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Original Article

N-cadherin expression is a potential survival mechanism of gefitinib-resistant lung cancer cells

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Abstract: Non-small cell lung cancer (NSCLC) is a major subtype of lung cancer and is the most common and fatal cancer worldwide. Specific tyrosine kinase inhibitors for epidermal growth factor receptor (EGFR), such as gefitinib, have been effective in some NSCLC patients and are being used in the clinical setting as pioneer molecularly targeted cancer drugs. However, many patients have not responded to these drugs, and have acquired resistance after long-term treatment. To identify other potential NSCLC molecular targets, we used DNA microarrays to examine gene expression profiles of gefitinib-resistant PC9/ZD cells that are derived from gefitinib-sensitive PC9 cells and harbor a threonine to methionine mutation at codon 790 (T790M) in EGFR, a known mechanism of acquired resistance to gefitinib. We found that N-cadherin expression was significantly upregulated in PC9/ZD cells compared with PC9 cells. Inhibition of N-cadherin expression by siRNA or treatment with antibodies against N-cadherin induced apoptosis of PC9/ZD cells in association with reduced phosphorylation of Akt and Bad, a proapoptotic protein. Moreover, inhibition of Akt expression by siRNA or treatment with an inhibitor for phosphatidylinositol (PI)-3 kinase reduced survival of PC9/ZD cells. In addition, we found several N-cadherin-expressing lung cancer cells that showed inherent resistance to gefitinib treatment and reduced survival owing to siRNA-induced inhibition of N-cadherin expression. Thus, it appears that N-cadherin maintains the survival of the gefitinib-resistant lung cancer cells via the PI-3 kinase/Akt survival pathway. From these results, we propose that N-cadherin signaling contributes, at least in part, to the survival mechanisms of gefitinib-resistant NSCLC cells and that N-cadherin is a potential molecular target in the treatment of NSCLC.

Keywords: NSCLC, EGFR, TKI, PC9, PI-3 kinase, Akt, microarray, gene expression profiling, Meta Gene profier

Introduction

Lung cancer is the leading cause of death of men and women worldwide [1]. Lung cancer is broadly divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), and the former represents approximately 85% of all lung cancers. While SCLC generally responds well to conventional regimens of chemotherapy and radiation therapy, NSCLC is largely refractory to these treatments. Recent research efforts have focused on the development of molecularly targeted drugs such as gefitinib (Iressa™), a tyrosine kinase inhibitor (TKI) specific to epidermal growth factor receptor (EGFR) [2-4]. While some patients responded dramatically to the drug, others did not.

Molecular studies have identified somatic mutations in *EGFR* as a major determinant underlying the dramatic clinical responses following gefitinib treatment [5-7]. Most of the *EGFR* mutations are either small deletions in exon 19 encompassing 5 amino acids at codons 746–750 (ELREA) or missense mutations resulting in the substitution of leucine with arginine at codon 858 (L858R) [8, 9]. Exon 19 deletion and L858R mutations cause increased and sustained EGFR phosphorylation and anti-apoptotic pathway activation without ligand stimulation. It is thus thought that gefitinib-sensitive lung cancer cells are dependent on or even addicted to

sustained EGFR signaling for their survival. The induction of apoptosis in cancer cells is a plausible mechanism of action of molecularly targeted drugs such as gefitinib [10].

Another problem is that even though patients initially respond to EGFR-TKI, they almost invariably become drug resistant. It was reported that a secondary mutation of the *EGFR* gene T790M was responsible for its acquired resistance [11, 12]. It is unlikely that cells harboring T790M EGFR are addicted to EGFR signaling since it was reported that irreversible EGFR-TKI should bind to T790M EGFR; however, it does not induce apoptosis in cells harboring the mutation [13, 14]. In addition, it was reported that amplification of the *MET* gene, a receptor tyrosine kinase, is yet another mechanism of acquired resistance to EGFR-TKI [15].

Although the clinical use of EGFR-TKI has raised hope for improved prognosis of NSCLC patients, there are still many patients who are inherently resistant to EGFR-TKI or become resistant after long-term treatment. Therefore, the identification of a new target for developing molecularly targeted drugs for NSCLC is important.

Epithelial cell-cell junctions provide tissue integrity, and the adherens junctions play a pivotal role in their activity. Cadherins, the major adhesion molecules in the adherens junctions, mediate Ca^{2+}-dependent cell-cell adhesion via their extracellular domains [16, 17]. Cadherin monomers are thought to dimerize on the surfaces of the cells from which they are expressed and then interact with homotypic dimers localized on the surfaces of neighboring cells to mediate cell-cell adhesion. The homophilically bound cadherins in various modes connect to the actin cytoskeleton by associating with catenins via their cytosolic domain. Epithelial cells typically express E-cadherin, whereas mesenchymal cells or neural cells express various cadherins including N-cadherin. During the developmental stages, such as gastrulation, epiblast cell ingression through the primitive streak, a phenomenon called cadherin switching, occurs in which E-cadherin loss and N-cadherin appearance take place in the epithelial-mesenchymal transition (EMT) process [18]. Cadherin switching also includes situations in which E-cadherin expression levels do not change significantly but the cells activate N-cadherin expression. Cadherin switching is thought to occur in cancers of epithelial origin. It is involved in changing tumor phenotypes into a more malignant state.

In the present study, we searched for a new molecular target for gefitinib-resistant NSCLC. We hypothesized that gefitinib-resistant cell survival is dependent on the activity of such molecular targets. We examined gene expression profiles of gefitinib-sensitive PC9 cells and gefitinib-resistant PC9/ZD cells derived from PC9 cells and identified N-cadherins as a candidate molecular target.

Materials and methods

Materials

Recombinant human EGF was purchased from Millipore (Billerica, MA, USA). Gefitinib was extracted from IressaÒ tablets (AstraZeneca, UK). The caspase inhibitor Z-VAD-FMK was purchased from MBL (Japan). The phosphatidylinositol (PI)-3 kinase inhibitor LY294002 was purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

A549, PC9, PC9ZD, H1650, H1975, H322, H157, PC3, PC14, II-18, Ma24, H520, PC10, H3255, and PC13 cells were grown in RPMI1640 medium (Nakarai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Kansas, MO, USA), 100 U/mL penicillin (Nakarai Tesque), and 100 mg/mL streptomycin (Nakarai Tesque). Cells were cultured at 37 °C in an atmosphere of 5% CO_2.

Western blotting

Cells were lysed in either lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM ethylene glycol tetraacetic acid [EGTA], 1 mM phenylmethylsulfonyl fluoride, 100 U/mL aprotinin, and 1 mM Na_3VO_4) or sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol). Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane

(Immobilon-P; Millipore, Billerica, MA). The membrane was blotted with a specific antibody and visualized with a chemiluminescence substrate (PerkinElmer, San Jose, CA, USA).

Antibodies

Anti-human EGFR antibodies were purchased from MBL. Anti-Met (25H2), anti-cleaved caspase-3 (Asp175), anti-cleaved-PARP (Asp 214), anti-phospho-Bad (Ser136), anti-phospho-Akt (Ser 473), and anti-Akt antibodies were purchased from Cell Signaling Technology. Anti-Actin (C4) antibody was purchased from Millipore. Anti-vimentin antibody (Clone9) was purchased from Dako Japan (Tokyo, Japan). Anti-E-cadherin and anti-b-catenin antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-N-cadherin (A-CAM) antibody was purchased from SIGMA (St. Louis, MO, USA). Anti-N-cadherin (H-63) and anti-Flg (C-15) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-α-tubulin (5-B-1-2) was purchased from Active Motif. Horseradish peroxidase (HRP)-conjugated anti-mouse/rat/rabbit immunoglobulin G (IgG) antibodies (Amersham Biosciences, Sweden) and HRP-conjugated anti-goat IgG antibodies (Santa Cruz Biotechnology) were used as secondary antibodies.

siRNA transfection

siRNA for N-cadherin (CDH2) (1, 5'-CUAACAGGGAGUCAUAUGGUGGAGC-TdT-3'; 2, 5'-AAUUAAGGGAGCUCAAGGACCCAGC-TdT-3'; and 3, 5'-UAAUGAAGAUACCAGUUGGAGGCUG-TdT-3'), siRNA for β-catenin (CTNNB1) (5'-UUACCACUCAGAGAAGGAGCUGUGG-TdT-3'), and StealthÔ RNAi Negative control duplexes (high GC content and low GC content) were purchased from Invitrogen (Carlsbad, CA, USA). siRNA for Akt (SignalSilenceÒ Akt siRNA II) was purchased from Cell Signaling Technology. Transfection was performed using LipofectamineÔ RNAiMAX (Invitrogen) according to the manufacturer's directions.

Cell growth assay

Cells were seeded on 96-well plates with the growth medium containing the materials to be tested followed by incubation at 37 °C for 24 or 72 h. Cell numbers were determined using a CellTiter 96® (Promega, Madison, WI, USA) according to the manufacturer's instructions. Low attachment culture dishes were purchased from CORNING (New York, NY).

Immunohistochemistry

To examine the apoptotic cells, PC9/ZD cells were seeded on 8-well culture slides. Transfection was performed with N-cadherin siRNA using LipofectamineÔ RNAiMAX, and the cells were incubated at 37 °C for 5 days. After incubation, the cells were fixed with 3.7% paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.1% (vol/vol) Triton X-100, and treated with 5% bovine serum albumin in PBS. The cells were first incubated with anti-cleaved-caspase 3 antibodies overnight at 4 °C and then incubated with an Alexa FluorÒ 488 goat anti-mouse IgG (Invitrogen) for 1 h. The samples were stained with 4',6-diamidino-2-phenylindole (DAPI) for the nuclear morphology assessment. The samples were then observed under a fluorescence microscope and photographed.

TUNEL assay

Apoptotic cells were detected using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) kit (Roche Diagnostics Co., Indianapolis). The samples were first stained with DAPI (Wako, Japan) for the nuclear morphology assessment and then observed under a fluorescence microscope and photographed.

RNA isolation

Total RNA was isolated from each sample using TRIzol® reagent (Invitrogen) according to the manufacturer's directions. The quality and integrity of the total RNA was evaluated on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA samples with an RNA integrity number (RIN) >8 were used for further analysis.

Microarray analysis

RNA samples were labeled using an Agilent Low RNA Input Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. In brief, amplification and labeling of 500 ng of total RNA was performed using Cyanine 3-CTP. Hybridization was carried out using the Gene Expression Hybridization Kit (Agilent Technologies) following the manufacturer's in-

structions. In brief, 1.65 mg of sample cRNA was subjected to fragmentation (30 min at 60°C) and then hybridization on a 44K Agilent Whole Human Genome Oligo Microarray (G41112F) (Agilent Technologies) in a rotary oven (10 rpm at 65 °C for 17 h). Slides were disassembled and washed in Agilent Gene Expression Wash Buffers 1 and 2 (Agilent Technologies) according to the manufacturer's instructions. Gene expression values measured with the microarrays for PC9 (N=3) and PC9/ZD (N=4) were normalized by a Box-Cox transformation (1=0.25) after a median-shift transformation, i.e., median value was set to 1, for each microarray.

Statistical analysis

Data in the graphs are represented as mean ± standard deviation (S.D.). The statistical significance was assessed with Student's t-test according to the variance between the two samples. Statistically significant differences are indicated with an asterisk ($P<0.05$) or double asterisks ($P<0.01$) in the figures.

Meta gene profiler analysis

In order to identify differentially expressed functional gene sets (e.g., pathways) between PC9 and PC9/ZD, we analyzed the gene expression profiles by Meta Gene Profiler (MetaGP) [19, 20]. In MetaGP, to calculate a p-value for a gene set, we integrated p-values of genes within the gene set; we used p-values for genes obtained from Welch's t-test between the two cell lines. For pathway gene sets, we used gene sets constructed from pathways in TRANSPATH database [21].

Results

N-cadherin expression is upregulated in gefitinib-resistant PC9/ZD cells

We used gefitinib-sensitive PC9 cells and gefit-

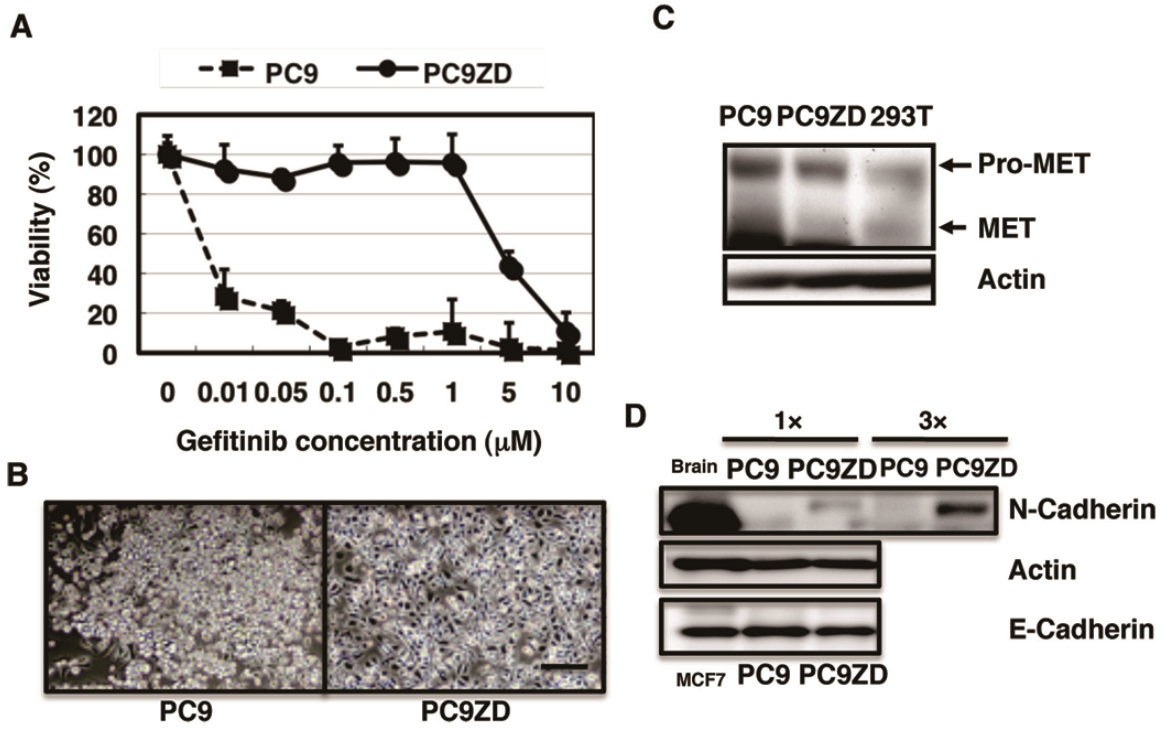

Figure 1. N-cadherin expression was upregulated in gefitinib-resistant PC9/ZD cells. (**A**) PC9/ZD human lung cancer cells derived from gefitinib-sensitive PC9 cells showed gefitinib resistance in a cell proliferation assay. The percentage of viable cells is shown relative to untreated controls. Experiments were performed 3 times with similar results. (**B**) The cell morphology of PC9 and PC9/ZD cells. Scale bar, 50μm. (**C, D**) Cells were lysed and the indicated proteins were detected by western blotting. Mouse brain lysates and MCF-7 cells (**D**) were used as positive controls of N-cadherin and E-cadherin, respectively. The amount of anti-N-cadherin antibodies for incubation was changed (x1 and x3).

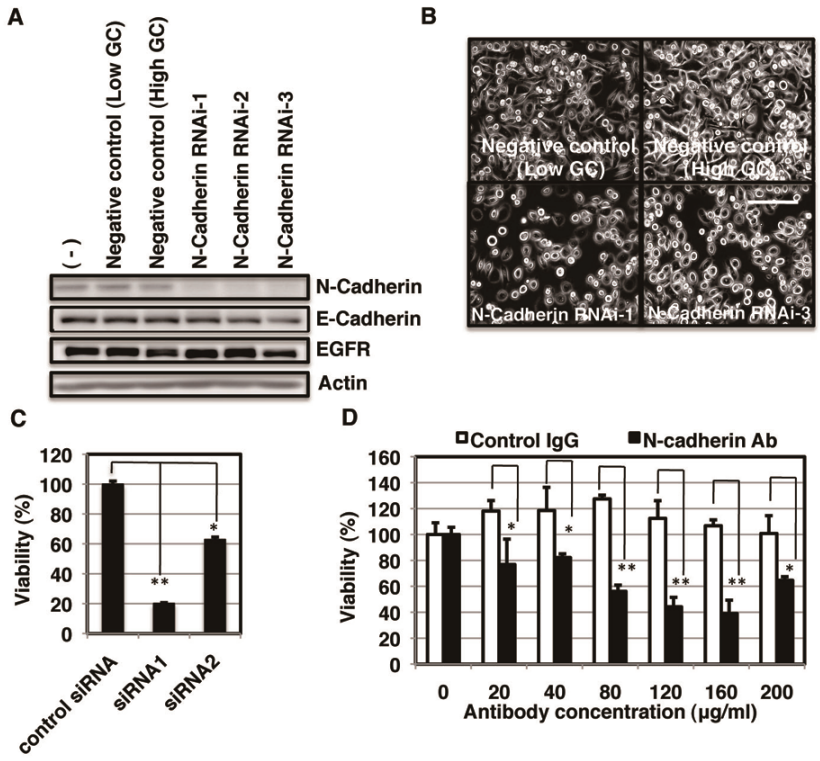

Figure 2. Cell viability was decreased by knockdown of N-cadherin expression or treatment with N-cadherin-neutralizing antibody in PC9/ZD cells. (**A**) Cells were lysed after siRNA transfection (48 h) and the indicated proteins were detected by western blotting. (**B**) Cell morphology. Scale bar, 50 μm. (**C**) The percentage of viable cells transfected with siRNA for N-cadherin is shown relative to control siRNA-transfected cells after a 5-day transfection. The results were obtained from at least 3 separate experiments and are indicated as mean (SD). (**D**) The percentage of viable cells treated with N-cadherin-neutralizing antibody or control IgG for 5 days is shown relative to untreated controls. The results were obtained from at least 3 separate experiments and are indicated as mean (SD). (*, $p < 0.05$; **, $p < 0.01$).

inib-resistant PC9/ZD cells derived from PC9 cells to examine a potential mechanism of gefitinib-resistant cell survival. PC9 cells harbor the exon 19 deletion for constitutive activation of EGFR tyrosine kinase. PC9/ZD cells generated from PC9 cells harbored a secondary T790M mutation for acquired resistance in addition to the exon 19 mutation in *EGFR* [22, 23]. We confirmed the gefitinib sensitivity of both cell lines (**Figure 1A**). Morphologically, many PC9 cells were floating, whereas the majority of the PC9/ZD cells were adherent to culture dishes in the regular culture condition (**Figure 1B**). MET expression levels were not increased in PC9/ZD cells compared to PC9 cells (**Figure 1C**).

We performed gene expression profiling of PC9 cells and PC9/ZD cells by using DNA microarrays. Since we found >100 genes showed a >10 -fold difference in expression levels between PC9 and PC9/ZD cells, it was difficult to find candidate key genes representing the differences between these cells by searching individual genes. We calculated differences in the molecular pathway networks between these cells by using Meta Gene profiler and ranked the pathways in the order of levels of difference [20]. Because the β-catenin and E-cadherin networks appeared close to the top of the list, we searched for the expression of individual genes that are contained in these pathway networks. We found that N-cadherin was expressed at most different levels between these cells at 70-fold higher amounts in PC9/ZD cells than in PC9 cells. At the protein level, N-cadherin expression was upregulated in PC9/ZD cells but not in PC9 cells, whereas E-cadherin expression was at similar levels in both cell lines (**Figure 1D**). These results suggest that cadherin switching occurred when PC9 cells were transformed to PC9/ZD cells.

N-cadherin inhibition induces apoptotic cell death in PC9/ZD cells

Upregulation of N-cadherin expression has been shown to promote anti-apoptotic activity in several tumor cells, including melanoma cells [24]. This raises a hypothesis that gefitinib-resistant PC9/ZD cells are addicted to N-cadherin signaling. To test this hypothesis, we knocked down N -cadherin expression using siRNAs in PC9/ZD cells (**Figure 2A**). Interestingly, N-cadherin knockdown changed the cell morphology from adherent to floating and the cells took on an unhealthy appearance (**Figure 2B**). These cells

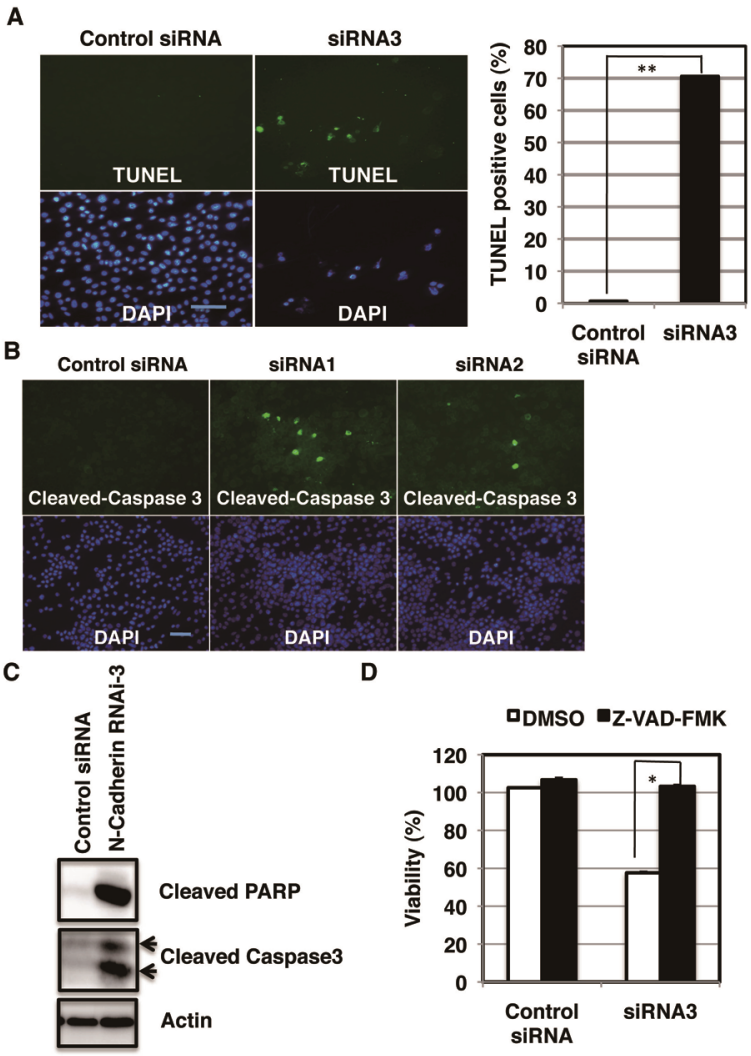

Figure 3. Knockdown of N-cadherin expression induced apoptotic cell death in PC9/ZD cells. (**A**) TUNEL-positive cells are stained green and the nuclei are shown by DAPI staining (left panel). The percentage of viable cells transfected with siRNA for N-cadherin is shown relative to control siRNA-transfected cells after a 5-day transfection (right panel). The results were obtained from at least 3 separate experiments and are indicated as mean (SD). (**B**) Cleaved caspase 3-positive cells are stained green and the nuclei are shown by DAPI staining. Scale bar, 50 μm. (**C**) Cells were lysed after a 5-day siRNA transfection, and the indicated proteins were detected by western blotting. (**D**) The percentage of viable cells transfected with siRNA for N-cadherin is shown relative to control siRNA-transfected cells cultured in the presence or absence of Z-VAD-FMK for 5 days. (*, $p < 0.05$).

showed decreased cell viability without gefitinib treatment (**Figure 2C**). Moreover, N-cadherin-neutralizing antibody treatment also led to decreased cell viability in a dose-dependent manner (**Figure 2D**). These results suggest that the PC9/ZD cells acquired an N-cadherin-dependent survival system.

We examined whether the decrease in cell viability seen after inhibition of N-cadherin expression was caused by apoptotic cell death. TUNEL staining showed that apoptosis was induced in N-cadherin knocked-down cells (**Figure. 3A**). In the process of apoptosis, procaspase-3 is cleaved by active caspase-9 or caspase-8 to become executioner caspase-3 [25]. Active caspase-3 then cleaves nuclear PARP into 2 fragments [26]. We found that caspase-3 and PARP were cleaved when N-cadherin was knocked down by siRNA (**Figures 3B** and **C**) and that DNA fragmentation was observed (data not shown). Furthermore, this decrease in cell viability was rescued by treatment with Z-VAD-FMK, a caspase inhibitor (**Figure 3D**). These data indicate that inhibition of N-cadherin expression led to apoptotic cell death in PC9/ZD cells.

N-cadherin induces Akt-dependent survival signals in PC9/ZD cells

To examine the mechanisms of N-cadherin-dependent cell survival in PC9/ZD cells, we analyzed the activation of Akt, a critical regulator of cell survival, by western blotting. As shown in **Figure 4A**, phosphorylated and activated Akt levels were significantly decreased by siRNA-induced inhibition of N-cadherin expression. Since Akt is a downstream substrate activated by PI-3 kinase, we evaluated whether PI-3 kinase is involved in N-cadherin-dependent Akt signaling in PC9/ZD cells. As shown in **Figure 4D**, treatment with a PI-3 kinase inhibitor (LY294002) induced significant decreases in cell viability in a dose-dependent manner. These observations suggest that N-cadherin mediates

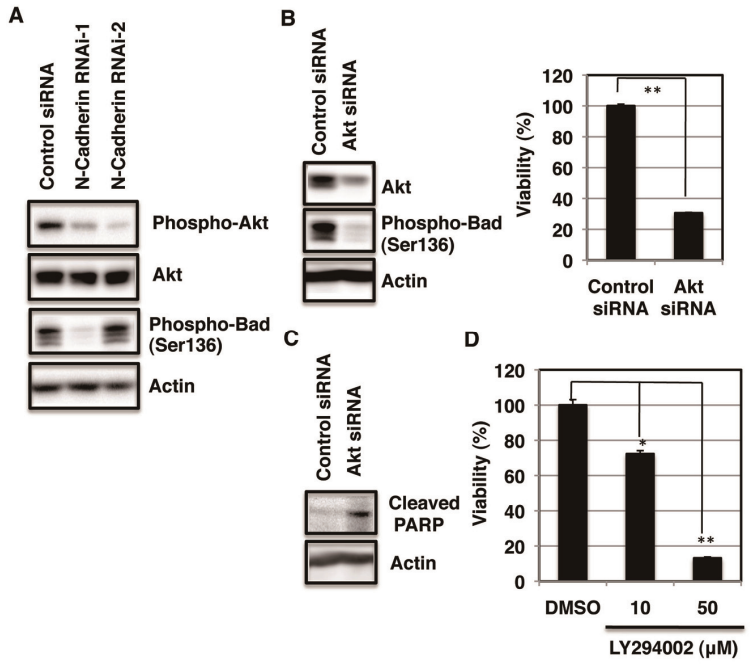

Figure 4. N-cadherin-dependent cell survival signals were mediated by the PI3K/Akt pathway. (**A-C**) Cells were lysed after a 3-day siRNA transfection, and the indicated proteins were detected by western blotting. (**B**) The percentage of viable cells transfected with siRNA for Akt is shown relative to control siRNA-transfected cells after a 5-day transfection (right panel). The results were obtained from at least 3 separate experiments and are indicated as mean (SD). (**D**) The percentage of viable cells treated with LY294002 is shown relative to dimethylsulfoxide (DMSO)-treated controls cultured for 5 days. The results were obtained from at least 3 separate experiments and are indicated as mean (SD) (*, $p < 0.05$; **, $p < 0.01$).

cell survival signaling via PI-3 kinase and Akt.

It is known that activated Akt phosphorylates Bad on Ser136, a proapoptotic protein, to reduce apoptotic activity [27]. We examined the possible role of Bad as a downstream substrate of Akt kinase activity using phosphospecific Bad antibodies. As shown in Figure 4A, N-cadherin inhibition resulted in decreased Bad phosphorylation on Ser136. To investigate whether Akt signaling plays a critical role in the survival of PC9/ZD cells, we examined the effect of Akt knockdown in PC9/ZD cells using siRNA. When Akt expression levels were decreased by Akt siRNA, cell viability was decreased (**Figure 4B**). We also confirmed that Akt inhibition led to apoptotic cell death with cleaved PARP (**Figure 4C**). Taken together, it appears that the N-cadherin-dependent cell survival signal is mediated by activation of the PI-3 kinase/Akt pathway involving Bad phosphorylation.

β-catenin is unlikely to be involved in N-cadherin-dependent survival signaling in PC9/ZD cells

The cytoplasmic region of N-cadherin binds to β-catenin, which in turn binds to α-catenin and interacts with actin filaments [16, 17]. The complex including N-cadherin and β-catenin seems to regulate adhesion function. β-catenin also functions as a transcriptional cofactor in the nucleus. We next examined whether β-catenin plays a role in N-cadherin-dependent survival signaling in PC9/ZD cells. We confirmed the interaction between N-cadherin and β-catenin using co-immunoprecipitation experiments with anti-N-cadherin antibodies (data not shown). We prepared the cytoplasmic and nuclear protein fractions and then examined β-catenin localization. The amounts of β-catenin in the nuclear and cytoplasmic fractions did not differ between N-cadherin knocked-down cells and control cells (**Figure 5A**, left panel). Moreover, knockdown of b-catenin using siRNA had no significant effects on cell viability in PC9/ZD cells (**Figure 5A**, middle and right panels). It is thus unlikely that β-catenin is significantly involved in N-cadherin-dependent survival signaling in PC9/ZD cells.

We then tested whether the homophilic bonds of N-cadherin were important for survival by inhibiting calcium-dependent adhesion with ethylene glycol tetraacetic acid (EGTA), a calcium-chelating reagent. The PC9/ZD cells floated and showed decreased cell viability after EGTA treatment (**Figure 5B**). We checked the effect of cell attachment on survival by culturing the cells on low-attachment dishes. As shown in **Figure 5C**, cell viability was significantly decreased in PC9/ZD cells, but not in PC9 cells, cultured in low-attachment dishes, compared

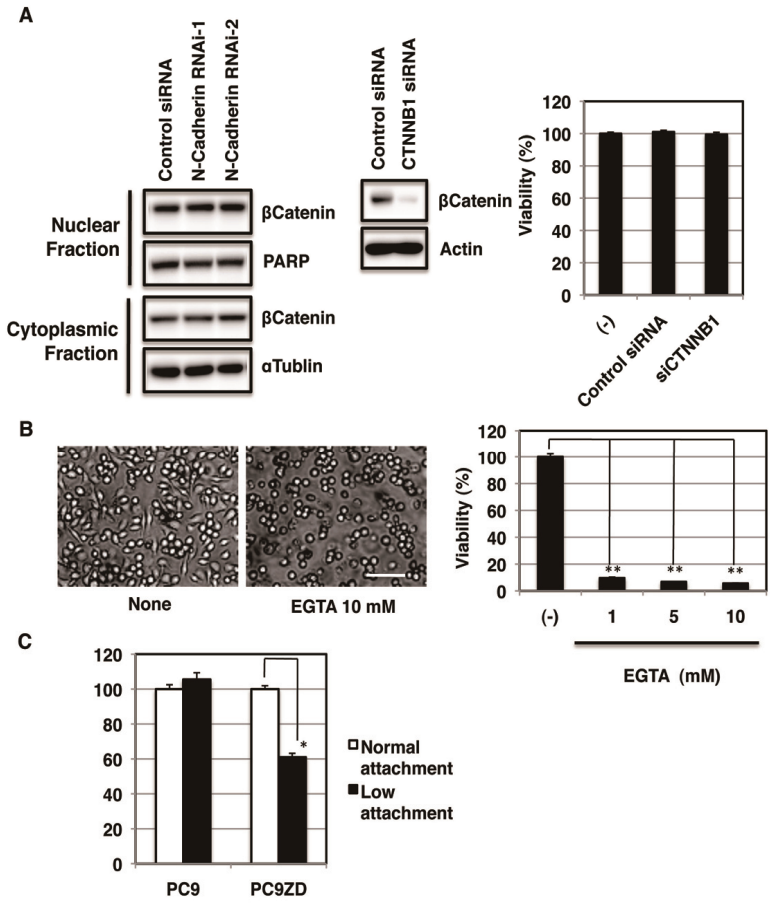

Figure 5. N-cadherin-dependent survival signals were independent of β-catenin activity. (**A**) Cells were lysed after a 5-day siRNA transfection, and the indicated proteins were detected by western blotting (left panel). Cells were lysed after a 48-h siRNA transfection, and the indicated proteins were detected by western blotting (middle panel). The percentage of viable cells transfected with siRNA for β-catenin is shown relative to control siRNA-transfected cells after a 5-day transfection (right panel). The results were obtained from at least 3 separate experiments and are indicated as mean (SD). (**B**) Cell morphology of PC9/ZD cells treated with or without ethylene glycol tetraacetic acid (EGTA) for 72 h (left panel). Scale bar, 50 μm. The percentage of viable cells treated with EGTA for 72 h is shown relative to untreated controls (right panel). (**C**) The percentage of viable cells cultured in low-attachment dishes is shown relative to those cultured in normal-attachment dishes. The results were obtained from at least 3 separate experiments and are indicated as mean (SD) (*, $p < 0.05$; **, $p < 0.01$).

with those cultured in normal attachment dishes. These results suggest that both homophilic bonds of N-cadherin and cell attachment are important for survival signaling in PC9/ZD cells.

N-cadherin inhibition affected the cell viability of gefitinib-resistant lung cancer cell lines

We finally examined whether N-cadherin signaling contributes to the survival of inherently gefitinib-resistant lung cancer cells. It has been reported that the EMT process predicts EGFR-TKI sensitivity in NSCLC [28-30]. Consistent with previous observations, N-cadherin and E-cadherin were expressed reciprocally in these cells, and the cells expressing E-cadherin were sensitive to gefitinib (H3255 and PC3); however, the cells expressing N-cadherin were resistant to gefitinib (A549, H157, and H322) (**Figure 6A** and **B**). We found that inhibition of N-cadherin expression using siRNA led to a significant decrease in viability in A549 and H322 cells but it was not significant in H157 cells (**Figures 6C** and **D**). These data suggest that N-cadherin signaling contributes at least partly to survival of gefitinib-resistant cells.

Discussion

In this study, we have shown that N-cadherin expression was significantly upregulated in gefitinib-resistant PC9/ZD cells harboring the acquired resistant mutation T790M in the *EGFR* gene and that inhibition of N-cadherin expression induced caspase-dependent apoptotic cell death in PC9/ZD cells. N-cadherin activates a cascade of PI-3 kinase/Akt/Bad signaling that results in cell survival. These data demonstrated that N-cadherin expression can mediate potent cell survival signals to the level at which cells depend on its activity for their survival. Furthermore, we demonstrated that N-cadherin can mediate cell survival signals in 2 lung cancer cell lines that show inherent gefitinib resistance but express significant amounts of N-

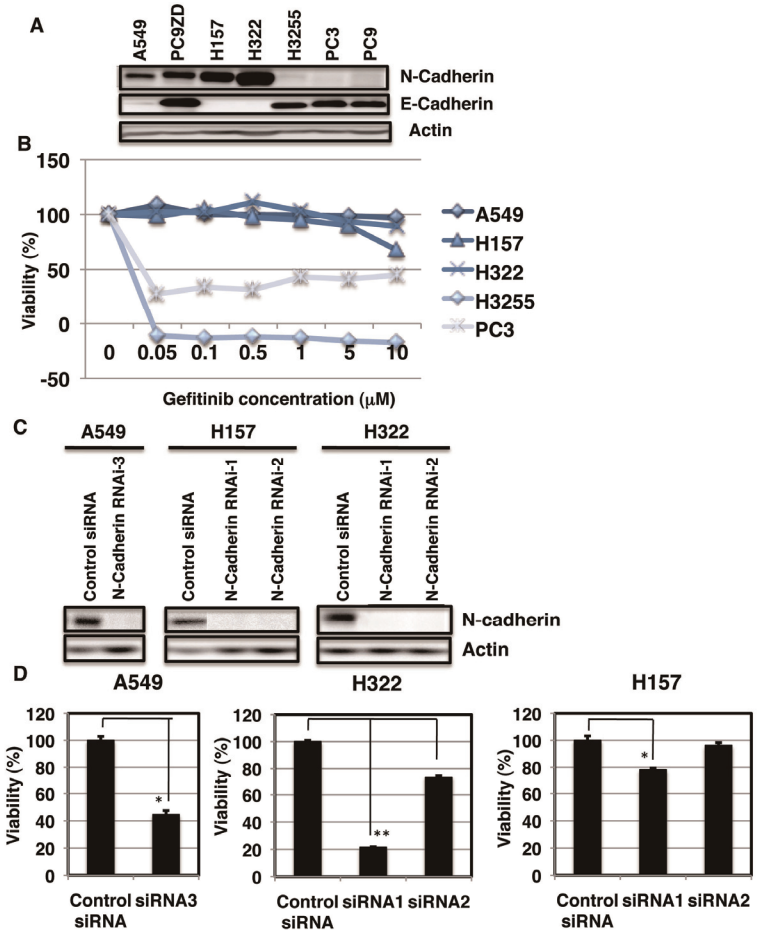

Figure 6. N-cadherin knockdown inhibited cell viability in N-cadherin-expressing gefitinib-resistant lung cancer cells. (**A**) Cells were lysed and the indicated proteins were detected by western blotting. (**B**) The profiles of gefitinib sensitivity in various lung cancer cell lines. The percentage of viable cells is shown relative to untreated controls. The results were obtained from at least 3 separate experiments and are indicated as mean (SD). (**C**) Cells were lysed after a 48-h siRNA transfection, and the indicated proteins were detected by western blotting. (**D**) The percentage of viable cells is shown relative to untreated controls. The results were obtained from at least 3 separate experiments and are indicated as mean (SD) (*, $p < 0.05$; **, $p < 0.01$).

cadherin.

We also found that the cells' dependence on N-cadherin signaling for their survival seemed to vary between cell lines. Therefore, N-cadherin is a promising molecular target of lung cancer; however, it would be important to determine what degree of N-cadherin survival signals is potent in cancer cells in individual lung cancer patients when treated with drugs that target N-cadherin. Drug sensitivity would differ in individual cancer tissues; this is routinely seen in all of the other molecularly targeted drugs that are used in clinical settings.

Many reports have stated that N-cadherin is involved in cancer cell motility and migration, which is involved in EMT and metastasis [18]. It was also reported that N-cadherin mediates tumor angiogenesis since endothelial cells endogenously express N-cadherin. A few studies have shown that N-cadherin is involved in melanoma, ovarian cancer, and prostate cancer cell survival. To the best of our knowledge, our study is the first to demonstrate that N-cadherin mediates survival signal in acquired or inherently gefitinib-insensitive lung cancer cells.

It remains unclear how N-cadherin homophilic binding activates the PI-3 kinase/Akt survival pathway. It was reported that p85 and p110, 2 subunits of PI-3 kinase, were coimmunoprecipitated with anti-N-cadherin antibodies in prostate cancer cells [31]. This suggests that PI-3 kinase is included in the N-cadherin complex and that one or both PI-3 kinase subunits may directly bind to molecules in the N-cadherin complex.

It was reported that activated Akt phosphorylates and inactivates the GSK3β that phosphorylates β-catenin for ubiquitination and degradation [32]. However, we observed a stable amount of β-catenin protein whose localization did not significantly change between the cyto-

plasm and the nucleus by N-cadherin stimulus. Therefore, it does not appear that this pathway was strongly activated by the N-cadherin stimulus in the PC9/ZD cells.

There have been efforts to develop molecularly targeted drugs against N-cadherin, including ADH-1, a polypeptide N-cadherin inhibitor [16]. Clinical trials using ADH-1 have shown that it has promising efficacy as a treatment option for cancer. Our data raises the possibility that ADH-1 is a useful treatment for EGFR-TKI-insensitive NSCLC. In order to test this possibility, we need to conduct extensive studies including *in vivo* tumorigenesis experiments using xenograft models among others. It would also be important to develop diagnostic methods such as biomarkers to identify appropriate NSCLC patients whose cancer tissues express N-cadherin and are sensitive to N-cadherin-targeted therapy.

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Disclosure of potential conflicts of interest

None

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