Abnormal expression of HNRNPA3 in multistep hepatocarcinogenesis

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Received February 28, 2020; Accepted September 8, 2020

DOI: 10.3892/ol.2020.12307

Abstract. Hepatocarcinogenesis is a multistep process involving progression from cirrhosis, to low-grade dysplastic nodule, to high-grade dysplastic nodule (HGDN) and, eventually, to hepatocellular carcinoma (HCC). Early detection of HCC is challenging as the differential diagnosis between HGDN and early HCC (eHCC) is difficult. The aim of the present study was to identify a novel biomarker to specifically differentiate between HGDN and eHCC, which may facilitate early diagnosis of HCC. Immunohistochemistry was performed to determine the expression of heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3) in cirrhosis, dysplastic nodules (DNs), well-differentiated HCC and progressed HCC. The staining was evaluated by assigning a staining intensity score of 0-3 and a percentage of positively stained cells score of 0-4. Receiver operator characteristic (ROC) curve analysis was used to assess the ability of HNRNPA3 expression to differentiate between DNs and HCC. HNRNPA3 expression increased in a stepwise trend in non-tumor hepatic tissue, DNs, eHCC and progressed HCC. ROC curves revealed that HNRNPA3 expression could be used to differentiate between HGDN and eHCC, particularly in combination with glypican 3 (GPC3), with a specificity of 100%. Moreover, HNRNPA3 expression was associated with HCC differentiation. In addition, high expression of HNRNPA3 was found to be associated with poor survival rates in patients with HCC. These findings demonstrated that HNRNPA3 combined with GPC3 is a helpful diagnostic biomarker in the differential diagnosis during the multistep process of hepatocarcinogenesis, particularly in the differential diagnosis between HGDN and eHCC. To the best of our knowledge, this is the first study to report the significance of HNRNPA3 in hepatocarcinogenesis and its potential role in carcinogenesis.

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Key words: differential diagnosis, dysplastic nodule, hepatocarcinogenesis, hepatocellular carcinoma, heterogeneous nuclear ribonucleoprotein A3

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults, with an incidence of ~850,000 new cases worldwide annually (1). Therefore, early detection of HCC is crucial for improving survival rates. Human HCC development is a multistep process characterized by progression from cirrhosis, to low-grade dysplastic nodule (LGDN), to high-grade dysplastic nodule (HGDN), to early HCC (eHCC) and, finally, to progressed HCC (2). A key step in early detection of HCC in the clinical setting is to differentiate between eHCC and dysplastic nodule (DN), as the transition from DN to eHCC denotes the earliest step of malignant transformation in the cirrhotic liver. The radiological observation of liver pathology has been developed for >30 years and has achieved significant advances in diagnosis (3). However, there are several reasons due to which imaging methods cannot accurately identify the stage of hepatocarcinogenesis. For example, the pathological characteristics of eHCC closely resemble those of HGDN, and a definitive pathological differentiation between the two is currently lacking (4). Moreover, most well-differentiated early-stage HCCs do not stain on angiography or retain lipiodol within the tumor, thereby making their diagnosis difficult (2). Whether Gd-EOB-DTPA-enhanced magnetic resonance or computed tomography imaging are sufficiently sensitive to detect eHCC is subject to dispute (5,6). Although some morphological criteria were put forward to distinguish DNs from eHCC, they are unreliable as diagnostic indicators to discriminate between the two, particularly for HGDN and eHCC, since they are close to each other in the stepwise morphological progression. Conventional immunohistochemistry (IHC) biomarkers, such as p53, E-cadherin and CD34, are widely used to confirm the presence of liver cancer, but cannot identify the specific HCC stage (7,8). Glypican 3 (GPC3) is a cell surface protein linked to the cell membrane via a glycosylphosphatidylinositol anchor and is highly expressed in HCC and certain other human cancers,

including melanoma and neuroblastoma (9). GPC3 staining was found to be negative or focally weakly expressed in HCC precursor lesions, but diffuse GPC3 staining is observed in the majority of HCCs (10). A growing body of evidence supports GPC3 as a novel biomarker for HCC, and its protein expression is associated with poor prognosis of patients with HCC (11,12). However, the sensitivity and specificity of GPC3 must be further optimized. Therefore, there is an urgent need for objective and effective markers that are sensitive to the differences between HGDNs and eHCCs.

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are defined as nuclear RNA-binding proteins that form complexes with pre-mRNA. It is widely accepted that the members of this protein family are known regulators of cell cycle progression, cell differentiation, cell cycle arrest and DNA damage. HNRNPA3 is a protein encoded in humans by the *HNRNPA3* gene and is a member of the hnRNP A/B family. HNRNPA3 is involved in RNA binding, mRNA transport and mRNA splicing via spliceosome. Although the detailed association between tumorigenesis and HNRNPA3 has not been fully elucidated, the HNRNP protein family members are closely associated with cancer regulation. The expression of APOBEC3B, a cytosine deaminase, increases with increasing levels of HNRNPA3 in cancer cell lines, and the possible underlying mechanism may be through telomere elongation (13). Altered expression of hnRNP A/B members, including A3, was found to antagonize alternative splicing factor/splicing factor 2 (ASF/SF2), a prototypical SR protein, in patients with non-small cell lung cancer (14). In light of these findings, hnRNP protein family members may be considered as valuable diagnostic/prognostic markers in cancer. Therefore, the aim of the present study was to investigate the role of HNRNPA3 in the process of hepatocarcinogenesis and to further validate its diagnostic/prognostic value for determining the differentiation degree of HCC. The results of the present study demonstrated that HNRNPA3 expression gradually increased in non-tumor hepatic tissue, DNs, eHCC and progressed HCC. It was confirmed that HNRNPA3 combined with GPC3 is a helpful diagnostic biomarker in the differential diagnosis during the multistep process of hepatocarcinogenesis, particularly in the differential diagnosis between HGDN and eHCC. In addition, HNRNPA3 expression was associated with HCC differentiation. High expression of HNRNPA3 was also demonstrated to be associated with poor survival rates in patients with HCC.

Materials and methods

Tissue samples. The study population comprised a total of 56 samples, including human DNs, well-differentiated, moderately and poorly differentiated HCC, with corresponding paired cirrhotic liver tissues. The samples were collected from patients undergoing surgery at Qilu Hospital, Shandong University (Jinan, China). The study protocol was approved by the Ethics Committee of Shandong University (approval no. 2012028). Patient cases were recorded at the Department of Pathology between March 2013 and June 2019. The Hepatitis B Virus (HBV) infection status of patients with HCC or DN were collected (Tables SI and SII). The histological slides were reviewed to confirm the diagnosis in all cases, which were classified as follows: i) Clinicopathologically typical HCC (n=48), tumors arising in men and women aged 43-89 years and displaying the typical histological characteristics of HCC. In a total of 48 samples, there were 38 progressed and 10 well-differentiated HCCs (including 7 eHCCs). eHCC was defined as being ≤2 cm in diameter, displaying a vaguely nodular pattern with indistinct margins and no tumor capsule (15). ii) DNs (n=8), nodules differing from the surrounding liver parenchyma with regards to size, color, texture and degree of bulging of the cut surface. LGDN was defined as a nodule exhibiting a mild increase in cell density with a monotonous pattern and/or clonal changes, whereas HGDN was defined as a nodule exhibiting marked cytological and architectural atypia. Few unpaired non-triadal arteries may be seen. Stromal and vascular invasion are absent (16).

IHC. The streptavidin-peroxidase-biotin method was employed (17). Tissue sections were fixed with 4% paraformaldehyde at room temperature for 14 h. Paraffin-embedded tissue sections were cut into 4-μm-thick sections, dewaxed in dimethylbenzene (twice, 20 min each time) and hydrated in gradient ethanol (100, 95, 85 and 75%, each for 5 min) at room temperature. The sections were treated according to the following method: Antigen retrieval with EDTA at 95˚C for 45 min (cat. no. ZLI-9069; OriGene Technologies, Inc.), endogenous peroxidase removed at room temperature for 10 min (cat. no. SP9000; OriGene Technologies, Inc), blocking with 10% goat serum at room temperature for 10 min (cat. no. SP9000; OriGene Technologies, Inc.). Tissue sections were subsequently incubated with primary antibodies against HNRNPA3 (1:75; cat. no. 25142-1-AP; ProteinTech Group, Inc.) or GPC3 (1.5 ml, ready-to-use; cat. no. MAB-0617; Fuzhou Maixin Biotech Co., Ltd.) at 4˚C for 12 h. Following the primary incubation, tissue sections were incubated with ready-to-use horseradish peroxidase-labeled secondary antibody (cat. no. SP9000; OriGene Technologies, Inc.) for 20-30 min at room temperature, DAB color reaction for 2 min or 45 sec at room temperature, respectively, counterstained with hematoxylin for 20 sec at room temperature, differentiation, dehydration and transparency. For negative controls, the primary antibody was replaced with PBS. Tissue sections were observed under a light microscope (magnification, x40).

Scoring. All the sections were evaluated by two observers blinded to the experimental groups. HNRNPA3 staining intensity was scored as follows: i) 0, negative; ii) 1, weak; iii) 2, moderate; and iv) 3, strong. The staining area (percentage of stained cells) was scored as follows: i) 0, 0%; ii) 1, 1-25%; iii) 2, 26-50%; iv) 3, 51-75%; and v) 4, 76-100%. These two scores were added together to produce the final IHC score in different samples. The suitable cut-off points were confirmed by receiver operating characteristics (ROC) curve analysis. GPC3 was considered as positive when moderate to strong nuclear, cytoplasmic and/or membranous staining was seen in ≥10% of tumor cells (18).

Statistical analysis. Statistical analysis was performed using Graphpad Prism 5 (Graphpad Software, Inc.). The Fisher's exact test was used to determine significant differences in expression of HNRNPA3 between HCC, DN and surrounding

normal tissues. The Kruskal-Wallis test (followed by post hoc Dunn's multiple comparison test) was used to compare IHC sum scores among non-tumorous tissue, DN and HCC. The Fisher's exact test was also used to analyze the association between HNRNPA3 expression and clinicopathological variables in DN and HCC (the χ^2 test was applied to the differentiation part due to limitations of statistical approach). The χ^2 test was used to analyze the association between HNRNPA3 expression and indicators that were subdivided into three groups, such as serum enzymes and tumor biomarkers in HCC. The Fisher's exact test was applied to find an association between HNRNPA3 expression and indicators that were subdivided into two groups. ROC curves were constructed and the area under the curve (AUC) was calculated to assess the ability of HNRNPA3 expression to discriminate these three different stages during progression of liver cancer, based on the highest Youden's index (sensitivity and 1-specificity). The prognosis rates of patients were calculated using the Kaplan-Meier method and differences between survival curves were examined using a log-rank test (19). P<0.05 was considered to indicate a statistically significant difference.

Database. The survival analysis of HNRNPA3 in patients with HCC according to The Human Protein Atlas (https://www.proteinatlas.org/). Different patient samples in The Human Protein Atlas were originally derived from The Cancer Genome Atlas (TCGA) database established by National Cancer Institute. Specific analysis methods are outlined in previous literature (19).

Results

HNRNPA3 expression increases in a stepwise trend during multistep hepatocarcinogenesis. To the best of our knowledge, the expression of HNRNPA3 in liver cancer has not been investigated before and the present study was the first to investigate the protein expression of HNRNPA3 using IHC in a total of 56 paraffin-embedded liver samples, including 8 cases of DNs, 48 cases of HCC and their paired non-tumor hepatic tissues. The immunostaining profiles of HNRNPA3 in liver samples (cirrhotic, DN and HCC), with HNRNPA3 staining the nucleus, are shown in Fig. 1. HNRNPA3 expression was found to be negative or low in non-tumor hepatic tissue, whereas its expression was high in DNs and highest in HCC (Fig. 1). Of the 48 cases of non-tumor hepatic tissue, only 1 case (2.08%) displayed positive HNRNPA3 expression. By contrast, 5 out of 8 cases of DNs were negative and the remaining cases (37.50%) were classified as positive (Table I). Intriguingly, 40 out of 48 HCC cases (83.33%) exhibited positive expression, whereas the remaining samples (16.67%) were negative.

Immunostaining for HNRNPA3 was performed on well-differentiated, moderately and poorly differentiated HCCs (Fig. 2). Well-differentiated HCC cases included 8 (80.00%) positive for HNRNPA3 expression and only 2 negative cases. In progressed HCC cases, HNRNPA3 immunoreactivity was predominately positive (84.21%) among the 38 samples. In addition, the present data revealed that HNRNPA3 expression increased in a stepwise trend from non-tumor tissue via DNs to well-differentiated HCC and advanced HCC (Table I, Fisher's exact test; Fig. 3, Kruskal-Wallis test; P<0.0001). It was also observed that the expression level of HNRNPA3 increased along with an increasing degree of tumor malignancy and was highly expressed in undifferentiated HCC samples (Fig. 2).

Clinical value of HNRNPA3 expression in the differential diagnosis between HGDN and eHCC. ROC curves were used to evaluate the ability of HNRNPA3 expression to differentiate between DN and HCC. The ROC curves revealed that the AUC value was up to 0.8216 (95% CI, 0.6896-0.9537; P=0.0038; Fig. S1A). ROC curves were also constructed to test the ability of HNRNPA3 expression to differentiate between non-tumor tissues and HCC. The ROC curves revealed that the AUC value was up to 0.9225 (95% CI, 0.8625-0.9826; P<0.0001; Fig. S1B). The same method was further applied to determine the ability of HNRNPA3 expression to discriminate between DN and well-differentiated HCC, with an AUC value of up to 0.8188 (95% CI, 0.6244-1.013; P=0.0235; Fig. S1C). A noteworthy finding was that well-differentiated HCC could be distinguished from HGDN by HNRNPA3 expression with an AUC value of 0.8083 (95% CI, 0.5934-1.023; P=0.0448; Fig. S1D). These data supported the value of the expression level of HNRNPA3 in distinguishing specific stages of hepatocarcinogenesis, from cirrhotic tissue to DN and HCC. The differentially upregulated HNRNPA3 expression between HGDN and well-differentiated HCC may therefore represent a specific biomarker for this developmental stage of hepatocarcinogenesis.

GPC3 immunostaining was negative in 87.5% (7/8) of DN samples, and only one sample exhibited positive expression. By contrast, 81.25% of HCCs (39/48) displayed positive expression of GPC3. The expression of GPC3 was markedly different between DN and HCC. The sensitivity and specificity of GPC3 expression for diagnosing HCC stage were 81.25 and 87.50%, respectively (Table SIII). Furthermore, HNRNPA3 and GPC3 were combined as a two-marker set in the differential diagnosis. The positive expression of both markers was not observed in DNs. Therefore, the two-marker set was used to improve the detection of HCC, with a sensitivity of 70.83% and a specificity of 100% in serial tests. The ROC yielded a larger AUC of 0.854 (95% CI, 0.734-0.934; P<0.0001) for HNRNPA3$^+$/GPC3$^+$ compared with that of GPC3 staining alone (AUC=0.844; 95% CI, 0.722-0.927; P<0.0001) in the diagnosis of HCC (Fig. S2A). When distinguishing between DN and well-differentiated HCC, a larger AUC of 0.850 (95% CI, 0.605-0.972; P<0.0001; Fig. S2B and Table SIII) and optimal specificity (100%) were obtained from the combination of the two markers compared with GPC3 staining alone (AUC=0.838; 95% CI; P<0.0001). In addition, HNRNPA3 or GPC3 staining alone did not have the highest diagnostic accuracy when distinguishing eHCC from DN. The HNRNPA3$^+$/GPC3$^+$ serial test exhibited an AUC of 0.857 (95% CI, 0.584-0.980; P<0.001; Fig. S2C and Table SIII), a sensitivity of 71.43% and a specificity of 100.0% in distinguishing eHCC from DN. Furthermore, the two-marker set may prove to be an effective biomarker for differentiating between eHCC and HGDN. The specificity of HNRNPA3 staining alone was 83.33%, whereas this increased to 100% with HNRNPA3$^+$/GPC3$^+$, with the AUC of the combined test

Table I. Expression of HNRNPA3 in non-tumorous hepatic tissue, DN and HCC.

Tissue samples	n	HNRNPA3 expression		P-value
		Negative, n (%)	Positive, n (%)	
Non-tumorous hepatic tissue[d]	48	47 (97.92)	1 (2.08)	=0.0075[a]
DN[d]	8	5 (62.50)	3 (37.50)	=0.0123[b]
HCC[d]	48	8 (16.67)	40 (83.33)	<0.0001[c]

HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HCC, hepatocellular carcinoma; DN, dysplastic nodule. [a]Difference between non-tumorous hepatic tissue and dysplastic nodule. [b]Difference between dysplastic nodule and hepatocellular carcinoma. [c]Difference between non-tumorous hepatic tissue and hepatocellular carcinoma. [d]Fisher's exact test was applied to find the association between HNRNPA3 expression and indicators that were divided into two groups.

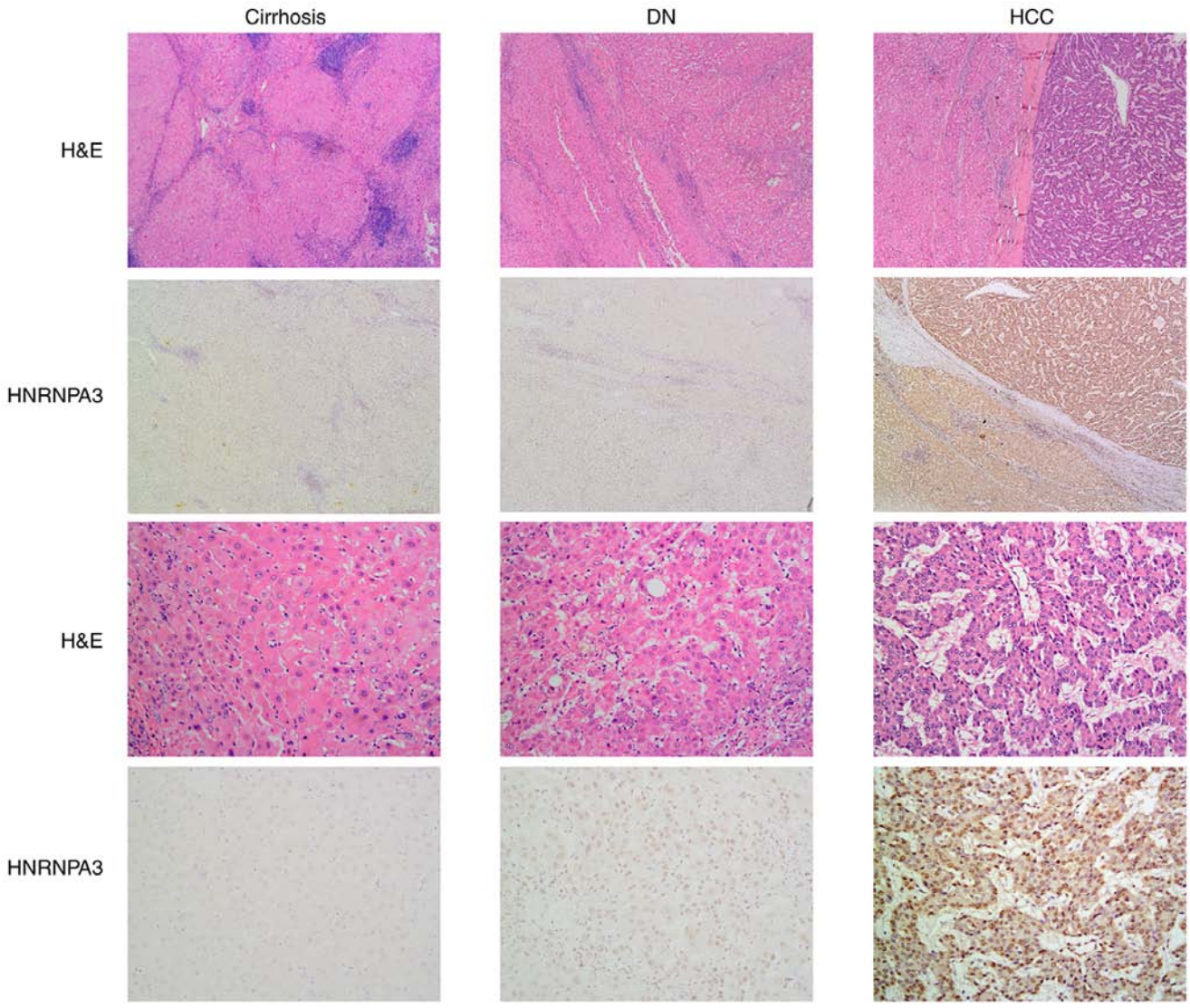

Figure 1. HNRNPA3 expression in cirrhosis, DN and HCC tissue samples. HNRNPA3 expression increased in a stepwise manner from cirrhosis (n=32), DN (n=8) to HCC (n=48), as detected by immunohistochemistry. Images on lane 1 and 2 are at x100 magnification. Images on lane 3 and 4 are at x400 magnification. HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; DN, dysplastic nodule; HCC, hepatocellular carcinoma.

at 0.857 (95% CI, 0.558-0.984; P=0.0001; Fig. 4A and Table II). Another notable discovery was that HNRNPA3 staining alone (AUC=0.906) was superior compared with GPC3 staining (AUC=0.844) or the combined assay (AUC=0.854) for distinguishing HCC from non-tumor tissue, and the sensitivity and specificity of HNRNPA3 staining were 83.33 and 97.92%,

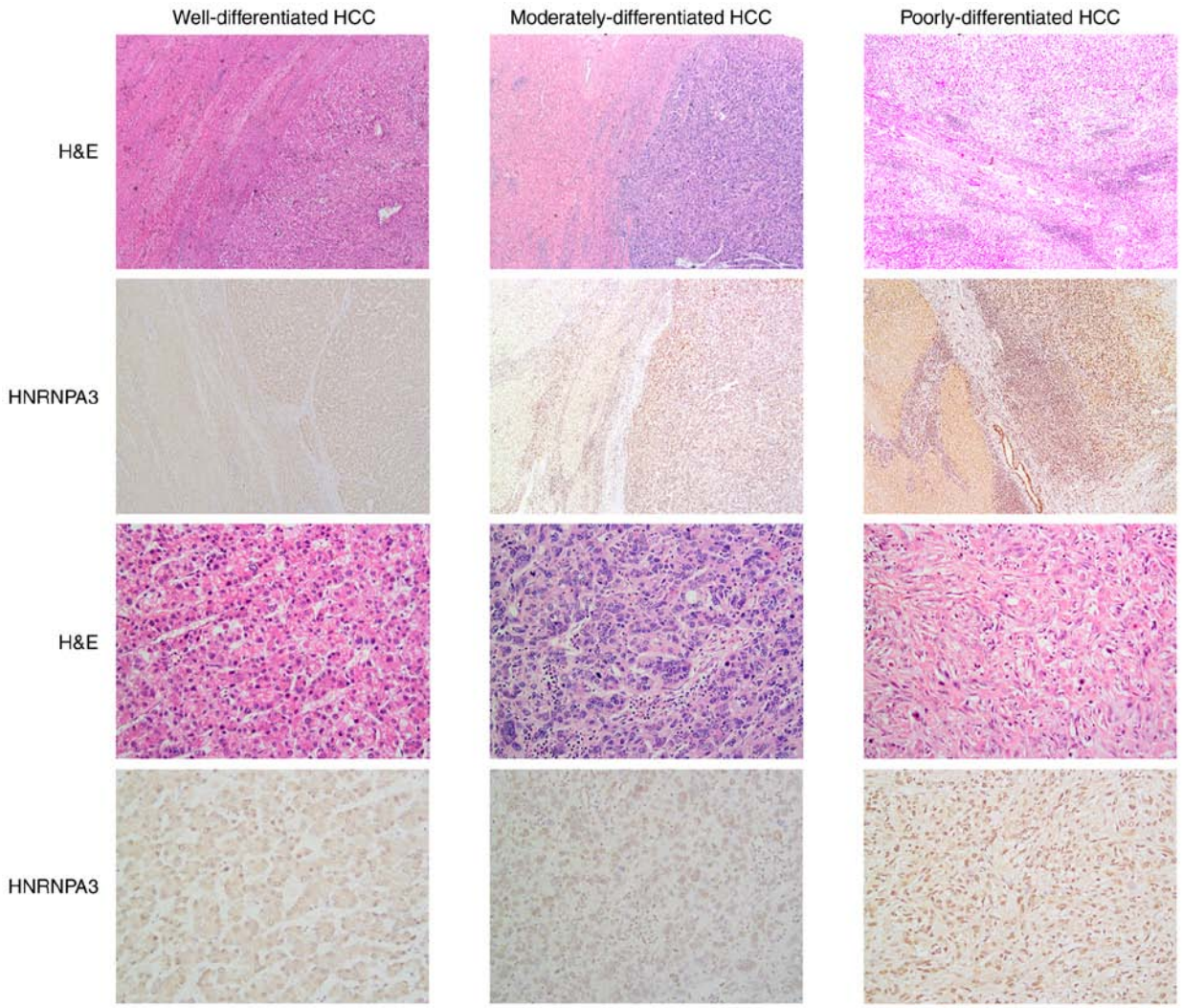

Figure 2. HNRNPA3 expression in well-differentiated HCC, moderately-differentiated HCC and poorly-differentiated HCC. HNRNPA3 expression was increased from well-differentiated HCC (n=10), moderately-differentiated HCC (n=32) to poorly-differentiated HCC (n=6), as detected by immunohistochemistry. Images on lane 1 and 2 are at x100 magnification. Images on lane 3 and 4 are at x400 magnification. HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HCC, hepatocellular carcinoma.

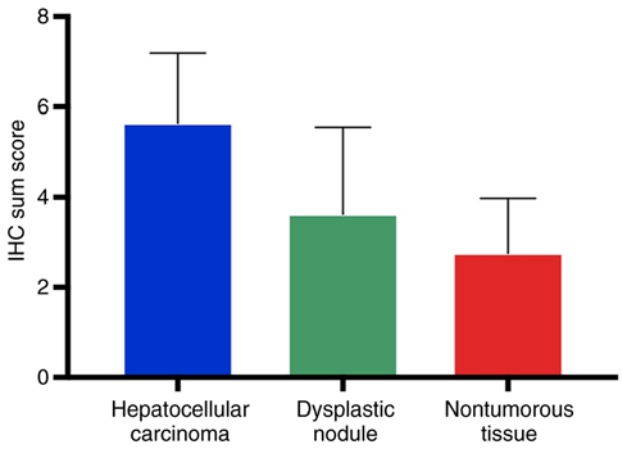

Figure 3. HNRNPA3 expression is higher in more aggressive liver tumors. IHC sum scores (0-7) were used to compare HNRNPA3 expression in different liver tissues. HNRNPA3 expression was significantly different in dysplastic nodule (n=8), non-tumorous tissue (n=48) and HCC (well-differentiated HCC and advanced HCC, n=48). Analysis was performed with a Kruskal-Wallis test followed by a post hoc Dunn's multiple comparison test (P<0.0001). HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HCC, hepatocellular carcinoma; IHC, immunohistochemistry.

respectively (95% CI, 0.829-0.956; P<0.0001; Fig. 4B, Table III). These results indicated that HNRNPA3 may be superior to conventional markers, such as GPC3, for differentiating HCC from non-tumor tissue.

HNRNPA3 expression is associated with tumor differentiation in HCC. To further investigate the clinical significance of HNRNPA3 in liver cancer, the association between HNRNPA3 expression and clinicopathological factors in HCC was investigated (Table IV). HNRNPA3 expression was found to be significantly associated with tumor differentiation (P=0.0472). However, no significant association was found between HNRNPA3 expression and other clinicopathological factors, including age, sex, cirrhosis, tumor number, tumor size, tumor biomarker levels and specific serum enzymes (Tables IV and SIV). There was also no significant association between HNRNPA3 expression and clinicopathological factors in DN (Table SV).

HNRNPA3 expression is associated with survival rate. According to the Human Protein Atlas, high expression of

Table II. Sensitivity, specificity, PPV and NPV for detection of early HCC (n=7) from HGDN (n=6) using HNRNPA3$^+$, GPC3$^+$ and combined HNRNPA3$^+$/GPC3$^+$.

Phenotype	Early HCC, n	HGDN, n	Sensitivity	Specificity	PPV	NPV
HNRNPA3$^+$	6	1	85.71	83.33	85.7	83.3
GPC3$^+$	5	1	71.43	83.33	83.3	71.4
HNRNPA3$^+$/GPC3$^+$	5	0	71.43	100.00	100.0	75.0

HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HCC, hepatocellular carcinoma; HGDN, high-grade dysplastic nodule; GPC3, glypican 3; NPV, negative predictive value; PPV, positive predictive value; +, positive expression.

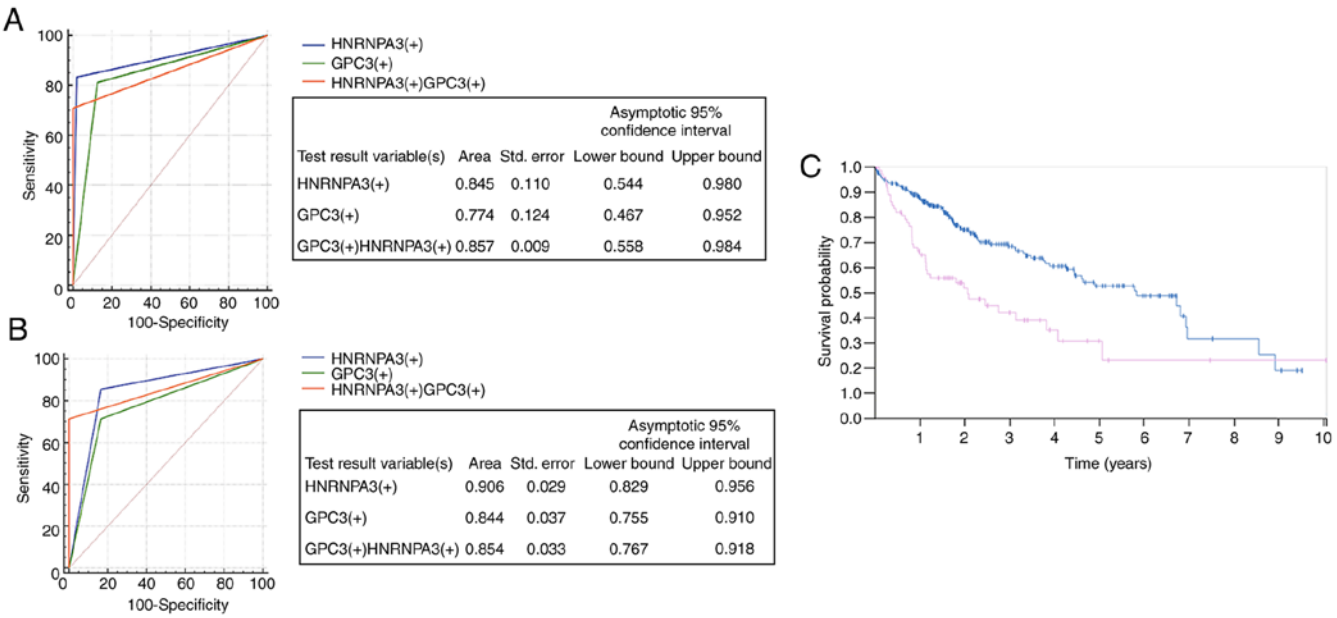

Figure 4. ROC curves confirmed the diagnostic value of HNRNPA3 expression in liver cancer, and survival analysis showed that high expression of HNRNPA3 indicates poor survival rate. (A) ROC curves were constructed to test the ability of HNRNPA3 expression to differentiate between HCC and non-tumorous normal tissue. (B) ROC curves were constructed to test the ability of HNRNPA3 expression to differentiate between HGDN and eHCC. (C) Survival analysis from Human Protein Atlas (P=0.000029) data. HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HCC, hepatocellular carcinoma; ROC, receiver operator characteristic; HGDN, high-grade dysplastic nodule; eHCC, early HCC; GPC3, glypican 3.

HNRNPA3 indicates a lower survival rate compared with that of patients with liver cancer exhibiting low expression of HNRNPA3 (Fig. 4C).

Discussion

HCC is the third most frequent cause of cancer-related mortality, and patients who are diagnosed at an earlier stage and receive effective treatment have improved overall survival (20). However, in the majority of cases, HCC is diagnosed at a late stage. HBV infection and Hepatitis C Virus infection are the most important risk factors for HCC. Due to the high infection rate of chronic HBV in China, the present study focused on HBV-related HCC. HBV-induced hepatocarcinogenesis is a multistep process progressing from cirrhosis, to LGDN, to HGDN, and finally to eHCC and progressed HCC (2). LGDN hepatocytes display minimal abnormalities, with well-defined boundaries, normal or slightly increased nuclear-cytoplasmic ratio and less nuclear atypia. Mitotic activity is absent and the portal tract is present. The thickness of the liver plate is 1-2 cell layers, it does not include pseudoglandular arrangements, and there is no obvious thickening. HGDNs display increased nuclear-cytoplasmic ratio, and more pronounced nuclear atypia and basophilic cytoplasm, the density of the cells is >twice the normal density, occasional mitotic figures may be seen, and the thickness of the liver plate is ≤3 cell layers. On microscopic observation there is a relative HGDN boundary, but this boundary is not clear on high magnification. Irregular trabecular hepatocytes are frequently arranged in HGDNs. Occasionally, pseudoadenoid arrangement may be seen (16). It is difficult to confirm whether the diagnosis is HGDN or well-differentiated HCC in the clinical setting, as they share similar pathological characteristics. The applicability of ultrasonography for diagnosing HCC in clinical practice is limited by the morphological similarity with tumors, operator dependency and deficient diagnostic accuracy (21). Furthermore, some widely accepted serum tumor markers, including α-fetoprotein, have not demonstrated satisfactory specificity and sensitivity in the diagnosis of HCC (22).

Table III. Sensitivity, specificity, PPV and NPV for detection of HCC (n=48) from non-tumorous tissue (n=48) using HNRNPA3$^+$, GPC3$^+$ and combined HNRNPA3$^+$/GPC3$^+$.

Phenotype	HCC, n	Non-tumorous tissue, n	Sensitivity	Specificity	PPV	NPV
HNRNPA3$^+$	40	1	83.33	97.92	97.6	85.5
GPC3$^+$	39	6	81.25	87.50	86.7	82.4
HNRNPA3$^+$/GPC3$^+$	34	0	70.83	100.00	100.0	77.4

HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HCC, hepatocellular carcinoma; GPC3, glypican 3; NPV, negative predictive value; PPV, positive predictive value; +, positive expression.

Table IV. Association between HNRNPA3 expression and clinicopathological factors in HCC.

		HNRNPA3 expression		
Variable	n	Negative	Positive	P-value
Age[a]				
≤55	14	3	11	
>55	34	5	29	0.6757
Gender[a]				
Male	41	6	35	
Female	7	2	5	0.3297
Tumor size[a]				
≤5	29	5	24	
>5	19	3	16	>0.9999
Tumor number (>1)[a]				
Yes	6	1	5	
No	42	7	35	>0.9999
Cirrhosis[a]				
Yes	32	5	27	
No	16	3	13	>0.9999
Differentiation[b]				
Well	10	2	8	
Moderate	32	3	29	
Poor	6	3	3	0.0472

HNRNPA3, heterogeneous nuclear ribonucleoprotein A3; HCC, hepatocellular carcinoma. [a]χ^2 test was used to investigate the association between HNRNPA3 expression and indicators that were subdivided into three groups. [b]Fisher's exact test was applied to find an association between HNRNPA3 expression and indictors that were subdivided into two groups.

HnRNPs are located at the border regions of chromatin to interact with newly synthesized nuclear RNAs (23). It is widely accepted that members of this protein family are known to act as regulators in cell cycle progression, cell differentiation, cell cycle arrest and DNA damage. HNRNP K has been demonstrated to coordinate with P53 in a mutually dependent manner under conditions of DNA damage, with knockdown of HNRNP K leading to defects in cell-cycle checkpoint arrest (24).

HNRNPA3 is a protein encoded in humans by the *HNRNPA3* gene and belongs to the hnRNP A/B family, along with other candidate transcription factors that interact with the regulatory region of the HOXC8 gene (25). *HNRNPA3* is located on chromosome 2, which is well known for its ability to regulate telomere length (26). It plays key roles in RNA binding, mRNA transport and mRNA splicing via spliceosome. Although the detailed association between tumorigenesis and HNRNPA3 has not been fully elucidated, the HNRNP protein family members are closely associated with cancer regulation (14,27). Moreover, HNRNPA3 has potential roles in carcinogenesis that have yet to be extensively investigated. Overexpression of another family member, HNRNPA1/A2, was found to control alternative splicing of the pyruvate kinase M gene and, thus, favor aerobic glycolysis to enhance tumorigenesis (27-29).

HNRNPA3 expression has not been investigated in the context of hepatocarcinogenesis before, and evaluation methods for HNRNPA3 using IHC are also unknown. Therefore, in the present study a semi-quantitative scoring criterion was employed in the IHC evaluation for HNRNPA3, considering both the staining intensity and percentage of positively stained cells. The subsequent verifications including ROC curves confirmed that this evaluation system is appropriate for HNRNPA3 analysis and diagnosing specific HCC stage. Based on the data and IHC results of the present study, HNRNPA3 expression was shown to increase in a stepwise manner from non-tumorous hepatic tissue via DN to HCC. It was verified by AUC that HCC was distinguishable from non-tumor tissue based on the changes in the expression of HNRNPA3, which appeared to be more effective compared with the currently available biomarkers. Moreover, HGDN and eHCC can be differentiated by the expression of HNRNPA3. GPC3 is highly expressed in HCC and is commonly used as a marker for differential diagnosis between HGDN and eHCC; however, it lacks sufficient sensitivity and specificity (9,10,12). Of note, the combined two-marker set (HNRNPA3$^+$/GPC3$^+$) was shown to increase the diagnostic accuracy between HGDN and eHCC, with a larger AUC and 100% specificity. Of note, more aggressive tumors, such as undifferentiated HCC, may be accompanied by high levels of HNRNPA3 expression. The Human Protein Atlas also supported that high expression of HNRNPA3 was associated with poor survival rate of patients with HCC. Therefore, HNRNPA3 may be considered as a potential diagnostic marker to identify specific stages during HCC development, and its overexpression may indicate a poor prognosis.

In regards to the association between HNRNPA3 expression and clinicopathological factors in HCC, the χ^2 test indicated that HNRNPA3 was associated with tumor differentiation. No such association was observed with serum enzyme levels, tumor biomarkers and HCC. However, the small sample size may limit the ability to evaluate the potential association between HNRNPA3 and clinicopathological factors in DN.

Collectively, the data of the present study revealed that the expression of HNRNPA3 increased in a stepwise manner from non-tumor cirrhotic tissue to DN and was the highest in HCC. HNRNPA3 combined with GPC3 may prove to be of value as a diagnostic tool to distinguish between HGDN and eHCC, which could provide a novel therapeutic strategy for pathologists to diagnose HCC with high accuracy. A high level of HNRNPA3 expression was also found to be associated with lower tumor differentiation and poor survival of patients with HCC. However, there is a limitation in the present study. Only the role of HNRNPA3 in the progression of HBV-related HCC was investigated. Further studies are warranted to reveal the potential significance and prognostic value of HNRNPA3 in HCV-related HCC.

Acknowledgements

Not applicable.

Funding

The present study was supported by The National Natural Science Foundation of China (grant nos. 81672842, 81872362 and 81802914) and The Taishan Scholars Program of Shandong Province (grant no. ts201511096).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

PG and HZ conceived and designed the present study. XR, MD and YD performed the experiments and drafted the initial manuscript. All authors have read and approved the final 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 present study was approved by the Ethics Committee of Shandong University (Jinan, China; approval no. 2012028). Written informed consent was provided by all participants prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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