Upregulated RACK1 attenuates gastric cancer cell growth and epithelial–mesenchymal transition via suppressing Wnt/β-catenin signaling

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Purpose: As there have been few studies on the effects of the receptor for activated C kinase 1 (RACK1) on gastric cancer (GC), we aimed to explore such effects and the mechanism that may be involved.

Patients and methods: Normal gastric epithelial cells and six GC cell lines were used to detect the mRNA expression of RACK1. Overexpressing RACK1 was transfected in HGC27 and MGC803 cells. The effects of overexpressing RACK1 on cell viability, migration, and invasion were determined by cell counting kit-8, wound scratch, and Transwell assay, respectively. The expressions of epithelial–mesenchymal transition (EMT) and Wnt/β-catenin signaling related genes were detected using quantitative real-time PCR or Western blot. Wnt pathway agonist LiCl was added into RACK1 overexpressing GC cells, and then cell viability, migration, and invasion were also detected.

Results: RACK1 was downregulated in GC cell lines. Under the circumstance that overexpressing RACK1 was successfully transfected in the two lowest RACK1-expressing GC cells, significant inhibition of cell viability, migration, and invasion, promotion to the mRNA and protein expression of E-cadherin, as well as a decrease in the N-cadherin and Snail expressions could be observed. Overexpressing RACK1 also enhanced the protein level of phosphorylation-β-catenin/β-catenin and attenuated c-Jun protein expression. Additionally, LiCl could partially reverse the inhibitory effects of cell viability, migration and invasion by overexpressing RACK.

Conclusion: We found RACK1 possibly inhibited epithelial–mesenchymal transition of GC cells through limitation of the Wnt/β-catenin pathway, thereby suppressing cell migration and invasion; RACK1 could also suppress cell growth.

Keywords: RACK1, gastric cancer, LiCl, Wnt/β-catenin signaling, epithelial-mesenchymal transition

Introduction

Gastric cancer (GC) is a heterogeneous, multifactorial disease. It poses a serious threat to the physical and mental health of human beings and brings heavy social and economic burden in both developed and developing countries. According to the GLOBOCAN series of the International Agency for Research on Cancer report, in 2012 there were about 951,000 new cases of GC and 723,000 deaths in the world, making it the fifth in morbidity and third in mortality of malignant tumor worldwide. Among them, more than 70% of GC cases occurred in developing countries, half of the world total cases occurred in East Asia, and the mortality rate of GC in East Asia was also the highest in the world. China was the main high incidence and high
mortality area of GC. According to statistics of cancer in China in 2015, the number of new cases of GC was about 679,100, and the death toll was about 489,000. Studies have shown that environmental and genetic factors play a key role in the development of GC, with highly relevant environmental factors such as smoking, Helicobacter pylori infection, high salt and smoked food intake, and non-steroidal anti-inflammatory drug abuse. However, the incomplete early screening and the limitation of current cancer treatment are the main reasons for the high mortality rate of GC. At present, the main treatment of GC is radical surgery combined with radiotherapy and chemotherapy. More than half of patients with GC undergoing radical resection had a local recurrence or distant metastasis or were diagnosed when the tumor spread, and the median survival time was rarely more than 12 months. In the case of metastatic GC, the 5-year survival rate was lower than 10%, and in GC without metastasis, the 5-year survival rate was only about 20%. Recent studies have found that early diagnosis combined with advanced surgical treatment can effectively improve the prognosis and survival rate of patients with GC, and early diagnosis is the most critical factor in treatment. Receptor for activated C kinase 1 (RACK1), as a scaffold protein interacting with protein kinase C (PKC), was first discovered as a receptor of PKCβ II. RACK1 can bind a variety of kinases and receptors through different WD40 sites and participate in many signal transduction pathways, including PKC signaling, cAMP/PKA signaling, MAPK signaling and Src signaling, and play an important role in immune response, cell growth, migration, and differentiation. In addition, studies have shown that RACK1 plays a role in regulating cell proliferation, apoptosis, and migration in many cancers.

To the best of our knowledge, the main mechanisms of RACK1 in GC are involved in nuclear factor-κB pathway and Wnt/β-catenin pathway. In the present study, we aimed to explore the effects of RACK1 on the biological function of GC cells and verify whether the effects of RACK1 on GC was through the Wnt/β-catenin pathway using Wnt pathway agonist LiCl.

Materials and methods

Cell culture

Normal gastric epithelial cells (GES-1 cells) and GC cell lines (SGC7901, BGC823, MKN45, AGS, HGC27, MGC803 cells) were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). GES-1 and MKN45 cells were cultured in DMEM (Beijing, Solarbio, China), and others were cultured in RPMI1640 medium (Solarbio, Beijing, China). All of them were incubated with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin and 50 µg/mL streptomycin (Gibco, Thermo Fisher Scientific) at 37°C with 5% CO₂.

HGC27 and MGC803 cells were selected to establish a LiCl-disposed cell model. Twenty mM LiCl was added into the medium after cell adherence and then cells were continuously cultured. The medium was changed every 1 d with 20 mM LiCl.

Cell transfection

HGC27 and MGC803 cells were seeded in 6-well plates (1.0×10⁵) for 24 h before transfection. Overexpressing RACK1 and negative control (NC) plasmids were synthesized by Invitrogen (Thermo Fisher Scientific). Lipofectamine 2000 (Invitrogen) was performed to determine transient transfection according to the manufacturer’s protocol. A total of overexpressing RACK1 or NC and lipofectamine 2000 were respectively added to Opti-MEM medium. Lipofectamine/overexpressing RNA mixtures were cultured at 20°C for 10 min and then added into Opti-MEM RPMI1640 medium. After 6 h culturing, the fluid was changed back to RPMI1640 medium containing 10% FBS.

Cell counting kit-8 (CCK-8)

Cell viability of GC cells after treatment or transfection was performed by CCK-8 assay. Cells were plated into 96-well plates at a seeding density of 1×10⁴ cells per well for 24 h. After the cells had been transfected for 12, 24, 48 h, 10 µL/well CCK-8 solution was added to each well and incubated for another 3 h at 37°C. OD was recorded at 450 nm by a microplate reader (Thermo Fisher Scientific).

Cell wound scratch assay

Cells were seeded in 12-well plates at 37°C incubation for 24 h. A wound was drawn using the sterile 10 ul pipette tip in the center of the plate, PBS was used to gently wash the cells 3 times, and then the serum-free medium was then added. Cell migration was observed by inverted microscope at 0 and 24 h. Scratch area was measured using Image J software.
Cell invasion assay
After transfection treatment, cells were resuspended in serum-free medium and 3×10⁴ cells/well were added into the upper chamber coated with matrigel. RPMI1640 medium containing 10% FBS was added in the lower 24-well chamber, and the cells were incubated for 24 h at 37°C with 5% CO₂. The cells were fixed with 4% formaldehyde for 20 min at 25°C and stained with 1% crystal violet for another 15 min. Invasion cells were counted at 200× magnification.

Quantitative real-time polymerase chain reaction (qRT-PCR)
RNA was isolated from cultured cells by use of Trizol reagent (Invitrogen) according to the manufacturer’s protocol. qRT-PCR was performed to detect RACK1, E-cadherin, N-cadherin, and Snail in different groups. Reverse transcription was performed by OmixScript TM RT reagent kit (Takara, Otsu, Japan) following the manufacturer’s instructions at 42°C for 60 min and 70°C for 15 min. qRT-PCR was performed by SYBR Fast qPCR Mix (Invitrogen) and primer sequences are listed in Table 1. RACK1 ran at 94°C for 7 min, 94°C for 1 min, 55°C for 45 s followed by 28 cycles of 72°C for 2 min and 72°C for 10 min; other samples ran with the following cycling parameters: 95°C for 2 min, 95°C for 15 s, 60°C for 1 min followed by 40 cycles of 72°C for 30 s and 72°C for 10 min. The above primers were synthesized by Sangon Biotech (Shanghai, China). Amplified products were electrophoresed through 2% agarose gels. The amount of RNA was calculated using the 2⁻ΔΔCT method.

Western blotting analysis
Proteins were collected from cultured cells in Radio Immuno Precipitation Assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The concentrations of proteins were detected via BCA protein kit (Beyotime). Aliquots of protein were separated by 12% SDS-PAGE and resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% milk in PBS with 0.1% Triton X-100 and incubated with different primary antibodies: anti-RACK1 antibody (ab62735, 1:1,000, 30 kDa, Abcam, Cambridge, MA, USA), anti-E-cadherin antibody (ab15148, 1:500, 135 kDa, Abcam, USA), anti-N-cadherin antibody (ab18203, 1:1,000, 125 kDa, Abcam), anti-Snail antibody (ab82846, 1:500, 68 kDa, Abcam), anti-phosphorylation (p)-β-catenin antibody (ab27798, 1:500, 86 kDa, Abcam), anti-β-catenin antibody (ab32572, 1:5,000, 92 kDa, Abcam), anti-c-Jun antibody (ab32137, 1:1,000, 43 kDa, Abcam) and anti-GAPDH (ab181602, 1:10,000, 36 kDa, Abcam, USA) overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Proteintech, Rosemont, IL, USA). Protein bands were detected with enhanced chemiluminescence (ECL, Thermo Fisher Scientific) and visualized by Quantity one (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis
Statistical analysis was performed by Prism Graphpad version 6.0 software. All data were presented as mean ± SD. Differences were performed using one-way ANOVA followed by Turkey’s multiple comparison. A P <0.05 was considered as significant.

Table 1 Primers used in qRT-PCR

| Gene       | Primer | Sequence             |
|------------|--------|----------------------|
| RACK1      | Forward| 5’-GGGGTCACTCCCACTTGTGTT-3’ |
|            | Reverse| 5’-AATCTGGCGGTTTGGTCAGAGG-3’ |
| E-cadherin | Forward| 5’-GCTGGACCGCAGAGTTTCC-3’ |
|            | Reverse| 5’-TCAAAATCCAAGCCGGTGT-3’ |
| N-cadherin | Forward| 5’-ATGGGAAATGAAAATCTTGAGGGC-3’ |
|            | Reverse| 5’-TGAAAAGCATTCTACACGGGAT-3’ |
| Snail      | Forward| 5’-TCTAGGGGCTGCGCTGCTGACAA-3’ |
|            | Reverse| 5’-ACATCTAGTGCGTGGTGGATTGCT-3’ |
| GAPDH      | Forward| 5’-AATCCCACATCACCACACCAGAC-3’ |
|            | Reverse| 5’-CTTCTCCATGTTGGTAAGAC-3’ |

Results
Expression of RACK1 in gastric normal cells and cancer cell lines
In order to explore the expression of RACK1 in GC, we detected mRNA level of RACK1 in gastric normal cells (GES-1 cells) and six cancer cell lines (SGC7901, BGC823, MKN45, AGS, HGC27, and MGC803 cell lines). As Figure 1 shows, the mRNA expression of RACK1 was significantly downregulated in the GC cell lines (BGC823 and MKN45, P<0.05; others, P<0.01) compared with GES-1 cells. HGC27 and MGC803 cell lines were selected to conduct the following experiments as RACK1 was expressed at a lower level in the two cell lines.
Upregulated expression of RACK1 inhibits cell viability, migration, and invasion in HGC27 and MGC803 cells

When overexpressing RACK1 was transfected into HGC27 (Figure 2A and B) and MGC803 cells (Figure 2D and E), mRNA and protein levels of RACK1 were determined to detect transfection efficiency of overexpressing RACK1. The results showed that both in HGC27 and MGC803 cells, the mRNA and protein levels of RACK1 were noticeably high expressed in RACK1 group in comparison to control or NC (P<0.01, Figure 2A, B and D, E). Compared to control or NC, overexpressing RACK1 could decrease the cell viability at 12, 24 and 48 h in the two cell lines. However, in HGC27 cells, the significantly decreased cell viability was mainly presented at 12 (P<0.05) and 48 h (P<0.01) (Figure 2C). In MGC803 cells, the remarkably decreased cell viability was mainly presented at 24 h (P<0.05) and 48 h (P<0.01) (Figure 2F). In regard to cell migration and invasion, wound scratch (Figure 3A and E) and Transwell assay (Figure 3C and G) were performed. In HGC27 cells, overexpressing RACK1 significantly decreased cell migration and invasion compared with control or NC (migration, P<0.01, Figure 3B; invasion, P<0.05, Figure 3D). In MGC803 cells, a similar tendency of an inhibitory effect of overexpressing RACK1 on migration and invasion was observed as in HGC27 cells (P<0.01, Figure 3F and H).

Figure 1 Downregulation of RACK1 in GC cell lines. qRT-PCR was performed to detect the mRNA expression of RACK1 in gastric normal cells (GES-1 cells) and cancer cell lines (SGC7901, BGC823, MKN45, AGS, HGC27, and MGC803 cells). Data were shown as mean ± SD in three independent experiments.

Note: Compared with gastric normal cells, *P<0.05, **P<0.01.

Abbreviations: GC, gastric cancer; qRT-PCR, quantitative real-time polymerase chain reaction.

Figure 2 The inhibitory effects of overexpressing RACK1 on cell viability in HGC27 and MGC803 cells. qRT-PCR (A) and Western blot (B) were used to detect the transfection efficiency of overexpressing RACK1 in HGC27 cells. (C) The effect of overexpressing RACK1 on cell viability at 12, 24, and 48 h was detected by CCK-8 assay in HGC27 cells. qRT-PCR (D) and Western blot (E) were used to detect the transfection efficiency of overexpressing RACK1 in MGC803 cells. (F) The effect of overexpressing RACK1 on cell viability at 12, 24, and 48 h was detected by CCK-8 assay in MGC803 cells. Expression of each protein in cells was following normalization with a loading control GAPDH. Data are shown as mean ± SD in three independent experiments.

Notes: Compared with control, *P<0.05, **P<0.01; compared with NC, ^P<0.05, ^^P<0.01.

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction; CCK-8, cell counting kit-8; NC, negative control.
Figure 3 The inhibitory effects of overexpressing RACK1 on cell migration and invasion in HGC27 and MGC803 cells. (A) Wound scratch assay was performed to detect the effect of overexpressing RACK1 on HGC27 cell migration at 0, 24 h. (B) Wound closure is shown as bar diagrams in HGC27 cells. (C) Transwell assay was used to detect the effect of overexpressing RACK1 on HGC27 cell invasion. (D) Invasion capability is shown as bar diagrams in HGC27 cells. (E) Wound scratch assay was performed to detect the effect of overexpressing RACK1 on MGC803 cell migration at 0, 24 h. (F) Wound closure is shown as bar diagrams in MGC803 cells. (G) Transwell assay was used to detect the effect of overexpressing RACK1 on MGC803 cell invasion. (H) Invasion capability is shown as bar diagrams in MGC803 cells. Data are shown as mean ± SD from three independent experiments.

Notes: Compared with control, *P<0.05, **P<0.01; compared with NC, "P<0.05, ""P<0.01.

Abbreviation: NC, negative control.
Upregulated expression of RACK1 regulates the expressions of epithelial–mesenchymal transition (EMT) related genes in HGC27 and MGC803 cells

The above experiments confirmed that upregulation of RACK1 could inhibit cell migration and invasion, so we further detected the expressions of EMT related genes. The results showed that there were no obvious differences in the expressions of EMT related genes in control and NC groups. Overexpressing RACK1 could noticeably increase the mRNA and protein levels of E-cadherin in HGC27 cells (P<0.01, Figure 4A, D and E). Meanwhile, both mRNA and protein expressions of N-cadherin and Snail showed a rapid reduction (P<0.01, Figure 4B–E) in RACK1 overexpressing HGC27 cells. In MGC803 cells, overexpressing RACK1 significantly increased the expression of E-cadherin and suppressed the expressions of N-cadherin and Snail in both mRNA and protein levels (P<0.01, Figure 4F–J).

Discussion

Increasing research is carried out on RACK1, especially in relation to cancer.33 Studies have shown that RACK1 can promote tumor invasion and metastasis and multiple cellular functions by PKC.34 At the same time, RACK1 binds to PKC and performs corresponding translation and regulation of ribosomes, thereby activating factors related to biological functions such as tumor invasion and metastasis.34 The abnormal expression of RACK1 is closely associated with the occurrence and development of tumor, and increased expression of RACK1 occurred in most tumors such as breast cancer, non-small cell lung cancer, liver cancer, and melanoma, indicating that it acts as an oncogene.35–38 In the present study, the finding showed that RACK1 was downregulated in several GC cell lines compared with normal gastric epithelial cells. The result was in accordance with Deng's report that RACK1 was a tumor suppressor gene of GC.39 Furthermore, it was expressed at a low level in GC and was associated with tumor invasion and low differentiation.39 Although Liu reported that RACK1 proteins were expressed at a low level in GC patients,40 and Chen reported that low expression of RACK1 predicted poor prognosis for overall five-year survival in all GC cases,29 our study lacked the information about RACK1 prognosis from TCGA database. We are planning to launch this analysis in a future study.

In addition, overexpressing RACK1 was successfully transfected into HGC27 and MGC803 cells, and showed that LiCl could significantly induce cell migration and invasion in comparison to RACK1 (P<0.01, Figure 6B and C). In the case of RACK1 combined with LiCl, a significant inhibitory tendency was observed in cell migration compared to that under LiCl treatment alone (P<0.01), while no noticeable reduction of invasion was observed compared to that under LiCl treatment alone (P>0.05). In MGC803 cells, compared to the RACK1 group, LiCl could also remarkably increase cell migration and invasion (P<0.01, Figure 6F and G). Overexpressing RACK1 could significantly decrease cell migration induced by LiCl (P<0.01). In the case of RACK1 combined with LiCl, a slight reduction of cell invasion was observed in comparison to that under LiCl treatment alone (P>0.05). All the above results indicated that Wnt/β-catenin signaling was the possible underlying mechanism of the inhibitory effects of overexpression RACK1 on cell growth and metastasis in GC cells.
Figure 4 The effects of overexpressing RACK1 on EMT-related genes. qRT-PCR was used to detect the overexpressing RACK1 on mRNA expressions of E-cadherin (A), N-cadherin (B) and Snail (C) in HGC27 cells. (D) Western blot was used to assess the overexpressing RACK1 on protein expressions of E-cadherin, N-cadherin, and Snail in HGC27 cells. (E) The three proteins were quantitative as bar diagrams in HGC27 cells. qRT-PCR was used to detect the overexpressing RACK1 on mRNA expressions of E-cadherin (F), N-cadherin (G) and Snail (H) in MGC803 cells. (I) Western blot was used to assess the overexpressing RACK1 on protein expressions of E-cadherin, N-cadherin, and Snail in MGC803 cells. (J) The three proteins were quantitative as bar diagrams in MGC803 cells. Expression of each protein in cells was following normalization with a loading control GAPDH. Data are shown as mean ± SD in three independent experiments.

Notes: Compared with control, *P<0.05, **P<0.01; ^ compared with NC, ^P<0.05, ^^P<0.01.

Abbreviations: EMT, epithelial–mesenchymal transition; qRT-PCR, quantitative real-time polymerase chain reaction; NC, negative control.
significant inhibitory effects on cell viability, migration, and invasion. We also detected the effects of overexpressing RACK on EMT genes in GC. To the best of our knowledge, EMT is a critical and necessary process in the early stage of tumor metastasis. In this process, tumor cells lose epithelial cell characteristics, including E-cadherin expression, intercellular adhesion, and epithelial cell polarity; and acquire mesenchymal cell characteristics, including migration and invasion. Our results found that overexpressing RACK1 regulated markers of EMT. Specifically, it inhibited the expression of N-cadherin and promoted E-cadherin expression. Meanwhile, overexpressing RACK1 suppressed Snail expression, which could induce the occurrence of EMT and downregulate E-cadherin expression in the progression of cancer.

In regard to the underlying mechanism of RACK1 on the above biological function, we explored the Wnt signaling. There is a study reported that RACK1 contributed to the occurrence of GC through activation of the Wnt pathway by increasing dissociation of the β-catenin complex. In our work, overexpression RACK1 could significantly increase the protein expression of p-β-catenin/β-catenin and decrease the protein expression of c-Jun. β-catenin is a key step in the Wnt pathway. The abnormal activation of Wnt/β-catenin signaling pathway leads to a significant increase in the accumulation of β-catenin in the nucleus, triggering excessive proliferation, and malignant transformation of cells. C-Jun is a member of the Jun gene family and is an oncogene. Takeda reported that in the activated Wnt pathway, β-catenin/TCF can bind to the c-Jun promoter and induce c-Jun transcription, which plays an important role in the development and progression of colorectal cancer. Therefore, we inferred the effect of overexpressing RACK1 on GC cells through inhibition of Wnt/β-catenin signaling.
Figure 6  The verification experiments of Wnt/β-catenin in GC cells. (A) The suppression effects of overexpressing RACK1 on increasing cell viability induced by LiCl in HGC27 cells. (B) The suppression effects of overexpressing RACK1 on increasing cell migration induced by LiCl in HGC27 cells. (C) The suppression effects of overexpressing RACK1 on increasing cell invasion induced by LiCl in HGC27 cells. (D) Cell migration and invasion in HGC27 cells were respectively performed by wound scratch and Transwell assay. (E) The suppression effects of overexpressing RACK1 on increasing cell viability induced by LiCl in MGC803 cells. (F) The suppression effects of overexpressing RACK1 on increasing cell migration induced by LiCl in MGC803 cells. (G) The suppression effects of overexpressing RACK1 on increasing cell invasion induced by LiCl in MGC803 cells. (H) Cell migration and invasion in MGC803 cells were respectively performed by wound scratch and Transwell assay. Data are shown as mean ± SD in three independent experiments.

Notes: Compared with control, *P<0.05, **P<0.01; compared with NC, *P<0.05, **P<0.01; compared with RACK1, *P<0.05, **P<0.01; compared with LiCl, *P<0.05, **P<0.01.

Abbreviations: GC, gastric cancer; NC, negative control.
overexpressing RACK1 on cell viability, migration, and invasion. RACK1 may inhibit the proliferation, migration, and invasion of GC cells by blocking the Wnt/β-catenin signaling pathway. There may be other pathways at the same time, and more in-depth research is needed.

In this study, there are still many limitations, for instance, a lack of research on cell mortality, experiments regarding the relation between cell migration/invasion and cell viability, as well as JNK activator, to validate the involved mechanism of Wnt/β-catenin signaling. With regard to this, we are planning to launch a comprehensive, confirmative and in-depth study to make up the limitations.

Conclusion
In this study, we found that RACK1 possibly inhibited EMT of GC cells through limitation of the Wnt/β-catenin pathway, thereby suppressing cell migration and invasion; and also inhibited cell growth. This study provides a theoretical basis for determining the anti-cancer effects of RACK1 and its application in the clinical study and targeted therapy of GC.

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Disclosure
The authors report no conflicts of interest in this work.

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