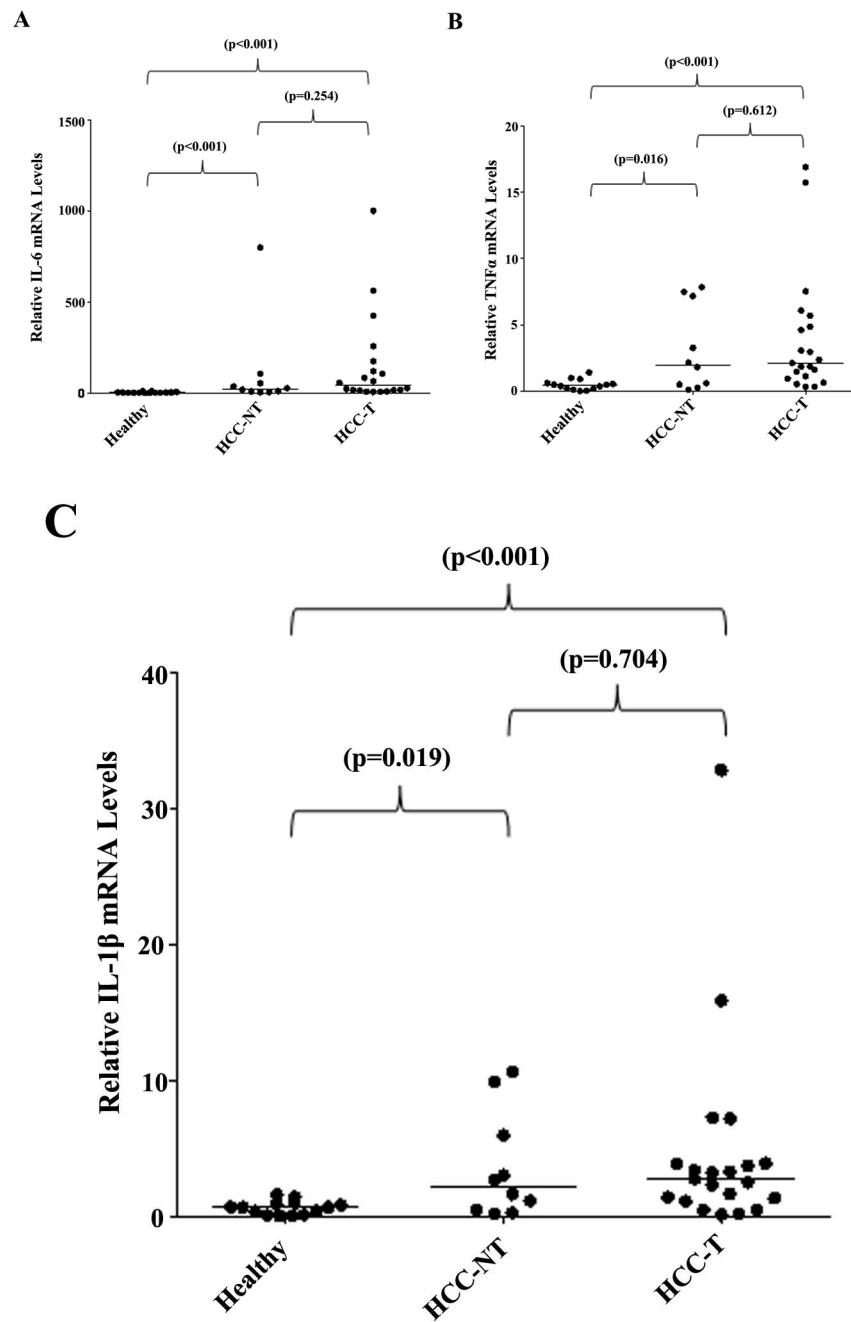


Fig. 7. Proinflammatory cytokines IL-1 β , IL-6 and TNF- α expression levels significantly elevated in HCC tissues

(A) The expression levels of IL-6, (B) TNF- α and (C) IL-1 β in healthy, HCC-T and HCC-NT tissues were quantified by TaqMan real-time PCR with GAPDH levels as internal standards. The median value of each group was indicated by a short line. Non-parametric Mann-Whitney test was used for pairwise comparison.

Table 1Relative expression of FXR α 1 at mRNA and protein levels

Sample type	FXR mRNA	FXR protein	
	FXR α 1 by RT-PCR (% of total FXR)	FXR α 1 by MS (% of total FXR)	FXR α 1 by 2DWB (% of total FXR)
Healthy	53	9	11
HCC-NT	71	10	7
HCC-T	100	85	59
HepG2	100	91	83