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RNF6 serves as a diagnostic hallmark of non-alcoholic fatty liver disease with hepatocellular carcinoma: a clinical research

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Abstract. – OBJECTIVE: Ring finger protein 6 (RNF6) has been identified to be associated with the progression of colorectal cancer, gastric cancer and breast cancer. Its role in hepatocellular carcinoma (HCC), however, remains largely unclear. This study aims to illustrate the prognostic potential of RNF6 in nonalcoholic fatty liver disease (NAFLD)-HCC.

PATIENTS AND METHODS: A total of 162 eligible NAFLD-HCC patients treated in our hospital from May 2017 to May 2019 were recruited. RNF levels in the collected tumor tissues and paracancerous tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and immunohistochemistry (IHC). Then, the influences of RNF6 on overall survival (OS) and recurrence-free survival (RFS) in NAFLD-HCC patients were explored. Potential clinical factors for the prognosis in NAFLD-HCC were finally analyzed.

RESULTS: RNF6 was upregulated in NAFLD-HCC tissues. Highly expressed RNF6 at both mRNA and protein levels predicted poor OS and RFS in NAFLD-HCC. RNF6 level, metastasis, tumor node metastasis (TNM) staging, and tumor grade were correlated with the prognosis in NAFLD-HCC.

CONCLUSIONS: Great effects have been made on improving the diagnostic rate. Here, highly expressed RNF6 was unfavorable to OS and RFS in NAFLD-HCC. As a result, RNF6 not only contributed to the diagnosis of NAFLD-HCC, but also predicted its prognosis and recurrence.

Key Words:
NAFLD-HCC, RNF6, OS, RFS.

Introduction

The prevalence and mortality of hepatocellular carcinoma (HCC) rank sixth and second, respectively[1]. In 2012, there were 782,500 onsets and 745,500 deaths of HCC throughout the world. HCC cases in China account for nearly 50% of global cases[2]. Nonalcoholic fatty liver disease (NAFLD) affects 30-40% adults, and notably, its incidence remains high in obese population[3]. It is estimated that there are NAFLD in 10% patients will develop into NAFLD-HCC. Therefore, NAFLD is a vital risk factor for HCC. Researches on HCC hallmarks have been well concerned. α-Fetoprotein (AFP) is used for HCC diagnosis, whereas its sensitivity ranges about 25-65%. As a result, American Association for the Study of Liver Diseases (AASLD) guidelines proposed that AFP should not be considered as the diagnostic hallmark owing to its low sensitivity in cancer diagnosis[4,5]. Novel hallmarks that can effectively and sensitively diagnose and predict the prognosis of HCC are urgently required.

The ring finger protein 6 (RNF6) is a ubiquitination enzyme located on chromosome 13q12[6]. It was initially considered as a tumor suppressor, but later has been discovered to induce abnormal ubiquitination and transcriptional activity of androgen receptors, displaying the cancer-promoting effect[7]. In colorectal cancer, RNF6 is identified to drive its progression by activating the Wnt pathway[8]. The proliferative ability of leukemia cells K562 is triggered by RNF6[9]. Moreover, RNF6 is found to be upregulated in breast cancer, indicating the potential role as an oncogene[10].

This study detected RNF6 level in NAFLD-HCC tissues by qRT-PCR and IHC. Its influences on the prognosis in NAFLD-HCC patients were mainly demonstrated.

Patients and Methods

Patients and Samples

HCC patients treated in the Bishan Hospital of Chongqing from May 2017 to May 2019 were recruited based on the British Society of Gastroenterology (BSG). Patients were included in the

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analysis if their age ≥ 18 at diagnosis and had disease information for ≥ 12 months from registration. Exclusion criteria include information missing, a record of alcohol abuse, drug treatment at any time prior to diagnosis and a history of other liver disease within the 12 months prior to diagnosis. At last, 864 HCC patients were eligible, and 162 NAFLD-HCC patients were included in this trial. A total of 162 NAFLD-HCC tissues and 62 paracancerous tissues were collected. Tumor node metastasis (TNM) staging of each subject was recorded[10]. HCC metastasis was confirmed by cancer cell infiltration to the distant organs. Overall survival (OS) was the duration from the date of diagnosis to the death or the latest follow-up. Recurrence-free survival (RFS) was the duration from the date of surgery to the initial recurrence or the latest follow-up. This study was approved by the Ethics Committee of the Bishan Hospital of Chongqing. Signed written informed consents were obtained from all participants before the study.

Immunohistochemistry (IHC)

HCC tissues and paracancerous tissues were fixed in 4% buffered formalin for 24 h and embedded with paraffin. Then, the tissues were sliced to sections with 4 μm thickness. Sections were deparaffinized and rehydrated through xylene and ethanol, which were autoclaved at 120°C for 10 min using 10 mM/L sodium citrate buffer (pH 6.0). Rabbit anti-RNF6 (ab204506, Abcam, Cambridge, MA, USA) was used for IHC.

Two pathologists were responsible for independently assessing the immunoreactivity of RNF6 in a blinded way. IHC staining intensity was graded 1-4 (1: 0-24%, 2: 25-49%, 3: 50-74% and 4: 75-100% of tumor cells, respectively). The cut-off value of RNF6 (>25% and ≤25% of tumor cells) was used to classify high-level and low-level group.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from HCC tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Transcriptor Reverse Transcriptase (Roche, Basel, Switzerland). Real Time-PCR was carried out using an SYBR Green master mixture (Roche, Basel, Switzerland) on LightCycler 480 Instrument. At last, the relative level of RNF6 was calculated by $2^{-\Delta\Delta Ct}$ method and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences were shown below: RNF6: Forward: 5'-CGCCTAACCCTGGTAACATCC-3', Reverse: 5'-TCGGGTTTGTTCGACTCACA-3'; GAPDH: Forward: 5'-GCAGGGGGGAGCCAAAAGGG-3'; Reverse: 5'-TGCCAGCCCCAGCGTCAAAG-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Differences between groups was compared using the *t*-test. Chi-square test was performed to analyze the relationship between RNF6 level and clinical factors in NAFLD-HCC patients. Kaplan-Meier curves were depicted for assessing survival, followed by log-rank test applied for comparing between curves. Cox proportional hazard regression analysis was introduced for multivariate analysis on potential risk factors that influenced OS and RFS in NAFLD-HCC. $p<0.05$ was considered statistically significant.

Results

Upregulation of RNF6 in NAFLD-HCC tissues

Both mRNA level ($p<0.001$) and immunoreactivity of RNF6 ($p=0.006$) were higher in NAFLD-HCC tissues than paracancerous tissues (Figure 1A). In particular, the mRNA level of RNF6 was much higher in stage III+IV NAFLD-HCC tissues than that in stage I+II ones ($p=0.004$). Identically, IHC staining obtained the same result ($p<0.001$) (Figure 1B). It was suggested that RNF6 is upregulated in NAFLD-HCC tissues and its level is increased with the worsen of tumor staging.

Influences of RNF6 on Clinical Factors in NAFLD-HCC Patients

The average age of recruited NALFD-HCC patients was 61.8 years old (39-89 years old), including 102 men (63%) and 60 women (37%). Based on the cut-off value of the mRNA level of RNF6 in NAFLD-HCC tissues, recruited patients were classified into high-level group (81/162, 50%) and low-level group (81/162, 50%). No significant differences in age, gender and BMI were found between groups, whereas significant differences were observed in metastasis ($p<0.001$), TNM staging ($p<0.004$) and tumor grade ($p=0.006$) (Table I).

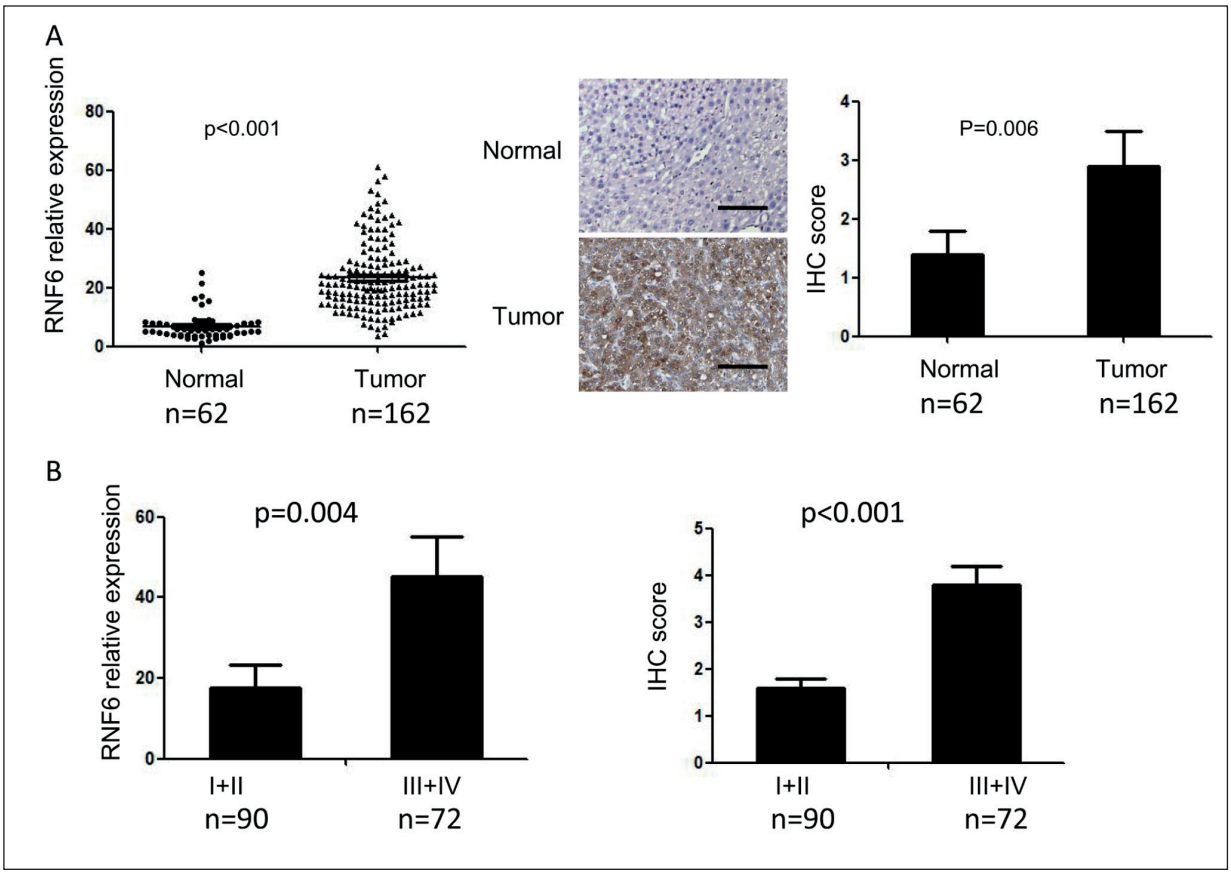

Figure 1. Upregulation of RNF6 in NAFLD-HCC tissues. **A**, The mRNA and protein levels of RNF6 in NAFLD-HCC tissues and paracancerous tissues detected by qRT-PCR and IHC, respectively (magnification: 400×). **B**, The mRNA and protein levels of RNF6 in stage I+II and stage III+IV NAFLD-HCC tissues. Scale bar = 50 μm.

Subsequently, patients were classified in the same way according to the semi-quantitive analysis on immunoreactivity of RNF6. Metastasis (p<0.001), TNM staging (p=0.002) and tumor grade (p=0.007) were significantly different between groups (Table II).

Influences of RNF6 on the Prognosis in NAFLD-HCC Patients

The 5-year follow-up data were collected for assessing the prognostic potential of RNF6 in NAFLD-HCC. There were 49 (30.2%) recurrent cases, 75 (46.2%) deaths and 87 (53.8%) surviv-

Table I. Primer sequences and product sizes in this research.

Polymorphism	Primer sequence (5'-3')	Product (bp)
rs128912	Forward: TCGATCCGATGTAGATGCT Reverse: ACGTGTAGTCGATGTCGTA	188
rs100321	Forward: CAGCTACGCTAGATCGATC Reverse: CAGCATGATAGCTAGCTGCA	105
rs129103	Forward: ACGATCGATCGTAGTGTAGC Reverse: ACGTAGCTGACTACGATCGT	215
TLR3	Forward: ACGATCGATCGTAGTGTAGC Reverse: ACGTAGCTGACTACGATCGT	156
GAPDH	Forward: ATTGCTAGGATCGTTTACCA Reverse: TGTAGTCGTAGTCGTGTAGT	129

Table II. Probe sequences of ligase reaction and product sizes of different TLR3 gene polymorphisms.

Polymorphism	Probe	Probe sequence (5'-3')
Frs128912	rs128912	P-ACGGTAGTCGTAGTTTTTTTTTTTTTTTTTT-FAM
	rs128912-A	TTTTTTTTTTCCCGTAGTCCCCATTTTTTTTTAT
	rs128912-T	TTTTTTTTTTTTTCAGCTACGACCGTTTTTTTTTAAA
rs100321	rs100321	P-AGCACACGTGTCAGCTTTTTTTTTTTTTT-FAM
	rs100321-C	TTTTTTTTTTTTTTTTTTTTTACACACGTAGCTAGTCG
	rs100321-T	TTTTTTTTTTTTTTTTTTTTTCAGCTAGTAGATGCTA
rs129103	rs129103	P-TAGCTGCTAGCTCCTTTTTTTTTTTTTTTTTT-FAM
	rs129103-C	TTTTTTTTTTTTTTTTTTTTTTTCACACGTAGATCG
	rs129103-G	TTTTTTTTTTTTTTTTTTTTTTTCACGTAGAGTGTGA

als. Among 87 survivals, 58 (35.2%) were recurrence-free survivals. High mRNA level of RNF6 was unfavorable to OS (p=0.003) and RFS (p=0.005) in NAFLD-HCC patients (Figure 2A). It was shown that the RFS was 48.9 and 123.5 months in high-level and low-level group, respectively (95% CI: 36.231-63.872 and 99.118-167.452, respectively). The OS was 68.4 and 159.7 months, respectively (95%CI: 49.332-91.435 and 133.554-179.221, respectively).

Identically, high protein level of RNF6 also predicted poor OS (p<0.001) and RFS (p=0.001) (Figure 2B). The RFS was 42.5 and 127.4 months, respectively (95% CI: 34.113-59.342 and 101.221-169.237, respectively). Besides, OS was 62.2 and 160.2 months, respectively (95% CI: 37.553-89.225 and 144.335-182.157, respectively).

The prognostic potential of RNF6 in NAFLD-HCC patients was assessed by multivariate analysis following adjustment of oth-

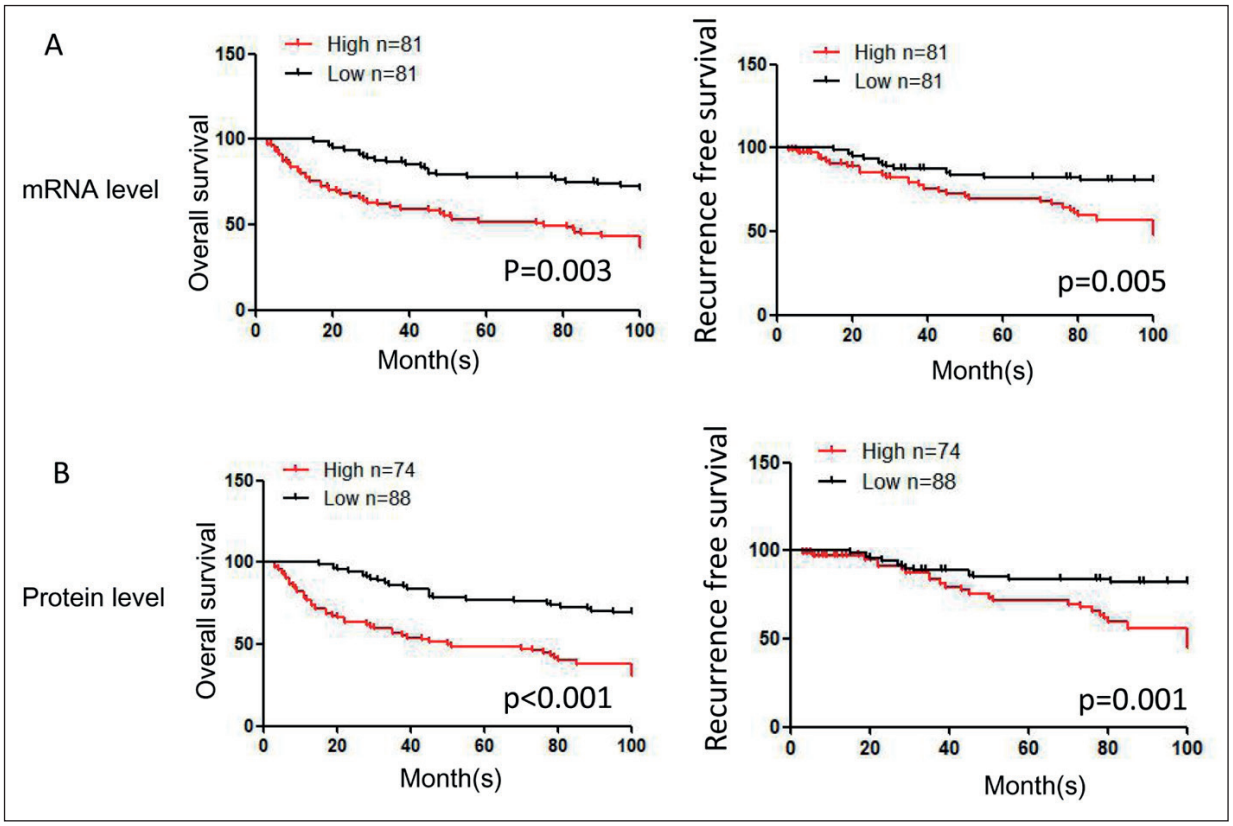

Figure 2. Influences of RNF6 on the prognosis in NAFLD-HCC patients. **A**, OS and RFS in NAFLD-HCC patients based on the mRNA level of RNF6. **B**, OS and RFS in NAFLD-HCC patients based on the protein level of RNF6.

Table III. Results of linkage disequilibrium test of TLR3 gene polymorphisms.

Polymorphism	r^2		
	rs128912	rs100321	rs129103
rs128912	–	0.002	0.051
rs100321	0.002	–	0.301
rs129103	0.051	0.301	–

er potentially prognostic parameters. It was found that the mRNA level of RNF6 was an independent prognostic factor for RFS in NAFLD-HCC (p=0.016). Highly expressed RNF6 also predicted shortened OS in NAFLD-HCC (p=0.011). In addition, the relative risk (RR) of tumor recurrence and death in NAFLD-HCC patients expressing high mRNA level of RNF6 was 3.161 (95% CI: 2.033-6.384) and 2.873 (95% CI: 1.894-5.022), respectively. Moreover, metastasis (p<0.001) and TNM staging (p=0.009) were also prognostic factors for OS in NAFLD-HCC (Table III).

Similarly, the protein level of RNF6 was closely linked to OS (p<0.001) and RFS (p=0.023) in NAFLD-HCC. The RR of tumor recurrence and death in NAFLD-HCC patients expressing high protein level of RNF6 was 3.113 (95%CI, 2.211-4.995) and 2.993 (95%CI, 1.788-5.662), respectively. As expected, metastasis (p<0.001) and TNM staging (p=0.006) were factors correlated with OS in addition to the protein level of RNF6 (Table IV).

Discussion

RNF6 used to serve as a tumor suppressor. The chromosome location of RNF6 contains many other tumor suppressors (i.e., p53 and PTEN)[11]. Later, RNF6 has been discovered to be an oncogene involved in many types of tumors, and it has been detected to be highly expressed in NAFLD-HCC tissues, especially advanced stage tissues, indicating that RNF6 is of significance during the progression of NAFLD-HCC.

The incidence of NAFLD is very high in obese people. With diet changes, and excessive intakes of processed food and high-fat food, the number of NAFLD cases increases with the popularization of obesity[12]. In this study, the highly expressed RNF6 was identified in NAFLD-HCC patients, whereas BMI was found to be unrelated to RNF6 expression in these patients. It is well known that RNF6 is responsible for protein ubiquitination. During the pathological process of NAFLD-HCC, cholesterol esters, rather than cholesterol, induce cancer cell proliferation[13]. The Wnt pathway can be activated by RNF6[8], which stimulates the storage of cholesterol esters[14]. Cholesterol is stored in the formation of cholesterol esters[15]. It is thereafter speculated that RNF6 may influence the progression of NAFLD-HCC by regulating cholesterol esters.

Table IV. Distribution of different genotypes of TLR3 gene polymorphisms in cataract patients.

Group	rs128912			rs100321			rs129103		
	AA	AT	TT	CC	CT	TT	CC	CG	GG
Cataract (n=80)	7.50%	30.00%	62.50%	22.50%	55.00%	22.50%	23.75%	51.25%	25.00%
Control (n=38)	23.68%	50.0%	26.31%	26.31%	44.74%	28.95%	31.58%	36.84%	31.58%
C^2	3.234			0.459			1.723		
p	0.002			0.326			0.081		

Table V. Distribution of alleles of TLR3 gene polymorphisms in cataract patients.

Group	rs128912		rs100321		rs129103	
	A	T	C	T	C	G
Cataract (n=80)	22.50%	77.50%	50.00%	50.00%	49.38%	50.62%
Control (n=38)	48.68%	51.32%	48.68%	51.32%	50.00%	50.00%
C^2	1.432		0.782		0.644	
p	0.000		0.114		0.412	

Conclusions

In this study, the oncogenic role of RNF6 in NAFLD-HCC was proven for the first time, which not only contributed to the diagnosis of NAFLD-HCC, but also predicted its prognosis and recurrence.

Conflict of Interests

The authors declare that they have no conflict of interests.

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