Impact of the Integrin Signaling Adaptor Protein NEDD9 on Prognosis and Metastatic Behavior of Human Lung Cancer

Shunsuke Kondo1, Satoshi Iwata2, Taketo Yamada4, Yusuke Inoue7, Hiromi Ichihara3, Yoshiko Ichikawa2, Tomoki Katayose6, Akiko Souta-Kuribara2, Hiroto Yamazaki2, Osamu Hosono2, Hiroshi Kawasaki2, Hirotoshi Tanaka2, Yuichiro Hayashi6, Michiie Sakamoto4, Kazunori Kamiya5, Nam H. Dang8, and Chikao Morimoto6

Abstract

**Purpose:** In a substantial population of non–small cell lung cancer (NSCLC), expression and activation of EGF receptor (EGFR) have been reported and is regarded as a novel molecular target. A growing body of evidence has shown the signaling crosstalk between EGFR and integrins in cellular migration and invasion. NEDD9 is an integrin signaling adaptor protein composed of multiple domains serving as substrate for a variety of tyrosine kinases. In the present study, we aimed at elucidating a role of NEDD9 in the signaling crosstalk between EGFR and integrins.

**Experimental Design:** Using NSCLC cell lines, we conducted immunoblotting and cellular migration/invasion assay in vitro. Next, we analyzed metastasis assays in vivo by the use of xenograft transplantation model. Finally, we retrospectively evaluated clinical samples and records of patients with NSCLCs.

**Results:** We showed that tyrosine phosphorylation of NEDD9 was reduced by the inhibition of EGFR in NSCLC cell lines. Overexpression of constitutively active EGFR caused tyrosine phosphorylation of NEDD9 in the absence of integrin stimulation. By gene transfer and gene knockdown, we showed that NEDD9 plays a pivotal role in cell migration and invasion of those cells in vitro. Furthermore, overexpression of NEDD9 promoted lung metastasis of an NSCLC cell line in NOD/Shi-scid, IL-2Rγnull mice (NOG) mice. Finally, univariate and multivariate Cox model analysis of NSCLC clinical specimens revealed a strong correlation between NEDD9 expression and recurrence-free survival as well as overall survival.

**Conclusion:** Our data thus suggest that NEDD9 is a promising biomarker for the prognosis of NSCLCs and its expression can promote NSCLC metastasis. Clin Cancer Res; 18(22); 6326–38. ©2012 AACR.

Introduction

Lung cancer is the leading cause of cancer-related mortality in men worldwide (1). Non–small cell lung cancer (NSCLC) constitutes more than 80% of lung cancer, whereas small cell lung cancer being around 13%. While surgical intervention is the therapeutic option in limited stage NSCLCs, relapse rate is very high, being around 40% within 5 years after surgical intervention with curative intent. Moreover, the prevalence rate of NSCLCs continues to grow, and 5-year survival rate after diagnosis is only 15% to 25%.

Large randomized trials showed that platinum-based adjuvant chemotherapy has modest survival advantage (HR, 0.6–0.8) for carefully selected patients with NSCLCs (2). Prognostic factor is a powerful tool to determine patients who may benefit from adjuvant chemotherapy, as well as the type of treatments which may benefit the patients. Besides tumor–node–metastasis (TNM) staging, which is the most important clinical prognostic factor for NSCLCs, several studies have examined gene expression profiles of NSCLCs, identifying molecular subtypes associated with patient outcome.

The EGF receptor (EGFR)/human epidermal receptor (HER) 1 is one such gene signature which has received increasing attention over the last decade. EGFR is a receptor tyrosine kinase (RTK; ref. 3) that frequently is overexpressed or harbors constitutively active mutations in NSCLCs. Its activation promotes tumor proliferation, invasion, and metastasis (4). The small molecule tyrosine kinase inhibitors (TKI) gefitinib and erlotinib target the ATP-binding
NEDD9 is phosphorylated at its tyrosine residues by integrins and other stimuli. Ligation of T- and B-cell antigen receptors (19, 20) caused tyrosine phosphorylation of NEDD9, resulting in the association of Crk, Crk-L, and C3G. Integrin- or integrin/TCR-elicited tyrosine phosphorylation of NEDD9 was mediated by focal adhesion kinase (FAK) and Src family tyrosine kinases (21, 22). Ectopic expression of NEDD9 conferred T cells with enhanced motility on the co-engagement of TCR/CD3 complex and β1-integrins (23, 24), suggesting a pivotal role of tyrosine-phosphorylated NEDD9 in TCR- and integrin-mediated cell motility. Recent work showed that NEDD9 expression correlates with metastatic behavior of several malignancies, including lung cancer, head and neck cancer, melanoma, and breast cancer (25–31).

In this study, we investigated the biologic significance of the link between NEDD9 and EGFR signaling pathway in NSCLCs by in vitro and in vivo approaches. Furthermore, we evaluated the clinical significance of NEDD9 expression in primary NSCLC tumor samples through a retrospective analysis. We showed that NEDD9 plays a pivotal role in cell metastasis and invasion of NSCLC cells, and expression of NEDD9 appears to be a promising biomarker for NSCLC prognosis.

Materials and Methods

Reagents and antibodies
Gefitinib was purchased from Biaffin GmbH & Co KG. Recombinant human EGF was purchased from R&D systems, Inc. Monoclonal antibodies (mAb) against FAK and BCAR1 were purchased from BD. Rabbit anti-phospho-FAK (Tyr-397) polyclonal antibody (pAb) was from Invitrogen. Mouse mAb against NEDD9 (2G9) was purchased from ImmuQuest Ltd. Rabbit pAb against NEDD9 was produced by MBL by immunizing synthetic peptide EYPSRYQKDVY-DIPPSH. Anti-phosphotyrosine antibody (4G10) and anti-c-myc tag mAb (9E10) were produced from the hybridoma center maintained at the University of Tokyo, Japan. Mouse mAb against NEDD9 (2G9) was purchased from ImmuQuest Ltd. Rabbit pAb against NEDD9 was produced by MBL by immunizing synthetic peptide EYPSRYQKDVY-DIPPSH. Anti-phosphotyrosine antibody (4G10) and anti-c-myc tag mAb (9E10) were produced from the hybridoma center maintained at the University of Tokyo, Japan. Mouse mAb against NEDD9 (2G9) was purchased from ImmuQuest Ltd. Rabbit pAb against NEDD9 was produced by MBL by immunizing synthetic peptide EYPSRYQKDVY-DIPPSH. Anti-phosphotyrosine antibody (4G10) and anti-c-myc tag mAb (9E10) were produced from the hybridoma center maintained at the University of Tokyo, Japan.

Cells, plasmids, and transfection procedures
293T cells and A549 cells were obtained from American Type Culture Collection. PC-9 cells harboring the gefitinib-sensitizing deletion mutation (ΔE746-A750) and PC-14 were kindly provided by Dr. F. Koizumi (National Cancer Center Hospital, Tokyo, Japan) and were maintained as described previously (32).

The plasmid vector pBabe puro EGFR wild-type and its constitutively active mutants EGFR (ΔIle748-A750) and PC-14 were kindly provided by Dr. F. Koizumi (National Cancer Center Hospital, Tokyo, Japan) and were maintained as described previously (32).

The plasmid vector pBabe puro EGFR wild-type and its constitutively active mutants EGFR (ΔIle748-A750) and PC-14 were kindly provided by Dr. F. Koizumi (National Cancer Center Hospital, Tokyo, Japan) and were maintained as described previously (32).
ΔSH3 (lacking aa 1–60), NEDD9 ΔSD (lacking aa 63–401), NEDD9 ΔC (lacking aa 406–834), NEDD9 ΔDYVYHL (lacking aa 629–834), NEDD9 ΔCC (lacking aa 637–834), NEDD9 AHHL (lacking aa 707–834), and NEDD9 F in which Y629 and Y631 were mutated into F. The plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) or FuGENE6 (Roche Applied Science) according to the manufacturer's instructions. In experiments using BCMG hygro vectors, transfected cells were selected in 0.2 mg/ml hygromycin B to establish stable transformants. The expression vector PMX-luc/neo for firefly luciferase was described elsewhere (34).

Gene knockdown by siRNA
To deplete endogenous NEDD9 or BCAR1, Stealth RNAi siRNAs were obtained from Life Technologies. The target sequence of siRNA for NEDD9 is 5'-UCCCAUGCGAGGAGCUCCGUCCACCU-3' and that for BCAR1 is 5'-GCCUCAAGUUUGGCAAGCUC-3'. As a control, we used Stealth RNAi siRNA negative control. The siRNAs were transfected using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions (Life Technologies).

Protein extraction, immunoprecipitation, and immunoblotting
Cells were serum-starved for 24 hours before the treatment with EGF (at a final concentration of 10 ng/ml). For suspension culture, HydroCell low cell binding culture dish (CellSeed Inc.) was used with serum-free medium. Cytoplasmic protein extracts were prepared by detergent lysis (1% Triton X-100) containing phosphatase and protease inhibitors. For immunoprecipitation, cell lysates were incubated with the appropriate first antibody at 4°C overnight and then with protein A Sepharose beads for 4 hours. After wash with lysis buffer, the beads were boiled in the SDS-PAGE loading buffer. The immunoprecipitates and cell lysates were separated by 8% SDS-PAGE gels and electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated serially with the primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibody and then developed by enhanced chemiluminescence system (GE Healthcare).

Cell migration and invasion assays
Cell migration assay was conducted as described previously using Transwell inserts (8-μm pore size; Corning Incorporated; ref. 24). Cells were placed in the upper chamber at 1 × 10^5 cells/ml in 100 μL of 0.6% bovine serum albumin (BSA)/RPMI-1640 medium. After 6 hours, the inserts were fixed and stained with Diff-Quick. The cells that had not migrated were removed from the upper surface of the inserts using cotton swabs. Images of 3 different high-power fields were captured from each insert, and the number of migratory cells was counted. To determine invasive potential, Matrigel invasion chamber (BD BioCoat) was used.

Animals
Five- to 6-week-old female NOD/Shi-scid, IL-2Rγ<sup>null</sup> mice (NOG mice; ref. 35) were supplied from Central Institute for Experimental Animals (Kawasaki, Japan) and maintained in a specific pathogen-free facility. All experiments were approved by and carried out following the guidelines of the Institute Animal Care and Use Committee of the University of Tokyo (Tokyo, Japan).

In vivo bioluminescence imaging
Human NSCLC cell line PC-14 was transplanted subcutaneously into the lumbar region on the dorsal side of NOG mice. On days 21 and 28, progression of the transplanted tumors was monitored by the following bioluminescence imaging (BLI) technique. α-Luciferin (Beetle Luciferin Potassium Salt; Promega) was used as the substrate for the luciferase expressed by PC-14 cells. The mice received an intraperitoneal injection of 150 mg/kg α-luciferin and placed in the light tight chamber of a cooled CCD camera system (IVIS Imaging System 100; Xenogen) in the prone position under isoflurane anesthesia. Dorsal, left lateral, ventral, and right lateral images were acquired from 10 minutes after α-luciferin injection with the CCD camera system. All luminescent images were collected with an exposure time of 1 minute and binning of 8.

Gross and microscopic pathology
The NOG mice undergoing xenograft transplantation were euthanized with carbon dioxide on day 28 after BLI. Major organs were examined for grossly visible changes. Lung tissues and the primary tumors were removed into 10% neutral-buffered formalin for histology. After formalin fixation, samples were processed into paraffin wax, sectioned at a nominal thickness of 5 μm, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Clinical specimens and immunohistochemical staining
Upon approval from the Institutional Review Board of Keio University (Tokyo, Japan), we selected 60 consecutive patients with lung adenocarcinoma treated with curative surgery resection between 1999 and 2001 from the Keio University Hospital (IRB number: 16–90). Immunohistochemical studies were conducted on 5-μm sections of formalin-fixed, paraffin-embedded tissue section of the primary tumors of lung. Antigen retrieval was conducted with 0.01 mol/L of boiled citrate buffer (pH 9.0) for 10 minutes. The slides were stained on the Dako Autostainer (Dako) using the EnVision (Dako) staining reagents. The sections were first blocked for endogenous protein binding and peroxidase activity with Dual Endogenous Block (Dako) for 10 minutes. The sections were serially incubated with a polyclonal antibody against NEDD9 (2 μg/ml) and EnVision+ Dual Link reagent for 30 minutes. The sections were then treated with 3,3’-diaminobenzidine (DAB) and hydrogen peroxide. A toning solution (DAB Enhancer, Dako) was used to enrich the final color. The sections were counterstained with hematoxylin, dehydrated, and
cells were cultured in the presence or absence of gefitinib. Tyrosine phosphorylation of NEDD9 in NSCLC cell lines. PC-9 and A549 were treated with EGF (10 ng/mL) for the indicated time period and then the cells were lysed by scraper in the lysis buffer. Lysates were immunoprecipitated with anti-NEDD9 pAb. The lysates and immunoprecipitates were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. BCAR1, anti-BCAR1 mAb; β-Actin, anti-β-actin mAb; EGFR, anti-EGFR pAb; FAK, anti-FAK mAb; NEDD9, anti-NEDD9 mAb; pFAK, anti-phospho-FAK Ab.

**Results**

**EGF enhances NEDD9 tyrosine phosphorylation**

To examine the role of NEDD9 in EGFR signaling pathway in NSCLCs, we first used NSCLC cell lines PC-9 and A549. As shown in Fig. 1A, stimulation with EGF elevated the level of tyrosine phosphorylation of NEDD9 in both PC-9 and A549 cells. Because PC-9 harbors activating mutation of EGFR (ΔE746-A750), the basal level of tyrosine phosphorylation of endogenous EGFR was significantly higher than that of A549 cells with the wild-type EGFR. A TKI for EGFR, gefitinib, abolished the increase in tyrosine phosphorylation of NEDD9 induced by EGF in those cells (Fig. 1A). Western blot analyses of EGF treated PC-9 cells and A549 cells are shown in Fig. 1A. Immunostaining for each sample was determined to be positive (defined as ≥30% positive cells) or negative (<30% positive cells) in a blind fashion by the participating pathologist. We compared 3 cutoff values for NEDD9 positivity (10%, 30%, and 50%) and chose 30%.

**Statistical analysis**

The χ² analysis was applied for the comparison of dichotomous variables. The Kaplan–Meier estimate was used for recurrence-free survival (RFS) and overall survival (OS) analysis, and the log-rank test was used to compare the difference. The Cox proportional hazards model was applied in univariate and multivariate survival analysis to test independent prognostic factors. The control groups of all statistical analyses were usually the first groups in the panels unless specified otherwise in the figure legends. All the statistical analyses were conducted by SPSS Statistics 17 (IBM Corporation) at the 0.05 level of significance.

Clinical investigation was conducted according to Declaration of Helsinki principles. The written informed consent was received from patients before inclusion in this study.

expression plasmid pSRα NEDD9 (NEDD9) and/or pME18S Fyn (Fyn) by FuGENE6. After 48 hours, the cells were lysed and immunoprecipitated with anti-NEDD9 pAb. Treatment with gefitinib (0.2 μmol/L) was conducted during 2 hours before the cell lysis. The immunoprecipitates were subjected to immunoblotting with the indicated antibodies. D, inhibition of EGFR by gefitinib modulates FAK, NEDD9, and BCAR1 in PC-9 cells. PC-9 cells were treated with 0.2 μmol/L gefitinib for the indicated time period. The cells were lysed by scraper in the lysis buffer. The lysates were then subjected to immunoblotting with the indicated antibodies. BCAR1, anti-BCAR1 mAb; β-actin, anti-β-actin mAb; EGFR, anti-EGFR pAb; FAK, anti-FAK mAb; NEDD9, anti-NEDD9 mAb; pFAK, anti-phospho-FAK Ab. E, constitutively active EGFR tyrosine phosphorolyses NEDD9 in 293T cells. 293T cells (6 × 10⁵ cells) were co-transfected with pBabe puro EGFR (wild-type or mutants) and pSRα-NEDD9 (wild-type) by FuGENE6. At 24 hours after transfection, the cells were detached by trypsin/EDTA and cultured in suspension with serum-starved condition. At 48 hours after transfection, the cells were lysed and immunoprecipitated with anti-NEDD9 pAb or anti-EGFR pAb. The immunoprecipitates were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Combination of the vectors is as follows: Vec/Vect, pSRα/NEDD9 (NEDD9) and/or pME18S Fyn (Fyn) by FuGENE6.
To assess the specificity of EGFR-mediated tyrosine phosphorylation of NEDD9, we next used the human embryonic kidney cell line 293T cells. As shown in Fig. 1C, gefitinib did not alter the level of tyrosine phosphorylation of NEDD9 caused by exogenous Fyn, an Src family tyrosine kinase in 293T cells. Time course experiments showed that the treatment of PC-9 cells with gefitinib downregulated not only tyrosine phosphorylation of EGFR but also that of FAK. Interestingly, the protein amounts of EGFR, NEDD9, and NEDD9 homologue BCAR1 were also reduced following the addition of gefitinib (Fig. 1D).

In NSCLCs, a variety of activating mutations or deletions have been found in EGFR to correlate with poor clinical outcome. To evaluate the effect of such gene alterations on the tyrosine phosphorylation of NEDD9 in vitro, we next conducted co-transfection analysis using 293T cells in suspension culture with serum starvation. As a result, co-transfection of constitutively active mutant EGFR D770N771 (EGFR NPG) promoted significant level of tyrosine phosphorylation of NEDD9 despite its relatively low level of protein expression, whereas other EGFR constructs including wild-type EGFR and the kinase-negative mutant D837A did not alter the level of tyrosine phosphorylation of NEDD9 (Fig. 1E). However, immunoprecipitation and co-localization analysis revealed that these EGFR constructs and NEDD9 did not co-precipitate and co-localize only marginally (data not shown). These results therefore suggest that the EGFR signaling pathway may modulate tyrosine phosphorylation of NEDD9 in an indirect manner.

Exogenous NEDD9 enhances migratory and invasive potential of NSCLC cell lines

We previously showed that transfected NEDD9 upregulated the motility of Jurkat T cell line on fibronectin (FN) and/or anti-CD3 mAb (23). To determine the association between the expression level of NEDD9 and migratory or invasive behavior of NSCLCs, we used full-length construct of NEDD9, a point mutant NEDD9 F1 in which the Src SH2-binding motif YDYVL was mutated to FDFVLH, and a series of deletion mutants: NEDD9 ASH3 lacking N-terminal SH3 domain (binding site for FAK and Pyk2); NEDD9 ΔSD lacking the substrate domain (binding site for Crk, Nck), NEDD9 ΔC lacking the C-terminal half, NEDD9 ΔYDHVL lacking from the C-terminal to the YDYVLH motif, NEDD9 ΔCC lacking from the C-terminus to the coiled-coil domain, NEDD9 ΔHLH lacking from the C-terminus to the helix-loop-helix domain (24). The structures of these mutants were schematically summarized in Fig. 2A. In Fig. 2B, we showed the protein expression of these constructs of NEDD9 in the A549 stable transformants.

As shown in Fig. 2C–F, gene transfer of the wild-type NEDD9 into PC-9 and A549 conferred significant enhancement of cell motility (Fig. 2C and D), as well as increasing their invasive potential into Matrigel, which is rich in basement membrane proteins (Fig. 2E and F). This enhancement of EGF- or EGF/integrin-mediated cell migration and invasion was markedly reduced by the addition of gefitinib, suggesting the importance of EGF/EGFR signaling in metastasis and invasion of NSCLCs. Of particular interest is that addition of gefitinib also reduced the fibronectin-mediated cell migration in the absence of exogenous EGF (Figs. 2C and D and 3C and D), suggesting a possible role for NEDD9 in the crosstalk of integrin and EGFR.

We next conducted migration assay of A549 cells with a set of NEDD9 mutants in the presence of EGF (Fig. 2G). As shown in the figure, deletion of SH3, SD domain, or the domains from C-terminus to SD abolished the motility-enhancing effect of NEDD9 completely. On the other hand, mutation or deletion of Src SH2-binding motif reduced this activity in a less efficient manner. The set of experiments involving serial C-terminal deletions suggest that the C-terminal region (CT) may regulate the biologic effect of NEDD9 to a certain degree. These results also show that exogenous expression of NEDD9 promotes cancer cell migration and invasion. In addition, the SH3, SD, and SR domains of NEDD9 are particularly important for EGF/integrin-induced cell motility.

Gene ablation of NEDD9 or BCAR1 reduces the migratory and invasive activity of NSCLC cell lines

To further confirm the enhancing effect of NEDD9 on cell motility and invasiveness of NSCLCs, we conducted gene knockdown studies involving endogenous NEDD9 and its related protein BCAR1 by transfecting PC-9 and A549 with their respective siRNA. Concerning to the off-targeting effect, we evaluated 3 siRNAs (siRNA-1: UCCCAUUGGAGGAGACUUGCUUCAGGU, siRNA-2: UCCCAUGGCAACCCGGUGAGACUUCUCU, and siRNA-3: CCLUCUAAUUGGACAUUGGUCUCCAGAUGG) for NEDD9 in their inhibitory effects and in cDNA rescue experiments and selected siRNA-1 (Supplementary Fig. S1).

As shown in Fig. 3, introduction of NEDD9-specific siRNA abolished not only fibronectin-induced cell migration and invasion but also EGF-induced cell invasion. The BCAR1-specific siRNA also reduced cell migration and invasion, although its inhibitory effect was less than that of NEDD9-specific siRNA in NSCLC cell lines.

Together, results of the gene transfer and gene knockdown experiments suggest that NEDD9 as well as BCAR1 seem to be indispensable factors for EGF as well as integrin-mediated cell motility and invasiveness in NSCLCs.

Gene transfer of NEDD9 into an NSCLC cell line promotes lung metastasis in vivo

Our data showing the enhancing effects of NEDD9 on cell motility and invasion of NSCLC cell lines led us to evaluate a xenograft transplantation model using immunodeficient mice (NOG mice) and BLI (34). For this purpose, we used the poorly differentiated human lung adenosquamous carcinoma cell line PC-14 which is negative for NEDD9 at the protein level (Fig. 4A). Figure 4B shows stable expression of transfected NEDD9 (wild-type) in PC-14 cells as long as 28 days after xenotransplantation into NOG mice. The bioluminescence images of xenograft-transplanted NOG mice revealed a positive signal in the lung region of mice transplanted with
Figure 2. Exogenous NEDD9 enhances migratory and invasive potential of NSCLC cell lines. A, structure of NEDD9 and its mutants. The secondary structure of NEDD9 is graphically shown. From 5', closed oval, c-myc tag; SH3, Src homology 3 domain; SD, substrate domain; SR, serine-rich region; C, coiled-coil domain; H, helix-loop-helix domain; CT, C-terminal region. B, protein expression of NEDD9 and its mutants in A549 NEDD9 transfectants. A549 cells were transfected with the indicated NEDD9 constructs. After establishing stable transformants, the cell lysates were subjected to immunoblotting with the indicated antibodies (c-myc, anti-c-myc mAb). Vec, BCMG hygro. C–F, transfection of wild-type NEDD9 promotes migratory activity of NSCLC cell lines. PC-9 (C and E) and A549 cells (D and F) were transfected with either empty vector BCMG hygro (closed bar) or BCMG hyg c-myc NEDD9 WT (open bar). The stable transformants were subjected to cell migration assay (C and D) or cell invasion assay (E and F) in the presence or absence of the indicated reagents. NT (nontreated), fetal calf serum (FCS), 10% in the lower chamber; EGF, 10 ng/mL in the lower chamber; FN, inserts were coated with 0.5 mg/mL of fibronectin; Gef, gefitinib in both chambers at 0.2 µmol/L. Statistical significance was evaluated by comparison with NT. *, P < 0.01; **, P < 0.001; *** P < 0.0001. G, motility-enhancing effect of a series of NEDD9 mutants. A549 NEDD9 transfectants were subjected to the cell migration assay in the presence of EGF (10 ng/mL). Statistical significance was evaluated by comparison with Vec (BCMG hygro).
The right lung and 1.42
lung and 10.33
found that subcutaneous injections of these cell lines
mice with PC-14 NEDD9 mutants (D
in NOG mice. We next conducted metastasis assays in NOG
lung metastasis of a xenograft-transplanted NSCLC cell line
suggest that overexpression of NEDD9 promotes
in vivo
case with the PC-14 NEDD9 transformant. These results
alone, a greater than 3 times higher signal intensity in the
transplanted with control PC-14 cells expressing luciferase
mean signal intensity of the ROI was 1.23
/C6
nal intensity of the ROI was 5.69
expressing luciferase and wild-type NEDD9, the mean sig-
In particular, for the mice transplanted with PC-14 co-
(photons/sec/cm²/steradian) in the ROI was determined.
A region of interest (ROI) of a fixed size was then set in
chest of these mice, and the mean signal intensity
PC-14 co-expressing luciferase and wild-type NEDD9, the mean signal intensity of the ROI was 5.69 \( \times 10^3 \pm 0.64 \) for the right lung and 10.33 \( \times 10^3 \pm 2.65 \) for the left lung, whereas the mean signal intensity of the ROI was 1.23 \( \times 10^3 \pm 1.03 \) for the right lung and 1.42 \( \times 10^3 \pm 0.66 \) for the left lung of mice transplanted with control PC-14 cells expressing luciferase alone, a greater than 3 times higher signal intensity in the case with the PC-14 NEDD9 transformant. These results suggest that overexpression of NEDD9 promotes in vivo lung metastasis of a xenograft-transplanted NSCLC cell line in NOG mice. We next conducted metastasis assays in NOG mice with PC-14 NEDD9 mutants (\( \Delta \)SH3 and \( \Delta C \)). We found that subcutaneous injections of these cell lines resulted in detectable tumors in NOG mice. However, the sizes of the primary tumors were less than tumors created by PC-14 vector transfectant and NEDD9 wild-type transfectant. Furthermore, by microscopic examination, we could not detect any metastatic lesions in the lungs of these mice, indicating that these NEDD9 mutants displayed a dominant-negative effect on cell metastasis as well as tumor growth (Fig. 4D and E).

**Expression of NEDD9 in human NSCLCs is associated with poor prognosis in lung cancer patients**

To determine the clinical relevance of NEDD9 expression in human NSCLCs, we evaluated NEDD9 expression in the primary lesions of human lung adenocarcinoma and also examined the available clinical information of 60 patients treated consecutively at Keio University Hospital with curative surgery between 1999 and 2001. Clinical characteristics of these patients are summarized in Supplementary Tables S1 and S2. By immunohistochemical method, significant expression of NEDD9 protein was identified in 46.7% (28...
of 60) of primary human lung adenocarcinoma tissues (Fig. 5A). Overexpression of NEDD9 was associated with increasing invasion into mediastinal (N2) lymph node ($P = 0.01$), pathologic lymphatic invasion ($P = 0.03$; Fig. 5B). However, no other statistically significant correlation was found in lung cancer between NEDD9 expression and other clinical parameters, such as age, sex, smoking history, and the extent of primary tumor (Table 1).

With a median follow-up time of 58.5 months (range, 7–91 months), the median RFS was significantly longer in the NEDD9-negative group (not reached) than in the NEDD9-positive group (23 months; $P < 0.001$; Fig. 5C). The HR for RFS was 4.24 [95% confidence interval (CI), 1.93–9.26] in the NEDD9-positive group. The median OS was also significantly longer in the NEDD9-negative group (not reached) than in the NEDD9-positive group (36 months; $P < 0.001$; Fig. 5C).

In univariate Cox analysis, the HR for OS was 5.35 (95% CI, 1.98–14.50) in the NEDD9-positive group. In addition, N2 invasion and pathologic lymphatic invasion were also significant predictors by univariate analysis. In multivariate Cox analysis, NEDD9 expression (HR, 3.88; 95% CI, 1.34–11.23; $P = 0.01$) and pathologic lymphatic invasion (HR,
4.94; 95% CI, 1.04–23.5; P = 0.04) were independent prognostic variables (summarized in Table 1). Taken together, these results suggest that the expression of NEDD9 closely correlates with venous and lymphatic invasion of cancer cells, and NEDD9 may also be a predictive biomarker for the recurrence and prognosis of human NSCLCs in the clinical setting.

Discussion

In the present study, we showed that tyrosine phosphorylation of NEDD9 was reduced by the inhibition of EGFR in NSCLC cell lines. A constitutively active mutant of EGFR promoted tyrosine phosphorylation of NEDD9 in the absence of integrin signaling. The gene transfer and gene knockdown studies revealed that NEDD9 plays a pivotal role in cell migration and invasion of NSCLC cell line. Overexpression of NEDD9 was shown to promote lung metastasis of an NSCLC cell line in a murine xenograft transplantation model. Finally, the evaluation of the clinical specimens of NSCLCs revealed a strong correlation between NEDD9 expression and RFS or OS, suggesting that NEDD9 is a promising prognostic biomarker in NSCLCs. This is the first study to show the clinical importance of NEDD9 as a prognostic factor as well as the crosstalk between EGFR and NEDD9 signaling pathways in NSCLCs.

Several protein tyrosine kinases (PTK) phosphorylate NEDD9, including FAK (21, 22), RAFTK/Pyk2 (20), Src family PTKs (20, 21), platelet-derived growth factor receptor (PDGFR; ref. 36), Abl (17), and Bcr-Abl (37), many of which are involved in cancer progression and growth. EGFR and PDGFR are RTKs (3) that crosstalk with integrins (8–10, 12), with EGFR physically associating with α5β1 (38) and PDGFR with αvβ3 (39). Engagement of EGFR modulates tyrosine phosphorylation of BCAR1, mediated partly by Src (9), whereas engagement of integrins induces tyrosine phosphorylation of EGFR, mediated by BCAR1 and Src (40, 41). FAK is also a necessary component for EGFR- and PDGFR-induced cell motility (42). These reports suggest that RTKs and integrins form a macromolecular signaling complex at least transiently and have reciprocally compensatory roles in contributing to cell migration and invasion. In this study, we show that NEDD9 may also be an integrator of EGFR and β1-integrin in phosphorylation-dependent signaling, leading to cell migration and invasion of NSCLCs. In this regard, it is possible that other molecules may be associated with EGFR in regulating NEDD9 tyrosine phosphorylation, and additional work is needed to further characterize the signaling complex involving NEDD9, EGFR, and β1-integrins in tumorigenesis and metastasis of NSCLCs.
PC-9 cells used in this study harbors an in-frame deletion in EGFR which causes the receptor to be constitutively activated as a result of structural change proximal to the ATP-binding site (43). These cells and a significant population of NSCLC tumor cells are dependent on the constitutively activated EGFR, thus blockade of the signal by gefitinib results in apoptotic cell death (6, 7). In the context of oncogene involvement in tumor growth, of particular...
interest is that blockade of EGFR signal caused not only dephosphorylation but also a reduction in the protein levels of focal adhesion resident proteins such as BCAR1 and FAK as well as NEDD9. Because these proteins have been reported to incur caspase-induced degradation in apoptosis (44), the results observed in this study may reflect a gefitinib-induced apoptotic process. Supporting this notion, gene transfer of NEDD9 into PC-9 and A549 cells conferred resistance to the chemotherapeutic reagents such as gefitinib-induced apoptotic process. Supporting this notion, gene transfer of NEDD9 into PC-9 and A549 cells conferred resistance to the chemotherapeutic reagents such as gefitinib, paclitaxel, and cisplatin (Supplementary Figs. S2 and S3). Future in-depth studies will be conducted to expand on these interesting data.

We and others previously reported that NEDD9 phosphorylation contributes to cell migration and invasion (22, 23, 36, 45). We now show the involvement of NEDD9 in EGFR-mediated cell migration and invasion of NSCLCs. Consistent with previous reports, our studies indicated that BCAR1 siRNA also caused significant reduction in EGFR-mediated cell motility of NSCLCs. Another member of Cas family, BCAR1/p130Cas, was independently identified as the primary gene that confers breast cancer cells with resistance to anti-estrogen (46). HER2/neu is a member of the EGFR family and is a notable therapeutic target of breast cancer and head and neck squamous cell carcinoma, processes which potentially involve novel upstream molecules such as hypoxia-inducible factor (28), N-terminal truncated carboxypeptidase E splice isoform (29), Wnt (31), and VEGF (50). Our analysis of the clinical records of patients undergoing surgical resection with curative intent of their NSCLCs (with a recurrence rate within 2 years of specimen collection of 43% being similar to the rate reported previously) to evaluate the potential clinical significance of NEDD9 expression in NSCLCs showed a significant correlation between NEDD9 expression and previously

| Table 1. Cox univariate and multivariate analysis of patients with NSCLCs |
|-----------------------------|-----------------------------|-----------------------------|
|                            | RFS                        | OS                         |
|                            | HR (95% CI)                | P                          | HR (95% CI)                | P                          |
| **Univariate analysis**    |                            |                            |                            |                            |
| NEDD9 (positive vs. negative) | 4.24 (1.93–9.26)           | <0.001                     | 5.35 (1.98–14.50)          | 0.001                     |
| Sex (male vs. female)      | 1.08 (0.53–2.20)           | 0.83                       | 1.3 (0.56–3.00)            | 0.54                       |
| Age (>65 y)                | 1.17 (0.58–2.38)           | 0.86                       | 1.11 (0.49–2.51)           | 0.8                       |
| Smoking history (smoker vs. non-smoker) | 1.19 (0.58–2.39) | 0.65                       | 1.25 (0.55–2.84)           | 0.59                       |
| pStage (III vs. I–II)      | 2.42 (1.20–4.91)           | 0.01                       | 3.1 (1.35–7.13)            | 0.01                       |
| N1 (positive vs. negative) | 1.45 (0.35–6.12)           | 0.61                       | 1.22 (0.16–9.09)           | 0.85                       |
| N2 (positive vs. negative) | 3.9 (1.91–7.97)            | <0.001                     | 4.8 (2.05–11.21)           | <0.001                     |
| Lymphatic invasion (positive vs. negative) | 5.83 (2.23–15.28) | <0.001                     | 10.1 (2.36–43.2)           | 0.002                     |
| Venous invasion (positive vs. negative) | 1.85 (0.85–4.06) | 0.12                       | 2.713 (1.17–6.29)          | 0.02                       |
| **Multivariate analysis**  |                            |                            |                            |                            |
| NEDD9 (positive vs. negative) | 3.08 (1.37–6.93)           | 0.006                      | 3.88 (1.34–11.23)          | 0.01                       |
| pStage (III vs. I–II)      | 0.78 (0.24–2.5)            | 0.67                       | 1.32 (0.38–4.47)           | 0.66                       |
| N2 (positive vs. negative) | 2.54 (0.76–8.46)           | 0.13                       | 1.84 (0.53–6.39)           | 0.34                       |
| Lymphatic invasion (positive vs. negative) | 4.84 (1.52–15.37) | 0.008                      | 4.94 (1.04–23.5)           | 0.04                       |
| Venous invasion (positive vs. negative) | 0.62 (0.26–1.44) | 0.26                       | 0.79 (0.31–1.99)           | 0.62                       |
identified pathologic prognostic factors. Expression of NEDD9 was associated with a significant increase in the risk of metastasis and recurrence, with a corresponding decrease in survival and worsened clinical outcome. Multivariate analysis also suggested that NEDD9 expression is an independent predictive factor for the recurrence of NSCLCs. To evaluate the NEDD9 expression in NSCLCs, we set 3 cutoff values (10%, 30%, 50%). Kaplan–Meier curves in the cases with 10% cutoff and 50% cutoff value are shown in Supplementary Fig. S4. When the cutoff value (% positive cells) was 10%, there was no statistically significant difference in OS and RFS. When the cutoff was 50%, the number of positive cases was only 6. When the cutoff was 30%, significant difference was observed in OS and RFS. It should be noted that these data were derived from a retrospective analysis and are likely to suffer from selection bias. Randomized control study or stratified analysis will be necessary in a future study to extend our present findings.

In conclusion, our present work suggests that NEDD9 is a predictive factor for recurrence and prognosis in NSCLCs. Although a variety of gene profiles have been reported to correlate with recurrence of NSCLCs, none has yet been definitely established (51). Because the clinical records evaluated in this study did not contain genetical information on EGFR mutation, KRAS mutation which may affect evaluation in this study did not contain genetical information on EGFR mutation, KRAS mutation which may affect the sensitivity to gefitinib (52), further comprehensive analysis is necessary in the next step. Optimal strategies to prevent recurrence and metastasis of NSCLCs may need to incorporate NEDD9 expression as one of the promising predictive factors and NEDD9 itself may be a novel therapeutic target for future NSCLC treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Kondo, S. Iwata, H. Kawasaki, H. Tanaka, C. Morimoto
Development of methodology: S. Kondo, S. Iwata, T. Yamada, H. Kawasaki
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kondo, T. Yamada, Y. Inoue, H. Ichihara, Y. Kichikawa, T. Katayose, A. Souta-Kuribara, H. Hayashi, K. Kaniya
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kondo, S. Iwata, T. Yamada, Y. Inoue, H. Ichihara, Y. Kichikawa, H. Yamazaki, H. Kawasaki, N.H. Dang
Writing, review, and/or revision of the manuscript: S. Kondo, S. Iwata, Y. Kichikawa, O. Hosono, H. Kawasaki, N.H. Dang, C. Morimoto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kondo, T. Yamada, Y. Inoue, H. Ichihara, A. Souta-Kuribara, H. Yamazaki, O. Hosono, M. Sakamoto
Study supervision: S. Iwata, H. Yamazaki, O. Hosono, H. Kawasaki, H. Tanaka, C. Morimoto

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Impact of the Integrin Signaling Adaptor Protein NEDD9 on Prognosis and Metastatic Behavior of Human Lung Cancer

Shunsuke Kondo, Satoshi Iwata, Taketo Yamada, et al.


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