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Research article

Low expression of RACK1 is associated with metastasis and worse prognosis in cholangiocarcinoma

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ABSTRACT

Background: Cholangiocarcinoma is a poorly prognostic malignant tumor, and the metastatic stage of cancer is not an early stage when diagnosed. Lymph node metastasis is common in the early stage. Ribosomal receptor for activated C-kinase 1 (RACK1) has found involved in the oncogenesis of various tumors and in the epithelial-mesenchymal transition (EMT). Nevertheless, its role in cholangiocarcinoma remains unknown.

Material and methods: The possible correlation between RACK1 and tumor prognosis was analyzed in cholangiocarcinoma patients. The GEO and TCGA databases were used to evaluate the level of *RACK1* in cholangiocarcinoma. The RBE and HCCC-9810 cell lines were used to examine the effects of RACK1 in the behavior of tumor cells *in vitro*.

Results: The Kaplan-Meier analysis indicated that low expression of RACK1 was associated with poor prognosis and RACK1 was negatively related to lymph node metastasis, which were verified in databases TCGA and GEO; downregulation of RACK1 via RNA interference correlated with changes in the expression of EMT biomarkers and promoted the migration of cholangiocarcinoma cell lines.

Conclusion: The protein expression of RACK1 is significantly higher in cholangiocarcinoma tissues than in peritumoral tissues, however, the high RACK1 expression indicates better overall survival and less risk for lymph node metastasis. *In vitro*, RACK1 may suppress the migratory ability of cholangiocarcinoma cells by inhibiting EMT.

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1. Introduction

Cholangiocarcinoma is a fatal malignancy in most patients with a slowly increasing incidence rate worldwide, and currently accounts for ~15% of all primary liver cancers and ~3% of gastrointestinal malignancies [1–3]. The prognosis of cholangiocarcinoma is still dismal and represents ~2% of all cancer-related deaths worldwide annually, due to lack of specific symptoms at early stage. There is not an early stage of cholangiocarcinoma at diagnosis mean that is not metastasized. The stage of tumor tissue can be T1-T2, but the metastatic stage of cancer is not an early stage. The average 5-year overall survival (OS) is ~10%–30%, while the median OS is ~10–28 months across all stages [4–6]. At the genomic, epigenetic and molecular levels, the high heterogeneity severely compromises the treatment efficacy of this disease and to achieve breakthrough in prognosis, it is necessary to deeply understand its molecular mechanism in cholangiocarcinoma [1]. The knowledge of causal mechanisms in cholangiocarcinoma has been improved in recent years. The main oncogenic networks in cholangiocarcinoma comprised WNT-CTNNB1, MYC, ERBB, TNF and VEGF signaling, availing cell survival signaling pathways in patients with poor OS. Stratified studies of cholangiocarcinoma based on prognosis using an integrated genomics approach have shown enhanced anti-apoptotic signaling, angiogenesis, signal transduction, and transcriptional control. The most clinically significant genomic breakthroughs in intrahepatic cholangiocarcinoma are the discovery of hotspot isocitrate dehydrogenase (IDH) mutations that cause an accumulation of the oncometabolite 2-hydroxyglutarate (2-HG), as well as the constitutive active gene fusion event between fibroblast growth factor receptor 2 (*FGFR2*) and many different partners. These alterations are important as they are driving current marker-based phase III clinical trials testing specific agents targeting these alterations. Furthermore, early lymph node metastasis deserves careful study. It has been reported that approximately 75% of cholangiocarcinoma patients found to have lymph node metastasis in early-stage (T1 and T2), which highly compromises the efficacy of all therapeutic options and indicates poor prognosis [5,7]. Tumor cells that have key phenotypic advantages: increased cell invasiveness and migration will be conducive to transfer. Epithelial–mesenchymal transition (EMT) plays a crucial role in the malignant tumor metastasis and in several tumors facilitates cell migration and invasion [7–9]. According to some studies, EMT is associated with several functional aberrations such as E-cadherin loss and SOX4 abnormality and can be reversed by the expression of claudin-1 [10,11]. The EMT-transcription factors, comprising SNAIL, ZEB and TWIST family, which are associated with poor prognosis in patients with cholangiocarcinoma regardless of the anatomical localization. Many signaling pathways, such as IL-6/JAK/STAT3/Twist-1 and MCT-1/miR-34a/IL-6/IL-6R, are abnormally activated during EMT in many types of cancersthe references in your manuscript are in a numbered format.

Ribosomal receptor for activated C-kinase 1 (RACK1) is one of the members from tryptophan-aspartate repeat protein family and acts as a scaffolding protein that nucleates key signaling complexes and regulates the shuttling proteins' function and subcellular location [12]. Recently, numerous studies investigated the relationship between RACK1 expression and cancer clinicopathological characteristics and patient prognosis, but its biological function was described as very heterogenous: both as tumor suppressor and more often as tumor promotor [13,14]. RACK1 is upregulated in various cancers, including ovarian, breast cancers and several gastrointestinal tumors, such as hepatic tumor, and usually higher expression of RACK1 is related to worse prognosis [15]. In oral squamous cell carcinoma, RACK1 promotes cancer progression via the NF-κB pathway [16]. In cervical cancer, RACK1 stimulates tumor invasion and lymph node metastasis via galectin-1 and imply that targeting RACK1/galectin-1 axis provides promising means for cervical cancer treatment [17]. Besides, in the context of breast cancer, up-regulation of RACK1 is an indicator of poor prognosis and directly related to the recurrence [18]; cortisol as well as glucocorticoids can induce cell migration also through the involvement of RACK1 [19]. Suppression of TRPM7 inhibits hypoxia-induced cell migration and invasion of androgen-independent prostate cancer cells by promoting the degradation of HIF-1α, with an underlying mechanism involving the oxygen-independent RACK1-mediated HIF-1α degradation in the proteasome thought increased phosphorylation of RACK1 [20]. By contrast, Chen et al. have reported that low RACK1 expression correlates with the cancer invasion/metastasis phenotype and that a loss of RACK1 expression can promote gastric cancer metastasis [21]. Down-regulation of RACK1 might be responsible for the occurrence of pancreatic cancer by activating the NF-κB pathway [22]. The precise underlying molecular mechanism remains to be elucidated.

Besides conflicting conclusions have been drawn from different studies, the evidence supporting the correlation between RACK1 and EMT remains controversial [12]. It is reported that RACK1 can inhibit EMT via the wnt/β-catenin pathway in gastric cancer but enhances EMT in glioma[[17,23]]. To date, how RACK1 affects cholangiocarcinoma remains unknown [1,24]. Hence, we aimed to investigate the function of RACK1 in cholangiocarcinoma, especially in relation to the migration ability of cholangiocarcinoma cells in this research.

2. Materials and methods

2.1. TCGA and GEO databases

The Cancer Genome Atlas (TCGA) data (RNA-seq V2 expression data, 36 tumor and 9 adjacent normal samples of intrahepatic cholangiocarcinoma) were downloaded from the genomic data commons (GDC) data portal (https://portal.gdc.cancer.gov/). The Gene Expression Omnibus (GEO) datasets GSE76311 (90 samples) and GSE107943 (27 samples) were downloaded from the GEO website (http://www.ncbi.nlm.nih.gov/geo/). The mRNA levels retrieved from TCGA were expressed in fragments per kilobase per million (FPKM). The mRNA levels retrieved from GSE76311 were expressed in transcripts per kilobase million (TPM), and the mRNA levels from GSE107943 were expressed in reads per kilobase million (RPKM).

2.2. Patients, tumor specimens, and cell lines

Clinical samples were obtained from 188 cholangiocarcinoma patients in Zhongshan Hospital (Shanghai, China). All the patients received radical surgery between January 2012 and December 2015. The including criteria were: (1) Cholangiocarcinoma was diagnosed by the department of pathology based on postoperative specimen, (2) the patient did not have distant metastasis before surgery, did not receive preoperative chemotherapy or radiotherapy, and (3) the patient underwent radical surgery. Patient data were excluded if the clinical records were incomplete or follow-up information was missing. All the characteristics and clinical parameters of these patients were collected from the medical records. This study was approved by the ethics committee of Fudan University (approval number: B2017-151R). All patients involved in this study were well informed about the study and provided consent to participate. Cell lines of human cholangiocarcinoma HCCC-9810 and RBE were purchased from the Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, P.R. China). Independent experiments were repeated for three times.

2.3. Immunohistochemistry staining

The construction of tissue microarray and the procedure of immunohistochemistry (IHC) staining were described as previously [25,26]. The primary antibody was anti-RACK1 antibody (1:1000; Santa Cruz Biotechnology, Lot # sc-17754). All immunohistochemistry data were evaluated based on the percentage of stained cells and staining intensity as described previously [26]. The percentage of staining was graded as: <5% (score 0), 5–25% (score 1), 26–50% (score 2), 51–75% (score 3), and 76–100% (score 4). The intensity of staining was graded as negative staining (score 0), weak staining (score 1), moderate staining (score 2), or strong staining (score 3). The total score was computed as 4 × (intensity score-1) + frequency score [27,28]. Then, x-tile software was used to determine the cut-off value of the IHC total score [29,30]. Accordingly, patients were stratified into high (total score 6–12) and low groups (total score 0–5).

2.4. siRNA, plasmids and transfections

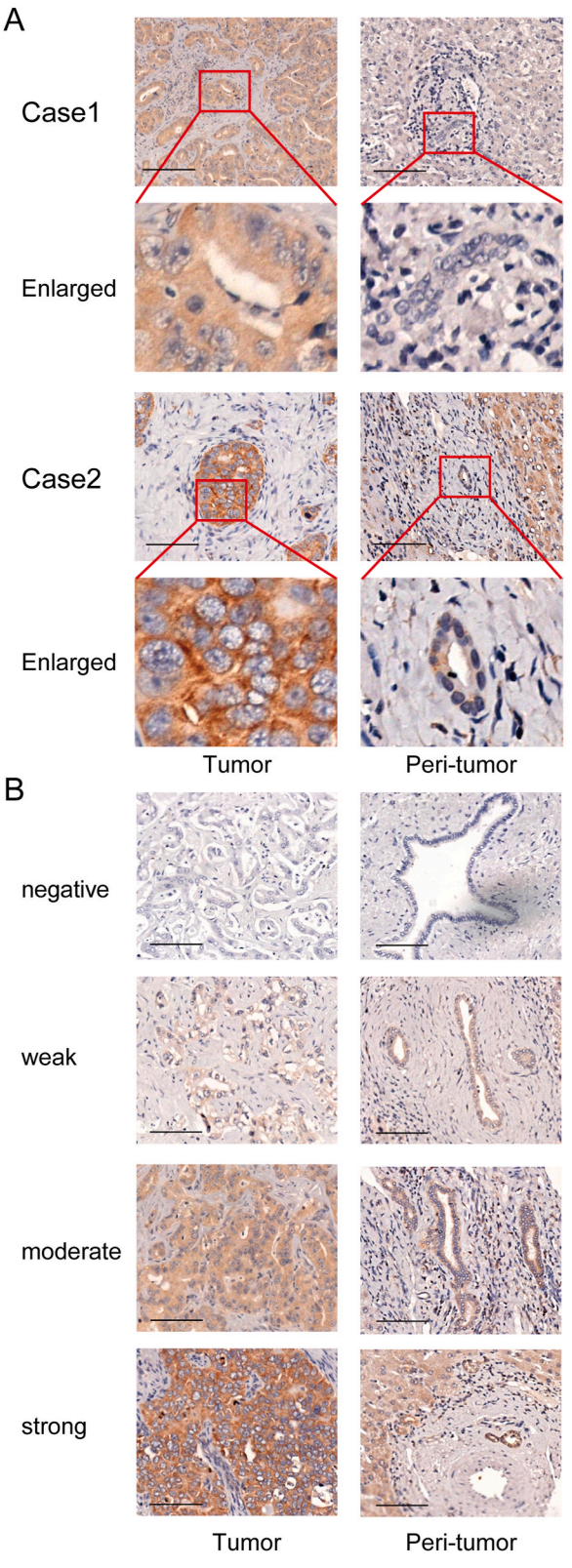
The cells (50–60% confluency) were transiently transfected using the lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) with small interfering RNAs (siRNAs) and this procedure was performed as described previously [17]. The siRNA specifically targeting human RACK1 mRNA was synthesized by Biomics Biotechnologies Co., Ltd (China). Sequences were as follows: siRACK1-1, 5′-CUCUGGAUCUCGAGAUAAA-3′; siRACK1-2, 5′-CCCACUUUGUUAGUGAUGU-3′; sequences of siRNA controls were as follows: 5′-UUCUCCGAACGUGUCACGUdTdT -3′; 3′-ACGUGACACGUUCGGAGAAdTdT -5′. The human wild-type RACK1 has been previously described [17]. The controls in non-overexpressing cells is empty vector. When cells grew to 50–60% confluency, the RACK1 plasmids and vectors were used to infect cells using lipofectamine 3000 and P3000 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

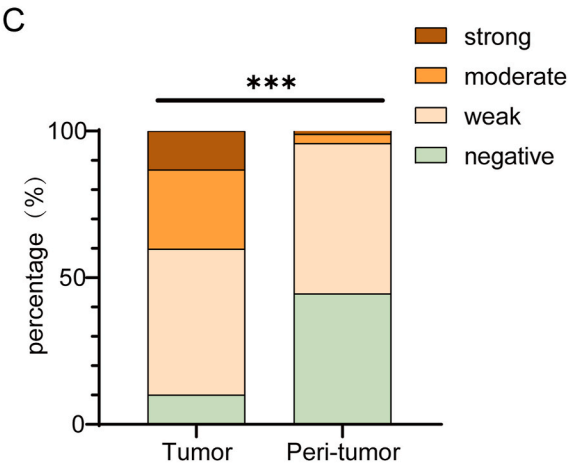
2.5. RNA extraction and real-time PCR

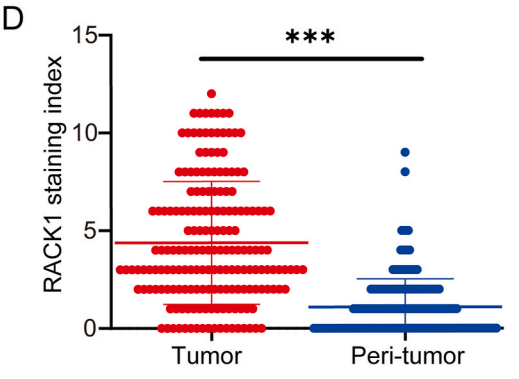
The RNA was isolated from cultured cells using the TRIzol reagent (Invitrogen) and the procedure was performed as described previously [17,31]. The total RNA (1.5 μg) was reverse-transcribed to cDNA with the PrimeScript RT Reagent Kit (Takara) in accordance with the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using ABI StepOne Plus (Applied Biosystems, Thermo Fisher Scientific) with the help of SYBR Premix Ex Taq (Takara, Japan) and calculated using β-actin as internal reference through the comparative CT ($2^{-\Delta\Delta CT}$) method. The sequences of real-time PCR primers were obtained from OriGene. The primer sequences of RACK1 were as follows: Forward Sequence: GCCATACCAAGGATGTGCTGAG; Reverse Sequence: CACAAGACACCCACTCTGAGTG. Primer sequences of β-actin were as follows: Forward Sequence: CACCATTGGCAATGAGCGGTTC; Reverse Sequence: AGGTCTTTGCGGATGTCCACGT.

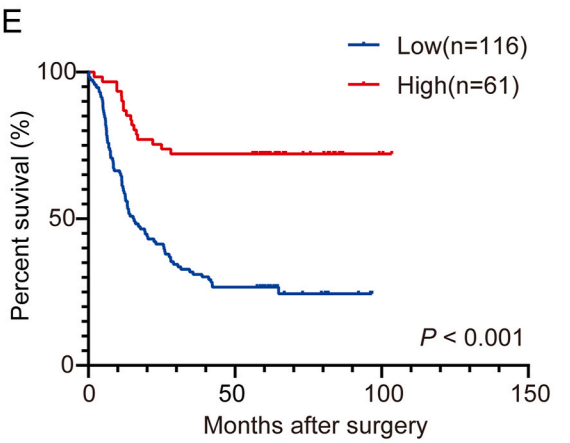
2.6. Western blot

Western blot analysis was done using the same protocol as described previously [17]. Protein concentrations of cell lysates were determined using a BCA Protein Assay Kit (Beyotime, USA). Protein samples were separated by 10% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with skim milk in Tris-buffered saline with Tween 20 (TBST) for 1 h and incubated overnight at 4 °C with primary antibodies. After washing with TBST, the membranes were incubated with secondary antibodies for 1 h at room temperature. Protein expression was assessed by an enhanced chemiluminescence assay. The results were recorded using an Image-Quant LAS 4000 mini imaging system (GE Healthcare, USA). The antibodies used were as follows: E-cadherin (2Q663) (1:1000; Santa Cruz Biotechnology, Lot # A0313); RACK1 (B-3) (1:2000; Santa Cruz Biotechnology, Lot # sc-17754); vimentin (D21H3) (1:1000; Cell Signaling Technology, Lot # 5741T); claudin-1 (D5H1D) (1:1000; Cell Signaling Technology, Lot # 13255T); zonula occludens protein-1 (ZO-1, D7D12) (1:1000; Cell Signaling Technology, Lot # 8193T); β-actin (C4) horseradish peroxidase (HRP) (1:2000; Santa Cruz Biotechnology, Lot # sc-47778); and HRP-conjugated secondary antibody (1:2000; Proteintech, Chicago, USA, Cat # SA00001-1 or SA00001-2).









	Low (n=116)	High (n=61)
Number of events, n (%)	86(74.1)	17(27.9)
Median survival	15.6	NA
HR (95% CI)	3.087(2.085-4.570) *P*<0.001	

(caption on next page)

Fig. 1. The immunohistochemistry result of RACK1 protein level in the clinical cholangiocarcinoma samples from Zhongshan Hospital A. Representative immunostaining images of RACK1 in 2 cholangiocarcinoma cases (scale bars = 100 μm). B. The representative figures of RACK1 expression intensities in tumor and adjacent normal tissues. The expression intensity was graded as negative, weak, moderate, or strong (scale bars = 100 μm). C. The RACK1 expression staining intensities in tumor tissues and peritumoral normal tissues. ($P < 0.001$). D. The composite expression score was used to evaluate the IHC staining intensity. The higher expression of RACK1 was observed in tumor tissues than in paired adjacent normal tissues ($P < 0.001$). E. The prognostic significance of RACK1 expression in Zhongshan Hospital's surveillance cohort. The OS of patients with high RACK1 expression was significantly longer than that of patients with low RACK1 expression ($P < 0.001$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2.7. Cell migration assay

This assay was based on Transwell chambers to analyze the migration ability of cholangiocarcinoma cells and was performed as described previously [17]. Approximately 30,000 RBE or HCCC-9810 cells were resuspended in 200 μl of a serum-free medium and seeded in the upper part of the Transwell chamber (Costar, Cambridge, MA, USA). Next, 600 μl of a complete growth medium was added into the lower chamber. After 24 h, non-invading cells on the upper side of the chamber were removed from the surface of the membrane by scrubbing, and the invading cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of metastatic cells was counted in five randomly selected microscopic fields of each filter.

2.8. Wound healing experiment

The cells (3×10^5 cells/well) were planted in 12-well plates, and when the cells reached to 90% confluency, the 200 μL sterile pipette tip was used to make a scratch in the plate. The cells were cultured with serum-free medium after gently rinsed three times with PBS. Photographs were taken and scratch widths were recorded at 0 h and 24 h after the formation of scratches.

2.9. Colony formation assay and CCK-8 assay

The cells (3×10^3 cells per well) were plated in 6-well plates and then cultured for 14 days to form colonies. After 14 days growing in culture conditions, the colonies were fixed with 4% paraformaldehyde for 20 min, stained with 0.1% crystal violet for 15 min, and then washed with PBS. Viable colonies larger than 0.5 mm were counted. Cell viability was analyzed using the Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) according to the manufacturer's protocols. The cells (5×10^3 per well) were seeded and cultured in 100 μL of medium into 96-well microplates (Corning, USA). Then, 90 μL Dulbecco's Modified Eagle Medium (DMEM) was mixed with 10 μL CCK-8 reagent to generate a working solution, of which 100 μL was added per well and incubated for 2 h. This assay was performed at 0 h, 24 h, 48 h, and 72 h.

2.10. Flow cytometry assay

To quantitatively assess the induced apoptotic cell death rate, annexin V-APC/7-amino-actinomycin (7-AAD) apoptosis analysis assay was performed according to the protocol presented by the manufacturer (Absin Bioscience Inc., China). The cells were harvested and then resuspended in 400 μL with 1 × binding buffer at a concentration of 1×10^6 cells/ml prior to addition of 5 μL of annexin V–APC. The cells were then gently vortexed and incubated for 30 min at 4 °C in the dark. Next, 5 μL of 7-AAD was added to each tube prior to incubation for another 5 min at room temperature in the dark. The percentage of apoptosis was analyzed using flow cytometry (BD, FACSCalibur, USA) within 1 h.

2.11. Statistical analysis

All analyses were performed in SPSS (version 18, Chicago, IL, USA) and R software (www.r-project.com). Data from the TCGA and GEO databases were analyzed in R using the "survminer" package. The differences in RNA expression between tumors and peritumoral normal tissues or between tumors with metastasis and tumors without metastasis were analyzed using the Wilcoxon test or unpaired *t*-test. The unpaired *t*-test was also used to compare the means of two groups. The correlations between clinical parameters, the expression of RACK1 in the IHC assay, and OS were analyzed using the Cox regression analyses. Kaplan–Meier analysis was carried out to determine the survival differences. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Expression and clinical significance of RACK1 in cholangiocarcinoma

The expression of RACK1 protein in cholangiocarcinoma was evaluated by IHC. A strong RACK1 protein expression was mostly associated with tumor tissues, while the absence of RACK1 protein expression was mostly associated with peritumoral normal bile duct epithelial cells (Fig. 1A and 1B). Then, the IHC data were evaluated based on the composite expression score. The RACK1 IHC staining score was rated as negative, weak, moderate, and strong in 10.0%, 49.7%, 27.0%, and 13.2% (negative: n = 19, weak: n = 94, moderate: n = 51, and strong: n = 25) of tumor tissues compared with that in 44.5%, 51.3%, 3.1%, and 1.0% (negative: n = 85, weak: n = 98, moderate: n = 6, strong: n = 2) of peritumoral normal tissues ($P < 0.001$; Fig. 1 C). The tumor tissues obtained higher scores

than the peritumoral normal tissues, and the difference was significant ($P < 0.001$; Fig. 1D). There is no correlation between paired RACK1 score in tumoral and peri-tumoral tissues ($P > 0.05$; Supplementary Fig. S1). However, the OS of patients with lower RACK1 expression level (the staining score 0–5) was significantly worse than that of patients with high RACK1 expression level (the staining score 6–12, $P < 0.001$; Fig. 1E). To further demonstrate the clinical significance of RACK1, the relationship between the RACK1 expression, clinical parameters and OS were analyzed. RACK1 expression was not significantly associated with patient age, sex, tumor size, cirrhosis and differentiation (all $P > 0.05$), but was significantly lower in cases with multiple tumor number ($P = 0.006$) and in the presence of lymph node metastasis ($P < 0.001$, Table 1). Results of the multivariate Cox regression analysis showed that lymph node metastasis and RACK1 expression were independently associated with OS (hazard ratio [HR]: 1.731, 95% confidence interval [CI]: 1.141–2.626, $P = 0.001$; HR: 0.842, 95% CI: 0.782–0.906, $P < 0.001$, respectively; Table 2).

3.2. The mRNA level of RACK1 in GEO and TCGA cholangiocarcinoma datasets

To verify the outcomes found previously, the expression of RACK1 protein were evaluated between tumor tissues with and without lymph node metastasis, and tumor number. The tumor tissues obtained lower scores in patients with lymph node metastasis than without, and the difference was significant ($P < 0.001$; Fig. 2A); the tumor tissues obtained lower scores in patients with multiple tumors than with single tumor, and the difference was significant ($P < 0.05$; Fig. 2B). The difference of RACK protein expression with different differentiations was not significant ($P > 0.05$; Fig. 2C). Next, to estimate the changes in the expression of *RACK1* in cholangiocarcinoma, the expression of *RACK1* in cholangiocarcinoma tissues and peritumoral normal tissues in two databases, GEO and TCGA, were analyzed. The *RACK1* gene expression was significantly higher in cholangiocarcinoma tissues than in the peritumoral normal tissues in datasets GSEA76311 and GSE107943 ($P < 0.001$; Fig. 2D and E). Next, the changes in *RACK1* expression between cholangiocarcinoma tissues and peritumoral normal tissues in TCGA database were analyzed. *RACK1* was significantly higher in cholangiocarcinoma tissues than that in peritumoral normal tissues ($P < 0.01$; Fig. 2F). Furthermore, the correlation between lymph node metastasis and the *RACK1* expression level in TCGA was analyzed. Interestingly, the expression level of *RACK1* was significantly lower in patients with lymph node metastasis compared with those without lymph node metastasis ($P < 0.05$; Fig. 2G). These results corroborated with our earlier observations, indicating that the expression of *RACK1* mRNA is significantly decreased in patients with cholangiocarcinoma with lymph node metastasis. As lymph node metastasis is one of the main causes of death in patients with progressive cholangiocarcinoma, further investigation focusing on the potential mechanism of RACK1 was carried out.

3.3. RACK1 depletion promoting cell migration in cholangiocarcinoma in vitro

To assess the role of RACK1 in the development and progression of cholangiocarcinoma at cellular level, the expression of RACK1 in RBE and HCCC-9810 cell lines was knocked down respectively, and the efficiency of RACK1 knockdown was verified by RT-PCR and Western blot analysis (Fig. 3A and B). Based on our observation that the low expression of RACK1 is correlated with cholangiocarcinoma lymph node metastasis in patient samples, we focused on examining whether the RACK1 expression level affects cholangiocarcinoma cell migration. Hence, a cell migration assay and a wound healing assay were performed. The knockdown of RACK1 was found to increase the migration of RBE and HCCC-9810 cells, and the difference was significant ($P < 0.001$, respectively; Fig. 3C and D). Next, the expression of EMT-related markers was examined. As shown in Fig. 3B, RACK1 knockdown suppressed the E-cadherin, ZO-1, and claudin-1 expression, but enhanced the vimentin expression. These findings suggest that RACK1 inhibits the lymph node metastasis of cholangiocarcinoma by negatively regulating the EMT. Meanwhile, the role of RACK1 in tumor progress was assessed, and colony formation and CCK-8 assays were performed. Results of the colony formation assay and CCK-8 assay of RBE and HCCC-9810 cell lines with knockdown of RACK1 did not differ significantly compared with those of controls (Fig. 4A and B). Meanwhile, results of the flow cytometry assay of RBE and HCCC-9810 with knockdown of RACK1 did not change significantly compared with those of controls (Fig. 4C). These results indicated that RACK1 depletion had no effect on cell proliferation.

Table 1
The relation between clinical parameters and the expression of RACK1.

Clinical parameters		Low (116)	High (61)	*P* value
Gender	Male	60 (51.7%)	38 (62.3%)	0.179
	Female	56 (48.3%)	23 (37.7%)	
Age (year)		60.9 ± 8.6	61.2 ± 11.5	0.877
Tumor size	≤5 cm	42 (86.2%)	19 (73.8%)	0.631
	>5 cm	74 (13.8%)	42 (26.2%)	
Tumor number	Single	79 (68.1%)	53 (86.9%)	0.006
	Multiple	37 (31.9%)	8 (13.1%)	
Lymph node metastasis	Absent	83 (71.6%)	60 (98.4%)	<0.001
	Present	33 (28.4%)	1 (1.6%)	
Cirrhosis	Absent	111 (95.7%)	59 (96.7%)	1.000
	Present	5 (4.3%)	2 (3.3%)	
Differentiation	Good	2 (1.7%)	1 (1.6%)	0.630
	Moderate	49 (42.2%)	21 (34.4%)	
	Poor	65 (56.0%)	39 (63.9%)	

Statistical analyses were performed with the Chi-square test or *t*-test. *$P < 0.05$ was considered statistically significant.

Table 2
Univariate and multivariate analysis for overall survival.

Characteristics	Univariate analysis				Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Gender	Male	1					
	Female	0.894	0.759–1.648	0.570			
Age (year)		0.995	0.976–1.013	0.572			
Lymph node metastasis	Absent	1					
	present	2.647	1.724–4.065	< 0.001	1.731	1.141–2.626	0.010
Tumor size	≤5 cm	1					
	>5 cm	1.643	1.103–2.445	0.014			
Tumor number	Single	1					
	Multiple	2.095	1.392–3.153	< 0.001			
Cirrhosis	Absent	1					
	present	1.148	0.422–3.120	0.787			
Differentiation	Good	1		0.779			
	Moderate	0.514	0.071–3.710	0.509			
	Poor	0.940	0.633–1.397	0.759			
RACK1		0.832	0.774–0.894	< 0.001	0.842	0.782–0.906	< 0.001

HR: Hazard Ratio, CI: Confidence Interval. *$P < 0.05$ was considered statistically significant.

3.4. *RACK1 overexpression reducing cell migration in cholangiocarcinoma in vitro*

Next, the expression of RACK1 in cell lines RBE and HCCC-9810 was overexpressed respectively, and the efficiency was verified by Western blot analysis (Fig. 5A). EMT-related markers were detected after RACK1 overexpression. As shown in Fig. 5A, RACK1 overexpression suppressed vimentin, whereas enhanced E-cadherin, ZO-1, and claudin-1 expression. Next, a cell migration assay and a wound healing assay were performed. Overexpression of RACK1 reduced RBE cell migration as compared with vehicle cells ($P < 0.001$, respectively; Fig. 5B and C). In summary, these data demonstrate that RACK1 may inhibit the progression of tumor by restraining metastasis.

4. Discussion

Cholangiocarcinoma is a relatively rare form of malignancy but with a high mortality rate; in recent years, there has been no obvious improvements in the treatment of this condition [2]. Although the roles of RACK1 have been investigated in several types of cancers with inconsistent results, no study has evaluated the role of RACK1 in the development cholangiocarcinoma. In this study, we found that the expression of RACK1 is significantly higher in cholangiocarcinoma tissues than in peritumoral normal tissues, and both Kaplan–Meier and Cox-regression analysis indicated that low expression of RACK1 was associated with poor prognosis, which was an interesting phenomenon. Meanwhile, RACK1 was negatively related to lymph node metastasis in cholangiocarcinoma without distant metastasis.

The expression of RACK1 is higher in tumors and the upregulation of RACK1 expression played a defensive role in cholangiocarcinoma. As to these unexpected results, we hypothesize that the high expression of RACK1 may take part in the cholangiocarcinogenesis or early stage of cholangiocarcinoma. RACK1 may induce tumor poor differentiation in the early stage of the disease, which needs further investigation; meanwhile, from the early stage to the middle and late stage, when the tumor is about to metastasize, the expression of RACK1 may decrease. The low RACK1 expression may trigger lymph node metastasis, thus increasing the degree of malignancy and causing recurrence and poor prognosis. For the protein processing of RACK1, it is mainly processed and degraded by ubiquitin-proteasome system. One research found a critical ubiquitin-conjugating enzyme E2T (UBE2T) that catalyzes the proteasomal degradation of RACK1 and then induces hyperactivation of Wnt/β-catenin signaling in gastric cancer [32]. Inhibiting ubiquitination may lead to stabilization of RACK1 at protein level and increased expression. Another research indicated that in ovarian cancer (OC), Smad ubiquitin regulatory factor 2 (SMURF2) was abnormal low expression, resulting in decreased ubiquitination of RACK1 and increased stability [33]. The development of cholangiocarcinoma is coordinated by complex interactions of extracellular ligands (such as pro-inflammatory cytokines, growth factors, bile acids, among others) present in the tumor microenvironment, increased expression and/or abnormal activation of cell surface receptors and disturbances of intracellular signaling pathways, ultimately leading to cell proliferation, survival, and genetic and/or epigenetic changes [1]; So we could think that a single gene *RACK1* need to produce the cancer-promoting mechanism under certain auxiliary conditions. The peritumoral environment varies among different types of tumors. RACK1 is highly expressed in normal hepatocytes [34], pancreatic ductal epithelial cells [35] and gastric epithelial cells, which has been implicated in the physiology. The RACK1 expression were significantly lower in pancreatic and gastric cancer tissue than in para-carcinoma normal tissue at the early stage. RACK1 play a crucial role as tumor suppressor in pancreatic cancer, gastric cancer and cholangiocarcinoma. However, we found the RACK1 expression was higher in tumor tissue, and the high affinity of RACK1 in liver tissue around bile ducts may affect the staining of normal bile ducts. RACK1 is upregulated in the other types of human cancers, such as colon cancer and lung cancer, and considered to play an important role as tumor promotor. Studies showed that RACK1 expression was progressively increased during the colonic epithelial carcinogenesis [36]. To summarize, the function of RACK1 might be dependent both on the cancer type and cellular context.

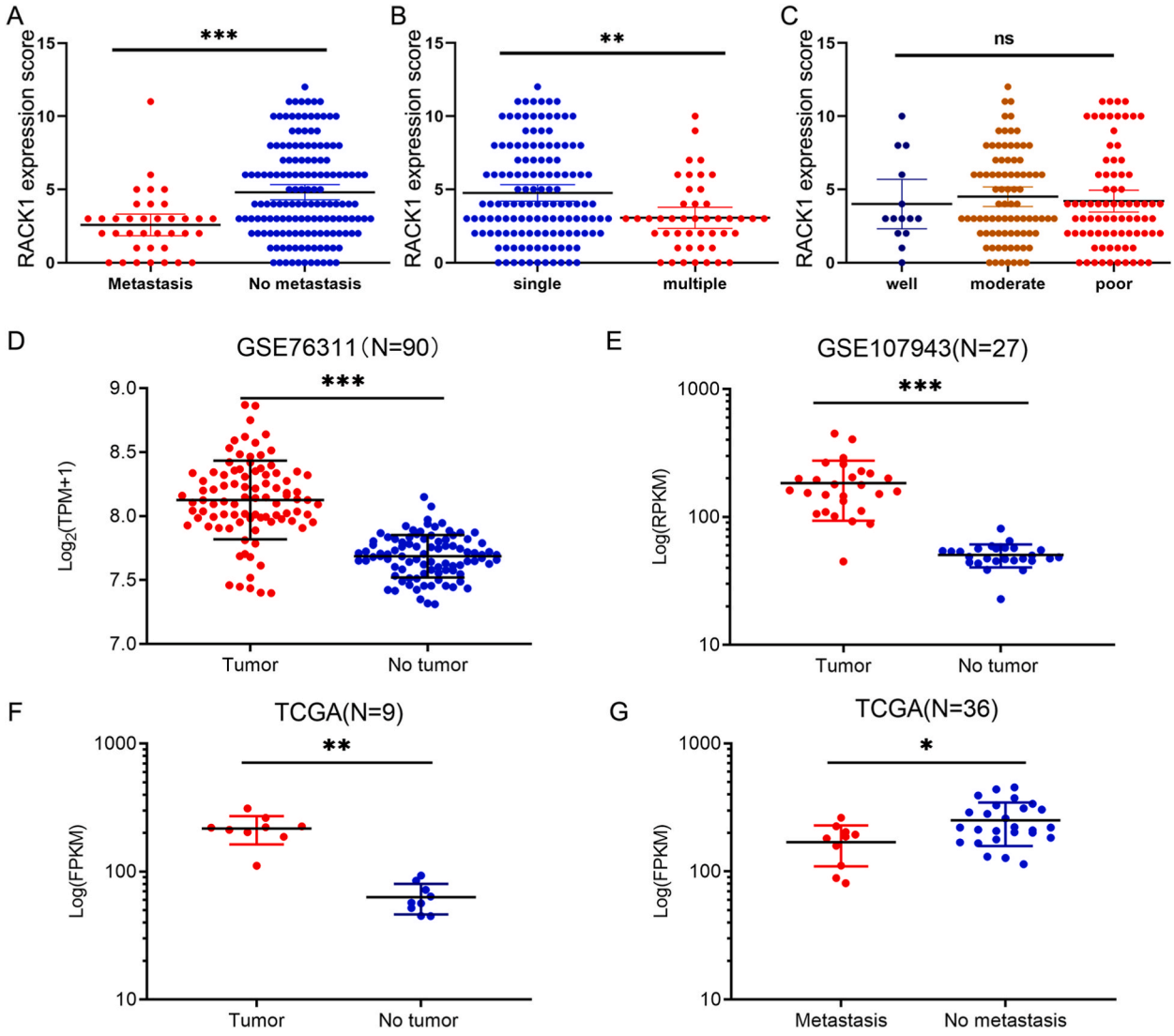


Fig. 2. The mRNA expression pattern of *RACK1* in GEO and TCGA cholangiocarcinoma datasets A. The RACK1 protein score in tumor tissues of patients with lymph node metastasis and without lymph node metastasis. The RACK1 scores were lower in patients with lymph node metastasis than in patients without lymph node metastasis ($P < 0.001$). B. The RACK1 protein score in tumor tissues of patients with different tumor number. The RACK1 scores were lower in patients with multiple tumors than with single tumor ($P < 0.05$). C. The RACK1 score in tumor tissues of patients with different differentiations was not significant ($P > 0.05$). D and E. The expression pattern of *RACK1* between tumor tissues and adjacent normal tissues in GEO datasets. Compared with the adjacent normal tissue, the aberrant expression of *RACK1* was observed in the tumor tissues in the GSE76311(N = 90, $P < 0.001$) and GSE107943 (N = 27, $P < 0.001$) datasets. F. The expression pattern of *RACK1* between tumor tissues and paired adjacent normal tissues in TCGA database. Compared with the adjacent normal tissue, the aberrant expression of *RACK1* was observed in the tumor tissues (N = 9, $P < 0.01$). G. The correlation between the lymph node metastasis and the *RACK1* expression level. The expression level of *RACK1* was significantly lower in patients with lymph node metastasis. (N = 36, $P < 0.05$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

At the same time, we find that other genes have similar phenomena published, such as chemokine ligand C-X-C motif chemokine ligand 11 (CXCL11) [37]. In that research, the authors found that CXCL11 expression in colon adenocarcinoma tumor tissues was upregulated and the upregulation of CXCL11 expression plays a defensive role in colon adenocarcinoma tumor development through correlating with antitumor immunity. However, the precise underlying molecular mechanism of the protein processing of RACK1 remains to be elucidated.

Based on our observation that the low expression of RACK1 is correlated with cholangiocarcinoma lymph node metastasis in our samples, we focused on examining whether the RACK1 expression level affects cholangiocarcinoma cell migration by EMT. We chose RBE and HCCC-9810 cells for *in vitro* study. RBE cells are considered to harbor Ras and IDH1 mutation, which do not exist in HCCC-9810 cells [38]. Our finding has shown that the RACK1 expression level affects cholangiocarcinoma cell migration by EMT independent from Ras and IDH1 mutation, and how RACK1 modulated the EMT phenotype of cholangiocarcinoma cells remains to be

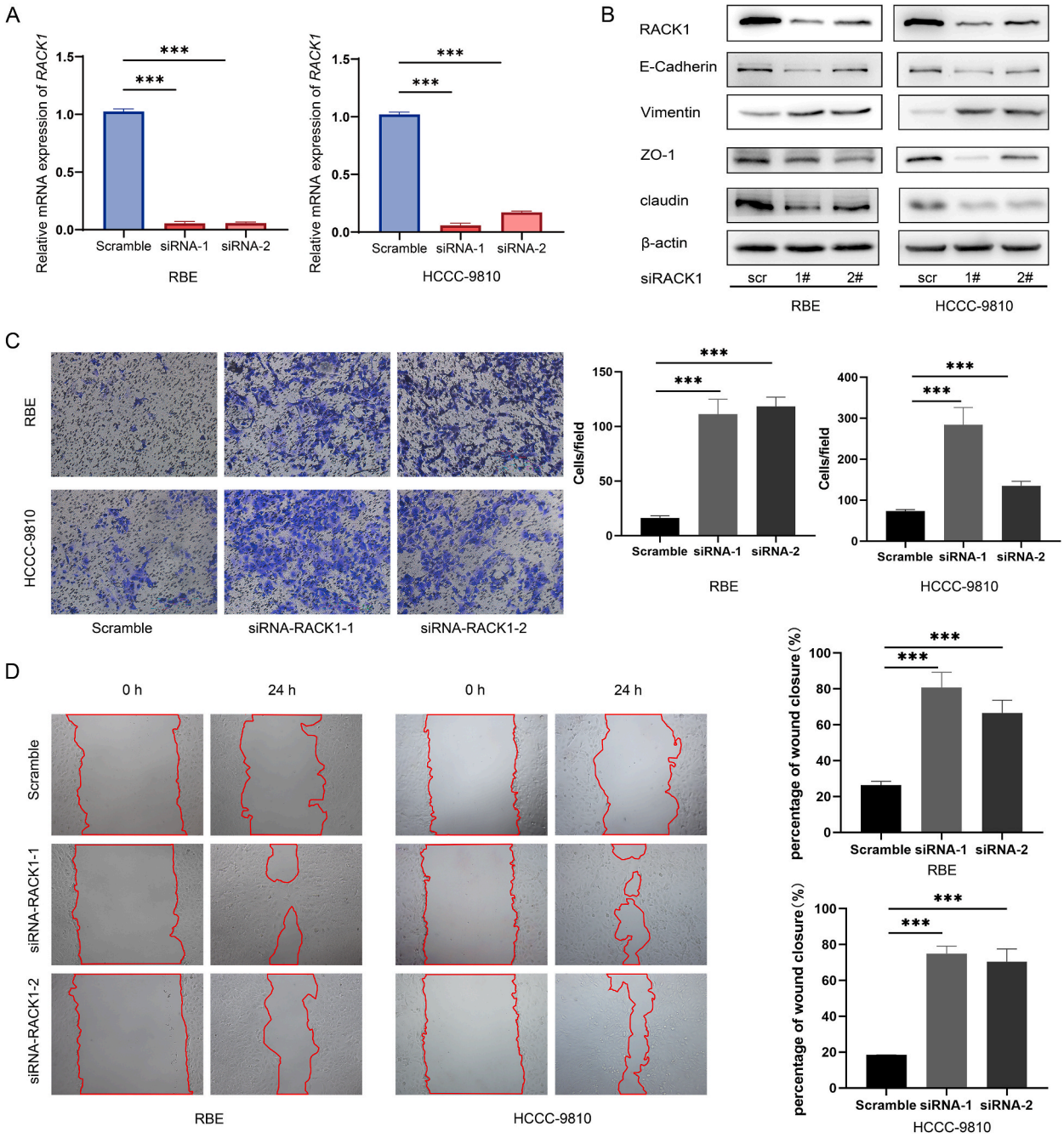


Fig. 3. The potential underlying mechanism for the biological effect of knockdown of RACK1 on cholangiocarcinoma (A) qRT-PCR results confirmed that the RACK1 mRNA expression was significantly lower in siRNA-1 and siRNA-2 RBE and HCCC-9810 cell lines compared with the control, siRNA-SCR RBE and HCCC-9810 cell lines. (B) The knockdown of RACK1 expression induced the downregulation of E-cadherin, ZO-1, and claudin and the upregulation of vimentin in RBE and HCCC-9810 cell lines. (C) Representative view and statistics of RBE and HCCC-9810 cell lines migrating from the upper Transwell chambers into the lower chambers. The knockdown of RACK1 expression promoted the migration of RBE and HCCC-9810 cell lines. (D) Representative view and statistics of RBE and HCCC-9810 cell lines migration on a wound healing assay. The knockdown of RACK1 expression promoted the migration ability of RBE and HCCC-9810 cell lines.

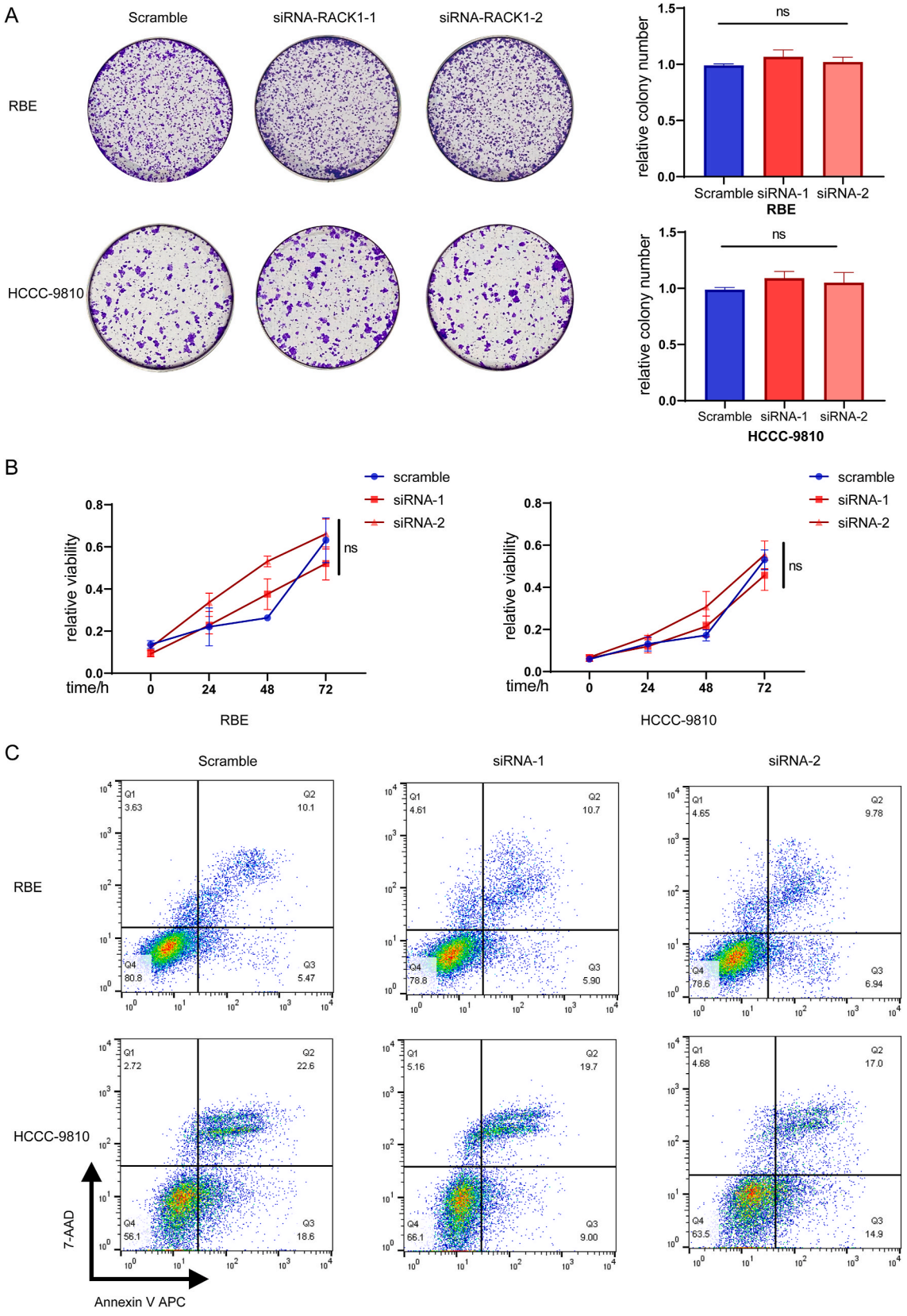


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Fig. 4. Effect of RACK1 depletion on the proliferation and apoptosis. (A) Representative view and statistics of RBE and HCCC-9810 cells in the colony formation assay. The knockdown of RACK1 expression had no significant effect on the colony number of RBE and HCCC-9810 cells. (B) Cell proliferation potential measured by CCK-8 assay. The knockdown of RACK1 expression had no significant effect on the cell viability of RBE and HCCC-9810 cells. (C) Cell apoptosis measured by flow cytometric analysis. The knockdown of RACK1 expression had no significant effect on the cell apoptosis of RBE and HCCC-9810 cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

determined. In other types of solid tumors such as gastric cancer and pancreatic carcinoma, a similar RACK1's inhibitory effect pattern has been observed [21,39]. Other studies presented several findings and indicated RACK1's dual effect on the EMT process in other types of solid tumors [23,39]. A previous study has shown that RACK1 inhibits the ubiquitination of E-cadherin (which is the key step in the EMT process) to stabilize it and facilitates the reassembly of E-cadherin-containing cell–cell contacts [40,41]. Vimentin, a mesenchymal protein, is reported to form a complex with RACK1 and to play an important part in lung adenocarcinoma metastasis [8,42]. In addition, there is evidence that tight-junction–related proteins claudins perform a pivotal function in EMT of cancer cells, but the members of this family of proteins may have either a stimulatory or inhibitory effect on EMT [10,43]. Moreover, evidence shows that the downregulation of another tight-junction–related protein, ZO-1, causes melanoma cells to acquire more epithelial characteristics [44].

This study has some limitations. Because the clinical correlations of RACK1 were derived from the two databases (GEO/TCGA) in which only a limited number of patients was documented, and the patients' data were obtained from a single center (Zhongshan Hospital), a larger-scale retrospective or prospective study is warranted to investigate the relationship between RACK1 and clinical parameters. Furthermore, the cases we provided were all received radical resection, so they only represent certain stage in the development of cholangiocarcinoma. Another potential limitation is that the expression of EMT-related markers changed when the expression of RACK1 was inhibited, but the underlying mechanism remains unknown. Thus, further studies should investigate this mechanism to determine the regulatory influence of RACK1 on EMT.

In summary, we found that the expression of RACK1 is significantly higher in cholangiocarcinoma tissues than in peritumoral normal tissues, and the high RACK1 expression indicates better OS and less opportunity to metastasis. *In vitro*, inhibiting the expression of RACK1 can promote EMT, whereas activation of RACK1 may be a novel therapeutic strategy in patients with progressive cholangiocarcinoma.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Fudan University (approval number: B2017-151R).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Data availability statement

The bioinformatics datasets generated and/or analyzed during the current study are available in The Cancer Genome Atlas repository, https://www.cancer.gov/tcga. GEO datasets GSE76311 and GSE107943 are available in GEO website (http://www.ncbi.nlm.nih.gov/geo/). The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Zhihui Gao: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Wentao Sun:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Xiaojian Ni:** Validation, Software, Resources, Funding acquisition, Data curation. **Wenze Wan:** Validation, Methodology, Formal analysis. **Tao Suo:** Data curation, Conceptualization. **Xiaoling Ni:** Resources, Investigation. **Han Liu:** Supervision, Investigation. **Na Li:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Formal analysis, Conceptualization. **Sheng Shen:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Houbao Liu:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

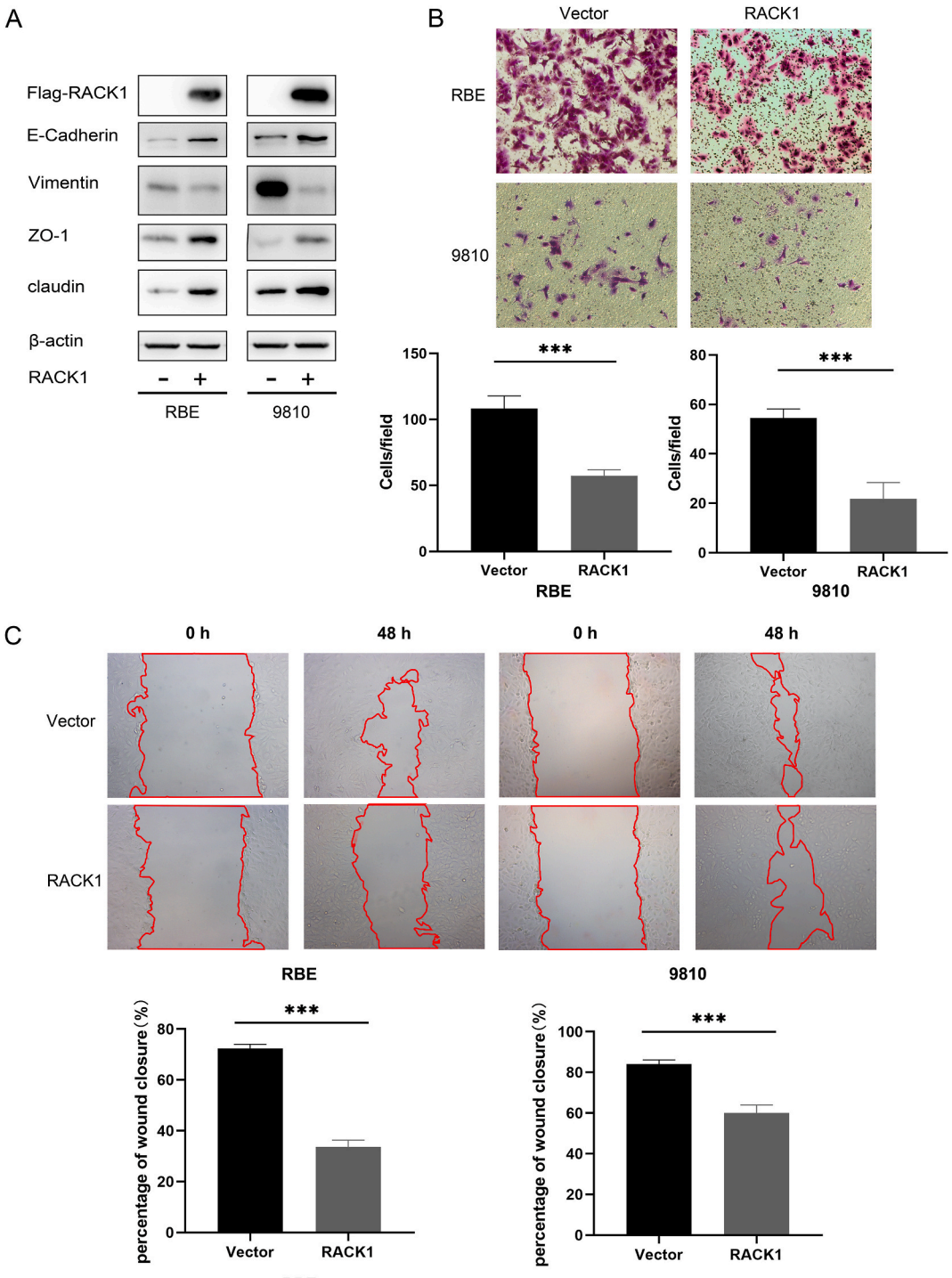

Fig. 5. The potential underlying mechanism for the biological effect of overexpression of RACK1 on cholangiocarcinoma (A) The overexpression of RACK1 expression induced the upregulation of E-cadherin, ZO-1, and claudin and the downregulation of vimentin in RBE and HCCC-9810 cell lines. (B) Representative view and statistics of RBE and HCCC-9810 cell lines migrating from upper Transwell chambers into lower ones. The overexpression of RACK1 expression inhibited the migration ability of RBE and HCCC-9810 cell lines. (C) Representative view and statistics of RBE and HCCC-9810 cell lines migration on a wound healing assay. The overexpression of RACK1 expression inhibited the migration ability of RBE and HCCC-9810 cell lines.

Abbreviations and Symbols

RACK1	Ribosomal receptor for activated C-kinase 1
EMT	epithelial–mesenchymal transition
OS	overall survival
2-HG	2-hydroxyglutarate
FGFR2	fibroblast growth factor receptor 2
IDH	isocitrate dehydrogenase
TCGA	The Cancer Genome Atlas
GEO	Gene Expression Omnibus
GDC	the genomic data commons
FPKM	fragments per kilobase per million
TPM	transcripts per kilobase million
RPKM	reads per kilobase million
IHC	immunohistochemical
HR	hazard ratio
CI	confidence interval
CXCL11	chemokine ligand C-X-C motif chemokine ligand 11
ZO-1	zonula occludens protein-1
UBE2T	ubiquitin-conjugating enzyme E2T
SMURF2	Smad ubiquitin regulatory factor 2

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27366.

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