

Basic Study

Hepatitis B virus X protein induces hepatic stem cell-like features in hepatocellular carcinoma by activating KDM5B

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Abstract**AIM**

To determine the role of hepatitis B virus X protein (HBx), HBx in regulating hepatic progenitor cell (HPC)-like features in hepatocellular carcinoma (HCC) and the underlying molecular mechanisms.

METHODS

We used a retrovirus vector to introduce wild type HBx or empty vector into HepG2 cells. We then used these cells to analyze cell proliferation, senescence, transformation, and stem-like features. Gene expression profiling was carried out on Affymetrix GeneChip Human U133A2.0 ver.2 arrays according to the manufacturer's protocol. Unsupervised hierarchical clustering analysis and Class Comparison analysis were performed by BRB-Array Tools software Version 4.2.2. A total of 238 hepatitis B virus (HBV)-related HCC patients' array data were used for analyzing clinical features.

RESULTS

The histone demethylase KDM5B was significantly

highly expressed in HBV-related HCC cases ($P < 0.01$). In HBV proteins, only HBx up-regulated KDM5B by activating c-myc. Hepatic stem cell (HpSC) markers (EpCAM, AFP, PROM1, and NANOG) were significantly highly expressed in KDM5B-high HCC cases ($P < 0.01$). KDM5B played an important role in maintaining HpSC-like features and was associated with a poor prognosis. Moreover, inhibition of KDM5B suppressed spheroid formation and cell invasion *in vitro*.

CONCLUSION

HBx activates the histone demethylase KDM5B and induces HPC-like features in HCC. Histone demethylases KDM5B may be an important therapeutic target against HBV-related HCC cases.

Key words: Hepatitis B virus X protein; Hepatocellular carcinoma; KDM5B; Progenitor cell; Tumorigenesis

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Core tip: The role of epigenetic regulation in cancer biology has been the subject of several studies. These chromatin structure modifiers have been increasingly shown to facilitate several steps of cancer progression. However, the epigenetic regulation of hepatocellular carcinoma has not been elucidated. We assumed that multifunctional protein hepatitis B virus X (HBx) protein, may affect epigenetic regulation of Hepatocellular carcinoma. We showed that HBx activated the histone demethylase KDM5B and induced HPC-like features in hepatocellular carcinoma (HCC) in this study. Our results suggested that histone demethylases may be an important therapeutic target against HBV-related HCC cases.

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INTRODUCTION

Chronic infection by hepatitis B virus (HBV) affects approximately 400 million people worldwide and is a leading cause of hepatocellular carcinoma (HCC), which is considered the sixth most common cancer in the world^[1,2].

HBV encodes the nonstructural hepatitis B virus X (HBx) protein, which is conserved among mammalian hepadnaviruses, suggesting an important function. HBx is the most critical protein for viral replication in hepatocytes and for the development of HCC. HBx is a multifunctional protein that has been shown to regulate

many transcription factors such as nuclear factor-kappa B, ATF-2, activator protein 1, cAMP response element binding protein^[3], Ras-Raf mitogen-activated protein kinase, extracellular signal-regulated kinase, phosphoinositol-3-kinase-protein kinase B/Akt^[4], and Wnt/beta-catenin pathway^[5]. These pathways are involved in a range of cellular functions including apoptosis, cell proliferation, cell cycle progression, and cytokine production. Therefore, it is proposed that HBx manipulates these cellular signaling pathways, resulting in the transformation of HBV-infected hepatocytes. Although extensive studies have focused on the roles of HBx in malignant transformation^[5-10], the molecular mechanisms underlying this process are not well elucidated.

KDM5B, which is also known as JARID1B or PLU1, is an H3K4me3 histone demethylase that is overexpressed in many types of cancer including breast^[11], prostate^[12], bladder^[13], and lung cancer^[14]. Histone modifiers, such as KDM5B, and their associated post-translational modifications are thought to be central to the determination of embryonic stem cell fate^[15,16]. Embryonic stem cell pluripotency and differentiation depend on the interactions between an array of chromatin modifiers and several downstream transcription factors. This stem cell epigenetic landscape has been implicated in tumor progression and chemoresistance.

The central hypothesis we explored here is that KDM5B maintains cancer stem cells in HCC.

MATERIALS AND METHODS

Human subjects

For gene expression analysis, we assessed three already published cohorts. Cohort 1 was 139 caucasian HCC cases. KDM5B expression data were derived from these specimens as described. Survival data linking to this cohort were kindly provided by Dr. Snorri Thorgeirsson at NCI^[17]. Cohort 2 was 238 cases who received surgical resection of HBV-related HCC at the Liver Cancer Institute of Fudan University. Their clinicopathologic characteristics and prognostic data were kindly provided by Dr. Xin Wei Wang at NCI^[16]. Cohort 3 was 89 Japanese HCC cases who received surgical resection at the Kanazawa University Hospital^[18].

Cell lines

Human HCC cell lines (HepG2, Huh7 and Hep3B) were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin-streptomycin. We used two immortalized human hepatocyte cell lines, TTNT16 cells (in which hTERT was introduced)^[19,20] and T5B cells [in which the SV40 large T antigen (LT) was introduced]^[21,22]. These cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum

Table 1 Real-time reverse-transcription polymerase chain reaction primers and probes designed and used in the experiments

PC-C (core)	FW	5'	CGAGGCAGGTCCTAGAA	3'
	RV	5'	CGGCGATTGAGACCTTCGT	3'
	probe	5'-FAM	AGAAGCTCCCTCGCCTCG	MGB-3'
PS-S (surface)	FW	5'	ACCCCAACAAGGATCATTGG	3'
	RV	5'	CGAATGCTCCCGCTCCTA	3'
	probe	5'-FAM	CAGAGGCAAATCAG	MGB-3'
HBx	FW	5'	TGTCAACGACCGACCTTGAG	3'
	RV	5'	CCCAACTCCTCCAGTCCTT	3'
	probe	5'-FAM	CATACTTCAAAGACTGTTTGT	MGB-3'
Pol (polymerase)	FW	5'	TCCGCTGCCGATCCAT	3'
	RV	5'	GCTGCGAGCAAAAACAAGCT	3'
	probe	5'-FAM	CTGCGGAACTCCT	MGB-3'
Pg RNA (Pregenomic RNA)	FW	5'	GCTCTGTATCGGAGGCCITA	3'
	RV	5'	TGAGTGCTGTATGGTGAGGAGAA	3'
	probe	5'-FAM	AGTCTCCGGAACATT	MGB-3'

Primers and probes for hepatitis B virus (HBV) type C.

and 1% penicillin-streptomycin.

Real-time reverse-transcription polymerase chain reaction analysis

Total RNA was subjected to quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). mRNAs were analyzed using TaqMan Gene Expression Assays in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA). All RT reactions were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). Probes used for the analyses were as follows: KDM5B, Hs00981910_m1; MYC, Hs00905030_m1 (Applied Biosystems), HBV proteins, shown in Table 1. The experiments were performed in triplicate. The TaqMan gene assay for actin was used to normalize the relative abundance of mRNA.

RNA interference

A small interfering RNA (siRNA) specific to KDM5B and a control siRNA were obtained from Nippon Gene. siRNA specific for c-Myc and a control siRNA were obtained from Invitrogen. Transfection was performed with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Invasion and cell migration assays

Cell migration and invasion were assessed using a Cytoselect 24-Well Cell Migration and Invasion Assay (CELL BIOLABS). For migration and invasion measurements, transfected Huh7 and Hep3B cells were seeded at 5.0×10^5 cells/well. The cells that invaded or migrated to the underside of the chamber were harvested and measured according to the manufacturer's protocol. Results were determined from triplicate wells in three independent experiments and expressed as a percentage relative to control.

Spheroid formation assays

For spheroid formation assays, single cell suspensions

of 2.0×10^3 cells were seeded in 6-well Ultra-Low Attachment Microplates (Corning, Corning, NY). The number of spheroids was measured at 10-14 d after seeding. Invasion assays were performed using BD BioCoat™ Matrigel Matrix Cell Culture Inserts and Control Inserts (BD Biosciences, San Jose, CA).

Immunohistochemistry analyses

Immunohistochemistry (IHC) was performed using Envision+ kits according to the manufacturer's instructions. An anti-KDM5B monoclonal antibody was used for detecting KDM5B. The staining area and intensities were evaluated in each sample and graded from 0-3 (0, 0-5%; 1, 5%-25%; 2, 25%-50%; 3, > 50%) and 0-2 (0, negative; 1, weak; 2, strong), respectively. The sum of the area and intensity scores of each marker (IHC score) were calculated. Samples were defined as marker-positive (IHC score ≥ 3) or -negative (IHC score ≤ 2).

Statistical analysis

Mann-Whitney, χ^2 , Fisher's exact, and Kruskal-Wallis tests were used to compare the clinicopathologic characteristics and gene expression data. The correlation of the gene expression data was evaluated by Spearman's rank correlation coefficient. Kaplan-Meier survival analysis with the log-rank test was performed to compare patients' survival. All analyses were performed using GraphPad Prism software 5.0.1 (GraphPad Software, San Diego, CA).

RESULTS

KDM5B expression in HBV-related HCC

We performed transcriptomic analysis of 139 retrospectively collected HCC (cohort 1) patients to assess whether KDM5B was associated with hepatocarcinogenesis. We evaluated and compared the expression of KDM5B in HCC cases developed from various backgrounds: chronic hepatitis B, chronic hepatitis

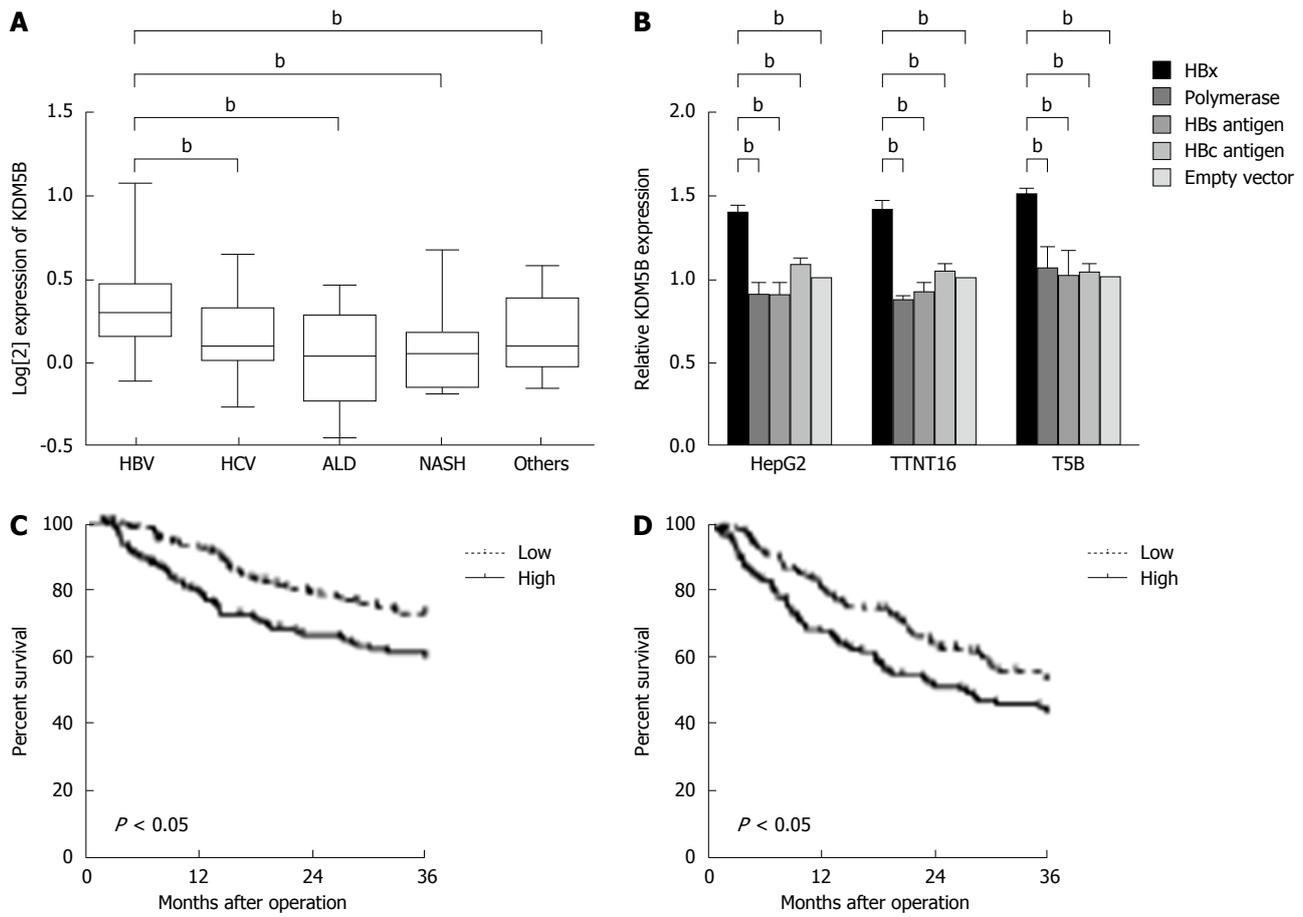


Figure 1 Heterogeneity of KDM5B expression in various hepatocellular carcinoma cases. A: Expression analysis of KDM5B-based quantitative RT-PCR data in 139 HCC samples. The horizontal lines in the boxplots represent the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles. A nonparametric test was used to compare the 5 patient groups of hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease (ALD), nonalcoholic steatohepatitis (NASH) and others respectively as indicated by *P* values; B: Expression of KDM5B gene in HepG2, TTNT16, and T5b cells. HBV proteins were introduced into these cells by a retroviral system; C: Kaplan-Meier plot of the overall survival of 238 hepatocellular carcinoma (HCC) cases based on their classification by KDM5B expression; D: Kaplan-Meier plot of recurrence-free survival of HCC cases based on their classification by KDM5B expression.

C, alcoholic liver disease, and nonalcoholic steatohepatitis. As shown in Figure 1A, KDM5B was significantly highly expressed in HBV-related HCC cases compared to other etiological-related HCC cases. Moreover, for HBV proteins, only HBx up-regulated KDM5B in HepG2 cells and immortalized human hepatocytes (TTNT16 and T5b cells) (Figure 1B). These results indicated that HBx was associated with HBV-related hepatocarcinogenesis through the activation of KDM5B.

To understand the effect of KDM5B on the survival of HBV-related HCC cases, we analyzed the microarray data of 238 HCC cases (cohort 2). Kaplan-Meier survival analysis revealed that the KDM5B high expression group had a significantly shorter overall survival ($P < 0.05$) and recurrence-free survival ($P < 0.05$) than the KDM5B low expression group (Figure 1C and 1D).

KDM5B regulates hepatic stem cell-like features in HCC

Previously, we identified two HCC subgroups, one resembling the gene expression signatures of hepatic stem cells (HpSCs) (referred to as HpSC-HCC) and the other similar to mature hepatocytes (referred to

as MH-HCC). HpSC-HCC had stem cell-like features and a poorer prognosis than MH-HCC cases. We hypothesized that KDM5B induced hepatic progenitor-like features in HBV-related HCC cases.

To determine whether KDM5B was functionally linked to the HpSC-like phenotype, we analyzed representative expression levels of HpSC markers in cohort 2 HCC cases. Consistently, HpSC markers, such as EpCAM, AFP, PROM1, and NANOG, were more abundantly expressed in KDM5B high expression HCC cases as compared to KDM5B low expression cases (Figure 2A). Moreover, KDM5B levels were positively correlated with EpCAM or AFP levels in 238 HCC cases (Figure 2B and C).

Next, to confirm that HBx upregulated KDM5B and HpSC markers in HCC cells, we introduced HBx-wt in HepG2 and Huh7 cells and evaluate the expression of KDM5B and HpSC markers. Introduction of HBx up-regulated the expression of KDM5B, EpCAM, AFP, PROM1 and NANOG in these cells (Figure 3A and B).

In addition, inhibition of KDM5B suppressed cell invasion (Figure 4A and B) and spheroid formation

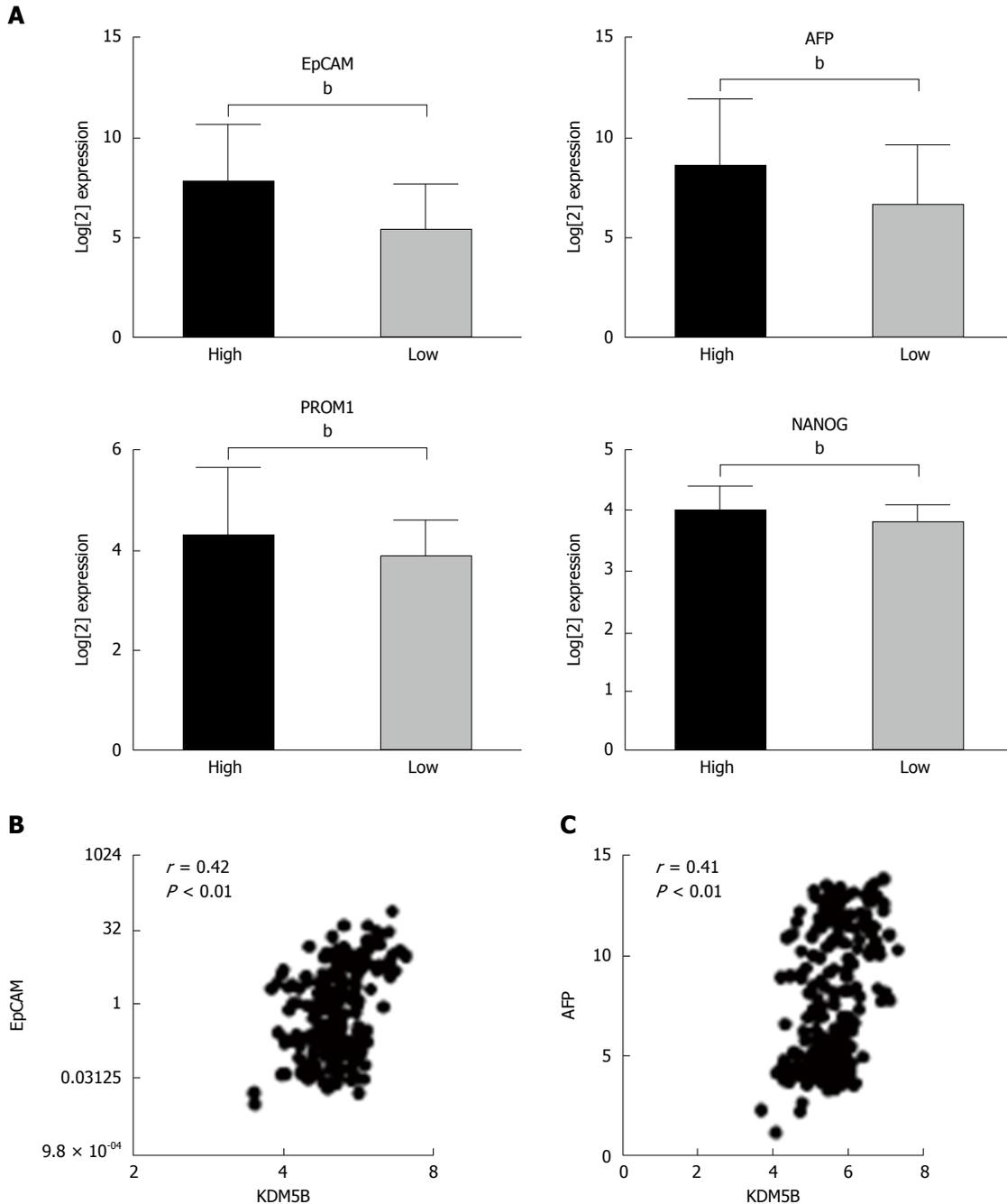


Figure 2 KDM5B is associated with HpSC markers in hepatocellular carcinoma cases. A: Expression analysis of HpSC markers (EpCAM, AFP, PROM1, and NANOG) in cohort 2 cases based on their classification by KDM5B expression; B: Spearman correlation analysis of KDM5B and EpCAM expression data as determined by mRNA arrays of cohort 2 cases; C: Spearman correlation analysis of KDM5B and AFP expression in 238 HCC cases.

(Figure 4C) in Hep3B and Huh7 cells. However, KDM5B did not affect cell proliferation or apoptosis in these cells as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide and transferase-mediated dUTP nick-end labeling assays (data not shown). These data indicated that KDM5B is an important molecule for maintaining HpSC-like features in HBV-related HCC cases.

The available Chip-Seq data (<http://genome.ucsc.edu/>) revealed that several transcriptional factors, such as c-Myc and TCF, preferentially bound to the immediate 5' upstream sequence of the predictive transcription

initiation site of KDM5B. Moreover, c-Myc is one of the transcription factors known to be activated by HBx. To determine whether c-Myc regulates KDM5B expression, we silenced c-Myc expression with a c-Myc-specific siRNA in Hep3B and Huh7 cells. Consistently, we found that inhibition of c-Myc resulted in the suppression of KDM5B and EpCAM expression (Figure 4D-F).

KDM5B predicts poor prognosis in HCC cases

We performed IHC analysis of cohort 3 cases. We confirmed the nuclear accumulation of KDM5B stained by an anti-KDM5B antibody (Figure 5A). After

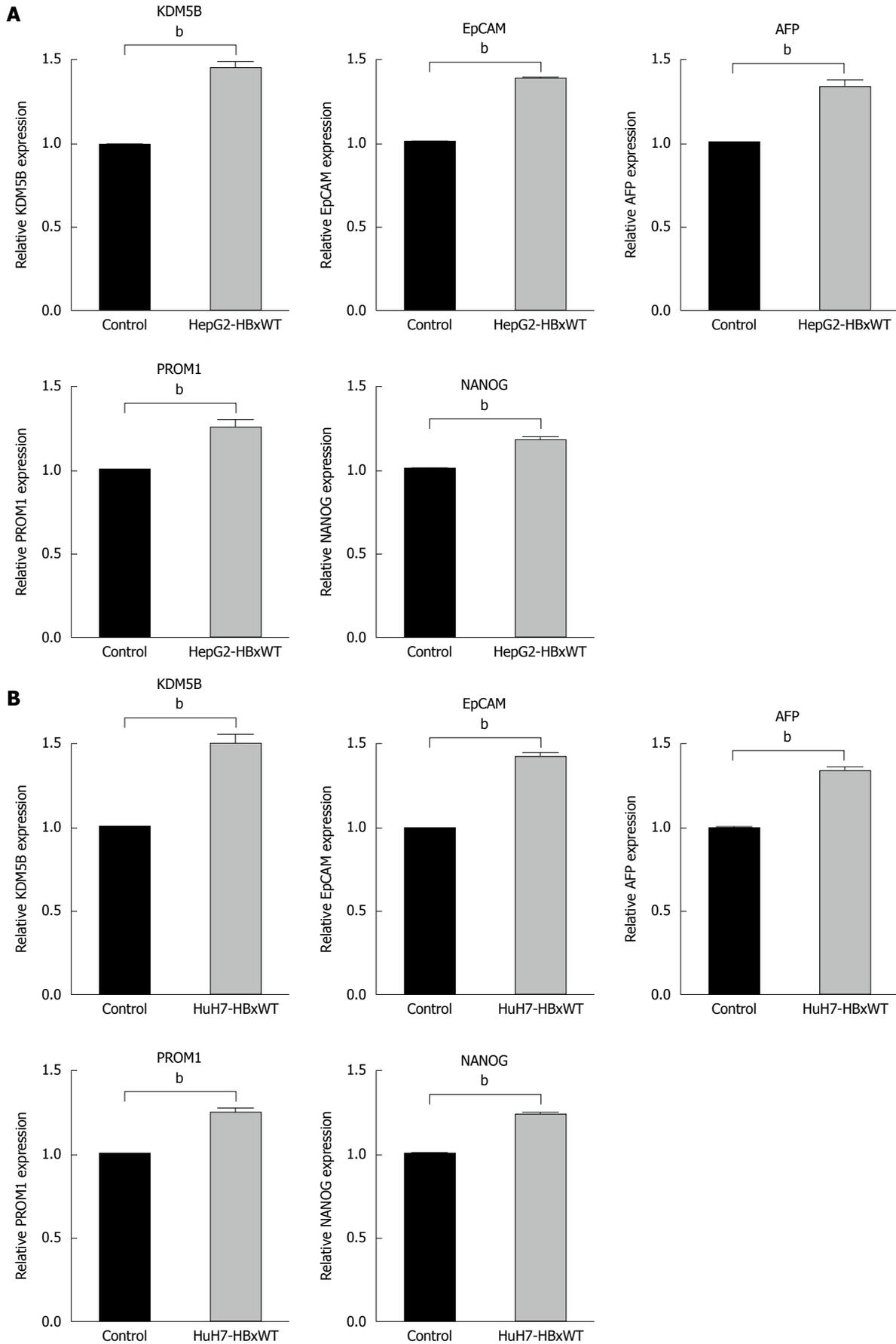


Figure 3 Introduction of hepatitis B virus X protein is associated with KDM5B and HpSC markers expression. A: Expression analysis of KDM5B and HpSC markers (EpCAM, AFP, PROM1, and NANOG) in HepG2 cells; B: Expression analysis of KDM5B and HpSC markers in Huh7 cells.

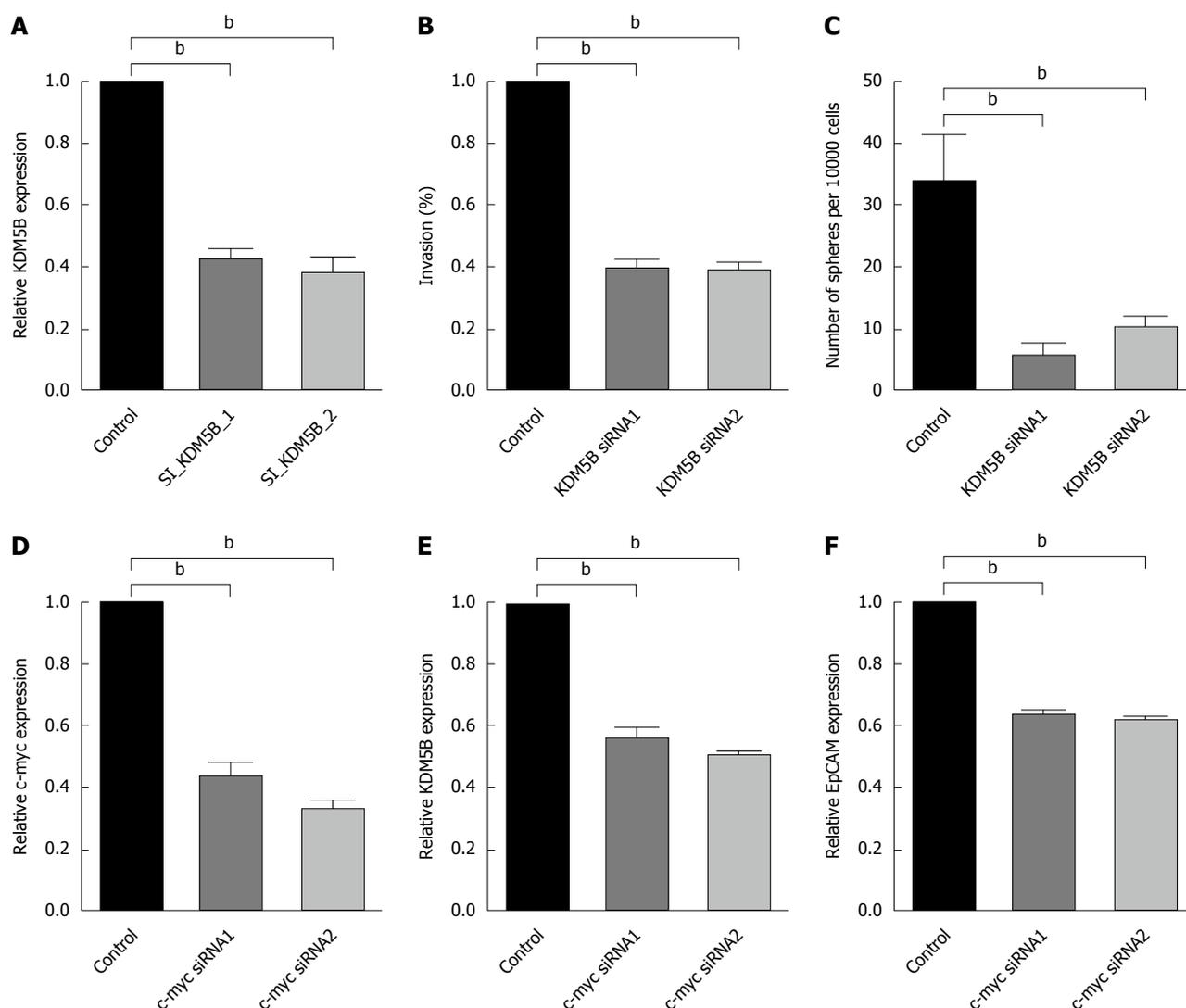


Figure 4 Inactivation of KDM5B is associated with stem-like features. Effect of KDM5B siRNA on KDM5B expression (A); Cell invasion of Huh7 cells transduced with KDM5B siRNA as determined by the Boyden chamber cell invasion assay (B); Spheroid formation of Huh7 cells transduced with KDM5B siRNA. A vertical axis indicates a total number of spheroids from 1000 cells (C); c-Myc mediated silencing of KDM5B and HpSC markers (D). Effect of c-Myc siRNA on the endogenous levels of c-Myc (D), KDM5B (E), and EpCAM (F) in Huh7 cells.

evaluating the clinicopathological characteristics of EpCAM-positive and -negative HCC cases, we found that EpCAM-positive HCCs were associated with a significantly high frequency of KDM5B-positive cases (Figure 5B). We further identified the survival outcome of these cases by Kaplan-Meier survival analysis. KDM5B-positive HCCs were associated with significantly lower overall survival outcomes within three years compared with KDM5B-negative HCCs ($P < 0.01$) (Figure 5C).

DISCUSSION

HBV is the smallest human hepatotropic DNA virus, which mainly infects host hepatocytes and causes a spectrum of pathological processes from acute hepatitis and chronic hepatitis, to serious end-stage liver diseases such as hepatic cirrhosis and primary HCC. Studies of its epidemiology and natural history

have shown that approximately 25% of chronic hepatitis B patients will develop HCC^[23,24]. Although the pathogenesis of HBV-related HCC has not been identified, many studies have suggested that HBx is one of the risk factors and is strongly implicated in hepatocarcinogenesis.

The role of epigenetic regulation in cancer biology, especially that of the histone lysine demethylases (KDMs), has been the subject of several studies^[25-27]. These chromatin structure modifiers have been increasingly shown to facilitate several steps of cancer progression^[28-30]. Several KDMs have been implicated in tumor growth, angiogenesis, invasion, metastasis, and tumor-related chemoresistance. KDM5B specifically removes methyl residues from methylated lysine 4 of histone 3, consequently repressing gene transcription. The cancer stem-like cell (CSC) hypothesis has drawn much attention^[31]. CSCs possess stem cell characteristics, including self-

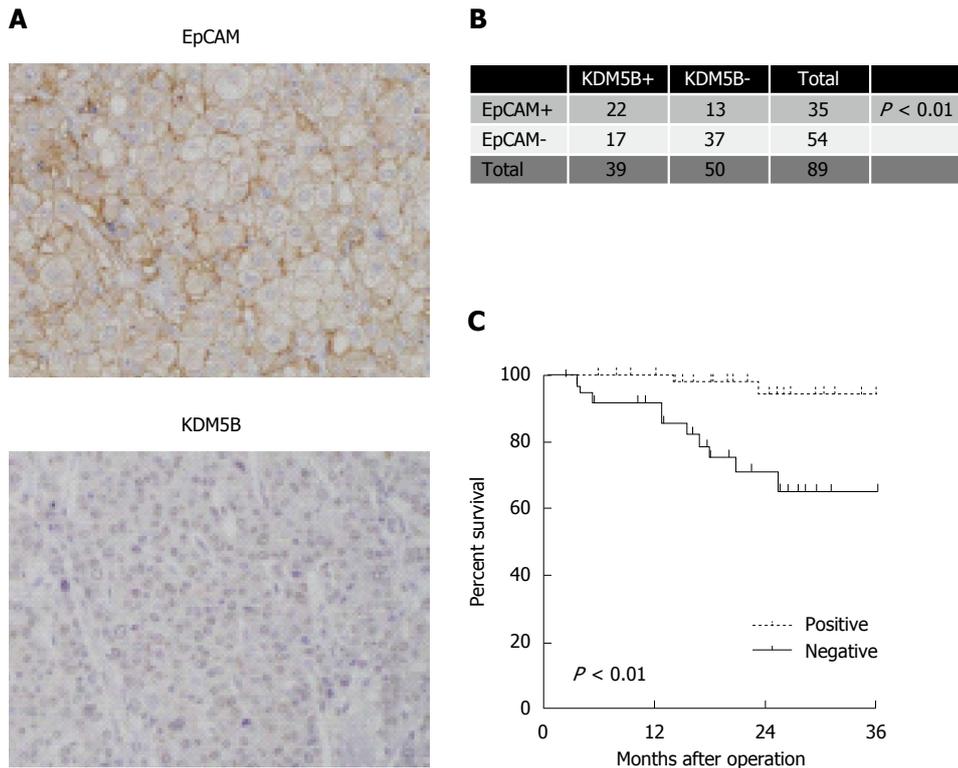


Figure 5 KDM5B protein expression is associated with EpCAM protein expression and poor prognosis. A: Photographs of immunohistochemistry staining with anti-KDM5B and EpCAM antibodies in 89 Japanese hepatocellular carcinoma (HCC) cases; B: Summary of KDM5B and EpCAM expression; C: Kaplan-Meier plot of overall survival of cohort 3 cases based on their classification by KDM5B expression.

renewal, chemotherapy resistance, and metastasis. On the basis of the role of KDM5B in cancer stem-like features, we hypothesized that KDM5B is actively involved in the poor prognosis of HCC patients.

In this study, we showed that KDM5B were associated with poor prognosis of HBV-related HCC. In various HBV proteins, HBx strongly up-regulated KDM5B expression in HBV-related HCC cases. Moreover, KDM5B expression is associated with the increased presence of CSC features, including enhanced tumor sphere formation, cell migration, and invasion, and is related with poor prognosis. The administration of a KDM5B inhibitor suppressed HpSC-like features in HBV-related cases. Therefore, we consider that KDM5B could be a potential therapeutic target against HBV-related HCC. Many researchers reported that HBx-MYC interaction was associated with HBV-related hepatocarcinogenesis^[32-34]. However, its mechanism is not yet clear. Our results indicated that KDM5B was a central molecule of HBx-MYC introduced hepatocarcinogenesis.

Recently, Wang *et al.*^[35] reported that KDM5B affected the poor prognosis of HCC cases through regulating p15 and p27. Similarly, in the present study, KDM5B was related to the poor prognosis of HCC patients. Our data show that KDM5B, which is activated by HBx, is a useful marker for poor prognosis in HBV-related HCC cases. Moreover, we demonstrated the possibility that suppression of KDM5B may improve the poor phenotype of HBV-related HCCs.

Our new knowledge is useful for the diagnosis of severe HCC patients. Histone demethylases KDM5B may be an important therapeutic target against HBV-related HCC cases.

In conclusion, HBx induced HpSC-like features in hepatocellular carcinoma by activating KDM5B. Moreover, HBx and KDM5B interaction related poor prognosis of hepatocellular carcinoma cases.

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COMMENTS

Background

The role of epigenetic regulation in cancer biology has been the subject of several studies. These chromatin structure modifiers have been increasingly shown to facilitate several steps of cancer progression. However, the epigenetic regulation of hepatocellular carcinoma has not been elucidated.

Research frontiers

Previous study showed that KDM5B affected the poor prognosis of hepatocellular carcinoma (HCC) cases through regulating p15 and p27. However, the relation between HCC background and KDM5B has not yet clear.

Innovations and breakthroughs

This is the first study evaluating that KDM5B, which is activated by HBx, a

useful marker for poor prognosis in HBV-related HCC cases. Moreover, the authors demonstrated the possibility that suppression of KDM5B may improve the poor phenotype of HBV-related HCCs.

Applications

HBx activated the histone demethylase KDM5B and induced hepatic progenitor cell (HPC)-like features in HCC. Presented results suggested that histone demethylases may be an important therapeutic target against HBV-related HCC cases.

Peer-review

Authors demonstrated that HBx activates the histone demethylase KDM5B and induces HPC-like features in HCC. This is the first study evaluating that KDM5B, which is activated by HBx, a useful marker for poor prognosis in HBV-related HCC cases. Moreover, they demonstrated the possibility that suppression of KDM5B may improve the poor phenotype of HBV-related HCCs.

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