

# Homeobox containing 1 inhibits liver cancer progression by promoting autophagy as well as inhibiting stemness and immune escape

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**Abstract.** Homeobox containing 1 (HMBOX1) is a novel transcription repressor that is significantly downregulated in human liver cancer tissues and cell lines, but the exact biological function of HMBOX1 in liver cancer is still unknown. We observed a negative association between HMBOX1 expression level and the clinical stages of liver cancer. HMBOX1 also increased the LC3 II/LC3 I ratio, the endogenous autophagy marker, and inhibited the p38/AKT/mTOR pathway. Furthermore, cancer stem cell specific genes, including CD133, KLF4, ESG1 and SOX2, were significantly downregulated upon HMBOX1 overexpression. Finally, the susceptibility of HepG2 cells to NK cell-mediated cytotoxicity was increased by HMBOX1 overexpression and weakened by siRNA-mediated inhibition of HMBOX1. All these findings indicated that HMBOX1 expression in hepatocytes could protect against the progression of liver cancer, and the underlying mechanisms may include promoting autophagy, inhibiting CSC phenotype and increasing the sensitivity of tumor cells to NK cell cytotoxicity. Therefore, HMBOX1 may be useful for developing new treatments for liver cancer.

## Introduction

Liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) in adults, and hepatoblastoma (HB) mainly in children, is one of the most malignant tumor types with poor response to drugs used at present against cancer (1-4). Traditional treatments for liver cancer include surgery, radio-frequency ablation and chemotherapy. However, liver cancer

is usually diagnosed at an advanced stage and the patients therefore miss the opportunity for surgical resection. Systemic chemotherapy via or trans-arterial chemoembolization is the second line of treatment, but the overall response rate is rather low due to the high chemoresistance of liver cancer cells (5,6). Therefore, dissecting the underlying mechanisms of liver cancer development and progression is essential for developing new therapeutic drugs and strategies.

HMBOX1 (homeobox containing 1), a novel human homeobox gene, was first isolated from the human pancreatic cDNA library. HMBOX1 has the atypical homeobox domain with 78 amino acids and a putative HNF1-N domain, and is classified into the HNF homeobox class of the Hmbox family (7-9). Recently, several studies have reported the possible biological functions of HMBOX1. Su *et al* demonstrated that HMBOX1 was the key factor in the differentiation of bone marrow stromal cells (BMSCs) to endothelial cells (ECs) by regulating IP-10 and Ets-1 (10). Ma *et al* reported that HMBOX1 regulated intracellular free zinc levels by interacting with MT2A, inhibiting apoptosis and promoting autophagy of VECs (11). In addition, HMBOX1 could directly bind to telomeric double-stranded DNA and helped in telomere maintenance in cells with ALT (alternative lengthening of telomeres) (12).

In our previous study, we found that HMBOX1 acted as a transcriptional repressor of interferon  $\gamma$  (IFN- $\gamma$ ) in natural killer (NK) cells (13,14). Furthermore, HMBOX1 was localized in both the cytoplasm and nucleus, and distributed widely in many tissues, including the liver. Notably, HMBOX1 was expressed in significantly lower levels in hepatic carcinoma tissues compared to adjacent healthy tissues (8,15). Therefore, HMBOX1 may play a role in the progression of liver cancer, although the exact biological function of HMBOX1 in liver cancer is still unknown.

In this study, we demonstrated that the expression level of HMBOX1 was negatively associated to the differentiation degree and clinical stage of liver cancer. Furthermore, we also revealed that HMBOX1 could significantly enhance autophagy and downregulate the 'stemness' genes in liver cancer cells. Notably, HMBOX1 overexpression increased the sensitivity of tumor cells to NK cell cytotoxicity and increased NK function. These results indicated that HMBOX1 may be

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a novel therapeutic target for human liver cancer that is worth investigating.

## Materials and methods

**Cell lines and cell culture.** The human natural killer cell line NK-92 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in a-MEM supplemented with 12.5% horse serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 12.5% fetal bovine serum (FBS), 100 U/ml rhIL-2, 0.1 mM P-mercaptoethanol and 0.02 mM folic acid. Human liver cancer cell line HepG2 (HCC/HB) (16), human hepatoma cell line PLC/PRF/5, human immortalized liver cell line HL7702 and murine hepatoma cell line Hepa1-6 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 medium supplemented with 10% FBS. All these cell lines were conserved in our laboratory.

**Liver cancer specimens.** Paraffin sections from 14 liver specimens of liver cancer patients, including 10 males and 4 females with the age of  $54.6 \pm 4.9$  years old, were procured from Qilu Hospital of Shandong University (Jinan, China) and written informed consents were obtained from all patients. The samples were collected from July 2014 to May 2015, and all of them were processed for routine histological examination and were classified by their degree of differentiation (poorly, moderately and well-differentiated) and clinical stage (I to III). The use of the liver specimens was approved by the Ethics Committee of Qilu Hospital of Shandong University and was consistent with the standards established by the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013).

**Animal model.** Dimethylnitrosamine (DEN, 25 mg/kg) were injected intraperitoneally into 2-week-old male C57BL/6 mice ( $n=10$ , 6.0–6.5 g) once a week for three times total, followed by 500  $\mu$ l/kg Carbon tetrachloride (CCL4) once a week for 12 times total. Mice at 42 weeks old were anesthetized by lidocaine (100 mg/kg) intraperitoneally. Mice with visible tumors ( $\geq 0.5$  mm) on the surface of the liver were used in this study. Tumor tissues and age-matched healthy liver tissues were collected and either fixed in 4% paraformaldehyde for further analysis.

Animal experiment protocols were approved by the Ethics Committee of Shandong University. Mice were housed in a temperature-controlled pathogen-free environment on a 12-h light/12-h dark cycle, and had access to food and water *ad libitum*.

**Immunohistochemistry.** After preconditioning, the sections were incubated overnight with a primary antibody in a humidified chamber at 4°C. Anti-HMBOX1 mAb (dilution 1:100; cat. no. 2A5F4 mAb) as previously described (8) was used as the primary HMBOX1 antibody. After washing with PBS, the sections were incubated with anti-mouse secondary antibody with no dilution (cat. no. SP-9002; Zsbio, Beijing, China) for 30 min at 37°C. The sections were washed again with phosphate-buffered saline (PBS) and stained with DAB solution (Zsbio) according to the manufacturer's protocol, and

the nuclei were dyed with hematoxylin. PBS was used in place of the primary antibody as a negative control.

**Immunofluorescence.** HepG2 cells transfected with pEGFP-N1-HMBOX1 or siRNA-HMBOX1 were cultured in 96-well plates for 6 h. After a 30-min fixation with 1% paraformaldehyde, the cells were blocked with 5% BSA at room temperature and incubated overnight with an LC3B antibody (dilution 1:500; cat. no. 2775; Cell Signaling Technology, Danvers, MA, USA) in a humidified chamber at 4°C. The cells were then washed with PBS and incubated with an anti-rabbit secondary antibody (dilution 1:200; cat. no. 8889; Cell Signaling Technology) for 1 h at 37°C in the dark. The nuclei were counterstained with DAPI and fluorescently-labeled cells were visualized at the requisite excitation wavelength (LC3B: 594 nm; DAPI: 340 nm) under a fluorescence microscope (Olympus TH4-200; Olympus Corp., Tokyo, Japan) at x400 magnification.

**Transfection and RNA interference.** Cells were transfected with either specific siRNA against HMBOX1 or the negative control using Lipofectamine™ 2000 (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The siRNAs (Target sequence: GGAAGTTCATATGGGAATA) were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

**Cytotoxicity assay.** The cytotoxic activity of NK-92 cells against HepG2 cells was assessed by MTT assay. HepG2 cells transfected with different plasmids were seeded in 96 well plates at  $1 \times 10^4$  cells/well 24 h after transfection. NK92 cells were added at a ratio of 10:1, 5:1 or 2.5:1, and incubated for an additional 6 h. MTT (Sigma-Aldrich, Merck; St. Louis, MO, USA) solution was prepared at 10 mg/ml and 20  $\mu$ l was added to the medium. The cells were incubated for another 4 h and the absorbance was determined at 570/630 nm using a multifunctional microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The percentage of cytotoxicity was calculated by the formula:  $\text{lysis (\%)} = 1 - (\text{OD}_{\text{E+T}} - \text{OD}_{\text{E}}) / \text{OD}_{\text{T}} \times 100\%$ ; E, effector cell group; T, target cell group; E+T, effector cell group and target cell group.

**Quantitative real-time PCR analysis.** Total RNA was extracted from cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and used to synthesize cDNA using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol.

The specific transcripts were detected via Real-time PCR (qRT-PCR) with SYBR Green Master Mix (Toyobo, Osaka, Japan) using an iCycleriQ Real-Time PCR system (Bio-Rad Laboratories, Inc.). The expression of specific transcripts was normalized to GAPDH levels. The qRT-PCR began with a step of denaturation (95°C for 25 sec), annealing (60°C for 20 sec) and extension (72°C for 30 sec) by 35 cycles. For the conventional RT-PCR, it began with a step of denaturation (95°C for 30 sec), annealing (60°C for 30 sec) and extension (72°C for 60 sec) by 35 cycles, and the production was separated by Gel electrophoresis (1.5%) with ethidium bromide. ImageJ software (version 1.4.3.67; National Institutes of

Health, Bethesda, MD, USA) was used for densitometry analysis. Forward and reverse primers were shown in Table 1.

**Western blotting.** Total protein was extracted from cells lysed with a RIPA lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl and 1 mM PMSF], and the concentrations were determined with BSA methods. The proteins (30 µg/lane) were separated by SDS-PAGE on a 10% polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (PVDF) (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBS/0.1% Tween-20 for 1 h at room temperature. Then the protein were probed with specific antibodies (1:1,000): anti-HMBOX1 (cat. no. ab97643; Abcam, Cambridge, UK), anti-p38 and anti-p-p38 (cat. nos. 8690 and 4511; Cell Signaling Technology), anti-AKT and anti-p-AKT (cat. nos. 4685 and 4060; Cell Signaling Technology), anti-mTOR and anti-p-mTOR (cat. nos. ab32028 and ab109268; Abcam), anti-LC3 (cat. no. 2775; Cell Signaling Technology) and β-actin (cat. no. sc-58673; Santa Cruz Biotechnology). The protein were incubated with secondary antibody (dilution 1:1,000; cat. nos. A0216 and A0208; Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at room temperature and the bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) and analyzed with Alpha Ease FC software (Bio-Rad Laboratories, Inc.).

**Flow cytometry.** The cells were phenotypically analyzed by flow cytometry with the following antibodies (1:10): PE-cy5-labeled anti-Fas (cat. no. 556641; BD Pharmingen BD Biosciences, San Diego, CA, USA), PE-conjugated anti-NKG2A (cat. no. FAB1059P; R&D Systems, Minneapolis, MN, USA), FITC-conjugated anti-NKG2D and PE-conjugated anti-PD-L1 (cat. nos. 11-5878-73 and 12-5983-42; eBiosciences, San Diego, CA, USA). The cells were stained with a saturating amount of the antibodies for 1 h at 4°C. After washing with PBS, the stained cells were acquired using a FACS Calibur system (BD Biosciences, San Jose, CA, USA) and analyzed with WinMDI 2.0 software (Scripps Research Institute, La Jolla, CA, USA).

**Statistical analyses.** All data are presented as the mean ± SD of three or more independent experiments. Statistical significance was calculated using Kruskal Wallis test, post hoc tests (Dunn's test, Mann Whitney U with Bonferroni's correction applied) and Student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference.

## Results

**HMBOX1 expression is correlated to the differentiation of liver cancer.** Our previous study (8) revealed that the expression of HMBOX1 in liver cancer was lower than that in adjacent non-cancerous tissues. To elucidate whether HMBOX1 expression was associated with the development of liver cancer, tissue specimens with varying degrees of differentiation and of different clinical stages were analyzed. As shown in Fig. 1A, HMBOX1 expression revealed a significant negative association with the degree of tumor differentiation (P<0.05) as well

Table I. Sequences of forward and reverse primers.

Gene	Sequence of primers (5'-3')
HMBOX1	Forward: AACCTGGCGCTACACTAAG Reverse: TCCTTTCTCCAGGTAAATCGAC
CD133	Forward: ACATGAAAAGACCTGGGGG Reverse: GATCTGGTGTCCAGCATG
Klf4	Forward: GCGGCAAACCTACACAAAG Reverse: CCCCCTGTGTTTACGGTAGT
NANOG	Forward: TTTGTGGGCCTGAAGAAACT Reverse: AGGGCTGTCCTGAATAAGCAG
Sox2	Forward: GCGAACCATCTCTGTGGTCT Reverse: GGAAAGTTGGGATCGAACAA
ESG	Forward: GCGCAGTATCACAGCCTTAAA Reverse: TCAATCTCTTGGCGATTTC
APOA1BP	Forward: TTCAGCGTGGACCAACTTATG Reverse: GGCCTTTTGGGGTAATAGATGG
CYP2W1	Forward: CCGATTTGACTACCGGGACC Reverse: CGTCCACATAGCTGCACAC
CYP46A1	Forward: GTGCGCTCCAGACTGTGTTT Reverse: CCAGGTCTATGACTCTCCGCT
CYP26B1	Forward: TGGACCTCCTCATTGAGAGCA Reverse: GGCATAGCCGCAAAGATCA
HPD	Forward: GAACCTCTAGCCTACAGGGG Reverse: TCTTTGTTCCAGGGGTTGAGC
Fas	Forward: TCTGGTTCTTACGTCTGTTGC Reverse: CTGTGCAGTCCCTAGCTTTCC
GAPDH	Forward: GAAGGTGAAGGTCTGGAGT Reverse: CATGGGTGGAATCATATTGGAA

as the clinical stage (Fig. 1B). Furthermore, both mRNA and protein levels of HMBOX1 were suppressed in the HepG2 and PLC/PRF/5 cell lines compared to the immortalized HL7702 hepatocyte cell line (Fig. 1C and D). In the mouse liver cancer model induced by DEN and CCL<sub>4</sub>, liver HMBOX1 expression level was also significantly decreased in cancer tissue compared with normal tissue (Fig. 2A). HMBOX1 expression in the murine Hepal-6 cell line was also lower than in primary murine hepatocytes (Fig. 2B and C). All these results indicated an important role of HMBOX1 in hepatocarcinogenesis.

**HMBOX1 promotes autophagy in liver cancer cells.** Autophagy is a common tumor suppression mechanism that prevents cellular damage and maintains cellular homeostasis. To elucidate the function of HMBOX1 on cell autophagy, HepG2 cells were transfected with either HMBOX1 siRNA or pEGFP-N1-HMBOX1 plasmids to suppress or overexpress HMBOX1 respectively. After 48 h of transfection, autophagy was detected by assessing the levels of autophagic protein, microtubule-associated protein 1 light chain 3B (LC3B). As shown in Fig. 3A, the expression of LC3B was increased in HepG2 cells overexpressing HMBOX1. To further validate

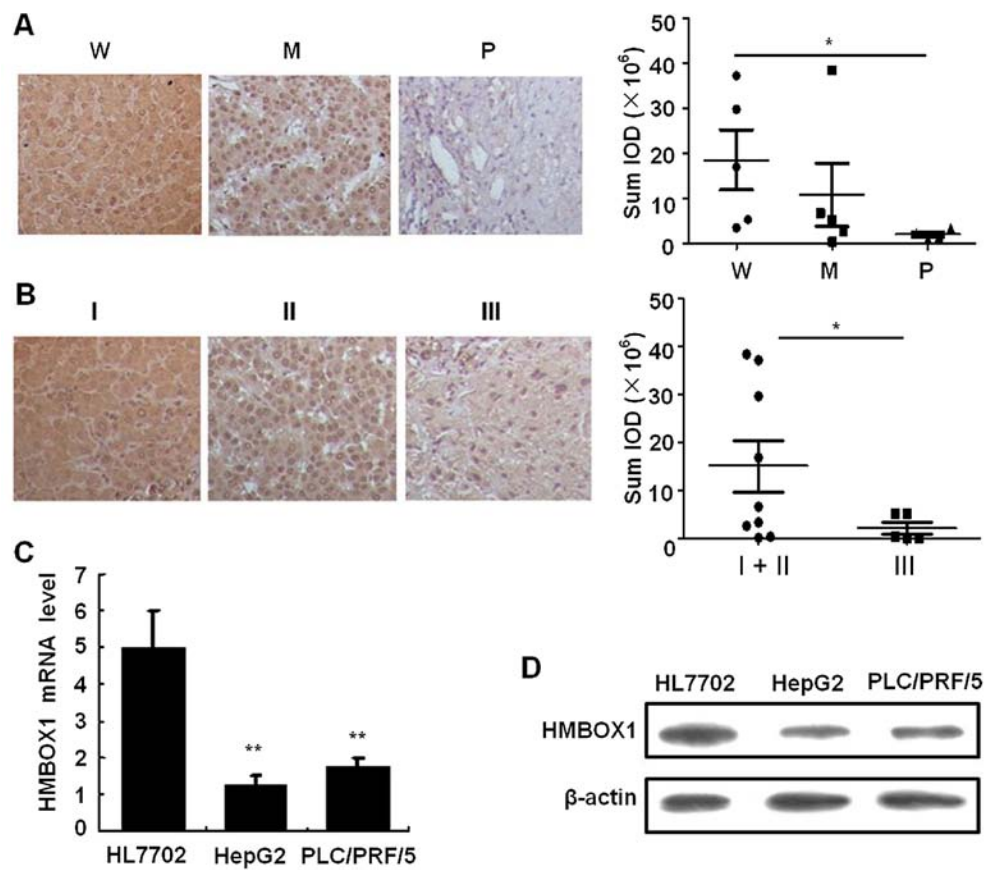


Figure 1. Expression of homeobox containing 1 (HMBOX1) is negatively associated to the differentiation and clinical stage of human liver cancer. (A) The relationship between HMBOX1 level and differentiation degree of liver cancer, which is classified as well-differentiated (W), moderately differentiated (M) and poorly differentiated (P). Kruskal-Wallis Test (non-parametric test) revealed a statistical difference between specimens with different degrees of differentiation. Then, post hoc tests (Dunn's test, Mann Whitney U with Bonferroni's correction applied) were used, and the results showed a significant difference between the poorly differentiated and the moderately differentiated group. (B) The relationship between HMBOX1 expression level and clinical stage of liver cancer (I-III). Kruskal Wallis Test was used to analyze the difference between stages I+II and III. Original magnification,  $\times 400$ . Grayscale values were quantified by Image Pro-Plus and values were indicated as relative density.  $^*P < 0.05$ . (C) The mRNA level of HMBOX1 in HL7702, HepG2 and PLC/PRF/5 cells was detected by real-time PCR. Each value represented the mean  $\pm$  SD of triplicate tests.  $^{**}P < 0.01$ . (D) The protein level of HMBOX1 in HL7702, HepG2 and PLC/PRF/5 cells was analyzed by western blotting and normalized against  $\beta$ -actin.

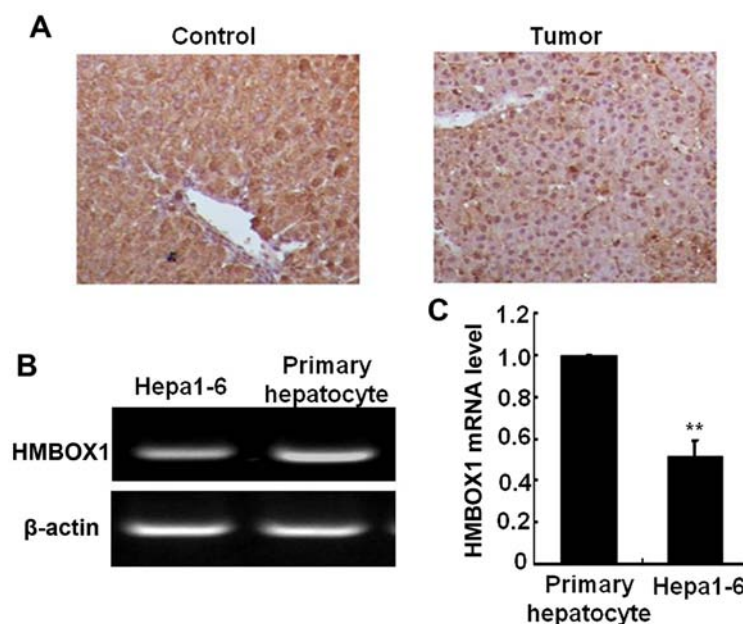


Figure 2. The expression of homeobox containing 1 (HMBOX1) is decreased in animal liver cancer. (A) The levels of HMBOX1 were decreased in tumor tissue ( $n=3$ ) compared with the control tissue ( $n=3$ ). (B) The mRNA level of HMBOX1 in mouse hepatoma cell line Hep1-6 and mouse primary hepatocytes was detected by RT-PCR. (C) Statistical results of HMBOX1 mRNA levels normalized against  $\beta$ -actin. Each value represented the mean  $\pm$  SD of triplicate tests.  $^{**}P < 0.01$ .



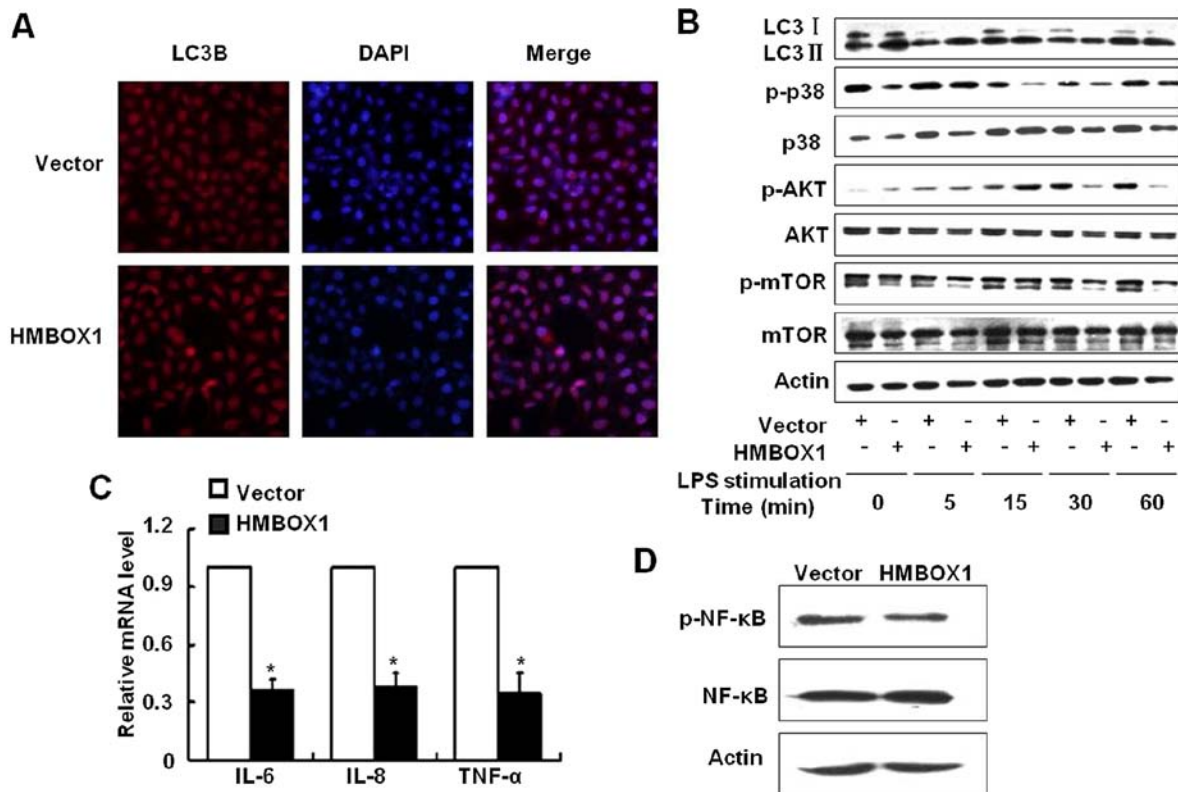


Figure 3. Homeobox containing 1 (HMBOX1) promotes autophagy in liver cancer cells. (A) Immunostaining of LC3B in HepG2 cells after transfection with pEGFP-N1 (Vector) or pEGFP-N1-HMBOX1 (HMBOX1). (B) HepG2 cells transfected with the pEGFP-N1-HMBOX1 plasmid were stimulated with 10  $\mu$ g/ml LPS for 0, 5, 15, 30 and 60 min. Western blotting was used to analyze LC3 II/LC3 I ratio, total and phosphorylated p38, and AKT and mTOR protein levels relative to  $\beta$ -actin. (C) The expression levels of IL-6, IL-8 and TNF- $\alpha$  mRNA in HepG2 cells transfected with control or the pEGFP-HMBOX1 plasmid were detected by real-time PCR 3 days after transfection. Each value represents the mean  $\pm$  SD of triplicate tests. \* $P$ <0.05. (D) Western blot analysis of total and phosphorylated NF- $\kappa$ B in HepG2 cells 48 h after transfection with pEGFP-N1 or pEGFP-N1-HMBOX1.

the effect of HMBOX1 on autophagic flux, HepG2 cells were stimulated with 10  $\mu$ g/ml lipopolysaccharide (LPS) and the LC3 II/LC3 I ratio was analyzed by western blotting. As shown in Fig. 3B, the LC3 II/LC3 I ratio was upregulated in HMBOX1 overexpressing HepG2 cells, clearly indicating that HMBOX1 promoted autophagy of HepG2 cells.

Mammalian target of rapamycin (mTOR) prevents mammalian cell autophagy (17,18), and is activated by the PI3K/protein kinase B (Akt) pathway kinases and the p38 mitogen-activated protein kinase (MAPK). HMBOX1 overexpression inhibited the phosphorylation of mTOR as well its upstream activators p38MAPK and AKT, indicating a possible underlying mechanism (Fig. 3B). HMBOX1 overexpression also suppressed the levels of pro-inflammatory IL-6, IL-8 and TNF- $\alpha$  (Fig. 3C), along with inhibiting NF- $\kappa$ B activation (Fig. 3D). These results indicated that HMBOX1 could suppress cancer development by upregulating autophagy, which inhibited cancer-associated inflammation.

**HMBOX1 inhibits expression of 'stemness' genes in liver cancer cells.** Considering that HMBOX1 expression is negatively associated to the differentiation of liver cancer, we hypothesized that HMBOX1 may affect the expression of genes regulating stemness and differentiation in liver cancer cells. Cancer stem cells (CSCs) are a subpopulation of tumor cells which resemble normal stem cells with respect to their ability to self-renew and differentiate into multiple cell types (19).

CD133, a CSC marker, is related to tumor initiation and progression, as well as colony formation ability and differentiation potential of the cells (13,14). Bahnassy *et al* reported that increased expression of CD133 in the liver tumor microenvironment promoted liver cancer progression (20). CSCs are also known to endogenously express stemness-related genes like OCT3/4, SOX2, NANOG and KLF4 in many cancers (21-23). To investigate whether HMBOX1 contributed to the regulation of stemness-related genes, their expression was analyzed by real-time PCR. As shown in Fig. 4A, the mRNA levels of CD133, KLF4, ESG1 and SOX2 were significantly reduced in HepG2 cells 3 days after pEGFP-N1-HMBOX1 transfection. Contrasting results were observed after HMBOX1 silencing, with an increasing tendency of the aforementioned genes level, however, no statistical difference was observed (Fig. 4B).

To gain deeper insights into the regulatory function of HMBOX1 in liver CSCs, mRNA microarray analysis was performed in HepG2 cells overexpressing HMBOX1. A cluster of liver metabolism-related genes was upregulated, including apolipoprotein A-1 binding protein (APOA1BP), cytochrome P450 proteins (CYP2W1, CYP46A1 and CYP26B1) and 4-hydroxyphenylpyruvate dioxygenase (HPD) (Fig. 4C). As anticipated, the mRNA levels of these genes were significantly downregulated upon HMBOX1 silencing (Fig. 4D). These results indicated a potential function of HMBOX1 in reversing the phenotype of liver CSCs to that of normal hepatocytes.

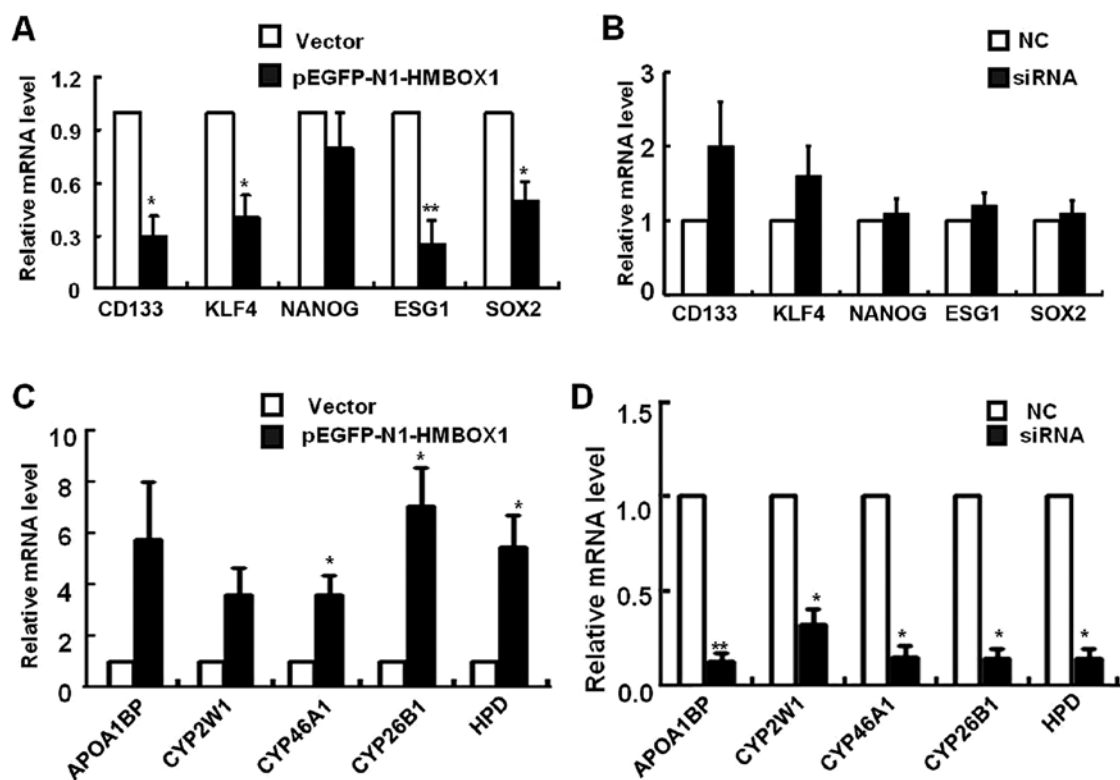


Figure 4. Homeobox containing 1 (HMBOX1) inhibits the expression of stemness genes in liver cancer cells. The mRNA levels of stemness genes in HepG2 cells transfected with (A) pEGFP-N1/pEGFP-N1-HMBOX1 plasmid for 3 days and (B) HMBOX1 siRNA or negative control. The mRNA expression of characteristic hepatocyte markers in HepG2 cells 48 h after being transfected with (C) pEGFP-N1/pEGFP-N1-HMBOX1 and (D) siRNA/NC. Samples were analyzed with real-PCR, and each value represents the mean  $\pm$  SD of triplicate tests. \*P<0.05, \*\*P<0.01.

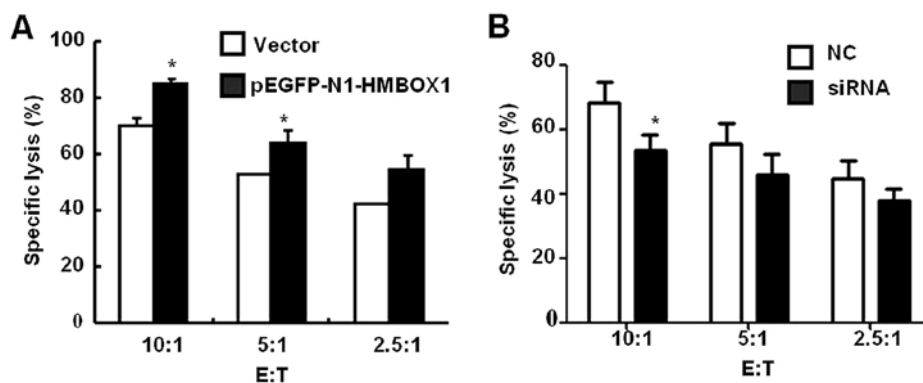


Figure 5. Homeobox containing 1 (HMBOX1) increases the sensitivity of liver cancer cells to NK cell-mediated cytotoxicity. The specific cytotoxic activity of NK cells against HepG2 cells transfected with (A) pEGFP-N1/pEGFP-N1-HMBOX1 and (B) HMBOX1 siRNA or negative control were analyzed by MTT assay. E, NK-92 cells; T, HepG2 cells. Each value represents the mean  $\pm$  SD of triplicate tests. \*P<0.05.

*HMBOX1 increases the sensitivity of liver cancer cells to NK cell-mediated cytotoxicity.* NK cells, the major cellular component of innate immunity, predominantly reside in the liver (24-28). Several studies have revealed significantly reduced cytotoxicity of NK cells obtained from liver perfusates of liver cancer patients (29), indicating that a functional defect of NK cells is responsible for the failure of antitumor immune responses (30). To elucidate whether HMBOX1 is involved in cancer-induced immune system escape, the human NK-92 cell line was used as the effector cell population. After co-incubation with the target HMBOX1 overexpressing or silencing HepG2 cells, NK cell cytotoxicity was detected by MTT assay. As shown in

Fig. 5A, HMBOX1 overexpression increased the sensitivity of HepG2 cells to NK cell cytotoxicity by 15-25%, while HMBOX1 knockdown aggravated the resistance of HepG2 cells to NK cell cytotoxicity (Fig. 5B). These findings indicated that low expression of HMBOX1 would help liver cancer cells escape NK cell-mediated immune surveillance.

*HMBOX1 regulates the expression of genes associated with NK-cell cytotoxic activity.* The interaction between active/inactive NK cell receptors and cancer cell surface ligands is the first step of NK cell-mediated target cell killing. Therefore, we determined whether HMBOX1 could influence

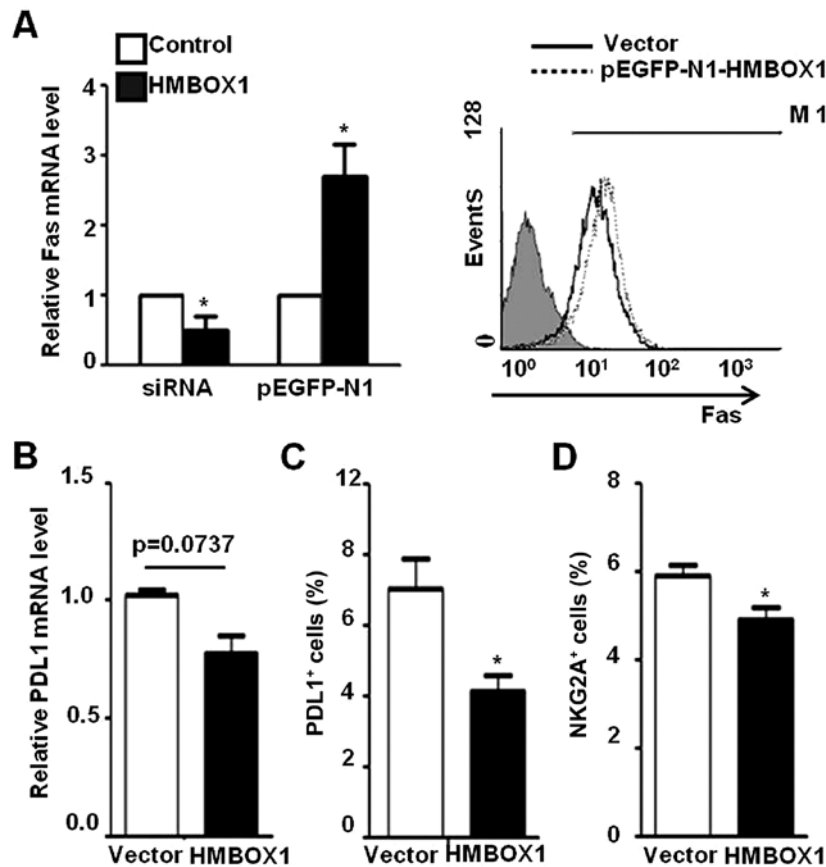


Figure 6. Homeobox containing 1 (HMBOX1) regulates the expression of genes associated with NK-cell cytolytic activity. (A) The mRNA and protein levels of Fas (CD95) were analyzed by real-PCR and FACS respectively 48 h after transfection. PD-L1 (B) mRNA and (C) protein levels were determined by real-PCR and FACS, respectively, 48 h after transfection. (D) NKG2A expression on NK-92 cells were determined by FACS after 12 h of co-incubation with HepG2 cells transfected with pEGFP-N1 (empty vector) or pEGFP-N1-HMBOX1 plasmids. Each value represents the mean  $\pm$  SD of triplicate tests. \* $P < 0.05$ .

the expression of factors involved in NK cell-mediated cytotoxicity. As shown in Fig. 6A, Fas expression was increased in HepG2 cells transfected with pEGFP-N1-HMBOX1, and decreased upon HMBOX1 silencing by siRNA. In addition, the expression of PD-L1 was decreased in HepG2 cells transfected with pEGFP-N1-HMBOX1 (Fig. 6B and C). However, no differences were observed in the expression levels of ULBP2 and HLA-A/B/C, as well as anti-inflammatory factors IL-10 and TGF- $\beta$  (data not shown). In addition, the inhibitory NKG2A receptor expressed on NK-92 cells was suppressed after co-incubation with HepG2 cells transfected with pEGFP-HMBOX1 (Fig. 6D) while no significant changes were observed with the activating receptor NKG2D (data not shown). These results indicated a protective role of HMBOX1 against liver cancer development via promotion of NK cell surveillance.

## Discussion

HMBOX1 is a novel transcription repressor whose structure and function have not been fully characterized. In a previous study, we found that the expression of HMBOX1 was lower in liver carcinoma cells (8,15), although the biological significance was unclear. In the present study, we found a significant negative association between high HMBOX1 expression and differentiation degree of liver cancer (Fig. 1), indicating a role of HMBOX1 in liver cancer development.

Autophagy is a normal cellular process that degrades dysfunctional and unnecessary components. Studies have revealed a role of autophagy in various human liver diseases (31-33). Earlier research demonstrated that autophagy exerts a suppressive function on tumors. During initial carcinogenesis, autophagy limited inflammation, p62 accumulation and oxidative stress response, thereby inhibiting genomic instability by maintaining cellular metabolic homeostasis (34). In addition, autophagy is known to inhibit inflammasome activation by removing endogenous sources of inflammasome agonists (35,36). Autophagy-deficient macrophages have insufficient microbial clearing capacity, resulting in increased bacillary burden and excessive pulmonary inflammation characterized by neutrophil infiltration, IL-17 response and increased IL-1 $\alpha$  levels (37). In the present study, we found that HMBOX1 overexpression inhibited inflammation and promoted autophagy via the p38MAPK/AKT/mTOR signaling pathway in HepG2 cells (Fig. 3). This suggests that downregulated HMBOX1 would reduce autophagy, thereby destroying the protective role of the latter in inflammatory regulation and ultimately leading to the progression of liver cancer.

Many studies have suggested a hierarchical organization of heterogeneous cancer cells with a rare subset of cancer cells with stem cell features, known as cancer stem cells (CSCs), at the apex. CSCs are defined as a group of cells with high tumorigenicity, metastasis, and resistance to chemotherapy

and radiation. Furthermore, CSCs have been implicated in tumor relapse after therapy (38-40). CSCs have also been detected in liver cancer tissues, and the role of CD133 as a CSC marker in liver cancer has been confirmed in several studies. CD133-expressing cancer cells are responsible for tumor initiation or progression, and display stem-cell-like properties such as colony-forming ability and multi-potent differentiation potential (41,42). Notably, stem cell-related genes like CD133, KLF4, ESG1 and SOX2 were inhibited with increased HMBOX1 expression (Fig. 4). Concomitantly, liver metabolism-related genes like HPD and cytochrome p450 were upregulated by HMBOX1 overexpression. These results indicated that HMBOX1 suppressed the stemness of CSCs and maintained the normal metabolic function of hepatocytes, which is indispensable for physiological functions of the liver.

The liver is an important organ of the immune system as it harbors a large number of innate immune cells. Studies have increasingly revealed that NK cells play a critical role in tumor immuno-surveillance and act as the first line of the defense against carcinoma cells. The recognition between the NK cell receptors and cancer cell surface ligands is the first step in NK cell-mediated cell killing. Liver cancer can inhibit antitumor immune responses in the host through various mechanisms (43). We observed that HMBOX1 overexpression improved NK cell-mediated antitumor immune responses (Fig. 5).

HMBOX1 mediated PD-L1 expression could be the underlying molecular mechanism which attenuates the immunosuppressive effect induced by PD-1/PD-L1. In addition, HMBOX1 also increased Fas expression in cancer cells, and consequently Fas/FasL-mediated apoptosis. Lastly, co-incubation of HMBOX1-overexpressing HepG2 cells and NK-92 cells downregulated NKG2A expression of the latter, which indirectly triggered NK cell-mediated antitumor response (Fig. 6).

Collectively, to the best of our knowledge, we revealed for the first time that HMBOX1 expression level was negatively associated with the development of liver cancer. HMBOX1 possesses several protective roles, which include promoting cell autophagy, downregulating stemness-related genes, increasing HepG2 sensitivity to NK cell cytotoxicity and enhancing the function of NK cells, all of which suppress the development of liver cancer. These findings indicated that the targeted use of HMBOX1 agonists may enhance the therapeutic effects on liver cancer, and presents a new perspective on the mechanism of development and clinical therapy of liver cancer.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article and are available from the corresponding author upon reasonable request.

### Authors' contributions

HZ performed the research on cell autophagy, stemness gene analysis and NK cell cytotoxic activity, and was a major contributor in writing the manuscript. HJ and QH analyzed and interpreted the liver cancer specimen data and NK cell-mediated cytotoxicity. JZ performed the final verification of the manuscript and was involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

The study was performed according to a protocol approved by the Ethics Committee of Qilu Hospital of Shandong University and was consistent with the standards established by the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013). Written informed consents were obtained from all patients. Animal experiment protocols were approved by the Ethics Committee of Shandong University.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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