Original Article HBx-related long non-coding RNA MALAT1 promotes cell metastasis via up-regulating LTBP3 in hepatocellular carcinoma

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Abstract: Though it is widely known that hepatitis B virus X protein (HBx) is involved in the progression of hepatocellular carcinoma (HCC), the underlying mechanisms are not entirely clear. In recent years, metastasis associated with lung adenocarcinoma transcript 1 (MALAT1), which is an oncogenic long non-coding RNA (IncRNA), has been proved to be associated with many kinds of tumors, including liver cancer. In this study, we demonstrated that MALAT1 was involved in the HBx-mediated hepatocarcinogenesis. Firstly, we found that expression of MALAT1 was strongly up-regulated in HCC tissues and was directly proportional to the expression of HBx. Moreover, in HBx transfected LO2 and HepG2 cells, MALAT1 was also up-regulated compared with non-transfected cells. Then, we observed up-regulated MALAT1 in HepG2 cells could promote cell invasion and migration, whereas knockdown of MALAT1 in HBx-expressing hepatic cells (HepG2-HBx) resulted in a markedly inhibition of cell invasion and migration both *in vitro* and *in vivo*. To further obtain a deeper understanding of the effect of MALAT1, we took latent transforming growth factor β -binding protein 3 (LTBP3) into account by using several assays such as RNA interference, luciferase, transwell and wound healing. Results showed that MALAT1 could promote tumor growth and metastasis by activating LTBP3, which could also be up-regulated by HBx. Meanwhile, the similar results were detected in nude mice. These findings could demonstrate an important mechanism of hepatocarcinogenesis through the signaling of HBx-MALAT1/LTBP3 axis, and may give a potential target for treatment of HCC.

Keywords: HBx, MALAT1, LTBP3, hepatocellular carcinoma, cell metastasis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies as well as the second leading cause of worldwide cancer related death [1-3]. Though treatment strategies like chemotherapy have significantly improved the survival rate of the patients, many problems such as the high incidence of cancer metastasis still exist. One of the major risk factor of HCC is chronic hepatitis B virus (HBV). Among various cancer related proteins, hepatitis B virus X protein (HBx) is considered as the key protein in the process of HBV-induced hepatocarcinogenesis [4, 5]. It has been detected with high frequency in patients who are suffering from liver cancer, chronic hepatitis and cirrhosis. HBx plays a multifunctional role in the development of HBV-related HCC, such as affecting cell growth, regulating cell apoptosis, and inhibiting nucleotide excision repair of damaged cellular DNA [6]. However, more detailed and deeper insights of HBx in HCC are still needed.

LncRNAs, which refer a class of non-proteincoding RNA transcripts longer than 200 nucleotides and do not contain an open reading frame [7], are involved in various physiological process, including cell proliferation, apoptosis, metastasis and invasion [8]. Several studies showed that IncRNAs are acted as oncogenes or tumor suppressors and closely related to HBx. With the development of high-throughput sequencing and bioinformatics, more and more IncRNAs have been discovered and are found to be abnormally expressed in HCC, such as PCNA-AS1 (antisense to PCNA) [9], HEIH (high

Name	Sequence
Sh-MALAT1	5'-CACCGCTGTGGAGTTCTTAAATATCTTCAAGAGAGATATTTAAGAACTCCACAGCTTTTTTG-3'
	5'-GATCCAAAAAAGCTGTGGAGTTCTTAAATATCTCTCTTGAAGATATTTAAGAACTCCACAGC-3'
Si-LTBP3	5'-GCAAGCAGGGCUUCUACUATT-3'
	5'-UAGUAGAAGCCCUGCUUGCTT-3'
HBx Forward	5'-CGTCCTTTGTTTACGTCCCG-3'
HBx Reverse	5'-GCAGATGAGAAGGCACAGAC-3'
MALAT1 Forward	5'-AAAGCAAGGTCTCCCCACAAG-3'
MALAT1 Reverse	5'-GGTCTGTGCTAGATCAAAAGGCA-3'
LTPB3 Forward	5'-CGGAACGGAGTGTGTGAGAA-3'
LTPB3 Reverse	5'-CTCGTCCACGTCCATCTCT-3'
GAPDH Forward	5'-GCTGTAGCCAAATCGTTGT-3'
GAPDH Reverse	5'-CCAGGTGGTCTCCTCTGA-3'

Table 1. The sequences for siRNA and qRT-PCR prime

expression in HCC) [10], MVIH (microvascular invasion in HCC) [11], HULC (highly up-regulated in liver cancer) [12] and metastasis associated with lung adenocarcinoma transcript 1 (MALAT1) [13]. MALAT1 is an oncogenic lncRNA which can stimulate cancer development, regulate cancer cell radio-sensitivity and chemosensitivity by diversity mechanisms, like acting as miRNA sponges, stimulating autophagy and enhancing epithelial-to-mesenchymal transition [14, 15]. Studies show that MALAT1 is upregulated in HCC, however either the deeper mechanisms or whether HBx is involved in the progress is still not clear [16].

Latent transforming growth factor *β*-binding proteins (LTBPs) are large secreted glycoproteins that regulate the bioavailability of transforming growth factor β (TGF β) [17]. Among the four subtypes, LTBP1, LTBP3 and LTBP4 bind covalently to TGF-β and are needed for the correct folding and the secretion of TGF-B [18]. Studies showed that LTBPs were involved in lots of diseases such as esophageal cancer [19], glaucoma and pseudoexfoliation syndrome [20], malignant mesothelioma [21] and so on. Recently, several studies demonstrated that MALAT1 could positively regulate LTBP3 transcription in mesenchymal stem cells by recruiting Sp1 to the LTBP3 promoter [22]. However, up to now, no study focuses on whether MALAT1 regulate HBV-induced hepatocarcinogenesis through activation of LTBP3.

In the present study, we aimed to study the role of MALAT1 in the development of hepatocellular carcinoma mediated by HBx. Our results showed that MALAT1 could promote cell metastasis of HCC mediated by HBx via up-regulating LTBP3 expression. These findings may provide new insights to the roles of MALAT1 in the development of HCC mediated by HBx.

Methods and materials

Cell culture and tissues

The LO2, LO2-HBx, and HepG2 cell lines were obtained from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS, Hyclone) and 1% PS (100 IU/ml penicillin, 100 μ g/ml streptomycin). All cells were incubated in a humidified atmosphere at 37°C with 5% CO₂.

The 20 paired HBV-related HCC and the adjacent liver tissue samples used in this study were obtained from patients who underwent radical resection at Xiangya Hospital (Central South University, Changsha, China). All tissues were confirmed histologically. This study was approved by the Ethics Committee of Xiangya Hospital.

Construction of plasmid, siRNA and cell transfection

LO2 and HepG2 cells were infected with the Lv-HBx and Lv-control viruses (GeneChem, Shanghai, China). For constructing MALAT1 and LTBP3, the full length of MALAT1 and LTBP3 were amplified and cloned into pcDNA-3.1 vectors (Invitrogen, USA), respectively [23]. Sh-MALAT1, si-HBx and si-LTBP3 were used for



Figure 1. HBx could up-regulate the expression of MALAT1 in hepatic tissues and cells. A. Expressions of HBx and MALAT1 in HCC and non-tumorous tissues; B. Relevance between HBx and MALAT1 in HCC tissues; C. Relevance between HBx and MALAT1 in non-tumorous tissues.

further studies, which were synthesized by GenePharma (Shanghai, China). The sequences of shRNA and siRNA were listed in **Table 1**. Lipofectamine 2000 (Invitrogen, USA) was used to transfect oligonucleotides and constructs into cell lines according to the manufacturer's instructions. The infection efficiency was confirmed by qRT-PCR or western blot 48 h after transfection.

Dual luciferase reporter assay

The LTBP3 promoter region (-1500/+200) was amplified and sub-cloned into the pGL3-basic luciferase vector (Promega, USA). LO2 and HepG2 cells cultured in 24-well plates were co-transfected with luciferase reporter plasmids and MALAT1 mimics/sh-MALAT1. After 48 h of transfection, luciferase activity was measured by using the Bright-Glo™ Luciferase Assay System (Promega, Cat. #E2610). All reactions were repeated in triplicate through at least three independent experiments.

RNA extraction and qRT-PCR

Detailed procedures used for total RNA extraction and were described elsewhere [24]. Primers used in qRT-PCR were listed in Table 1.

Western blotting (WB)

Western blotting was used to test the expression of MALAT1, HBx, LTPB3; and EMT related proteins E-cadherin, N-cadherin, vimentin. β -actin was used as a control. Detailed procedures of WB were described elsewhere [23].

In vitro cell invasion and migration assays

Transwell assay was used to determine cell invasion as described elsewhere [23]. Briefly,

cell suspension containing 5×10^5 cells/ml was prepared in serum-free media, followed with 300 µl cell suspension. Then, 500 µl of DMEM containing 10% FBS was added and cells were incubated for 24 h, stained with 0.1% crystal violet, and then counted and photographed. Cell migration ability was tested using the scratch wound assay and then seeded into 6-well plates for 24 h. Cell layers were scratched using a 200 µl pipette tip to form wound gaps, and then maintained in DMEM with 10% FBS. The cells were photographed at 0 and 48 h to record the wound width. All experiments were independently repeated in triplicate.

Tumor growth analysis in vivo

Male BALB/C nude mice (4-6 weeks) were purchased from the Animal Center of Silaike Jingda (Hunan, China). All animals were housed in micro-isolator cages with free access to food and water according to the Guide for the Care and Use of Laboratory Animals. The whole study was approved by the Institutional Animal Care Committee of Third Xiangya Hospital. HepG2-HBx cells (1×107/ml, 0.1 ml) transfected with shMALAT1 (or siLTBP3) and sh/si-ctrl vector were subcutaneously injected into the left back and right back of 4-5 per group mice, respectively. The sizes of tumors were recorded every 5 days and all mice were sacrificed 1 month after injection. The tissues were collected, photographed and the weights of tumors were measured.

Statistical analysis

The measurement data was expressed by mean \pm SD. Independent continuous variables was compared using the t-test and categorical data was compared using the chi square test or Fisher exact test. When *P*-value was less



Figure 2. HBx could up-regulate the expression of MALAT1 in hepatic cells. (A, B) Expressions of HBx (A) and MALAT1 (B) in the four cell lines, LO2, LO2-HBx, HepG2 and HepG2-HBx; (C) Influence of over expression of HBx on MALAT1 in LO2 cells; (D) Influence of silencing of HBx on MALAT1 in HepG2-HBx cells. *P<0.05 and **P<0.01.

than 0.05 it was considered to be statistically significant. All calculations were made using SPSS 18.0.

Results

HBx could up-regulate the expression of MALAT1 in hepatic tissues and cells

To examine whether MALAT1 was regulated by HBx, we detected the expression levels of HBx and MALAT1 in HCC (n=20) and non-tumorous (n=17) tissues by qRT-PCR. Results showed that the expression levels of MALAT1 was significantly increased in HCC tissues compared with non-tumorous tissues (**Figure 1A**). Meanwhile, we found that the mRNA levels of MALAT1 was positively correlated with HBx, indicating that HBx infection would up-regulate the expression of MALAT1 (**Figure 1B** and **1C**). Thus, we constructed cell lines which could stably express HBx and found that MALAT1 was significantly upregulated in cells stably expressing HBx (**Figure 2A** and **2B**).

To further investigate the influence of HBx on MALAT1 in LO2 cells, pcDNA3.1-HBx was transfected into LO2 cells under concentrations of 1 µg/ ml and 2 µg/ml respectively. Results of qRT-PCR and western blotting showed that expression of MALAT1 was significantly increased with the over expression of HBx, and the effect was dosedependent (Figure 2C). Then si-HBx of different concentrations (1 μ g/ml and 2 μ g/ml) and si-ctrl was transfected into HepG2-HBx cells respectively. Results showed that when treated with si-HBx, expression of MALAT1 in Hep-G2-HBx cells was significantly decreased and the effect was also dose-dependent (Figure 2D). These results demonstrated that HBx in hepatocellular carcinoma cells could up-

regulate the expression of MALAT1, which meant the expression of MALAT1 would significantly increase with the increasing expression of HBx. And the effect was in a dose dependent manner.

Influence of MALAT1 on cell invasion and migration in vitro

To further investigate the influence of MALAT1 on cell invasion and migration, MALAT1 over expression model was established through HepG2 cells transfected with pcDNA3.1-MALAT1. Also si-MALAT1 and si-control were transfected into HepG2-HBx to establish the down expression model of MALAT1. Results showed that expression of MALAT1 treated by pcDNA3.1-MALAT1 in HepG2 cells was significantly higher than that in the control cells (**Figure 3A**). And when treated with si-MALAT1,





Figure 3. MALAT1 could promote cell invasion and migration *in vitro*. A. Expression of MALAT1 in HepG2 cells treated with pcDNA3.1-MALAT1 and in HepG2-HBx cells treated with si-MALAT1 and si-ctrl, respectively; B. Cell migration for HepG2 HepG2-HBx cells when were transfected with pcDNA3.1-MALAT1 and Si-MALAT1 alone, pcDNA3.1 and si-ctrl as control, respectively; C. Cell invasion for HepG2 and HepG2-HBx cells when were transfected with pcDNA3.1-MALAT1 alone, pcDNA3.1 and si-Ctrl as control, respectively; O. Cell invasion for HepG2 and HepG2-HBx cells when were transfected with pcDNA3.1-MALAT1 alone, pcDNA3.1 and si-Ctrl as control, respectively. D. Western blotting analysis of EMT maker proteins E-cadherin, N-cadherin, vimentin in different cell lines.



Figure 4. MALAT1 could promote tumor growth *in vivo*. A, B. Tumor volumes and size of mice treated with si-MALAT1 HepG2-HBx cells and the control cells, respectively; C. Tumor weights of mice treated with si-MALAT1 HepG2-HBx cells and the control cells, respectively; D. Expression of MALAT1 in mice treated with si-MALAT1 HepG2-HBx cells and the control cells, respectively.

expression of MALAT1 in HepG2-HBx cells decreased significantly (**Figure 3A**). These results indicated the successful establishment of MALAT1 over/down-expression cells.

The above models were used in the in vitro assay of cell invasion and migration. Results showed that HBx could significantly enhance the cell migration of HepG2 cells compared with the HepG2-HBx cells. Additionally, cell migration was apparently promoted when MALAT1 was over expressed and was significantly inhibited when MALAT1 decreased (Figure 3B). Meanwhile, we found that the invasion ability of HepG2 cells was enhanced when HBx and MALAT1 were over expressed, but the invasion ability of HepG2-HBx cells was decreased when HBx was knockdown (Figure 3C). Western blotting results showed that EMT related protein levels of N-cadherin and vimentin significantly increased but level of E-cadherin significantly decreased when MALAT1 was over-expressed in the HepG2 cells. Meanwhile, expression of N-cadherin and vimentin also decreased with the decrease of MALAT1, while expression of E-cadherin increased when expression of MALAT1 was reduced in HepG2-HBx cells (Figure 3D). These results suggested that HBx and MALAT1 could enhance the cell invasion and migration of HCC cells *in vitro*.

MALAT1 could promote tumor growth in vivo

To further observe the effects of MALAT1 in tumor growth, we took in vivo xenograft model experiment. As shown in Figure 4A-C, silencing MALAT1 (si-MALAT1) significantly decreased the tumor volumes and weights compared with the control (si-NC). Meanwhile qRT-PCR confirmed that the expression levels of MALAT1 were down-regulated in the si-MALAT1 tumor tissues (Figure 4D). This finding suggested that MALAT1 could promote tumor growth in vivo.

MALAT1 could up-regulate the expression of LTBP3

To study the possible mechanism of the above influence of MALAT1 in HCC, firstly, we analyzed the information of MALAT1 from NCBI gene database (www.ncbi.nlm.nih.gov/gene). Results showed that MALAT1 located at 11q-13.1., which was 32.0 kb away from LTBP3 and possessed opposite transcriptional direction with it (Figure 5A). Then, we confirmed that the expression of LTBP3 was significantly higher in HCC tissues than in non-tumor tissues (Figure 5B) and was positively correlated with the expression of MALAT1 (Figure 5C). Similar results were confirmed in LO2 and HepG2 cells. As showed in Figure 5D and 5E, expression of LTBP3 significantly increased when MALAT1 was over expressed in LO2 cells (Figure 5D). However, when HepG2 cells were transfected with si-MALAT1, expression of LTBP3 significantly decreased compared with the control groups (Figure 5E). Then, dual luciferase assay showed that the over-expression of MALAT1 could increase the promoter activity of LTBP3 in LO2 cells, whereas knockdown of MALAT1 led to an obvious decrease of LTBP3 promoter activity in HepG2 cells (Figure 5F).



Figure 5. MALAT1 could up-regulate the expression of LTBP3. A. Location of MALAT1 and LTBP3 analyzed by NCBI gene database; B. Expression of LTBP3 in HCC and non-tumorous tissues; C. Relevance between MALAT1 and LTBP3; D. Expression of LTBP3 in LO2 cells treated and untreated with MALAT1, respectively; E. Expression of LTBP3 in HepG2 cells treated with si-MALAT1 and the controls; F. Luciferase activity of HepG2 cells treated with si-MALAT1 and the controls; and LO2 cells treated with pcDNA3.1-MALAT1 and pcDNA3.1.

Thus, we concluded that MALAT1 was able to up-regulate LTBP3 in hepatoma cells.

MALAT1 could promote tumor growth and metastasis by up-regulating LTBP3

To investigate the mechanism of the promotion effect of MALAT1 on tumor growth and metastasis, we knocked down the LTBP3 in HepG2 cells (si-ctrl as the control, **Figure 6A**). Then, we observed that silencing LTBP3 significantly inhibited cell migration and invasion (**Figure 6B** and **6C**). What's more, when transfected with both si-MALAT1 and pcDNA3.1-LTBP3 in HepG2 cell line, cell migration and invasion ability were also enhanced significantly compared with cells only transfected with si-MALAT1, which demonstrated that LTBP3 was required for the MALAT1-enhanced cell invasion and migration of HepG2 (**Figure 6B** and **6C**). Besides, western blotting results of EMT maker proteins E-cadherin, N-cadherin, vimentin showed that when silencing LTBP3, the expression of N-cadherin and vimentin decreased and expression of E-cadherin increased (**Figure 6D**). These results could further demonstrate that the promotion effect of MALAT1 on tumor growth and metastasis was through the regulation of LTBP3.

HBx promotes HCC metastasis via MALAT1/LTBP3 axis





Figure 7. HBx enhanced cell metastasis through up-regulating LTBP3. A. Expression of LTBP3 in different cell lines, LO2-HBx and HepG2-HBx cell lines treated with si-HBx, si-HBx+MALAT1, si-ctrl respectively and untreated LO2, LO2-HBx, HepG2 and HepG2-HBx cells. B, C. Cell invasion and migration assay for si-Ctrl, si-HBx and si-HBx+MALAT1 cells.

Furthermore, tumorigenicity assay showed that when LTBP3 was silenced in HepG2 cells, the tumor volumes and weights were significantly decreased compared with the control. Interestingly, the tumorigenicity inhibitory function of si-MALAT1 was partially attenuated by over-expressed LTBP3 (**Figure 6E** and **6F**).

HBx enhanced cell metastasis through upregulating LTBP3

Relationship between HBx and LTBP3 in the process of cell metastasis was also studied. si-HBx, si-LTBP3, si-HBx+LTBP3 and si-ctrl were used to transfect both LO2-HBx and HepG2-HBx cell lines respectively. Western blotting results showed that LTBP3 was significantly increased in LO2-HBx (or HepG2-HBx) cells compared with LO2 (or HepG2) cells. Meanwhile, silencing HBx in LO2-HBx and HepG2-HBx cells resulted in the decrease of LTBP3. Interestingly, the over-expression of MALAT1 was able to rescue the LTBP3 expression levels in both LO2-HBx and HepG2-HBx cells transfected with si-HBx (Figure 7A). The result suggested that HBx could up-regulate LTBP3 via MALAT1. Moreover, we found that silencing HBx could block the cell migration and invasion in HepG2-HBx cells, whereas it was partially rescued by the over-expression of MALAT1 (Figure 7B and 7C). Thus, we concluded that elevated MALAT1 by HBx could promote the metastasis of hepatoma cells through up-regulation of LTBP3.

Discussion

HBV is a major risk factor of HCC, and HBx plays a key role in the process of HBV-induced hepatocarcinogenesis, but how it affects the tumor development in HCC needs more illumination. Previous studies suggested that lots of IncRNAs were involved in HBx related HCC, including MALAT1 [4, 5, 25]. It has been proved that MALAT1 could promote tumor growth and cell metastasis in lots of cancers, such as malignant melanoma [22], osteosarcoma [26] and pancreatic cancer [27]. Recently, MALAT1 level was observed to be associated with liver damage and could predict development of HCC [13]. However, whether MALAT1 was involved in the hepatocarcinogenesis mediated by HBx was poorly understood. Thus in this study, for the first time we focused on

investigating the role of MALAT1 in HBxassociated HCC and the possible mechanisms in tumorigenesis and metastasis of HCC. Firstly, we detected the expression of MALAT1 in hepatic tissues and HCC. Results showed that the expression of MALAT1 was significantly increased in HCC tissues and cells, which was in consistent with other studies [13]. Then we found that HBx could up-regulate the expression of MALAT1 in both hepatic tissues and HCC, which meant MALAT1 expression level was positively associated with the mRNA level of HBx in HCC cells. And this is the first time the relationship of HBx and MALAT1 in HCC was determined. Secondly, we investigated effects of MALAT1 on tumor growth and cell invasion and migration both in vitro and in vivo. Results showed that MALAT1 could promote HepG2-HBx cells migration and invasion both in vitro and in vivo. These results could further demonstrated the function of MALAT1 in tumor growth and metastasis in HBx-associated HCC.

Generally, IncRNAs regulated downstream genes expression through direct, indirect or epigenetic ways. As one of IncRNAs, previous studies also showed that MALAT1 could regulate several down-stream genes, including CD133 [28], miR-23c [29], and LTBP3 [22]. It has been proved that LTBPs are involved in cancer development, and MALAT1 could positively regulate LTBP3 transcription in mesenchymal stem cells [22]. Thus to further investigate possible mechanisms of the above influence of MALAT1 in HBx-associated hepatocarcinogenesis, we hypothesized whether MALAT1 affected HBxassociated HCC through regulation of LTBP3. Results showed that in both hepatic tissues and HCC, MALAT1 could up-regulate the expression of LTBP3. What's more, the promotion effects on tumor growth and metastasis, cell invasion and migration were also found to be induced by LTBP3. At last, we preliminarily studied the relationship between HBx and LTBP3 and found expression of LTBP3 could be reduced by si-HBx and the effect could be moderated by the over-expression of MALAT1. Therefore, HBx is able to enhance cell metastasis through up-regulating LTBP3.

In conclusion, in this study we demonstrated that HBx could up-regulate long non-coding RNA MALAT1 in HCC, and MALAT1 could further influence the expression of LTBP3, resulting in promotion of development and metastasis of HCC. These results may provide new insights for the roles of IncRNAs in HBx-associated hepatocarcinogenesis.

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Disclosure of conflict of interest

None.

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