Original Article

RACK1 as a potential prognostic biomarker and regulator of epithelial-mesenchymal transition in non-small cell lung cancer

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Abstract: Background: The prognostic value of the receptor for activated C kinase 1 (RACK1) in different tumors has been identified. However, few studies have assessed the prognostic significance of RACK1 in non-small cell lung cancer (NSCLC). Methods: RACK1 levels in normal lung and NSCLC tissues were determined by immunohistochemical staining. The correlation between RACK1 levels and clinicopathological variables was analyzed. The prognostic value of RACK1 for recurrent-free survival (RFS) and overall survival (OS) was analyzed using the Kaplan-Meier survival method and multivariable Cox regression. RACK1 was knocked-down in NSCLC cell lines by siRNA, and its biological function was examined by the wound healing assay and transwell assay. The levels of the epithelial-to-mesenchymal transition (EMT) markers were detected by western blot. Results: RACK1 was overexpressed in NSCLC tissues compared to those in non-neoplastic tissues, and high levels of RACK1 correlated with the pT classification and pN classification (P < 0.05). RACK1 was an independent prognostic risk factor for RFS (HR: 1.83; 95% CI: 1.17-4.30) and OS (HR: 1.76; 95% CI: 1.23-4.17). In vitro experiments showed that RACK1 promoted cell migration and invasion via EMT in NSCLC cells. Conclusion: RACK1 associates significantly with poor prognosis in NSCLC, promotes tumor progression, and is involved in EMT of NSCLC.

Keywords: RACK1, non-small cell lung cancer, prognosis, epithelial-to-mesenchymal transition

Introduction

Lung cancer is the most frequent cause of cancer-related deaths throughout the world [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 80-85% of all cases of lung cancer. Despite advances in NSCLC therapy, the prognosis for patients with advanced NSCLC remains poor [2]. Studies on the molecular biology of human cancers suggested that several molecules associated with angiogenesis or metastasis produced more aggressive malignant tumors during subsequent tumor progression [3, 4]. Considering these facts, it is essential and necessary to understand the pathogenic factors involved in NSCLC progression and identify biomarkers related to prognosis.

The receptor for activated C kinase 1 (RACK1), was first reported as a binding partner of protein kinase C (PKC) in 1991 [5]. RACK1 is likely to be essential for cellular functions and is expressed in a variety of tissues such as the ovary, lung, and liver [6]. Recent studies focused on the clinical significance of RACK1 in malignant tumors, and an increasing number of reports have demonstrated that RACK1 functions as an independent prognostic factor for patients with oral squamous cell carcinoma [7], esophageal cancer [8], and breast cancer [9]. However, there is limited information regarding the predictive significance of RACK1 in NSCLC.

The epithelial-mesenchymal transition (EMT) is a critical physiological process, which is often deregulated during cancer metastasis [10, 11]. Activation of EMT is associated with dissolution of intercellular junctions through the internalization and down-regulation of epithelial markers such as zonula occludens (ZO)-1 and E-cadherin, and up-regulation of mesenchymal markers, including N-cadherin and vimentin [12, 13].
It is one of the most important processes via which tumor cells acquire invasive and migratory abilities. EMT is a reversible process that often occurs at the invasive front of many metastatic cancers [14]. Recently, Wang et al. showed that RACK1 regulated the expression of EMT-related genes in esophageal squamous cell carcinoma cells and promoted cell invasion and migration [8]. However, further investigation is required for determining the correlation of RACK1 levels with the levels of EMT molecular markers in lung cancer specimens and understanding its prognostic significance.

In the present study, we sought to determine the role of RACK1 in NSCLC prognosis and further explore whether RACK1 contributed to the malignant progression of NSCLC via EMT.

Materials and methods

Patients and their follow-up treatments

The study was approved by the ethics committee of the Chinese People’s Liberation Army (PLA) General Hospital. Ninety-two patients, who underwent thoracic surgical procedures at the Nanlou Respiratory Diseases Department from February 2010 to November 2014, were included in the study. Only patients with NSCLC who did not have metastasis and were suitable for surgery were included in present study. In addition, 17 non-neoplastic samples from individuals who underwent surgical resection in the hospital from 2010 to 2014 were used as control. All the specimens were obtained before chemotherapy or radiotherapy, immediately frozen in liquid nitrogen, and stored at -80°C till further analysis. Samples were analyzed with the consent of the patients who signed the informed consent form. The cohort included 52 male and 40 female patients with a mean age of 57.3 years. The clinical characteristics were obtained from the medical records of the patients. The medical conditions of the patients were followed till April 2016. All the patients were assessed for overall survival from the time of surgery.

Figure 1. RACK1 levels in non-small cell lung cancer (NSCLC) tissues. A: Representative immunohistochemical staining of RACK1. a, b: Low levels of RACK1 in NSCLC tissues; c, d: High levels of RACK1 in NSCLC tissues. B: The immunohistochemistry results showed that RACK1 was overexpressed in lung cancer tissues compared to that of non-tumor tissues (P < 0.05).
Recurrent-free survival (RFS) was defined as tumor recurrence at the site of initial resection, ipsilateral hilar or mediastinal nodes.

**Immunohistochemical analysis**

Sections of formalin-fixed, paraffin-embedded NSCLC tissue samples were cut into 4 μm thick slices, deparaffinized, heated in citrate buffer, treated with 0.3% hydrogen peroxide, and rehydrated. After blocking with normal goat serum, the sections were incubated with anti-RACK1 polyclonal antibody (LifeSpan BioSciences, Inc., Seattle, USA) overnight at 4°C. Then, the sections were incubated with biotinylated secondary antibodies and rinsed in phosphate-buffered saline. Subsequently, the sections were incubated with streptavidin-horse radish peroxidase (HRP) for 30 min at room temperature, visualized by reaction with 3,3’ dianinobenzidine (DAB), and counterstained with hematoxylin. Finally, they were dehydrated, transparented, covered with coverslips, and mounted for microscopic examination. The staining intensity of RACK1 was scored using a four-point grade scale described below: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining of cancer cells. In the current study, 0-1 was considered as low levels of RACK1 and 2-3 was considered as high levels of RACK1, respectively.

**Cell culture**

The normal human bronchial epithelial cell line 16HBE, and human small cell lung cancer cell lines A549, NCI-H1703, NCI-H1299, and NCI-H520 were cultured in Roswell Park Memorial Institute (RPMI 1640) media (Gibco, Invitrogen Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone Inc., USA) and 100 μg/mL penicillin and streptomycin.

**Real-time (quantitative) polymerase chain reaction (qPCR)**

Total RNA from the cultured cells was extracted using the Trizol reagent (Takara, China) and reversed transcribed. qPCR analysis was performed using the ABI 7300 system (Applied Biosystems, CA, USA). Each well contained 10 μL Power SYBR Green PCR master mix (Applied Biosystems), 1 μL each primer, and 1 μL template. GAPDH was used as an endogenous control for data normalization. Sequences of RACK1 primers used are: Forward, 5'-CTACCTGGCCCGTCC-3'; Reverse, 5'-AGCTCATGTTCCAGGACC-3'. All samples were analyzed in triplicates. The fold change for RACK1 expression was obtained using the 2-ΔΔCt method.

**Vectors and cell transfection**

The siRNAs that was directed against human RACK1 (RACK1-siRNA) and a non-targeting negative control siRNA (NC-siRNA) were synthesized by GenePharma Co. Ltd. (Shanghai, China) and confirmed using enzyme digestion. The sequences of RACK1 siRNAs were as follows: S1 (5'-CUACCAUGAGUAAGATUAT-3')

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**Table 1. Demographic, clinical characteristics and RACK1 expression**

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>RACK1 expressiona</th>
<th>P valueb</th>
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<td>III-IV</td>
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a: RACK1 high group (n = 56) is defined as cases with moderate and strong expression; RACK1 low group (n = 36) is defined as cases with weak or negative expression. b: Pearson χ² test was used to derive P-values.
Prognostic value of RACK1 in NSCLC

Figure 2. Kaplan-Meier analysis of overall survival and recurrent-free survival in lung cancer patients. A: Patients positive for RACK1 showed poorer overall survival. B: Patients positive for RACK1 showed poorer recurrent-free survival. The log-rank test was used to calculate P values.

and S2 (5'-GAUTCCGAAGCAUUAUAUAAT-3'), the NC-siRNA: (5'-UCGUCCGACAUATCCGUUGUT-3'). Cells were plated into the 6-well plates and incubated for 24 h followed by transfection with 100 nmol/L RACK1 siRNA by the LipofectamineTM 2000 (Invitrogen, USA) according to the manufacturer’s instructions. After 48 h transfection, transfected cells were harvested for analysis.

Western blot

Cells were transfected with either NC siRNA or RACK1 siRNA. Total protein was extracted using the RIPA buffer (Beyotime, China) and quantified using the bicinchoninic acid (BCA) protein assay kit. Equal amounts of protein were resolved upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were blocked for 1 h in 5% skim milk dissolved in TBST. Then, the membranes were incubated overnight with anti-RACK1, anti-E-cadherin, anti-ZO1, anti-N-cadherin, anti-vimentin (1:1000 dilution, Abcam), and anti-GAPDH (1:2000 dilution, Abcam) antibodies at 4°C, followed by incubation with appropriate HRP-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, USA) for 1 h at room temperature. The transferred proteins were detected using an enhanced chemiluminescence (ECL) system following the manufacturer’s protocol.

Cell migration and invasion assay

Approximately 10⁶ cells were added into 6-well plates. After transfection, a cell scratch spatula was used to scratch the cell. Plates were washed using PBS three times and the cells were incubated at 37°C for 48 h, examined and photographed under a microscope (Olympus Corp., Tokyo, Japan) 0 h and 48 h after wounding. Each cell condition was assayed in triplicate.

For the invasion assay, cells were plated in the top chamber on the extracellular matrix gel (Sigma, E1270)-coated membranes (8-μm pore size, BD Biosciences, USA). The cells were cultured overnight and transfected with NC siRNA or RACK1 siRNA, and trypsinized after 48 h, followed by culturing in serum-free Dulbecco’s modified Eagle medium (DMEM) at a density of 1 × 10⁴ cells per insert. The cells in the upper chamber were gently removed with a cotton
swab and the inserts were stained with crystal violet. The images were photographed and assessed at × 200 magnification under a microscope (Olympus BX53, Japan). Three independent experiments were performed.

**Statistical analysis**

The statistical analyses were performed using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Group differences were assessed by Pearson’s chi-square test or Fisher’s exact test, as appropriate. The survival curves were generated with the Kaplan-Meier method and were compared using the log-rank test. Multivariate analyses were performed using Cox’s proportional hazards regression model. P < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Association of RACK1 levels with clinicopathological variables of NSCLC**

Immunohistochemistry was used to determine the levels of RACK1 in 92 paraffin-embedded tumor slides and 17 non-tumor lung tissue slides. Cytoplasmic and nucleoli staining was observed in the normal lung tissue (Figure 1Aa) and human NSCLC tissue samples (Figure 1Ab-Ad). High levels of RACK1 were detected in 56 (60.86%) cancer tissues and 3 (17.64%) non-tumor lung tissues (Figure 1B). Moreover,
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Statistical analysis demonstrated that RACK1 levels correlated with the demographic and pathological factors of the tumors (Table 1). Notably, there was a statistically significant association between RACK1 and the pT (P = 0.016) and pN classification (P < 0.001), which indicated that deeper tumor invasion (T2-4) and higher extent of lymph node metastasis (N1-3) were characterized by higher RACK1 levels compared to samples without these features.

Overexpression of RACK1 is associated with poor prognosis in NSCLC

The prognostic significance of RACK1 was determined, and the results of the Kaplan-Meier survival analysis showed that the overall survival (OS) of patients with high RACK1 levels was significantly shorter than those of patients with low RACK1 levels (P = 0.034, Figure 2A). Remarkably, patients with higher RACK1 levels showed a worse recurrent-free survival (RFS) rate (P = 0.020, Figure 2B). Furthermore, a Cox proportional hazards model was applied to estimate the effect of RACK1 expression on survival. Among the clinical factors, abundant RACK1 was a strong independent prognostic factor for RFS (HR: 1.83 (95% CI: 1.17-4.30), P < 0.001) and OS (HR: 1.76 (95% CI: 1.23-4.17), P < 0.001). High lymph node metastatic status (N1-3) correlated significantly with shorter RFS (HR: 1.29 (95% CI: 1.04-2.07), P = 0.028) and OS (HR: 1.33 (95% CI: 1.06-1.92), P = 0.017). The details are summarized in Table 2.

RACK1 is critical for the invasiveness and migratory abilities of lung cancer cells

In vitro experiments were designed to investigate the mechanism underlying the involvement of RACK1 in NSCLC progression. The wound healing assay and transwell assay were performed to determine whether RACK1 was involved in the invasion and migration of lung cancer cells. qPCR and western blot analyses showed that RACK1 levels were significantly higher in NSCLC cell lines compared to that in the normal human bronchial epithelial cell line (Figure 3A, 3B) (P < 0.05). RACK1 levels were lower in the RACK1 siRNA-treated group compared to the NC siRNA-treated or control group for A549 and NCI-H1299 cells (P < 0.05, Figure 4A). The results of the wound healing assay and transwell assay demonstrated that cell migratory and invasive abilities were significantly inhibited by knocking down of RACK1 (Figure 4B, 4C).

Role of RACK1 in epithelial-mesenchymal transition in NSCLC

EMT plays an essential role in tumor invasion and metastasis during cancer progression. Consequently, western blot was performed to analyze the alterations in the levels of EMT markers when RACK1-specific siRNA was used to suppress the expression of RACK1 in NSC-
Prognostic value of RACK1 in NSCLC

LC cells. RACK1 knockdown increased the levels of E-cadherin and ZO-1, whereas the levels of N-cadherin and vimentin decreased in the transfected AD549 and NCI-H1299 cells (Figure 5). This suggested that RACK1 may promote metastasis by inducing EMT in NSCLC cell lines.

Discussion

As lung cancer is one of the most aggressive malignancies, and metastasis is the leading cause of lung cancer-associated mortality, it is essential to elucidate the mechanisms of lung cancer metastasis for improving the disease prognosis. RACK1, originally identified by its ability to bind to protein kinase C, is a pivotal scaffold and an adapter protein that regulates cell motility via multiple intracellular signal transduction pathways. As a result, RACK1 has effects on multiple cellular processes, such as cell proliferation, apoptosis, and migration, and its deregulation may contribute to tumorigenesis [16, 17]. In the present study, we used immunohistochemistry to determine the level of RACK1 in NSCLC tissues, and analyzed its correlation with clinical and prognostic character-
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Statistics of the disease. Our data revealed that RACK1 levels correlated with the T-stage of tumors; lymph node metastasis and survival analyses suggested that RACK1 positivity in patients with NSCLC correlated significantly with poor OS and RFS. Furthermore, RACK1 affected cell migration and invasion via EMT in NSCLC cell lines.

In fact, RACK1 has been demonstrated to be involved in a variety of malignant tumors. Peng et al. suggested that RACK1 expression is higher in glioma tissues than that in the normal brain tissues, and correlated positively with glioma malignancy [18]. In gastric cancer, dysregulation of RACK1 modulated the autocrine activity of the proinflammatory cytokine IL-8 to promote cancer progression [19]. In addition, the levels of RACK1 were determined by qPCR, western-blot, and immunohistochemistry in colorectal carcinoma tissues, which demonstrated that RACK1 levels correlated positively with the extent of differentiation and lymph node metastasis in patients with colorectal carcinoma [20]. Another study showed that RACK1 is highly expressed in pancreatic ductal adenocarcinoma, and multivariate analyses showed that high RACK1 levels was an independent prognostic factor for patients survival [21]. Importantly, Zhong et al. detected high levels of RACK1 in pulmonary adenocarcinoma, and RACK1 expression correlated with higher incidence of lymph node metastasis and lower differentiation than RACK1-negative tumors [22]. However, the clinical significance of RACK1 in other types of NSCLC is still unknown. We determined the levels of RACK1 in NSCLC containing adenocarcinoma, squamous cell carcinoma, and others in this study. RACK1 was overexpressed in tumor tissues compared to those in non-tumor tissues. Clinicopathological significance analysis suggested that RACK1 levels correlated with the pT and pN classification. Subsequent survival analysis revealed that abundant RACK1 was a strong independent prognostic factor for RFS (HR: 1.83) and OS (HR: 1.76).

RACK1 is known to promote cancer cell invasion and migration in a variety of cancers. Knocking down of RACK1 markedly reduces the activity of the Src/Akt signaling pathway, indicating that RACK1 is a novel and promising therapeutic target for the treatment of glioma [18]. Similarly, Lin et al. found that down-regulation of RACK1 in epithelial ovarian cancer cells

Figure 5. Effect of RACK1 on the expression of EMT markers in small cell lung cancer cells. Western blot was utilized to assess the changes in the levels of EMT markers in (A) A549 cells and (B) NCI-H1299 cells transfected with RACK1 or NC siRNA. *P < 0.05 compared to the NC siRNA group.
significantly suppressed proliferation, migration, and invasion in vitro, and tumor growth in vivo via suppression of the constitutive phosphorylation of Akt and MAPK [23]. These indicated that RACK1 acts through multiple intracellular signal transduction pathways. Two recent studies indicated that forced down-regulation of RACK1 significantly suppressed the migratory abilities and invasiveness via inhibition of the expression of EMT markers, such as N-Cadherin, E-cadherin, and vimentin, and thus acts as a valuable prognostic biomarker in cancers [8, 24]. Here, we showed that RACK1 was involved in the migration and invasion of NSCLC cells, and altering of RACK1 levels by specific siRNA treatment prevented EMT in both A549 and NCI-H1299 cells. This indicated that RACK1 contributes to the malignant progression of NSCLC through EMT and may be a useful biomarker for prognosis in patients with NSCLC.

However, the present study is not without limitations. We hypothesize a mechanism where RACK1 participates in the regulation of metastasis by affecting the expression of key EMT-related genes, which is well-established in several other studies. In addition, RACK1 may target other genes or pathways, which is currently unknown as we investigated the changes in the levels of only EMT markers in this study. The mechanism by which RACK1 regulates EMT is still unanswered. Future studies would contribute to our understanding of the precise underlying mechanisms through which RACK1 affects the prognosis of NSCLC.

Conclusion

In summary, we suggest that RACK1 is overexpressed in NSCLC tissues and cells. RACK1 could predict poor prognosis and regulate progression of NSCLC via EMT.

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Disclosure of conflict of interest

None.

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