Podoplanin-Positive Cancer-Associated Fibroblasts in the Tumor Microenvironment Induce Primary Resistance to EGFR-TKIs in Lung Adenocarcinoma with EGFR Mutation

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Abstract

Purpose: The biologic characteristics of microenvironmental constituents, especially cancer-associated fibroblasts (CAF), can be key regulators of the cellular sensitivity to molecular-targeted therapy. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) have marked therapeutic effects against non–small cell lung cancer (NSCLC) with EGFR mutations, but some patients have exhibited primary resistance to EGFR-TKIs. We recently reported that podoplanin-positive fibroblasts are associated with a tumor-promoting phenotype of CAFs in lung adenocarcinoma. The aim of this study was to evaluate whether the susceptibility of NSCLC to EGFR-TKIs could be affected by podoplanin-expressing CAFs.

Experimental Design: We evaluated the EGFR-TKI sensitivity of EGFR-mutant lung adenocarcinoma cell lines cocultured with podoplanin-expressing CAFs. We also examined the association between the expression of podoplanin in CAFs in surgical specimens and EGFR-TKI response of postoperative recurrent patients with EGFR mutations (N = 106).

Results: Lung adenocarcinoma cell lines became more resistant to EGFR-TKI when cocultured with podoplanin-expressing CAFs, compared with control CAFs in vitro. The knockdown of podoplanin expression on CAFs cancelled the resistance to EGFR-TKIs in cancer cells. Compared with control CAFs, the cancer cells that were cocultured with podoplanin-positive CAFs continued to exhibit significantly higher p-ERK levels after treatment with gefitinib. Furthermore, postoperative recurrent patients with podoplanin-positive CAFs had a significantly lower overall response rate to EGFR-TKIs compared with those with podoplanin-negative CAFs (53% vs. 83%; P < 0.01).

Conclusions: Podoplanin-positive CAFs play an important role in primary resistance to EGFR-TKIs and may be an ideal therapeutic target for use in combination therapy with EGFR-TKIs.

Introduction

Epidermal growth factor receptor (EGFR) mutations have recently been reported to be a predictive factor for the efficacy of EGFR tyrosine kinase inhibitors (EGFR-TKI) in patients with advanced non–small cell lung cancer (NSCLC; refs. 1–5). EGFR-TKIs induce a significant response and the prolongation of progression-free survival (PFS), compared with standard first-line cytotoxic chemotherapy, in patients with NSCLC with EGFR activating mutations, such as exon 19 deletions and L858R point mutations (1–7). However, the clinical efficacy of EGFR-TKIs differs among patients with NSCLC harboring EGFR mutations, and about 20% to 30% of patients exhibit primary resistance to EGFR-TKIs despite having tumors that harbor EGFR mutations. In addition, patients who respond initially later develop resistance to EGFR-TKIs after varying periods of time (8, 9). Therefore, the differential efficacy of EGFR-TKIs as first-line chemotherapy and acquired resistances to EGFR-TKIs in patients with the EGFR mutation are major problems in the management of EGFR-mutated lung cancer.

The molecular mechanisms associated with acquired resistance to EGFR-TKIs have been well documented (10–12). On the other hand, there are only a few reports on the mechanism of the primary resistance to EGFR-TKIs. Wang and colleagues (13) reported that hepatocyte growth factor (HGF) confers an intrinsic resistance to EGFR-TKI by activating MET, which restores the phosphorylation of downstream MAPK/ERK1/2 and the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway in lung adenocarcinoma with EGFR mutations. Ng and colleagues (14, 15) also reported that a BIM-deletion polymorphism is associated with intrinsic resistance to EGFR-TKIs.

The biologic characteristics of microenvironmental constituents have recently been reported to be key regulators of resistance...
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Translational Relevance
Cancer-associated fibroblasts (CAF) reportedly influence tumor progression via the secretion of extracellular matrix (ECM) proteins, proteases, cytokines, and growth factors. Recently, the significance of CAFs was reported to be associated with resistance to molecular-targeted therapy. We recently reported that podoplanin-positive fibroblasts are associated with a tumor-promoting phenotype of CAFs in lung adenocarcinoma. This study shows that lung adenocarcinoma cell lines harboring epidermal growth factor receptor (EGFR)-activating mutation became more resistant to EGFR-tyrosine kinase inhibitor (EGFR-TKI) when cocultured with podoplanin-expressing CAFs, compared with control CAFs in vitro. Furthermore, 106 postoperative recurrent patients with podoplanin-positive CAFs had a significantly lower EGFR-TKI response compared with those with podoplanin-negative CAFs. Our studies show that podoplanin-positive CAFs play an important role in primary resistance to EGFR-TKIs and may be an ideal therapeutic target for use in combination therapy with EGFR-TKIs.

to anticancer drugs (16–19). However, which types of noncancerous cells play important roles in the resistance mechanism remain unclear. Cancer-associated fibroblasts (CAF) reportedly influence tumor progression via the secretion of ECM proteins, proteases, cytokines, and growth factors (20–25). Recently, the significance of CAFs was reported to be associated with resistance to molecular-targeted therapy. Straussman and colleagues (16) reported that HGF secreted by CAFs induced intrinsic resistance to an RAF inhibitor in BRAF-mutant melanoma. However, the heterogeneity of CAFs is well known, and different types of CAFs are thought to have distinct functional contributions to the sensitivity of cells to molecular-targeted therapy.

We previously demonstrated that CAFs expressing podoplanin (PDPN) enhance tumor progression in a mouse model and that podoplanin expression in CAFs predicts a poorer outcome in patients with lung adenocarcinoma (26–28). Interestingly, CAFs expressing podoplanin are observed more frequently in poorly differentiated adenocarcinoma (solid with mucin subtype; ref. 28). Furthermore, among patients with patients with lung adenocarcinoma harboring EGFR mutations, a poorly differentiated histology was significantly associated with primary resistance to EGFR-TKIs (29). Considering these results, we hypothesized that podoplanin-expressing CAFs were correlated with primary resistance to EGFR-TKIs. In this study, we evaluated whether the susceptibility to EGFR-TKIs could be affected by cross-talk with podoplanin-expressing CAFs.

Materials and Methods

Cell culture and reagents
The EGFR-mutant human lung adenocarcinoma cell lines PC-9 (del E746_A750) and HCC827 (del E746_A750) were purchased from the European Collection of Cell Culture and the American Type Culture Collection at 2011 and 2013, respectively, and stocked in liquid nitrogen (30–32). In this study, we resuscitated these cell lines from the stocks for the experiment and used in fewer than 6 months after resuscitation. All cells had been routinely tested and were negative for Mycoplasma. The PC-9 and HCC827 cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma), 1% glutamine, and antibiotics (1% penicillin and streptomycin; Sigma). The cultures were incubated at 37 °C in an atmosphere containing 5% CO2. Gefitinib was obtained from Tocris Bioscience.

Fibroblast culture
CAFs were prepared from human lung cancer tissue, as previously reported (33, 34). The characteristics of the adenocarcinoma cases from which primary CAFs were obtained are shown in Supplementary Table S1.

Transfection
The lentiviruses were produced using 293T cells transfected with PCAG-HIV, CMV-VSV-G-RSV-Rev, and either human-WT-PDPN, PDPN-Del IC vector (CSII-CMV-BA-E1B2-Venus; ref. 35), CSII-CMV-mRFP1 vector (RIKEN BioResource Center), or podoplanin short hairpin (sh) RNA vector (CS-H1-shRNA-EG, RIKEN BioResource Center; ref. 27). Transfection was performed using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Vector-containing medium was filtered through a 0.45-mm filter, and 8 μg/ml (final concentration) of polybrene (Sigma) was added for target cell transduction. PC-9 and HCC-827 were labeled with mRFP, and the CAFs were labeled with Venus (Supplementary Fig. S1).

The podoplanin mRNA level in CAFs overexpressing PDPN-WT (CAF-PDPN-WT) was shown in Supplementary Fig. S2.

Coculture of cancer cells with CAFs
The Venus-labeled CAFs (2 × 104 cells) were plated into each well of 12-well plates, together with mRFP-labeled cancer cells (2 × 104 cells) on day 1. On day 2, the medium was replaced with fresh medium, and the cells were treated with gefitinib (0.3 μmol/L) or dimethylsulfoxide (DMSO). On day 5, the number of mRFP-labeled cancer cells was measured using a FACSCaliber (BD Biosciences) to discriminate the cancer cells from the CAFs (Fig. 1A). The percentage of cell number was determined relative to untreated controls without CAFs. Each experiment was performed at least three times, each with triplicate samples.

Coculture of cancer cells and CAFs in separate chambers
To evaluate whether the CAF-mediated resistance to gefitinib involved the secretion of soluble factors, cancer cells and CAFs were cultured separately in Transwell chambers with 1-μm pore filters (BD Biosciences). The cancer cells were placed in the bottom chamber, and the CAFs were placed in the top chamber. The number of cancer cells was measured using the same method as described in the previous section.

Real-time RT-PCR
Cells were washed with PBS and suspended in 1 mL of TRIzol (Invitrogen), and then stored at −80 °C. Total RNA was purified from thawed samples using standard techniques, and cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa), according to the manufacturer’s instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in a Smart Cycler System (TaKaTa) using SYBR Premix Ex Taq (TaKaRa) and Real-time PCR Primers for HGF (forward, 5′-ACTGGACACCAGTGCTATAGA-3′ and reverse, 5′-CAGTGACTGATGCATATTGA-3′). podoplanin
Figure 1.
Podoplanin-positive CAFs induce gefitinib resistance in lung cancer cells. A, the mRFP-labeled cancer cells (PC-9 or HCC-827, line) and the Venus-labeled CAFs (dot) were discriminated using flow cytometry. B, total cell numbers of mRFP-labeled PC-9 or mRFP-labeled HCC-827 cells treated for 72 hours with gefitinib in the presence or absence of Venus-labeled CAF-Ctrl and PDPN-WT. The error bars show the mean ± SD. Asterisk, *P* < 0.05 according to the Student *t* test. C, schema showing the coculture system. The cancer cells and CAFs were plated in the same well (left). The tumor cells and CAFs were separated by Transwell chambers (right). D, total number of mRFP-labeled PC-9 or HCC-827 cells treated with gefitinib for 72 hours in the presence or absence of Venus-labeled CAF-Ctrl and PDPN-WT in the separate coculture system. The error bars show the mean ± SD. Asterisk, *P* < 0.05 according to the Student *t* test.
Podoplanin-Positive CAFs Induce Resistance to EGFR-TKI

Nonlabeled PC-9 and Venus-labeled CAFs were plated 1 day before gefitinib at a density of 3.4 × 10^5 cells each in a 10-cm plate. One and 24 hours after gefitinib treatment, the cells were fixed by the addition of BD Phosflow Fix Buffer at room temperature for 15 minutes and then permeabilized in ice-cold BD Perm Buffer III for 24 hours at −20°C. The samples were washed with BD statin buffer and stained for 1 hour with conjugated phospho-Akt XP (Ser473; Alexa Fluor-647; Cell Signaling Technology), phospho-p44/42 MAPK XP (Erk1/2; Thr202/Tyr204; Alexa Fluor-647; Cell Signaling Technology), and IgG isotype control (Alexa Fluor-647; Cell Signaling Technology). A fluorescence-activated cell sorting (FACS) analysis was performed 1 and 24 hours after treatment with gefitinib. We selected a region of the Venus-negative cells as cancer cells for analysis. The relative mean fluorescence intensity (MFI) of p-Akt and p-ERK was defined as the MFI of each group divided by the MFI of PC-9 without gefitinib.

Measurement of phospho-Akt and ERK1/2

Nonlabeled PC-9 and Venus-labeled CAFs were plated 1 day before gefitinib. We selected a region of the Venus-negative cells as cancer cells for analysis. The relative mean fluorescence intensity (MFI) of p-Akt and p-ERK was defined as the MFI of each group divided by the MFI of PC-9 without gefitinib.

Postoperative recurrence in patients with lung adenocarcinoma harboring an EGFR-activating mutation who were treated with an EGFR-TKI

Between January 2002 and December 2012, 134 postoperative recurrence patients with lung adenocarcinoma harboring an EGFR-activating mutation were treated with an EGFR-TKI (250 mg/day of gefitinib or 150 mg/day of erlotinib, administered orally) at our institution. We selected 105 patients who were treated with EGFR-TKIs as first-line chemotherapy. To evaluate the association between a podoplanin-positive CAF status and the response to EGFR-TKIs in this study, we defined assessable patients as follows: presence of evaluable lesions according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (36).

Histopathologic analysis and evaluation of clinicopathologic factors

All the surgical specimens were fixed with 10% formalin or absolute methanol and were embedded in paraffin. Serial 4-μm sections were stained using hematoxylin and eosin. Two observers (T. Yoshida and G. Ishii) who were unaware of the clinical data independently reviewed all the pathologic slides. The histologic diagnosis was based on the 2004 World Health Organization histologic classification. In addition, lung adenocarcinomas were classified according to the 2011 IASLC/ATS/ERS International Multidisciplinary Classification of lung adenocarcinomas into the following subtypes: lepidic predominant subtype, papillary predominant subtype, acinar predominant subtype, micropapillary predominant subtype, and solid predominant subtype (37). The predominant subtype was defined as the histologic component that comprised the largest percentage among the components.

Analysis of EGFR status and EGFR-TKI response

We evaluated the activating EGFR mutations (deletions in exon 19, L858R point mutation, L861Q point mutation, and G719 missense point mutations) using either the direct sequencing method, Cycleave PCR (SRL), the peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp method (Chemical Medicine), or a PCR-Invader Assay (BML). The objective tumor response was assessed according to the RECIST version 1.1 (36). The objective response rate (ORR) was calculated as the total percentage of patients with a complete response (CR) or a partial response (PR). A clinical evaluation of PFS was measured from the start of the first-line chemotherapy to the earliest identifiable sign of disease progression, as determined using CT or MRI imaging using the RECIST, or any cause of death.

Immunohistochemistry

Immunohistochemistry was performed according to a previously reported method (28, 38). Tissue sections were stained overnight at 4°C with a mouse anti-human podoplanin antibody (D2-40; Signet Laboratories) at a final dilution of 1:50. The slides were subsequently incubated with EnVision (Dako) for 1 hour at room temperature.

Statistical analysis

All the statistical analyses were performed using JMP for Windows version 9 statistical software package (SAS Institute). The χ² test or the Fisher exact test was used to determine the statistical significance of differences between two groups. The PFS and overall survival (OS) were estimated using the Kaplan–Meier method, and differences in the variables were calculated using the log-rank test. A multivariate regression analysis was conducted according to the Cox proportional hazard model. All the P values were two-sided, and a level of 5% was considered statistically significant, unless otherwise specified. This study was approved by the Institutional Review Board of the National Cancer Center (Kashiwa, Chiba, Japan).

Results

Coculture with CAF PDPN-WT induces gefitinib resistance in lung cancer cell lines

PC-9 was highly sensitive to gefitinib, and the total cell number after gefitinib treatment for 72 hours was reduced to 28.2% ± 1.3% (mean ± SD) of the total cell number without gefitinib treatment. When PC-9 cells were cocultured with Venus-labeled CAF (CAF-Ctrl), 42.9% and 1.3% (mean ± SD) of the total cell number were resistant to gefitinib (P = 0.001). When cocultured with CAF-PDNP-WT, the number of cells resistant to gefitinib further increased significantly to 57.2% ± 3.6% (Fig. 1B, left). CAF-Ctrl and CAF-PDNP-WT did not affect growth of cocultured PC-9 in the absence of gefitinib. This resistance as a result of CAF-PDNP-WT was also shown when these cells were cocultured with another EGFR-mutated lung adenocarcinoma cell line, HCC-827 (Fig. 1B, right). Furthermore, we examined the same assay using five other primary CAFs and obtained similar results when PC-9 cells were cocultured with four of the five CAFs and when HCC-827 cells were cocultured with three of the five CAFs (Supplementary Fig. S3).

Separated coculture with CAF PDNP-WT did not affect gefitinib resistance

To determine whether the resistance to gefitinib was mediated by direct contact between the CAFs and the cancer cells, cancer cells and CAFs were cultured separately using Transwell chambers (Fig. 1C). The total cell number of PC-9 cells after gefitinib treatment in the absence of CAFs was 30.0% ± 1.3%. When the PC-9 cells were separately cultured with CAFs-Ctrl or CAFs-PDNP-WT, the total
cell number of PC-9 cells was 29.4% ± 3.0% and 27.2% ± 2.1%, respectively, and no significant difference was observed between the two groups (Fig. 1D). These results suggested that the resistance to gefitinib induced by CAF-PDPN-WT was mediated by direct contact between the CAFs, and not by soluble factors.

Podoplanin in CAF is a functional protein involved in the resistance of cancer cells to gefitinib

To confirm whether podoplanin is a functional protein involved in the resistance of cancer cells to gefitinib, podoplanin expression in CAF was decreased by specific shRNA-targeting podoplanin (CAF-sh-PDPN). The expression level of podoplanin in the transduced cells was confirmed using FACS (Fig. 2A). The PC-9 cells that were cocultured with CAF-sh-PDPN displayed significantly less resistance to gefitinib than those cocultured with CAF-sh-luciferase (sh-PDPN 1: 38.3% ± 3.0%, sh-PDPN 3: 37.1% ± 2.1% vs. sh-luciferase: 51.0% ± 3.2%; P < 0.05). This phenomenon was also confirmed using HCC-827 cells (sh-PDPN 1: 19.4% ± 2.7%, sh-PDPN 3: 17.1% ± 0.3% vs. sh-luciferase: 40.6% ± 3.1%; P < 0.05; Fig. 2B). CAF-sh-PDPN and CAF-sh-luciferase did not affect growth of cocultured PC-9 and HCC-827 in the absence of gefitinib (Fig. 2C).

We used CAFs expressing a podoplanin mutation in which the cytoplasmic domain of podoplanin was deleted (CAF-PDPN-Del.IC). The PC-9 and HCC-827 cell numbers in the presence of CAF-PDPN-Del.IC were significantly lower than those in the presence of CAF-PDPN-WT (PC-9: 39.9% ± 4.6% vs. 59.9% ± 3.2%, HCC-827: 21.7% ± 1.7% vs. 49.9% ± 2.0%; Fig. 2D).

Phosphorylation status of ERK and Akt after gefitinib treatment

Both the MAPK pathway and the PI3K pathway have been suspected of being involved in primary and acquired resistance to EGFR-TKIs. We assessed the phosphorylation of ERK and Akt in PC-9 cells cocultured with CAFs after 1 hour of gefitinib treatment. Figure 3A contains a histogram showing the phosphorylation of Akt and ERK in cancer cells treated with gefitinib and without gefitinib. The relative MFIs of the p-Akt level and the p-ERK level in PC-9 cells treated with gefitinib were 0.46 ± 0.04 and 0.14 ± 0.01, respectively. On the other hand, the relative MFIs of the PC-9 cells after gefitinib treatment in a coculture with control CAFs increased to 0.59 ± 0.08 (p-Akt) and 0.59 ± 0.04 (p-ERK). The relative MFIs of p-Akt and p-ERK after gefitinib treatment further increased to 0.69 ± 0.11 and 0.80 ± 0.02, respectively, when the cells were cocultured with CAF-PDPN-WT (Fig. 3B and C). The relative MFI of p-ERK after coculture with CAF-PDPN-WT, in particular, was significantly higher than that after coculture with CAF-Ctrl (P = 0.03). Without gefitinib treatment, the relative MFIs of the p-ERK and p-Akt level of PC-9 cocultured with CAFs-PDPN-WT were not different from those of PC-9 cocultured with CAFs-Ctrl. The relative MFIs of p-Akt and p-ERK at 24 hours after treatment with gefitinib exhibited similar results (data not shown).

Association between podoplanin expression of CAFs and EGFR-TKI response

We next examined the association between the podoplanin expression of CAFs in surgical specimens and the EGFR-TKI response. As for podoplanin expression in the CAFs, a specimen was judged to be positive if at least 10% of the fibroblasts showed an unequivocal reaction for podoplanin that was equal to that of lymphatic endothelial cells, according to a previously described definition (Fig. 4A and B; refs. 19, 28). The number of the cases according to the podoplanin-positive CAF area/stromal area (%) is shown in Supplementary Fig. S4. The patient characteristics are listed in Table 1. When examined according to the EGFR mutation status, exon 19 deletion, L858R point mutation, and minor mutations, such as G719X and L861Q, were detected in 46, 56, and 4 of the patients, respectively. The pathologic characteristics of the surgical specimens are summarized in Supplementary Table S2. Of the 106 specimens, 57 were positive for podoplanin in CAFs. In a response evaluation of EGFR-TKIs, 70 of the 106 patients with evaluable lesions according to the RECIST criteria (version 1.1) had PR, 24 patients had stable disease, 10 patients had progressive disease, and 2 patients were not evaluable; the ORR was 66% for all the patients. The patients with podoplanin-positive CAFs had a poorer response rate than those with podoplanin-negative CAFs (53% vs. 83%; P < 0.01; Table 2), indicating that patients with lung adenocarcinoma with podoplanin-expressing CAFs had a significantly poorer response to treatment with EGFR-TKIs. In addition, the DFS in the patients with podoplanin-positive CAFs was shorter than that of patients with podoplanin-negative CAFs [9.6 vs. 15.6 months; HR, 1.763; 95% confidence interval (95% CI), 1.146–2.713; P = 0.0099; Fig. 4C]. The univariate and multivariate analysis showed that podoplanin expression in CAFs was the predictive factor of the response duration with EGFR-TKI (podoplanin-positive CAFs: HR, 1.999; 95% CI, 1.187–3.416; P = 0.009; Supplementary Table S3). Next, we evaluated the correlation between podoplanin expression status and response to EGFR-TKIs in patients with NSCLC common mutations, exon 19 deletion, and L858R (N = 102). The outcome of overall response rate and DFS is shown in Supplementary Table S4 and Supplementary Fig. S5. Although not significant, clinical response to EGFR-TKIs in the patients with podoplanin-positive CAFs was worse than those with podoplanin-negative CAFs according to EGFR common mutations, exon 19 deletion, and L858R.

Discussion

Although the underlying mechanisms explaining how CAFs modulate drug resistance remain poorly understood, a few previous reports have shown that HGF secreted by CAFs influences resistance to anticancer drugs, especially oncoprotein-targeted drugs, such as EGFR-TKIs, in NSCLC tumors harboring EGFR mutations (13) and to a RAF inhibitor in melanoma harboring BRAF mutations (28). However, CAFs represent a heterogeneous cell population with diverse biologic properties. In this study, we found that a special subpopulation of CAFs, podoplanin-expressing CAFs, influenced the resistance to EGFR-TKIs in cancer cells in vitro. Moreover, among patients with lung adenocarcinoma harboring EGFR-activating mutations, cases with the presence of podoplanin-expressing CAFs had a relatively poor response to EGFR-TKIs. This study is the first report to indicate that certain types of CAFs can influence the primary resistance to EGFR-TKIs in cancer cells.

In this study, the knockdown of podoplanin expression on CAFs cancelled the resistance to EGFR-TKIs in cancer cells. Moreover, CAFs expressing a podoplanin mutation, in which the cytoplasmic domain of podoplanin was deleted, did not reduce the sensitivity to EGFR-TKIs. These findings indicated that podoplanin, especially the cytoplasmic domain on CAFs, plays functionally important roles in the resistance of cancer cells to EGFR-
Podoplanin in CAFs is a functional protein involved in the resistance of cancer cells to gefitinib. A, expression of podoplanin in control or podoplanin shRNA-infected CAFs. The expression of podoplanin on the cell surface was detected using flow cytometry. Total cell numbers of mRFP-labeled PC-9 and HCC-827 after treatment with gefitinib when cocultured (B and C) with CAF-sh-PDPN or CAF-sh-luciferase and (D) with CAF-PDPN-WT or CAF-PDPN-Del.IC. The error bars show the mean ± SD. Asterisk, $P < 0.05$ according to the Student t test.

Figure 2.
Podoplanin-Positive CAFs Induce Resistance to EGFR-TKI
TKIs. Resistance to EGFR-TKIs was not observed when cancer cells and podoplanin-positive CAFs were cocultured separately, suggesting that direct contact between CAFs and cancer cells is required for this phenomenon. Furthermore, there was no apparent difference between podoplanin-positive CAFs and control CAFs regarding the HGF expression levels, which are reportedly associated with the resistance of cells to EGFR-TKIs (Supplementary Fig. S6).

The molecular mechanism explaining how podoplanin-positive CAFs cause resistance to EGFR-TKIs remains unclear. One possibility is that intracellular RhoA activation in podoplanin CAFs influences the resistance to EGFR-TKIs. We previously reported that RhoA activity was higher in podoplanin-positive fibroblasts, resulting in the promotion of tumor engraftment (35). RhoA activation also influences ECM remodeling and regulates gene expression through various transcription factors, such as nuclear transcription factor kappa-β (39). We evaluated whether CAF expressing a constitutive active form of RhoA (G14VRhoA) promotes resistance to gefitinib. However, the CAFs with this mutation were unable to promote resistance to gefitinib (data not shown). The second possibility was that podoplanin-positive CAFs interact directly with PC-9 cells through CLEC-2, the only known receptor for podoplanin described so far (40). However, PC-9 cells were negative for CLEC-2 when examined using quantitative RT-PCR (data not shown), suggesting that podoplanin-positive CAFs did not bind directly to cancer cells through CLEC-2. Although CAF-PDPN-Del.IC decreased the level of resistance to gefitinib to a level similar to that observed for CAF-Ctrl, the possibility that the cytoplasmic deletion might have induced a conformational change in the extracellular domain of podoplanin, resulting in an

Figure 3.
Phosphorylation status of ERK and Akt after gefitinib treatment. A, flow cytometry analysis of p-Akt and p-ERK levels in PC-9 cells at 1 hour after gefitinib treatment in the presence or absence of Venus-labeled CAF-Ctrl and PDPN-WT. B and C, relative MFI of p-Akt and p-ERK after gefitinib treatment. The error bars show the mean ± SD. Asterisk, *P < 0.05 according to the Student t test.

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ineffectual signal transduction via podoplanin ligands other than CLEC-2 on the tumor cells, cannot be excluded. Therefore, we think the possibility that signal transduction through the ligand for podoplanin causes the resistance to EGFR-TKIs of cancer cells after direct contact with CAFs.

To further examine the molecular mechanism, we evaluated the p-Akt and p-ERK levels in PC-9, which are associated with the downstream signaling pathway of several receptor tyrosine kinases, such as EGFR. Cancer cells cocultured with podoplanin-positive CAFs continued to exhibit significantly high p-ERK levels after treatment with gefitinib, compared with the levels observed after coculturing with control CAFs. This phenomenon suggests that podoplanin-positive CAFs influence the intracellular signaling pathway, especially the MAPK signaling pathway, in cancer cells. Mink and colleagues (41) reported that gefitinib treatment caused >50% inhibition of the pMAPK signal on HCC-827 in coculture with the CAFs isolated from gefitinib sensitive tumors, whereas the presence of the CAFs isolated from gefitinib resistant tumors resulted in less than a 15% inhibition. On the other hands, no significant changes were observed in p-Akt level. These outcomes were, in part, consistent with this study.

Table 1. Patient characteristics (N = 106)

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<tr>
<td></td>
<td>Positive N (%)</td>
<td>Negative N (%)</td>
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<td>Number of patients</td>
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<tr>
<td>Gefitinib</td>
<td>53 (93)</td>
<td>48 (98)</td>
<td>0.370</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>4 (7)</td>
<td>1 (2)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Overall response to EGFR-TKI for patients with podoplanin-positive CAFs (N = 57) and podoplanin-negative CAFs (N = 49)

<table>
<thead>
<tr>
<th>Objective response</th>
<th>Podoplanin expression of CAFs</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive N (%)</td>
<td>Negative N (%)</td>
<td>P</td>
</tr>
<tr>
<td>ORR</td>
<td>53%</td>
<td>85%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CR or PR</td>
<td>30</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>17</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: NE, not evaluable; PD, progressive disease; SD, stable disease.
Finally, we evaluated the association between podoplanin expression in CAFs and the EGFR-TKI response in patients with postoperative recurrences harboring EGFR mutations. As a result, the overall response rate in the patients with podoplanin-positive CAFs was significantly lower than that of patients with podoplanin-negative CAFs. The PFS period in the patients with podoplanin-positive CAFs was shorter than that of patients with podoplanin-negative CAFs; these results were consistent with the in vitro results. It remains to be unclear whether this survival difference truly relates to the resistance to EGFR-TKIs or other aspects of tumor biology. We evaluated time to recurrence in this cohort. In both podoplanin-positive and PDPN-negative patients, there was no significant difference in time to recurrence (data not shown). Therefore, we think that podoplanin CAFs truly modulates the survival time and PFS in patients treated with EGFR-TKIs. However, this study included retrospective clinical data, and did not include the other resistance mechanisms to EGFR-TKIs. In fact, the resistant to EGFR-TKIs has been reported to associate with various mechanisms, such as EGF R T790M, MET amplification, and a BLM-deletion polymorphism, etc. Future studies are thus required to confirm the predictive significance of podoplanin-positive CAFs on the response to EGFR-TKIs in validated populations using a multicenter trial.

It would be of obvious important to investigate whether the sensitivity-modulatory effect of different patient-derived CAFs is linked to clinical response. In this study, we used CAFs from 6 patients with adenocarcinoma. Only 1 of these 6 patients had postoperative recurrence and received gefitinib treatment. Therefore it is difficult to evaluate this problem and accumulation of the further cases is required. In conclusion, this study showed that podoplanin-positive CAFs were useful for predicting a response to EGFR-TKIs in patients harboring EGFR mutations. These findings suggest that the effect of chemotherapy may depend on not only the cancer cells, but also the tumor microenvironment surrounding the cancer cells. Our results provide novel insights into a new mechanism of resistance to targeted drugs, although the exact mechanism responsible for the podoplanin CAF-mediated resistance is not fully understood. In the future, combination treatment with targeted drugs for both cancer cells, and podoplanin-positive CAFs might be important for overcoming resistance to EGFR-TKIs or for strengthening the effect of EGFR-TKIs.

Disclosure of Potential Conflicts of Interest

Y. Ohe reports receiving speakers bureau honoraria from AstraZeneca, Chuigai, Daichi-Sankyo, Lilly, ONO, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: T. Yoshida, G. Ishii, S. Neti, H. Ohmatsu, A. Ochiai
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Yoshida, G. Ishii, K. Goto, S. Neti, K. Yoh, S. Umemura, S. Matsumoto
Writing, review, and/or revision of the manuscript: T. Yoshida, G. Ishii, K. Goto, K. Nagai, A. Ochiai

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