Am J Transl Res 2020;12(11):7236-7248
www.ajtr.org /ISSN:1943-8141/AJTR0107849

Original Article

RCE1 deficiency enhances invasion via the promotion of epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma

Mengxian Tu[1,2*], Xinyi Wang[2,3,4*], Peng Chen[2], Jinying Li[5], Xiaojun Luo[2], Lu He[3], Wei Huang[5], Jian Hong[1,2], Chen Qu[1]

[1]Department of Pathophysiology, School of Medicine, Jinan University, Guangzhou 510630, Guangdong, China; [2]Department of Abdominal Surgery, Traditional Chinese Medicine-Integrated Hospital, Southern Medical University, Guangzhou 510315, Guangdong, China; [3]Guangzhou Medical University Affiliated Cancer Hospital, Guangzhou 510315, Guangdong, China; [4]Department of Oncology, Zhongshan City People's Hospital, Zhongshan 528403, Guangdong, China; [5]Department of Gastroenterology, The First Affiliated Hospital of Jinan University, Guangzhou 510630, Guangdong, China. [*]Equal contributors.

Received January 14, 2020; Accepted October 8, 2020; Epub November 15, 2020; Published November 30, 2020

Abstract: Ras converting CAAX endopeptidase 1 (RCE1) is an integral membrane protease involved in cell proliferation, differentiation, and carcinogenesis. RCE1 plays opposite roles in different tumor types; however, the actual biological function of RCE1 in hepatocellular carcinoma (HCC) is unknown. Here, we aim to investigate the prognostic value and molecular function of RCE1 in HCC. We performed immunohistochemistry in 20 normal human liver, 216 HCC, and 216 adjacent non-tumorous tissues and analyzed the expression change and clinical value of RCE1. Additionally, *in vitro* and *in vivo* studies were performed to investigate the role of RCE1 in regulating HCC proliferation, invasion, and metastasis. We found decreased RCE1 expression in HCC tissues. Moreover, the RCE1 expression level was negatively correlated with pathological parameters characteristic of early recurrence ($P < 0.044$) and the serum alpha-fetoprotein (AFP) level ($P < 0.018$). Survival analysis indicated that reduced RCE1 expression was a predictor of poor outcomes in patients with HCC. Functional studies showed that the knockdown of RCE1 promoted proliferation, migration, and invasion of HCC cells, while RCE1 overexpression suppressed these effects. *In vivo* studies further confirmed that the stable knockdown of RCE1 resulted in more rapid tumor growth and an increased number of lung metastatic nodules. Mechanistically, we found that RCE1 deficiency induced epithelial-mesenchymal transition (EMT) via activation of the P38 signaling pathway. Collectively, these results indicate that RCE1 deficiency enhances invasion via promoting epithelial-mesenchymal transition. The downregulation of RCE1 in HCC tissues predicts an unsatisfactory prognosis for patients with HCC.

Keywords: Hepatocellular carcinoma, RCE1, tumor suppressor, epithelial-mesenchymal transition, prognosis

Introduction

Hepatocellular carcinoma (HCC) is a malignancy that has a huge impact on global health [1]. Despite an overall decline in the incidence of most cancers during the last several decades, the incidence rates of HCC have continued to increase rapidly, by 3%-4% per year [2]. Radical resection is the mainstay of treatment; however, metastasis and recurrence of HCC compromise the efficiency of surgery [3-6]. Therefore, identifying molecular indicators of metastasis and recurrence of HCC is crucial for predicting the progression and prognosis of HCC.

Ras converting CAAX endopeptidase 1 (RCE1) is a type of integral membrane protease that was first identified in *Saccharomyces cerevisiae* [7, 8]. RCE1 plays a key role in processing CAAX-type prenylated proteins, including those of the Ras superfamily of small GTPases, the γ-subunit of heterotrimeric GTPases, nuclear laminas, and several other protein kinases [9-12]. RCE1 participates in cell proliferation, differentiation, and carcinogenesis through the regulation of these proteins [13-15].

RCE1 promotes the proliferation of fibroblasts and hematopoietic cells [16-18] and plays dis-

tinct roles in different cancer types. Ectopic expression of RCE1 has been shown in urologic neoplasms [19]. Moreover, RCE1 has been reported to regulate endoplasmic reticulum stress, maintaining the survival of renal carcinoma cells [20]. In contrast, it has also been reported that RCE1 suppresses the invasion of colorectal carcinoma via the regulation of P38 activity, and that low RCE1 expression predicts the poor prognosis of patients with colorectal carcinoma [21]. Despite recent evidence regarding the function of RCE1 in different cancer types, the biological function of RCE1 in HCC remains unclear. Thus, we analyzed the expression of RCE1 in HCC tissues, evaluated the prognostic value of RCE1 expression, and investigated its molecular function.

Materials and methods

Patient samples and murine liver fibrosis/HCC tissues

With approval from the Research and Ethics Committee of the Affiliated Tumor Hospital of Guangzhou Medical University, 216 paired HCC and adjacent non-tumorous tissues were collected from patients who underwent curative liver resection at the Affiliated Tumor Hospital of Guangzhou Medical University from September 2006 to June, 2010. All the patients were followed-up until September 30th, 2013. Twenty normal hepatic tissues were obtained from patients who underwent resection due to the presence of benign hepatic lesions. Informed consent was obtained from each patient. The study endpoints included overall survival (OS) and time to recurrence (TTR). All murine liver fibrosis/HCC tissues were retained from previous studies. Six-week-old male C57BL/6 mice received 0.1 mL 40% CCl_4 (in olive oil) gavage three times a week for 8 or 18 weeks to establish liver fibrosis or HCC models, respectively. Fibrotic tissues were collected from mice receiving CCl_4 gavage for 8 weeks. HCC tissues were collected from mice receiving CCl_4 gavage for 18 weeks.

Histology and immunohistochemistry

Formalin-fixed, paraffin-embedded liver tissue samples were cut into 4 μm-thick sections. Hematoxylin-eosin (H&E) and immunohistochemistry (IHC) staining was performed on the sections according to standard procedures. For IHC, liver sections were stained with the following antibodies: rabbit polyclonal antibodies against RCE1 (1:100; GeneTex, Irvine, CA, USA), Ki-67 (1:100, ZA-0502; ZSGB-BIO, Beijing, China) and E-cadherin (1:100, ZA-0565; ZSGB-BIO), and monoclonal antibodies against Vimentin (1:100, ZM-0260; ZSGB-BIO). Expression levels were measured according to the following rules, as previous reported [22]: Both the extent and intensity of immunostaining were taken into consideration. Staining intensity was scored from 0 to 3 (0 for negative, 1 for weak staining, 2 for moderate staining, and 3 for strong staining), and the extent of staining was scored from 0% to 100%. The final quantitation of each staining was obtained by multiplying the two scores. The median IHC score (1.5) was selected as the optimal cut-off value to classify high and low RCE1 expression groups.

Cell culture and lentiviral infection

SMMC7721 and Hep G2 cells were cultured in DMEM medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Life Technologies) at 37°C and a humidified atmosphere of 5% CO_2. Lentiviral particles carrying the full-length RCE1 gene coding sequence or RCE1 shRNA were purchased from GeneChem (Shanghai, China), the sequences were as follows: sh-RCE1-1, 5'-ATTCCTTCTGCAATTACAT-3' (sense), sh-RCE1-2, 5'-CCACTGATGCAGCTCTCTA-3' (sense). The cells were infected with lentivirals according to the manufacturer's protocol. Puromycin (1 μg/mL; GeneChem) was used for stable cell line selection.

Real-time PCR

Total RNA was extracted from the indicated cells with a total RNA extraction kit according to the protocol provided by the manufacturer (OMEGA, Norcross, GA, USA). Random hexamers and a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan) were used to synthesize the cDNA templates. The mRNA expression levels of the indicated genes were evaluated by RT-qPCR using SYBR Green qPCR mix (TakaRa) according to the manufacturer's protocol. PCR primers were synthesized by Thermo Fisher (Guangzhou, China) and their sequences are shown in **Table 1**. The qPCR conditions were as follows: Predenaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec and 60°C for 40 sec.

Table 1. Primers for reverse transcription-quantitative PCR

Primer name	Sequence (5'-3')
E-cadherin	Forward: TCGACACCCGATTCAAAGTGG
	Reverse: TTCCAGAAACGGAGGCCTGAT
Vimentin	Forward: TGGCCGACGCCATCAACACC
	Reverse: CACCTCGACGCGGGCTTTGT
N-cadherin	Forward: GCGCGTGAAGGTTTGCCAGTG
	Reverse: CCGGCGTTTCATCCATACCACAA
Snail	Forward: AAGGATCTCCAGGCTCGAAAG
	Reverse: GCTTCGGATGTGCATCTTGA
ZEB1	Forward: TACAGAACCCAACTTGAACGTCACA
	Reverse: GATTACACCCAGACTGCGTCACA
RCE1	Forward: TCTCTGCCATTCCTTCTGCA
	Reverse: CCAAAAGCACACAAAGGGGA
GAPDH	Forward: TGCCAAATATGATGACATCAAGAA
	Reverse: GGAGTGGGTGTCGCTGTTG

The results of the real-time PCR were analyzed by $2^{-\Delta\Delta CT}$. The housekeeping gene GAPDH was used to normalize the results. Each experiment was performed independently in triplicate.

Western blot analysis

The cells were lysed in RIPA buffer (Invitrogen, Carlsbad, CA, USA) with a protease inhibitor. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Primary antibodies were used for immunodetection with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) and enhanced chemiluminescence reagents (Millipore). The primary antibodies used for western blotting were as follows: rabbit polyclonal antibodies against RCE1 (1:500, ab62531; Abcam, Cambridge, UK), N-cadherin (1:1000, 4061p; Cell Signaling Technology), Vimentin (1:1000, 5714p; Cell Signaling Technology), Snail (1:1000, 3879p; Cell Signaling Technology), and β-actin (1:1000, 4970p; Cell Signaling Technology), and monoclonal antibodies against E-cadherin (1:1000, 564186; BD Biosciences, San Jose, CA, USA).

Cell proliferation and colony formation assays

The Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan) was used to assess cell viability. Briefly, the indicated cells were seeded at a density of 3,000 cells/well in a 96-well plate, and after 2.5 hours of incubation with 10 μl of CCK-8 reagent, at specified time points, optical density was measured at 450 nm. Three independent experiments were performed. For colony formation assays, stable cells were seeded into 6-well plates with 500 cells per well, then incubated at 37°C for 14 days. After most of the single cells had expanded to > 50-cell colonies, colonies were fixed with methanol, stained with 0.1% crystal violet, and counted.

Wound healing assays

For the wound-healing assay, the indicated cells were seeded into a 6-well plate; straight lines were delineated at the bottom of the 6-well plate after incubation for 12 hours. Then, the width was respectively measured after 0, 24, 48, 72, and 96 hours.

Cell migration and invasive assays

Cell motility was assessed by cell migration and invasion assays using Transwell chambers with or without Matrigel (BD Biosciences). Approximately 1×10^5 cells suspended in 100 μl of serum-free medium were added to the upper chambers of the Transwell plate covers with or without Matrigel (8%) for invasion and migration assays, respectively. The bottom chambers were filled with 500 μl DMEM medium with 20% FBS. After 15-20 h (for migration) or 24-48 h (for invasion) of incubation, cells that migrated or invaded were stained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min, and counted.

Immunofluorescence

Briefly, cells were washed twice with cold PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100/PBS, and incubated in blocking buffer. Then, the indicated cells were respectively stained with E-cadherin (1:500; ZSGB-BIO) or Vimentin (1:500; ZSGB-BIO), and detected by secondary antibodies labeled with Alexa Fluor 555 (Invitrogen, Bar Harbor, ME, USA) according to the manufacturer's instructions. Nuclei were stained with DAPI (Thermo Fisher Scientific).

Tumor xenografts and lung metastasis model

In all, 2×10^6 logarithmically growing SMMC7721 cells, with or without RCE1 knockdown, was suspended in 200 μl of DMEM medium. Next, the suspension was injected into the right flanks of 4-6-week-old female nude mice (N = 6). Tumor volumes were measured and recorded every 3 days and the tumors were resected from the mice, weighed, and photographed 4 weeks after injection.

For the lung metastasis model, 6×10^6 cells resuspended in 50 μl of PBS were injected into 4-6-week-old female nude mice through the tail vein (total of 12 mice were randomly allocated to one of two groups). All mice were sacrificed 6 weeks later, and the lungs were resected for counting metastatic nodules. Nude mice were purchased from the Guangdong Medical Lab Animal Center (Foshan, China). All animal studies were conducted in accordance with the principles and procedures outlined in the Southern Medical University Guide for the Care and Use of Animals.

Statistical analysis

All results are presented as the mean ± SD. χ^2 tests were used to analyze the association between RCE1 and clinicopathological parameters. Survival curves were constructed using the Kaplan-Meier method and analyzed by the log-rank test. The Cox regression model was used for the univariate and multivariate analyses to identify significant prognostic factors. The Student's *t*-test was used for comparisons. Statistical analyses were performed using SPSS version 22.0 (IBM, Armonk, NY, USA) and $P < 0.05$ was considered statistically significant.

Results

Expression profile of RCE1 in HCC tissues

To investigate expression changes in RCE1 in the HCC development process, we performed IHC staining of RCE1 in normal murine liver, CCl_4-induced mouse liver fibrosis, and HCC tissues. RCE1 expression was significantly reduced in tumor tissues compared with matched adjacent non-tumorous tissues, fibrotic tissues, and normal liver tissues. Moreover, RCE1 expression was reduced in fibrotic liver tissues compared with normal liver tissues (**Figure 1A**). Next, we examined RCE1 expression by immunostaining in 216 human HCC specimens (including tumor and matched adjacent non-tumorous tissues) and 20 normal human hepatic tissue specimens. Similarly, we found that RCE1 expression was dramatically decreased in HCC tissues compared with matched adjacent non-tumorous tissues and normal liver tissues (**Figure 1B**). We detected high expression of RCE1 in 80/216 (37.04%) HCC tissues compared with 150/216 (69.44%) adjacent matched tissues. Besides, high expression of RCE1 was observed in almost all the 20 normal hepatic tissues. These data indicated that RCE1 expression was decreased in HCC tissues compared with non-tumor liver tissues ($P < 0.001$; **Figure 1C**). In addition, we analyzed the correlation between RCE1 expression and clinicopathological parameters. The results showed that decreased RCE1 expression was positively correlated with the elevated level of serum alpha-fetoprotein (AFP) and early recurrence of HCC (**Table 2**).

Reduced RCE1 expression promotes the proliferation of HCC cells

Next, we investigated the molecular function of RCE1 in HCC cells. We detected RCE1 expression in six human hepatocellular carcinoma cell lines by RT-qPCR and immunoblotting (**Figure 2A**). We observed relatively high RCE1 expression in SMMC7721 and relatively low RCE1 expression in Hep G2 cells. The proliferation and metastatic ability of SMMC7721 cells is reportedly lower than that of Hep G2 cells [23, 24]. Hence, we knocked down RCE1 expression in SMMC7721 cells, and overexpressed RCE1 in Hep G2 cells. We found that depletion of RCE1 enhanced the proliferation and colony formation of HCC cells. In contrast, abnormal expression of RCE1 inhibited the proliferation and colony formation of HCC cells (**Figure 2B** and **2C**). To further validate the biological function of RCE1 *in vivo*, SMMC7721 cells with stable knockdown of RCE1 and corresponding control cells were subcutaneously injected into nude mice. We found that silencing RCE1 expression in SMMC-7721 cells significantly promoted the enlargement of the tumor xenografts (**Figure 2D**). The xenografts of RCE1 knockdown SMMC7721 cells were larger than those of control cells (**Figure 2E**). Consistently, IHC

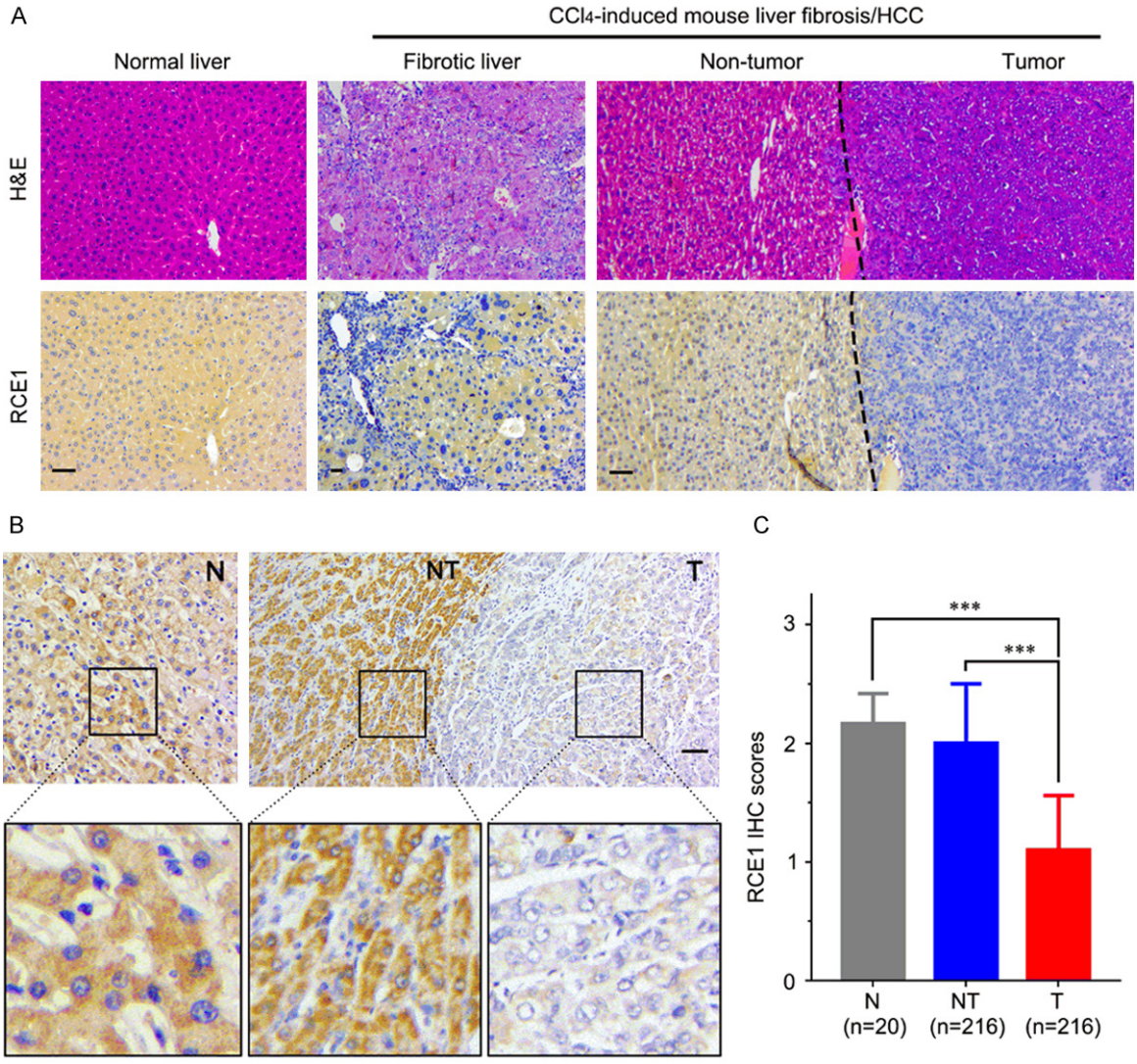

Figure 1. RCE1 expression is dramatically decreased in HCC tissues. A. RCE1 expression in mouse normal liver and CCl_4-induced mouse liver fibrosis/HCC tissues was detected by immunohistochemistry (IHC) staining. Representative images of H&E and IHC staining are shown. Scale bars, 50 μm. B. Representative images of IHC staining for RCE1 in normal human liver, HCC, and adjacent non-tumorous tissues. Scale bars, 50 μm. C. RCE1 expression levels (IHC score) were compared among normal liver, HCC, and adjacent non-tumorous tissues based on the IHC results. Data are presented as means ± SD. ***$P < 0.001$.

assays performed on serial sections of subcutaneous xenografts also showed that RCE1 expression negatively correlated with expression of Ki67, a biomarker of proliferating cells (**Figure 2F**). These results further confirmed that RCE1 inhibited proliferation of HCC cells.

RCE1 inhibits migration and invasion capacity of HCC cells

Our data indicated that low RCE1 expression was related to early recurrence of HCC, which suggests that RCE1 regulates the invasion capacity of HCC cells. Thus, we examined whether knockdown or overexpression of RCE1 affected the migration and invasion capacity of HCC cells. In wound-healing assays, we found that RCE1 silencing increased the migratory activity of SMMC721 cells. Meanwhile, overexpression of RCE1 suppressed the migratory activity of Hep G2 cells (**Figure 3A** and **3B**). Transwell assays, with or without Matrigel, further confirmed that downregulation of RCE1 enhanced the migration and invasion capacity of HCC cells and overexpression of RCE1 suppressed the migration and invasion capacity of HCC cells (**Figure 3C** and **3D**). Moreover, *in vivo* tail vein assays of

Table 2. Correlation of RCE1 expression with clinicopathological features in 216 patients with hepatocellular carcinoma

Characteristics	n	RCE1		*P* value
		Low	High	
AFP (ng/ml)				
≤ 20	68	35 (51.50%)	33 (47.83%)	**0.018**
> 20	148	101 (68.20%)	47 (31.58%)	
GGT (U/l)				
≤ 50	58	32 (55.20%)	26 (44.80%)	0.151
> 50	158	104 (66.80%)	54 (34.20%)	
HBsAg				
Negative	24	14 (58.30%)	10 (41.70%)	0.659
Positive	189	119 (63.00%)	70 (37.00%)	
Child-Pugh score				
A	200	127 (63.50%)	73 (36.50%)	0.634
B	14	9 (57.1%)	6 (42.90%)	
Tumor number				
Single	166	103 (62.00%)	63 (38.00%)	0.612
Multiple	50	33 (66.00%)	17 (34.00%)	
Tumor size (cm)				
≤ 5	99	56 (56.60%)	43 (43.40%)	0.081
> 5	116	79 (68.10%)	37 (31.90%)	
Tumor capsule				
No/incomplete	164	102 (62.20%)	62 (37.8%)	0.484
Complete	11	8 (72.70%)	3 (27.30%)	
Liver cirrhosis				
No	79	52 (65.80%)	27 (34.20%)	0.509
Yes	137	84 (61.30%)	53 (38.70%)	
Tumor thrombus				
No	179	110 (61.50%)	69 (38.50%)	0.312
Yes	37	26 (70.30%)	11 (29.70%)	
Early recurrence*				
No	89	49 (55.10%)	40 (44.90%)	**0.044**
Yes	127	87 (68.50%)	40 (31.50%)	

Bold values (P < 0.05) are statistically significant. *Early recurrence indicates tumor were detected within 2 years after surgical operation.

cancer metastasis suggested that knocking down RCE1 expression resulted in an increase of lung metastatic nodules (**Figure 3E** and **3F**). Taken together, these results indicate that RCE1 inhibited invasion and metastasis of HCC cells, both *in vivo* and *in vitro*.

RCE1 suppresses epithelial-mesenchymal transition of HCC cells via regulation of the P38 signaling pathway

Epithelial-mesenchymal transition (EMT) plays an important role in tumor progression, and especially in tumor cell metastasis [25]. We examined the expression of several EMT-related hallmarks in HCC cells with RCE1 up- or downregulation. We found that the mRNA expression of mesenchymal markers included N-cadherin, Vimentin, Snail, ZEB1, and Twist, were markedly increased in RCE1 depleted SMMC7721 cells, while the epithelial marker E-cadherin was significantly downregulated (**Figure 4A**). In contrast, the opposite was seen when RCE1 was overexpressed in Hep G2 cells (**Figure 4B**). Moreover, western blot (**Figure 4C**) and Immunofluorescence (**Figure 4D**) analyses further confirmed the qPCR result. In addition, IHC performed on the xenograft specimens also indicated that downregulation of RCE1 suppressed epithelial-mesenchymal transition (**Figure 4E**). Collectively, these results indicate that RCE1 might inhibit invasion and metastasis via the suppression of EMT in HCC cells.

Concurrently, we found that phosphorylated P38 protein was upregulated in SMCC7721 cells with RCE1 knockdown, but downregulated in RCE1-overexpressing Hep G2 cells (**Figure 4C**). Studies have reported that activation of the P38 signaling pathway promotes EMT in HCC cells. Thus, these results suggest that RCE1 might suppress EMT in HCC cells via inhibiting P38 activation.

Reduced RCE1 expression is associated with poor outcomes in patients with HCC

Furthermore, we analyzed the prognostic value of RCE1 expression in patients with HCC. Survival analysis suggested that patients with low RCE1 expression exhibited a significantly poorer overall survival ($P < 0.001$; **Figure 5A**) and higher cumulative recurrence rate ($P < 0.010$; **Figure 5B**) than patients with high RCE1 expression. Univariate Cox analysis indicated that tumor size, serum alpha-fetoprotein (AFP) level, tumor number, tumor thrombus, as well as RCE1 expression level were predictive factors for the overall survival (OS) and time to recurrence (TTR) of patients with HCC. A multi-

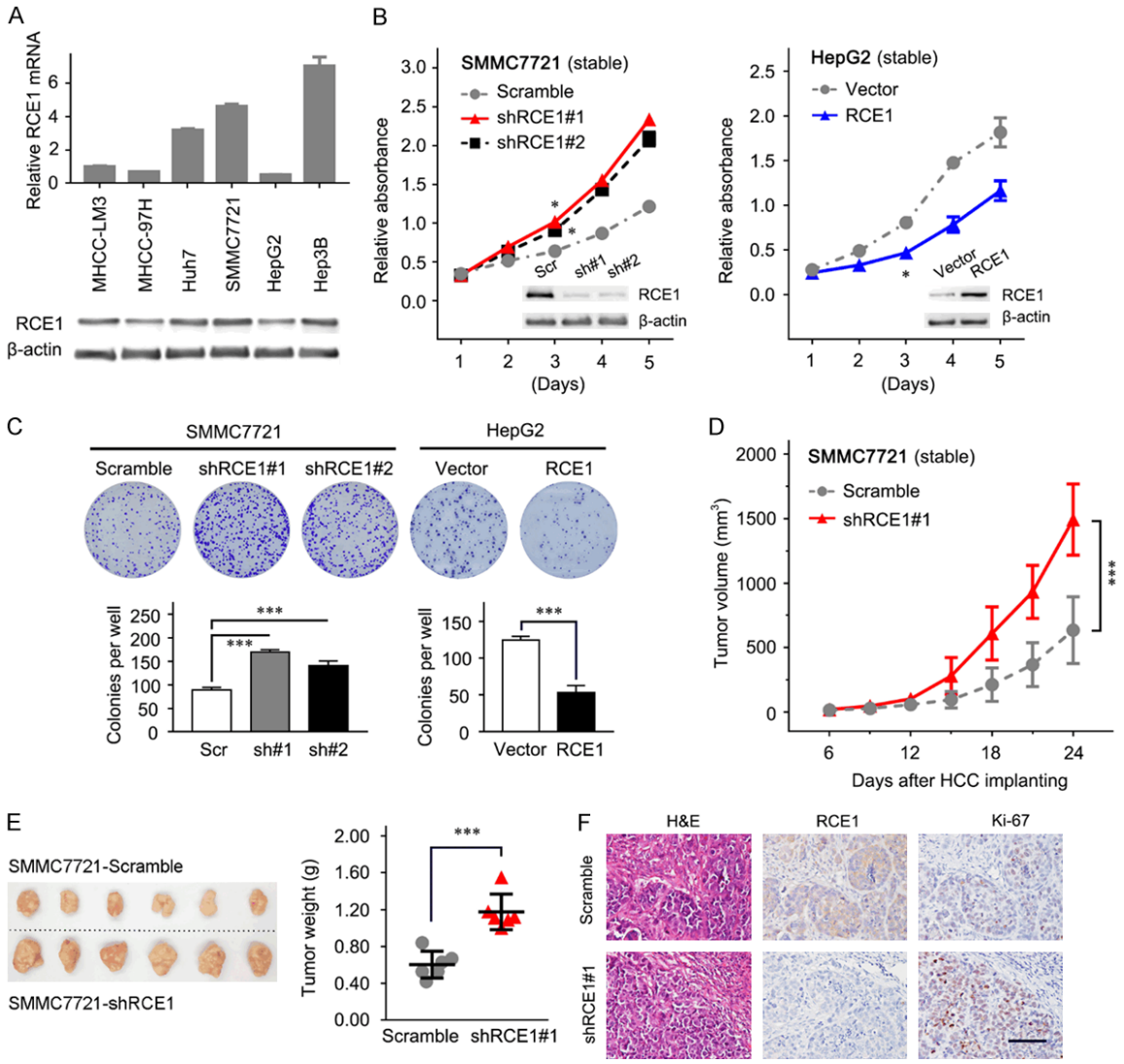

Figure 2. Reduced RCE1 expression promotes proliferation of HCC cells *in vitro* and *in vivo*. A. mRNA and protein levels of RCE1 expression in the indicated HCC cell lines were detected by real-time qPCR and western blotting. B. CCK8 assays were performed to analyze cell viability in RCE1 knockdown SMMC7721 cells and RCE1-overexpressing Hep G2 cells. C. Colony formation assays were performed to detect the colony forming ability of the indicated cells. Representative images of the colonies from the indicated cells are shown in the upper panel, and the quantification of colony numbers is shown in the lower Panel. D. *In vivo* growth of SMMC7721 cells with or without RCE1 knockdown. The tumor volumes were measured and recorded every 3 days. E. Representative images of subcutaneous xenograft tumors of the indicated cells. Weights of the xenograft tumors are shown in the right panel. F. Serial sections of the xenograft tumors were subjected to IHC staining for RCE1, and Ki67. Scale bars, 100 μm. Data are presented as means ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

variate Cox model including these parameters further indicated that RCE1 expression was an independent predictive factor for the OS (**Figure 5C**) and TTR (**Figure 5D**) of patients with HCC.

Prognostic value of RCE1 expression in AFP-negative patients with HCC

Currently, serum AFP level is widely used to diagnose HCC and evaluate HCC recurrence risk. However, the application of AFP in the early diagnosis of HCC is limited because there are no significant changes in serum AFP in one third of the patients with HCC. Correlation analysis suggested an interesting finding in that RCE1 expression in HCC tissues was closely related to the serum AFP level in patients with HCC ($P < 0.018$; **Table 2**). To further determine if RCE1 expression had a particular prognostic value for AFP-negative patients with HCC, we performed Kaplan-Meier

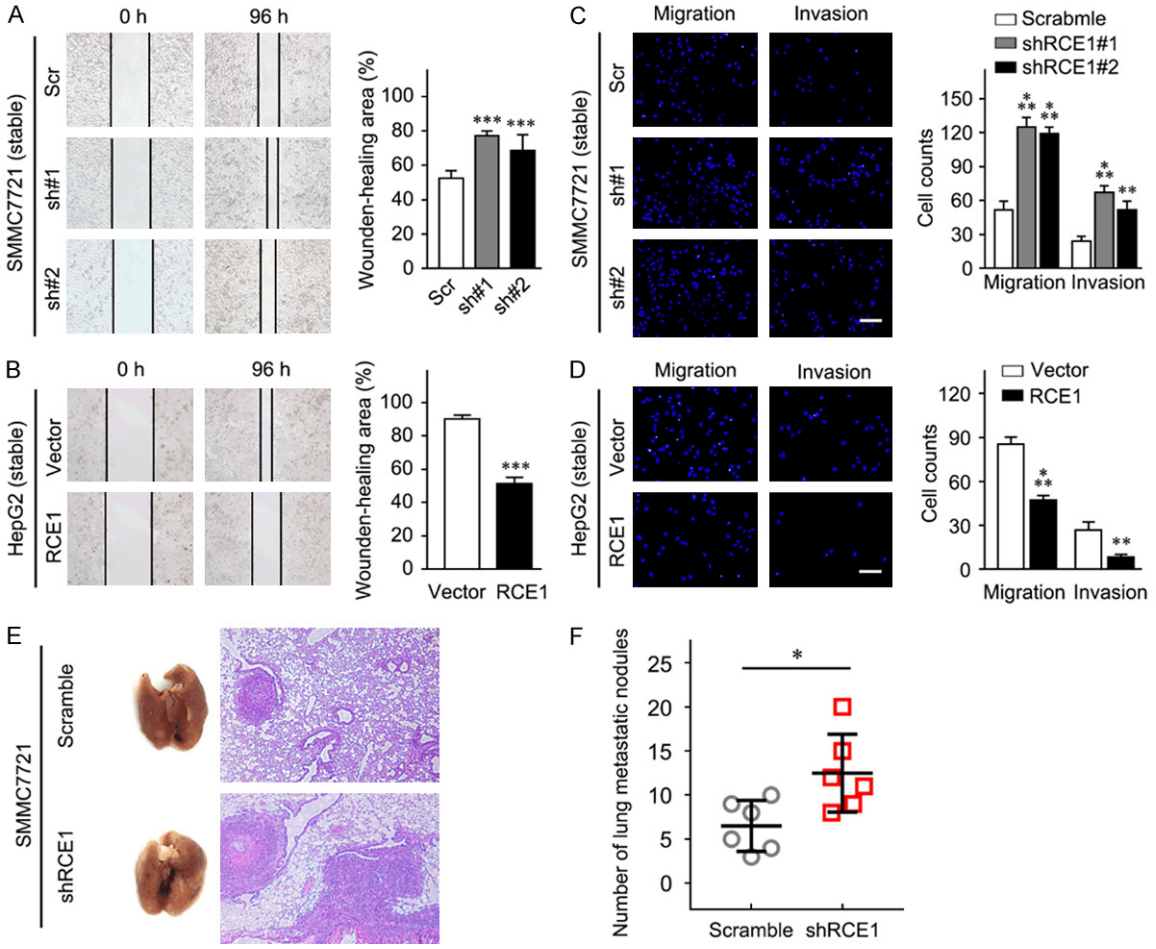

Figure 3. Reduced RCE1 expression promotes migration and invasion of HCC cells. A and B. Wound healing assays were performed to analyze the migration capacity in RCE1 knockdown SMMC7721 cells and RCE1-overexpressing Hep G2 cells. C and D. Transwell assays were conducted, with or without Matrigel, to determine the migration and invasion capacities of the indicated cells. Scale bars, 100 μm. E and F. Representative images of lung metastatic tumors obtained from an *in vivo* lung metastasis model. The number of lung metastasis nodules is shown in the left panel (N = 6). All data are presented as means ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

survival analysis based on RCE1 expression in AFP-positive (serum AFP > 20 ng/ml) and AFP-negative (serum AFP ≤ 20 ng/ml) patients with HCC. The results showed that low RCE1 expression predicted shorter survival time and higher recurrence risk in both AFP-positive (**Figure 6A** and **6B**) and AFP-negative (**Figure 6C** and **6D**) patients with HCC. However, due to the limitation of the cohort size, the *P* value was greater than 0.05 in the test for time to recurrence in AFP-negative patients. These data suggest that RCE1 could be a candidate prognostic predictor for both AFP-positive and AFP-negative patients with HCC.

Discussion

The prognosis of patients with HCC is limited by the high incidence of recurrence and metastasis. Early detection of recurrence and metastasis followed by timely and effective treatment may result in better life expectancy [26]. Therefore, effective and sensitive biomarkers that predict the risk of recurrence and metastasis are critical for HCC surveillance. Here, we report that RCE1 expression is frequently lost in HCC, and is related to poor prognosis.

In this study, we found that RCE1 expression was significantly reduced in HCC tissues compared with matched adjacent non-tumorous tissues. Survival analysis indicated that the reduced RCE1 expression was an independent prognostic factor for high recurrence risk and poor prognosis of patients with HCC. These data suggested that RCE1 has potential as a prognosis predictor for HCC surveillance. In addition, we analyzed the prognostic value of

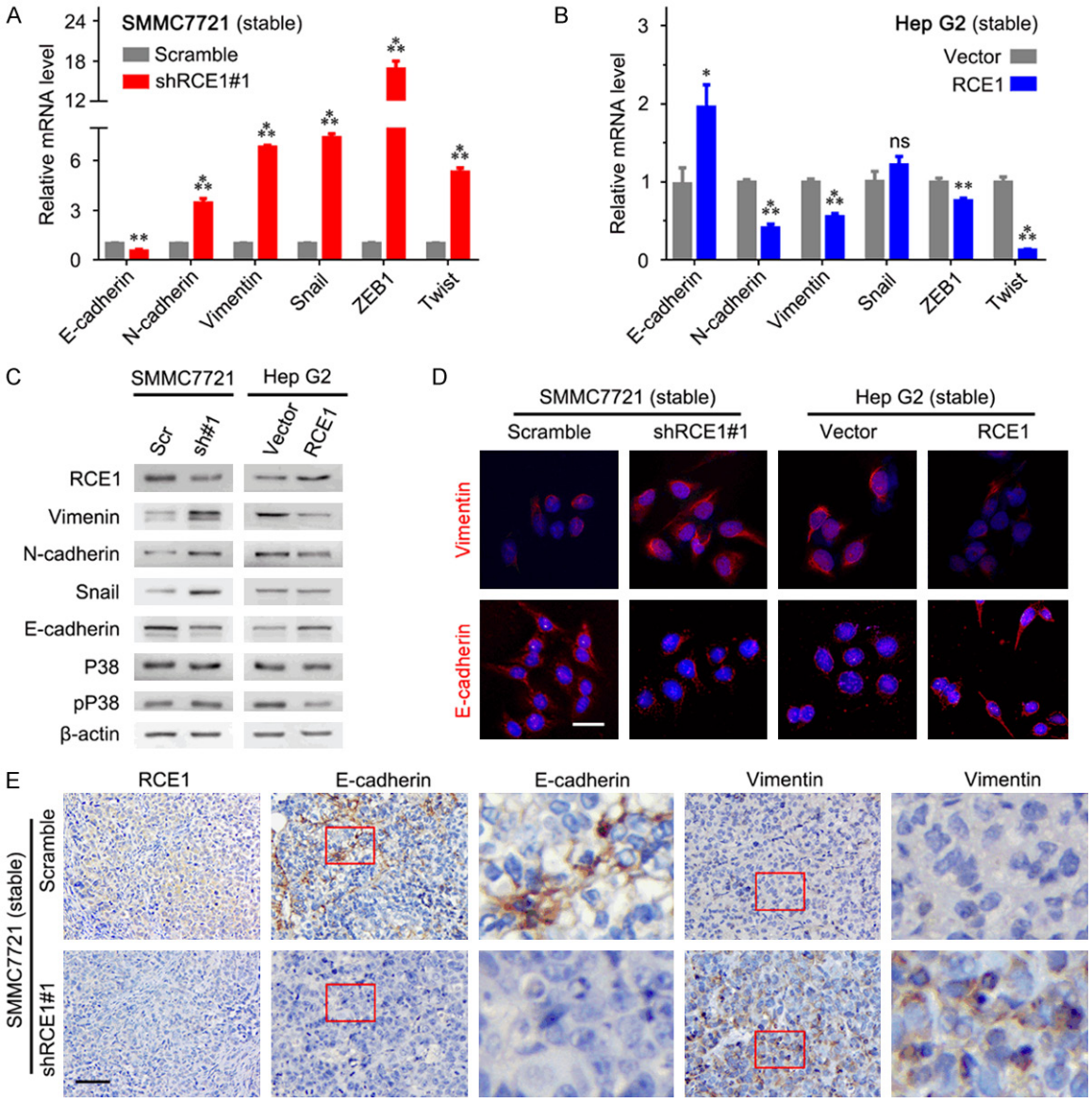

Figure 4. RCE1 suppresses epithelial-mesenchymal transition (EMT) of HCC cells. A and B. mRNA expression of E-cadherin, N-cadherin, Vimentin, Snail, ZEB1, and Twist in the indicated cells. C. Protein levels of RCE1, Vimentin, N-cadherin, Snail, E-cadherin, pP38, and β-actin in RCE1 knockdown SMMC7721 cells and RCE1-overexpressing Hep G2 cells. β-actin was used as a loading control. D. Representative immunofluorescence images of E-cadherin and Vimentin of the indicated cells. Scale bars, 25 μm. E. Representative IHC staining images of RCE1, E-cadherin and Vimentin in xenograft tumors. Scale bar, 100 μm. All data are presented as means ± SD. **P < 0.01; ***P < 0.001; ns: not significant.

RCE1 in AFP-negative patients with HCC. Serum AFP is a widely recognized tumor marker used in the diagnosis and management of HCC. It can be used to evaluate the recurrence and metastasis risk in patients with HCC with curative resection [27, 28]. However, patients with HCC who are positive for AFP account for only 60-70% of all the patients with HCC [29]. Thus, it is imperative to find a method of predicting early recurrence in AFP-negative patients with HCC after resection. Several markers, such as AFP-L3 [30], GPC3 [31, 32], Dickkopf-1 [33, 34] and PKIV-II [35], have shown potential to complement AFP for use in the early diagnosis of HCC recurrence. However, these markers are not fully applicable to clinical practice. In our study, we found that RCE1 had prognostic predictive value both in

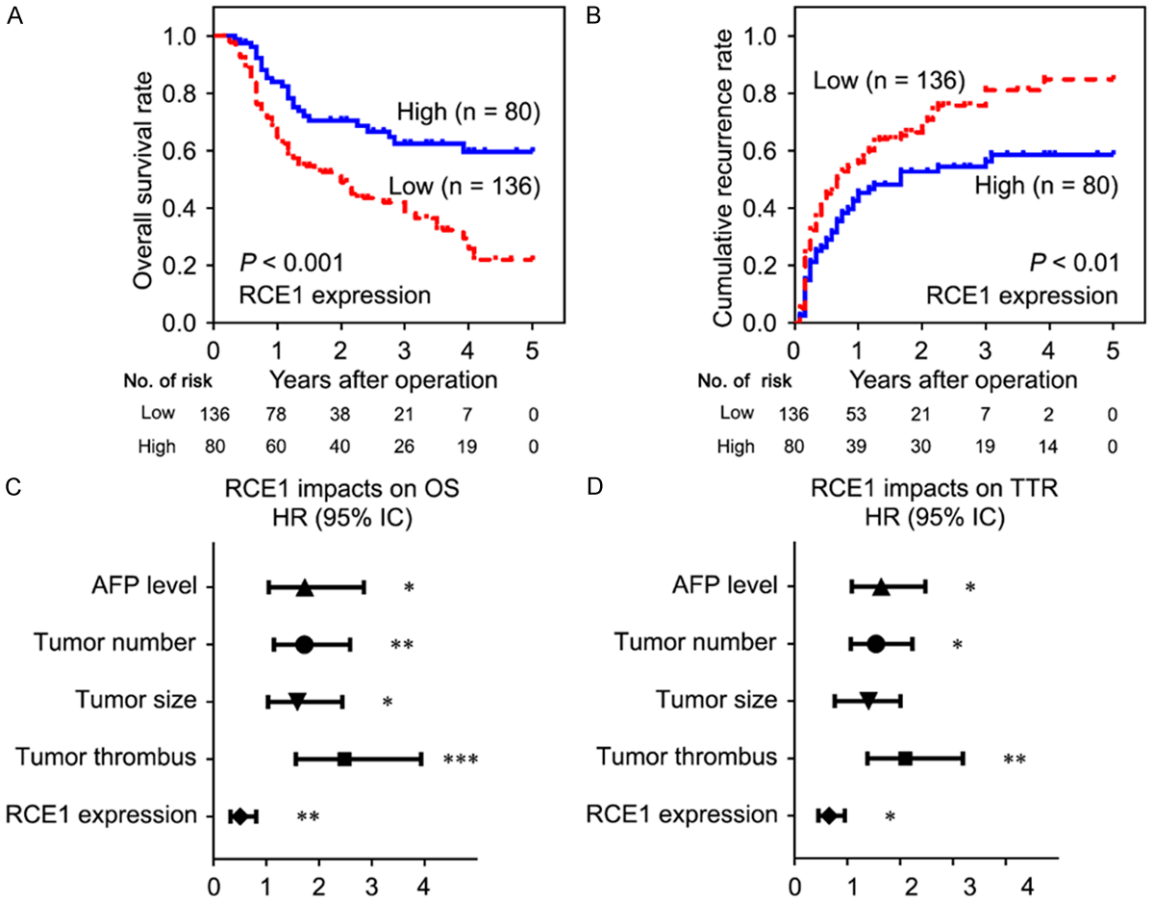

Figure 5. Reduced RCE1 expression correlates with the poor prognosis of patients with HCC. A and B. Kaplan-Meier curves for overall survival and cumulative recurrence rate according to RCE1 expression in 216 HCC samples. The number of patients remaining in the risk set is presented underneath the figure. C and D. Forest plots show the hazard ratios (HR) of the indicated pathologic parameters for OS and TTR of patients with HCC. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

AFP-negative and AFP-positive patients with HCC. Thus, RCE1 expression may be an auxiliary diagnostic and prognostic prediction tool for AFP-negative patients with HCC, although more studies are needed to further confirm its application value.

Functionally, we found that RCE1 suppressed proliferation and invasion of HCC cells both *in vitro* and *in vivo*. Moreover, RCE1 negatively regulated the expression of mesenchymal markers and positively regulated epithelial markers. These data suggest that RCE1 might exert its tumor suppressor function via the inhibition of EMT, which is an important process that facilitates HCC metastasis [36, 37]. We also found that RCE1 expression negatively correlated with the phosphorylation of P38. Evidence indicated that the activation of the P38 signaling pathway closely correlated with EMT and enhanced the invasion capacity of different types of cancer including HCC [38-40]. Hence, we speculate that RCE1 might exert a tumor-suppressing function via regulation of the P38 signal pathway. However, further studies are needed to investigate the detailed molecular mechanisms.

In addition, we observed that RCE1 expression was decreased in fibrotic liver tissues compared with normal liver tissues. The strong association with liver fibrosis is an outstanding feature of HCC development, and about 90% of HCC cases develop fibrotic or cirrhotic livers [41]. Hence, RCE1 downregulation might be an early event in HCC development. Previous studies have reported that RCE1 negatively regulates TGF-β1 expression, which induces

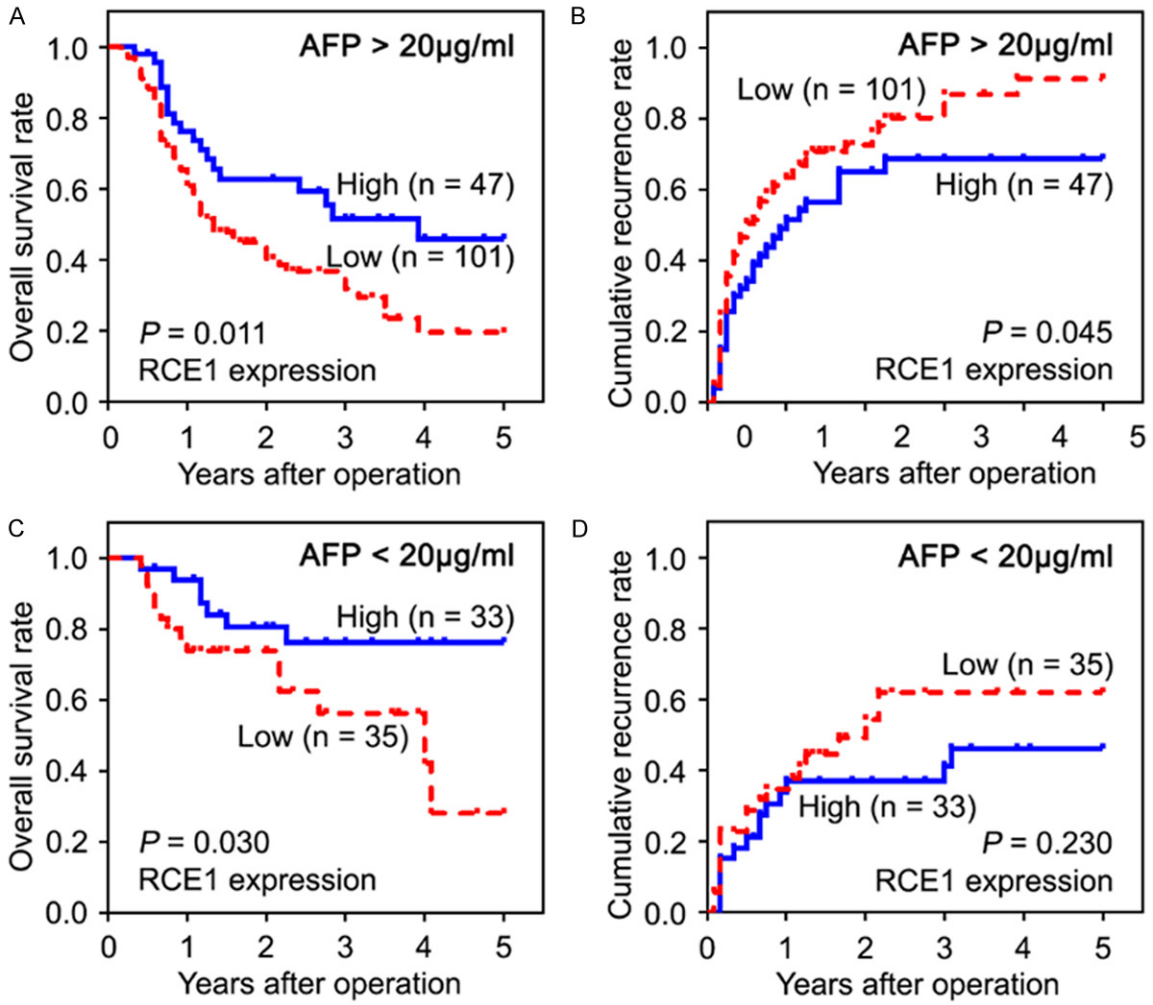

Figure 6. Prognostic value of RCE1 expression in AFP-negative patients with HCC. Overall survival and cumulative recurrence rate according to the RCE1 expression level in AFP-positive patients with HCC (A and B) and AFP-negative patients with HCC (C and D).

liver fibrosis by activating hepatic stellate cells and promotes hepatocarcinogenesis via cross-talk with integrins. Reduced RCE1 may increase TGF-β1 expression, and thus promote liver fibrosis and HCC development.

Taken together, our study demonstrated that RCE1 suppressed EMT and inhibited the proliferation and metastasis of HCC cells. The downregulation of RCE1 in HCC tissues is an independent prognostic factor of poor prognosis in patients with HCC. RCE1 shows promising diagnostic potential to help predict prognosis and thus direct postsurgical therapy for HCC.

Acknowledgements

This study was supported by Yangcheng Scholar Program (1201561579 to J. Hong); National Natural Science Foundation of China (814 72265 to J. Hong; 81602067 to L. He; 81802423 to C. Qu).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chen Qu, Department of Pathophysiology, School of Medicine, Jinan University, No. 601 West Huangpu Road, Guangzhou 510630, Guangdong, China. E-mail: cheyne1988@outlook.com

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and

mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.
[2] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2017. CA Cancer J Clin 2017; 67: 7-30.
[3] Bruix J, Reig M and Sherman M. Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma. Gastroenterology 2016; 150: 835-853.
[4] Desai JR, Ochoa S, Prins PA and He AR. Systemic therapy for advanced hepatocellular carcinoma: an update. J Gastrointest Oncol 2017; 8: 243-255.
[5] Clark T, Maximin S, Meier J, Pokharel S and Bhargava P. Hepatocellular carcinoma: review of epidemiology, screening, imaging diagnosis, response assessment, and treatment. Curr Probl Diagn Radiol 2015; 44: 479-486.
[6] Acloque H, Adams MS, Fishwick K, Bronner-Fraser M and Angela Nieto M. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. J Clin Invest 2009; 119: 1438-1449.
[7] Boyartchuk VL, Ashby MN and Rine J. Modulation of ras and a-factor function by carboxyl-terminal proteolysis. Science 1997; 275: 1796-1800.
[8] Michaelis S and Barrowman J. Biogenesis of the saccharomyces cerevisiae pheromone a-factor, from yeast mating to human disease. Microbiol Mol Biol Rev 2012; 76: 626-651.
[9] Otto JC, Kim E, Young SG and Casey PJ. Cloning and characterization of a mammalian prenyl protein-specific protease. J Biol Chem 1999; 274: 8379-8382.
[10] Winter-Vann AM and Casey PJ. Opinion-post-prenylation-processing enzymes as new targets in oncogenesis. Nat Rev Cancer 2005; 5: 405-412.
[11] Manolaridis I, Kulkarni K, Dodd RB, Ogasawara S, Zhang Z, Bineva G, Reilly NO, Hanrahan SJ, Thompson AJ, Cronin N, Iwata S and Barford D. Mechanism of farnesylated CAAX protein processing by the intramembrane protease Rce1. Nature 2013; 504: 301-305.
[12] Fueller F, Bergo MO, Young SG, Aktories K and Schmidt G. Endoproteolytic processing of RhoA by Rce1 is required for the cleavage of RhoA by Yersinia enterocolitica outer protein T. Infect Immun 2006; 74: 1712-1717.
[13] Mohammed I, Hampton SE, Ashall L, Hildebrandt ER, Kutlik RA, Manandhar SP, Floyd BJ, Smith HE, Dozier JK, Distefano MD, Schmidt WK and Dore TM. 8-Hydroxyquinoline-based inhibitors of the Rce1 protease disrupt Ras membrane localization in human cells. Bioorg Med Chem 2016; 24: 160-178.
[14] Burrows JF, Kelvin AA, McFarlane C, Burden RE, McGrattan MJ, De la Vega M, Govender U, Quinn DJ, Dib K, Gadina M, Scott CJ and Johnston JA. USP17 regulates Ras activation and cell proliferation by blocking RCE1 activity. J Biol Chem 2009; 284: 9587-9595.
[15] Bergo MO, Ambroziak P, Gregory C, George A, Otto JC, Kim E, Nagase H, Casey PJ, Balmain A and Young SG. Absence of the CAAX endoprotease rce1: effects on cell growth and transformation. Mol Cell Biol 2002; 22: 171-181.
[16] Bergo MO, Wahlstrom AM, Fong LG and Young SG. Genetic analyses of the role of RCE1 in RAS membrane association and transformation. I Methods Enzymol 2008; 438: 367-389.
[17] Aiyagari AL, Taylor BR, Aurora V, Young SG and Shannon KM. Hematologic effects of inactivating the Ras processing enzyme Rce1. Blood 2003; 101: 2250-2252.
[18] Wahlstrom AM, Cutts BA, Karlsson C, Andersson KM, Liu M, Sjogren AK, Swolin B, Young SG and Bergo MO. Rce1 deficiency accelerates the development of K-RAS-induced myeloproliferative disease. Blood 2007; 109: 763-768.
[19] Huang L, Li M, Wang D, He J, Wu W, Zeng Q, Li J, Xiao M, Hu J, He Y, Li Y, Mai L and Liu W. Overexpressed Rce1 is positively correlated with tumor progression and predicts poor prognosis in prostate cancer. Hum Pathol 2016; 47: 109-114.
[20] Li J, Wang D, Liu J, Qin Y, Huang L, Zeng Q, Xiao M, Hu J, Yang Q, He J, Mai L, Li Y and Liu W. Rce1 expression in renal cell carcinoma and its regulatory effect on 786-O cell apoptosis through endoplasmic reticulum stress. Acta Biochim Biophys Sin 2017; 49: 254-261.
[21] Shi B, Zhou X, He L, Liang M, Luo Y and Jiang P. Reduced RCE1 expression predicts poor prognosis of colorectal carcinoma. BMC Cancer 2017; 17: 414.
[22] He L, Zhou X, Qu C, Hu L, Tang Y, Zhang Q, Liang M and Hong J. Musashi2 predicts poor prognosis and invasion in hepatocellular carcinoma by driving epithelial-mesenchymal transition. J Cell Mol Med 2014; 18: 49-58.
[23] Zhong L, Fu XY, Zou C, Yang LL, Zhou S, Yang J, Tang Y, Cheng C, Li LL, Xiang R, Chen LJ, Chen YZ, Wei YQ and Yang SY. A preclinical evaluation of a novel multikinase inhibitor, SKLB-329, as a therapeutic agent against hepatocellular carcinoma. Int J Cancer 2014; 135: 2972-2983.
[24] Zhang W, Sun HC, Wang WQ, Zhang QB, Zhuang PY, Xiong YQ, Zhu XD, Xu HX, Kong LQ, Wu WZ, Wang L, Song TQ, Li Q and Tang ZY. Sorafenib down-regulates expression of HTATIP2 to promote invasiveness and metastasis of orthotopic hepatocellular carcinoma tumors in mice. Gastroenterology 2012; 143: 1641-1649.

[25] Thiery JP, Acloque H, Huang RYJ and Angela Nieto M. Epithelial-mesenchymal transitions in development and disease. Cell 2009; 139: 871-890.
[26] Mei Y, Yang JP and Qian CN. For robust big data analyses: a collection of 150 important pro-metastatic genes. Chin J Cancer 2017; 36: 16.
[27] Mehta A and Singal AG. Hepatocellular carcinoma surveillance: does alpha-fetoprotein have a role? Gastroenterology 2015; 149: 816-817.
[28] Marrero JA and Feng Z. Alpha-fetoprotein in early hepatocellular carcinoma. Gastroenterology 2010; 138: 400-401.
[29] Sauzay C, Petit A, Bourgeois AM, Barbare JC, Chauffert B, Galmiche A and Houessinon A. Alpha-foetoprotein (AFP): a multi-purpose marker in hepatocellular carcinoma. Clin Chim Acta 2016; 463: 39-44.
[30] Choi JY, Jung SW, Kim HY, Kim M, Kim Y, Kim DG and Oh EJ. Diagnostic value of AFP-L3 and PIVKA-II in hepatocellular carcinoma according to total-AFP. World J Gastroenterol 2013; 19: 339-346.
[31] Jia X, Liu J, Gao Y, Huang Y and Du Z. Diagnosis accuracy of serum glypican-3 in patients with hepatocellular carcinoma: a systematic review with meta-analysis. Arch Med Res 2014; 45: 580-588.
[32] Li J, Wang T, Jin B, Li W, Wang Z, Zhang H, Song Y and Li N. Diagnosis accuracy of serum glypican-3 level in patients with hepatocellular carcinoma: a systematic review with meta-analysis. Int J Biol Markers 2018; 33: 353-363.
[33] Yang H, Chen GD, Fang F, Liu Z, Lau SH, Zhang JF, Lau WY and Yang LY. Dickkopf-1: as a diagnostic and prognostic serum marker for early hepatocellular carcinoma. Int J Biol Markers 2013; 28: 286-297.
[34] Jang ES, Jeong SH, Kim JW, Choi YS, Leissner P and Brechot C. Diagnostic performance of alpha-fetoprotein, protein induced by vitamin k absence, osteopontin, dickkopf-1 and its combinations for hepatocellular carcinoma. PLoS One 2016; 11: e0151069.
[35] Kim DY, Paik YH, Ahn SH, Youn YJ, Choi JW, Kim JK, Lee KS, Chon CY and Han KH. PIVKA-II is a useful tumor marker for recurrent hepatocellular carcinoma after surgical resection. Oncology 2007; 72: 52-57.
[36] Lamouille S, Xu J and Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014; 15: 178-196.
[37] De Craene B and Berx G. Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 2013; 13: 97-110.
[38] Wang B, Zhang L, Zhao L, Zhou R, Ding Y, Li G and Zhao L. LASP2 suppresses colorectal cancer progression through JNK/p38 MAPK pathway meditated epithelial-mesenchymal transition. Cell Commun Signal 2017; 15: 21.
[39] Nieto MA, Huang RY, Jackson RA and Thiery JP. EMT: 2016. Cell 2016; 166: 21-45.
[40] Chen X, Zhang S, Wang Z, Wang F, Cao X, Wu Q, Zhao C, Ma H, Ye F, Wang H and Fang Z. Supervillin promotes epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma in hypoxia via activation of the RhoA/ROCK-ERK/p38 pathway. J Exp Clin Cancer Res 2018; 37: 128.
[41] El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011; 365: 1118-1127.