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Serum exosomal *hnRNPH1* mRNA as a novel marker for hepatocellular carcinoma

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Abstract

Background: Distinctive exosomal contents could be useful for cancer diagnosis and prognosis. However, little is known about whether serum exosomal heterogeneous nuclear ribonucleoprotein H1 (*hnRNPH1*) mRNA is a satisfactory biomarker for hepatocellular carcinoma (HCC).

Methods: Two hundred and ninety-one participants divided into four age- and gender-matched groups, including a HCC group (n = 88), a liver cirrhosis (LC) group (n = 67), a chronic hepatitis B (CHB) group (n = 68) and a healthy control group (n = 68), were enrolled. Serum exosomal *hnRNPH1* mRNA and *GAPDH* mRNA were measured using TaqMan real-time PCR, and the relative expression levels were calculated. Receiver operating characteristic (ROC) curves were constructed to evaluate the effectiveness of *hnRNPH1* mRNA alone and in combination with α -fetoprotein (AFP) in the diagnosis of HCC. The correlation between *hnRNPH1* mRNA levels and clinicopathological characteristics and overall survival (OS) in HCC was determined.

Results: The serum exosomal *hnRNPH1* mRNA levels in HCC patients were remarkably higher than in the other groups ($p < 0.05$). The *hnRNPH1* mRNA discriminated HCC from CHB with an area under the ROC curve (AUC) of 0.865, with sensitivity of 85.2% and specificity of 76.5% at cut-off value of 0.670. The AUC for *hnRNPH1* mRNA in combination with AFP was further improved. The exosomal *hnRNPH1* mRNA levels in HCC patients were associated with the Child-Pugh classification, portal vein tumor emboli, lymph node metastasis, TNM stage and OS ($p < 0.05$).

Conclusions: These findings suggested that serum exosomal *hnRNPH1* mRNA could be an effective marker for HCC in high HBV prevalence areas.

Keywords: exosome; hepatocellular carcinoma; heterogeneous nuclear ribonucleoprotein H1; messenger RNA.

Introduction

In China, chronic hepatitis B virus (HBV) infection is the main cause of hepatocellular carcinoma (HCC), and the prevalence of HBV infection in patients with HCC is approximately 80% [1–3]. Once the diagnosis is definite, the patients with HCC often proceed to the progressive stage or occurrence in distal metastasis, precluding the opportunity for rational surgery for these patients. Currently, the most commonly used marker for HCC is serum α -fetoprotein (AFP); however, approximately 50% of HCC patients are negative for this marker. Hence, it is important for clinical scientists to identify ideal HCC markers, particularly for HBV-related HCC.

Exosomes are small vesicles of approximately 30–100 nm in diameter, which selectively pack mRNA, non-coding RNA, DNA, protein, etc. and can be secreted by a wide variety of cells. In functional studies, exosomes play an important role in signaling between the cells including physiological and pathological cells [4–6]. Reflecting these characteristics, exosomes could potentially be applied in the diagnosis and prognosis assessment of tumors. In a previous study, we pooled four pairs of sera from untreated-HCC patients and healthy subjects into one pair of sample, respectively, extracted total exosomal RNA, and used RNA sequencing (RNA-seq) to screen the differentially expressed transcripts (unpublished data). In the exosomal mRNAs data (see Supplementary Table S1) produced by RNA-seq, the results revealed heterogeneous nuclear ribonucleoprotein H1 (*hnRNPH1*) mRNA as a significantly upregulated transcript in HCC patients. HnRNPH1 is a type of RNA binding protein and splicing factor, which stimulates the dissociation and polyadenylation of mRNA precursors and plays a decisive role in alternative splicing [7]. Studies have shown that *hnRNPH1* is highly expressed in esophageal squamous cell carcinoma, prostate cancer and other tumor tissues and is associated with cell differentiation [8–10]. These findings indicate that serum exosomal *hnRNPH1* mRNA could represent a novel “liquid biopsy” assay for HCC.

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Therefore, in the present study, we detected the relative expression levels of serum exosomal *hnRNPH1* mRNA in HCC, liver cirrhosis (LC) and chronic hepatitis B (CHB) patients and healthy subjects. A receiver operating characteristics (ROC) curve was constructed to evaluate the effectiveness of the diagnosis for HCC. In addition, we discuss the relationship between the *hnRNPH1* mRNA and the clinicopathological characteristics and overall survival (OS) of patients with HCC.

Materials and methods

Patients

A total of 223 patients, including 88 cases of HCC, 67 cases of LC and 68 cases of CHB among patients who visited Hangzhou First People's Hospital, were enrolled in the present study between January 2015 and June 2016. HCC was diagnosed based on ultrasound, computed tomography (CT), serum AFP and histopathological examination. LC was diagnosed based on ultrasound and CT examination and further characterized according to patients with or without portal hypertension and hypersplenotrophy. CHB was diagnosed based on patients with HBV surface antigen (HBsAg)-positive serum for more than 6 months. Sixty-eight cases of healthy subjects were collected from the physical examination center of Hangzhou First People's Hospital. The participants with autoimmune hepatitis, alcoholic liver disease, Wilson's disease, other types of viral hepatitis and other major diseases were excluded. All participants were age- and gender-matched.

The present study was approved through the Medical Ethics Committee of Hangzhou First People's Hospital, and all participants completed an informed consent process.

Blood specimens

Four milliliters of blood were drawn from each participant in the fasting state on the first visiting day. Two milliliters of serum were separated after centrifugation at $3000\times g$ for 5 min within 8 h, and 2 mL of serum was subsequently obtained after centrifugation at $10000\times g$ for 5 min. The prepared serum samples were stored at -70°C until further use.

Serum exosome separation and total RNA extraction

A 1-mL aliquot of serum was transferred to a new tube, and 200 μL of total exosome isolation reagent (Thermo Fisher Scientific Co., Ltd, Lithuania) was added. The serum/reagent mixture was mixed well by pipetting up and down until a homogenous solution was obtained. The sample was incubated at 4°C for 30 min, followed by centrifugation at $10,000\times g$ for 10 min at room temperature. The obtained pellet was resuspended in 200 μL of $1\times$ PBS solution. Total exosomal RNA was extracted and purified using a commercial kit (Thermo Fisher Scientific Co., Ltd, Lithuania), and eventually 50 μL of total RNA was

acquired. The above procedures were strictly performed according to the manufacturer's instructions.

TaqMan real-time PCR

The primers and probes for exosomal *hnRNPH1* mRNA and *GAPDH* mRNA amplification were designed using Express v3.0 software (Applied Biosystems Co., Ltd, USA), and synthesized at Shanghai Hui-rui Biological Technology Co., Ltd. The following primer and probe sequences were used: forward primer for *hnRNPH1* 5'-CGCCCGCCTGTTG-3', reverse primer for *hnRNPH1* 5'-TGCAGT-GAGCCGAGATTGTG-3', and probe for *hnRNPH1* 5'-FAM-ACAGTCTCACTTTATCGCCAGGCTGG-TAMRA-3'; forward primer for *GAPDH* 5'-GAGCTGAACGGGAAGCTCAC-3', reverse primer for *GAPDH* 5'-GCCTGCTTACCACCTTCTT-3', and probe for *GAPDH* 5'-FAM-ACAGTCTCACTTTATCGCCAGGCTGG-TAMRA-3'. The reaction system comprised 10 μL of $2\times$ PCR buffer, 0.4 μL of DNA polymerase II, 0.4 μL of reverse transcriptase mixture, 0.4 μL of each primer, 0.8 μL of probe, 2 μL of RNA template and deionized water to the total volume of 20 μL (TaKaRa Co., Ltd, China). The ABI Prism[®] 7500 PCR instrument (Applied Biosystems Co., Ltd, USA) was used for detection. The following reaction conditions were used: the first stage (50°C for 15 min), the second stage (95°C for 5 min), and the third stage, including 45 cycles (95°C for 15 s, 60°C for 40 s). The relative expression level of *hnRNPH1* mRNA was calculated using the $2^{-\Delta\text{Ct}}$ method as previously described [11]. Each sample was assessed in duplicate, with an average of the two duplicates used for analysis.

Statistical analyses

Non-parametric variables were described as median and interquartile range (IQR) and examined using the Mann-Whitney U or Kruskal-Wallis H-test. Furthermore, exosomal *hnRNPH1* mRNA relative expression levels were illustrated as box plots. Categorical variables were compared using the χ^2 -test. ROC curves were constructed to determine the feasibility of *hnRNPH1* mRNA alone and in combination with AFP in the diagnosis of HCC. OS curves were estimated using the Kaplan-Meier method. Differences were considered significant at the α level of 0.05 for two-sided tests. All data analyses were performed using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Serum exosomal *hnRNPH1* mRNA relative expression levels

Serum exosomal *hnRNPH1* mRNA relative expression levels in the HCC group at 0.970 (0.764, 1.386) were significantly higher than those in the LC group at 0.671 (0.393, 1.346), the CHB group at 0.354 (0.175, 0.637) and the healthy control group at 0.390 (0.226, 0.495) ($p=0.012$,

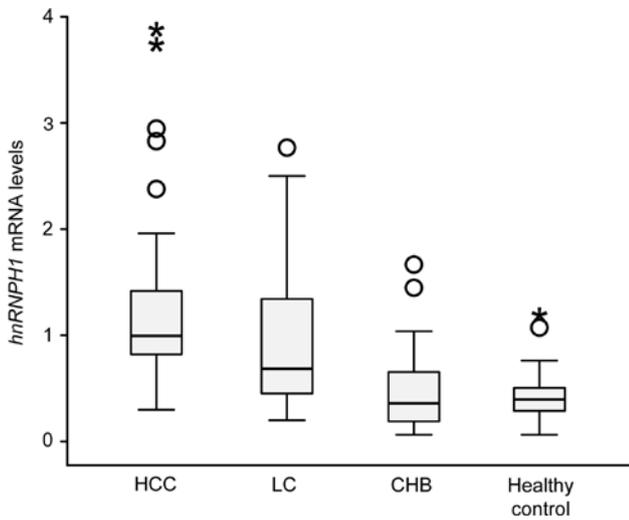


Figure 1: Box plots of the serum exosomal *hnRNPH1* mRNA relative expression levels in four groups.

The serum exosomal *hnRNPH1* mRNA relative expression level in the HCC group at 0.970 (0.764, 1.386) was significantly higher than that in the LC group at 0.671 (0.393, 1.346), the CHB group at 0.354 (0.175, 0.637) and the healthy control at 0.390 (0.226, 0.495) ($p=0.012$, $p=0.006$ and $p=0.004$, respectively). Compared with the CHB group and healthy control, the serum exosomal *hnRNPH1* mRNA levels in the LC group were also obviously higher ($p=0.009$ and $p=0.008$, respectively). Notably, no difference between CHB group and healthy control was observed ($p=0.591$).

$p=0.006$ and $p=0.004$, respectively). Compared with the CHB group and healthy control, the serum exosomal *hnRNPH1* mRNA levels in the LC group were also obviously higher ($p=0.009$ and $p=0.008$, respectively). Notably, no difference between the CHB group and healthy control was observed ($p=0.591$) (Figure 1).

Effectiveness of exosomal *hnRNPH1* mRNA relative expression levels in identifying HCC

To determine whether exosomal *hnRNPH1* mRNA could be a potential diagnostic marker for HCC in high HBV prevalence areas, ROC curve analyses were performed. The exosomal *hnRNPH1* mRNA discriminated HCC patients from CHB patients with an AUC of 0.865 (95% CI = 0.808–0.922, $p=0.003$). At a cut-off value of 0.670, the sensitivity and specificity of detection was 85.2% and 76.5%, respectively. Serum AFP at the cut-off value of 20 ng/mL yielded an AUC of 0.785 (95% CI = 0.694–0.845, $p=0.007$) with 69.3% sensitivity and 87.9% specificity. Furthermore, the AUC for exosomal *hnRNPH1* mRNA in combination with AFP was 0.891 (95% CI = 0.873–0.939, $p=0.005$) with 87.5% sensitivity and 84.8% specificity (Figure 2A).

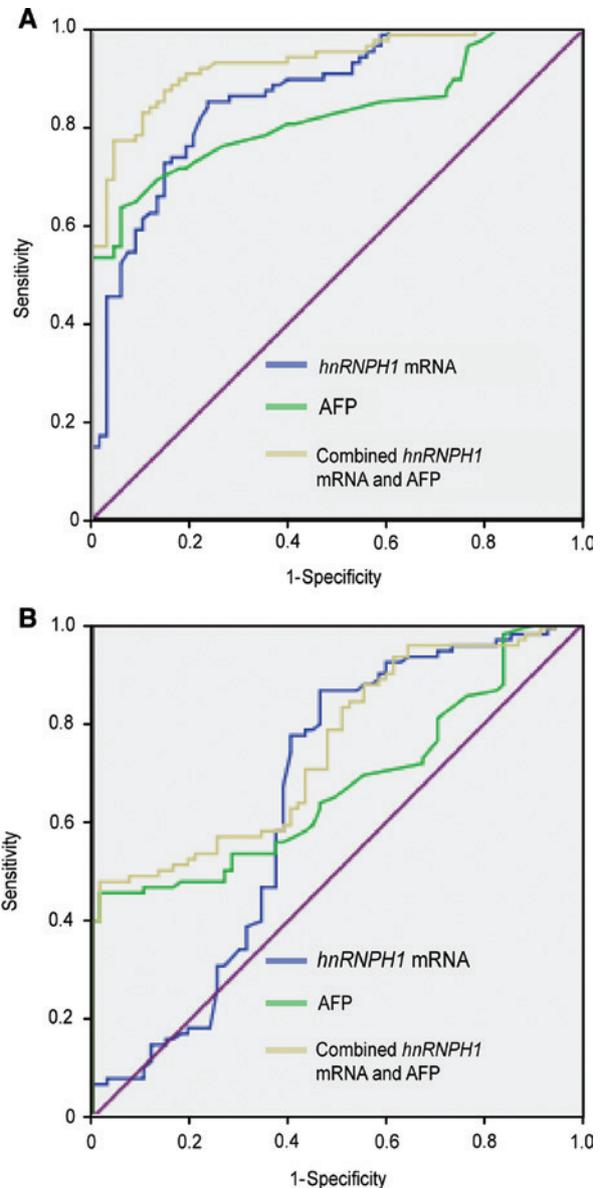


Figure 2: Receiver operating characteristics (ROC) curve analyses using serum exosomal *hnRNPH1* mRNA for discriminating HCC patients from CHB or LC patients.

(A) Exosomal *hnRNPH1* mRNA at a cut-off value of 0.670 yielded an AUC (the area under the ROC curve) of 0.865 (95% CI = 0.808–0.922, $p=0.003$) with 85.2% sensitivity and 76.5% specificity for discriminating HCC patients from CHB patients. Serum AFP at a cut-off value of 20 ng/mL yielded an AUC of 0.785 (95% CI = 0.694–0.845, $p=0.007$) with 69.3% sensitivity and 87.9% specificity. The AUC for exosomal *hnRNPH1* mRNA in combination with AFP was 0.891 (95% CI = 0.873–0.939, $p=0.005$) with 87.5% sensitivity and 84.8% specificity. (B) Similarly, exosomal *hnRNPH1* mRNA discriminated HCC patients from LC patients with an AUC of 0.647 (95% CI = 0.553–0.741, $p=0.002$) with 86.4% sensitivity and 54.0% specificity. Serum AFP yielded an AUC of 0.674 (95% CI = 0.590–0.758, $p=0.006$) with 46.6% sensitivity and 88.3% specificity. The AUC for exosomal *hnRNPH1* mRNA in combination with AFP was 0.749 (95% CI = 0.674–0.825, $p=0.001$) with 50.3% sensitivity and 91.0% specificity.

Similarly, exosomal *hnRNPH1* mRNA discriminated HCC patients from LC patients with an AUC of 0.647 (95% CI=0.553–0.741, $p=0.002$) with 86.4% sensitivity and 54.0% specificity. Serum AFP yielded an AUC of 0.674 (95% CI=0.590–0.758, $p=0.006$) with 46.6% sensitivity and 88.3% specificity. The AUC for exosomal *hnRNPH1* mRNA in combination with AFP was 0.749 (95% CI=0.674–0.825, $p=0.001$) with 50.3% sensitivity and 91.0% specificity (Figure 2B).

Relationship between exosomal *hnRNPH1* mRNA relative expression levels and the clinicopathological features of HCC patients

We examined the clinicopathological features, including gender, age and other clinical parameters, according to the exosomal *hnRNPH1* mRNA relative expression status. Significant associations between the Child-Pugh classification, portal vein tumor emboli, lymph node metastasis and TNM stage and exosomal *hnRNPH1* mRNA relative expression levels ($p<0.05$) were observed, and gender, age, HBsAg, histological grade and cirrhosis showed no associations with exosomal *hnRNPH1* mRNA ($p>0.05$) (Table 1).

Association between exosomal *hnRNPH1* mRNA relative expression levels and HCC survival

In the present study, the clinical cases for 88 HCC patients were followed for 48 months. According to the median exosomal *hnRNPH1* mRNA level (0.970), HCC patients were divided into two subsets. Patients whose exosomal *hnRNPH1* mRNA levels were higher than 0.970 were classified into the high-level subset ($n=58$), and patients whose exosomal *hnRNPH1* mRNA levels were lower than 0.970 were classified into the low-level subset ($n=30$). The results of the Kaplan-Meier analysis demonstrated that the exosomal *hnRNPH1* mRNA level is a prognostic factor in HCC patients. High exosomal *hnRNPH1* mRNA levels were associated with worse OS in HCC patients ($\chi^2=5.206$, $p=0.023$) (Figure 3).

Discussion

The exchange of exosomal packages between the cells could influence the tumor microenvironment and contribute to the tumor development [4–6, 12]. Exosomes are easy to obtain from nearly all body fluids, including blood,

Table 1: Relationship between serum exosomal *hnRNPH1* mRNA levels and the clinicopathological characteristics of HCC patients.

Characteristics	n	<i>hnRNPH1</i> mRNA	p-Value
Gender			
Male	59	0.977 (0.742, 1.404)	0.404
Female	29	0.963 (0.780, 1.368)	
Age, year			
<50	26	0.966 (0.772, 1.374)	0.863
≥50	62	0.974 (0.753, 1.410)	
HBsAg			
Positive	61	0.982 (0.631, 1.405)	0.305
Negative	27	0.961 (0.785, 1.315)	
Child-Pugh classification			
A	12	0.793 (0.737, 1.306)	0.003
B	11	0.893 (0.769, 1.384)	
C	11	1.118 (0.775, 1.492)	
Tumor size, cm			
<5	21	0.963 (0.650, 1.355)	0.361
≥5	67	0.977 (0.770, 1.428)	
Histological grade			
I+II	37	0.959 (0.725, 1.328)	0.159
III+IV	51	0.993 (0.781, 1.416)	
Portal vein tumor emboli			
Present	40	1.118 (0.784, 1.590)	0.004
Absent	48	0.883 (0.644, 1.295)	
Lymph node metastasis			
Present	20	1.107 (0.809, 1.483)	0.006
Absent	68	0.865 (0.737, 1.355)	
TNM stage			
I+II	16	0.827 (0.619, 1.165)	0.002
III+IV	72	1.114 (0.803, 1.493)	
Cirrhosis			
Present	34	1.002 (0.733, 1.455)	0.278
Absent	54	0.957 (0.782, 1.364)	

HCC, hepatocellular carcinoma. Child-Pugh classification for 34 cases of HCC with cirrhosis was evaluated. The histological examination was conducted by referencing the standard Edmondson grade. Tumor stage was classified according to the TNM criteria of the International Union Against Cancer. Serum exosomal *hnRNPH1* mRNA levels are presented as the median and interquartile range (IQR).

urine, saliva, semen and ascites. From the perspective of exploring novel tumor markers, exosomes could assuredly undertake this role, as these vesicles are stable, can easily be used for dynamic tracking, and particularly, selectively pack bioactive molecules. Exosomal microRNAs (miRNAs) have been recommended as diagnostic and prognostic markers for lung cancer, esophageal squamous cell carcinoma, prostate cancer, breast cancer and glioblastoma [13–18]. Comparison of the exosomal proteins and non-coding RNAs in the diagnosis of cancers showed that the application of exosomal mRNAs was limited.

The hnRNPs (heterogeneous nuclear ribonucleoproteins) are RNA-binding proteins that complex with

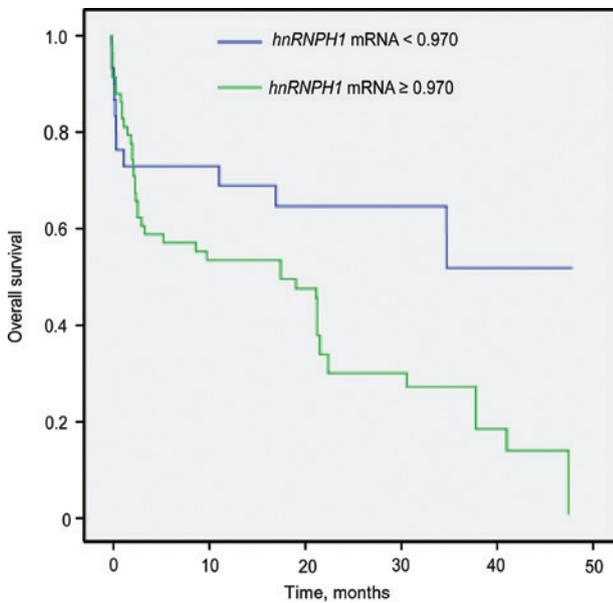


Figure 3: Kaplan-Meier analysis of the overall survival (OS) of 88 HCC patients. Eighty-eight HCC patients were followed for 48 months. According to the serum exosomal *hnRNPH1* mRNA levels, 58 HCC patients were stratified to the serum high-level subset (more than 0.970), and 30 HCC patients were stratified to the serum low-level subset. Kaplan-Meier analysis showed that high serum exosomal *hnRNPH1* mRNA levels were associated with worse OS ($\chi^2 = 5.206$, $p = 0.023$).

heterogeneous nuclear RNA and play key roles in multiple aspects of nucleic acid metabolism, including the packaging of nascent transcripts, alternative splicing and translational regulation. Moreover, the dysregulation of alternative pre-mRNA splicing has been implicated in carcinogenesis [19–21]. *HnRNPA1* is a major factor associated with various types of cancer and metastasis. This molecule is markedly overexpressed in lung cancer tissues and is associated with tumor proliferation [22]. Similarly, *hnRNPA2/B1* is significantly upregulated in patients suffering from lung cancer and may be a marker for the early detection of lung cancer [23]. Sun et al. [8] suggested that the levels of *hnRNPH1* mRNA were obviously increased in esophageal squamous cell carcinoma tissues and decreased in non-tumorous tissues ($p = 0.0026$); the high levels of *hnRNPH1* mRNA were associated with poorly differentiated tumors ($p = 0.0287$). A recent study showed that *hnRNPH1/H2* mediated the switch in expression from high-activity fructokinase (KHK)-C to the low-activity KHK-A isoform. Above all, KHK-A acts as a protein kinase that phosphorylates and activates phosphoribosyl pyrophosphate synthetase 1 (PRPS1) to promote pentose phosphate pathway-dependent de novo nucleic acid synthesis and HCC occurrence [24]. So far, the molecular

mechanism of exosomal *hnRNPH1* mRNA participation in the formation and development of cancer remains obscure.

In the present study, we screened exosomal *hnRNPH1* mRNA as a candidate marker for HCC diagnosis using the TaqMan real-time PCR method. The results revealed that the levels of serum exosomal *hnRNPH1* mRNA in HCC patients were remarkably higher than those in other groups. Exosomal *hnRNPH1* mRNA discriminated HCC from CHB with an AUC of 0.865 with 85.2% sensitivity and 76.5% specificity at a cut-off value of 0.670. The effectiveness of *hnRNPH1* mRNA combined with AFP (≥ 20 ng/mL) was further improved.

In addition, we examined the relationship between serum exosomal *hnRNPH1* mRNA levels and the clinicopathological characters in HCC. The results indicated that high levels of serum exosomal *hnRNPH1* mRNA were associated with Child-Pugh classification, portal vein tumor emboli, lymph node metastasis and TNM stage, which represent more progressive clinical phenotypes. Moreover, the association of serum exosomal *hnRNPH1* mRNA levels with the prognosis of HCC patients was assessed, and the results showed that patients with high serum exosomal *hnRNPH1* mRNA levels exhibited worse OS than those with low *hnRNPH1* mRNA levels, suggesting that the level of serum exosomal *hnRNPH1* mRNA has important predictive value in HCC prognosis classification.

Although the differences of serum exosomal *hnRNPH1* mRNA levels have been described in detail among HCC, LC, CHB patients and healthy subjects, there are some limitations in this study because of a lack of clinical data. For example, it is need to compare the results between patients with chronic active hepatitis B and those with inactive infection; the difference between patients with compensated cirrhosis and those with decompensated cirrhosis is worth to be discussed also, and it would be of interest to evaluate the differences in CHB patients with various fibrosis stages.

In conclusion, these findings suggest that serum exosomal *hnRNPH1* mRNA may be a novel biomarker for HCC patients in high HBV prevalence areas.

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