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Original Article

Aberrant expression and prognostic value of RacGAP1 in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is the most common type of liver cancer, which is characterized by rapid progressive development, high degree of malignancy, high post-surgical recurrence, extremely poor prognosis and high mortality, since most HCC patients are identified at a late stage upon diagnosis. Identification of novel biomarkers for early diagnosis and predicting prognosis of HCC is therefore of urgent need, which may potentially improve the prognosis and mortality of HCC cases. Rac GTPase-activating protein 1 (RacGAP1) has been shown to correlate with the biological behaviors and poor prognosis of multiple cancers; however, the role of RacGAP1 in the development, progression and prognosis of HCC remains unknown to date. The major purpose of this study was to examine the associations of RacGAP1 with the clinicopathological characteristics and prognosis of HCC. The RacGAP1 protein was determined in 93 HCC specimens, 39 liver biopsy specimens of hepatic cirrhosis patients, 30 liver biopsy specimens of hepatitis patients and 18 normal liver specimens using immunohistochemistry, and the associations of RacGAP1 protein expression with the clinicopathological features and survival of HCC patients were evaluated. Immunohistochemical staining revealed negative RacGAP1 expression in normal liver specimens, and liver biopsy specimens of hepatitis and hepatic cirrhosis patients, while 44.1% (41/93) of HCC specimens were positive for RacGAP1 ($P < 0.05$). In the HCC specimens, RacGAP1 was predominantly expressed in the cytoplasm, and partially co-localized in the nucleus and cytoplasm. The RacGAP1 expression was found to correlate with the tumor size, pathological grading, TNM stage, vascular tumor thrombus and lymph node metastasis of HCC (all P values < 0.05), but not with patients' gender or age (both P values > 0.05). Univariate analysis showed a significantly lower survival rate in HCC patients with positive RacGAP1 expression, vascular invasion and advanced TNM stage, while multivariate analysis revealed that high RacGAP1 expression was an independent prognostic factor in HCC patients. The results of this study demonstrate that aberrant expression and abnormal localization of RacGAP1 in the cytoplasm and nucleus may correlate with the development and progression of HCC, and RacGAP1 may have a promising value to serve as a biomarker for malignant biological behaviors and poor prognosis of HCC.

Keywords: Hepatocellular carcinoma, RacGAP1, prognosis, aberrant expression, clinicopathological characteristics

Introduction

Primary liver cancer is a common malignant tumor in the gastrointestinal system, in which hepatocellular carcinoma (HCC) consists of approximately 90% [1-3]. Worldwide, 748,000 new HCC cases are diagnosed per year, and HCC is currently found to rank third in the causes of cancer-related deaths [4]. HCC is characterized by rapid progressive development, high degree of malignancy, extremely poor prognosis and high mortality, since approximately 80% of the HCC patients are identified at a late stage upon diagnosis, and a high post-surgical recurrence and metastasis is detected [5]. Identification of novel biomarkers for early diagnosis and predicting prognosis of HCC is therefore of urgent need, which may potentially improve the prognosis and mortality of HCC cases [6-8].

As a member of GTPase-activating protein family, RacGTPase-activating protein 1 (RacGAP1), also termed MgcRacGAP or Cyk4, interacts with the small G protein, a member of RhoGTPases that binds to GTP, and stimulates GTP hydrolyzation, which promotes the transformation into an inactive state that binds to GDP, thereby

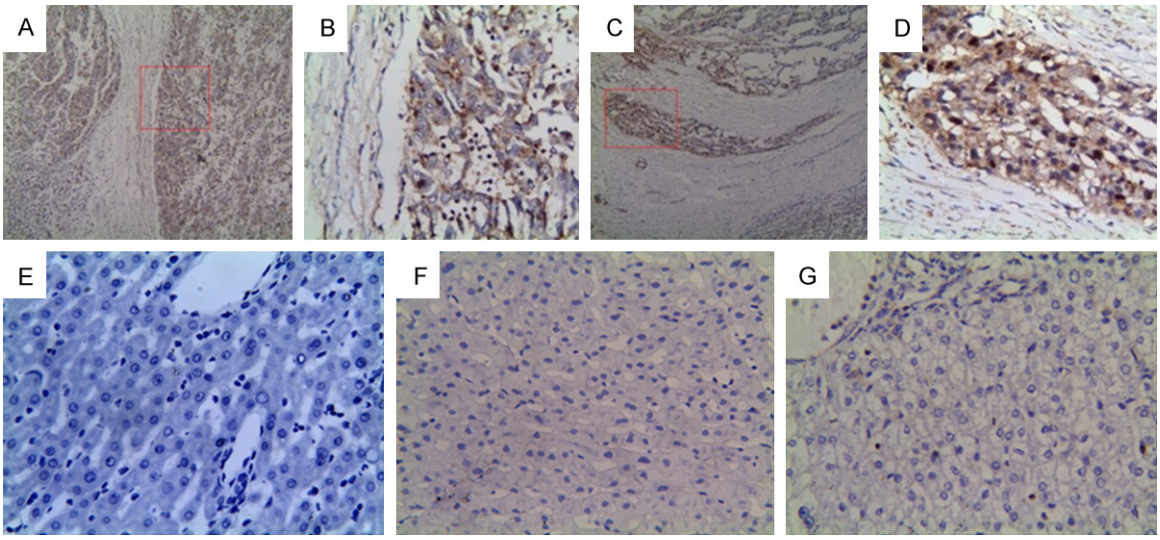

Figure 1. Immunohistochemical staining for RacGAP1 in live specimens. A. Cytoplasmic RacGAP1 expression (× 100); B. Cytoplasmic RacGAP1 expression (× 400); C. Cytoplasmic/nuclear RacGAP1 co-expression (× 100); D. Cytoplasmic/nuclear RacGAP1 co-expression (× 400); E. RacGAP1 expression in normal liver tissues; F. RacGAP1 expression in liver biopsy specimens of hepatitis (× 400); G. RacGAP1 expression in liver biopsy specimens of hepatic cirrhosis (× 400).

resulting in negative regulation of Rho protein [9-11]. In vertebrate cells, RacGAP1 is found to promote the formation of a central spindle protein complex, which plays an indispensable role in cell division [12-15]. In addition, RacGAP1 is involved in cell transformation, movement, migration and transfer [16-18]. It has been recently demonstrated that RacGAP1 plays a critical role in the development and progression of multiple cancers, and RacGAP1 expression was found to correlate with the biological behaviors and poor prognosis of malignant tumors [19-24]. To our knowledge, however, the associations of RacGAP1 with the clinicopathological characteristics and prognosis of liver cancer remain unknown till now. The current study was therefore designed to detect and compare the RacGAP1 expression between HCC and non-HCC tissues, and assess the associations of RacGAP1 with the clinicopathological characteristics and survival of HCC, so as to evaluate the role of RacGAP1 in the development and progression of HCC and the correlation between RacGAP1 and prognosis of HCC.

Materials and methods

Ethical approval

This study was approved by the Ethical Review Committee of the First Affiliated Hospital of Bengbu Medical College (permission No.: BYFY2013-0027). Signed informed consent was obtained from all participants or their guardians following a detailed description of the purpose of the study.

Specimens

A total of 93 HCC patients with complete medical records that underwent treatment in the First Affiliated Hospital of Bengbu Medical College during the period from January, 2008 through December, 2012, were enrolled in this study. The subjects included 78 men and 15 women, and had a median age of 55 years (range, 33 to 72 years). There were 42 cases with a tumor diameter of 5 cm or less, and 51 cases with a tumor diameter of > 5 cm; 12 cases with lymph node metastasis and 81 cases without lymph node metastasis; 8 cases with portal vein tumor thrombus and 85 cases without portal vein tumor thrombus. All patients underwent surgical resection of tumors, and no pre-surgical chemotherapy or radiotherapy was given. According to the TNM Staging System developed by the American Joint Committee on Cancer (AJCC), there were 25 cases with stage I, 19 cases with stage II, and 49 cases with stage III HCC; and there were 9 cases with grade I, 59 cases with grade II, 24 cases with grade III and 2 cases with grade IV HCC using the Edmondson grading system. The 93 surgi-

Table 1. Correlation between RacGAP1 expression and clinic-pathological features of HCC

Characteristic		Number of cases	RacGAP1 expression		χ^2	*P*
			+	-		
Gender	Male	78	35	43	0.121	0.728
	Female	15	6	9		
Age (years)	< 60	76	33	43	0.075	0.785
	≥ 60	17	8	9		
Tumor size (cm)	≤ 5	42	11	31	9.950	0.002*
	> 5	51	30	21		
Pathologic grade	I/II	67	23	44	9.257	0.002*
	III/IV	26	18	8		
TNM staging	I	25	5	20	8.379	0.015*
	II	19	9	10		
	III	49	27	22		
Vascular invasion	Yes	12	9	3	5.342	0.021*
	No	81	32	49		
Lymph node metastasis	Yes	8	7	1		0.020*
	No	85	34	51		

*$P < 0.05$.

cally resected HCC specimens were collected for the subsequent determinations, while 39 liver biopsy specimens of hepatic cirrhosis patients, 30 liver biopsy specimens of hepatitis patients, and 18 normal liver specimens collected from adults with traumatic liver resection or accidental death served as controls.

Immunohistochemistry

The HCC specimens, liver biopsy specimens of hepatic cirrhosis, liver biopsy specimens of hepatitis and normal liver specimens were fixed in 10% paraformaldehyde, embedded in paraffin wax, and cut into sections with 4 μm in thickness. Then, sections were dewaxed, hydrated, antigen-retrieved in citrate buffer, boiled and cooled naturally. After twice repetition of these procedures, sections were incubated in mouse anti-human RacGAP1 monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA) at 4°C overnight, while those incubated in PBS served as negative controls. Subsequently, sections were incubated in biotin-conjugated goat anti-mouse secondary antibody at 37°C for 1 h, added with streptavidin-biotin complex (SABC), visualized with DAB and counterstained with hematoxylin. Positive staining and intracellular localization of RacGAP1 was visualized under an optical microscope by two independent pathologists. The presence of yellow-brown granules in cell nucleus or cytoplasm was identified as positive RacGAP1 staining, while absence of yellow-brown granules was defined as negative staining. Ten fields of vision were randomly selected from each section, and 100 cells were observed in each field of vision. The percentage of positively stained cells was calculated. A < 10% proportion of positive cell staining was defined as negative RacGAP1 expression, while the percentage of positive cell staining > 10% or greater was considered as positive expression [25].

Statistic analyses

All clinical data were entered into Microsoft Excel 2007 (Microsoft; Redmond, WA, USA), and all statistical analyses were performed using the statistical software SPSS version 17.0 (SPSS, Inc.; Chicago, IL, USA). Differences of proportions were tested for statistical significance with chi-square test or Fisher's exact text, and the overall survival was estimated using a Kaplan-Meier method. The correlation between RacGAP1 expression and clinic-pathological features of HCC patients were evaluated using chi-square test, while the associations of RacGAP1 expression and clinic-pathological features with the clinical prognosis were examined with Kaplan-Meier method and log-rank test. Factors affecting the overall survival of HCC patients were identified using Cox regression analysis. A *P* value < 0.05 was considered statistically significant.

Results

RacGAP1 expression in HCC and non-HCC specimens

Immunohistochemical staining revealed that RacGAP1 protein was localized in the cytoplasm and nucleus, and positive RacGAP1 expression appeared pale brown. Among the 93 HCC specimens, positive RacGAP1 staining was determined in the cytoplasm of 31

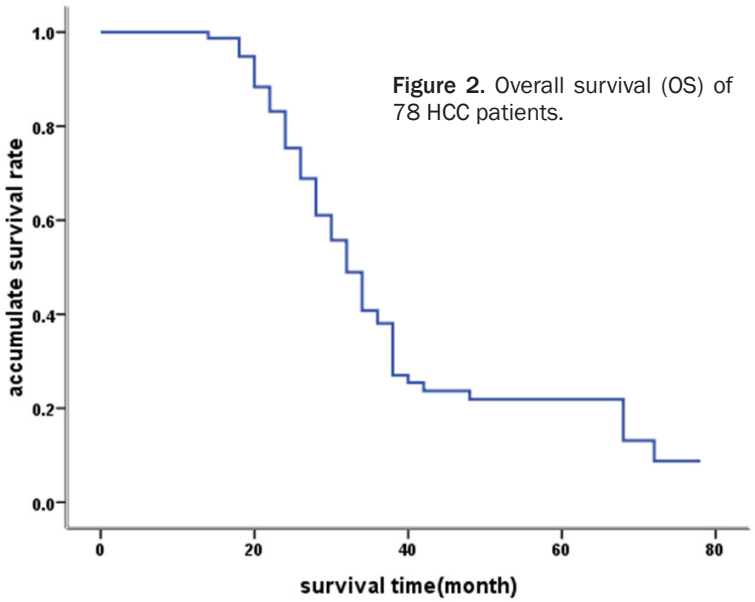

Figure 2. Overall survival (OS) of 78 HCC patients.

specimens (**Figure 1A** and **1B**), and positive expression was co-localized in the cytoplasm and nucleus of another 10 specimens (**Figure 1C** and **1D**), while negative RacGAP1 expression was detected in normal liver specimens (**Figure 1E**), or liver biopsy specimens of hepatitis (**Figure 1F**) or hepatic cirrhosis patients (**Figure 1G**). There was a significant difference in the RacGAP1 expression between HCC and non-HCC specimens (44.1% vs. 0; $\chi^2 = 49.668$, $P < 0.01$).

Correlation between RacGAP1 expression and clinicopathological characteristics of HCC

RacGAP1 expression was found to correlate with the tumor size ($P = 0.002$), pathological grading ($P = 0.002$), TNM stage ($P = 0.015$), presence of vascular tumor thrombus ($P = 0.021$) and development of lymph node metastasis of HCC specimens ($P = 0.02$), while no associations of RacGAP1 expression were detected with HCC patients' gender or age (both P values > 0.05) (**Table 1**).

Associations of RacGAP1 expression and clinicopathological features with survival of HCC patients

Among the 93 HCC patients, 78 cases underwent post-surgical follow-up, including 3 cases lost for follow-up and 14 cases survived until the ending of this study. The 78 patients had a median survival of 31.2 months (range, 13.1 to 72.0 months), and the overall survival curve for the 78 cases is shown in **Figure 2**.

Kaplan-Meier survival analysis showed a significant reduction of the survival rate in the HCC patients with positive RacGAP1 expression in relative to those with negative expression (**Figure 3A**; $P = 0.000$), in the patients with a tumor size of > 5 cm as compared to those with a tumor size of < 5 cm (**Figure 3B**; $P = 0.043$), in the patients with vascular invasion in relative to those without vascular invasion (**Figure 3C**; $P = 0.0013$), and in the patients with advanced clinical stage as compared to those with early stage (**Figure 3D**; $P = 0.0014$).

Factors affecting the prognosis of HCC

RacGAP1 expression and the clinicopathological characteristics of HCC patients, including HCC patients' gender, age, tumor size, clinical stage, histological grading, hepatic cirrhosis and vascular tumor thrombus, were included in the Cox regression model to identify the factors affecting the prognosis of HCC patients. Univariate analysis showed no significant associations of patients' gender, age, tumor size or lymph node metastasis with the prognosis (all P values > 0.05), while RacGAP1 expression, vascular tumor thrombus and clinical stage significantly correlated with the prognosis of HCC patients (all P values < 0.05) (**Table 2**).

RacGAP1 expression, tumor size, vascular tumor thrombus and clinical stage were included in the Cox regression analysis to further examine the factors affecting the prognosis of HCC patients, and multivariate analysis revealed that RacGAP1 expression was a major risk factor affecting the median survival of HCC patients ($P = 0$), indicating RacGAP1 expression is an independent prognostic factor of HCC (**Table 3**).

Discussion

RacGAP1, a member of GTPase-activating protein family, has shown prognostic and predic-

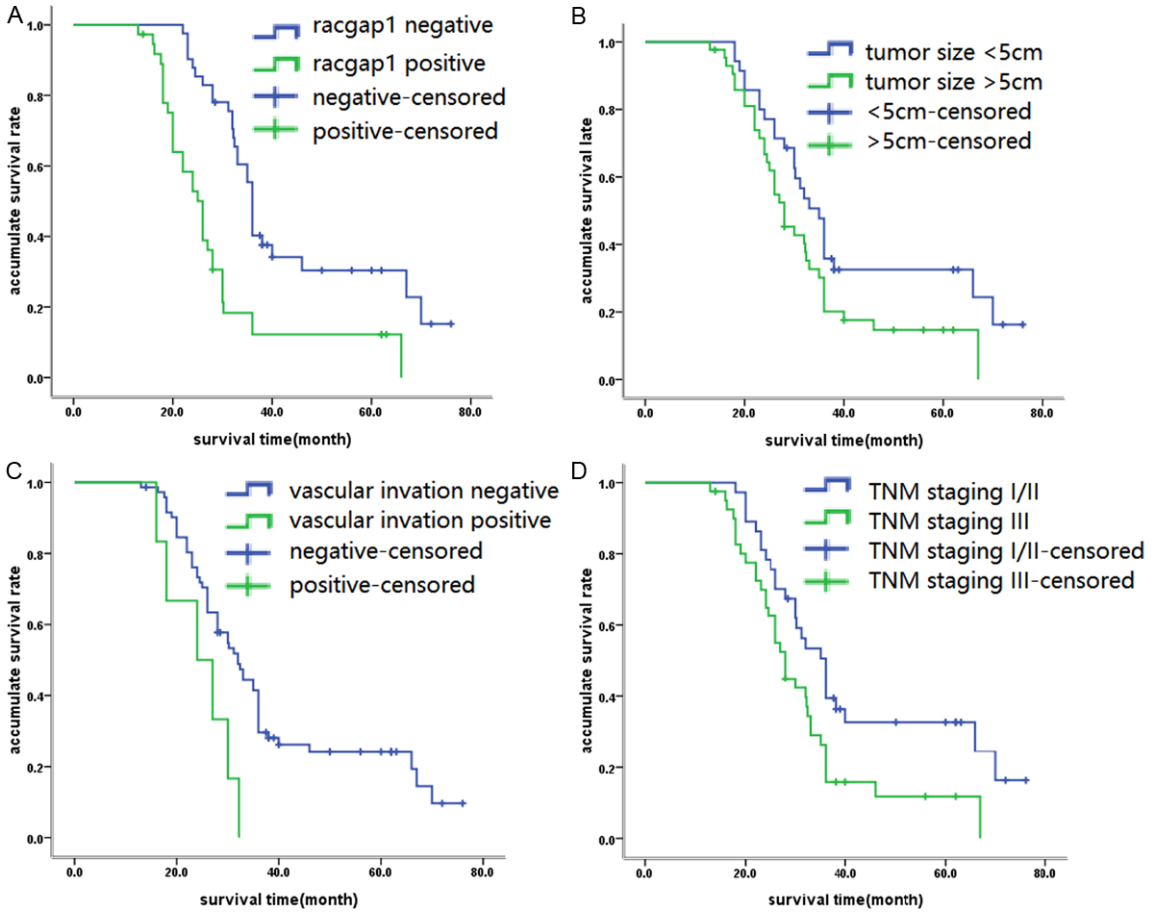

Figure 3. The survival curves of HCC patients with various levels of RacGAP1 and clinic-pathological parameters. A. Survival curves based on RacGAP1 expression; B. Survival curves based on tumor size; C. Survival curves based on vascular invasion; D. Survival curves based on clinical stage.

tive significance in breast cancer [22, 26, 27], colorectal cancer [28, 29], gastric cancer [21] and squamous cell carcinoma [30], and it has been identified as a biomarker of epithelial ovarian cancer [31], non-small-cell lung cancer [32], meningioma [20], and bladder cancer [23]. In addition, RacGAP1 upregulation was found to strongly correlate with the early recurrence of hepatocellular carcinoma [33]. However, there is little knowledge pertaining to the potential role of RacGAP1 in the clinicopathological characteristics and prognosis of liver cancer to date.

In the current study, immunohistochemical staining showed no RacGAP1 expression in normal liver specimens, liver biopsy specimens of hepatitis or liver biopsy specimens of hepatic cirrhosis, and high expression (44.1%) in HCC specimens, suggesting that HCC may play a potential role in the development of HCC. Previously, RacGAP1 expression was detected in Hep3B and MHCC97-H HCC cells, as revealed by Western blotting assay, and RacGAP1 was found to be associated with the increase in the invasion of HCC cell lines [33], which also supports our findings regarding the potential correlation between RacGAP1 expression and liver cancer development. In addition, increasing evidence demonstrates higher expression in cancer tissues than in non-cancer tissues [21, 22, 30]. Taking these findings together, it is hypothesized that RacGAP1 is involved in the regulation of cancer cell function.

Our findings showed aberrant localization of RacGAP1 in HCC tissues, indicating that RacGAP1 is predominantly localized in cytoplasm and partly co-localized in cytoplasm and nucleus. It is supposed that the alteration of RacGAP1 localization may be associated with the development of HCC. In normal tissues or

Table 2. Univariate analysis (Cox regression model) of prognostic factors in 78 HCC patients

Variable		Number of cases	Median OS (months)	Univariate analysis		
				HR	95% *CI*	*P* value
Gender	Male	66	32.0	0.820	0.415-1.621	0.569
	Female	12	27.2			
Age (years)	< 60	63	32.5	1.404	0.743-2.654	0.296
	≥ 60	15	26.0			
Tumor size (cm)	≤ 5	35	35.2	1.673	0.994-2.817	0.053
	> 5	43	28.6			
Vascular invasion	Yes	6	24.0	2.817	1.183-60710	0.019*
	No	72	32.5			
Lymph node metastasis	Yes	4	28.5	0.897	0.315-2.557	0.839
	No	74	32.0			
Pathologic grade	I/II	71	32.3	1.452	0.587-3.643	0.427
	III/IV	7	28.6			
TNM staging	I/II	37	36.2	1.864	1.110-3.131	0.019*
	III	41	28.5			
Hepatic Cirrhosis	Yes	34	28.5	0.755	0.451-1.267	0.288
	No	44	35.4			
RacGAP1 expression	Positive	41	26.0	2.875	1.701-4.859	0.000*
	Negative	37	36.5			

*$P < 0.05$.

cells, RacGAP1 was reported to promote central spindle formation and play a critical role in mitosis in cytoplasm [12-15]. In the cultured SPC-A-1 lung cancer cells, RacGAP1 was found to be predominantly present in the nucleus of non-mitotic cells, but showed a diffuse distribution in the cytoplasm of mitotic cells (metaphase) and at the contractile ring between two separating daughter cells (telophase), indicating that RACGAP1 is required for cytokinesis [32]. Taking our findings together, it is hypothesized that relative weak proliferation, normal mitosis occurs and low RacGAP1 expression is detected in normal liver, hepatitis and hepatic cirrhosis tissues, resulting in failure in detection of RacGAP1 expression by immunohistochemistry. During the progressive aggravation of hepatitis and cirrhosis and the subsequent carcinogenic process, acceleration in cell proliferation, increase in mitosis, remarkable elevation of RacGAP1 expression and RacGAP1 entry into nucleus are present, which are jointly involved in the development and progression of HCC. However, further studies are required to investigate the exact mechanisms underlying the potential role of RacGAP1 in the development and progression of HCC. In cancer specimens from primary colorectal cancer patients, immunohistochemistry revealed that RacGAP1 expression was found to be present in both the nucleus and cytoplasm at different amounts, and the localization of RacGAP1 was associated with the prognosis of colorectal cancer [28]. These findings demonstrate the importance of altered RacGAP1 localization in cancer development and prognosis.

In this study, we found that RacGAP1 expression positively correlated with tumor size, vascular invasion, lymph node metastasis, TNM staging and histological grading, suggesting that RacGAP1 may indicate the biological behaviors of liver cancer. In low-differentiation HCC, high RacGAP1 expression, and high malignancy, invasion, recurrence and metastatic rate of HCC was detected, while low RacGAP1 expression and low malignancy, invasion, recurrence and metastatic rate of HCC was observed in high-differentiation HCC. Univariate analysis showed a significantly shorter median survival in HCC patients with positive RacGAP1 expression than in those with negative RacGAP1 expression, and a significantly reduced median survival in HCC patients with vascular invasion and advanced clinical stage as compared to those with absence of vascular invasion and early clinical stage. Multivariate Cox regression analysis revealed that high RacGAP1 expression was an independent prognostic factor in HCC patients. It has been shown that high RacGAP1 protein expression is associated with lymph node metastasis, higher clinical stage and shorter survival in colorectal cancer patients, and *in vitro* assay demonstrates that high RacGAP1 expression increases the invasion of colorectal cancer cells; and therefore, RacGAP1 was identified as a potential biomarker for identifying patients with lymph node metastasis and poor prognosis in colorectal

Table 3. Multivariate analysis (Cox regression model) of prognostic factors in 78 hepatocellular carcinoma patients

Variable	β	SE	Wald	Exp(B)	95% *CI*	*P*
Negative RacGAP1 expression vs. positive RacGAP1 expression	1.178	0.285	17.142	3.249	1.860-5.677	0.000
Tumor size ≤ 5 cm vs. > 5 cm	0.381	0.394	0.933	1.463	0.676-3.169	0.334
Non-vascular invasion vs. vascular invasion	0.269	0.474	0.323	1.309	0.517-3.314	0.570
Clinical stage I/II vs. III	0.456	0.387	1.390	1.578	0.739-3.368	0.238

cancer [29]. Saigusa and colleagues reported that RacGAP1 expression at the invasive front in gastric cancer was significantly correlated with factors reflecting tumor progression and poor prognosis, as detected by immunohistochemistry [21]. In addition, high RacGAP1 expression was reported to strongly correlate with the recurrence of meningioma [20] and hepatocellular carcinoma [33]. These findings demonstrate the strong associations of high RacGAP1 expression with high invasion and poor prognosis of cancers.

To date, the exact mechanism underlying the involvement of RacGAP1 in the liver cancer development, invasion and metastasis remains unclear. It is hypothesized that the following mechanisms may be responsible for the role RacGAP1 in cancer development, progression and metastasis. (1) RacGAP1 mediates cancer cell division, transformation, migration, invasion and transfer through interacting with Rac1, Cdc42 and RhoA [34, 35]. (2) The Rho-Rac interaction may lead to epithelial-mesenchymal transition, which increases the invasion and transfer of tumor cells [36]. (3) RacGAP1 may regulate cytoskeleton and transcriptional signaling pathway via PRC1, thereby enhancing cell movement, proliferation and survival, which finally promotes tumor progression and metastasis [33]. In addition, siRNA-induced knockdown of RacGAP1 expression showed significantly decreased proliferation, migration and invasion of colorectal cancer cells [29]. Further studies to investigate the mechanisms underlying the role of RacGAP1 in the biological behaviors of HCC cancers are warranted.

In summary, the results of this study, for the first time, demonstrate upregulation of RacGAP1 protein expression and aberrant localization of RacGAP1 in cytoplasm and nucleus during the progression from hepatitis to HCC, and aberrant RacGAP1 expression may be a promising biomarker for the malignant biological behaviors and poor prognosis of HCC. More importantly, the elucidation of the mechanisms underlying the role of RacGAP1 in HCC development and progression may provide new insights into the identification of novel targets used for HCC diagnosis and treatment. However, the present study is a retrospective analysis recruiting small samples. Further studies with larger sample size and extension of follow-up period are required to validate the findings from the current study.

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Disclosure of conflict of interest

None.

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