Silencing of Rac1 modifies lung cancer cell migration, invasion and actin cytoskeleton rearrangements and enhances chemosensitivity to antitumor drugs

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Abstract. Rac1, an intracellular signal transducer, regulates a variety of cell functions, including the organization of the cytoskeleton, cell migration, and invasion. Overexpression of Rac1 has been reported in several human cancers. However, the underlying mechanisms are not well understood. In the present study, we evaluated the possibility of Rac1 as an appropriate molecular target for cancer gene therapy. The expression of Rac1 in 150 primary non-small cell lung cancer tissues (NSCLC) and 30 normal paraneoplastic lung tissues was determined by immunohistochemical staining, and the correlation of Rac1 overexpression with clinicopathological factors was evaluated. Overexpression of Rac1 was detected in 94 of 150 lung cancer specimens, the incidence rate being higher than that in normal lung tissue specimens. In addition, overexpression of Rac1 was also associated with poor differentiation, high TNM stage, and lymph node metastasis in NSCLC patients. Moreover, RNAi-mediated suppression of Rac1 expression reduced lamellipodia formation, migration and invasion potential of a lung cancer cell carcinoma cell line, 801D. Down-regulation of Rac1 expression also reduced the expression of Pak1. NSC23766, an inhibitor of Rac1 activity, could also inhibit lung cancer cell migration, invasion and induce rearrangements of the actin cytoskeleton. Furthermore, the suppression of Rac1 expression also sensitized cells to antitumor drugs. These results indicate that the overexpression of Rac1 is tightly associated with an aggressive phenotype of lung cancer cells. Therefore, we proposed that Rac1 could be a potential molecular target of gene therapy by RNAi-targeting in lung cancer cells.

Introduction

Accumulating evidence indicates that small GTPase-dependent cell signaling is important for malignant migration and the motility process (1). The Rho family GTPases belong to the Ras superfamily of small GTPases that serve as molecular switches. Activation of Rho family GTPases regulates a wide range of cellular functions. Rac1 is an important GTPase that has been implicated in many cellular processes, such as cytoskeleton rearrangement, cell adhesion, and transcriptional activation, and is believed to be involved in cancer cell migration, invasion, and metastasis (2,3). A number of investigations indicate that Rac1 plays an important role in malignant transformation. Rac1 overexpression has been found in breast carcinoma, gastric carcinoma, oral squamous cell carcinoma (SCC), non-small cell lung carcinoma, and testicular germ cell tumors. Rac1 overexpression has also been considered as an independent predictor of adverse outcome of these carcinomas (4,5). Consistent with these reports, suppression of Rac1 protein expression and disruption of its function significantly reduced lung metastasis in a mouse colorectal adenocarcinoma model (6). Furthermore, Rac1 silencing by small hairpin RNA could reverse the metastatic behavior of human glioblastoma cells and fibrosarcoma HT1080 cells (7,8). Taken together, the observed effects of Rac1 overexpression and silencing on the cell malignant transformation indicate a role for Rac1 in regulating tumor metastasis and progression.

In addition, Rac1 can interact with various specific effectors to coordinate the activation of a multitude of signaling cascades that influence diverse physiological outcomes. The Pak (p21-activated kinase) serine/threonine kinases have recently been found to be key regulators of cytoskeletal remodeling, cell motility, and cell proliferation, with a role in both carcinogenesis and cellular invasion (9). It has been reported that Pak1, the best
characterized member of this family, shows increased expression and activity in human cancers (9,10). Multiple signaling pathways converge to promote activation of Pak1 through both small GTPases and several of the tyrosine kinases. In turn, activated Pak1 regulates diverse cellular functions. Pak1 binds to Rac1 in a GTP-dependent manner, after which activated Pak1 regulates cellular functions such as cytoskeletal dynamics, cell adhesion, and transcription (9).

However, the mechanisms of Rac1-mediated tumor development still require further elucidation. In the present study, we showed that overexpression of Rac1 was widespread in primary lung cancer patients. Knocking down of Rac1 expression by short hairpin RNA (shRNA) against Rac1 suppressed lung cancer cell migration, invasion and induced rearrangements of the actin cytoskeleton in lung cancer cells. Rac1 suppressed lung cancer cell migration through Pak1-dependent cell signaling pathways. All these results suggested that Rac1 may be an adjuvant gene therapy strategy to chemotherapy.

Materials and methods

Reagents and cell lines. Rac1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated Pak1 and total Pak1 antibodies, MT1, IA3 and TRITC-phalloidin were purchased from Sigma. Human lung cancer cell lines (95D, 801D, PG, A549 and 95C) and human bronchial epithelial cells (BEAS-2B) (a gift from Dr Guohua Lu, Zhejiang University) were cultured in RPMI-1640 medium at 37˚C in 5% CO2. Rac1-shRNA (Rac1 target sequence 5’-GCGATCCGAGCAGTCCTCT-3’) plasmid (pGPU6-Neo-Rac1) and empty plasmid vector (pGPU6/GFP/Neo-control) were obtained from Zhe Jiang Chinese Medical University. Nedisalene was obtained from Huali Jin Gang Pharmaceuticals Ltd., Inc., Dalian, P.R. China. Nedaplatin was obtained from the 117th Hospital of PLA. The diagnosis of lung cancer, histological grading and pathological staging were re-evaluated with respect to histologic subtype, differentiation, and tumor stage, following collection. SCC was identified in 55 of the cases (56 well-differentiated, 64 moderately differentiated, and 30 poorly differentiated). Lymph node metastases were identified in 80 of the 150 patients. The TNM staging system of the International Union Against Cancer (12) was used to classify specimens as stages I (n=58), II (n=57), and III (n=35). Immunohistochemical staining and evaluation were described in previous studies (13).

Cell transfections. The shRNA expression plasmids were transfected into 801D cells using Lipofectamine 2000 (Invitrogen, USA) as described previously (14). Stable GFP-positive clones were obtained by flow sorter. The expanded cells were then used for subsequent studies. Rac1 expression was determined by Western blotting.

Migration and invasion assay. Wound healing experiment and Transwell insert (24-well insert; pore size, Corning, USA) assays were used to explore the effect of Rac1 on the migration and invasion, respectively, of 801D cells as previously described (14). Briefly, for the wound healing experiment, cells were grown to confluence wounded using a pipette tip and photographed at 0 h and subsequent time points. Cell migration was evaluated by measuring the width of the wound at the identical position. For the invasion assay, the lower chambers of matrigel-coated invasion plates were used. Cells (50,000) were added to the upper chamber in serum-free medium and invasion at 37˚C towards 10% FBS-containing growth media was determined after 24 h. Cells that invaded through the membrane were fixed, stained with crystal violet stain and counted with light microscopy. All experiments were carried out in triplicate.

Cell proliferation analysis. To analyze the effect of Rac1 on 801D cell proliferation activity, the MTT assay was performed. Briefly, cells were plated into 96-well plates at 5,000 cells/well, cultured in 10% FBS RPMI-1640 medium at 37˚C for various durations and proliferation activity was measured using a colorimetric assay.

Western blot analysis. Cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride) as previously described (15). Briefly, the lysates were resolved on 10% SDS-PAGE gels, then transferred to PVDF membranes, blocked with 5% non-fat milk, probed with primary and corresponding secondary antibodies and detected by an ECL reagent.

Drug-sensitivity assay. To assess the chemosensitivity to anti-tumor drugs, the cells were seeded in triplicates on 96-well plates at 5,000 cells/well and incubated for 24 h. The medium was then removed and replaced with fresh medium containing varying concentrations of drugs. After 24 h, cells were treated...
with MTT as described above. The inhibition ratio was calculated. The assay was repeated three times.

Statistical analysis. SPSS version 11.5 for Windows was used for all analyses. The χ²-test was used to examine possible correlations between the expression profiles of Rac1, and their all-abnormal expression, as well as for the comparison with clinicopathological factors. The data were expressed as mean ± SD and one-Way ANOVA test was used to determine the significance of the multiple comparisons. The Kaplan-Meier method was used to estimate the probability of patient survival, and the log-rank test was used to evaluate differences in survival between patient subgroups. Differences were considered significant when P<0.05.

Results

Immunohistochemical analysis of Rac1 expression in lung cancer samples. To assess the role of Rac1 in lung cancer, we analyzed its expression pattern in 150 lung cancer cases and 30 normal paraneoplastic lung tissues (>1.5 cm away from the tumor). Among these 30 cases of normal lung tissues, weak to no expression was observed (Fig. 1A); However, 94/150 (62.67%) lung cancer samples were found to be Rac1-positive, (Fig. 1B-E), suggesting that Rac1 was overexpressed in human lung cancer. Rac1 high expression was significantly associated

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Figures

Figure 1. Expression of Rac1 in lung cancer tissues and its relation to overall survival. (A) Negative immunostaining for Rac1 in normal lung tissues. In contrast, Rac1 showed strong cytoplasmic staining in (B) lung adenocarcinoma and (C) lung squamous cell carcinoma (SCC), weak cytoplasmic staining in (D) lung adenocarcinoma and (E) lung SCC. (F) Kaplan-Meier survival curves were constructed, and the difference between the Rac1-positive and Rac1-negative groups was analyzed by log-rank test. Magnification, x200.
with lymph nodal metastasis, TNM staging and differentiation, but not correlated with other factors (such as age, gender, tumor size and histological type) (Table 1). Additionally, Kaplan-Meier analysis revealed that the 5-year survival rate of patients with Rac1-negative expression was 32.14%, which was significantly higher than that of patients with Rac1-positive expression (17.02%; \( \chi^2 = 12.85, P<0.005 \)) (Fig. 1F). These results showed that Rac1 expression levels could serve as a prognosis and metastasis marker in lung cancer patients.

Expression of Rac1 in human lung cancer cell lines with different metastasis potentials. To clarify the functional relationship between Rac1 expression and the ability of tumor metastasis in human lung cancer cell lines PG, 801D, 95D, A549 and 95C, Rac1 protein levels were detected by Western blot analysis in these cell lines (Fig. 2A). Of the five human

Figure 2. Expression of Rac1 in various lung cancer cells. Five human lung cancer cell lines (PG, 801D, 95D, A549 and 95C) were selected, Rac1 protein expression was detected by Western blotting (A). Deletion of Rac1 inhibits the expression of Rac1 in 801D cells (B).

Figure 3. Transwell insert and wound healing assay showing that Rac1 regulates cell migration and invasion in vitro. After initial equilibrium, 801D cells were suspended in fresh medium without fetal bovine serum were added to the insert. (A) The migratory cell number of 801D transfected with Rac1 was significantly less than that of 801D cells transfected with negative control or un-transfected. (B) Confluent cell monolayers were wounded with a pipette tip. Wound closure was monitored by microscopy at the indicated times. (C) For the invasion assay, the inserts were coated with ECM and then repeated as for the migration assay. The invasive cell number of 801D transfected with Rac1 was also significantly lower than that of 801D cells transfected with negative control. Effects of NSC23766 treatment on the invasion of lung cancer cells is very similar to the effects of Rac1-shRNA. *P<0.05 compared with control.
lung cancer cell lines, Rac1 expression was the strongest in the highly metastatic cells 801D and PG, and the weakest in the lowly-metastatic 95C cells, suggesting that Rac1 expression is positively associated with metastatic potentials of human lung cancer cells. Interestingly, Rac1 expression in human bronchial epithelial cells (BEAS-2B) was very weak. In addition, expression of Rac1 protein decreased significantly in 801D cells with transfected Rac1-shRNA plasmid compared to the cells with shRNA-control, as shown by Western blot analysis (Fig. 2B). Together, these results suggested that Rac1 was overexpressed in highly metastatic lung cancer cells. Thus, 801D cells were selected to further investigate the effect of specific silencing of 801D on metastasis behaviors such as tumor cell invasion and cell motility.

**Rac1 silencing suppresses 801D cell migration and invasion.** Increased cell motility and invasion of carcinoma cells are key steps in the metastatic cascade. We next examined the role of Rac1 in the cell motility ability of 801D cells. Wound healing experiment results showed that Rac1-shRNA expressing 801D cells exhibited reduced migration ability compared with the control shRNA cells or the cells with no transfectant (Fig. 3A). We then assayed the polarized migration of cells using the scratch-wound model. As shown in Fig. 3B, cells transfected with Rac1-shRNA closed the scratch-wounds more slowly than cells untreated or transfected with negative control. Consistent with the migration results, the silencing of Rac1 in Rac1-shRNA expressing 801D cells significantly inhibited their invasion (Fig. 3C). These data demonstrated the importance of Rac1 in lung cancer cell migration and invasion.

**NSC23766, a Rac1 inhibitor, inhibits lung cancer cell migration and invasion.** This experiment was performed to determine whether inhibition of lung cancer cell migration is mediated through Rac1 expression. For this purpose, equal numbers of 801D cells were subjected to the cell migration assay after treatment with 100 µM NSC23766, a Rac1 inhibitor for 24 h. Treatment of the cells with NSC23766 resulted in a reduction in the cell migration and invasion capacity of lung cancer cells as compared with non-NSC23766-treated controls (Fig. 3). These data suggest that the inhibition of constitutive levels of Rac1 expression in the presence of NSC23766 resulted in inhibition of migration and invasion.

**Effects of depletion of Rac1 on 801D cell proliferation.** To rule out the possibility that the stimulation of migration following Rac1 inhibition was due to stimulation of proliferation, we performed cell survival/proliferation assays in 801D cells under the same conditions as our migration assay. Results of these assays revealed no significant change in proliferation of shRNA-transfected cells (Fig. 4) as well as pharmacological Rac1 inhibitor-treated cells (data not shown), suggesting that the dramatic inhibition in cell migration caused by depletion of Rac1 is not due to a decrease in cell growth.

**Rac1 is essential for rearrangements of the actin cytoskeleton in lung cancer cells.** Because actin cytoskeletal reorganization is considered to be one of the most important functions of Rho GTPases, we stained untreated and NSC23766-treated 801D cells or shRNA-transfected cells for F-actin with TRITC-conjugated phalloidin and visualized the staining under a confocal microscope. As shown in Fig. 5A and B, 801D-negative control cells and 801D cells showed a cross-linked actin network (A and B). Deletion of Rac1 reduced the appearance of a cross-linked actin network in 801D-shRNA cells (C). Deletion of Rac1 inhibited the EGF-induced lamellipodia and pseudopodia formation (D), but this phenomenon was not shown in 801D cells and 801D-negative control cells (E and F). Cells treated with 100 µM NSC23766 showed distinct actin cytoskeleton changes (G and H).

**The Rac1-Pak1 signaling pathway is involved in lung cancer cell migration and invasion.** To determine the role of Pak1 in...
Rac1-mediated lung cancer cell migration, we first examined Pak1 phosphorylation in 801D cells with 100 ng/ml EGF treatment and then tested whether inhibition of Rac1 alters Pak1 phosphorylation in response to EGF. As shown in Fig. 6A, an increase of Pak1 phosphorylation was observed following EGF treatment, indicating that EGF induces the phosphorylation of Pak1 in 801D cells. Conversely, depletion of Rac1 by shRNA transfection abolished EGF-induced phosphorylation of Pak1.

We then examined whether Pak1 activity correlates with lung cancer migration. To address this question, we examined whether depletion of Pak1 by IPA3, a Pak1 inhibitor, alters EGF-induced lung cancer cell migration and invasion. As shown in Fig. 6B, depletion of Pak1 levels significantly attenuated EGF-induced cell migration and invasion compared to the control cells.

Sliencing of Rac1 in 801D cells resulted in increased chemosensitivity to elemene and nedaplatin. We next investigated whether inhibition of Rac1 by RNAi affected the sensitivity of 801D cells to the antitumor drugs, such as nedaplatin. As shown in Fig. 7A, the chemosensitivity in 801D/Rac1-siRNA cells was markedly increased at all concentration examined compared with 801D cells. There was no significant difference between 801D/Rac1-control cells and 801D cells. Similar results were confirmed for the antitumor drug elemene (Fig. 7B), which indicates that this observation seems not to be drug-specific or observed by chance.

**Discussion**

Emerging evidence is beginning to reveal the diverse and profound role of Rac1 in regulating tumor metastasis and the tumorigenic process, raising the possibilities that Rac1 may be a useful prognostic indicator (4,5,7,8). Expression of Rac1 protein has been reported to be greater in testicular germ cell tumors of higher stages than of lower stages (16,17). It has also been shown that the increased expression of Rac1 is related...
to higher TNM stages of gastric carcinoma (18,19). These findings suggest the role of Rac1 overexpression in human tumors and the correlation of Rac1 overexpression with a malignant phenotype. In the current study, we examined the expression of Rac1 in patients with non-small cell lung cancer (NSCLC) and compared it to clinicopathological parameters by immunohistochemistry. We found that Rac1 expression was correlated with lymphatic metastasis and TNM stages, while no association was observed between Rac1 expression and any other clinicopathologic parameters. Additionally, Kaplan-Meier analysis revealed a significant association of Rac1 expression with 5-year survival rates. These observations are well-correlated with the recent studies demonstrating that overexpression of Rac1 plays a crucial role in progression and metastasis of lung cancer cells (5,20).

Different studies have indicated contradictory roles for Rac1 in tumor progression. For example, although in most human malignancies Rac1 down-regulation was shown to result in inhibition of migration (17,19,22,23), migration was shown to be stimulated in the aggressive breast cancer cells where Rac1 was knocked down (21,24). These issues indicate a complex network of Rac1 in the tumor process that results in a particular biological outcome in each tumor type. In our experiments, we found that Rac1 expression positively associated with the metastatic potential of human lung cancer cells. Knocking down of Rac1 expression by shRNA against Rac1 suppressed lung cancer cell migration and invasion. Similarly, NSC23766, an inhibitor of Rac1 activity, could inhibit lung cancer cell migration and invasion. Our results indicate that Rac1 plays a key role in the vigorous migration and invasion of lung cancer cells.

The Rho GTPases are reported to be key regulators of actin dynamics that lead to organized actin-based structures associated with cell migration. Activated Rac1 stimulates lamellipodia formation (22). In the present study, we reported the roles of Rac1 in the organization of the actin cytoskeleton and cell migration in 801D cells. Our results showed that shRNA-mediated deletion of Rac1 resulted in fewer cross-linked actin networks and strongly inhibited actin-positive membrane ruffles, lamellipodia, and pseudopodia formation. Conversely, expression of the Rac1 protein in control 801D cells led to the formation of lamellipodia. Treatment with a Rac1 inhibitor resulted in distinct changes in morphology as well as actin cytoskeletal changes in these cells, which is very similar to the effects of Rac1-shRNA. In addition, we also determined the roles of Rac1 in the rearrangements of the actin cytoskeleton by stimulating cells with EGF. EGF increased lamellipodia and pseudopodia formation in vector control, but not in Rac1-shRNA 801D cells. These results are consistent with the reported observations that depletion of Rac1 in colorectal carcinoma cells strongly inhibited rearrangements of the actin cytoskeleton (14,18). Consistent with our findings, the activation of Rac1 is required for the EGF-induced morphological changes by regulating the organization of the actin cytoskeleton (23).

However, the mechanism by which Rac1 increases the invasive potential remains unclear. Rac1 and Pak1 have recently been shown to be key regulators of cancer cell signaling networks, and there are several lines of evidence linking Rac1 and Pak1 to the acquisition of a migratory, invasive, and metastatic phenotype and to a variety of processes that occur in tumors, including cell transformation, survival, invasion, metastasis, and angiogenesis (9,10,12,13). Pak1 binds to Rac1 in a GTP-dependent manner, after which activated Pak1 regulates cellular functions such as cytoskeleton dynamics, cell adhesion, and transcription (10,12). Furthermore, several growth factors including the epidermal growth factor (EGF) could activate Pak1 and subsequently promote tumor cell invasion (24). To address this question, we examined Pak1 phosphorylation levels in 801D cells after treatment with 100 ng/ml EGF, and found an increase of Pak1 phosphorylation. Conversely, depletion of Rac1 by shRNA transfection abolished EGF-induced phosphorylation of Pak1. Additionally, IPA3, a Pak1 inhibitor, could alter EGF-induced lung cancer cell migration and invasion, which indicates that the Rac1-Pak1 pathway may be a potential therapeutic target for the prevention of tumor invasion and metastasis by inhibition of this signaling pathway.

Nedaplatin has been developed as a second-generation platinum complex with pronounced clinical antitumor activity against solid tumors but with lower nephrotoxicity than cisplatin (25). Elemene (1-methyl-1-vinyl-2, 4-diisopropenylcyclohexane) is a novel anticancer drug extracted from the traditional Chinese medicinal herb Rhizoma zedoariae. It has been effectively used in China to treat certain types of tumors (26), which displayed low toxicity with no observable liver, kidney, or bone marrow toxicities. Therefore, for this investigation, we used these two chemotherapeutic drugs. The MTT results revealed that down-regulation of Rac1 also increased chemosensitivity to the antitumor drugs elemene and nedaplatin in 801D cells. To our knowledge, this is the first report describing a relationship between a cytoskeletal-related protein and the sensitivity to elemene and nedaplatin, which are widely used in the treatment of solid tumors. This observation raised the possibility that the level of expression of Rac1 in tumor samples could be correlated with sensitivity to elemene and nedaplatin in the clinic. Studies of Rac1 in samples of elemene and nedaplatin-treated patients are thus warranted.

In summary, our data demonstrate that suppression of Rac1 could decrease the invasive potential of lung cancer and induce the rearrangements of actin cytoskeleton in vitro. Pak1, a major common downstream effector for Rac1, also could play a critical role in Rac1-mediated invasion and metastasis. Furthermore, Rac1 was found to be overexpressed in tumor tissues, and its expression was directly correlated with tumor stage, lymph node metastasis, and patient survival. We also showed that down-regulation of Rac1 enhanced lung cancer cell sensitivity to the chemotherapeutic drugs elemene and nedaplatin. Our findings indicate that Rac1 may be a potential target for therapeutic anti-cancer drugs and we provide new insight into the development of gene therapy technology to treat patients with lung cancer.

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References


