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Original Article

Decreased cytoplasmic expression of Raptor correlates with disease progression and unfavorable prognosis in hepatocellular carcinoma

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Abstract: The purpose of the present study is to explore the correlation between regulatory associated protein of mTOR (Raptor) and clinicopathologic features in hepatocellular carcinoma (HCC), including patient survival. Immunohistochemistry was used to examine the expression of Raptor in 90 HCC tissues and peritumoral liver tissues. The relationship between tumor Raptor expression and clinicopathologic characteristics was analyzed. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. The significance of various survival variables was analyzed using multivariate Cox proportional hazards model. We found that Raptor protein was detected in cytoplasmic compartment. Significantly lower Raptor expression was observed in HCC compared to peritumoral liver cells (P=0.048). The tumor expression levels of Raptor significantly inversely correlated with clinical stage (P=0.026). Patients with high Raptor expression had better recurrence-free survival (P=0.010). Further, we observed that Raptor expression was positively associated with recurrence-free survival of HCC patients with tumor capsule (P=0.043) and without portal vein tumor thrombus (P=0.033) classifications. Finally, we found that Raptor was an independent prognostic factor of recurrence-free survival for patients with HCC (P=0.042). To conclude, our results support that decreased cytoplasmic expression of Raptor is a potentially unfavorable factor in the progression and prognosis of HCC.

Keywords: Raptor, hepatocellular carcinoma, immunohistochemistry

Introduction

HCC is the most frequent form of the hepatobiliary malignancies all over the world. Partial hepatectomy is a potentially curative therapy for patients with resectable HCC [1]. However, HCC recurrence rates at 5 years following surgery have been reported to exceed 70% [2, 3]. Risk factors for HCC include viral infections caused by hepatitis B virus, hepatitis C virus, alcoholic cirrhosis and inherited liver diseases [4, 5]. Chronic hepatitis B viral infection is the main cause of HCC in Asia and Africa, while hepatitis C viral infection in Europe, Japan, and North America [6, 7]. On the molecular level, many epigenetic and genetic events have been associated with the pathogenesis of HCC, including inactivation of tumor suppressors such as p53 and activation of epigenetic targeting of cancer-related genes such as JNK1 and Wnt signaling [8-11]. However, the molecular mechanisms of HCC invasion and metastasis are not yet fully understood.

The PI3K/Akt/mTOR pathway is activated in a number of human neoplasms, accompanied by shorter overall as well as recurrence-free survival [12]. This pathway plays an important role in the regulation of cellular functions, including cell survival, cell proliferation, cell motility, protein synthesis, and autophagy [13]. The mechanistic target of rapamycin (mTOR), a serine/threonine kinase that regulates cell growth and metabolism, exists in two distinct functional complexes: mTORC1 and mTORC2. mTORC1 consists of mTOR, Raptor, mLST8 and PRAS40 while mTORC2 consists of mTOR, Rictor, mLST8, Sin1 and PROTOR [14].

Raptor, the regulatory associated protein of mTOR, interacts with mTOR and mTORC1 substrates S6K1 and 4EBP1 [15-18]. With mTORC1,

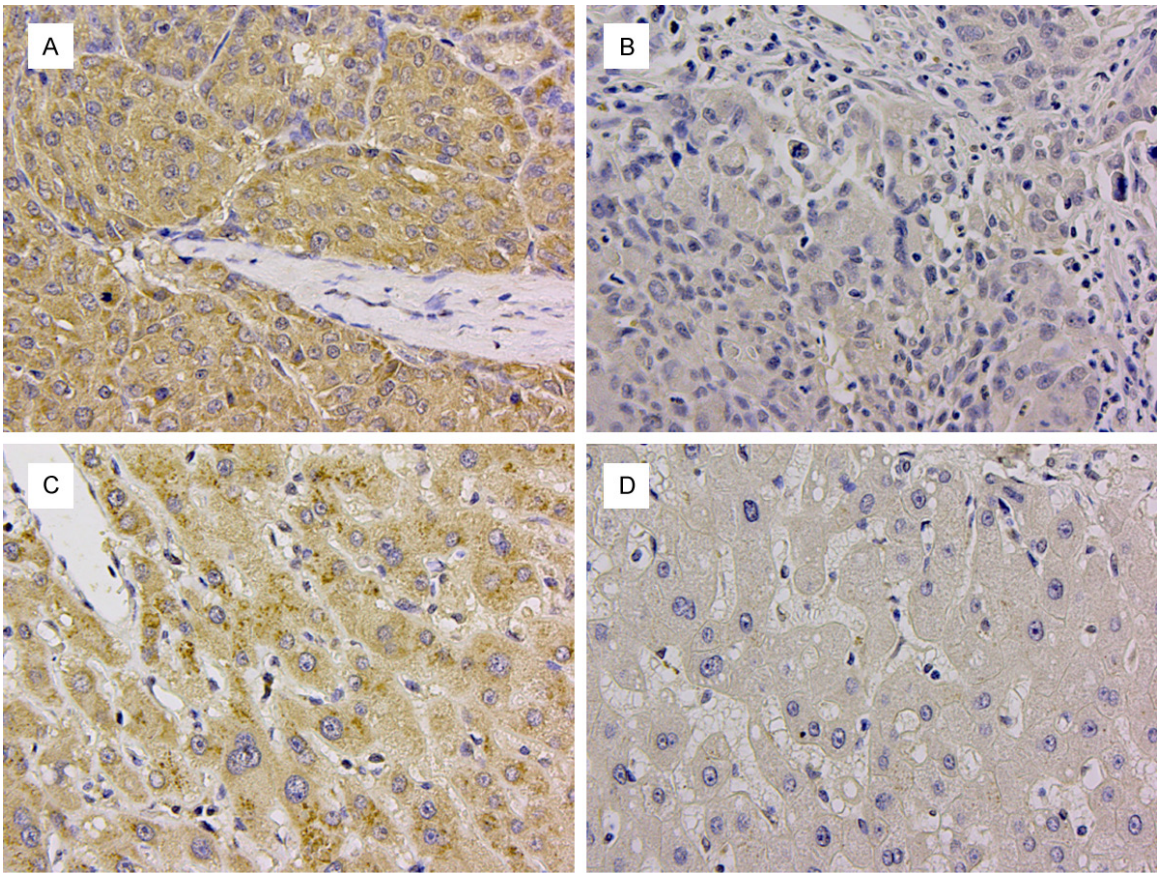

Figure 1. Raptor expression in hepatocellular carcinoma and peritumoral liver tissues. High (A) and low (B) expression of Raptor was demonstrated in HCC (400×). High (C) and low (D) expression of Raptor was demonstrated in peritumoral liver tissues (400×).

phosphorylation of S6K1 and 4EBP1 coordinately improves protein synthesis and promotes cell growth [19, 20].

In order to clarify the role of Raptor in the pathogenesis of hepatocellular carcinoma, we investigated the correlation of tumor Raptor protein expression with clinicopathologic features including survival. We found that the expression level of Raptor protein was lower in HCC tissues than that in peritumoral liver tissues. Lower expression of Raptor was associated with poor prognosis of HCC. Our results suggest that decreased cytoplasmic expression of Raptor is a potential unfavorable factor in the progression and prognosis of HCC.

Materials and methods

Sample collection

A tissue array including 90 paired paraffin-embedded HCC and peritumoral liver tissues were purchased from the National Engineering Center for BioChips in Shanghai, China. All patients with HCC underwent surgery during 2002 to 2008 in Taizhou Hospital of Zhejiang Province. Patients' age ranged from 27 to 84 years old. The clinical follow-up time of patients ranged from 7 to 72 months. For the use of these clinical materials for research purposes, prior consent from the patients and approval from the Ethics Committees of Taizhou Hospital of Zhejiang Province were obtained. All specimens had confirmed pathological diagnosis and were staged according to the American Joint Committee on Cancer (AJCC) (the seventh edition) staging system for HCC.

Immunohistochemistry

A tissue array with 90 paired paraffin-embedded HCC and peritumoral liver tissues was deparaffinized in 100% xylene and rehydrated in descending ethanol series (100%, 90%, 80%, and 70% ethanol) and water according to standard protocols. Heat-induced antigen

Table 1. Protein expression of Raptor between HCC and peritumoral normal liver samples

Group	Cases (n)	Protein expression (n)		*P* value
		High expression	Low expression	
Cancer	90	58 (64.4%)	32 (35.6%)	0.048
Normal	90	70 (77.8%)	20 (22.2%)	

Table 2. Correlation between the clinicopathologic characteristics and cytoplasmic expression of Raptor protein in HCC

Characteristics	N	Raptor expression (%)		*P*
		High	Low	
Age (y)				
<50	38	25 (65.8%)	13 (34.2%)	0.82
≥50	52	33 (63.5%)	19 (36.5%)	
Gender				
Male	74	48 (64.9%)	26 (35.1%)	0.858
Female	16	10 (62.5%)	6 (37.5%)	
Clinical stage				
I-II	56	41 (73.2%)	15 (26.8%)	0.026
III	34	17 (50%)	17 (50%)	
ALT (umol/L)				
<40	41	29 (70.7%)	12 (29.3%)	0.254
≥40	49	29 (59.2%)	20 (40.8%)	
AFP (ug/L)				
<20	27	18 (66.7%)	9 (33.3%)	0.773
≥20	63	40 (63.5%)	23 (36.5%)	
TB (umol/L)				
<20	74	45 (60.8%)	29 (39.2%)	0.121
≥20	16	13 (81.3%)	3 (18.7%)	

retrieval was performed in 10 nM citrate buffer at 100°C for 2 min. Endogenous peroxidase activity and non-specific antigen were blocked with peroxidase blocking reagent containing 3% hydrogen peroxide and serum, followed by incubation with rabbit anti-human Raptor antibody (1:100) (Abcam, Cambridge, MA, USA) overnight at 4°C. After using phosphate-buffered saline to wash, the tissue array was incubated with biotin-labelled goat anti-mouse/rabbit antibody at room temperature for 10 minutes at room temperature and was subsequently incubated with streptavidin-conjugated horseradish peroxidase (HRP) (Maixin Inc, China). The peroxidase reaction was developed with 3,3-diaminobenzidine (DAB) chromogen solution in DAB buffer substrate. The tissue array was then counterstained with haematoxylin, mounted in neutral gum and analyzed using a bright-field microscope.

Evaluation of staining

The tissue array immunohistochemically stained for Raptor was reviewed and cytoplasmic staining scored separately by two pathologists blinded to the clinical parameters. Staining intensity was scored as described previously [21]. The extent of staining, defined as the percentage of positive staining areas of tumor cells or peritumoral liver tissues in relation to the whole tissues area, was scored on a scale of 0-4 as the following: 0, <10%; 1, 10-25%; 2, 26-50%; 3, 50-75%; and 4, >75%. The sum of the staining-intensity and staining-extent scores was used as the final staining scores for Raptor (0-7). For statistical analysis, final staining scores of 0-5 and 6-7 were respectively considered to be low and high expression.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. The χ^2 test was used to verify the relationship between the levels of Raptor expression and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. The significance of various variables in survival was analyzed using multivariate Cox proportional hazards model. A value of *P* less than 0.05 was considered statistically significant.

Results

Immunohistochemical analysis of Raptor protein expression in HCC and peritumoral liver tissues

We measured the expression levels and cellular localization of Raptor protein on a tissue array including 90 paraffin-embedded HCC and peritumoral liver samples. Specific Raptor protein staining was detected in the cytoplasm of malignant and peritumoral liver cells (**Figure 1**). Furthermore, Raptor cytoplasmic expression was significantly decreased in HCC compared to peritumoral liver samples. We observed that in 77.8% (70/90) of peritumoral liver samples, Raptor protein was highly expressed. In comparison, only 64.4% (58/90) of HCC samples had highly expressed Raptor protein, signifi-

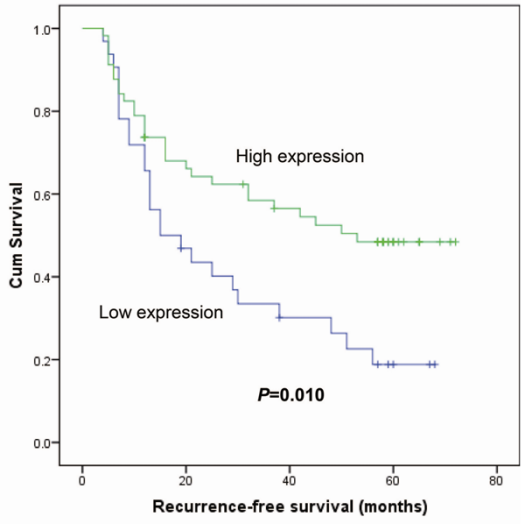

Figure 2. Cytoplasmic expression of Raptor was an unfavorable factor for recurrence-free survival in HCC. Low expression of Raptor protein was correlated with the shorter recurrence-free survival time for HCC patients (P=0.010).

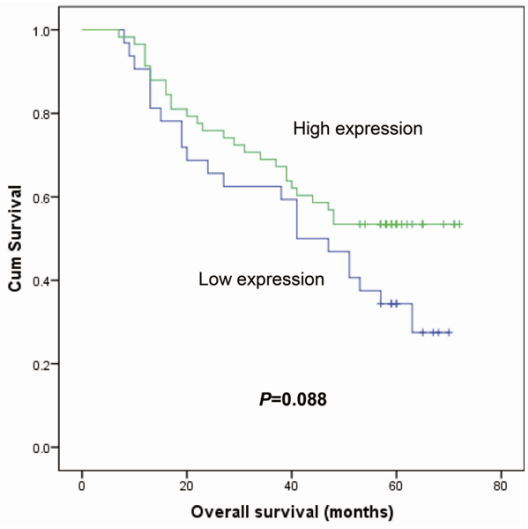

Figure 3. Cytoplasmic expression of Raptor was an unfavorable factor for overall survival in HCC. Low expression of Raptor protein was correlated with the shorter overall survival time for HCC patients (P=0.088).

cantly lower than that in the peritumoral liver samples (*P*=0.048) (**Table 1**).

The association between the clinicopathological characteristics and Raptor expression in HCC patients

As shown in **Table 2**, we did not find out the significant correlation between tumor Raptor expression and the patients' age, gender, serum alpha-fetoprotein (AFP) and total bilirubin (TB) level in HCC patients. However, we observed that tumor Raptor cytoplasmic expression inversely correlated with clinical stage (I-II vs. III) (*P*=0.026) in 90 HCC cases (**Table 2**).

Raptor expression is positively associated with recurrence-free survival of HCC

To investigate the prognostic value of tumor Raptor expression for HCC, we assessed the association between the levels of Raptor expression and patient survival using Kaplan-Meier analysis with the log-rank test. In 90 HCC cases with prognosis information, we observed that the level of Raptor cytoplasmic protein expression was significantly correlated with recurrence-free survival. Patients with low expression of Raptor had worse recurrence-free survival than those with high expression (*P*=0.010) (**Figure 2**). Additionally, patients with low expression of Raptor had worse overall survival than those with high expression, but not significantly (*P*=0.088) (**Figure 3**).

Raptor expression is positively associated with recurrence-free survival of HCC patients based on Tumor capsule and portal vein tumor thrombus

We further analyzed the correlation between Raptor expression and survival prognosis for HCC patients by strata analysis against tumor capsule and portal vein tumor thrombus. These results indicated that Raptor protein expression was significantly associated with recurrence-free survival for HCC patients based on with tumor capsule (*P*=0.043) and without portal vein tumor thrombus (*P*=0.033) (**Figure 4**). In these groups, patients with low expression of Raptor had worse recurrence-free survival than those with high expression.

Cytoplasmic expression of Raptor is an independent prognosis factor of recurrence-free survival in HCC patients

Univariate analysis showed that clinical stage, serum alanine aminotransferase (ALT) level and tumor Raptor expression were all significantly associated with recurrence-free survival of HCC patients (*P*=0.012, *P*=0.007, and *P*=0.012, respectively). To determine the potential of Raptor cytoplasmic expression as an independent prognostic factor for HCC, we per-

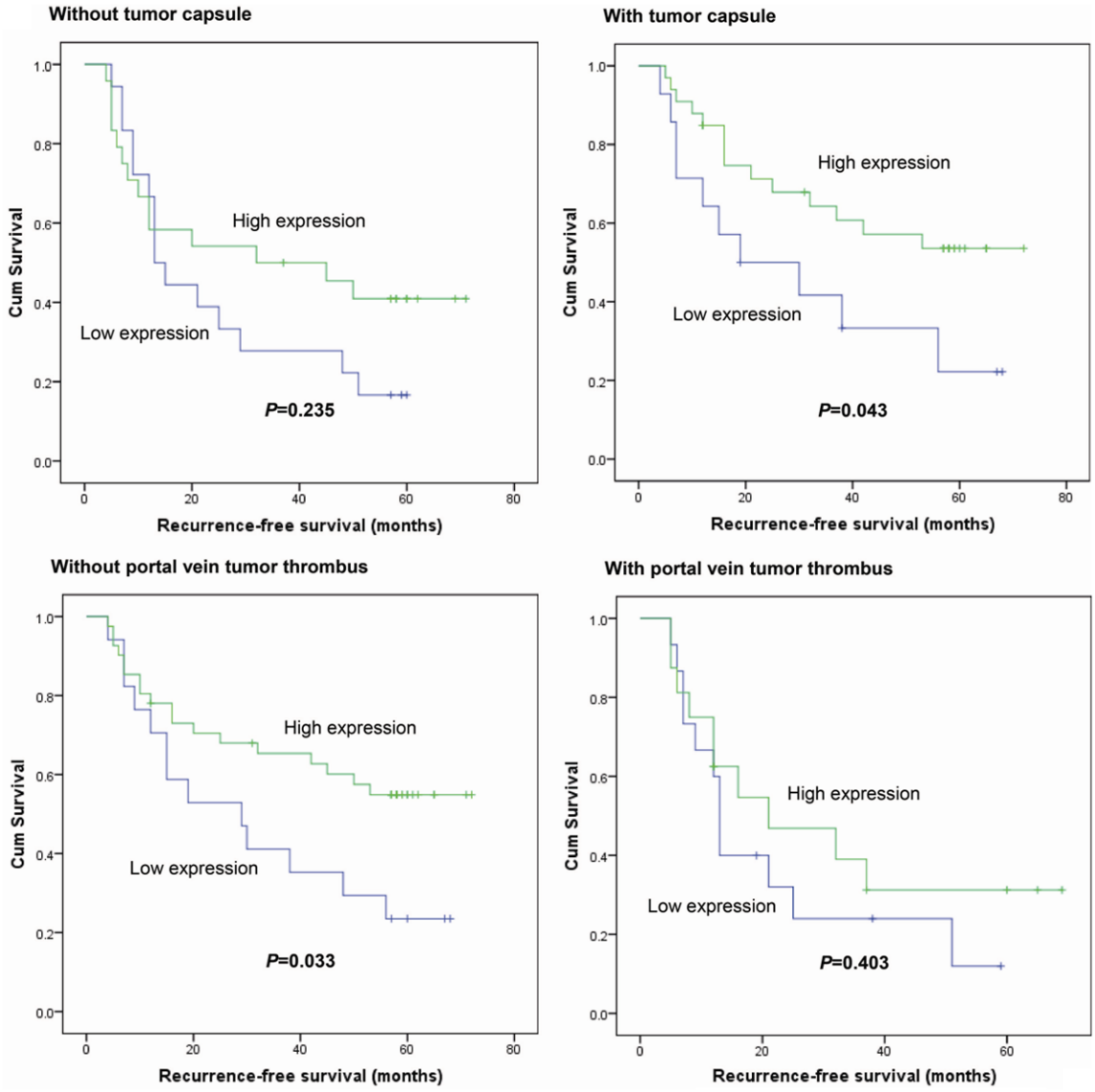

Figure 4. The correlation of Raptor expression with recurrence-free survival time for HCC patients stratified by tumor capsule and portal vein tumor thrombus classification. Patients with a higher expression of Raptor protein had a longer recurrence-free survival time in the HCC patients with tumor capsule and without portal vein tumor thrombus.

formed multivariate analysis of Raptor protein expression levels adjusted for clinical stage and ALT of HCC patients. These results showed that the level of Raptor expression was an independent prognostic factor of recurrence-free survival for HCC (P=0.042) (**Table 3**).

Discussion

HCC is one of the most common malignant cancers with poor prognosis. The high recurrence rate after surgery is the main cause of death in the HCC patients. The presence of vascular invasion is considered as a strong predictor of HCC recurrence [22]. Clinical trials have reported that there were low response rates with cytotoxic chemotherapy [23-25]. As the preferred approach for advanced HCC patients with adequate liver function, Sorafenib could only provide 2.4 months longer median overall survival than placebo [25].

Activation of PI3K/Akt/mTOR signaling through mutation of pathway components occurs in a majority of cancers [26, 27]. In a study, hepatocyte-specific Raptor ablation strongly potentiated hepatocarcinogenesis in mice [28]. It has been discovered that Raptor staining mainly

Table 3. Summary of univariate and multivariate Cox regression analysis of recurrence-free survival

Parameter	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age						
<50 versus ≥50 years	0.962	0.987	0.569-1.712			
Gender						
Male versus female	0.179	1.676	0.789-3.563			
AFP						
<20 versus ≥20 ug/L	0.226	0.686	0.372-1.263			
TB						
<20 versus ≥20 umol/L	0.777	1.109	0.541-2.273			
ALT						
<40 versus ≥40 umol/L	0.007	0.452	0.254-2.273	0.057	0.554	0.302-1.018
Clinical stage						
I-II versus III	0.012	0.493	0.285-0.853	0.09	0.603	0.336-1.082
HCC Raptor expression						
High expression versus low expression	0.012	2.001	1.162-3.446	0.042	1.796	1.021-3.160

displayed in the cytoplasm of colorectal cancer, prostate cancer, and renal cell carcinoma [29-31]. Similarly, our study presented that Raptor expressed in the cytoplasmic of the HCC and peritumoral liver tissues. It was revealed that down-regulation of Raptor in two paclitaxel-resistant ovarian cancer cell lines when compared with paclitaxel-sensitive cells [32]. However, in colorectal carcinoma (CRC), the correlation with the Raptor expression and patients' survival is controversial. It was reported that CRC patients with dominant Raptor expression had a better overall survival than those with dominant Rictor expression [29]. In contrast, it was observed that Raptor is overexpressed in colorectal cancers and associated with distant metastases, higher mortality and worse prognosis in CRCs in other studies [33, 34].

Recent report has shown that the HCC patients with early recurrence had significantly higher tumor Rictor/Raptor ratios compared with those with long-term recurrence-free survival [35]. These suggested that upregulated Raptor may play a protected role during HCC progression. However, the correlation between cytoplasmic Raptor expression and clinical features has not been reported in HCC.

In this study, we found that Raptor protein expressed in HCC samples was significantly lower than peritumoral liver samples. Although cytoplasmic expression of Raptor level was not associated with most clinical features, such as age, gender, serum AFP, TB, and ALB level, it was inversely correlated with clinical stage. This hints that low cytoplasmic expression of Raptor promotes the clinical progression of HCC and further supports the role of Raptor as a potential tumor suppressor in HCC.

In this investigation, we presented evidence that cytoplasmic expression of Raptor protein in HCC was positively correlated with patients' recurrence-free survival time. The results showed that the patients with low expression of Raptor had significantly worse recurrence-free survival time than those with high expression. Furthermore, we also observed a trend of significantly shorter overall survival in patients with low cytoplasmic expression of Raptor compared with high expression (P=0.088). Our results suggest that low cytoplasmic expression of Raptor is a clinically relevant biomarker for HCC prognosis, especially for the recurrence-free survival.

It is well known that invasion and metastasis of tumor cells are the key causes of death for HCC patients. We assessed survival prognosis by strata analysis against clinical features associated with invasion and metastasis including tumor capsule and portal vein tumor thrombus. Interestingly, we observed that cytoplasmic expression of Raptor protein was significantly associated with the recurrence-free survival time for the HCC patients with tumor capsule and without portal vein tumor thrombus.

Patients with low cytoplasmic expression of Raptor protein in these groups had a shorter recurrence-free survival time.

Finally, we analyzed the possibility of Raptor cytoplasmic expression as an independent prognostic factor for HCC. Based on univariate analysis, HCC patients' recurrence-free survival is inversely proportional to clinical stage, and serum ALT level but positively correlated with Raptor cytoplasmic expression. Multivariate analysis showed that Raptor cytoplasmic expression was an independent predictor of recurrence-free survival for HCC patients regardless of patient disease status.

In summary, our study demonstrates that decreased cytoplasmic expression of Raptor may be involved in the clinical progression and poor prognosis of HCC patients. Due to the limited patient sample size in our investigation, further studies are needed to verify these findings and establish the role of Raptor as a reliable clinical predictor of outcome for HCC patients.

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Disclosure of conflict of interest

None.

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