Research Article

Hepatitis B Virus X Protein-Induced RORγ Expression to Promote the Migration and Proliferation of Hepatocellular Carcinoma

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Received 8 July 2019; Revised 4 September 2019; Accepted 13 September 2019; Published 3 November 2019

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1. Introduction

The retinoic acid receptor-related orphan receptors (RORs) are members of nuclear receptor superfamily, including three subtypes (RORα, RORβ, and RORγ), which are a class of ligand-dependent transcriptional factors [1]. Previous studies have revealed that RORs have critical roles in the regulation of physiological and pathological processes, such as development, circadian rhythm, and cellular metabolism, especially in the immune response regulation [2–5]. Besides, RORyt is also a member of the ROR family, which is different from RORγ in the N-terminal [6]. RORyt is overexpressed in the thymus, and RORγ is normally expressed in the kidney, liver, skeletal muscles, adipose tissue, pancreas, and so on [7, 8]. The pathogenetic mechanism studies have revealed that RORyt regulates the differentiation of Th17 cells, which is a kind of cells that secrete interleukin 17 (IL-17), a regulator of proinflammatory signaling [9, 10]. Summary of the previous reports confirm the role of RORyt in the regulation of immune-inflammatory effect. However, the study of RORγ in the regulation of signaling pathway remains unclear.

In recent years, the study of RORγ in cancer has received more attention. Muscat and colleagues demonstrated RORγ expression is downregulated in breast tumor tissues RORγ negatively regulates TGFβ/EMT and MaSC pathways, and agonist targeting RORγ could inhibit the migration and proliferation of breast cancer cells [11, 12]. However, its expression was reported to be highly expressed in non-small-cell lung cancer, its expression has a positive correlation with the lymph node metastasis, and the high-expression of RORγ showed a poor prognosis [13], suggesting that RORγ may perform a different function in different cancers. More interestingly, another study in breast cancer revealed that the high RORγ expression represented a low survival rate [14]; therefore, the different functions of RORγ were illuminated in breast cancer. And a similar phenomenon was reported in melanoma. Kupper’s report showed the growth of melanoma cells was inhibited in the RORγ-deficient mice [15]. RORγ expression showed inverse
correlation with melanoma progression [16]. Accordingly, the explanation of different functions in cancers may be due to the tumor microenvironment, and the study of RORγ in cancers is worthy of further research.

Hepatocellular carcinoma (HCC) is a main malignant tumor in the digestive system, contributed to the fifth leading cause of cancer-related death [17]. RORγt was reported to be overexpressed in peripheral blood mononuclear cells of HCC patients [18], and the contribution of RORγ in HCC still has not been reported. In this work, we firstly examined the expression of RORγ in HCC and evaluated the potential mechanism. As hepatitis B virus (HBV) infection is a major risk factor for HCC and hepatitis B virus X protein (HBx) is a major protein in the occurrence and development of HCC [19–21], whether RORγ is involved in the regulation of HBV-related HCC remains unclear. To formulate the hypothesis, we examined the expression of RORγ in the tumor and adjacent tissues and found the hypomethylation of RORγ in the liver tumor, and RORγ expression was further enhanced in the HCC patients with HBV infection. The most important work is that HBx could induce RORγ expression by promoting its transcriptional function. The biological function study demonstrated overexpression of RORγ can enhance the migration and growth activity of liver cancer cells. Our finding provides new insights into the role of RORγ in HCC.

2. Materials and Methods

2.1. Clinical Liver Cancer Tissues and Cell Lines. The liver cancer tissues and corresponding adjacent tissues were collected in the department of hepatobiliary surgery, the Guangxi Zhuang Autonomous Region People’s Hospital, from the patients who have not undergone any treatment, including chemotherapy and radiotherapy. The tissues were used for the analysis of the RORγ expression. The study was approved by the institutional research ethics committee at the Guangxi Zhuang Autonomous Region People’s Hospital (GuangXi Science and Technology-2018-30). All patients signed the written consent.

2.2. Cell Culture. The HepG2, SMMC-7721, HEK293T, and Huh7 cell lines were obtained from ATCC (Rockville, MD, USA). HCCLM3 cells were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM) (GIBCO, USA), supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) with heat inactivation and 100 U/mL penicillin (GIBCO, USA). The cells were cultured in a humidified incubator with 37°C and 5% CO2. The passenger of cell lines was no more than 10 times, and liquid nitrogen was used for the storage of cells.

2.3. Western Blotting. The clinical tissues were homogenized with tissue homogenization treatment and centrifuged at 12000 g/min at 4°C for purification of protein. The cells were lysed with RIPA lysis buffer containing the protease inhibitor cocktail (Yeasen, Shanghai, China) on ice for 30 min. The lysis was centrifuged at 4°C for 15 min, and the supernatant was collected and subjected to BCA assay for protein concentration evaluation. A total of 30 μg protein was subjected to SDS-PAGE and transferred to the PVDF membrane. The membrane was incubated with primary antibody overnight and subjected to the second antibody at room temperature for 1 h. The antibodies are as follows: anti-RORγ (Abcam, Cambridge, USA, ab798007), anti-GAPDH (Proteintech, USA, 60004-1-lg), anti-Myc (Santa Cruz, CA, USA, 9E10), anti-HBx (Abcam, Cambridge, USA, ab203540), and anti-β-actin (Proteintech, USA, 23660-1-AP). The enhanced chemiluminescence (ECL) system (Yeasen, Shanghai, China) was used to visualize the protein band. The Quantity One software (Bio-Rad, CA, USA) was used to quantify protein expression.

2.4. Bioinformatics Analysis of TCGA Database. The Cancer Genome Atlas (TCGA) program for liver cancer [22] was assessed in this study. A total of 59 normal liver tissues and 97 liver tumor tissues were compared in its mRNA levels. The relative expression levels were evaluated by the median-centered intensity method. The methylation analysis was analyzed with 450k methylation array, and the ratio of the methylation levels between liver tumor and corresponding adjacent normal tissues was calculated. And, the median value of methylation level was subjected to those normal liver tissues due to deficiency. Also, the correlation between the methylation level and gene mRNA expression was examined in liver tumor tissues and adjacent normal tissues.

2.5. Oncomine Analysis. The Guichard Liver microarray data were selected from the Oncomine portal [23]. A total of 86 normal liver tissues and 99 liver tumor tissues were analyzed by a reporter (01-150058071), and the data type was mRNA level. Furthermore, 81 cases of liver cancer patients with hepatitis virus infection information were classified into two groups, 48 patients without HBV infection and 33 patients with HBV infection.

2.6. Quantitative Real-Time Reverse Transcription PCR (qRT-PCR). The cells were washed with cold phosphate buffer solution (PBS) twice and collected with a cell scraper. The cells were lysed with TRIzol RNA extraction reagent (Invitrogen, Gaithersburg, MD) for 10 min at room temperature, and trichloromethane was subsequently added. After vortex oscillation, the reaction solution was stored at 4°C for 10 min and centrifuged at 4°C for 10 min. The supernatant was collected and mixed with isopropanol to precipitate total RNA. With 10 min incubation, the solution was centrifuged for 10 min, and the collected RNA was washed with 75% ethanol. The first-strand cDNA was synthesized with SuperScript reverse transcriptase (Invitrogen, MD, USA). The SYBR green-quantitative polymerase chain reaction (PCR) system (TaKaRa, Shiga, Japan) was used to examine the mRNA levels of indicated genes in the StepOne Real-time PCR system (Applied Biosystems, USA). The relative gene expression was calculated by $2^{-ΔΔCT}$. The
primer sequences are as follows: RORy forward, 5′-GTG
GGG ACA AGT CTG TCT G-3′, RORy reverse, 5′-AGT
GCT GCC ATG TTC TGC G-3′, the amplicon size is 156 bp.
GAPDH forward, 5′-AGG TCG GAG TCA ACG GAT TT-
3′, GAPDH reverse, 5′-ATC TCG CTC CTG GAA GAT
GG-3′. GAPDH is a housekeeping gene to normalize the
relative gene expression.

2.7. Luciferase Reporter Assay. The luciferase reporter system
of RORy was conducted as follows. The promoter sequence
of RORy was obtained for Genecopoeia (Rockville, MD,
USA), a web portal for promoter region clones (http://www.
genecopoeia.com) [24]. The detailed RORy promoter se-
quence in this study contains the core promoter sequence
of RORy, which was reported previously [8]. The following
primers were used for amplification: forward primer (up-
stream position-1492), 5′-CCC TCG AGC TCC CCT GCA
CTC CGC CGC C-3′, reverse primer (downstream position
+93), and 5′- CCC AAG TTT GGT GCC GTC CTG GCT
GAPDH C-3′. The sequence was inserted into a pGL3-basic
plasmid (Promega, USA) with XhoI and HindIII restriction
site. The genomic DNA of HepG2 cells was used as a
template to amplify the promoter sequence of RORy. The
HepG2 and Huh7 cells were transfected with HBx-over-
expressing plasmid or siRNA against HBx (Sigma, Saint
Louis, USA), combined with the luciferase reporter plasmid
of RORy and Rellina plasmid. After transfection for 24 h,
the cells were collected and washed with cold PBS twice.
The dual-luciferase reporter system (Promega, USA) was used
to examine the luciferase activity. In detail, the collected cells
were lysed with a reporter lysis buffer for 30 min on ice. After
lysis, the supernatant was transferred into white 96-well
microplates, and the firefly luciferase solution was added,
followed by the addition of Rellina luciferase substrate. The
microplate was then placed into a microplate reader to
examine the luciferase reporter activity.

2.8. Construction of Stable Overexpressing Cell with RORy and
HBx. The full sequence of RORy and HBx was cloned into a
pCDH-CMV-MCS-EF1-Puro vector. The cDNA of HepG2
cells was used as a template for RORy amplification, and
the cDNA of HBx was cloned from a pGFP-HBx plasmid
(Addgene, Cat.No.65463). The primer of RORy is as follows:
forward, 5′- GCT CTA GAA TGG ACA GGG CCC CAC
AGA G-3′, reverse, 5′- CGG AAT TCT CAC TTG GAC
GAC GCC CCC ACA GG-3′, and the restriction sites were XbaI
and EcoRI. Then, the lentivirus plasmid of RORy or HBx
combined with the packaging plasmid pCMV-DR8.91 and
pCMV-VSV-G was transfected into HEK293T cells. For
further incubation with 48 h, the lentiviral particles were
collected, and the particles were condensed with lentivirus
concentration solution. Also, the quality of particles was
evaluated with the viral titer, and the qualified lentiviral
particles were stored in an ultra-low temperature freezer.
Then, the viral particle containing RORy and HBx was
applied to incubate the indicated cells, supplemented with
polybrene at the concentration of 5 μg/mL. The puromycin
selection (Thermo Scientific, Madison, WI, USA) was
carried out for GeneCopoeia (Rockville, MD, USA), a web portal for promoter region clones (http://www.
genecopoeia.com) [24]. The detailed RORy promoter se-
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+93), and 5′- CCC AAG TTT GGT GCC GTC CTG GCT
gapdh C-3′. The sequence was inserted into a pGL3-basic
plasmid (Promega, USA) with XhoI and HindIII restriction
site. The genomic DNA of HepG2 cells was used as a
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lysis, the supernatant was transferred into white 96-well
microplates, and the firefly luciferase solution was added,
followed by the addition of Rellina luciferase substrate. The
microplate was then placed into a microplate reader to
examine the luciferase reporter activity.

2.9. Wound-Healing Assay. The cells were plated in 6-well
plates and cultured for 24 h and then subjected to the
lentiviral transduction particles packing RORy over-
expression and control vector system for 12 h transfection.
The scratches were produced across the cell monolayers with
yellow tips. And the cells were washed with PBS solution for
three times to remove the shedding cells. Then, the cells were
cultured in fresh culture medium for another 48 h, and the
confluence of cells was recorded with PrimoStar microscope
(Zeiss, Jena, Germany). The migration index was calculated
with the ratio of the scratch area between RORy over-
expression and vector cells by Image J software (US National
Institutes of Health, Bethesda, MD, USA).

2.10. CCK-8 Cell Viability Assay. The HepG2 cells were
seeded in 96-well plates at a density of 5000 cells per well,
and the cells were cultured in the indicated culture medium,
supplemented with lentiviral particles of RORy over-
expression and control vector. The cells were cultured for
a different time, and before 3 h of indicated detection time, 10 μL CCK-8 reagent (Yeasen, Shanghai, China) was added
into the cells, and the absorbance value was detected by a
microplate reader (Thermo Scientific, Madison, WI, USA) at
450 nm.

2.11. Colony Formation Assay. HepG2 cells overexpressing
RORy and with vector control cells were seeded in a 3.5 cm
dish (500 cells/dish) and cultured for 2 weeks in DMEM
(containing 10% FBS). After washing twice gently with PBS,
cells fixed with 4% paraformaldehyde and stained with
crystal violet. The number of foci containing ≥50 cells was
calculated at 40X magnification using an optical microscope
(Zeiss, Jena, Germany).

2.12. Statistical Analysis. Data are represented as mean ±
standard deviation (SD), which were acquired in at least
three independent experiments. The statistical significances
of differences were analyzed by using analysis of variance or
Student’s t-test.

3. Results

3.1. Overexpression of RORy in Liver Cancer Patients. A
previous study revealed that RORy has a different regulation
mechanism in different cancers. The study of RORy in liver
cancer has not been reported yet. However, RORy as a
truncated variant was reported overexpressed in peripheral
blood mononuclear cells of HCC patients. Then, we ex-
amined the RORy expression of liver cancer. As seen in
Figure 1(a), compared with 86 cases of normal liver tissues,
RORy mRNA levels were significantly increased in the 99
cases of liver tumor tissues. And a similar result was con-
firmed in the TCGA liver database (Figure 1(b)). To further
confirm the overexpression of RORy in the liver tumor
tissues, 3 pairs of patients with liver cancer in tumor tissues and corresponding adjacent normal tissues were subjected to western blotting; the protein expression of RORγ was also enhanced remarkably (Figures 1(c)–1(d)). These results revealed that RORγ was significantly increased in liver tumor at both protein and mRNA levels, suggesting that RORγ may play a potential role in the occurrence and development of liver cancer.

3.2. Downregulation of RORγ Promoter Methylation Activity in Liver Tumor. In our bioinformatics and western blotting analysis of RORγ in liver cancer, we confirmed that RORγ was overexpressed in the liver tumor tissues. Next, we tried to explore the overexpression of RORγ in liver cancer, as methylation regulation was reported as an important regulatory mechanism for gene expression. Then, we hypothesized that methylation regulation may play an important role in the overexpression of RORγ in liver tumor tissues.

The TCGA analysis of the RORγ promoter methylation activity of liver tumor tissues and normal liver tissues revealed that 62% of liver cancer patients express hypomethylation (Figure 2(a)). To accurately evaluate the promoter methylation levels of RORγ in tumor and normal liver tissues, we selected 49 cases of patients with complete methylation levels both in tumor and corresponding normal tissues. The result showed that the promoter methylation levels of RORγ were notably decreased in the tumor tissues (Figure 2(b)). To directly evaluate the association between methylation levels and gene expression levels of RORγ in liver cancer patients, as the potential correlation between the gene expression and methylation level, we analyzed the correlation of RORγ promoter methylation levels and its mRNA expression of 190 cases of liver tumor tissues and 50 cases adjacent normal liver tissues. The results showed that both in tumor and normal liver tissues, the promoter methylation levels of RORγ were negatively correlated with its mRNA expression (Figures 2(c)–2(d)). Taking this, we...
believed that the promoter methylation regulation might be an important reason for the overexpression of RORc in liver tumor tissues.

3.3. Liver Cancer Patients with HBV Infection Representing Higher RORc Expression. RORc was overexpressed in liver tumor. In order to deeply study the expression of RORc in different subtypes of liver cancers, as hepatitis virus infection is a major risk factor of HCC, especially the hepatitis B virus (HBV) infection widely occurs in Asia, we examined the RORc expression in hepatitis virus infection or negative infection in liver cancer patients. Interestingly, we found the expression of RORc was higher in these liver cancer patients with HBV infection than in those without hepatitis virus infection (Figure 3(a)). And, a similar phenomenon was revealed in the analysis of the TCGA database (Figure 3(b)). These results suggested that hepatitis virus infection might
mediate the RORγ expression through regulation of its coactivators in liver cancer.

3.4. HBx Increased the Expression of RORγ. Considering the higher expression of RORγ in those liver cancer patients with HBV infection, HBV may be involved in the regulation of RORγ. And hepatitis B virus X (HBx) protein is the most important factor of HBV-mediated liver cancer progression. Thus, we hypothesized that HBx may play an important role in the regulation of RORγ. To confirm the hypothesis, HBx-overexpressing plasmid was transfected into the HepG2 cells and examined the expression of RORγ. As the results showed, HBx can increase the protein levels of RORγ in a dose-dependent manner (Figures 4(a) and 4(b)). Next, we also examined the transcriptional activity of RORγ with or without HBx. The RT-PCR result revealed that HBx can enhance the mRNA level of RORγ (Figures 4(c) and 4(d)). These results suggest that HBx may increase the RORγ expression through the promotion of the transcriptional activity.

3.5. Promoter Activity of RORγ Was Activated by HBx. As described earlier in this study, HBx can increase the expression of RORγ in protein and mRNA levels, suggesting that HBx could regulate RORγ through the transcriptional regulation mechanism. To confirm the hypothesis, we conducted the luciferase reporter of RORγ promoter. As Figures 5(a) and B showed, HBx could increase the promoter activity of RORγ in HepG2 and Huh7 cells in a dose-dependent way. Similarly, the promoter activity of RORγ was also enhanced in the stable HBx-overexpressing cells (Figures 5(c) and 5(d)). To further confirm the regulation of HBx on the promoter activity of RORγ, The HepG2-HBx and Huh7-HBx cells, which were stably overexpressing HBx, were subjected to HBx siRNA, and the efficiency of knockdown was confirmed at the protein level by western blotting (Figure 5(e)). Furthermore, we observed the luciferase activity of RORγ promoter was significantly decreased in a dose-dependent manner (Figures 5(f) and 5(g)). Thus, HBx could activate the promoter activity of RORγ. Taken these results, RORγ was overexpressed in liver tumors, and HBx could increase RORγ expression, we believed that HBx can increase RORγ expression through activation of promoter activity.

3.6. RORγ Promotes the Migration of Liver Cancer Cells. RORγ was overexpressed in liver tumor tissues, and the RORγ expression was mediated by the HBx, an important oncogene in the development of liver cancer; these data suggest that RORγ may act as a cancer promoter in liver cancer. To further validate the function of RORγ, RORγ was detected in HepG2, Huh7, SMMC7721, and HCCLM3 cells. And HCCLM3 represented the highest expression of RORγ (Figures 6(a) and 6(b)), and as the previous report, among the four cell lines, HCCLM3 was the most malignant grade with higher migration activity [25]. Thus, whether RORγ has a potential role in the migration process of liver cancer cells is unclear. Then, HepG2 cells were selected to the next study. The lentiviral particles of overexpressing RORγ and control vector were confirmed with RT-PCR (Figure 6(c)), and the effect of RORγ on the migration activity of liver cancer cells was evaluated by wound-healing assay. The results showed that overexpression of RORγ could significantly promote the migration of HepG2 cells after incubation with lentiviral particles for 24 h. And for another 24 h culture, the migration index was further enhanced (Figures 6(d) and 6(e)).

3.7. RORγ Enhances the Proliferation Activity of Liver Cancer Cells. RORγ has a potential role in the migration activity of liver cancer.
Figure 4: HBx can upregulate the expression of RORγ. (a, b) The HepG2 cells were transfected with Myc-HBx plasmid in increased concentration, and western blotting was subjected to analyze the protein expression of RORγ. (c, d) HepG2 cells were transfected with HBx-overexpressing and vector plasmids, and the mRNA levels of RORγ were examined with RT-PCR. The Quantity One software was used for quantitative analysis. The data are expressed as mean ± standard deviation (SD), n = 3. **P < 0.01 was considered statistically significant.

Figure 5: Continued.
liver cancer cells, and the effect of RORγ on the proliferation activity was examined. As Figure 7(a) revealed, cell viability showed no significant difference after treatment with RORγ-overexpressing lentiviral particles for 12 h. After treatment with the above for 24 h, cell viability was enhanced by overexpression of RORγ (Figure 7(a)). To confirm the effect of RORγ on the regulation of proliferation in liver cancer cells, the colony formation assay was used in this study. The results showed the colony number was increased after overexpression of RORγ. Thus, the cell viability and colony formation assay both represented that RORγ accelerated the proliferation activity of liver cancer cells.

**Figure 5**: HBx can increase the promoter activity of RORγ. (a) HepG2 and (b) Huh7 cells were transfected with pGL3-RORγ, complemented with HBx plasmid. After 24 h transfection, the cells were subjected to analyze the reporter activity with the luciferase reporter system. (c) The stable overexpressing HBx cells and the vector controls of HepG2 and (d) Huh7 cells were transfected with pGL3-RORγ reporter plasmid and Rellina plasmid, and the luciferase activity was detected by the dual-luciferase reporter system. (e) The knockdown efficiency of HBx was assessed in the HBx-stable overexpressing cells of HepG2 and Huh7. (f) The stable HBx-overexpressing HepG2 and (g) Huh7 cells were transfected with pGL3-RORγ reporter plasmid and cotransfected with siRNA against HBx. With 24 h culture, the promoter activity of RORγ was detected by the luciferase reporter value. The data are expressed as mean ± standard deviation (SD), n = 4. **P < 0.01, ***P < 0.001 were considered statistically significant.
4. Discussion

The current study of RORγ in cancers remains still unclear. RORγ was reported as an oncogene in non-small-cell lung cancer and melanoma cancer [13, 15]. However, RORγ was also described as a tumor suppressor gene in breast cancer, suggesting that RORγ performs a different function in different organs [11, 26]. But the recent study also revealed that even in the same type of cancer, RORγ was reported to perform a different function. Considering RORγt as an important regulator in the immune regulation network, the immune system was a crucial aspect of the tumor microenvironment. Similarly, RORγ is different from RORγt at the N-terminal, and RORγ is also selectively expressed in the Th17 cells and involved in the control of Th17 differentiation [27]. These contribute to the different functions in cancers. Up to date, about the function of RORγ in liver cancer was not reported yet. In this study, we intended to study the expression of RORγ and its significance in liver cancer cells. The previous study showed that RORγt was highly expressed in peripheral blood lymphocyte of liver cancer patients [18], RORγ as a similar isoform, the expression and its significance have not been reported, and then we explored the expression of RORγ in liver cancer. The bioinformatics analysis and western blotting showed that RORγ was highly expressed in the liver tumor tissues compared with the adjacent normal liver tissues (Figure 1). More interestingly, we firstly found that the methylation levels of RORγ in liver tumor tissues were downregulated compared with the normal liver tissues (Figures 2(a) and 2(b)). Similar to the current study, the regulation of methylation in the gene expression was an important method [28]. Also, the correlation analysis confirmed that the overexpression of RORγ in the liver tissues was due to its hypomethylation levels of the promoter (Figures 2(c) and 2(d)), as numerous studies have revealed that the aberrant DNA methylation was deeply involved in the occurrence and development of some cancers [1, 29, 30]. In this study, we showed that the aberrant expression of RORγ in liver cancer tissues and the methylation regulation contributed to the overexpression of RORγ in liver tumor tissues.
As hepatitis B virus (HBV) infection is a major risk factor of liver cancer [31], hepatitis B virus X protein (HBx) is a major protein for HBV-related liver cancer [32]. And the former study revealed that RORc may be involved in the development of liver disease [33]. To evaluate the correlation between HBx and RORc, we test the correlation between both the molecules. The results showed RORc expression was further increased in the HBV-positive liver cancer tissues (Figure 3). And overexpression of HBx in liver cancer cells, the expression of RORc was significantly increased in both protein and mRNA levels (Figure 4). The transcriptional regulation is an essential manner for gene levels. Then, we hypothesized that HBx could promote the transcriptional activity of RORc. And the results also confirmed our hypothesis. Furthermore, to confirm the results, we also knockdown HBx in the HBx-stable expressing cells (Figure 5). And these results demonstrated that RORc was a novel regulator in HBV-positive liver cancer. However, given that HBx was a coactivator of transcription regulation, HBx could activate some signaling pathways, so it is an important direction to study the regulation of HBx on RORc in further studies. Another important question is that the methylation level of RORc promoter was downregulated in the liver tumor tissues, and the promoter methylation regulation might contribute to the overexpression of RORc in liver tumor tissues, and we also reported HBx mediated the RORc expression. Previous reports show that HBx can regulate some genes via DNA methylation, especially in the methylation of the promoter [34, 35]. Then, whether HBx could affect the promoter methylation of RORc needs further evaluation.

To confirm the significance of RORc in liver cancer, we evaluated the function of RORc in the regulation of migration and proliferation activity in liver cancer cells. The wound-healing assay revealed that overexpression of RORc could enhance the migration activity of liver cancer cells (Figure 6). Furthermore, the cell viability and colony assays indicated that RORc has positive regulation in the process of proliferation (Figure 7). These functional tests initially evidenced that RORc performed as an oncogene in the liver cancer cells.

In conclusion, this study demonstrated that RORc was highly expressed in the liver cancer tissues and the disorder of methylation is an important factor contributing to the overexpression of RORc. More interestingly, we firstly revealed that RORc was close with HBV-related liver cancer and HBx could increase the expression of RORc through the promotion of promoter activity. And in vitro studies also showed RORc was a positive regulator in the migration and proliferation of liver cancer cells, suggesting that RORc was a novel target for liver cancer therapy, especially for the HBV-positive liver cancer.

Data Availability
Data are available from the corresponding author.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Acknowledgments
This study was supported by the key projects of Guangxi Natural Science Foundation (2013GXNSFDA019019) and the Project of “139” Plan for Training High Level Cadre Talents in Guangxi Medicine. The project was also supported by the Guangxi special fund project for Cultivating
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