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Clinical significance of down-regulated *HINT2* in hepatocellular carcinoma

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Abstract

To study the clinical significance of *HINT2* expression in patients with HCC.

We investigated *HINT2* mRNA expression in tumors and adjacent non-tumor hepatic tissues from 106 HCC patients using quantitative real-time PCR. Appropriate statistical methods were then applied to assess the relationships between the *HINT2* mRNA level and clinical parameters.

HINT2 was significantly down-regulated in HCC ($P < .0001$). No significant correlation was found between *HINT2* expression and clinicopathological factors in HCC patients. A Kaplan–Meier survival curve showed that *HINT2* expression is related to recurrence-free survival ($P < .05$). Multivariate analyses revealed that tumor size and *HINT2* expression are risk factors for HCC recurrence.

HINT2 is down-regulated in HCC, and low *HINT2* expression predicts earlier tumor recurrence. *HINT2* expression may serve as a prognostic indicator of recurrence in HCC.

Abbreviations: HCC = hepatocellular carcinoma, *HINT2* = histidine triad nucleotide-binding 2, HR = hazard ratio, SPSS = statistical package for the social sciences, TCGA = the cancer genome atlas.

Keywords: *HINT2*, hepatocellular carcinoma, recurrence-free survival, prognostic indicator

1. Introduction

Hepatocellular carcinoma (HCC) is the second most frequently diagnosed gastrointestinal tumor worldwide and one of the most lethal cancers, especially in less developed countries.[1,2] Most HCCs originate from chronic hepatic diseases, especially chronic hepatitis viral infections, such as hepatitis C virus in North America and hepatitis B virus in Asia.[3] Hereditary liver disease and non-alcoholic fatty liver disease are also associated with HCC.[4] HCC development is a long-lasting process involving genetic changes that accumulate over time.[5] Although comprehensive treatments for HCC patients are available, their long-term survival remains dismal, owing to repeated recurrence and other therapies.[6] Additionally, most patients with better survival rely on early diagnosis by serological tests and imaging. Histidine triad nucleotide-binding 2 (*HINT2*), a member of the HINT superfamily, exists extensively in several mammalian organs, including the liver, pancreas, and adrenal gland.[7] It is reported that the *HINT2* protein is localized exclusively in the mitochondrial matrix of liver cells.[8]*HINT2* knock-out experiments confirmed that *HINT2* is related to lipid and glucose homeostasis in mitochondria and cells.[9] Furthermore, *HINT2* is significantly down-regulated in colon carcinoma tissues,[10] and down-regulation of *HINT2* induces colon carcinoma cell migration and invasion by promoting hypoxia inducible factor-2α.[11] High expression of *HINT2* induced by progesterone or calcitriol plays a critical role in the inhibition of endometrial cancer cell growth by pathways that involve cell cycle arrest and apoptosis.[12] In brief, *HINT2* may function as a tumor suppressor in certain types of carcinoma. Although a significant effect by *HINT2* on cancer pathobiology is evident, its clinical significance in HCC remains poorly elucidated.

In this study, we investigated *HINT2* mRNA expression in tumor tissues and adjacent non-tumor tissues from HCC patients and analyzed the correlations between *HINT2* expression and clinical and pathological parameters.

2. Materials and Methods

2.1. Clinical samples and follow-up

A total of 106 paired tissues from different patients who underwent curative hepatectomy at the First Affiliated Hospital of Zhejiang University were selected. All consent forms were signed by the patients, and this research was authorized by the Ethics Committee

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of the First Affiliated Hospital of Zhejiang University. All patients met the pathological diagnostic criteria for HCC, and no patient received chemotherapy or radiation therapy before surgery. Each patient was followed up for 5 years or until death.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA concentration and purity were assessed at 260 and 280 nm. cDNA was synthesized from total RNA (2 μg) using M-MLV Reverse Transcriptase (Promega, San Luis Obispo, CA, USA) following the manufacturer's instructions.

2.3. Quantitative real-time PCR

Quantitative real-time PCR was performed on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Dimer Eraser Kit (Takara Biotechnology, Dalian, Liaoning, China). Amplification reactions containing 1 μl cDNA template, 0.3 μl of the forward and reverse primer each (10 μM), 0.2 μl 50 × ROX Reference Dye II (Takara) and 5 μl 2 × SYBR Premix Dimer Eraser were mixed and brought to a total volume of 10 μl. The primer sequences were as follows: 5′-GGACACCTACTCCTTGTGGC-3′ (forward) and 5′-CCCATCGTTGATCACAAGTCG-3′ (reverse) for *HINT2* and 5′-CTTAGTTGCGTTACACCCTTTC-3′ (forward) and 5′-CACCTTCACCGTTCCAGTTT-3′ (reverse) for β-actin. Amplification reactions proceeded as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95°C for 5 s, 55°C for 30 s and 72°C for 34 s. Relative quantification was performed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method, with β-actin as the internal control. To evaluate data reproducibility, all real-time PCRs were performed in triplicate.

2.4. The Cancer Genome Atlas (TCGA) data analysis

The results of prognosis analysis of HINT2 mRNAs expression datasets were validated in the TCGA datasets. TCGA-hepatic cancer mRNA data and clinical data (level 3) of the corresponding patients (365 tumor tissue) were downloaded from the TCGA Data portal. We used median value of mRNA expression level as the cutoff value to divide the data into low and high group. The expression analyses were carried out using BRB-ArrayTools (version 4.5, National Cancer Institute, Bethesda, MD, USA). As a result, a dataset of 471 HCC patients was generated for further statistical analysis.

2.5. Statistical analysis

Continuous variables are presented as means ± standard deviation. The paired *t* test was used to analyze differences in *HINT2* mRNA expression between paired tissues. Pearson Chi-Squared test was used to compare categorical variables, and Student *t* test was used for continuous variables. As the median is not affected by extreme values (outliers), we chose to divide the patients into high and low *HINT2* expression groups according to the median *HINT2* expression value. The Kaplan–Meier method was performed for survival curves, and the log-rank test was used to compare differences. Multivariate analysis was performed using the Cox proportional hazard regression model. A two-tailed P value $< .05$ was considered statistically significant. Statistical Package for the Social Sciences (SPSS 20.0 for Windows, SPSS, Chicago, IL, USA) was used for all statistical analyses. Graphs were created using GraphPad Prism (ver. 6.01; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Down-regulation of HINT2 mRNA in HCC

We performed quantitative real-time PCR in 106 paired samples of human HCC and corresponding non-tumor hepatic tissues to determine differences in *HINT2* mRNA expression. *HINT2* mRNA was down-regulated in approximately 70.8% of the paired tissue samples (Fig. 1A). The paired *t* test further demonstrated that *HINT2* was significantly ($P < .0001$) down-regulated in tumor tissues compared with the non-tumor counterparts (Fig. 1B).

3.2. Correlations between clinicopathological parameters and HINT2 expression

The HCC patients were divided into 2 groups according to the median *HINT2* expression value, high and low *HINT2*

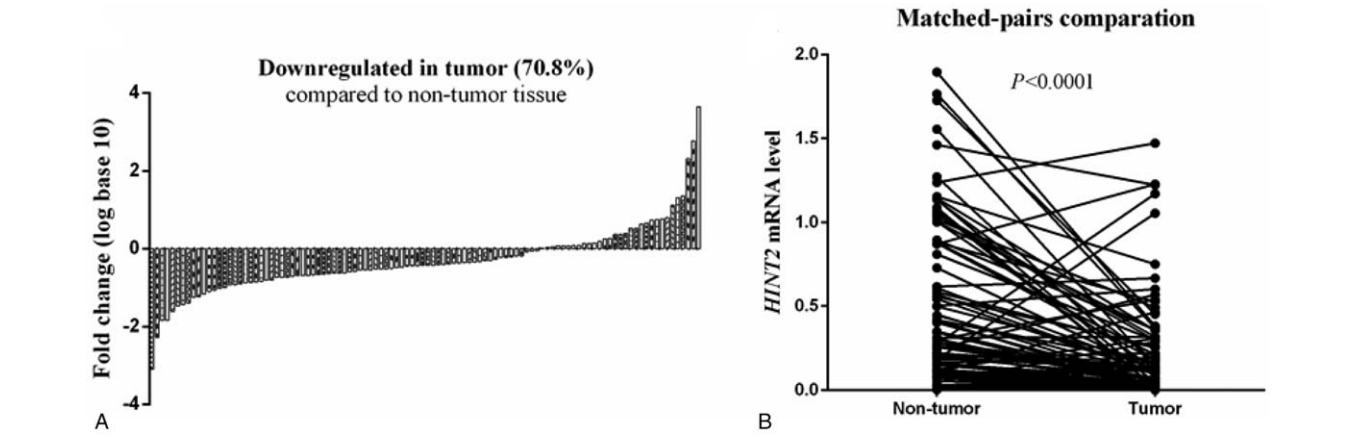

Figure 1. Expression of HINT2 mRNA in hepatocellular carcinoma (HCC) tumor and corresponding non-tumor tissues. (A) HINT2 was down-regulated in most HCC tumor (70.8%) tissues compared with the paired non-tumor tissues. (B) The paired T-test revealed that HINT2 was significantly down-regulated ($P < 0.0001$) in tumor compared with non-tumor counterpart tissues.

Table 1

Correlation between *HINT2* expression in tumor tissue with clinicopathological factors in hepatocellular carcinoma patients.

Variables	*HINT2* expression		*P* value
	Low n = 53	High n = 53	
Age (year)	56.4 ± 11.3	56.1 ± 10.8	.917
Gender (female/male)	3/50	8/45	.113
HBsAg (no/yes)	10/43	8/45	.607
HBV-DNA replication (no/yes)	33/20	30/23	.555
Liver cirrhosis (no/yes)	24/29	25/28	.846
Preoperative AFP	5207.5 ± 14417.7	5216.5 ± 14088.0	.722
Tumor number (1/>1)	11/42	13/40	.644
Tumor size (cm)	6.6 ± 3.2	6.5 ± 3.5	.510
PV or VI invasion (no/yes)	42/11	43/10	.771
Lymph node metastasis (no/yes)	44/9	46/7	.589
Intrahepatic metastasis (no/yes)	37/16	45/8	.065
Liver capsular invasion (no/yes)	41/12	40/13	.950
TNM stage (I/II-IV)	18/35	19/34	.839
Differentiation (well/moderate or poor)	23/30	29/24	.246

HBsAg = hepatitis B surface antigen, HBV-DNA = hepatitis B virus deoxyribonucleic acid, AFP = alpha-fetoprotein, PV = portal vein, VI = intrahepatic vein.

expression, with 53 patients in each group. The clinicopathological variables of each group are listed in Table 1, while all patients' demographic and clinicopathological characteristics are given in Supplementary Table 1, http://links.lww.com/MD/D398. There was no significant correlation between any clinicopathological parameter and *HINT2* mRNA expression.

3.3. Prognostic significance of HINT2 expression

We evaluated the prognostic significance of *HINT2* mRNA expression by Kaplan–Meier survival curves, which revealed no significant relationship between overall survival and *HINT2* expression ($P > .05$) (Fig. 2A). However, further analysis demonstrated that patients with high *HINT2* expression in their tumor tissues have substantially longer recurrence-free survival compared with patients with low *HINT2* expression ($P < .05$) (Fig. 2B). The TCGA data analysis verified a better survival with no HCC recurrence in high *HINT2* mRNA expression group (Fig. 2D), while overall survival was not affected by *HINT2* mRNA expression (Fig. 2C). Univariate analysis found that tumor size (<5 vs ≥5 cm), tumor size (<8 vs ≥8 cm) and *HINT2* expression were prognostic of recurrence-free survival (Table 2). Multivariate analysis using the Cox proportional hazards model showed that tumor size (<5 vs ≥5 cm; hazard ratio [HR] = 2.224, $P = .025$) and *HINT2* expression (HR = 0.560, $P = .047$) were independent prognostic factors in HCC patients (Table 2).

4. Discussion

HINT proteins are the most conserved members of the HINT family.[13] *HINT2* was initially identified as a functional gene involved in mitochondrial import signaling in yeast.[14] Korsisaari and colleagues found that *HINT2* was not required for murine development.[15] There may be other HINT family members that functionally compensate for the loss of *HINT2*.[16,17] Lenglet et al validated that *HINT2* serves as a regulator of steroid formation by a calcium ion-independent pathway in human adenocarcinoma cells.[8] A recent study showed that *HINT2* triggers mitochondrial calcium ion influx by regulating a mitochondrial calcium ion uniporter.[18] *HINT2* also plays a role in hepatic steatosis and hepatocellular energy metabolism.[19,20] Our research confirmed that *HINT2* mRNA was significantly down-regulated in HCC tumor tissues compared with corresponding non-tumor tissues among 106 paired samples, consistent with a previous study on HCC.[7] However, we did not find any significant correlation between *HINT2* expression and clinical parameters in HCC patients, although our findings revealed that *HINT2* plays an important role in diverse biological processes of cellular metabolism. Considering our limited number of samples, we cannot exclude sampling error; therefore, additional samples are needed to verify this conclusion. Our survival analysis showed that HCC patients with high *HINT2* expression have a long recurrence-free survival period, suggesting that *HINT2* expression is a potential prognostic biomarker of HCC recurrence. Because most cases of tumor recurrence are attributed to the dissemination of metastatic HCC cells,[21,22] we speculate that low expression of *HINT2* may promote invasion and metastasis of HCC. We find no relation between *HINT2* expression with overall survive, it may due to that there is no uniform method for postoperative treatment of liver cancer, thus, the postoperative treatments for patients and time to follow-up are different, which can deeply affect the overall survive of patients. As shown in the multivariate analysis, tumor size (<5 vs ≥5 cm) and *HINT2* expression are independent prognostic factors in HCC patients.

To date, several genes including castor zinc finger 1 (*CASZ 1*), cell division cycle associate 5(*CDCA5*) and *P16* serve as prognostic biomarkers for HCC.[23–25] Like many other cancers, HCC development is a multistep process involving altered expression of several genes. *HINT2* is likely one of these genes,

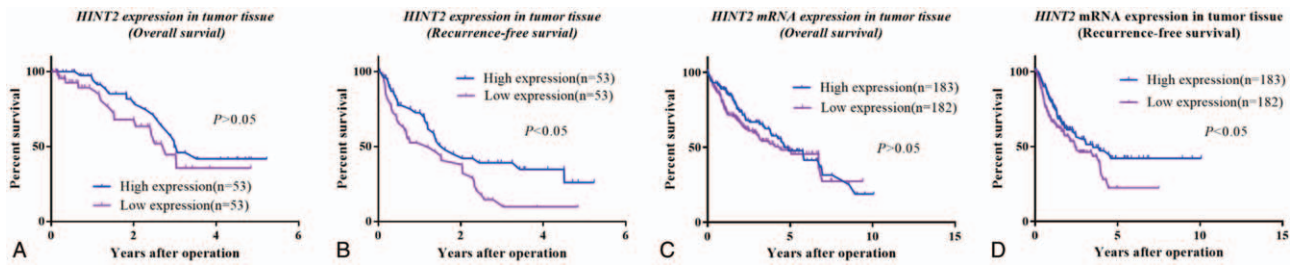

Figure 2. Survival curves of HCC patients with high and low *HINT2* expression were plotted using Kaplan–Meier analysis, and the differences in survival curves were evaluated by the log-rank test. (A) No significant difference in overall survival was found, but (B) recurrence-free survival rates differed significantly between the high and low *HINT2* expression groups. The TCGA data analysis revealed (C) overall survival was also not affected by *HINT2* expression, while (D) high *HINT2* expression group had better recurrence-free survival than low *HINT2* expression group.

Table 2

Risk factor analysis of recurrence-free survival in tumor tissue.

Prognositic factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (<60/≥60)	0.961 (0.578–1.600)	.880		
Gender (female/male)	0.969 (0.416–2.257)	.941		
HBsAg (no/yes)	0.977 (0.464–2.061)	.952		
HBV-DNA replication (no/yes)	1.250 (0.730–2.143)	.416		
Liver cirrhosis (no/yes)	0.923 (0.552–1.544)	.760		
Preoperative AFP (<20/≥20 ng/ml)	0.955 (0.567–1.607)	.862		
Preoperative AFP (<400/≥400 ng/ml)	0.870 (0.506–1.496)	.615		
Tumor size (<5/≥5 cm)	2.163 (1.247–3.754)	.006	2.224 (1.104-4.480)	.025
Tumor size (<8/≥8 cm)	1.939 (1.121–3.356)	.018	0.927 (0.404-2.127)	.859
PV or VI invasion (no/yes)	1.206 (0.638–2.278)	.564		
PVTT (no/yes)	1.376 (0.676–2.801)	.379		
Lymph node metastasis (no/yes)	1.102 (0.522–2.327)	.798		
Intrahepatic metastasis (no/yes)	1.849 (0.990–3.454)	.540		
Liver capsular invasion (no/yes)	1.234 (0.652–2.336)	.518		
TNM stage (I/II-IV)	1.681 (0.976–2.896)	.061		
Differentiation (well/moderate or poor)	0.242 (0.741–2.081)	.410		
HINT2 expression (low/high)	0.567 (0.339–0.950)	.031	0.560 (0.317–0.991)	.047

HBsAg = hepatitis B surface antigen, HBV-DNA = hepatitis B virus deoxyribonucleic acid, AFP = alpha-fetoprotein, PV = portal vein, VI = intrahepatic vein, PVTT = portal vein tumor thrombus, *HINT2* = histidine triad nucleotide binding protein 2.

but may not be as efficient as the tumor suppressors P53 and Rb.[26] However, our findings along with those of other current studies are not sufficient to recommend *HINT2* expression as a valuable predictor of HCC recurrence. Many problems should be taken into consideration for a new biomarker. The mow level of *HINT2* expression in HCC tissues does not make it a reliable biomarker. Unfortunately, recent studies have shown that there is no circulating cell-free *HINT2* DNA in blood samples.[27,28] In addition, based on our limited number of samples, it was difficult to extract a normal baseline value and to exclude other effects to minimize false-positive results.[29] Therefore, further studies are necessary to verify the sensitivity and specificity of *HINT2* expression as a prognostic factor before its clinical application. Evaluating the protein expression of *HINT2* by immunohistochemical staining in HCC samples is indispensable, because various factors may impact the process of mRNA translation. Future investigations should focus on methods to up-regulate *HINT2* expression in HCC, for example, certain types of drugs or proteins. Stabilization and even up-regulation of *HINT2* expression may result in elevated sensitivity to mitochondrial apoptosis in HCC,[7] hence improving the efficacy of chemotherapy and molecular targeted therapies.

In conclusion, we showed that *HINT2* expression is down-regulated in most HCC tissues. As a potential prognostic marker, low expression of *HINT2* in the tumor tissues of HCC patients may imply early recurrence after hepatectomy.

Author contributions

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Validation: Xiao-Hui Qian.
Visualization: Ling-Hui Chen.
Writing – original draft: Xiao-Hui Qian.
Writing – review & editing: Xiao-Hui Qian.

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