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High Expression of Retinoblastoma-Binding Protein 2 (RBP2) in Patients with Hepatocellular Carcinoma and Its Prognostic Significance

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Background: Recently, some studies have found that retinoblastoma-binding protein 2 (RBP2) is involved in the development and progression of many kinds of malignant tumors. This study aimed to explore the expression level of RBP2 in hepatocellular carcinoma (HCC) and its prognostic significance.

Material/Methods: Immunohistochemical analysis was used to evaluate the RBP2 expression level in 130 HCC patients and adjacent normal tissues. Tumor angiogenesis was marked by CD31 and vascular endothelial growth factor (VEGF) staining. Kaplan-Meier and Cox regression analyses were performed to examine the relationship between RBP2 expression and prognosis of HCC patients.

Results: RBP2 expression was significantly higher in HCC tissues (positive expression rate: 72.3%, 94/130). Increased RBP2 expression was dramatically associated with AFP level (P=0.016), degree of differentiation (P=0.000), and TNM stage (P=0.035). Moreover, tumors with RBP2-positive expression showed significantly higher intratumoral MVD than those with RBP2-negative expression (P=0.000). Kaplan-Meier analysis revealed RBP2-positive expression was related to decreased disease-free survival (DFS) (P=0.000) and overall survival (OS) (P=0.000). Furthermore, RBP2 was an independent poor prognostic factor of DFS and OS (P=0.029 and 0.010, respectively) as demonstrated by multivariate analysis.

Conclusions: Increased RBP2 expression, as an independent poor prognostic factor for DFS and OS of HCC patients, is closely related to tumor angiogenesis. RBP2 is expected to become a new potential therapeutic target for HCC.

MeSH Keywords: Angiogenesis Inducing Agents • Carcinoma, Hepatocellular • Prognosis • Retinoblastoma-Binding Protein 2

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Background

Hepatocellular carcinoma (HCC), a common malignant tumor of the digestive tract, is the third-leading cause of cancer death in the world [1] and ranks second in malignant tumor mortality in China [2]. Liver cancer has obvious vascular characteristics, the tumor cells of which can produce a variety of vascular growth factor to promote angiogenesis. Based on the above characteristics, anti-tumor angiogenesis strategy research and exploration in HCC patients is particularly necessary and has important clinical significance.

The emergence of targeted drugs presents new hope for the treatment of cancer patients, with higher specificity and relatively minor adverse effects [3]. In liver cancer, sorafenib [4] and regorafenib [5] are clinically proven to be effective oral agents, but the effect is still very limited. At present, there are a large number of molecular-targeted drugs. One such drug is apatinib, which is still in clinical trials and its efficacy is uncertain. In view of this, a currently popular liver cancer research focus is the molecular mechanism underlying the development of HCC and establishing a more effective targeted therapy.

Histone modification plays a key role in tumor progression, including angiogenesis [6]. For example, mixed-lineage leukemia 1 (MLL1), as the histone methylase, plays an important role in tumor growth and angiogenesis. Histone deacetylase 3 (HDAC3) can act as a negative regulator of angiogenesis factor. Retinoblastoma-binding protein 2 (RBP2) is a newly discovered histone demethylase that can participate in the development and progression of cancer [7,8]. Recently, studies have shown that RBP2 also plays an important role in the angiogenesis of cancer [9,10]. However, the biological and clinical significance of RBP2 in HCC patients remain largely unknown.

Therefore, in the present study, immunohistochemical staining was done to examine the expressions of RBP2, VEGF, and CD31-labeled microvessel density (MVD) in HCC and corresponding adjacent normal tissues. We also investigated RBP2 expression in HCC and its relationships with patient clinicopathological features, prognosis, and angiogenesis.

Material and Methods

Patients and samples

The tissue samples were collected from 130 patients diagnosed with HCC after curative operation at Renmin Hospital of Wuhan University from August 2009 to December 2012. Tumor staging was established on the basis of the sixth edition of the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC). All patients' clinicopathological parameters are summarized in Table 1. The study was authorized by the Ethics Committee of Renmin Hospital of Wuhan University and abided by the Declaration of Helsinki. Written informed consent was provided by all patients.

Immunohistochemical protocol and analysis

Immunohistochemical staining was done by a two-step method. RBP2, VEGF, and CD31 antibodies were used (the concentration of all antibodies was 1: 100). In brief, the procedure was: (1) Fix tumor tissues with 10% formalin at room temperature; (2) Rinse the tissue with running tap water to eliminate the formaldehyde; (3) Dehydrate the tissues in EtOH baths; (4) Clear the tissue twice in xylene; (5) Melt the paraffin prior to adding the tissue; (6) Pour melted paraffin into a paraffin block mold; (7) Section the paraffin-embedded tissue block in 4-μm-thick slices; (8) Float the tissue sections onto clean glass slides and microwave at 65°C for 15 min, and then store overnight at room temperature; and (9) Establish a negative control by using PBS to replace the primary antibody. Immunohistochemical scores were classified according to a published report [11].

MVD counts

MVD counts were labeled by CD31-positive staining vascular endothelial cells. After scanning an immunostained section at low magnification (×40), the regions with maximum number of dramatically marked microvessels stained with anti-CD31 were selected, and microvessels were counted at higher power (×100). All sections were evaluated by 2 pathologists independently.

Statistics and data analysis

Statistical analysis was done using SPSS 19.0 software. The relationship between RBP2 expression and clinicopathological characteristics was examined by Pearson X^2 test or Fisher test. Kaplan-Meier and Cox regression models were used to determine the survival rates and for multivariate analysis. Statistical significance was defined as $P < 0.05$.

Results

RBP2 expression and correlation with clinicopathological parameters in HCC

Immunohistochemistry was used in 130 cases of HCC and corresponding adjacent normal tissues to detect the clinicopathological and prognostic values of RBP2 in HCC. RBP2 protein staining was mainly located in the cytoplasm (Figures 1A, 2A). RBP2 positive expression rate was 72.3% (94/130) in HCC

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Table 1. Relationships between RBP2 protein expression in HCC tissues and clinicopathological variables.

Variables	Total	RBP2 expression			
		Negative (n=36)	Positive (n=94)	χ^2	*P*
Gender					
Male	106	33	73	3.393	0.065
Female	24	3	21		
Age at surgery (yeas)					
≤60	90	24	66	0.154	0.695
>60	40	12	28		
Tumor size (cm)					
≤5	62	21	41	2.260	0.133
>5	68	15	53		
HbsAg					
Negative	19	5	14	0.021	0.885
Positive	111	31	80		
Cirrhosis					
No	10	2	8	0.320	0.572
Yes	120	34	86		
Child-Pugh					
A	124	36	88	2.409	0.121
B	6	0	6		
AFP (ng/ml)					
≤20	44	18	26	5.802	0.016
>20	86	18	68		
Degree of differentiation					
Well/moderate	71	35	36	36.463	0.000
Poor and not	59	1	58		
TNM stage					
I/II	99	32	67	4.446	0.035
III/IV	31	4	27		

tissues (Figure 1). The correlations of RBP2 expression with clinicopathological factors are summarized in Table 1. Elevated RBP2 expression was dramatically related to AFP level (P=0.016), degree of differentiation (P=0.000) and TNM stage (P=0.035).

Relationship between RBP2 and VEGF protein expression

VEGF staining was mainly located in the cytoplasm (Figure 2B); 93 of the 130 HCC tissues were VEGF-positive (71.5%), and the positive rate of both RBP2 and VEGF was 57.7% (75/130). Furthermore, Pearson's test showed a significant relationship between expression of RBP2 and VEGF in tumor tissues (r=0.295, P=0.001; Table 2).

Correlation between RBP2 and MVD in HCC

MVD was counted by examining the CD31 staining to assess the correlation between RBP2 and angiogenesis (Figure 2C).

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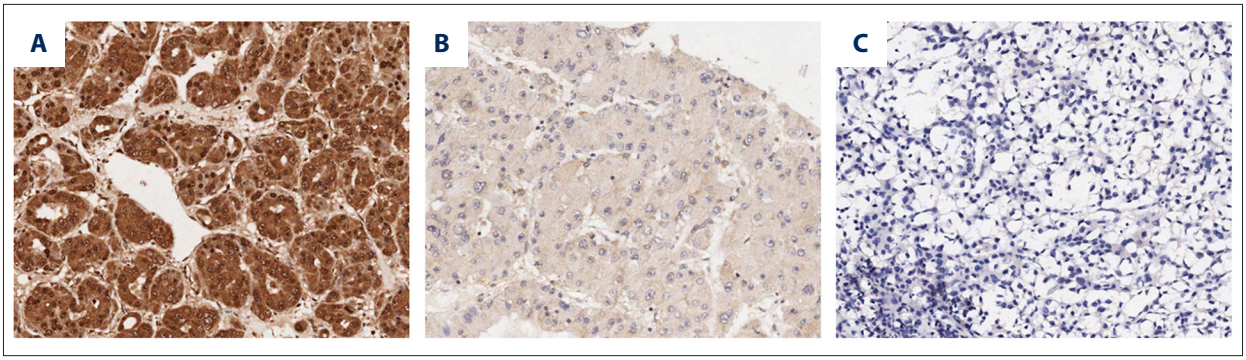

Figure 1. Immunochemical staining of RBP2 in HCC tissues. (**A**) High positive expression of RBP2; (**B**) Low positive expression of RBP2; (**C**) Negative expression of RBP2. (with 100× magnification).

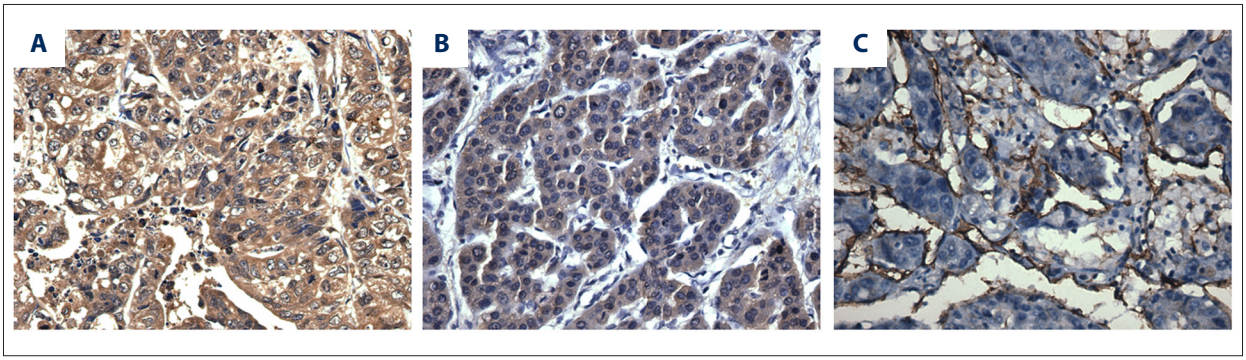

Figure 2. (**A–C**) Positive co-expression of RBP2, VEGF, and CD31 in HCC tissues, confirmed by immunochemical staining (400× magnification).

Table 2. Expression correlation of RBP2 and VEGF in HCC tissues.

Group	RBP2 expression		*r*	*P*-value
	Positive	Negative		
VEGF expression			0.295	0.001
Positive	75	18		
Negative	19	18		

RBP2-positive HCC tissues had an evidently higher MVD than in RBP2-negative tissues (P=0.000; Figure 3).

Relationship between RBP2 and prognosis

Survival analysis showed that RBP2 expression was inversely related to the survival of HCC patients. In comparison to those with negative RBP2 expression, DFS and OS times were significantly decreased in RBP2-positive patients (P=0.000, Figure 4A and P=0.000, Figure 4B, respectively).

Prognostic values in HCC patients

Univariate analysis was performed to reveal that RBP2 expression, degree of differentiation, and TNM stage had significant prognostic impacts on DFS and OS (Tables 3, 4). Furthermore, Cox analysis showed that RBP2 expression was an independent prognostic parameter for DFS (P=0.029) and OS (P=0.010) (Tables 5, 6).

Discussion

RBP2 belongs to the JARID family and can remarkably demethylate H3K4me2 and H3K4me3 [12]. Accumulating evidence demonstrated that RBP2 is abnormally expressed in many kinds of malignant tumors such as gastric cancer [9], non-small cell lung cancer (NSCLC) [10], and liver cancer [13]. These findings show that the function of RBP2 is mainly associated with the epithelial-mesenchymal transition (EMT), migration, invasion,

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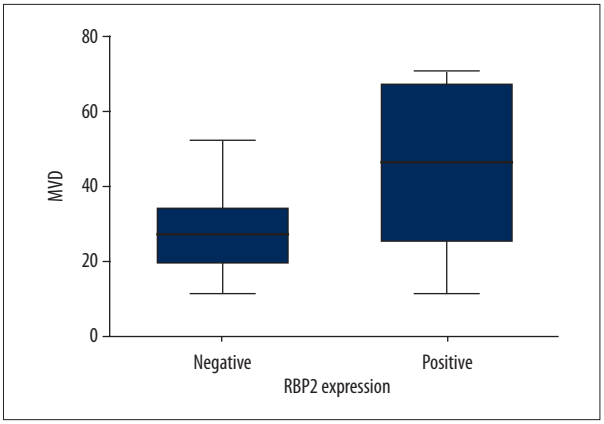

Figure 3. Intratumoral microvessel density (MVD) in relation to RBP2 protein immunoreactivity. HCC patients with RBP2-positive expression showed significantly higher intratumoral MVD than in patients with RBP2-negative expression (*P*=0.000).

and cell proliferation of cancer. However, whether RBP2 expression is related to HCC angiogenesis and its prognostic value still remain unclear. In the present study, our preliminary findings demonstrated that RBP2 was highly expressed in HCC tissues. Moreover, further results showed that RBP2-positive expression was remarkably related to the AFP level, degree of differentiation, and TNM stage. The above data suggest a pivotal role for RBP2 in progression and development of HCC.

Accumulating research demonstrates that overexpression of VEGF is associated with aggressive behavior and unfavorable prognosis of cancer [14,15]. Moreover, several studies have demonstrated that increased VEGF expression and MVD are significantly correlated with poorer prognosis in HCC [16,17]. In our study, a remarkable positive relationship between expression of RBP2 and VEGF was found. In comparison to those with negative RBP2 expression, patients with positive RBP2 expression had a significantly higher MVD, suggesting that RBP2 is involved in HCC tumor angiogenesis, possibly in cooperation with VEGF. Recently, Li et al. [9] found that RBP2 can directly bind to the promoter of VEGF to regulate its expression and promote the angiogenesis of gastric cancer by histone H3K4 demethylation. Qi et al. [10] found that RBP2 can promote HIF-1α-VEGF-induced angiogenesis of NSCLC via the AKT pathway. The AKT signaling pathway plays an important regulatory role in many cellular survival pathways, primarily in angiogenesis and tumorigenesis, through regulation of VEGF [18]. These results suggest that RBP2 may be engaged in promoting VEGF expression through PI3K/AKT/HIF-1α signaling. Furthermore, Fan et al. [19] recently reported that miR-34a promotes the osteogenic differentiation of hASCs via the RBP2/NOTCH1/CYCLIN D1 coregulatory network. Therefore, further detailed research is needed to elucidate the role of RBP2 in angiogenesis of HCC.

Next, we explored the clinical significance in prognosis of RBP2 in HCC. Compared to those with RBP2-negative expression, patients with RBP2-positive expression have decreased DFS and OS, as shown by Kaplan-Meir analysis. Univariate and multivariate analyses showed that RBP2 was an independent unfavorable predictor of DFS and OS in HCC patients.

There are several limitations in the present study. Firstly, it was a relatively small-sample, retrospective study, possibly leading to a selective bias. Secondly, we only used immunohistochemical

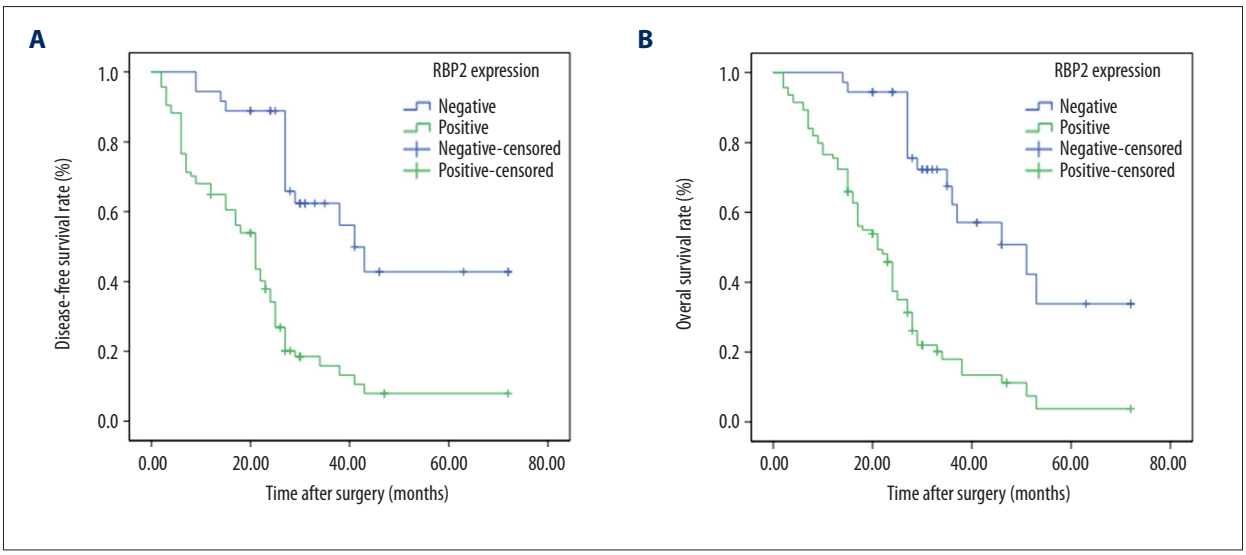

Figure 4. Kaplan-Meier analysis of disease-free survival (DFS) and overall survival (OS) curves of HCC patients based on RBP2 expression as positive or negative. (**A**) DFS curve of HCC patients based on RBP2 expression; (**B**) OS curve of HCC patients based on RBP2 expression.

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Table 3. Univariate analysis of the correlation between clinicopathological parameters and disease free survival time of HCC patients.

Variable	Mean survival time (m)	95% CI	P
Gender			
Male	32.256	20.793–43.719	0.843
Female	28.854	24.038–33.669	
Age at surgery (yeas)			
≤60	30.910	25.438–36.382	0. 319
>60	22.316	17.729–26.902	
Tumor size (cm)			
≤5	33.435	26.636–40.234	0.120
>5	24.766	19.587–29.945	
HbsAg			
Negative	29.906	25.081–34.731	0. 307
Positive	20.632	15.972–25.291	
Cirrhosis			
No	29.291	24.689–33.893	0.755
Yes	23.600	18.195–29.005	
Child-Pugh			
A	29.791	25.287–34.294	0.034
B	12.667	4.671–20.662	
AFP (ng/ml)			
≤20	32.007	30.650–43.410	0.127
>20	28.649	23.388–33.910	
Degree of differentiation			
Well/moderate	32.825	27.433–38.217	0.013
Poor and not	18.965	13.862–24.069	
TNM stage			
I/II	39.352	32.666–46.038	0.000
III/IV	16.549	13.397–19.701	
RBP2 expression			
Negative	47.323	38.148–56.228	0.000
Positive	22.140	17.965–26.315	

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Table 4. Univariate analysis of the correlation between clinicopathological parameters and overall survival time of HCC patients.

Variable	Mean survival time (m)	95% CI	P
Gender			
Male	32.341	21.511–43.171	0.943
Female	30.505	26.228–34.781	
Age at surgery (yeas)			
≤60	31.999	26.799–37.199	0. 389
>60	27.550	22.750–32.350	
Tumor size (cm)			
≤5	34.738	28.539–40.938	0.082
>5	27.087	22.083–32.092	
HbsAg			
Negative	31.652	27.185–36.119	0. 361
Positive	23.592	17.896–29.288	
Cirrhosis			
No	30.650	26.405–34.894	0.665
Yes	28.700	21.558–35.842	
Child-Pugh			
A	31.220	27.112–35.328	0.104
B	15.667	5.529–25.804	
AFP (ng/ml)			
≤20	37.030	30.650–43.410	0.016
>20	28.649	23.388–33.910	
Degree of differentiation			
Well/moderate	33.340	28.324–38.356	0.034
Poor and not	23.492	17.595–29.389	
TNM stage			
I/II	40.293	34.167–46.420	0.000
III/IV	19.677	16.115–23.239	
RBP2 expression			
Negative	48.395	40.477–56.344	0.000
Positive	23.670	20.000–27.341	

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Table 5. Multivariate analysis of the correlation between clinicopathological parameters and disease free survival time of HCC patients.

Covariates	HR	95% CI for HR	*P*
Gender (Male *vs.* Female)	0.739	0.413–1.324	0.310
Age (≤60 *vs.* >60 cm)	0.622	0.385–1.005	0.052
Tumor size (≤5 *vs.* >5 cm)	0.610	0.378–1.985	0.043
HbsAg (negative *vs.* positive)	1.654	0.813–3.362	0.165
Cirrhosis (No *vs.* Yes)	1.127	0.410–3.097	0.817
Child-Pugh (A *vs.* B)	0.547	0.185–1.618	0.276
AFP (≤20 *vs.* >20 ng/ml)	0.906	0.547–1.500	0.700
Differentiation (Well/moderate *vs.* Poor and not)	0.835	0.494–1.411	0.501
TNM stage (stage I/II *vs.* III/IV)	0.309	0.168–0.569	0.000
RBP2 expression (negative *vs.* positive)	0.476	0.244–0.925	0.029

Table 6. Multivariate analysis of the correlation between clinicopathological parameters and overall survival time of HCC patients.

Covariates	HR	95% CI for HR	*P*
Gender (Male *vs.* Female)	0.828	0.460–1.491	0.529
Age (≤60 *vs.* >60 cm)	0.722	0.718–1.624	0.177
Tumor size (≤5 *vs.* >5 cm)	0.592	0.365–0.960	0.034
HbsAg (negative *vs.* positive)	1.998	1.000–3.989	0.050
Cirrhosis (No *vs.* Yes)	0.531	0.192–1.466	0.222
Child-Pugh (A *vs.* B)	0.745	0.253–2.192	0.593
AFP (≤20 *vs.* >20 ng/ml)	0.707	0.425–1.176	0.182
Differentiation (Well/moderate *vs.* Poor and not)	0.945	0.554–1.611	0.835
TNM stage (stage I/II *vs.* III/IV)	0.377	0.208–0.681	0.001
RBP2 expression (negative *vs.* positive)	0.414	0.211–0.812	0.010

staining, which is a semi-quantitative method, to examine the expression of relative antibodies. Finally, the detailed underlying molecular mechanisms were not explored, which needs to be elucidated in our further studies.

Conclusions

Our preliminary findings demonstrated that increased RBP2 expression is closely related to HCC angiogenesis and is an independent adverse prognostic factor. RBP2 is expected to become a new potential therapeutic target for HCC.

Conflicts of interest

None.

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