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Original Article

High RNF40 expression indicates poor prognosis of hepatocellular carcinoma

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Abstract: Human RING-finger protein 40 (RNF40) is reported as an E3 ligase of H2B ubiquitination. RNF40 needs to couple with its homolog RNF20 to format a complex to regulate DNA double strand break (DSB) response and chromatin stability. Deficient expression of RNF40 might cause incorrect DNA repair and contribute to genomic instability, leading to an abnormal transcriptional program. Incorrect DSB repair and aberrant gene transcription play important roles in tumorigenesis. The role in primary hepatocellular carcinoma (HCC), however, remains unclear. In this study, we selected 103 cases of HCC for immunohistochemistry to explore the role of RNF40 in HCC. The relationship between RNF40 expression and clinicopathological features of HCC was evaluated. RNF40 was mainly localized in the nucleus, where the percentage of low and high staining of RNF40 in tumor tissues was 50.4% (53/103) and 49.6% (50/103), respectively. By contrast, in para-normal tissues the percentage was 92.2% (95/103) and 7.8% (8/103) respectively. Expression of RNF40 in tumor tissues was significantly higher than that in para-normal tissues (P<0.01). Expression of RNF40 had significant association with AFP and TNM tumor stage (both P<0.01). However, age, gender, Hepatitis B Virus infection, liver cirrhosis, tumor size, tumor number, differential stage, and tumor thrombosis were not associated with RNF40 expression. Meanwhile, HCC patients with high expression of RNF40 had lower 5 year overall survival rates and disease-free survival rates (P<0.05). RNF40 is, potentially, an independent prognostic factor for survival in HCC.

Keywords: RNF40, hepatocellular carcinoma, immunohistochemistry, prognosis

Introduction

Primary liver cancer is one of the most common malignancies around the world, of which hepatocellular carcinoma (HCC) accounts for approximately 90% [1]. HCC has been reported to be the fifth most common cancer and second most common cause of cancer-related deaths [2, 3]. The nature of aggressive invasion, rapid progression, and difficulty of early diagnosis makes HCC so deadly. Most patients are already locally advanced at diagnosis, leading to unsuitable aggressive treatment for them. Moreover, HCC has a high risk of recurrence even when radical treatment is conducted. Therefore, it is important to understand the molecular mechanisms of development and progression of HCC in order to improve the therapeutic effect.

In recent years, an explosion of information concerning effects of histone modifications on gene transcription have been seen, including ubiquitination, acetylation, methylation, and phosphorylation [4]. Monoubiquitination of histone H2B is one of those important modifications associated with active chromatin [5]. It is required for methylation of histone H3 on lysine 4 (K4), thus, playing an important role in regulating cell proliferation [4], DNA double strand break (DSB) [6], cell differentiation [7], and cell cycle [8]. RING-finger protein 40 (RNF40), also named HuBre1B, has been identified as one of the responsible E3 ubiquitin ligases of H2B [5, 9, 10]. Database search and alignment revealed that it contains α-helical coiled-coil structure and a high conservative sequence of RING domain lies at the extreme C terminus [5]. Until now, RNF40 has been report-

ed as an important ligase for H2B ubiquitination [11], also known as an important partner for RNF206 to regulate DNA double-strand breaks (DSB) repair [12]. The ability of tumor cells to repair DNA damage, particularly DSB, is a major determinant of cancer progression and resistance to treatment [13]. On the other hand, it has been reported that RNF40 plays a critical role in regulating breast cancer cell [10] and prostate cancer cell proliferation [14, 15]. However, limited information is available regarding RNF40 and HCC. Therefore, we conducted immunohistochemistry of 103 HCC cases to investigate the relationship between RNF40 and HCC.

Materials and methods

Patients and tissue samples

This study was conducted with approval of the Ethical and Scientific Committees of Sir Run Run Shaw Hospital, Zhejiang University (Hangzhou, China). All patients were informed that resected specimens would be kept and possibly used for scientific research. We promised that their personal privacy would be protected.

A total of 103 HCC patients that underwent hepatectomy, between January 2006 and December 2010, were enrolled including 85 men and 18 women. Ages of patients ranged from 28 to 79 years (mean, 53 years). Patients that had received preoperative radiotherapy, chemotherapy, or immunotherapy before surgery were excluded. For all samples, we used each's own para-normal tissue as normal controls. Tumor stage was graded according to the 7th edition of International Union Against Cancer (UICC) tumor-node-metastasis (TNM) system. All patients were followed for up to 60 months or until their death.

Immunohistochemical technique

Immunohistochemistry was performed using GTVision^{TM} detection kit (Gene Tech, Shanghai), according to manufacturer instructions. Specimens obtained from surgical resection were fixed in 10% formalin prior to being processed in paraffin. All hematoxylin-eosin stained sections were reviewed and confirmed by two experienced pathologists, according to World Health Organization (WHO) classification guidelines. A representative section for each case was selected for immunohistochemical analysis.

Selected sections were dewaxed, hydrated with dimethylbenzene and a gradient concentration of alcohol, and then washed with deionized water and phosphate-buffered saline (PBS). Next, an antigen retrieval process was performed with 0.01 M citrate buffer (pH 6.0 Química Contemporânea, Diadema, Brazil) before blocking Endogenous peroxidase activity by 0.3% hydrogen peroxide for 15 minutes. Sections were incubated in preimmunized goat serum for 0.5 hours and then incubated overnight at 4°C refrigerator using the following primary antibody: anti-RNF40 (Atlas, rabbit polyclonal IgG, 0.2 mg/ml, 1:750 dilution, cat. No. HPA041330). The next day, after rewarming, sections were incubated with a secondary antibody of GTVision^{TM}/HRP, Rabbit/Mouse (ENV) reagent. Finally, ChemMateDAB + chromogen was used to visualize the reaction, followed by counterstaining with hematoxylin.

Evaluation of staining

Slide staining intensity and percentage of positive cells were evaluated by two independent investigators three times. A positive expression result was indicated by brown-yellow or brown granular deposits at corresponding antibody expression sites. Positive expression of RNF40 was located in the nucleus. Intensity of staining was scored in the following four categories: 0, negative; 1, weak; 2, moderate; and 3, strong staining. Similarly, the percentage of positive cells was also scored in 3 categories: 1, 0%-25%; 2, 25%-50%; 3, 50%-100%. The two scores were subjugated to obtain an immunoreactivity score (IRS) value of RNF40 expression ranging from 0 to 12. Specimens were divided into two categories, according to IRS: <6, low expression and 6-12, high expression.

Statistical analysis

Statistical analysis was carried out using SPSS 22.0 software package (SPSS, IBM, Chicago, IL, USA) by a third analyst, not participating in the experiment. The relationship between RNF40 expression and clinicopathologic features was estimated by Chi-square analysis or Fisher's exact test. Kaplan-Meier method was used to analyze survival curve and differences were judged by log-rank test. Disease-free sur-

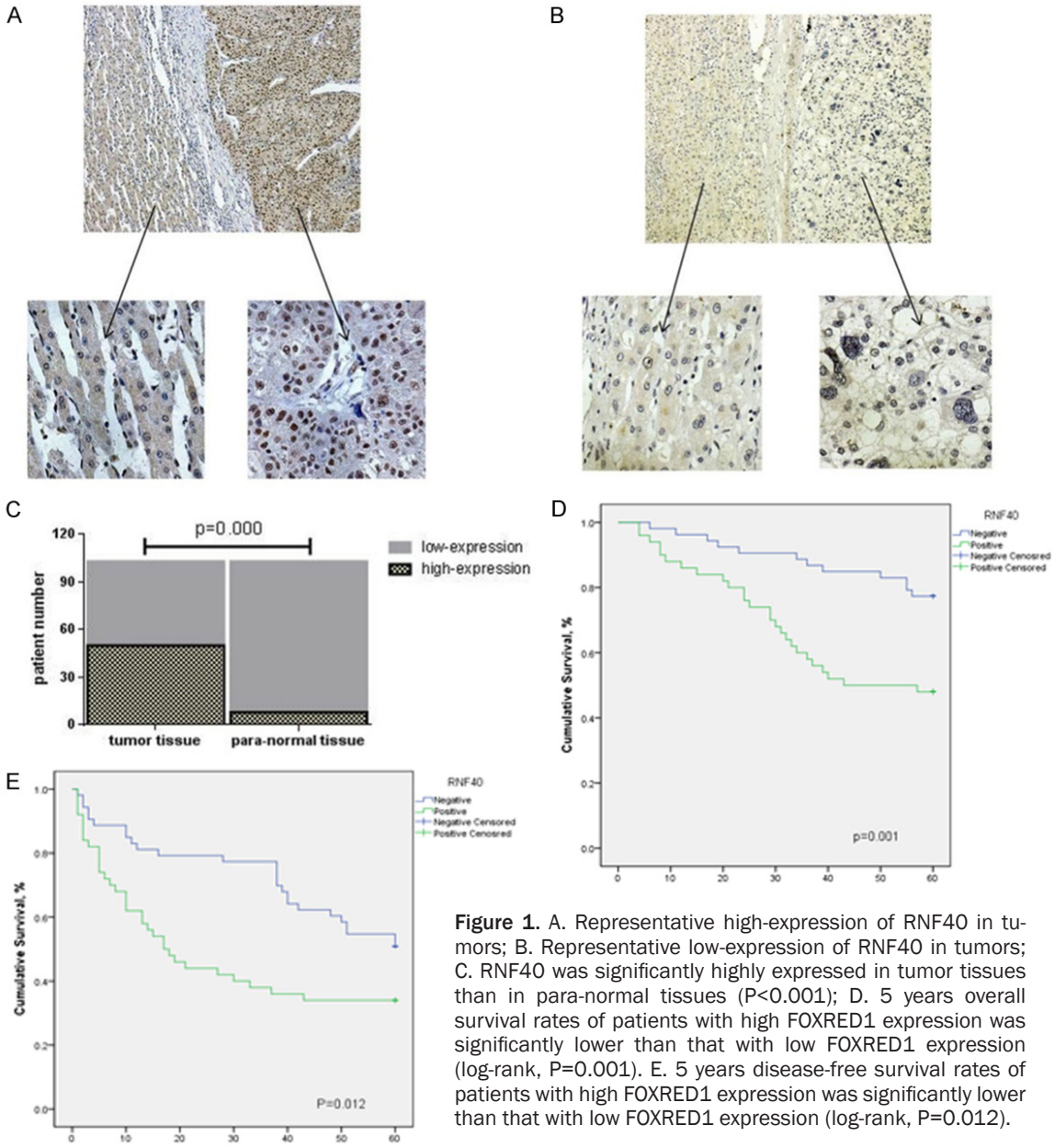

Figure 1. A. Representative high-expression of RNF40 in tumors; B. Representative low-expression of RNF40 in tumors; C. RNF40 was significantly highly expressed in tumor tissues than in para-normal tissues (P<0.001); D. 5 years overall survival rates of patients with high FOXRED1 expression was significantly lower than that with low FOXRED1 expression (log-rank, P=0.001). E. 5 years disease-free survival rates of patients with high FOXRED1 expression was significantly lower than that with low FOXRED1 expression (log-rank, P=0.012).

vival (DFS) was defined as the time from surgery to time of HCC recurrence and overall survival (OS) was defined as time from surgery to death of any cause. Cox's proportional hazards regression model was utilized for univariate and multivariate analyses. Multivariate analysis was performed depending on the Cox proportional hazards model for variables with P<0.05, examined in univariate analysis. Estimated relative risks of dying was expressed as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). P<0.05 was considered statistically significant.

Results

Expression of RNF40 in HCC

We performed immunohistochemical staining of RNF40 in 103 paired tissue samples, located in the nucleus. Representative immunostaining results of RNF40 in HCC tissues are shown in **Figure 1A**, **1B**). The percentage of low and high staining of RNF40 in tumor tissues was 50.4% (53/103) and 49.6% (50/103), respectively. The percentage of high staining was only 7.8% (8/103), meanwhile, that of low

Table 1. Clinical analysis of RNF40 expression in histopathological immunoreactivity in tumor tissues

Variables	RNF40 immunoreactivity		*P*-value
	Low (%)	High (%)	
Gender			0.090
Male	47 (88.7)	38 (76.0)	
Female	6 (11.3)	12 (24.0)	
Age, years			0.063
Median			
<53	20 (37.7)	28 (56.0)	
≥53	33 (62.3)	22 (44.0)	
Hepatitis B Virus			0.132
No	11 (20.8)	5 (10.0)	
Yes	42 (79.3)	45 (90.0)	
AFP, ng/mL			0.001
<400	41 (77.4)	23 (46.0)	
≥400	12 (22.6)	27 (54.0)	
Liver cirrhosis			0.598
No	25 (47.2)	21 (42.0)	
Yes	28 (52.8)	29 (58.0)	
Tumor size, cm			0.716
<5	31 (58.5)	31 (62.0)	
≥5	22 (41.5)	19 (38.0)	
Tumor number			0.470
Single	48 (90.6)	43 (86.0)	
Multiple	5 (9.4)	7 (14.0)	
Differential stage			0.062
Well/moderately	32 (60.4)	21 (42.0)	
Poorly	21 (39.6)	29 (58.0)	
Tumor thrombosis			0.065
No	49 (92.5)	40 (80.0)	
Yes	4 (7.5)	10 (20.0)	
TNM stage*			0.007
I + II	52 (98.1)	41 (82.0)	
III + IV	1 (1.9)	9 (18.0)	

*Distribution compared by Fisher's exact test.

staining was 92.2% (95/103) in paranormal tissues. In addition, RNF40 was found to be more highly expressed in tumors than paranormal tissues with significant differences, according to IRS value (P<0.01) (**Figure 1C**).

Relationship between RNF40 expression and clinicopathological feature in HCC patients

To evaluate association between RNF40 protein and HCC progression, we analyzed correlation between RNF40 expression and clinicopathological parameters of HCC patients (**Table 1**). High expression of RNF40 was found to be associated with AFP level (P<0.01) and tumor TNM stage (P<0.01) while no association was detected between RNF40 expression and gender, age, hepatitis B virus infection, liver cirrhosis, tumor size, numbers, thrombosis, and differential stages. In addition, Kaplan-Meier survival indicated that, both, overall survival times and disease free survival times were significantly shorter in positive expression patients than those in negative (**Figure 1D**, **1E**, both P<0.01).

Univariate analysis explained that tumor size (P<0.01), TNM stage (P<0.01), as well as RNF40 expression (P<0.01) was associated with overall survival time (**Table 2**). Results of multivariate analysis definitized that RNF40 expression (HR: 3.01, 95% CI 1.45-6.28, P<0.01) and tumor size (HR: 2.40, 95% CI 1.19-4.88, P<0.01) were independent prognostic factors for survival in HCC (**Table 2**).

Discussion

In this study, we initially found that RNF40 was highly expressed in tumor tissues. We also found that RNF40 expression in HCC tumor tissues is associated with elevated AFP and advanced TNM tumor stage. However, no significant differences were found between RNF40 expression and age, gender, hepatitis B virus infection, liver cirrhosis, tumor size, tumor numbers, differential stage, and tumor thrombosis. Five-year overall survival rates and disease-free survival rates of patients with high RNF40 expression were significantly lower than those of patients with low RNF40 expression. Such findings indicate that RNF40 could be an independent prognosis factor for HCC.

To the best of our knowledge, there have been only a few studies reporting association between RNF40 expression and cancer bioactivity. As we know, tumor cells enjoy DNA damage repairing and particularly DSB for cancer progression and resistance to treatment [13, 16]. RNF40 was found to be coupled with its family homolog RNF20 in order to form a heterodimer and stabilize each other [11, 17-19]. RNF40/20 dimer is linked to elongating RNAPII via the WW domain containing adapter with coiled-coil (WAC) protein that binds directly to pSer2 [20].

Table 2. Associations of clinical information with overall survival of hepatocellular carcinoma

Variables		Death (N=38)	Univariate Model		Multivariate Model	
			HR (95% CI)	*P* value	HR (95% CI)	*P* value
RNF40	High expression		2.96 (1.49-5.87)	0.002	3.01 (1.45-6.28)	0.003
Gender	Female		1.11 (0.49-2.52)	0.805		
Age, years	≥53		0.77 (0.41-1.45)	0.412		
Hepatitis B Virus	Yes		0.83 (0.36-1.88)	0.647		
AFP, ng/mL	≥400		1.39 (0.73-2.65)	0.314		
Liver cirrhosis	Yes		1.17 (0.61-2.23)	0.634		
Tumor size, cm	≥5		2.34 (1.24-4.45)	0.009	2.40 (1.19-4.88)	0.015
Tumor number	Multiple		1.30 (0.51-3.34)	0.581		
Differential stage	Poorly		1.26 (0.66-2.38)	0.483		
Tumor thrombosis	Yes		2.11 (0.97-4.61)	0.061		
TNM stage	III + IV		3.24 (1.42-7.40)	0.005	1.19 (0.46-3.09)	0.721

Phosphorylation of Ser2 is needed for H2B ubiquitination, both *in vivo* and *in vitro* [17, 20], thus, leading to decompressing the 30 nm chromatin fiber and facilitating DSB repair [19]. Soo Lee et al. reported novel RAP80-binding partner TRAIP [TRAF (tumor necrosis factor receptor-associated factor)-interacting protein], also known as RNF206, directly interacts with RNF40/RNF20 heterodimer to regulate recruitment of the damage signaling machinery and promote homologous recombination through the C terminus [12]. On the other hand, RNF40 knockdown has resulted in decreased H3K56ac and decreased recruitment of facilitate chromatin transcription (FACT) complex to chromatin following DSB [21]. Taken together, RNF40 acts as an important regulator in DSB repair. This might be a tumorigenesis factor.

Aberrant chromatin states of cancer cells are tightly correlated with abnormal transcriptional program [22]. Abnormal expression of RNF40/20 contributes to genomic instability, suggesting that RNF40/20 might play a role in the initial step of carcinogenesis [18]. Prenzel et al. reported that loss of RNF40 activity can sharply decrease ERα-dependent cell differentiation, which may be a critical step in development of hormone-independent breast cancer [10]. In addition, knockdown of RNF40 could activate AKT and ERK cell proliferation pathways, indicating that RNF40 regulates estrogen-independent cell proliferation of breast cancer cell [10]. Jääskeläinen et al. [14] and Hahn et al. [15] reported that RNF40 has the ability to interact with the androgen receptor (AR) by both functional and physical approaches, thus, modulating its transcriptional activity. Depletion of RNF40 strongly retards the growth of LNCaP cells, a kind of prostate cancer cell, due to decreased expression of several cycle promoters [14, 15]. Although our data indicates that RNF40 plays a role in regulating HCC, further mechanistic exploration is still required.

Conclusion

Our study demonstrates that the status of RNF40 expression might be a prognostic factor for HCC patients. Targeting this molecular mechanism could be a potential strategy for treatment of HCC.

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Disclosure of conflict of interest

None.

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