A mutant of HBx (HBxΔ127) promotes hepatoma cell growth via sterol regulatory element binding protein 1c involving 5-lipoxygenase

Qi WANG1, Wei-ying ZHANG1, Li-hong YE2,*, Xiao-dong ZHANG1,*

Department of 1Cancer Research, Key Laboratory of Molecular Microbiology and Technology of Ministry of Education, Institute for Molecular Biology, and 2Biochemistry, the Key Laboratory of Bioactive Materials, Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, China

Aim: Previously, we identified a natural mutant of hepatitis B virus X gene (HBx) with a deletion from 382 to 401 base pairs (termed HBxΔ127), which could potently enhance growth of hepatoma cells. In this study, we further investigated the mechanism of increased hepatoma cell growth that was mediated by HBxΔ127.

Methods: We examined the effect of HBxΔ127 on the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) and fatty acid synthase (FAS) in a model of HepG2-XΔ127 (or H7402-XΔ127) cells, which was stably transfected with HBxΔ127 gene in a human hepatoma HepG2 (or H7402) cell line.

Results: Relative to wild type HBx, HBxΔ127 was able to potently activate SREBP-1c at the levels of promoter activity, mRNA and protein by a luciferase reporter gene assay, RT-PCR and Western blot analysis. Then, using the treatment with MK886, a specific 5-lipoxygenases (5-LOX) inhibitor, (or 5-LOX siRNA) we identified that 5-LOX was responsible for the upregulation of SREBP-1c by luciferase reporter gene assay, RT-PCR and Western blot analysis. Because FAS was a target gene of SREBP-1c, we further showed that HBxΔ127 was able to strongly activate the promoter activity of FAS and upregulated the mRNA expression level of FAS as well, by luciferase reporter gene assay and RT-PCR. In function, flow cytometry analysis revealed that FAS contributed to the growth of hepatoma cells that was mediated by HBxΔ127, using cerulenin (a FAS inhibitor).

Conclusion: HBxΔ127 promotes hepatoma cell growth through activating SREBP-1c involving 5-LOX.

Keywords: HBxΔ127; proliferation; sterol response element binding protein-1c; fatty acid synthase; 5-lipoxygenase

Acta Pharmacologica Sinica (2010) 31: 367–374; doi: 10.1038/aps.2010.5; published online 22 February 2010
It has been reported that changes in fatty acid metabolism in cancer cells interfere with normal cell cycling. The endogenous synthesis of fatty acid is usually minimal in normal cells because of sufficiently high levels of dietary fat. As a major transcription factor for lipogenic gene expression, sterol regulatory element binding protein-1c (SREBP-1c) may be involved in the development of tumors. SREBP-1c belongs to a basic helix-loop–helix leucine zipper SREBP-1 family of transcription factors, which activate genes involved in the synthesis of cholesterol and fatty acids, as well as their uptake from plasma lipoproteins. However, the role of HBxΔ127 in the regulation of SREBP-1 is unclear. Recent studies demonstrate that arachidonic acid metabolites are associated with cancer development. Our laboratory previously found that COX-2 and 5-LOX were involved in the proliferation and migration of breast cancer LM-MCF-7 cells. Therefore, we hypothesize that arachidonic acid metabolism may be involved in the regulation of SREBP-1c mediated by HBxΔ127. It has been reported that FAS is regulated by SREBP-1, which binds to sterol regulatory elements located in the promoter region of FAS. Studies have shown that the FAS gene is highly upregulated in various types of human malignancies, while this gene is expressed at minimal or undetectable levels in most normal tissues. In vitro, inhibition of FAS induces apoptosis in a variety of human cancer cells including breast, prostate, colon, and ovarian cell lines. It has been reported that the increased expression of FAS, together with the high proliferative index of breast cancer cells, is associated with a nine-fold increase in the risk of patient mortality.

Recently, we have shown that HBxΔ127 is able to promote cell growth that involves the activation of FAS. In the present study, we further investigate the mechanism of increased hepatoma cell growth mediated by HBxΔ127 and the role of SREBP-1, a regulatory element of FAS. Our data show that HBxΔ127 has a greater capacity to stimulate SREBP-1c, relative to wild type HBx, in the promotion of hepatoma cell growth. Interestingly, 5-LOX plays a role in the increased expression of SREBP-1c in HepG2-XΔ127 cells. Thus, our findings provide new insight into the mechanism of increased hepatoma cell growth that is mediated by HBxΔ127.

Materials and methods

Cell culture

Hepatoma HepG2 cells, HepG2-P (pCMV-Tag2B vector stably transfected cell line), HepG2-X (HBx stably transfected cell line) and HepG2-XΔ127 (HBxΔ127 stably transfected cell line) were maintained in Dulbecco's modified Eagle's medium (Gibco, Santa Clara, CA, USA) in 5% CO2 at 37 °C. Hepatoma H7402 cells, H7402-P (pCMV-Tag2B vector stably transfected cell line), H7402-X (HBx stably transfected H7402 cell line) and H7402-XΔ127 (HBxΔ127 stably transfected cell line) were cultured in RPMI Medium 1640 (Gibco) in 5% CO2 at 37 °C.

Reagents and plasmids

MK886 and indomethacin (Indo) were purchased from Sigma-Aldrich (St Louis, MO, USA). Cerulenin was purchased from Fermentek Ltd (Jerusalem, Israel). The siRNA targeting the mRNA of 5-LOX and the negative control siRNA were designed and synthesized by RiboBio (Guangzhou, China). Plasmids pCMV-X, pCMV-XA127, pSilencer3.0-X and pEGFP-C2 have been previously described. SREBP-1c-571-Luc-WT (a human SREBP-1 promoter luciferase reporter plasmid), pFAS-WT-Luc (a FAS promoter luciferase reporter plasmid) and pFAS ΔSRE-Luc (an SRE deleted FAS promoter reporter plasmid) were obtained from Dr Q LIU (University of Saskatchewan, Canada).

RNA interference experiment

HepG2-XΔ127 (or H7402-XΔ127) cells were transfected with a pSilencer-X vector, which produces the siRNA that targets HBxΔ127 mRNA (targeting nucleotides 271 to 290 of HBx mRNA) or control siRNA. Small interference RNA (siRNA) targeting bases 315 to 335 of the human 5-LOX mRNA (NM_000698) were introduced into HepG2-XΔ127 (or H7402-XΔ127) cells according to the manufacturer’s instructions. Each experiment included controls containing the transfection reagent with control siRNA. RT-PCR and Western blot analysis were performed 48 h after the transfection.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Extraction of total RNA from cells and reverse transcription were carried out as described previously. To confirm the stable expression of the HBxΔ127 gene, we used specific primers for the HBxΔ127 gene (forward primer, 5'-ATGGCTGCTAGGCTGCTG-3' and reverse primer, 5'-TTAAATCTCCTCCAACTCCT-3'). Specific primers were used for FAS (forward, 5'-GGTCTTGGAGATGCTGCTG-3' and reverse, 5'-AAATGCGCAAACCGCGTGTG-3') and SREBP-1c (forward, 5'-CCGCGCTTACCATAAGCTGCAC-3' and reverse, 5'-GACTGGTCTCTACTCTCAATG-3'). As a control, β-actin was amplified with specific primers (forward, 5'-AGCGGGGATCTGGTGCGT-3' and reverse, 5'-CAGGGTGATCGTGCCGCC-3').

Western blot analysis

Cells were washed three times with ice-cold PBS and extracted directly in lysis buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol). Equal amounts of protein (30 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane for 90 min. The membrane was blocked in blocking buffer (PBS, 5% skim milk, 0.1% Tween 20) at room temperature for 2 h, and membranes were then incubated at 4 °C overnight with a SREBP-1c-specific antibody (Santa Cruz Biotechnology, Delaware Avenue, CA, USA) and anti-HBx antibody (Abcam, Cambridge, UK). For protein loading controls, the amount of β-actin protein was also determined using a β-actin-specific antibody (Cell Signaling Technology, Danvers, MA, USA). The membranes were washed three times in PBST.
(PBS, 0.1% Tween 20) and incubated for 1 h with the appropriate secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG). The membranes were then washed three times, and the bands were visualized using ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Luciferase reporter gene assays
Transfected cells were harvested after 48 h. Luciferase activity was determined using the dual-Luciferase Reporter® Assay System (Promega, Madison, WI, USA) on a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA), according to the manufacturer’s instructions. The pCMV-Tag 2B empty vector, pGL3-basic plasmid and mock transfection were used as controls. Luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity. All experiments were performed at least three times.

Flow cytometry analysis
The detailed procedures of flow cytometry analysis were performed as previously described[6]. Briefly, HepG2-XΔ127 (or H7402-XΔ127) cells were grown in serum-free DMEM for 12 h and then treated with 10 μg/mL cerulenin for 12 h. At the end of incubation, cells were harvested, washed twice in PBS, and resuspended in 200 μL of PBS. Then, 2 mL of ice-cold 70% ethanol was added, and the cells were fixed overnight at 4 °C. Next, 100 μL of RNaseA (1 mg/mL) and 100 μL of propidium iodide (100 mg/mL) were added to the cell suspensions, and cells were incubated at 37 °C for 30 min, followed by analysis of cell proliferation using a FACSscan flow cytometer (Becton, Dickinson, San Jose, CA, USA). Cell proliferative index (PI) is the sum of the S and G2/M phase activities of the cell cycle expressed as a fraction of the total cell population, ie, PI=[(S+G2/M)/(G0+G1+S+G2/M)]×100[6]. Data are representative of 3 independent experiments.

Statistical analysis
Statistical analyses were performed using SigmaPlot 2001 (Systat Software Inc, Richmond, CA. http://www.systat.com). Statistical significance was assessed by comparing the mean±SD using Student’s t test. A P value of <0.05 was considered statistically significant.

Results
HBxΔ127 has a greater capacity to stimulate SREBP-1c relative to wild type
HBx SREBP-1c is able to activate the transcription of lipogenic genes, including fatty acid synthase (FAS), and plays a critical role in cancer cell survival and proliferation. To further identify the role of SREBP-1c in the promotion of hepatoma cell growth mediated by HBxΔ127, we examine the effect of HBxΔ127 on the regulation of SREBP-1c in more detail. The data show that both wild type HBx and HBxΔ127 are able to upregulate SREBP-1c at the level of mRNA, protein and promoter activity of SREBP-1c in HepG2-X (or H7402-X) and HepG2-XΔ127 (or H7402-XΔ127) cells, as indicated by RT-PCR (Figure 1A), immunoblot analysis (Figure 1B) and luciferase reporter gene assay (P<0.01, Student’s t test, Figure 1C, 1D). However, HepG2-XΔ127 (or H7402-XΔ127) cells exhibit higher expression levels of SREBP-1c, compared to HepG2-X (or H7402-X) cells. Interestingly, treatment of cells with pSilencer3.0-X (RNAi that targets HBx mRNA) abolishes the upregulation of SREBP-1c (Figure 1A–1D). As a control, the transfection efficiency of pSilencer3.0-X plasmid is monitored.

Figure 1. HBxΔ127 has a greater capacity to stimulate SREBP-1c relative to wild type HBx. (A, B) HepG2-X (or H7402-X) cells and HepG2-XΔ127 (or H7402-XΔ127) cells were transfected with pSilencer 3.0-X plasmid for 48 h. (A) The mRNA levels of SREBP-1c were detected by RT-PCR. (B) Immunoblot analysis to detect SREBP-1c (top panel) and β-actin protein levels (bottom panel). (C, D) HepG2-X (or H7402-X) cells and HepG2-XΔ127 (or H7402-XΔ127) cells were co-transfected with or without pSilencer 3.0-X plasmid and SREBP-1c-571-Luc-WT. Data are representative of 3 independent experiments. Values represent means±SD. n=3. 3P<0.05, 3P<0.01 (Student’s t test).
by co-transfection with 0.2 µg pEGFP-C2 plasmid (Figure S1, S2, S3). Our data suggest that HBxΔ127 has a greater capacity to activate SREBP-1c relative to wild type HBx.

5-LOX is responsible for the activation of SREBP-1c
We find that pre-treatment of cells with 20 µmol/L MK886, a specific 5-LOX inhibitor, abolishes the increase in upregulation of SREBP-1c at the level of mRNA (Figure 2A), protein (Figure 2B) and promoter activity (P<0.01, Student’s t test, Figure 2C) in HepG2-XΔ127 (or H7402-XΔ127) cells. However, treatment of cells with Indo, a COX-2 inhibitor, does not affect the mRNA expression level of SREBP-1c (Figure 2A). In addition, we find that decreasing 5-LOX levels by treatment of cells with siRNA significantly attenuates the mRNA and protein levels of SREBP-1c in HepG2-XΔ127 cells (or H7402-XΔ127 cells) (Figure 2D), which is consistent with the above observation. Thus, our data suggest that 5-LOX plays an important role in the HBxΔ127-mediated upregulation of SREBP-1c.

HBxΔ127 upregulates the transcriptional activity of FAS
It has been reported that one of the target genes of SREBP-1c is FAS, which is primarily regulated at the transcriptional level. In our study, we find that HBxΔ127 significantly activates the promoter activity of FAS (P<0.01, Student’s t test, Figure 3A, 3B). Furthermore, HBxΔ127 also upregulates the expression level of FAS mRNA in HepG2-XΔ127 (or H7402-XΔ127) cells, as indicated by RT-PCR (Figure 3C). Treatment of cells with RNAi that targets HBx mRNA (pSilencer3.0-X) abolishes the upregulation of FAS that is mediated by HBxΔ127 (Figure 3C). Because SREBP-1 is a major transcriptional regulator for FAS, presumably through binding to the SRE site in the promoter region of FAS, we examined the promoter activity of FAS using FAS-ΔSRE–Luc, a truncated FAS promoter-luciferase reporter that lacks the SREBP binding site (ΔSRE) using a reporter gene assay. Our data show that the luciferase activity of FAS is barely detectable by the pFAS-ΔSRE–Luc promoter reporter (Figure 3A, 3B), indicating that SREBP-1c fails to bind to the FAS promoter due to deletion of the SRE.

HBxΔ127 enhances hepatoma cell growth through FAS
Here, we examine the role of SREBP-1c and FAS in the HBxΔ127-dependent increase in proliferation of hepatoma cells that we described previously. Following treatment with 10 µg/mL cerulenin, a FAS inhibitor, we investigate the

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**Figure 2.** 5-LOX is involved in the upregulation of SREBP-1c mediated by HBxΔ127. (A) HepG2-XΔ127 (or H7402-XΔ127) cells were incubated with MK886 (an inhibitor of 5-LOX) or Indo (an inhibitor of COX-2) at the indicated concentrations, and the expression of SREBP-1c mRNA was determined by RT-PCR. (B) The expression of SREBP-1c protein was determined by Western blot analysis in HepG2-XΔ127 (or H7402-XΔ127) cells that were treated with or without MK886 (5, 10 and 20 µmol/L) for 6 h. (C) The promoter activity of SREBP-1c was determined in HepG2-XΔ127 (or H7402-XΔ127) cells that were treated with or without MK886 (5, 10 and 20 µmol/L) for 6 h by luciferase reporter gene assay. Data are representative of 3 independent experiments. Values represent means±SD. n=3. *P<0.01 vs control (Student’s t test). (D) HepG2-XΔ127 (or H7402-XΔ127) cells were transfected with 100 nmol/L 5-LOX siRNA or control siRNA for 48 h, and the mRNA and protein levels of SREBP-1c were then detected.
effect of FAS activity on the proliferation of HepG2-XΔ127 (or H7402-XΔ127) cells by flow cytometry analysis. The results show that inhibition of FAS significantly decreases cell proliferation of HepG2-XΔ127 (or H7402-XΔ127) cells, as indicated by the percentage of cells in S phase and PI values ($P<0.05$ vs control, Student’s $t$ test, Figure 4), which suggests that FAS is involved in the HBxΔ127-mediated increase in hepatoma cell growth.

**Discussion**

Although expression of HBx is detected in almost all HBV-infected hepatocytes, there are a small percentage of HBV-infected patients develop HCC. A number of studies have reported that a frequently occurring HBx mutation is the deletion of the COOH-terminus in tissue samples of patients with HCC\(^3\)\(^-\)\(^5\). These data suggest that COOH-terminal truncations of HBx may play a critical role in hepatocarcinogenesis. Ma, *et al* proposes that full-length HBx contains two functional domains: the oncogenic domain (the NH\(_2\)-terminal domain) and the proapoptotic domain (the COOH-terminal domain). Since there is a balance between these two opposing functions, a COOH-terminal deletion may alter the balance between

**Figure 3.** Chemical structure of AVE8134 HBxΔ127 upregulates the transcriptional activity of FAS. The transcriptional activity of FAS in HepG2-XΔ127 (A) and H7402-XΔ127 (B) cells was detected by the luciferase reporter gene assay. The data are representative of 3 independent experiments. Values represent means±SD. $n=3$. $P<0.01$ (Student’s $t$ test). (C) The expression level of FAS mRNA was detected by RT-PCR, which was abolished by RNAi targeting HBxΔ127 mRNA using pSilencer3.0-X plasmid.

**Figure 4.** HBxΔ127 enhances hepatoma cell growth through FAS. FACS analysis shows the proliferation index (PI) and S phase fraction of HepG2-X△127 cells (A), HepG2-X△127 cells treated with 10 μg/mL cerulenin (B), H7402-X△127 cells (C), and H7402-X△127 cells treated with 10 μg/mL cerulenin (D). Data are representative of 3 independent experiments.
HBx functional domains in the regulation of cell proliferation and apoptosis, viability, and transformation. Truncated HBx has been shown to effectively increase the tumorigenicity of HepG2 and MIHA cells[23]. The studies have also provided evidence that HBx mutants with a COOH-terminal deletion increase cell proliferation, relative to wild type HBx protein[7–9]. Furthermore, a comparison of expression profiles between COOH-terminally truncated HBx and wild type HBx using cDNA microarrays showed that 5 genes involved in increasing cell proliferation were upregulated, while 3 genes that decrease cell proliferation were downregulated. These data support the hypothesis that a COOH-terminal truncation of HBx plays a critical role in promoting cell proliferation[23]. Our previous study demonstrated that HBxΔ127 played a key role in regulating transcriptional activity and controlling cell viability and proliferation[8]. In the present study, we focus on investigating the underlying mechanism that contributes to the cell growth mediated by HBxΔ127.

Wild type HBx protein induces the activation of lipogenic transcription factor SREBP-1c[23–25]. Recently, we have reported that HBxΔ127 is able to promote cell growth, which involves the activation of FAS[18]. Thus, in the present study we hypothesize that SREBP-1 may also be involved in the promotion of cell growth that is mediated by HBxΔ127. To that end, we examine the effect of HBxΔ127 on the expression of SREBP-1c. Our results demonstrate that the expression of SREBP-1c is upregulated at the level of promoter, mRNA and protein in hepatoma HepG2-X (or HCC H7402-X[33]) and HepG2-XA127 (or H7402-XA127) cells (Figure 1). These data suggest that the effect of HBxΔ127 on the regulation of SREBP-1c is similar in both hepatoma and HCC cell lines. However, HepG2-XA127 (or H7402-XA127) cells exhibit higher expression levels of SREBP-1c, compared to HepG2-X (or H7402-X) cells (Figure 1), which suggests that HBxΔ127 has a greater ability to enhance cell growth, compared to wild type HBx, through SREBP-1c activation. These data are consistent with our previous study, which report that HBxΔ127 is more sensitive than wild type HBx in the transactivation and promotion of cell growth[8].

Using cDNA microarrays, Liu et al demonstrated that the expression profile mediated by HBx with a truncated COOH-terminus was quite different than that of wild type HBx, where most of the differentially expressed genes are involved in transcriptional regulation, oncogenesis, cell junction maintenance, signal transduction, metabolism and the immune response[23]. Furthermore, HBx can function by interacting with cellular proteins and signal transduction pathways. The majority of HBx-interacting proteins are transcription factors, such as AP-1, NF-kB, ATF/CREB, and C/EBP[27–30]. HBx protein also binds to the TATA-binding protein TFIIB and subunit 5 of RNA polymerase II[31, 32], which are likely the targets of HBx in transcription regulation. Importantly, most of these proteins bind to HBx through a common region that overlaps the COOH-terminus. Therefore, the COOH-terminal truncation of HBx would result in a loss of function with respect to these binding proteins. We propose that the loss of protein binding function may be related to the alteration of wild type HBx, which may explain why this mutant has higher expression levels of SREBP-1c, relative to wild type HBx.

We next examine the mechanism involved in the upregulation of SREBP-1c that is mediated by HBxΔ127. It has been reported that cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) are overexpressed during multistage tumor progression in many neoplastic disorders, including lung, breast, pancreatic cancers and HCC with an integrated HBx gene in host hepatocytes[33]. Several studies have confirmed that metabolites of 5-LOX are able to enhance cell proliferation and increase cell survival[34]. A recent study shows that the expression levels of FAS, COX-2, and 5-LOX plays important roles in mediating breast cancer progression[35]. According to our previous report[13], we present the hypothesis that arachidonic acid metabolism and SREBP-1c expression may contribute to the high proliferation of hepatoma cells that is mediated by HBxΔ127. Interestingly, we find that treatment of cells with 20 μmol/L MK886 (a specific inhibitor of 5-LOX) and 5-LOX siRNA abolishes the upregulation of SREBP-1c at the level of promoter, mRNA and protein in HepG2-XA127 (or H7402-XA127) cells (Figure 2). However, treatment of cells with Indo fails to affect the mRNA level of SREBP-1c (Figure 2A), which suggests that 5-LOX, rather than COX-2, is responsible for the HBxΔ127-mediated upregulation of SREBP-1c.

Several studies have reported that FAS expression is primarily regulated at the transcriptional level by SREBP-1c[36]. The level of fatty acid synthesis is low in normal tissues, but it is high in common human tumors. The preferential expression of FAS in cancer cells has recently been exploited as a target for anticancer chemotherapy. For example, an inhibitor of FAS significantly repressed human breast and prostate tumor[37]. It is recently reported that FAS expression in LNCaP prostate cancer cells is markedly elevated by androgens in an indirect pathway that involves SREBP[38]. In addition, it has been reported that SREBP-1 is involved in the regulation of FAS in tumor cells[39]. Since HBxΔ127 mediates SREBP-1c expression, we investigate the effect of HBxΔ127 on the regulation of FAS in hepatoma cells. Our data demonstrate that HBxΔ127 upregulates the expression of FAS at the level of transcriptional activity and mRNA (Figure 3). In addition, we demonstrate that SREBP-1c fails to bind to the SRE region of the FAS promoter due to the deletion of the SRE in the pFASΔSRE-Luc plasmid (Figure 3A, 3B). Thus, we conclude that HBxΔ127 is able to indirectly activate the transcriptional activity of FAS through the SREBP-1c-FAS pathway. Furthermore, we use flow cytometry analysis to demonstrate that 10 μg/mL cerulenin (an inhibitor of FAS) can significantly decrease the cell proliferation of HepG2-XA127 (or H7402-XA127) cells (Figure 4), which is consistent with similar results in human breast and prostate cancer[37].

In summary, we conclude that HBxΔ127 is able to enhance the growth of hepatoma cells via activation of SREBP-1c in a 5-LOX-dependent mechanism of action. Our findings provide new insight into the mechanism involving the promotion of HBxΔ127-mediated hepatoma cell growth.
Acknowledgments
This work was supported by grants from the National Basic Research Program of China (No 973 Program, No 2007CB914804, No 2007CB914802, No 2009CB521702) and the National Natural Science Foundation of China (No 30670959).

The authors thank Dr Q LIU (University of Saskatchewan, Canada) for providing the plasmids: pFAS-WT-Luc, pFAS-ΔSRE-Luc, and SREBP-1c-571-Luc-WT.

Author contribution
Xiao-dong ZHANG and Li-hong YE designed research; Qi WANG and Wei-ying ZHANG performed the research, analyzed data and wrote the paper.

Abbreviations
HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein; SREBP-1c, sterol regulatory element binding protein 1c; FAS, fatty acid synthase; AA, arachidonic acid; 5-LOX, 5-lipoxygenases; COX, cyclooxygenase; Indo, indomethacin

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