

Collagen type I induces EGFR-TKI resistance in EGFR-mutated cancer cells by mTOR activation through Akt-independent pathway

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Primary resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) is a serious problem in lung adenocarcinoma patients harboring *EGFR* mutations. The aim of this study was to examine whether and how collagen type I (Col I), the most abundantly deposited matrix in tumor stroma, affects EGFR-TKI sensitivity in EGFR-mutant cells. We evaluated the EGFR-TKI sensitivity of *EGFR*-mutated cancer cells cultured with Col I. Changes in the activation of downstream signaling molecules of EGFR were analyzed. We also examined the association between the Col I expression in tumor stroma in surgical specimens and EGFR-TKI response of postoperative recurrence patients with EGFR mutations. Compared to cancer cells without Col I, the survival rate of cancer cells cultured with Col I was significantly higher after EGFR-TKI treatment. In cancer cells cultured with and without Col I, EGFR-TKI suppressed the levels of phosphorylated (p-)EGFR, p-ERK1/2, and p-Akt. When compared to cancer cells without Col I, expression of p-P70S6K, a hallmark of mTOR activation, was dramatically upregulated in cancer cells with Col I. This activation was maintained even after EGFR-TKI treatment. Simultaneous treatment with EGFR-TKI and mTOR inhibitor abrogated Col I-induced resistance to EGFR-TKI. Patients with Col I-rich stroma had a significantly shorter progression-free survival time after EGFR-TKI therapy (238 days vs 404 days; $P < .05$). Collagen type I induces mTOR activation through an Akt-independent pathway, which results in EGFR-TKI resistance. Combination therapy using EGFR-TKI and mTOR inhibitor could be a possible strategy to combat this resistance.

KEYWORDS

collagen type I, EGFR-activating mutation, EGFR-TKI, lung adenocarcinoma, mTOR

1 | INTRODUCTION

Epidermal growth factor receptor (EGFR) activating mutations such as exon 19 deletions and L858R point mutations are predictive factors for the effect of EGFR tyrosine kinase inhibitors (EGFR-TKIs) in patients with advanced non-small-cell lung cancer (NSCLC). Epidermal growth factor receptor tyrosine kinase inhibitors prolong progression-free survival (PFS) in patients with NSCLC compared to standard first-line cytotoxic chemotherapy.¹⁻⁷ However, 20%-30% of the patients show primary resistance to EGFR-TKI, although they harbor EGFR activating mutations.^{8,9} Moreover, initial responders to EGFR-TKI treatment acquire resistance and develop recurrence after varying periods.¹⁰⁻¹² Therefore, the primary and acquired resistance to EGFR-TKI in patients with EGFR activating mutations is a critical problem associated with EGFR-TKI treatment.

EGFR T790M mutation, Met amplification, hepatocyte growth factor (HGF) overexpression, epithelial-mesenchymal transition (EMT), and transformation to small-cell lung cancer¹⁰⁻¹² have been reported as acquired resistance factors to EGFR-TKI. However, the molecular mechanism of primary resistance is still poorly understood. Park et al¹³ reported that CRIPTO1 expression is associated with primary resistance to EGFR-TKI. Faber et al and Ng et al^{14,15} reported a correlation between BIM deletion and primary resistance to EGFR-TKIs. Yano et al and Engelman et al^{16,17} showed that the HGF-MET pathway induces resistance to gefitinib.

The biochemical characteristics of the tumor microenvironment were delineated as key regulators of resistance to anticancer drugs.¹⁸⁻²¹ The cancer stroma is comprised of various types of immune cells, endothelial cells, fibroblasts, and extracellular matrix (ECM).²²⁻²⁴ Within the ECM, collagen type I (Col I) is the most abundantly deposited matrix in the cancer stroma and is produced mainly by fibroblasts. Collagen type I plays an important role in homeostasis in normal tissue as well as cell growth, invasion, and metastasis in cancer tissue.^{25,26} Furthermore, Col I has been reported to impact drug sensitivity.

Collagen type I contributes to gemcitabine resistance in pancreatic cancer through membrane type I-matrix metalloproteinase-mediated high mobility group A2 expression.²⁷ Moreover, integrins such as the Col I receptor in cancer cells promote Src-Akt pathway activation and induce erlotinib resistance in lung cancer cells.²⁸ However, little has been reported on the influence of Col I on the EGFR-TKI sensitivity of cancer cells.

In the current study, we examined whether Col I can influence the resistance of cancer cells to EGFR-TKI. We also explored the molecular mechanism associated with resistance induced by Col I.

2 | MATERIALS AND METHODS

2.1 | Cell lines and reagents

The EGFR mutant (Δ E746-A750) human lung adenocarcinoma cell lines PC-9 and HCC 827 were obtained from the ECACC (UK) and ATCC (Manassas, VA, USA), respectively. The EGFR WT human lung

adenocarcinoma cell lines A427, A549, and H1299 cells were obtained from the ATCC.

PC-9, HCC827, A427, and H1299 cells were maintained in RPMI-1640 (Sigma-Aldrich, MO, USA) supplemented with 10% FBS (Life Technologies, NY, USA), 1% penicillin-streptomycin (Sigma-Aldrich), and L-glutamine (Life Technologies). A549 cells were maintained in DMEM/F-12 (Sigma-Aldrich) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

2.2 | Fluorescence labeling of cells

Lentiviruses were produced using 293T cells transfected with pCAG-HIV, pCMV-VSV-G-RSV-Rev, and either CSII-CMV-mRFP1 or CS-CDF-CG-PRE (Riken BioResource Center, Japan), using the Lipofectamine 2000 reagent (Invitrogen, NY, USA) according to the manufacturer's instructions. Virus-containing medium was filtered through a 0.45- μ m filter, and 8 μ g/mL (final concentration) polybrene (Santa Cruz Biotechnology, Dallas, TX, USA) was added for target cell transduction as previously reported.²¹

2.3 | Cell culture on collagen gel

An aliquot of 100 μ L collagen solution (Nitta Gelatin, Japan) was added to the 0.4- μ m pore membrane culture insert in a 24-well plate (BD Bioscience, NJ, USA). This was incubated for 24 hours at 37°C to polymerize. PC-9-monomeric red fluorescent protein (mRFP) or HCC-827-mRFP cells (8.5×10^3) were plated onto the polymerized collagen gels. The upper and lower chambers of each well were filled with growth medium (Figure S1A).

The control consisted of cells directly seeded onto the membrane culture insert. This system enables the measurement of both the cytotoxic response and morphophenotypic changes of cancer cells (Figure S1B,C).

2.4 | Measurement of cell survival ratio

We first examined whether the fluorescence area ratio correlated with the number of seeded cells in this system. Varying numbers of PC9-mRFP cells (5×10^4 , 1.5×10^5 , 2.5×10^5 , and 3.5×10^5 cells/mL) were plated onto a membrane culture insert. At 12 hours post seeding, the fluorescent area of mRFP-labeled cells was measured using BZ-9000 (Keyence, Japan). The cell number and corresponding fluorescent area significantly correlated in this system. The model in both the absence and presence of collagen revealed a perfect positive correlation (r^2 value $\geq .97$ and r^2 value $\geq .99$, respectively) (Figure S2). The number of mRFP-labeled cancer cells were calculated using the above formula. Each experiment was carried out at least three times.

2.5 | Epidermal growth factor receptor-TKI treatment

On day 1 after seeding, the medium was replaced with fresh medium with gefitinib (0.3 μ mol/L) (Tocris Bioscience, UK) or dimethyl

sulfoxide (DMSO) (Sigma-Aldrich). The cell survival ratio compared to that of the DMSO-treated control was calculated following 72 hours of exposure to gefitinib. To assess the effects of mTOR inhibitor, we used rapamycin (Tokyo Chemical Industry Co., Japan) (final concentration 8 $\mu\text{mol/L}$) and everolimus (Selleckchem, Houston, TX, USA) (final concentration 1 $\mu\text{mol/L}$); 8 $\mu\text{mol/L}$ rapamycin and 1 $\mu\text{mol/L}$ everolimus was added to the culture medium.

2.6 | Real-time RT-PCR

Cells were washed with PBS and suspended in 1 mL TRIzol (Invitrogen), 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 with gDNA Eraser (TaKaRa), according to the manufacturer's instructions. Real-time RT-PCR was carried out in a Smart Cycler System (TaKaRa) using SYBR Premix Ex Taq (TaKaRa) and real-time PCR primers. Information regarding the primers used is shown in Table S1.

2.7 | Tissue sections

To examine the morphophenotypic changes in cancer cells, we created tissue sections from culture cells onto membrane of culture inserts. Membranes were fixed in 10% formalin (Wako, Japan) and embedded in paraffin, using Tissue-Tek VIP and Tissue-Tek TEC Systems (Sakura Finetek, Japan). The sections were cut to 4- μm thickness and H&E) staining was carried out.

2.8 | Immunohistochemical staining

Immunostaining was undertaken using 4- μm serial tissue sections. The sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Primary antibodies used in this study are listed in Table S2. After antigen retrieval, individual slides were incubated overnight at 4°C with primary antibody. After washing with PBS, the slides were incubated with Envision+ System HRP Labelled Polymer (Dako, CA, USA) for 30 minutes at room temperature. After washing with PBS (LSI Medience, Tokyo, Japan), the color reaction using HRP was developed for 10 minutes in 5% 3,3'-diaminobenzidine in imidazole-HCl buffer (pH 7.6) containing hydrogen peroxide. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated, and mounted.

2.9 | Evaluation of doxorubicin uptake by PC-9 cells

The cellular uptake quantity of anticancer drug was determined by the intracellular fluorescence intensity of doxorubicin (Kyowa Hakko, Japan). PC9-GFP cells were seeded in Col I. On day 1, medium was replaced with fresh medium with containing doxorubicin (6 $\mu\text{mol/L}$). After 3 hours of exposure, we measured the red fluorescence intensity of GFP-positive cells. Fluorescence images were acquired using

the LSM 710 confocal system mounted on a Zeiss Axio Observer Z1 microscope (Carl Zeiss, NY, USA).

2.10 | Western blot analysis

Cells were washed by PBS (Sigma-Aldrich) and were lysed in lysis buffer supplemented with phosphatase inhibitor cocktails (cOmplete Mini and PhosphoSTOP; Roche, Germany) for 30 minutes on ice. Cell debris was removed by centrifugation at 15490 g for 30 minutes at 4°C , and the protein solution was decanted to a new tube. The protein solution was inspissated using Amicon Ultra-4 units, with 10 kDa cut-off regenerated cellulose membranes (Millipore, MA, USA). Concentrated protein quantity was measured by Bradford assay. The protein was separated on a 7.5% or 10% SDS polyacrylamide gel and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The EGFR (#4267), phosphorylated (p-)EGFR (#3777), Akt (#4691), p-Akt (#4060), ERK (#4695), p-ERK (#4370), p70S6K (#2708), and p-p70S6K (#9205) antibodies for Western blotting were all purchased from Cell Signaling Technology (Beverly, MA, USA).

2.11 | Clinical samples

All specimens were collected after obtaining written comprehensive informed consent from each patient. This study was carried out with the approval of the Institutional Review Boards of the National Cancer Center (Kashiwa, Japan) (approval no. 2014-070). All methods were carried out in accordance with the approved guidelines.

2.12 | Histopathological analysis and evaluation of the association between Col I and EGFR-TKI response

Postoperative disease-recurrent patients with lung adenocarcinoma harboring an EGFR-activating mutation were treated with gefitinib between January 2002 and December 2012.^{21,29} We selected 87 patients who were treated with gefitinib as first-line chemotherapy. Patient characteristics are listed in Table S3. We identified EGFR activating mutations (deletions in exon19, L858R point mutation, L861Q point mutation, or G719 missense point mutations) through either direct sequencing using Cycleave PCR (SRL Diagnostics, India), the peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR-clamp method (LSI Medience), or a PCR-Invader Assay (Third Wave Technologies, WI, USA). We carried out a clinical evaluation of PFS lasting from the initiation of gefitinib treatment to the appearance of the earliest identifiable sign of disease progression, as determined by evaluation data from computed tomography or MRI imaging according to the RECIST guidelines, or until occurrence of death from any cause. We objectively quantified the positive rate of Col I by image analysis using ImageJ. In the current study, the Col I-rich stroma group was defined if $>50\%$ of the tumor area showed specifically positive reaction for Col I.³⁰ The distribution of patients according to

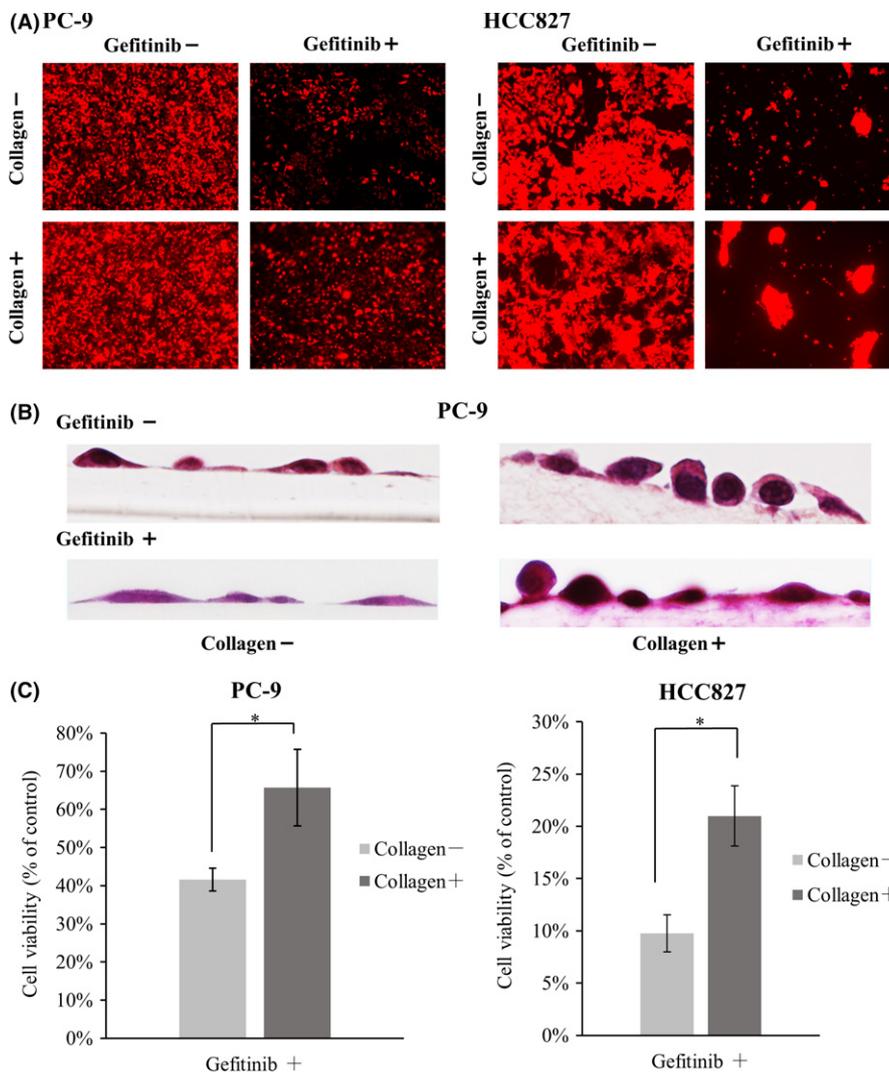


FIGURE 1 Collagen type I (Col I) induced gefitinib resistance in lung adenocarcinoma cells. A, Fluorescence images of monomeric red fluorescent protein (mRFP)-labeled PC-9 (left) and HCC827 cells (right) before and after gefitinib treatment with (+) or without (-) Col I. B, Morphological features of mRFP-labeled PC-9 cells in the presence (right) or absence (left) of Col I. C, Viability of mRFP-labeled PC-9 (right) and HCC827 cells (left) treated with gefitinib in the presence or absence of Col I. Error bars show the mean \pm SD. * $P < .05$, Student's *t*-test

the expression level of Col I is shown in Figure S3. The median Col I positive rate was 25.68.

2.13 | Statistical analysis

The significance of the differences between any two groups was evaluated by Student's *t*-test, and a difference was considered significant at $P < .05$. The recurrence-free survival and PFS rates were estimated using the Kaplan–Meier method, and differences in the variables were calculated by the log-rank test. A P -value $< .05$ was considered to indicate statistical significance, unless stated otherwise. All statistical analyses were undertaken using EZR (Saitama Medical Center of the Jichi Medical University, Shimotsuke, Japan), a graphical user interface for the R programming language that was developed by the R Foundation for Statistical Computing. More precisely, EZR is a modified version of R-commander (Free Access), created by importing statistical functions frequently used in biostatistics into the latter.

3 | RESULTS

3.1 | Collagen type I induces gefitinib resistance in EGFR-mutant lung cancer cells

Gefitinib treatment decreased the survival of PC-9 cells ($40.8 \pm 3.0\%$, mean \pm SD). Conversely, when PC-9 cells were cultured with Col I, $64.1 \pm 10.5\%$ of cells survived after gefitinib treatment ($P = .007$). Without gefitinib, Col I did not affect the growth of PC-9 cells (Figure 1). Moreover, Col I increased the viable cell number when PC-9 cells were treated with 0.8 and 1 $\mu\text{mol/L}$ gefitinib (Figure S4). We used another EGFR-mutated lung adenocarcinoma cell line, HCC-827, for further analysis. The total HCC-827 cell number in the absence of Col I following gefitinib treatment was reduced to $9.8 \pm 1.8\%$. However, when HCC-827 cells were cultured with Col I, $21.0 \pm 2.9\%$ of the HCC-827 cells survived ($P = .009$; Figure 1A,C).

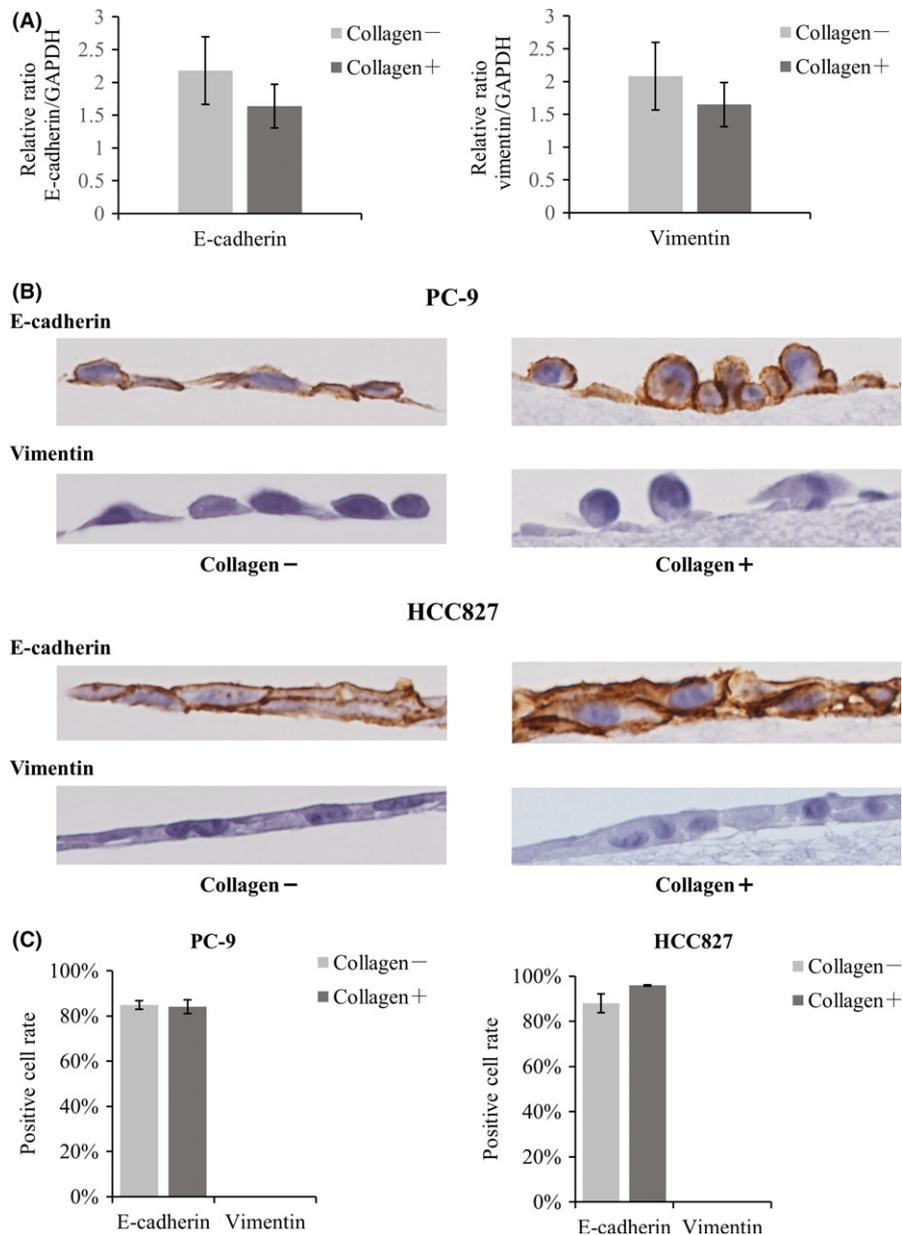


FIGURE 2 Immunophenotype of lung cancer cells cocultured with collagen type I. A, Relative *E-cadherin* and *vimentin* mRNA levels of PC-9 cells with or without collagen type I as determined by RT-PCR assays. B, Immunohistochemical staining for *E-cadherin* and *vimentin* in PC-9 and HCC827 cells with or without collagen type I. C, Frequency of *E-cadherin*- and *vimentin*-positive cells with or without collagen type I.

3.2 | Morphophenotypic analysis of PC-9 cells cultured with Col I

PC9 cells cultured with Col I were phenotypically more rounded and tall compared to PC-9 cells cultured without Col I (Figure 1B). The average area per cell in the absence of Col I was $30.9 \pm 4.8 \mu\text{m}^2$ ($n = 100$) and that in the presence of Col I was $48.9 \pm 4.7 \mu\text{m}^2$ ($n = 100$) (Figure S5). The size of PC-9 cells cultured with Col I was significantly larger than that of PC-9 cells in the absence of Col I. This phenomenon was also observed in HCC-827 cells.

Next, we examined the phenotypic changes in PC-9 and HCC827 cells cultured with Col I. We investigated EMT-related markers, as Col I reportedly promotes EMT in cancer cells.³¹ The mRNA expression of *E-*

cadherin and *vimentin* was not upregulated in PC-9 cells with Col I (Figure 2A). Immunohistochemical staining revealed the rate of *E-cadherin* positivity in the absence and presence of Col I was $84.9 \pm 1.9\%$, and $84.1 \pm 2.9\%$, respectively. Conversely, the rate for *vimentin* positivity in the absence of Col I was 0%. Similarly, in the presence of Col I, none of the PC-9 cells were *vimentin*-positive (Figure 2B,C). Similar results were obtained with HCC-827 cells. Taken together, the current results indicate that Col I did not induce EMT of PC-9 and HCC-827 cells.

3.3 | Drug uptake rate in PC-9 cells cultured with Col I

We considered the possibility that Col I can alter drug uptake ability. Therefore, as gefitinib does not have autofluorescence, we used

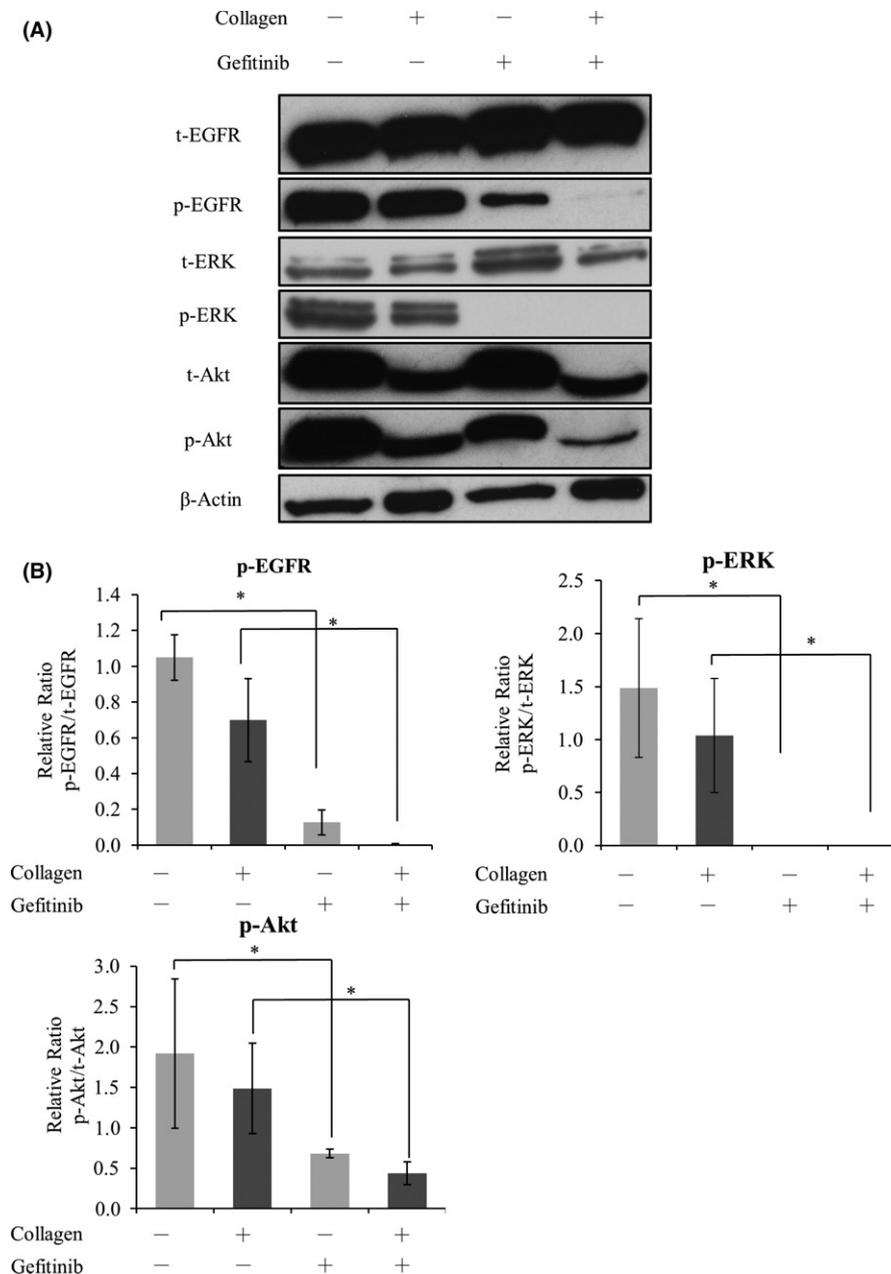


FIGURE 3 Changes in the activation of epidermal growth factor receptor (EGFR), ERK, and Akt with (+) or without (-) collagen type I (Col I) by gefitinib. A, EGFR, ERK, and Akt phosphorylation (p-) and total protein (t-) in PC-9 cells cultured with or without Col I. B, Quantitative analysis of EGFR, ERK, and Akt phosphorylation

doxorubicin, which does. The average red fluorescence intensity was 71.2 in the absence of Col I and 73.5 in the presence of Col I. The rate of drug absorption by the cells was 78.2% in the absence of Col I and 78.3% in its presence. Therefore, Col I does not affect drug uptake in the PC-9-GFP cells (Figure S6).

3.4 | Phosphorylation status of ERK and Akt after gefitinib treatment

Both the MAPK and PI3K pathways are active in primary and acquired resistance to gefitinib. Therefore, we assessed the phosphorylation of key downstream signaling proteins EGFR, ERK, and

Akt in PC-9 cells cultured with or without Col I after 24 hours of gefitinib treatment. Gefitinib treatment induced the suppression of p-EGFR, p-ERK, and p-Akt expression (Figure 3). The phosphorylation levels of Stat3, focal adhesion kinase (FAK), and p38 did not differ in PC-9 cells cultured with or without Col I (Figure S7).

3.5 | Collagen type I induces mTORC1 activation in EGFR-mutant lung cancer cell lines

We hypothesized that the Col I-induced resistance in EGFR-mutated cells was caused by activation of mTOR complex 1 (mTORC1), a downstream molecule of Akt. Previous studies

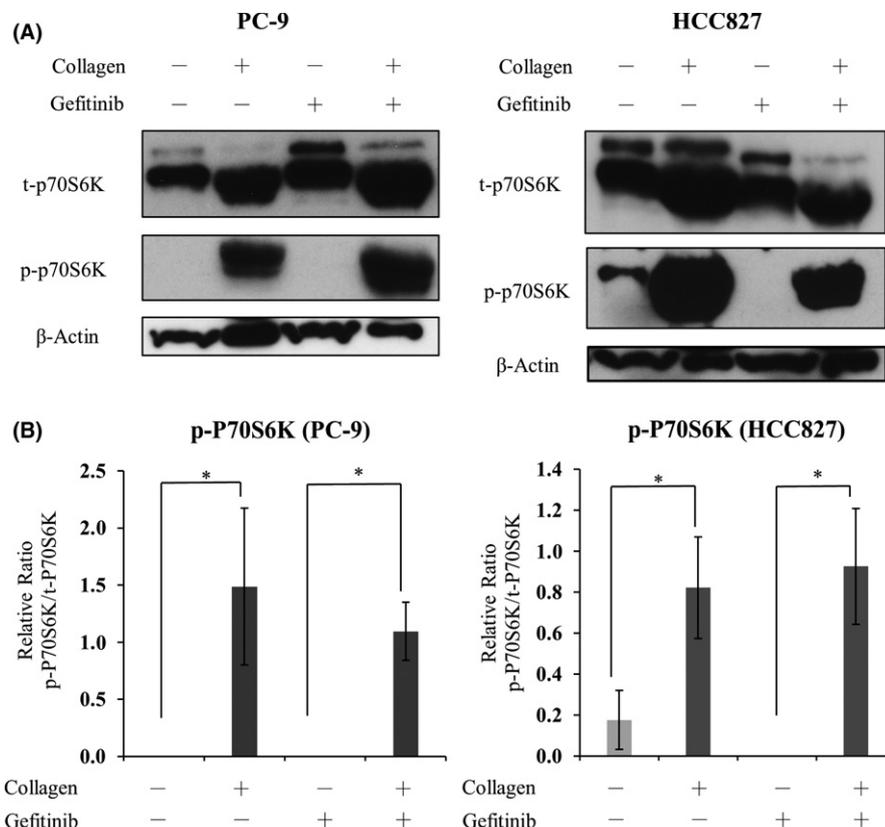


FIGURE 4 Changes in the activation of p70S6K with (+) or without (-) collagen type I. A, Phosphorylated (p-) and total protein (t-) p70S6K expression in PC-9 cells and HCC-827 cells cultured with or without collagen type I. B, Quantitative analysis of p70S6K phosphorylation

reported that the size of mTORC1-activated cells is increased, which supports our hypothesis.³² Therefore, we evaluated the phosphorylation status of p70S6K, which is an indicator of mTORC1 activation. In the presence of Col I, phospho-p70S6K expression was drastically upregulated in both PC-9 and HCC827 cells. This activation was observed even after gefitinib treatment (Figure 4).

3.6 | Suppression of mTORC1 activation in combination with gefitinib treatment leads to synthetic lethality in EGFR-mutant lung cancer cell lines

We first assessed whether treatment with the mTOR inhibitor rapamycin could reduce cell size. The average size of PC-9 cells in the absence of Col I was $33.2 \pm 5.1 \mu\text{m}^2$, and rapamycin treatment did not change the size of cells ($31.5 \pm 4.9 \mu\text{m}^2$). In contrast, in the presence of Col I, rapamycin significantly reduced cell size ($51.5 \pm 8.3 \mu\text{m}^2$ vs $25.6 \pm 5.3 \mu\text{m}^2$; $P = .001$) (Figure 5A,B).

We next examined whether the combination of EGFR-TKI and rapamycin could abrogate Col I-induced resistance. Rapamycin alone had little effect on cell number in both the Col I (-) and Col I (+) groups ($90.7 \pm 7.8\%$ and $96.1 \pm 0.8\%$, respectively). As expected, treatment with gefitinib alone was not effective in the Col (+) group (Col I [-], $45.0 \pm 1.7\%$; Col I [+], $71.5 \pm 2.9\%$; $P < .001$).

Simultaneous treatment with gefitinib and rapamycin treatment reduced cell numbers to $29.0 \pm 5.5\%$ in the absence of Col I. Even in the presence of Col I, this combination treatment significantly reduced the survival rate to the same extent as that in the Col I (-) condition ($33.9 \pm 1.9\%$) (Figure 5C). We investigated the combination effect of gefitinib and everolimus, another mTORC1 inhibitor for PC-9 cells, cultured with Col I. The combination treatment of gefitinib and everolimus significantly reduced the viable cell number to the same extent as without Col I (Figure 5D).

3.7 | Association between Col I expression within the tumor and PFS after EGFR-TKI treatment

Using human lung adenocarcinoma samples from 87 patients with postoperative recurrence who were previously treated with gefitinib, we next examined whether Col I deposition causes resistance to gefitinib treatment. We separated these patients into two groups based on Col I deposition in the total tumor area: (i) Collagen-rich group (>50% Col I-positive area, $n = 6$); and (ii) control group ($\leq 50\%$ Col I-positive area, $n = 81$) (Figure 6A). The postoperative recurrence-free survival was similar between the Collagen-rich and control groups ($P = .904$; Figure 6B). Conversely, the PFS of the Collagen-rich group after gefitinib treatment was significantly shorter compared to that of the control group ($P = .049$; Figure 6C).

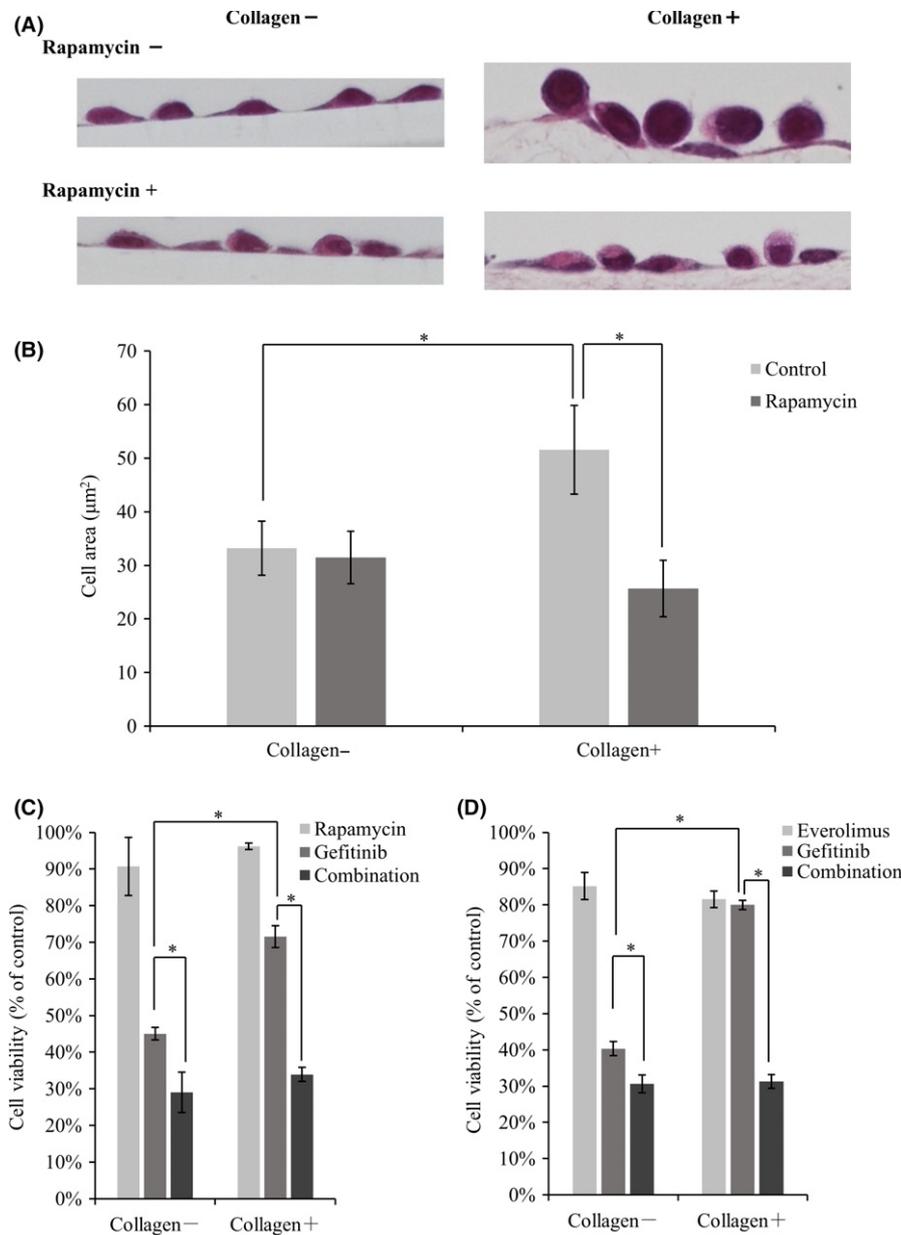


FIGURE 5 Effects of mTOR inhibition alone and with combined suppression of epidermal growth factor receptor (EGFR) and mTOR. A, Morphological features of monomeric red fluorescent protein (mRFP)-labeled PC-9 cells before and after rapamycin treatment in the presence (+, right) or absence (-, left) of collagen type I (Col I). B, Quantitative analysis of mRFP-labeled PC-9 cell size before and after rapamycin treatment and with or without Col I. C, Cell viability following combination treatment with gefitinib and rapamycin with or without Col I. D, Cell viability following combination treatment with gefitinib and everolimus with or without Col I

4 | DISCUSSION

Primary and acquired resistance is a critical problem associated with the consideration of EGFR-TKI treatment. Although several molecular mechanisms of acquired resistance have been reported, the causes of primary resistance are poorly understood. In the current study, we found that Col I induces EGFR-TKI resistance in cancer cells through mTOR activation. However, this activation was induced by EGFR-ERK1/2- and Akt-independent signaling pathways. This is the first study to elucidate the molecular mechanism by which Col I impacts EGFR-TKI resistance. Furthermore, the current results

suggest the efficacy of combination therapy of EGFR-TKI and mTOR inhibitor to relieve Col I-mediated resistance.

In this study, we found that mTOR activation plays a key role in Col I-mediated resistance. Activation of mTOR has been reported to induce anticancer drug resistance and proliferation in several cancers.³³⁻³⁵ However, whether this mTOR activation by Col I is specific for EGFR activating mutation-harboring cells was unknown. We addressed this question using the EGFR WT lung adenocarcinoma cells A549, A427, and H1299. Subsequently, Col I-mediated mTOR activation was confirmed in all EGFR WT cell lines. Therefore, Col I-mediated mTOR activation is a universal phenomenon (Figure S8).

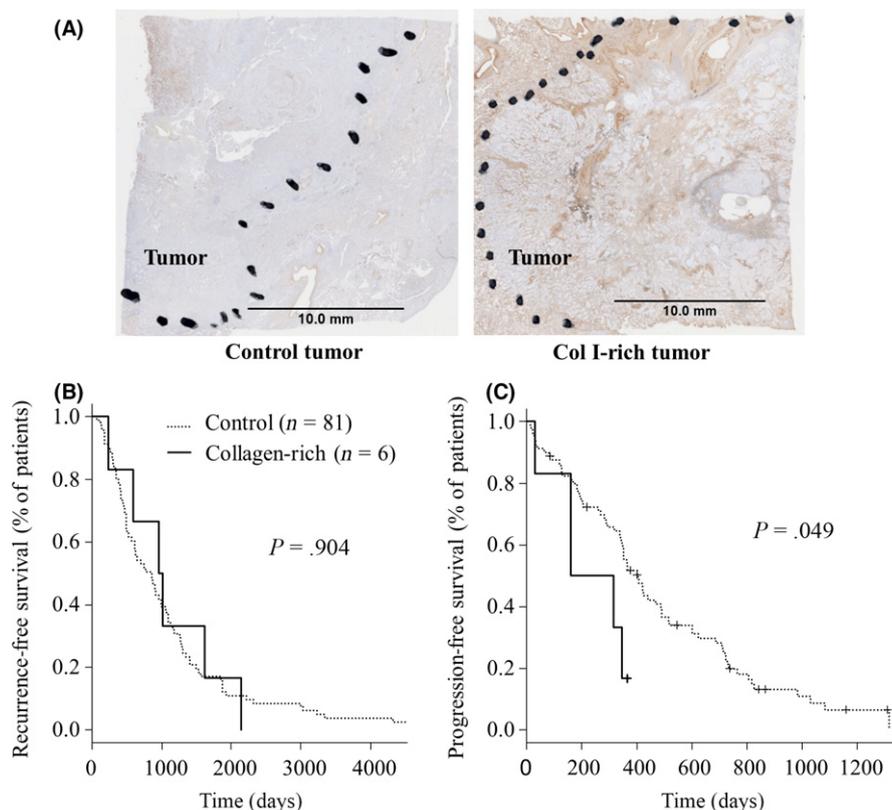


FIGURE 6 Progression-free survival according to collagen type I (Col I) deposition in patients with lung adenocarcinoma treated with gefitinib. A, Immunohistochemical features of control (left panel) and Col I-positive (right) groups. In the control group, the proportion of Col I was 4.4%. In the Col I-rich group, the proportion of Col I was 66.5%. B, Recurrence-free survival of epidermal growth factor receptor (EGFR)-activating mutation-harboring lung adenocarcinoma patients with high Col I deposition vs control subjects. C, Progression-free survival of gefitinib-treated EGFR-activating mutation-harboring lung adenocarcinoma patients in Col I-rich vs control subjects

Although it is widely known that the PI3K–Akt and MAPK signaling pathways activate mTOR,³⁶ our current study indicates that Col I could activate mTOR through a signaling pathway-independent mechanism. Moreover, the p-Akt antibody used in this study is specific for Serine 473. Serine 473 delivers the phosphorylation for direct mTORC2.³⁷ Therefore, Col I-mediated mTOR activation was not seemingly caused by mTORC2–Akt phosphorylation. There are only a few reports on mTOR activation through PI3K–Akt- and MAPK-independent signaling.^{38,39} Integrin is well known as the receptor of Col I, however, our data suggest that the integrin-mediated pathway was not associated with Col I-induced mTOR activation, as FAK, a cytoplasmic tyrosine kinase that plays critical roles in integrin-mediated signaling, was not activated by Col I (Figure S5A,B).^{40,41} There are two possible explanations to this phenomenon. One is that the receptors for Col I, other than integrin, could induce Col I-mediated mTOR activation.

Alternatively, it should be noted that mTOR functions as an amino acid sensor. It is also a conventional adjustment factor corresponding to intracellular and extracellular nutrition.^{42,43} Collagen type I is a matrix containing large quantities of amino acids. Therefore, release of amino acids from degraded Col I might cause mTOR activation. Elucidation of this molecular mechanism requires further investigation.

We evaluated the association between Col I deposition within the tumor and EGFR-TKI response in patients with postoperative recurrence harboring EGFR-activating mutations. The PFS of the Col I-rich group after gefitinib treatment was significantly shorter compared to that of the control group, supporting current *in vitro* results. However, we could not measure mTOR activation in cancer cells directly by immunohistochemical analysis because of the lack of an appropriate antibody. Future consideration will be needed in order to detect this beneficial biomarker. The limitations of our analysis using patient specimens include the lower number of Col I-rich cases. Moreover, we could not exclude the possibility that Col I expression status in the primary resected tumors was also preserved, even in the recurrent tumors. The examination of the recurrent cases will be important.

In conclusion, we showed that Col I induces mTOR activation in EGFR-activating mutation-harboring cancer cells through an EGFR–ERK-1/2- and Akt-independent pathway, which results in EGFR-TKI resistance. These findings suggest that Col I acts as not only a “physiological barrier” for drug delivery but also as a “signal provider” for resistance to molecular targeted therapy. Recently, PEGPH20, which can degrade stromal hyaluronic acid, has been studied in combination with anticancer agents in pancreatic cancer. The combination of PEGPH20 and paclitaxel/gemcitabine showed a

beneficial overall response rate compared to monotherapy using paclitaxel/gemcitabine in pancreatic cancer patients highly expressing hyaluronidic acid.⁴⁴ To overcome the effects of ECM composition in cancer tissue, targeting the tumor ECM could be a promising method. Our current study provides a new insight into understanding how ECM, especially Col I, regulates the drug resistance process. This study also suggests a novel strategy for increasing the therapeutic effect of EGFR-TKI.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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