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*Original Article*

# Down-regulation of HOPX associates with poor prognosis of human hepatocellular carcinoma

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**Abstract:** HOPX has been reported to function as tumor suppressor in a variety of cancers. However, the role of HOPX is still unknown in hepatocellular carcinoma (HCC). In this study, we investigated the expression of HOPX aiming to define their prognostic significance in HCC. A total of 198 patients were included in this study and immunohistochemically evaluated for HOPX. Quantitative real time PCR(qRT-PCR) and western blot were performed to assess HOPX expression in HCC cell lines as well as 10 HCC tissues with paired non-cancerous tissues. Down-regulation of HOPX was found in HCC cell lines and HCC tissues by qRT-PCR and western blot. Patients with low HOPX expression had significantly poorer prognosis than those with high expression. Multivariate analysis identified HOPX as an independent predictor for the outcome of HCC patients. In addition, patients with lower HOPX expression exhibited more aggressive clinicopathologic features, including larger tumor size, more advanced stage and vascular invasion. HOPX may performed as a tumor suppressor in HCC. Our data suggested for the first time that HOPX status may correlate with the development of HCC and predict poor prognosis of these patients, providing rationale for developing a novel target therapy against HCC.

**Keywords:** Hepatocellular carcinoma, HOPX, prognosis

## Introduction

Hepatocellular carcinoma (HCC) is one of the top 10 prevalent and mortal cancers worldwide [1]. Despite the evolving of various treatments including surgery, radiofrequency ablation and transarterial chemoembolization, the prognosis of HCC patients are still poor due to the high recurrence rate and death rate [2-4]. It is essential to explore novel diagnostic measures for early detection and efficient therapeutic targets to improve the strategy of HCC.

HOP homeobox (HOPX), also known as HOP, NECC1, LAGY or OB1, was initially identified as an essential element for cardiac development [5, 6]. HOPX has been reported to be critical for the differentiation of multiple types of cells including T cells [7], lung alveolar cells [8] and keratinocytes [9]. HOPX expressed ubiquitously in various normal tissues, but is epigenetically silenced in many malignancies, such as lung cancer [8], gastric cancer [10], colorectal cancer [11], nasopharyngeal carcinoma [12], breast cancer [13] as well as head and neck cancer [14]. Although methylation of HOPX promoter has been implicated in its inactivation, the underlying mechanism has yet to be fully defined [8, 10-14]. Moreover, restored HOPX expression suppresses tumor metastasis and enhances chemosensitivity [12]. Above findings suggest that HOPX acts as a tumor-suppressor in a variety of cancer. However, the association between HOPX and HCC remains unknown.

In this study, we firstly examined the expression of HOPX in HCC and analyzed its correlation with clinical features to provide possible rationale for practical use.

## Materials and methods

### *Cell culture*

Human liver cell line HL-7702 and HCC cell lines MHCC97L, HepG2, MHCC97H, Huh7 and HCCLM3 were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cells were maintained in DMED (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), penicillin (100 units/ml) and streptomycin (100 units/ml) at 37°C in humidified 5% $CO_2$ incubator.

### *Patients and specimens*

Fresh tumor tissues with paired non-cancerous liver tissues of 10 HCC patients ranging from 34 to 68 year old were obtained during hepatoectomy from Nanfang Hospital between 2014 and 2017 for real-time PCR and western blot analysis. None of these pathologically confirmed patients received radiotherapy or chemotherapy before surgery. A total of 198 paraffin-embedded HCC samples with detailed follow-up clinical data from Nanfang Hospital and Traditional Chinese Medicine-Integrated Hospital between 2007 and 2017 were also included in this study for immunohistochemical anslysis. Clinical and pathological data of the 198 patients with HCC were collected, including age, tumor size, Child-Pugh scores and vascular invasion, et al. Written Ethics Approval and Patient Consent from the Research Ethics Committee of Nanfang Hospital and Traditional Chinese Medicine-Integrated Hospital were obtained.

### *Quantitative real-time PCR (qRT-PCR) analysis*

Total RNA was extracted using Trizol reagent (TaKaRa, China) according to the manufacturer's instructions. Then mRNA was reversely transcribed into cDNA using PrimeScript RT reagent Kit (TaKaRa). To evaluate the mRNA level of HOPX, qRT-PCR was performed on a strataneMx3005P qRT-PCR System using SYBR Green qRT-PCR master mix (TaKaRa). GAPDH was used as the internal control. The primers used in qRT-PCR assay were listed as follows: HOPX-F 5-CACCACGCTGTGCCTCAT-3, HOPX-R 5-CCATTTCTGGGTCTCCTCC-3, GAPDH-F 5-ACCCAGAAGACTGTGGATGG-3, GAPDH-R 5-TCTAGACGGCAGGTCAGGTC-3. All samples were normalized to internal controls and fold changes were calculated based on relative quantification ($2^{-\Delta\Delta Ct}$).

### *Western blot analysis*

Protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots then were probed with primary antibodies against GAPDH (Abcam, ab181602), HOPX (Abcam, ab195974), followed by HRP (horseradish peroxidase)-labeled secondary antibodies. The hybridization signal was detected using enhanced chemiluminescence (ECL). GAPDH was used as a loading control.

### *Immunohistochemistry (IHC)*

After deparaffinization and rehydration, paraffin-embedded sections were subjected to high pressure for 2 min for antigenic retrieval. Then the slides were incubated overnight at 4°C with anti-HOPX antibody (Abcam, ab106251) before developing with DAB for 2 min. The expression level was calculated using following equation: staining index = staining intensity × percentage of positive cells. The staining intensity was defined as following: 0, no staining; 1, weak, light yellow; 2, moderate, yellow brown; and 3, strong, brown. The proportion of positive cells was defined as follows: 1, < 10%; 2, 10-35%; 3, 35-70%; 4, > 70%. The staining index was scored at 0-12. Staining index score ≥ 6 was indicated as high HOPX expression, while < 6 was indicated as low expression. Two pathologists examined and scored IHC results without knowing the clinical characteristics and prognosis.

### *Statistical analysis*

Statistical analyses were performed using SPSS19.0. Fisher's exact test or $\chi^2$-test was used to compare categorical variables, and Student's t-test was used to analyze continuous variables. The Kaplan-Meier method was used to estimate cumulative survival rates, and differences in survival rates were assessed with log-rank test. Variables suggesting to be prognostic factors in univariate analysis ($P < 0.05$) were subjected to multivariate analysis using a COX proportional-hazard regression model. $P < 0.05$ was considered to indicate statistical significance.

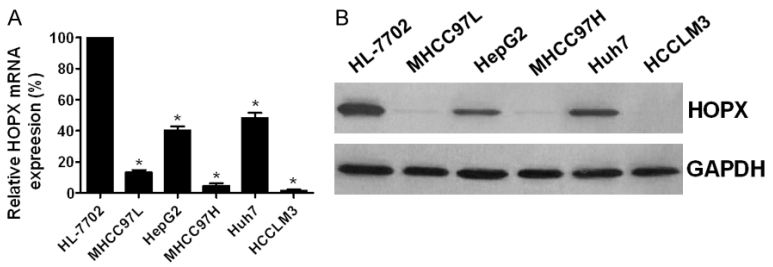


**Figure 1.** Reduced HOPX expression in HCC cell lines. The expression of HOPX was examined by qRT-PCR (A) and western blotting (B); $*P < 0.01$ compared with HL-7702.

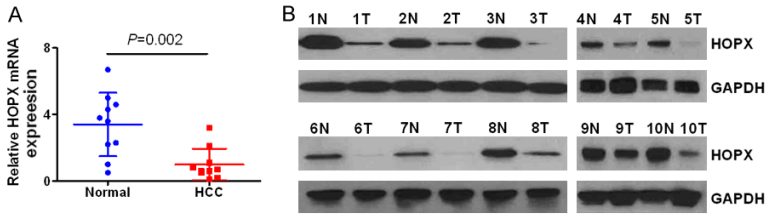


**Figure 2.** Decreased HOPX expression in HCC Tissues. The expression of HOPX was examined by qRT-PCR (A) and western blotting (B) in 10 pairs of HCC tissues and adjacent normal tissues.

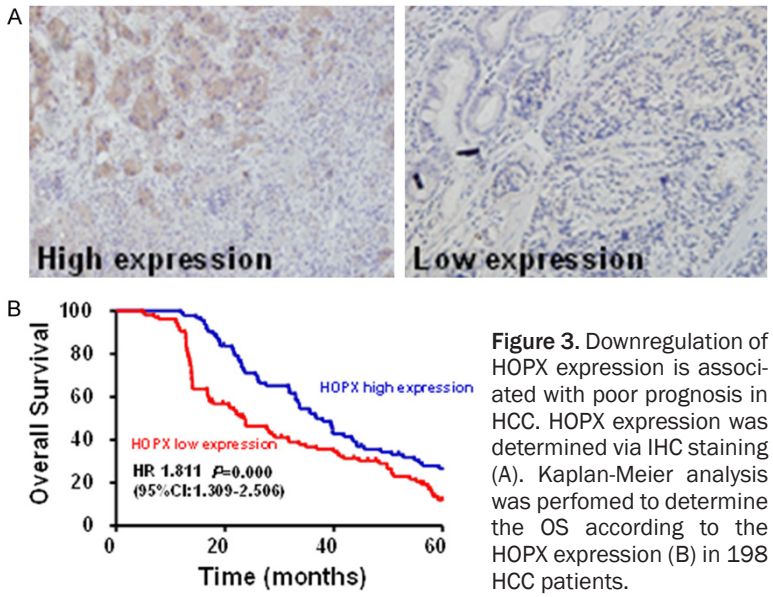


**Figure 3.** Downregulation of HOPX expression is associated with poor prognosis in HCC. HOPX expression was determined via IHC staining (A). Kaplan-Meier analysis was perfomed to determine the OS according to the HOPX expression (B) in 198 HCC patients.

## Results

*The expression of HOPX was decreased in HCC cell lines*

To determine the expression of HOPX in HCC cell lines, qRT-PCR and western blot were performed in human hepatocyte cell line HL-7702 and HCC cell lines MHCC97L, HepG2, MHCC97H, Huh7 and HCCLM3. Both HOPX mRNA (**Figure 1A**) and protein (**Figure 1B**) levels were significantly down-regulated in HCC cell lines. Notably, HOPX expression in HepG2 and Huh7 were much higher than that in MHCC-97L, MHCC-97H and HCCLM3. Moreover, the low-metastatic cell lines HepG2 and Huh7 exhibited stronger HOPX expression than the high-metastatic cell lines MHCC97L, MHCC97H and HCCLM3.

*HOPX expression was down-regulated in HCC tissue*

We subsequently investigated the expression of HOPX in 10 HCC tissues and paired non-cancerous tissues. Among the 10 HCC tissues, decreased HOPX expression were detected in all tumor tissues in comparison to paired non-cancerous tissues at both mRNA (**Figure 2A**) and protein level (**Figure 2B**). These data suggested that HOPX downregulation was accompanied with occurrence of HCC and might serve as a tumor suppressor.

*Less HOPX expression correlated with more aggressive phenotype in HCC patients*

To evaluate the association of HOPX expression with tumor behaviors, comparisons of the clinicopathlogic features with HOPX expression were made. We examined the expression of HOPX in 198 paraffin-embedded HCC samples with immunohistochemical analysis. 93 of 198 (47%) paraffin-embedded HCC tissues showed high HOPX expression, while 105 of 198 (53%) HCC tissues showed low HOPX expression. In HCC specimens, HOPX was located in the cytoplasm (**Figure 3A**), which is similar to previous results in other malignancies [8, 10-15]. The association between HOPX expression and the clinipa-

**Table 1.** Correlation between HOPX expression and clinical features of 198 HCC patients

| Factors | HOPX (n=198) | | $X^2$ | *P* |
|---|---|---|---|---|
| | Low expression (n=105) | High expression (n=93) | | |
| Age (years) | | | 0.005 | 0.941 |
| ≤ 60 | 48 (52.7%) | 43 (47.3%) | | |
| > 60 | 57 (53.3%) | 50 (46.7%) | | |
| Gender | | | 0.942 | 0.332 |
| Male | 63 (50.4%) | 62 (49.6%) | | |
| Female | 42 (57.5%) | 31 (42.5%) | | |
| AFP (ng/ml) | | | 1.091 | 0.296 |
| ≥ 20 | 72 (50.7%) | 70 (49.3%) | | |
| < 20 | 33 (58.9%) | 23 (41.1%) | | |
| Tumor size (cm) | | | 4.244 | 0.039 |
| ≥ 5 | 65 (59.6%) | 44 (40.4%) | | |
| < 5 | 40 (44.9%) | 49 (55.1%) | | |
| Child-Pugh | | | 2.412 | 0.120 |
| A | 76 (50.0%) | 76 (50.0%) | | |
| B | 29 (63.0%) | 17 (37.0%) | | |
| BCLC stage | | | 12.266 | 0.002 |
| A | 43 (42.6%) | 58 (57.4%) | | |
| B | 25 (54.3%) | 21 (45.7%) | | |
| C | 37 (72.5%) | 14 (27.5%) | | |
| Hepatitis history | | | 0.003 | 0.956 |
| Yes | 85 (53.1%) | 75 (46.9%) | | |
| No | 20 (52.6%) | 18 (47.4%) | | |
| Vascular invasion | | | 11.863 | 0.001 |
| Yes | 34 (75.6%) | 11 (24.4%) | | |
| No | 71 (46.4%) | 82 (53.6%) | | |
| Liver cirrhosis | | | 3.285 | 0.070 |
| Yes | 64 (48.5%) | 68 (51.5%) | | |
| No | 41 (62.1%) | 25 (37.9%) | | |

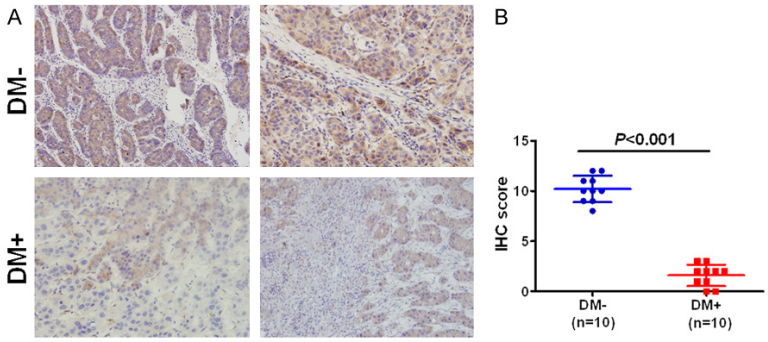


**Figure 4.** Attenuated HOPX expression is associated with distant metastasis in HCC patients. Immunohistochemical staining (A) and statistical analysis (B) of HOPX in primary HCC tissues without or with distant metastasis. DM+, HCC tissues with distant metastasis; DM-, HCC tissues without distant metastasis.

thological characteristics was shown in **Table 1**. Patients with less HOPX expression exhibited more aggressive phenotypes, such as more lesions with the more vascular invasion and larger tumor size (**Table 1**). Notably, HOPX protein levels were substantially lower in the HCC tissues with distant metastasis than those without metastasis (**Figure 4A**, **4B**).

*Low HOPX expression was an independent predictive factor for poor prognosis of HCC patient*

The median follow-up time for overall survival (OS) was 48 months for all patients included in this study. Patients with low HOPX expression had significantly poorer OS than those with high HOPX expression [HR, 1.811; 95% CI, 1.309-2.506, **Figure 3B**; **Table 2**]. The median OS were 39.9 and 31.8 months for patients with high HOPX expression and low HOPX expression, respectively. The COX proportional hazards model was employed to determine the independent factors for OS. In univariate analysis, HOPX expression status (HR, 1.811; 95% CI, 1.309-2.506), tumor size (HR, 3.798; 95% CI, 2.648-5.449), BCLC (Barcelona-Clí Liver Cancer) stage (HR, 3.474; 95% CI, 2.400-5.031) and vascular invasion (HR, 2.615; 95% CI, 1.714-3.989) were significantly correlated with OS (**Table 2**). Variables showing significant difference in univariate analysis were subjected to multivariate analysis. Notably, HOPX expression status was shown to be an independent prognostic factor for OS. Pa-

**Table 2.** Univariate analysis and Multivariate analysis of prognosis factors associated with overall survival

| Variables | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* | HR | 95% CI | *P* |
| Age, years (> 60 vs ≤ 60) | 0.992 | 0.715-1.377 | 0.964 | | | |
| Gender (Female vs male) | 1.259 | 0.936-1.693 | 0.128 | | | |
| AFP, ng/ml (≥ 20 vs < 20) | 1.188 | 0.655-1.984 | 0.454 | | | |
| Tumor size, cm (> 5 vs ≤ 5) | 3.798 | 2.648-5.449 | 0.000 | 6.119 | 3.991-9.381 | 0.000 |
| Child-Pugh (B vs A) | 1.433 | 0.775-2.681 | 0.248 | | | |
| BCLC stage (B+C vs A) | 3.474 | 2.400-5.031 | 0.000 | 6.468 | 4.288-9.756 | 0.000 |
| Hepatitis history (yes vs no) | 1.265 | 0.943-1.697 | 0.117 | | | |
| Vascular invasion (yes vs no) | 2.615 | 1.714-3.989 | 0.000 | 1.239 | 0.883-1.737 | 0.215 |
| Liver cirrhosis (yes vs no) | 0.954 | 0.699-1.301 | 0.764 | | | |
| HOPX (Low vs high expression) | 1.811 | 1.309-2.506 | 0.000 | 5.051 | 3.453-7.390 | 0.000 |

tients with low HOPX expression were nearly five times more likely to suffer from death than those with high HOPX expression (HR, 5.051; 95% CI, 3.453-7.390). Besides, tumor size (HR, 6.119; 95% CI, 3.991-9.381) and BCLC stage (HR, 6.468; 95% CI, 4.288-9.756) were recognized as independent prognostic factors for OS (**Table 2**).

## Discussion

Here we described that HOPX is constitutively down-regulated in HCC cells as well as in tumor specimens. More importantly, we firstly examined the prognostic effect of HOPX expression in HCC patients and found that decreased HOPX expression are correlated with high risk of poor outcomes. Multivariate analysis further supported our conclusion that HOPX expression was an independent prognostic factors for HCC patients. Therefore, the assessment of HOPX expression may provide additional information for evaluating patients' prognosis and could be a potential target for HCC treatment.

Like many other genes such as sirtuins [15] and NFAT transcription factor genes [16], HOPX serves as an oncogene or tumor suppressor in a tissue-specific pattern. The loss of HOPX expression has been implicated in many malignancies [8, 10-14], suggesting that HOPX might be a promising biomarker for the prediction of cancer outcomes. On the other hand, HOPX upregulation also has been reported in sarcoma and pancreatic cancer, and connect to tumor metastasis [17, 18]. However, there is little knowledge regarding the roles of HOPX in HCC. In current study, we found that HOPX expression was significantly decreased in HCC cell lines and HCC tissues, especially in HCC tissues from patients with distant metastasis. In addition, patients with low HOPX expression were more likely to exhibit aggressive clinicopathologic features and poorer OS, suggesting that HOPX might be a promising biomarker for predicting HCC patients' outcome, which might facilitate the selection of more appropriate individual therapies. Therefore, we assumed that HOPX act as a tumor suppressor in HCC.

It is well known that HOPX plays an important role in the development of normal cells and tissues, such as the epidermal cells [19] and the heart [5]. However, its function and the molecular mechanism in malignant disease remains unclear. In lung adenocarcinoma, HOPX was one of the critical nodes in a lineage-selective pathway that directly links effectors of airway epithelial specification to inhibition of metastasis. Knockdown of HOPX furnished cell to display basal epithelial characteristics, which was not sufficient to induced EMT and had little effect on the WNT signaling pathway [8]. In NPC, HOPX suppressed metastasis by mediating epigenetic silencing of SNAIL through the enhancement of histone H3K9 deacetylation in the promoter of SNAIL. Notably, the restoration of HOPX promoted expression of epithelial markers and inhibited mesenchymal markers expression, indicating that HOPX partially represses EMT in NPC [12]. Thus, whether HOPX participate in the EMT and metastasis of HCC are worthy further investigation and identify the functional mechanism of HOPX might

provide important clues leading to new molecular therapeutic targets in HCC treatment.

In summary, we demonstrated that HOPX down-regulation in HCC is associated with tumor aggressiveness and maybe a predictor for unfavorable outcomes. These findings highlight the importance of HOPX as a tumor suppressor of HCC, indicating that HOPX may serve not only as a prognostic factor but also as a therapeutic target.

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## Disclosure of conflict of interest

None.

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