

Tumor LXR Expression is a Prognostic Marker for Patients with Hepatocellular Carcinoma

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Received: 30 September 2016 / Accepted: 27 April 2017 / Published online: 16 May 2017
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Abstract Liver X receptor (LXR) activation exerts an anti-tumor effect. However, whether the tumor LXR expression has prognostic significance in hepatocellular carcinoma (HCC) patient has not been addressed yet. Primary HCC and the adjacent non-tumor tissues were obtained from 169 patients who underwent routine curative surgical treatment. All patients were followed for prognosis analyses. Tumor LXR was detected by immunohistochemical analysis. In *in vitro* study, several HCC cell lines were cultured for cellular protein detection of LXR and other cytokines, including nuclear factor kappa (NFκB), Matrix metalloproteinases 2 and 9 (MMP-2 and -9). Meanwhile, the invasion ability of cultured HCC cell lines was performed. We found that LXR expression status in tumor samples is associated with the clinical characteristics, such as tumor stage and metastasis, of HCC patients. Prognosis analysis shows that tumor LXR expression status is closely related to the post-operative outcome in HCC patients who underwent surgical treatment. Patients with low LXR expression have a significantly lower mean 5-year overall survival rate and mean overall survival period than those with high LXR level. Our *in vitro* data reveal that HCC cell lines

had increased NF-κB, MMP2, MMP9 and invasive ability than normal cell line, which are suppressed by LXR activation via NFκB pathway. Our data suggest that LXR could be used as a biomarker for HCC prognosis. Further study is warranted to explore the molecular mechanism under which LXR regulates tumor behaves.

Keywords Liver X receptor · Hepatocellular carcinoma · Prognosis · Invasion

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies with poor prognosis [1] [2] [3]. Many HCC patients developed early recurrence or metastasis after surgical resection. The traditional serum marker for HCC, such as alpha-fetoprotein (AFP), does not meet the clinical need due to its low sensitivity and specificity. Therefore, the identification of new markers with higher sensitivity and specificity remains important.

Liver X receptor (LXR) belongs to the nuclear receptor family of ligand-dependent transcription factors and regulates cholesterol, glucose, fatty acid metabolism, and inflammatory responses in mammal cells [4]. Previous studies reported that LXR activation exerts an anti-tumor effect on breast, prostate, ovarian, lung, skin, and colorectal cancer cells, suggesting that LXR is a potential target in cancer prevention and treatment [5] [6] [7] [8]. A recent study revealed that LXR mediates hepatitis B virus X protein induced lipogenesis in hepatitis B virus-associated hepatocellular carcinoma; and there is a significant increase in the expression of LXR in HCC in comparison with adjacent non-tumorous nodules in human HBV associated HCC specimens [9]. However, whether the tumor

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LXR expression has prognostic significance in HCC patient has not been addressed yet.

Methods

Tissue Samples

Primary HCC and the adjacent non-tumor tissues were obtained from 169 patients who underwent routine curative surgical treatment at our hospital from May 2009 to May 2014. HCC was diagnosed by ultrasonography, computed tomography, and confirmed by liver biopsy. Among these patients, 114 patients were infected with HBV and 55 patients with HCV. Patients who had received radiotherapy or chemotherapy prior to surgery were excluded. Patient's information including gender, age, tumor stage, size, cirrhosis and metastasis status were collected from medical charts. These patients were followed up after discharger from hospital. Overall survival (OS) was defined as the interval between treatment and death or last observation of surviving patients.

The study protocol was approved by the institute ethics committee, and informed consent was acquired from all patients and donors before the study commenced.

Immunohistochemical Analysis

Immunohistochemical (IHC) staining was performed using Dako Envision system (Dako, Carpinteria, CA) according to the manufacturer's instructions. Briefly, 4 μ m formalin-fixed paraffin-embedded sections were deparaffinized, followed by antigen retrieval by heating sections in a microwave oven. The sections were then incubated with rabbit polyclonal anti-LXR α antibody (1:100; Millpore Inc. USA) overnight at 4 °C. After sequential incubation with biotinylated anti-rabbit secondary antibody and streptavidin-horse-radish peroxidase, reaction product was developed with Diaminobenzidine (DAB). Negative control was composed of mixture with no primary antibody but normal goat serum instead. Two pathologists unaware of study protocol were invited to score the slides independently.

Immunoreactivity was scored for the extent and intensity of the nuclear staining. Extent of staining was scored as follows: 0, no positive cells; 1, < 25% positive cells; 2, 25–50% positive cells; 3, 50–75% positive cells and 4, > 75% positive cells. Intensity was scored as follows: 0, no positive staining; 1, weak staining; 2, moderate staining and 3, strong staining. Multiplying extent by intensity gave the LXR staining scores from low expression (0–6) to high expression (> 6) accordingly.

Cell Culture and Treatment

Several HCC cell lines, including HepG2, SMMC-7721, Bel-7404, and human hepatocyte cell line HL-7702, were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Cells were treated with LXR activator GW3965 (20 μ M for 6 h; Invitrogen, Carlsbad, USA) or NF κ B inhibitor, BAY 11–7082 (5 μ M, 6 h).

Protein Extraction and Western-Blotting Analysis

After siRNA transfection, cells were harvested in RIPA lysis buffer (Thermal Scientific inc. USA). Proteins (10 μ g) were separated by SDS-PAGE, transferred to Hybond-C Extra membranes (Amersham, UK), and immunoblotted using antibodies against human LXR α , NF κ B, Matrix metalloproteinases 2 and 9 (MMP-2 and -9), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (all from Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Invasion Assay

Cells were incubated in the upper chambers of 24-well transwell plates (Corning Incorporated, New York, NY, USA), which were coated with 50% Matrigel (BD Biosciences, USA). After 18 h incubation, invaded cells were stained with 0.5% crystal violet, examined by bright field microscopy (OLYMPUS, Japan), and photographed. Invasion rate was quantified by counting the invaded cells in five random fields per chamber under the fluorescence microscope.

Statistical Analysis

Statistical analyses were performed using a statistical software package (SPSS19.0, Chicago, IL). The chi-square test was used to analyze the relationship between tumor LXR α expression and clinical characteristics. Survival times were evaluated using the Kaplan-Meier survival curves, and compared by the log-rank test. The significance of various variables for survival was analyzed by multivariate survival analysis using Cox's regression model. *P* value less than or equal to 0.05 was considered to be statistically significant.

Results

LXR α Expression in HCC Tissues

Our immunohistochemical analysis show that LXR α is predominantly expressed in nuclei. LXR α expression level in

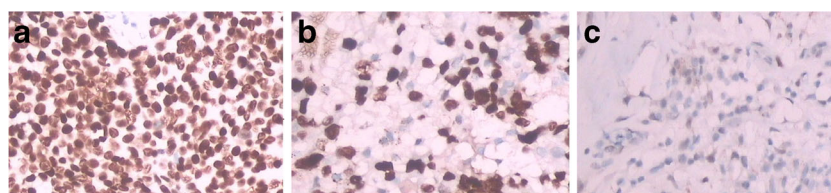


Fig. 1 Typical immunohistochemical analysis for LXRA. LXRA was predominantly expressed in nuclei detected by LXRA immunohistochemical analysis. LXRA expression is high in normal

noncancerous tissues (Fig. 1a.), but is much lower in HCC sample (Fig. 1b). Typical images for high LXRA expression in HCC sample; and Fig. 1c. for low LXRA expression in HCC sample)

HCC patient tissues are high in normal noncancerous tissues, but significantly low in HCC HCC samples (Fig. 1). Representative images of LXRA staining in HCC patient tissues and adjacent noncancerous tissues are shown in Fig. 1. Of the 169 HCC patients, 104 patients had staining scores lower than 6, while 65 had staining scores over or equal to 6. The LXRA expression levels was not related to age, sex, tumor size and cirrhosis, but correlated to tumor stage and metastasis (Table 1).

Correlation of LXRA Expression with HCC Overall Survival Rate

The HCC patients were followed for up to 60 months. The 5-year overall survival rates for patients with low and high LXRA expression were 32.4% and 45.7%, respectively ($P = 0.001$, Fig. 2). The overall survival period of the patients with low LXRA expression was significantly lower than that

of high LXRA expression group (19.5 ± 3.5 vs. 25.7 ± 3.6 , month, $P < 0.001$ by log rank analysis).

We next employed the Cox proportional hazards mode to determine the effects of the independent factors, including tumor LXRA expression status, gender, age, tumor size, serum AFP, tumor size, liver cirrhosis, stage, tumor metastasis, and tumor differentiation. Univariate Cox proportional hazards mode screened that LXRA expression, serum AFP, tumor size, tumor metastasis and TNM stage as possible prognostic factors of survival (Table 2). In the multivariate Cox proportional hazards mode, LXRA and tumor metastasis were identified to have significant correlation with clinical outcome of HCC patients.

Our in vitro data show that all HCC cell lines had a dramatically lower LXRA expression level compared to normal liver cell line HL-7702 (Fig. 3a), which is consistent with what we observed in HCC patients.

Contrast to the reduced LXRA expression, our western blot assay show that HCC cell lines had considerable increases in cytokines related to tumor invasiveness, including NF- κ B, MMP2 and MMP9, than normal cell line. We noted that

Table 1 The association between LXRA expression status and clinical features of HCC patients

Feature	Low LXRA (N = 104)	High LXRA (N = 65)	P value
Gender			
Male	81	50	0.514
Female	23	15	
Age			
≥ 55	79	49	0.537
< 55	25	16	
Cirrhosis			
Yes	81	51	0.514
No	23	14	
TNM stage			
I-II	40	53	< 0.001
III-IV	64	12	
Metastasis			
Present	62	20	< 0.001
Absent	42	45	
Tumor size			
≥ 5 cm	14	13	0.18
< 5 cm	90	52	

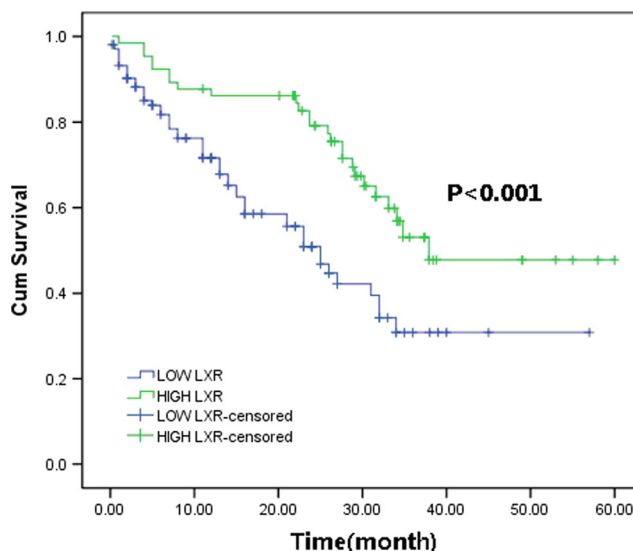


Fig. 2 Prognosis analyses in HCC patients by using Kaplan-Meier curves and log rank tests. The 5-year overall survival rates for patients with low and high LXRA expression were 32.4% and 45.7%, respectively ($P = 0.001$, Fig. 2). The overall survival period of the patients with low LXRA expression was significantly lower than that of high LXRA expression group (19.5 ± 3.5 vs. 25.7 ± 3.6 , $P < 0.001$ by log rank analysis)

Table 2 Cox regression analysis of patients with HCC

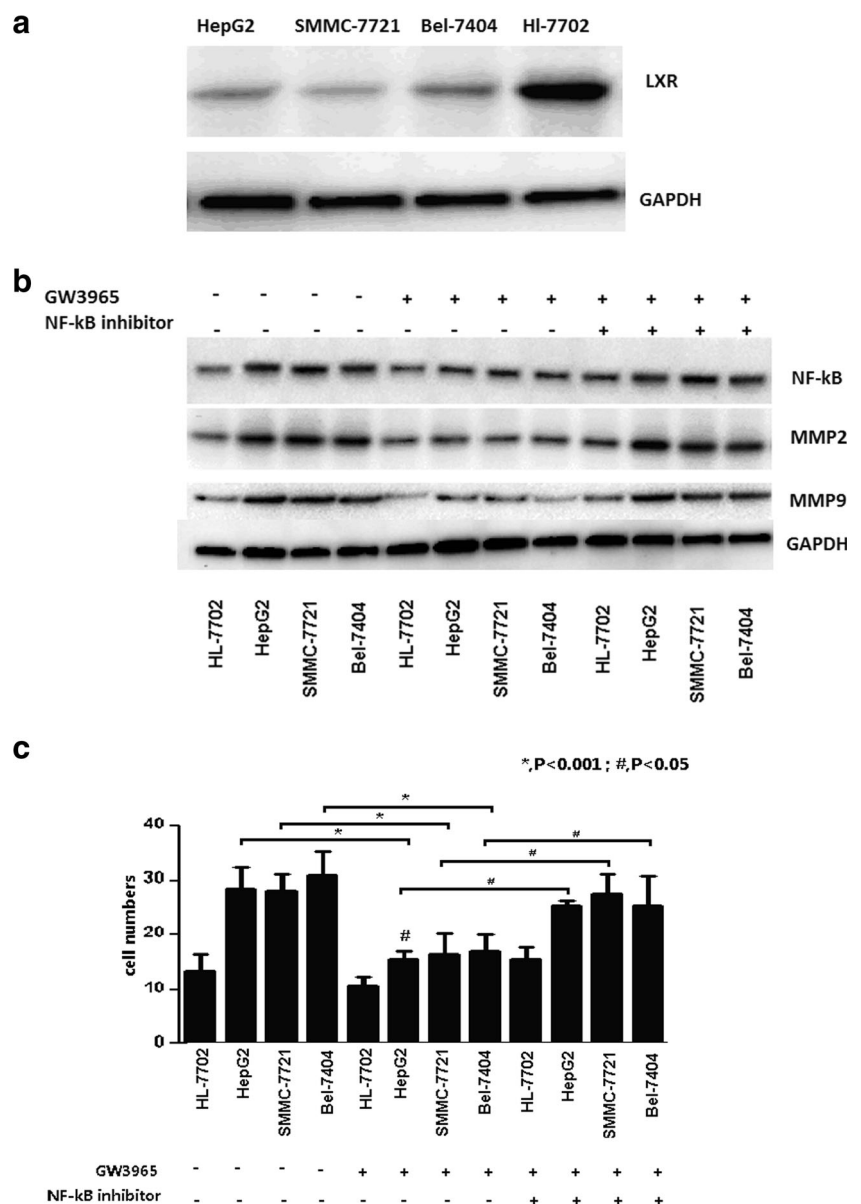
Variables	Univariate		P value	Multivariate		P value
	HR	CI (95%)		HR	CI (95%)	
LXR	0.345	0.125 ~ 0.675	<0.001	0.387	0.176–0.623	<0.001
Serum AFP	2.113	1.275 ~ 4.045	0.009	1.615	0.869 ~ 3.789	0.069
Cirrhosis	1.623	1.545–3.454	0.015	1.453	0.786–3.443	0.058
Metastasis	3.336	2.412 ~ 6.456	<0.001	2.987	2.001 ~ 6.012	<0.001
TNM stage	1.489	1.021 ~ 3.982	0.027	1.332	0.887 ~ 3.775	0.052

LXR activation by GW3965 significantly decreased NF-κB, MMP2 and MMP9 levels (Fig. 3b). When these cells received co-incubation of GW3965 and NFκB inhibitor, we observed the NFκB inhibitor abrogate effect of GW3965 on MMP2 and

MMP9, suggesting NFκB is involved in the pro-invasive effect of LXR activation.

Tumor cell invasion assays showed that the numbers of cells passing through Matrigel in HepG2, SMMC-7721 and

Fig. 3 **a** LXR expression in HCC cells and normal cells by western blot assay. Compared to normal cell line HL-7702, all three HCC cell lines had significantly lower LXR expressions. **b** Western blot assays show that LXR activation induced NF-κB, MMP2 and MMP9 expressions, which was abolished by NFκB inhibitor. **c** Invasiveness assays showed LXR activation by GW3965 treatment significantly reduced the invasive ability of HCC cell lines, while NFκB inhibitor abrogated this effect



Bel-7404 was significantly higher than that in the HL-7702 (Fig. 3b). LXR activation by GW3965 treatment significantly reduced the invasive ability of HCC cell lines. However, Notably, the addition of NF κ B inhibitor abolished the effect of GW3965 on the invasive ability of HCC cell lines (Fig. 3c).

Discussion

In this study, we found that tumor LXRA expression status is associated with the clinical characteristics, such as tumor stage and metastasis, of HCC patients. More importantly, tumor LXRA level is closely related to the post-operative outcome in HCC patients. Patients with low LXR expression have a significantly lower mean 5-year overall survival rate and mean overall survival period than those with high LXR level, suggesting tumor LXRA can be used as a marker for HCC prognosis. Our in vitro data reveal that HCC cell lines had increased NF- κ B, MMP2, MMP9 and invasive ability than normal cell line, which are suppressed by LXR activation via NF κ B pathwa. This finding provides a novel insights into the molecular mechanisms for the anti-tumor effect of LXR.

Accumulating evidences suggest that LXR plays a critical role in cell proliferation and tumor development [10]. Previous reports show that LXR reduces proliferation of human colorectal cancer cells. LXR-deficient mice display increased proliferation in the colonic epithelial, whereas activation of LXR inhibits epithelial proliferation [11]. In human breast cancer cell lines, activation of LXR could significantly inhibit proliferation via PI3K/AKT pathway [5]. It is possible that the altered levels of LXR in different HCC stages differentially impact cancer cell proliferation and thus tumor development.

Despite the anti-tumor activity of LXR has been reported in many studies, the underlying mechanisms and signaling pathways remain elusive. It has been reported that LXR and its ligands could regulate metabolic genes, cell cycle regulatory proteins, hormone signaling, and apoptosis in cells [8]. We demonstrated in this study that LXR activation suppressed the expression of MMP-2 and MMP-9 in HCC cell lines, along with antagonism of the NF κ B, a protein complex that controls transcription of DNA, cell proliferation and cell survival. Treatment with NF κ B inhibitor abolished the LXR-dependent suppression of MMP-2 and -9 expressions. These results suggest LXR may act against cancer at least in part by regulating of MMPs through NF κ B signaling pathway.

MMPs are Zn²⁺-containing endopeptidases that degrade extracellular matrix proteins during normal and pathogenic tissue turnover. Activation of MMPs has been implicated in development, invasion and metastasis of cancer, including HCC [12] [13] [14] [15]. In murine peritoneal macrophages, LXR has been shown to regulate MMP-9 expression via antagonism of the NF κ B signaling pathway [16]. Moreover,

treatment with LXR agonists GW3965 and TO901317 suppressed the levels of MMP-9 in brains after experimental stroke and lead to neuroprotection and reduced neuroinflammation (Morales 2008, circulation). In line with these previous findings, our data confirmed the effect of LXR activation on MMPs repression via NF κ B in HCC cell lines. Future study is required to confirm these results in human.

Several limitations should be addressed. Firstly, the sample size is relative small and enrolled only Chinese patients, thus the conclusion of this study need to be validated with further study with larger sample size. Secondly, we found that LXR mediate MMP2 and MMP9 expression via NF κ B. Further in vitro study is warranted to explore the molecular mechanism mediating the interaction between LXR and NF κ B.

Conclusion

Our data reveal a correlation of LXR expression levels with HCC stage and metastasis and suggest the potential role of LXR as a prognostic marker for HCC patients. Further more, we have linked activation of LXR to NF κ B-dependent suppression of MMPs in HCC cell lines, providing important novel insights into the molecular mechanisms for LXR to inhibit cancer development.

Acknowledgements This study was supported by a grant from Shanxi Medical University.

Author's Contribution HL and QH conceive and design this study. HL, XG and SQ enrolled patients and collected data. QH analyze and interpretation of data, and drafting the article.

GUARANTOR Qingxing Huang.

Compliance with Ethical Standards

Competing Interest None.

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