Upregulation of miR-124-3p by Liver X Receptor Inhibits the Growth of Hepatocellular Carcinoma Cells Via Suppressing Cyclin D1 and CDK6

Dan Zhong, PhD¹, Xilin Lyu, MS¹, Xiaohong Fu, MS¹, Peng Xie, MB¹, Menggang Liu, MD², Fengtian He, PhD¹, and Gang Huang, PhD¹

Abstract
MiR-124-3p has been identified as a novel tumor suppressor and a potential therapeutic target in hepatocellular carcinoma (HCC) through regulating its target genes. However, the upstream regulatory mechanisms of mir-124-3p in HCC has not been fully understood. The transcription factor liver X receptor (LXR) plays a critical role in suppressing the proliferation of HCC cells, but it is unclear whether LXR is involved in the regulation of mir-124-3p. In the present study, we demonstrated that the expression of mir-124-3p was positively correlated with that of LXR in HCC, and the cell growth of HCC was significantly inhibited by LXR agonists. Moreover, activation of LXR with the agonists up-regulated the expression of mir-124-3p, and in turn down-regulated cyclin D1 and cyclin-dependent kinase 6 (CDK6) expression, which are the target genes of mir-124-3p. Mechanistically, miR-124-3p mediates LXR induced inhibition of HCC cell growth and down-regulation of cyclin D1 and CDK6 expression. In vivo experiments also confirmed that LXR induced miR-124-3p expression inhibited the growth of HCC xenograft tumors, as well as cyclin D1 and CDK6 expression. Our findings revealed that mir-124-3p is a novel target gene of LXR, and regulation of the miR-124-3p-cyclin D1/CDK6 pathway by LXR plays a crucial role in the proliferation of HCC cells. LXR-miR-124-3p-cyclin D1/CDK6 pathway may be a novel potential therapeutic target for HCC treatment.

Keywords
liver X receptor, miR-124-3p, liver cancer, cell growth, signal pathway

Abbreviations
CCK-8, Cell Counting Kit-8; CDK6, cyclin-dependent kinase 6; DMSO, dimethyl sulfoxide; HCC, hepatocellular carcinoma; LXR, liver X receptor; LXRE, liver X receptor response element; miRNA, microRNA; qRT-PCR, quantitative real-time polymerase chain reaction; WB, western blot.

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Introduction
MicroRNAs (miRNAs) are ~22-nucleotide-long noncoding RNAs that normally function by inhibiting translation or performing degradation of the target mRNA. Given their participation in the carcinogenesis of liver cancer, they have been introduced as promising therapeutic targets for cancer treatments. MicroRNA-124-3p has been identified as a tumor suppressor in multiple cancers including breast cancer, bladder cancer, gastric cancer, and ovarian cancer. Especially, it was down-regulated in HCC and had a close

¹ Department of Biochemistry and Molecular Biology, College of Basic Medical Sciences, Army Medical University (Third Military Medical University), Chongqing, China
² Department of Hepatobiliary Surgery, Daping Hospital (Army Medical Center), Army Medical University (Third Military Medical University), Chongqing, China

Corresponding Authors:
Fengtian He and Gang Huang, Department of Biochemistry and Molecular Biology, College of Basic Medical Sciences, Army Medical University (Third Military Medical University), Chongqing 400038, China.
Emails: hefengtian66@163.com; cqhuanggang@aliyun.com
correlation with both of the clinical stage and the prognosis of patients with HCC. Moreover, previous studies have confirmed that miR-124-3p alleviates the progression of HCC by inhibiting the expression of its target genes, such as MAPK14, RELA, CDK2, CDK4, and SPI1. Therefore, up-regulating the expression of miR-124-3p should be beneficial in the treatment of HCC. However, the precise mechanism to regulate miR-124-3p expression in HCC is poorly understood. Fan et al reported that the expression of miR-130a-3p was up-regulated by liver X receptor (LXR) in HUVECs and attenuated endothelial barrier dysfunction. However, it is unclear whether LXR can improve the expression of miR-124-3p.

LXR is a member of the nuclear receptor family, it has 2 isoforms, named LXRα (NR1H3) and LXRβ (NR1H2). As a transcription factor, LXR carries out vital cellular functions in cholesterol transport and a variety of malignancies, which makes it become a highly druggable therapeutic target. The synthetic agonists T0901317 and GW3965 can activate LXR, which are most widely used in research. Recently, accumulating evidence demonstrated that LXR was a potential prognostic marker and exerted significant anti-tumor effect in HCC. Furthermore, many genes related with cell proliferation (FOXM1, SOCS3), invasion, and migration in HCC are directly regulated by LXR. However, limited knowledge is available concerning whether LXR affects the proliferation of HCC cells by modulating miR-124-3p, which needs to be studied further.

Previously, our studies revealed that activation of LXR inhibited the proliferation of HCC cells by repressing cyclin D1 expression, and it was reported that miR-124-3p was one of the direct regulators of CDK6 in medulloblastoma. Interestingly, cyclin D1 and its binding partner CDK6 form the cyclin D1/CDK6 active complex, which promotes the progression of the cell cycle. Moreover, cyclin D1 and CDK6 were found to be overexpressed in HCC tissues and accelerated the cell cycle progression of HCC cell lines. Therefore, it is interesting to clarify whether miR-124-3p mediates LXR regulated cyclin D1 and CDK6 expression in HCC.

In the present study, we for the first time demonstrated that the expression of miR-124-3p was positively correlated with that of LXR in HCC, and activation of LXR up-regulated the expression of miR-124-3p. Mechanism investigation revealed that miR-124-3p mediates LXR induced inhibition of HCC cell growth and down-regulation of cyclin D1 and CDK6 expression. Furthermore, LXR induced miR-124-3p expression inhibited the growth of HCC xenograft tumors, as well as cyclin D1 and CDK6 expression in vivo. These results indicate that the LXR-miR-124-3p-cyclin D1/CDK6 axis may be a novel pathway to suppress the proliferation of HCC cells, which may be a new potential target for HCC therapy.

Materials and Methods

HCC Tissues and Adjacent Noncancerous Specimens

Five pairs of HCC tissues and adjacent noncancerous specimens were obtained from the Department of Hepatobiliary Surgery, Daping Hospital, Army Medical University (Chongqing, China). Fresh identified cancer tissues were collected and frozen into liquid nitrogen immediately after taken out of the operating room. Then the extraction of total RNA and reverse transcription of cDNA were operated according to the manufacturer’s instructions.

Reagents

GW3965, T0901317, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was from Dojindo (Kumamoto, Japan). Lipofectamine 2000, Optim-medium, M-MLV, Oligo dT, dNTPs, and DTT were purchased from Invitrogen (Carlsbad, CA, USA). All-in-One miRNA quantitative reverse transcriptase PCR Detection Kit was offered by GeneCopoeia (Guangzhou, China). TRizol was from Invitrogen Life Technologies (Carlsbad, CA, USA). SYBR Green Mix was bought from Takara (Dalian, China). RIPA buffer and BCA kit were provided by Beyotime Biotechnology (Shanghai, China). The protease inhibitor cocktail was from Roche (Switzerland). Anti-CDK6 was purchased from Santa Cruz (Delaware, CA, USA), while Anti-Cyclin D1 was purchased from Abcam (Cambridge, UK) and Anti-z-tubulin was purchased from Zhongshan jinqiao (Beijing, China). Anti-miR-124-3p and anti-miR-NC mimics were synthesized by Sangon Biotech (Shanghai, China).

Cell Culture and Transfection

Human HCC cell lines HepG2 and Hep3B were purchased from American Type Culture Collection (Manassas, VA, USA). The other HCC cell lines including Huh7, PLC, SMMC-7721, and relatively normal hepatic cell line L02 were purchased from China Center for Type Culture Collection (Wuhan, China). All cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco Company, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco Company, Grand Island, NY, USA), penicillin (100 U/mL) and streptomycin (100 mg/mL) from Beyotime Biotechnology (Shanghai, China) at 37 °C in 5% CO2 incubator. Briefly, when HepG2 and SMMC-7721 cells were grown to 85% confluence in 75 cm² flask, they were seeded in 6-well plates and 96-well plates overnight, then the anti-miR-124-3p or anti-miR-NC were transfected into cells using lipofectamine 2000 according to instructions. After 12 hours, the cells were treated with LXR agonists or vehicle control for 24 h, and then the corresponding assays were performed.

CCK-8 Assay

HepG2 cells were seeded in 96-well plates and cultured overnight, then the cells were treated with LXR agonist T0901317, GW3965, and DMSO for 48 h, respectively. Where after, 10 μL/well of cell counting kit-8 (CCK-8) reagent was mixed into the plate and incubated at 37 °C for 30 min to 1 h. Then
measured the absorbance at 450 nm (OD 450) of formazan dye by a microplate reader (Molecular Devices, Sunnyvale, CA, USA), which represented the survival of HCC cells. The OD 450 value of test group was normalized with that of the control group. Each experiment was done in triplicate.

**Quantitative Real-Time PCR (qRT-PCR) Assay**

Total RNA was extracted according to the manufacturer’s instructions of Trizol (Invitrogen Life Technologies, CA, USA). After that, reverse transcriptase M-MLV was used to synthesize the first-strand cDNA. β-actin was used as an internal control for mRNA determination. The expression of miR-124-3p was examined with All-in-One miRNA quantitative reverse transcriptase PCR Detection Kit (GeneCopoeia, Guangzhou, China) under the guidance of protocol and normalized with U6 small nuclear RNA (U6 snRNA). Relative expression was calculated with normalization to β-actin or U6 values by using the 2^−ΔΔCt method. Reactions were repeated minimum of 3 times.

**Western-Blot Assay**

Cells and tissues were lysed by RIPA buffer and the concentration was measured with BCA kit. The denaturalized proteins were subsequently analyzed using SDS-PAGE and monoclonal antibody against cyclin D1 and CDK6. The α-tubulin was used as a loading control. The HRP labeled goat anti-rabbit and goat anti-mouse IgG were used as secondary antibodies. The enhanced chemiluminescence detection reagents (Pierce, Dallas, TX, USA) were used to detect the signal.

**Knockdown of the Target Genes by siRNAs**

After grown to 80-90% confluence, the HepG2 cells were transiently transfected using lipofectamine 3000 (Invitrogen Life Technologies, CA, USA) with siRNAs. Six hours later, the cells were cultured in DMEM containing 0.5% fetal bovine serum in the presence or absence of LXRs agonists for 36 h. The transfected cells were subsequently analyzed using SDS-PAGE and monoclonal antibody against cyclin D1 and CDK6. The α-tubulin was used as a loading control. The HRP labeled goat anti-rabbit and goat anti-mouse IgG were used as secondary antibodies. The enhanced chemiluminescence detection reagents (Pierce, Dallas, TX, USA) were used to detect the signal.

**In Vivo Experiment**

Those 4-week-old male nude mice were purchased from Laboratory Animal Center and cared under the guidelines and regulations of the Animal Care and Ethics Committee of Army Medical University (Chongqing, China). 2 × 10^6 HepG2 cells in 100 μL phosphate-buffered saline were subcutaneously injected into the right axilla of each nude mouse. The mice were randomly divided into test group and control group when palpable tumors formed in them. The test group was intraperitoneally injected GW3965 (30 mg/kg) every 2 days for 16 days-while the control group was treated with DMSO instead of LXR agonist GW3965. After that, all mice were sacrificed in a peaceful way and the xenograft tumors were harvested. The volume of tumor was calculated according to the formula of volume = width^2 × length × 1/2. The mRNA and protein levels of target genes in xenografts were measured by qRT-PCR and western blot separately.

**Statistical Analysis**

The Statistical Package for the Social Sciences (SPSS) 13.0 was used for assessment. Data are reported as mean ± SD. Student’s t-test was applied to analyze the significance of differences, and the significance is presented as *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

**miR-124-3p Expression is Positively Correlated With LXR in HCC**

To investigate the roles of miR-124-3p in HCC, we first determined the expression of miR-124-3p in HCC tissues and cell lines by qRT-PCR. As shown in Figure 1A, compared with the corresponding adjacent noncancerous tissues, the expression of miR-124-3p was significantly down-regulated in HCC tissues, which was consistent with previous studies and GEO dataset analysis.13,30 Moreover, the level of miR-124-3p was also lower in HCC cell lines (HepG2, Hep3B, Huh7, PLC, and SMMC-7721) than that in the relatively normal hepatic cell line L02 (Figure 1B). Furthermore, the prognostic significance of miR-124-3p expression in HCC was evaluated using the Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service&cancer=pancancer_mirna). As shown in Figure 1C, miR-124-3p high expression was found to be correlated to significantly better overall survival for HCC patients. These results suggested that miR-124-3p performed as a potential tumor suppressor in HCC. Moreover, compared with normal adjacent tissues, the expression level of LXRα was lower in HCC tissues (Figure 1D), which was consistent with the result in the TCGA liver cancer RNA-seq dataset (Figure 1E). Particularly, the expression of LXRα was positively correlated with that of miR-124-3p in HCC tissues (Figure 1F). However, there was no significant difference of LXRβ in HCC tissues (Figure 1G), while its expression was even higher in HCC tissues in TCGA dataset (Figure 1H). Together with our former studies,22,23 we confirmed that LXRα was the key factor as a suppressor in HCC, but not LXRβ. Taken together, these results indicated that LXR might be a potential regulator of miR-124-3p.

**Activation of LXR Upregulates the Expression of miR-124-3p**

To evaluate whether miR-124-3p is up-regulated by LXR in HCC, HepG2 cells were treated with LXR agonist GW3965 or T0901317 at indicated dose for 48 h, respectively. Then the expression of miR-124-3p was measured by qRT-PCR. As shown in Figure 2A-B, the expression of miR-124-3p was up-regulated by GW3965 and T0901317 in a dose-dependent manner. Furthermore, knockdown of LXRα by siRNA
decreased the GW3965 or T0901317 mediated upregulation of miR-124-3p in HepG2 cells (Figure 2C and D). These results demonstrated that LXR was involved in the regulation of miR-124-3p.

To investigate whether miR-124-3p mediates the anti-tumor effect of LXR in HCC cells, we antagonized miR-124-3p by transfecting anti-miR-124-3p mimics in HepG2 cells. As shown in Figure 3A, activation of LXR by GW3965 and
T0901317 dramatically inhibited the growth of HepG2 cells in a dose-dependent manner, respectively. Inhibition of miR-124-3p by anti-miR-124-3p mimics obviously attenuated the GW3965 and T0901317 induced inhibition of HCC cell growth (Figure 3B-C). These data indicated that miR-124-3p was one of the crucial factors in the anti-HCC effect of LXR.

miR-124-3p Mediates LXR Induced Downregulation of Cyclin D1 and CDK6
To further clarify the molecular mechanism of LXR-induced miR-124-3p expression which inhibited the growth of HCC cells, the target genes of miR-124-3p including cyclin D1 and CDK6 were analyzed. GEPIA (Gene Expression Profiling Interactive Analysis) database showed that the expression of cyclin D1 and CDK6 was higher in the HCC tumor tissues than that in the normal tissues, which was contrary to the miR-124-3p expression in HCC. Further experiments revealed that the expression of cyclin D1 and CDK6 was markedly inhibited by miR-124-3p mimics in HepG2 and SMMC-7721 cells (Figure 4B). Moreover, the mRNA levels of cyclin D1 and CDK6 were significantly down-regulated by GW3965 and T0901317 in HepG2 and SMMC-7721 cells in a dose-dependent manner (Figure 4C-F), which is consistent with the protein levels of cyclin D1 and CDK6 (Figure 4G-H). On the contrary, inhibition of miR-124-3p by anti-miR-124-3p abolished the GW3965-mediated suppression of miR-124-3p target genes, such as cyclin D1 and CDK6 (Figure 4I). These results suggested that LXR inhibited the expression of cyclin D1 and CDK6 in a miR-124-3p-dependent manner.
LXR Induced mir-124-3p Expression is Involved in the Suppression of HCC Xenograft in Vivo

The above results clearly elucidated that activation of LXR induced up-regulation of miR-124-3p which played an important role in LXR-mediated inhibition of HCC cell growth in vitro. Subsequently, we detected the effect of LXR on miR-124-3p expression and tumor growth in vivo. Our previous study confirmed that both GW3965 and T0901317 can inhibit the growth of HCC xenograft tumors with similar effects. Therefore, we used GW3965 in the animal model in this study. As shown in Figure 5A-B, treatment with GW3965 dramatically decreased the growth of HCC xenografts in nude mice compared with the vehicle treated group. Consistent with the in vitro results, the expression of miR-124-3p was markedly increased in GW3965 treatment group mice (Figure 5C).

Figure 4. Activation of LXR represses cyclin D1 and cyclin-dependent kinase 6 (CDK6) expression by upregulating miR-124-3p. A. The expression of cyclin D1 and CDK6 was analyzed by using GEPIA database. B. Western blot was used to detect expression of cyclin D1 and CDK6 in HepG2 and SMMC-7721 cells transfected with miR-124-3p mimics. C-F. qRT-PCR was used to analyze the expression of cyclin D1 and CDK6 in HepG2 and SMMC-7721 cells during LXR activation by its agonist GW3965 or T0901317, *p < 0.05, **p < 0.01. G-H. Western blot was used to analyze the expression of cyclin D1 and CDK6 in HepG2 and SMMC-7721 cells during LXR activation by its agonist GW3965 or T0901317. I. Western blot was used to detect expression of cyclin D1 and CDK6 in HepG2 and SMMC-7721 cells treated with indicated LXR agonists and antagonir of miR-124-3p. All data are representative of 3 independent experiments and expressed as the mean ± SD.
Remarkably, this was accompanied by the down-regulation of cyclin D1 and CDK6 expression in the xenografts (Figure 5D). These results suggested that the anti-HCC effect of LXR was through upregulating miR-124-3p, which inhibited the target gene expression of miR-124-3p in vivo.

Discussion

HCC is one of the most common causes of cancer-related death worldwide, with less accurate markers for early diagnosis and efficient treatment. Nucleic acid-based drugs such as miRNAs may have promising therapeutic potential for HCC treatment. MicroRNAs are pivotal participants and regulators in the development and progression of HCC. In this study, we focused on miR-124-3p which was a tumor suppressor in HCC and its decrease resulted in a more aggressive and poor prognostic phenotype of patients with HCC. Interestingly, we found that activation of LXR with its agonists significantly up-regulated the expression of miR-124-3p, and miR-124-3p strongly affected the function of LXR against HCC in return.

In most circumstances, LXR binds with retinoic X receptor (RXR) to form heterodimer after activation, and then the heterodimer LXR/RXR binds to specific DNA sequences called LXR response elements (LXREs) and regulates gene expression. In the present study, we had tried to explore the mechanism by which LXR up-regulated miR-124-3p expression. Unfortunately, online analysis (https://www.nubiscan.unibas.ch/) predicted that there were no potential LXREs in the miR-124-3p gene promoter region. Therefore, miR-124-3p might not be a direct target of LXR. Since several reports revealed that long non-coding RNAs, such as NEAT1 and OGFRP1, could directly bind to miR-124-3p to inhibit its function in HCC, we should determine whether LXR modulates miR-124-3p through long non-coding RNAs in our further study.

In the present study, cyclin D1 and CDK6 were found to be the candidate target genes of miR-124-3p in HCC and participated in the anti-HCC mechanism of “LXR-miR-124-3p” signal pathway. Moreover, it was reported that cyclin D1 and CDK6 were direct targets of miR-206 which was also a tumor suppressor in HCC. Therefore further study is needed to clarify whether miR-206 is involved in the “LXR-cyclin D1/CDK6” signal pathway in HCC. Interestingly, the up-regulation of cyclin D1 and CDK6 accelerated cell cycle progression and cell proliferation was faster than that of CDK6 alone. Therefore, we were excited to find that the elevated miR-124-3p decreased cyclin D1 and CDK6 expression, which meant we revealed a more effective regulator to control cell growth than those controlling only one of these 2 genes. However, further study is still needed to clarify whether miR-124-3p affects the formation of cyclin D1/CDK6 complex.

Growing evidence revealed that miR-124-3p up-regulation decreased the expression of both cyclin D1 and CDK6 in tumors such as malignant glioma and ovarian cancer, but the
underlying mechanism by which miR-124-3p regulates cyclin D1 and CDK6 is unknown. We predicted the potential targets of miR-124-3p in miRbase, Targetscan, and PicTar databases and found that CDK6 has been reported as a direct target of miR-124-3p. However, there is no evidence showed that miR-124-3p can directly bind to cyclin D1. Recent studies showed that miR-124-3p repressed cyclin D1 expression by targeting several genes such as Fra-2, EZH2, AKT3, and several signaling pathways such as Notch pathway, JAK-STAT3, and Wnt/β-catenin. Since we obtained the similar in vitro results via using HepG2 and SMMC-7721, we used only HepG2 to form xenograft tumors. Certainly, the results would be more consummate if both of HepG2 and SMMC-7721 were used to do the xenograft experiments. Whatever, HepG2 should be more consummate if both of HepG2 and SMMC-7721 were used to do the xenograft experiments.

In conclusion, our present study demonstrated that activation of LXR promoted miR-124-3p expression, which significantly inhibited the growth of HCC cells via suppressing cyclin D1 and CDK6 expression. These findings provide new insights into the mechanisms of HCC development, which may be a novel potential therapeutic target for HCC therapy.

Authors’ Note
GH and FTH conceived of the study and carried out its design. DZ, XLL, XHF and PX performed the experiments and the statistical analyses. MGL provided the Human tissues. DZ and XLL wrote the paper. GH and FTH revised the paper. All authors read and approved the final manuscript.

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Author Contribution
Dan Zhong and Xilin Lyu contributed equally to this work.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD
Gang Huang https://orcid.org/0000-0001-6850-940X

Ethics Statement
All procedures involving animals were performed in accordance with the Animal Management Rules of the Ministry of Health of the People’s Republic of China (No. 55, 2007) and the guidelines for the Laboratory Animal Welfare and Ethics Committee of Army Medical University (No. 20170002, Chongqing, China). Human HCC samples were from Daping Hospital, Army Medical University. The study was approved by the ethics committee of Army Medical University (Chongqing, China).

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