

RAC1 inhibition as a therapeutic target for gefitinib-resistant non-small-cell lung cancer

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Non-small-cell lung cancer (NSCLC) accounts for almost 80% of lung cancer and the median survival of metastatic NSCLC is only around 1 year, even when treated with platinum chemotherapy.⁽¹⁾ An alternative therapy is molecular target therapy, such as epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (EGFR-TKI), which target mutant EGFR.^(2–4) EGFR is overexpressed in approximately 50% of NSCLC patients and is also known to be correlated with tumor growth and poor prognosis.^(5,6) In addition, specific EGFR mutations (e.g. $\Delta E746-A750$ and L858R) causing aberrant activation have been seen in approximately 10% of NSCLC.^(7–10) Current phase-III clinical studies have also shown that EGFR-TKI are more beneficial as a first-line therapy than combination therapies of other chemotherapies in the aspect of progression-free survival, especially in NSCLC patients harboring EGFR mutations.^(2–4) Despite such apparent clinical benefits, EGFR targeting therapies are now confronting the problem of acquired resistance after long-term treatment by the mechanisms of secondary mutations such as EGFR T790M, MET amplification or hepatocyte growth factor (HGF) overexpression.^(11–13)

Some pathways are activated in the downstream of EGFR signaling, such as the PI3K-Akt pathway, the MEK1/2-ERK1/2 pathway and the Ras-related C3 botulinum toxin substrate1 (RAC1) pathway. Both the PI3K-Akt pathway and the MEK1

Although epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (EGFR-TKI), including gefitinib, provide a significant clinical benefit in non-small-cell lung cancer (NSCLC) patients, the acquisition of drug resistance has been known to limit the efficacy of EGFR-TKI therapy. In this study, we demonstrated the involvement of EGF-EGFR signaling in NSCLC cell migration and the requirement of RAC1 in EGFR-mediated progression of NSCLC. We showed the significant role of RAC1 pathway in the cell migration or lamellipodia formation by using gene silencing of RAC1 or induction of constitutive active RAC1 in EGFR-mutant NSCLC cells. Importantly, the RAC1 inhibition suppressed EGFR-mutant NSCLC cell migration and growth *in vitro*, and growth *in vivo* even in the gefitinib-resistant cells. In addition, these suppressions by RAC1 inhibition were mediated through MEK or PI3K independent mechanisms. Collectively, these results open up a new opportunity to control the cancer progression by targeting the RAC1 pathway to overcome the resistance to EGFR-TKI in NSCLC patients.

/2-ERK1/2 pathway are involved in the survival, proliferation and anti-apoptotic signals from EGFR in NSCLC.^(14–16) In fact, dual blockade of PI3K and MEK pathway can sufficiently induce apoptosis of NSCLC cell lines.^(17,18) Even though these two survival pathways are well-understood, the signal related to NSCLC progression and metastasis has not been elucidated. One of the candidates is the RAC1 pathway. RAC1 is a member of the Rho family of small GTPases and its overexpression is correlated with a poor prognosis in NSCLC.^(19,20) RAC1 has been implicated in a wide variety of cellular processes, including cytoskeletal reorganization, gene transcription, axonal guidance, cell proliferation and cell transformation.⁽²¹⁾ RAC1 is also known to promote lamellipodia formation at the leading edge of migrating cells, which are involved in cancer metastasis;^(22,23) however, it is not well understood whether RAC1 is involved in the migration and metastasis of NSCLC cells.

In this study, we identify that RAC1 is an important downstream molecule of EGF-EGFR signaling involved in NSCLC cell migration. We also demonstrate the significant role of the RAC1 pathway in cell migration or lamellipodia formation in PC-9 cells using RAC1 siRNA or transducing constitutively active mutants of RAC1 in PC-9 cells. Importantly, a RAC1-specific inhibitor NSC23766 suppressed the cell migration and growth of gefitinib-resistant NSCLC cells even in the presence of MEK and PI3K inhibitors. These

results indicate that RAC1 mediates EGFR-induced cell growth and migration of PC-9 cells through a MEK-independent or PI3K-independent mechanism. In summary, we propose a new therapeutic strategy of targeting the RAC1 pathway to control disease progression of EGFR-mutant NSCLC to overcome resistance to EGFR-TKI.

Materials and Methods

Reagents and plasmid preparation. An EGFR-tyrosine kinase inhibitor, gefitinib, was purchased from Cayman Chemical (Ann Arbor, MI, USA) and a PI3K inhibitor (LY294002), a MEK inhibitor (U0126), a p38 inhibitor (SB203580) and a RAC1 inhibitor (NSC23766) were purchased from Merck Millipore (Billerica, MA, USA). Recombinant human EGF and HGF were purchased from Pepro Tech (Rocky Hill, NJ, USA).

Human RAC1 cDNA was amplified by RT-PCR from cDNA derived from A549 cells and inserted into pcDNA3.1-HAHA vector (a kind gift from David E. Fisher, MGH, Boston, MA, USA). Point mutations of G12V and Q61L⁽²⁴⁾ were introduced by following the protocol of the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), and named as pcDNA3.1-HA/RAC1^{G12V} and -HA/RAC1^{Q61L}, respectively.

Cell cultures. PC-9 and RPC-9 cells were kind gifts from Dr Kiura (Okayama University, Japan). A549, PC-9 and RPC-9 cells were cultured in RPMI 1640 Medium (Invitrogen, Carlsbad, CA, USA) with 10% FBS (ICN Biomedicals, Aurora, OH, USA), 2 mM L-glutamine (Life Technologies, Gaithersburg, MD, USA), 100 units/mL penicillin and 100 µg/mL streptomycin in 5% CO₂ at 37°C. For preparation of PC-9 HGF cells, PC-9 cells were treated with 100 ng/mL HGF for 24 h. PC-9, RPC-9 or PC-9 HGF cells were treated with 10–10,000 nM gefitinib, 20 µM LY294002, 10 µM U0126, 10 µM SB203580 or 50/100 µM NSC23766 for the indicated time.

For knockdown experiments, 50 nM ON-target plus SMART pool siRAC1 or negative control #2 (Thermo Scientific, Rockford, IL, USA) were transfected in PC-9 cells using Lipofectamine RNAiMAX reagent (Life Technologies) and the transfected cells were subjected to a migration assay or western blotting after 72 h.

For transient transfection, pcDNA3.1-HA/RAC1^{G12V}, -HA/RAC1^{Q61L} or vector control plasmids with pEGFP-C1 (Clontech, Palo Alto, CA, USA) were co-transfected into PC-9 cells using Lipofectamine 2000 reagent (Life Technologies) and the transiently transfected cells were subjected to a migration assay, western blotting or phalloidin staining after 48 h.

Wound healing assay and migration assay. For the wound healing assay, PC-9 cells (2.2×10^5 cells/well) were seeded into a 24-well plate and cultured overnight. Then, confluent cells were scratched using pipette tips and treated with 10–10,000 nM gefitinib. After scratching, the scratched width was measured at 0 and 24 h.

The migration assay was performed as described previously.⁽²⁵⁾ Briefly, membrane filters (Whatman, Maidstone, UK) were attached to Transwell chambers (Costar, Cambridge, MA, USA) and the lower surface was pre-coated with 1.25 µg laminin (Iwaki, Tokyo, Japan). Pre-treated cells (5×10^4 cells/200 µL in RPMI 1640 medium with 0.1% BSA) were added to the upper compartment of the chamber. After incubation for 9 h, the migrated cells were stained by hematoxylin and eosin and counted manually under a microscope at 50× magnification. For detection of EGFP⁺-invaded cells, filters were fixed

with 4% paraformaldehyde and stained with VECTASHIELD mounting media with DAPI (Vector Laboratories, Burlingame, CA, USA).

Cell counting and WST-1 assay. For cell counting to check the proliferation, PC-9 cells were seeded into a six-well plate (8×10^4 cells/well). After overnight culture, cells were treated with 300 nM gefitinib. After additional 24, 48 or 72 h incubation, cells were collected and the number of live cells were counted manually under a microscope by trypan blue staining.

For the WST-1 assay, A549, PC-9, RPC-9 and PC-9 HGF cells were seeded into a 96-well plate (2 or 5×10^3 cells/well). After overnight culture, cells were treated with 100 µM NSC23766, 10–10,000 nM gefitinib or 20 µM LY294002/10 µM U0126. After an additional 24 or 72 h incubation, cells were subjected to the WST-1 assay using a Cell Counting Kit (Dojindo, Kumamoto, Japan).

Western blotting and pull-down assay. Whole cell lysates were prepared as described previously.⁽²⁶⁾ Primary antibodies used were Phospho-EGFR (Tyr1068), Phospho-Akt (Ser473), Phospho-ERK1/2 (Thr202/Tyr204), Phospho-p38 (Thr180/Tyr182), Phospho-Rac1/cdc42 (Ser71), cyclin D1 from Cell Signaling Technology (Beverly, MA, USA), EGFR, Akt, ERK1/2, p38α, α-tubulin from Santa Cruz Biotechnology (Santa Cruz, CA, USA), an anti-RAC1 antibody from Merck Millipore, an anti-EGFP antibody from Clontech and an anti-HA antibody from Roche (Indianapolis, IN, USA). The active RAC1 was assessed using the Rac1 Activation Assay kit (Merck Millipore) according to the manufacturer's instructions. The pulled-down cell lysates were subjected to western blotting.

Phalloidin staining. Pre-treated PC-9 cells were cultured in chamber slides, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with rhodamine-conjugated phalloidin (Invitrogen) for 20 min at room temperature in the dark. After mounting using VECTASHIELD mounting media with DAPI, the images were analyzed using a Carl Zeiss LSM 700 confocal microscope (Carl Zeiss, Le Pecq, France).

Annexin-V/7-AAD staining. Apoptotic cell number was determined using the MUSE Annexin V and Dead Cell Kit (Merck Millipore, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, after cells were treated with RAC1 inhibitor (100 µM NSC23766) for 24 and 48 h, all cells were collected and diluted with phosphate-buffered saline (PBS) containing 1% BSA as a dilution buffer to a concentration of 5×10^5 cells/mL. 100 µL of cell suspension was then added to 100 µL MUSE Annexin V and dead cell reagent, incubated for 20 min at room temperature, and analyzed using the MUSE Cell Analyzer (Merck Millipore).

In vivo subcutaneous xenograft model. Female 5-week-old C.B-17/lcrHsd-Prkdc^{scid} mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments were approved and performed according to the guidelines of the Care and Use of Laboratory Animals of the University of Toyama. RPC-9 cells were inoculated subcutaneously (5×10^6 cells/100 µL PBS/mouse) into the mouse flank. Mice in each group received NSC23766 in 20% DMSO (2.5 mg/kg/day) or vehicle by intraperitoneal administration every day. Intraperitoneal administration of 20% DMSO is known to be a tolerated dose^(27,28) and we did not observe any side effects with our treatment protocol.⁽²⁹⁾ The tumor volume was assessed every 2 days starting from day 4. The primary tumor was measured using a caliper square along the longer (i) and shorter (ii) axis, and

tumor volume was calculated using the following formula: tumor volume (mm^3) = $ab^2/2$. Upon the termination of the experiment on day 20, tumors were excised and weighed. Mice body weight was recorded every day for monitoring any side effects of the treatment.

Results

Epidermal growth factor receptor signaling regulates the cell migration of non-small-cell lung cancer cells. To understand the role of EGFR signaling in other than NSCLC cell growth or survival, we firstly investigated the effects of EGFR signaling on cell migration by using an *in vitro* wound healing assay. In human NSCLC cell line PC-9 expressing mutant EGFR ($\Delta E746-A750$), which has constitutively activated EGFR signaling without extrinsic ligand stimulation,⁽³⁰⁾ we found that the migratory ability was clearly impaired after gefitinib treatment at 300 nM for 24 h; that in the condition cell growth was not affected (Figs 1a,S1). Given that the migration of the A549 cell line expressing wild-type EGFR was increased after recombinant EGF stimulation (data not shown), both cell intrinsic and extrinsic EGFR signaling regulated the cell migration of NSCLC cells in addition to regulating their cell growth and survival.

RAC1 is essential for epidermal growth factor receptor-mediated cell migration. To identify the downstream molecular mechanism that regulates the migration of PC-9 cells under EGFR activation, we next examined the chemical inhibition of various signaling pathways in PC-9 cell migration. Among four compounds tested that are known to inhibit EGFR-related signaling pathways (PI3K, MEK, p38 and RAC1), only RAC1 inhibitor (NSC23766) suppressed PC-9 cell migration at a similar level to gefitinib (Fig. 2a). Considering that RAC1 is a member of the Rho family of small GTPase, we next examined the effect of gefitinib on the expression level of RAC1-GTP, which is an active form of RAC1, in PC-9 cells. Interestingly, we observed both the reduction of RAC1-GTP and the induction of RAC1 Ser71 phosphorylation in PC-9 cells after gefitinib treatment besides the reduced phosphorylation of molecules associated with cell growth and survival, such as p38,

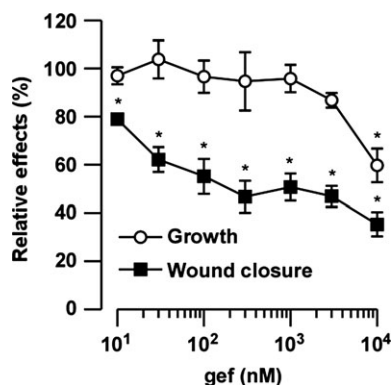


Fig. 1. Epidermal growth factor receptor (EGFR) signaling regulates the cell migration of non-small-cell lung cancer cells. PC-9 cells were incubated for 24 h with 10–10,000 nM gefitinib (gef) after with or without scratching. After 24 h incubation, relative growth (open circle) to vehicle control (–) was determined by WST-1 assay and relative wound closure (closed square) was calculated from the scratched width filled after 24 h incubation compared with at 0 h and normalized to vehicle control (–). Data are the means \pm SD of at least three independent experiments. * $P < 0.01$ by one-way ANOVA followed by the Bonferroni *post-hoc* test compared with vehicle control.

Akt and ERK1/2 (Fig. 2b). In addition to such inactivation of RAC1 molecules, the formation of lamellipodia, known as an important cellular function of RAC1, was diminished after gefitinib treatment in PC-9 cells (Fig. 2c). We further directly confirmed the essential role of RAC1 in NSCLC cell migration by knocking down RAC1 protein using siRNA against RAC1 (Fig. 2d). Given that the phosphorylation of p38, Akt and ERK1/2 was not affected by knocking down RAC1 in PC-9 cells, RAC1 likely regulates the cell migration of PC-9 cells apart from the conventional downstream cell survival pathway of EGFR signaling.

Constitutively active RAC1 attenuates anti-migratory effect of gefitinib. To further determine whether RAC1 suppression is involved in the suppression of cell migration upon gefitinib treatment, we generated PC-9 cells overexpressing constitutive active forms of RAC1, RAC1^{G12V} or RAC1^{Q61L}.⁽²⁴⁾ As shown in Figure 3(a), the reduced cell migration of PC-9 cells by gefitinib treatment was diminished in RAC1^{G12V} or RAC1^{Q61L} overexpressing PC-9 cells. Furthermore, the reduction of lamellipodia formation (Fig. 3b) by gefitinib treatment was also diminished in both RAC1^{G12V} and RAC1^{Q61L} overexpressing cells. RAC1 overexpression was confirmed by detection of HA tag (Fig. 3c). Collectively, these results strongly support that RAC1 activity is critically involved in EGFR-mediated cell migration of PC-9 cells.

RAC1 inhibition is a therapeutic target of gefitinib-resistant non-small-cell lung cancer. Considering the clinical significance of resistance to gefitinib therapy in NSCLC, we sought to determine whether RAC1 inhibition is clinically applicable to targeting gefitinib-resistant NSCLC. To examine the effect of RAC1 inhibitor on gefitinib-resistant NSCLC, we used two different types of gefitinib-resistant cells: RPC-9 cells, which have a secondary EGFR mutation, and PC-9 HGF cells, which are PC-9 cells pretreated with HGF for 24 h to functionally mimic gefitinib resistance through HGF overexpression or MET amplification.^(12,13) Both RPC-9 and PC-9 HGF cells have shown gefitinib resistance on molecular levels.^(13,31) Although gefitinib did not suppress cell migration in gefitinib-resistant cells, RAC1 inhibitor significantly attenuated the cell migration of RPC-9 cells and PC-9 HGF cells (Fig. 4a). In concert with the inhibition of cell migration, we observed the inactivation of RAC1 after treatment with NSC23766 in both RPC-9 cells and PC-9 HGF cells (Fig. 4b). Considering that gefitinib and NSC23766 did not inhibit the migration and the RAC1 activity of A549 cells (Fig. 4a,b), which have wild-type EGFR, RAC1 inhibition might not affect the cell migration machinery of NSCLC other than the constitutive active EGFR signaling cascade.

To further explore whether RAC1 inhibition could affect the proliferation of gefitinib-resistant NSCLC, we tested the effect of NSC23766 in a long-term *in vitro* growth assay of A549, PC-9, RPC-9 and PC-9 HGF cells. As shown in Figure 4(c), RAC1 inhibitor NSC23766 could largely reduce the proliferation of gefitinib-resistant cells at a similar level to their parental PC-9 cells. In addition, the cell growth was slightly suppressed in A549 cells with NSC23766, as described previously.⁽³²⁾ Importantly, NSC23766 inhibited the cell proliferation of gefitinib-resistant RPC-9 and PC-9 HGF cells even in the presence of MEK and PI3K inhibitors (Fig. 4c). Furthermore, in PC-9 cells treated with NSC23766, we could detect the cyclin D1 reduction, one of the markers of cell cycle arrest, and the cell death (Fig. S2). These results suggest that RAC1 is a potential therapeutic target for gefitinib-resistant NSCLC through inhibiting both cell growth and

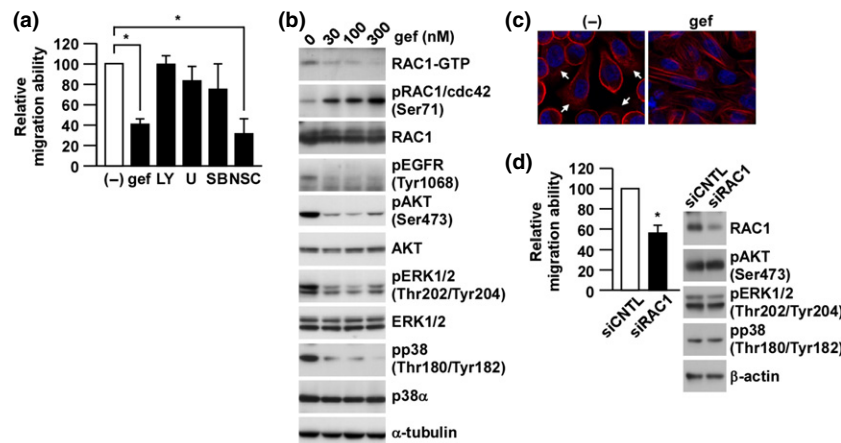


Fig. 2. RAC1 is essential for epidermal growth factor receptor (EGFR)-mediated cell migration. (a) PC-9 cells were pretreated with various inhibitors (gef: 300 nM gefitinib, LY: 10 μ M LY294002, U: 5 μ M U0126, SB: 10 μ M SB203580, NSC: 50 μ M NSC23766) targeting the downstream molecules of EGFR signaling for 24 h and subjected to migration assay. Relative migration ability was calculated after counting the migrated cells and normalized to the control. Data are the means \pm SD of at least three independent experiments. * P < 0.01 by one-way ANOVA followed by the Bonferroni *post-hoc* test. (b) PC-9 cells were treated with various concentrations of gefitinib (gef) for 24 h and whole cell lysates were subjected to western blotting. RAC1-GTP was detected after pull-down assay. (c) PC-9 cells were treated with 300 nM gefitinib (gef) for 24 h and stained with rhodamine-conjugated phalloidin and DAPI. Images were collected using a confocal fluorescence microscope at 40 \times magnification. White arrows indicate lamellipodia. (d) PC-9 cells transfected with the indicated siRNA for 72 h were subjected to migration assay or western blotting. Other conditions are similar to (a) or (b). Data are the means \pm SD of at least three independent experiments. * P < 0.01 by Student's *t*-test.

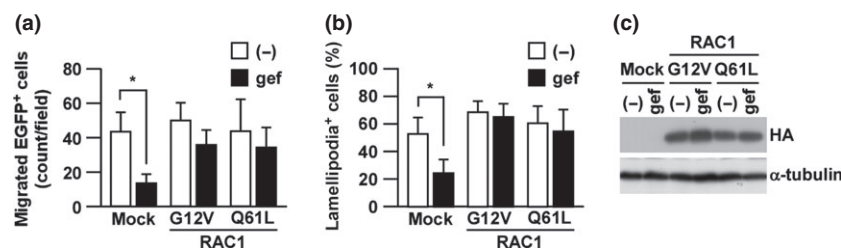


Fig. 3. Constitutively active RAC1 attenuates anti-migratory effect of gefitinib. (a) PC-9 cells were co-transfected with RAC1^{G12V} or RAC1^{Q61L} expression vectors and EGFP expression vector. After 24 h, cells were treated with 300 nM gefitinib (gef) for 24 h and subjected to migration assay. Migrated EGFP⁺ cells were counted manually under a fluorescence microscope at 50 \times magnification. Data are the means \pm SD of at least three independent experiments. * P < 0.01 by two-way ANOVA followed by the Bonferroni *post-hoc* test. (b) Lamellipodia⁺ cells in EGFP⁺ cells were counted at 20 \times magnification after staining with rhodamine-conjugated phalloidin and DAPI. Other conditions are similar to (a). Data are the means \pm SD of at least three independent experiments. * P < 0.01 by two-way ANOVA followed by the Bonferroni *post-hoc* test. (c) PC-9 cells were treated similarly to (a). Whole cell lysates from the transfected PC-9 cells were subjected to western blotting.

migration through MEK-independent or PI3K-independent mechanisms.

RAC1 inhibitor suppresses gefitinib-resistant non-small-cell lung cancer tumor growth *in vivo*. Finally, to investigate whether RAC1 inhibition could suppress tumor growth *in vivo*, we inoculated a human gefitinib-resistant NSCLC cell line, RPC-9 cells, subcutaneously into mice. Consistent with *in vitro* data, the RAC1 inhibitor, NSC23766, significantly reduced RPC-9 tumor size and weight compared to vehicle control (Fig. 5) without any loss of the body weight of mice (data not shown). This suggested that NSC23766 could inhibit gefitinib-resistant NSCLC tumor growth without severe side effects.

Discussion

In this study, we determined the importance of RAC1 for cell migration and growth in the mutant EGFR-expressing NSCLC cell line, PC-9 cells. Importantly, the small molecule RAC1 inhibitor NSC23766 could effectively suppress both the cell migration and growth even in gefitinib-resistant RPC-9 and PC-9 HGF cells *in vitro*, and suppress gefitinib-resistant

NSCLC tumor growth *in vivo*, implying that RAC1 inhibition is an attractive therapeutic target in gefitinib-resistant NSCLC.

Several EGFR-tyrosine kinase inhibitors (EGFR-TKI), including gefitinib, have been approved as molecular-targeted anti-cancer drugs for EGFR-mutant NSCLC and are expected to provide a significant clinical benefit in NSCLC patients. Despite the substantial responses to EGFR-TKI in NSCLC patients, there is a clinical obstacle in that mutant EGFR-expressing NSCLC frequently acquires drug resistance to EGFR-TKI through secondary EGFR mutation, HGF overexpression or MET amplification, therefore limiting the efficacy of EGFR-TKI.^(11–13) Thus, it is critical to identify an alternative strategy to inhibit tumor growth and metastasis in gefitinib-resistant NSCLC to establish a new therapeutic approach against resistance to EGFR-TKI. Similar to its limited efficacy in the inhibition of tumor growth,^(11–13) we observed a limited effect of gefitinib on cell migration in gefitinib-resistant NSCLC cells (Fig. 4a), suggesting that EGFR-mediated signaling regulates cellular motility in PC-9 cells in addition to providing a growth signal. Importantly, we clearly demonstrated that a RAC1 inhibitor, NSC23766, was

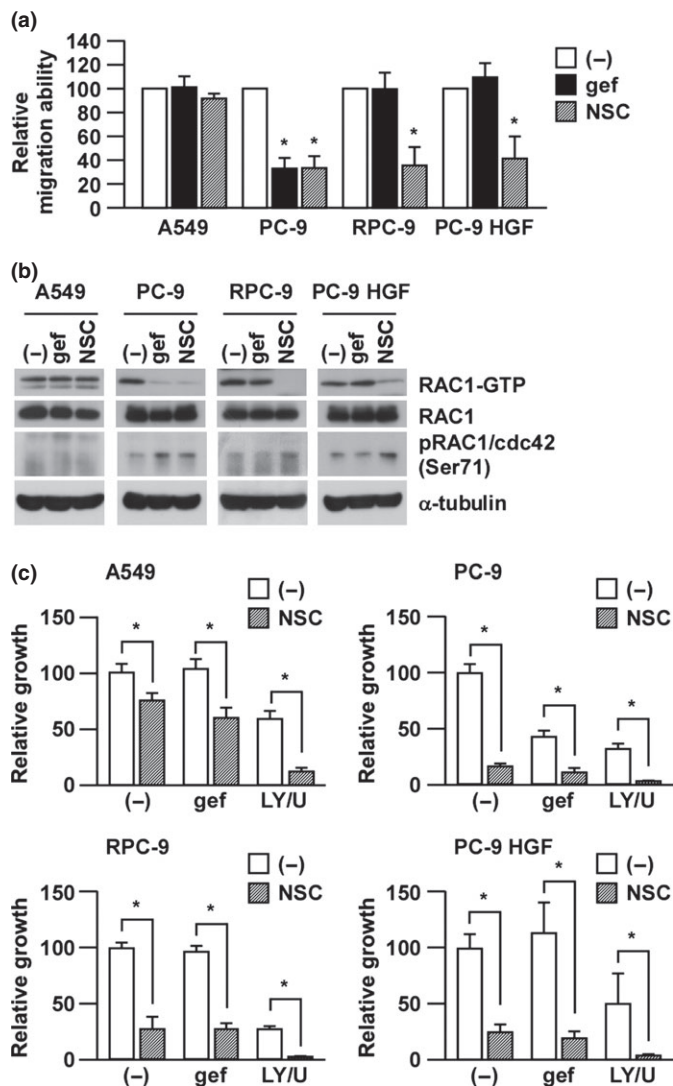


Fig. 4. RAC1 inhibition is a therapeutic target for gefitinib-resistant non-small-cell lung cancer (NSCLC). (a) A549, PC-9, RPC-9 cells and PC-9 cells were treated with 100 ng/mL HGF (PC-9 HGF) were treated with 300 nM gefitinib (gef) or 50 μ M NSC23766 (NSC) for 24 h, and were subjected to migration assay. Migrated cells were counted and normalized with vehicle control (-). Data are the means \pm SD of at least three independent experiments. * P < 0.01 by two-way ANOVA followed by the Bonferroni *post-hoc* test compared with vehicle control. (b) A549, PC-9, RPC-9 cells and PC-9 HGF were treated similarly to (a). Whole cell lysates were subjected to western blotting. (c) A549, PC-9, RPC-9 cells and PC-9 hepatocyte growth factor (HGF) were treated with 100 μ M NSC23766 (NSC) and/or 300 nM gefitinib (gef) or 20 μ M LY294002/10 μ M U0126 (LY/U) for 72 h, then subjected to WST-1 assay. Relative cell growth was normalized to vehicle control (-). Data are the means \pm SD of at least three independent experiments. * P < 0.01 by two-way ANOVA followed by the Bonferroni *post-hoc* test compared with vehicle control.

able to suppress cell migration and proliferation not only in parental gefitinib-sensitive PC-9 cells but also in gefitinib-resistant cells (Fig. 4). In addition, NSC23766 could suppress gefitinib-resistant NSCLC tumor growth *in vivo* (Fig. 5). These results strongly support that RAC1 is an attractive target to inhibit tumor progression in NSCLC patients to overcome resistance to EGFR-TKI.

Our results also indicate the independence of the RAC1-mediated signaling pathway from other downstream pathways

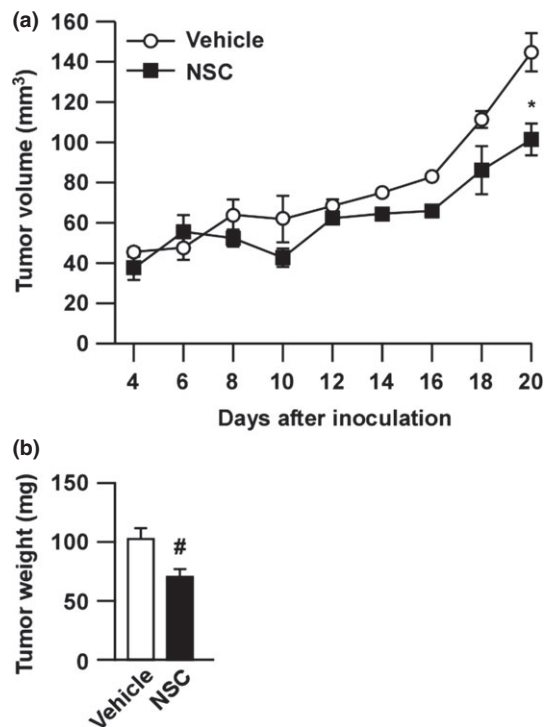


Fig. 5. RAC1 inhibitor suppressed gefitinib-resistant non-small-cell lung cancer (NSCLC) tumor growth *in vivo*. (a) RPC-9 cells were subcutaneously inoculated into mice. Mice received NSC23766 in 20% DMSO (2.5 mg/kg/day) (closed square) or vehicle (open circle) by intraperitoneal administration every day. Tumor volume was measured at the indicated times. Data represented as the mean \pm SD (n = 3). * P < 0.01 by two-way ANOVA followed by the Bonferroni *post-hoc* test. (b) At day 20 after inoculation, tumors were excised and tumor weight was weighed. Data represented as the mean \pm SD (n = 3). # P < 0.05 by Student's *t*-test.

of EGFR-mediated signaling, such as the PI3K-Akt pathway or the MEK1/2-ERK1/2 pathway, which do not seem to be involved in the cell migration of PC-9 cells (Fig. 2a). Thus, we speculated that the EGF-EGFR signal pathway enhances cell migration directly through RAC1 activity. We also could detect no change to Akt and ERK1/2 phosphorylation after knocking down of RAC1 or treatment of RAC1 inhibitor by western blotting (Fig. 2d and data not shown). Given that the RAC1 inhibitor showed further growth inhibition even in the presence of MEK inhibitor and PI3K inhibitor (Fig. 4c), multiple kinase inhibition targeted RAC1, MEK and PI3K could be an attractive strategy to complementally suppress both the growth and metastasis of NSCLC cells, including those resistant to EGFR-TKI.

In addition to EGFR-mutant NSCLC cells, the cell growth in EGFR-wild type cells could be also suppressed by RAC1 inhibitor, NSC23766 (Fig. 4c), although its suppression was not very significant compared to that in EGFR-mutant NSCLC cells. RAC1 inhibitor has been shown to suppress the cell growth in EGFR-wild type NSCLC cells though the induction of cell cycle arrest,⁽³²⁾ which is supported by the reduction of the cyclin D1 in EGFR-mutant NSCLC cells (Fig. S2). Moreover, the induction of cell death was also detected in EGFR-mutant cells with RAC1 inhibitor treatment (Fig. S2). These findings suggest that the EGFR-RAC1 axis is more critical in NSCLC cells with EGFR active mutations through the induction of cell cycle arrest and cell death.

Regarding the clinical implication of targeting the RAC1 pathway in NSCLC therapy, it has been reported that overexpression of RAC1 is often detected in NSCLC specimens and that there is an association between RAC1 expression with TNM stage and lymph node metastasis of NSCLC.⁽¹⁹⁾ Considering that RAC1-dependent regulation of cell growth and migration is known to be relevant in the non-NSCLC type of cancers with aberrant EGFR activity,^(33–37) RAC1 generally plays an important role in cancer progression and metastasis. RAC1 is also known to be required as a downstream molecule of many other receptor tyrosine kinases besides EGFR, including ErbB2 in breast cancer cells.^(38,39) Indeed, we observed the suppression of cell migration in ErbB2-overexpressing human breast cancer cell line BT-474 breast cancer cells by RAC1 inhibitor (data not shown); therefore, the involvement of RAC1 in cancer progression should not be a specific target of the EGFR-NSCLC axis, but rather could be generally applicable to other cancer-associated dysregulation in RTK. Collectively, the present study opens up the new therapeutic potential of target-

ing the RAC1 pathway to control disease progression of EGFR-mutant NSCLC to overcome resistance to EGFR-TKI.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. 300 nM of gefitinib affects the cell growth in PC-9 cells for long time but not for 24 h.

Fig. S2. RAC1 inhibitor suppresses the expression of cyclin D1 and induces the cell death.