Research Article

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# Upregulated FoxM1 expression induced by hepatitis B virus X protein promotes tumor metastasis and indicates poor prognosis in hepatitis B virus-related hepatocellular carcinoma

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**Background & Aims**: Forkhead box M1 (FoxM1) is a master regulator of tumor metastasis that plays an important role in the development of hepatocellular carcinoma (HCC). However, whether or not FoxM1 contributes to the progression of HBV-associated HCC (HBV–HCC) remains unknown. Therefore, we aimed at investigating the clinicopathologic significance of FoxM1 in HBV–HCC and the potential role of FoxM1 in hepatitis B virus X (HBx)-mediated invasiveness and metastasis.

**Methods**: The expression of FoxM1 and its functional targets matrix metalloproteinase-7 (MMP-7), RhoC, and Rho-kinase 1 (ROCK1) in human HBV–HCC tissues was detected by immunohistochemistry. Luciferase reporter, chromatin immunoprecipitation, and electrophoretic mobility shift assays were used to measure the transcriptional regulation of *FoxM1* promoter by HBx. The effect of FoxM1 on HBx-mediated invasiveness and metastasis was analyzed by transwell assays and an orthotopic metastatic model.

**Results**: FoxM1 overexpression correlated with multiple malignant characteristics and indicated poor prognosis of HBV–HCC patients. FoxM1 expression was an independent factor affecting the recurrence and survival of patients with HBV–HCC after surgical resection. FoxM1 promoted hepatoma cell invasion and metastasis by promoting MMP-7, RhoC, and ROCK1 expression, while FoxM1 overexpression was associated with elevated expressions of these proteins in HBV–HCC tissues. HBx upregulated FoxM1 expression through the ERK/CREB pathway, and FoxM1 inhibition significantly decreased HBx-enhanced hepatoma cell invasion *in vitro* and lung metastasis *in vivo*.

**Conclusions**: We report a new molecular mechanism for HBV-associated hepatocarcinogenesis that involves the activation of FoxM1 expression by HBx through the ERK/CREB pathway, thereby leading to invasion and metastasis of hepatoma cells.


Keywords: Forkhead box M1; Hepatitis B virus X; Metastasis; Hepatocellular carcinoma.

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*Abbreviations:* FoxM1, forkhead box M1; HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; HBV, hepatitis B virus; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; OS, overall survival; TTR, time to recurrence; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation analysis; ERK, extracellular signal-regulated kinase; CREB, cAMP-response element-binding protein.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide, with nearly 600,000 deaths annually [1]. Chronic hepatitis B virus (HBV) infection is a major risk factor for HCC development. Over 50% of HCC cases are attributable to persistent HBV infection [2]. Metastasis is the major cause of death in HBV-related HCC (HBV–HCC), in which hepatitis B virus X protein (HBx) is thought to play a key role [3]. It has been reported that HBx facilitates hepatoma cell invasion and metastasis through upregulation of matrix metalloproteinases (MMPs) expression and repression of fibronectin expression [4,5]. Mark A. Feitelson's and our laboratories previously reported that HBx promoted HCC invasion and progression by upregulating VEGFR3 and β-catenin expression and downregulating E-cadherin expression [6–8]. These studies indicate that HBx contributes to the metastasis of HBV–HCC. However, the complex mechanism of metastasis in HBV–HCC is far from being understood.

FoxM1 is a member of the forkhead box (Fox) transcription factor family known as an oncogene in several aggressive cancers [9]. FoxM1 levels were dramatically decreased in adult tissues, but FoxM1 expression was reactivated by oncogenic signaling pathways and reactive oxygen species, resulting in the malignant progression of numerous cancers [10,11]. We had previously reported that hypoxia induced FoxM1 expression and FoxM1 overexpression accelerated the growth and survival of cancer cells under hypoxic stress [12]. Interestingly, several recent studies reported that FoxM1 was a master regulator of tumor metastasis [13]. FoxM1 induced an epithelial-mesenchymal-like transition (EMT) phenotype and increased cell migration by transactivating MMP-2, 9 and JNK1 expression. It also induced

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a premetastatic niche at the distal organ of metastasis by transactivating lysyl oxidase (LOX) and lysyl oxidase-like 2 (LOXL2) expressions [13–16]. More interestingly, it has been reported that conditionally deleted *FoxM1* mouse hepatocytes failed to proliferate and were highly resistant to HCC development in response to diethylnitrosamine (DEN) [17]. Furthermore, a $p19^{ARF}$ peptide that significantly inhibited FoxM1 transcriptional activity efficiently diminished HCC proliferation and induced apoptosis of the HCC region in *FoxM1* transgenic mice [18]. In addition, a recent study has reported that FoxM1 overexpression was associated with poor prognosis in HCC patients [19]. Another study has reported that FoxM1 overexpression predicted worse clinical outcome in HCC patients after orthotopic liver transplantation [20]. These studies indicate that FoxM1 plays an essential role in the development of HCC. However, whether or not FoxM1 contributes to the progression of HBV–HCC remains unknown, and the molecular basis of how FoxM1 promotes HCC metastasis needs further investigation.

HBx is a multifunctional protein that activates many viral and cellular genes, modulates cellular signal transduction pathways, and regulates cell proliferation, apoptosis and invasion [21]. HBx does not bind DNA directly but regulates gene expression by transactivating multiple transcription factors including AP-1, NF-kB, HIF-1, and CREB [22]. These interactions provide molecular mechanisms by which HBx facilitates the development of HBV-associated HCC. HBx has been strongly implicated in tumor invasion and metastasis during hepatocarcinogenesis. This has raised the question of whether HBx could activate the expression of FoxM1 during HBV infection, resulting in the stimulation of tumor cell metastasis.

In the present study, we provide the first evidence that HBx facilitates hepatoma cell invasion *in vitro* and metastasis *in vivo* by activating FoxM1 expression. The HBx-induced upregulation of FoxM1 involves the ERK/CREB signaling pathway.

## Materials and methods

A detailed description of this section can be found in the online Supplementary Material.

## Results

### *FoxM1 overexpression is associated with multiple malignant characteristics and indicates poor prognosis in HBV–HCC patients after surgical resection*

To explore whether FoxM1 could be an important factor in determining clinical outcomes of HBV–HCC patients, we examined the expression of FoxM1 in 306 paired HBV–HCC samples by immunohistochemistry. Positive immunoreactivity for FoxM1 was observed primarily in the nucleus (Fig. 1A). Most HBV–HCC samples (201/306, 65.7%) were found to be positive for FoxM1. In contrast, less than half of the adjacent liver tissues (62/306, 20.3%) expressed FoxM1 ($p$ <0.01, Fisher's exact test). The mRNA levels of *FoxM1* were significantly increased in tumors compared to adjacent non-tumorous tissues. *FoxM1* expression increased 2-fold more in CHB and LC samples compared to normal liver samples (Fig. 1B), which suggested that the overexpression of FoxM1 was an early event associated with HBV-related carcinogenesis.

In addition, 34 HCV-positive HCC, 10 HBV and HCV co-infection HCC, and 56 non-viral infected HCC were used to detect FoxM1 expression by immunohistochemistry. As shown in Supplementary Table 2, among the 34 HCV-infected HCC tissues, 11 (32.4%) had positive FoxM1 expression. Among the 56 non-viral infected HCC tissues, 18 (32.1%) had positive FoxM1 expression. Correlation regression analysis indicated that the overexpression of FoxM1 was significantly correlated with HBV infection ($p$ <0.001).

FoxM1 expression levels were detected in several human HCC cell lines with varying metastatic capabilities. Real-time PCR and Western blot assays showed that FoxM1 expression increased progressively from normal liver cells, to low metastatic HCC cells, and finally to highly metastatic HCC cells (Fig. 1C).

Correlation regression analysis indicated that positive expression of FoxM1 was significantly correlated with maximal tumor size, loss of tumor encapsulation, microvascular invasion, malignant differentiation, and tumor-node-metastasis (TNM) stage in HBV–HCC patients (Table 1). Kaplan–Meier analysis showed that patients with positive expression of FoxM1 had shorter overall survival and higher recurrence rates than those with negative expression of FoxM1 (Fig. 1D). Multivariate Cox proportional hazards model showed that FoxM1 expression level was an independent and significant factor for recurrence and survival in HBV–HCC patients after surgical resection (Table 2). Taken together, these data indicated that FoxM1 might contribute to the progression of HBV-associated hepatocarcinogenesis.

### *FoxM1 promotes hepatoma cell invasion in vitro and distant lung metastasis in vivo*

To determine the role of FoxM1 in the migration and invasion of HCC cells, we established two stable cell lines, named SMMC7721 LV-FoxM1 and HCCLM3 LV-shFoxM1, after lentivirus transduction. Transwell assay showed that upregulation of FoxM1 expression using lentivirus LV-FoxM1 significantly enhanced the migration and invasion capacities of the SMMC7721 cells (low metastatic potential). In contrast, silencing endogenous FoxM1 expression using lentivirus LV-shFoxM1 in HCCLM3 cells (high metastatic potential) significantly reduced cell migration and invasion (Fig. 1E).

To further explore the role of FoxM1 in HCC metastasis *in vivo*, cell lines were transplanted into the livers of nude mice, which closely mimicked the mechanisms of human HCC metastasis after the formation of primary HCC. Tumor metastasis in mice was monitored by an imaging system detecting the luciferase signal. Eight weeks after orthotopic implantation, bioluminescent imaging (BLI) showed the presence of lung metastasis in the mouse implanted with SMMC7721 LV-FoxM1 cells, while no metastasis occurred in the mouse implanted with SMMC7721 LV-control cells. The mouse implanted with HCCLM3 LV-shcontrol cells showed lung metastasis, whereas no metastasis was detected in the mouse implanted with HCCLM3 LV-shFoxM1 cells (Fig. 1F, black rectangular boxes indicate the lung metastatic nodules). Histological analysis further confirmed that upregulation of FoxM1 expression in SMMC7721 cells significantly increased the incidence of intra-