

Pirfenidone may revert the epithelial-to-mesenchymal transition in human lung adenocarcinoma

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Abstract. The epithelial-to-mesenchymal transition (EMT) in cancer is associated with invasion, metastasis and chemoresistance. Recent studies have revealed the increased expression of programmed death-ligand 1 (PD-L1) in cells undergoing EMT. The underlying mechanism of EMT involves transforming growth factor- β (TGF- β) and fibroblast growth factor-2 (FGF-2). Pirfenidone and the known EMT-suppressor nintedanib suppress pulmonary fibrosis partially through suppression of TGF- β . The present study aimed to determine whether pirfenidone has the potential to induce EMT-reversion, using nintedanib as a reference. The human lung adenocarcinoma cell lines A-549, HCC-827, and PC-9 were treated with TGF- β and FGF-2 to induce EMT. The EMT-induced cells were further treated with pirfenidone or nintedanib. Phenotypic alterations associated with EMT were assessed by examining the following: i) The expression levels of E-cadherin, vimentin, fibronectin and slug, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and fluorescent immunohistochemistry; ii) cell motility via wound-healing assays; and iii) the expression of PD-L1 using RT-qPCR. The combination of TGF- β and FGF-2 successfully induced EMT in all three cell lines, characterized by a significant reduction in E-cadherin expression in the A-549 and HCC-827 cells, increased expression levels of vimentin, fibronectin, slug and PD-L1, and increased cell motility in all three cell lines. Pirfenidone and nintedanib reverted all of these phenotypes, with the exception of unaltered E-cadherin expression in all three cell lines, and inconsistent expression of vimentin in the HCC-827 and PC-9 cells. Thus, pirfenidone and nintedanib have the ability to induce EMT-reversion in human lung adenocarcinoma.

Introduction

The epithelial-to-mesenchymal transition (EMT) serves important roles in embryonic development, cancer invasion, metastasis and chemoresistance (1-3). EMT cells demonstrate lower expression levels of epithelial markers, including E-cadherin, and higher expression levels of mesenchymal markers, including vimentin and fibronectin (1,2). These expressional changes occur through the upregulation of transcriptional factors, including the zinc finger proteins, zinc finger E-box binding homeobox (ZEB)1 and 2, the basic helix-loop helix protein Twist, and the Snail family, including Snail and Slug (1,2). Although the underlying mechanisms remain unclear, transforming growth factor- β (TGF- β) and fibroblast growth factor-2 (FGF-2) constitute the main EMT-inducing factors in numerous types of cancer (1,4,5). Due to its roles in cancer invasion, metastasis and chemoresistance, the inhibition or reversion of the EMT has been regarded as a promising strategy for treating cancer. Several agents, including a mechanistic target of rapamycin inhibitor (6) and metformin (7), have been reported to suppress EMT in lung cancer. However, effective approaches for inhibiting the EMT have not been established.

Recently, the anti-fibrotic agents pirfenidone and nintedanib have been approved in numerous countries for the treatment of idiopathic pulmonary fibrosis (IPF). Randomized controlled clinical trials have demonstrated that pirfenidone suppresses the deterioration of the percentage forced vital capacity (FVC), with manageable toxicities, in patients with IPF (8,9). Furthermore, pirfenidone has been revealed to suppress lung fibrosis through the downregulation of TGF- β , platelet-derived growth factor (PDGF) and collagen synthesis in a hamster model (10-12). In addition, it was shown to suppress liver fibrosis through the downregulation of TGF- β (13,14). Nintedanib has been demonstrated to suppress the deterioration of FVC and the incidence of acute exacerbation in patients with IPF (15,16). Additionally, it has been revealed to inhibit fibrosis through the downregulation of extracellular matrix proteins in pulmonary fibroblast cells (17). The expression levels of the receptor tyrosine kinases of PDGF, FGF and vascular endothelial growth factor were observed to be inhibited following treatment with nintedanib (17). Recently, nintedanib was demonstrated to inhibit the early signaling of TGF- β by suppressing the phosphorylation of the TGF- β type II receptor (18).

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Numerous studies have reported that the relative risk of lung cancer among patients with IPF is 7-14 times higher compared with that in patients without IPF (19-21). Under such circumstances, these agents may soon be widely used for the treatment of patients with lung cancer accompanied by IPF. Thus, elucidating the potential effects of these agents on lung cancer cells is required. Nintedanib, which is assumed to suppress TGF- β and FGF, was recently reported to suppress the EMT in ovarian cancer cells *in vitro* (22). As pirfenidone also has the ability to suppress TGF- β , it may be useful for suppressing or reverting the EMT. Therefore, the present study aimed to evaluate the effect of pirfenidone on the EMT in human lung cancer and compare its efficacy to the activity of nintedanib.

Materials and methods

Cells and reagents. The human lung adenocarcinoma cell lines A-549, HCC-827 (American Type Culture Collection, Manassas, VA, USA) and PC-9 (Riken Cell Bank, Tsukuba, Japan) were used throughout the present study. HCC-827 and PC-9 cells have a deletion in exon 19 (del E746-A750) of the epidermal growth factor receptor gene. Cells were cultured as a monolayer in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Pirfenidone (Sigma-Aldrich; Merck KGaA) was dissolved in water at a concentration of 1.0 M and stored at -20°C. Nintedanib (Selleck Chemicals, Houston, TX, USA) was dissolved in DMSO (Sigma-Aldrich; Merck KGaA) at a concentration of 10 mM and stored at -20°C. Prior to use in the experiments, each agent was diluted in RPMI-1640 medium with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Mouse monoclonal anti-fibronectin antibody (cat. no. F3648) was purchased from Sigma-Aldrich (Merck KGaA). The anti-E cadherin antibody was purchased from BD Biosciences (cat. no. 610181; Franklin Lakes, NJ, USA). Anti-mouse IgG Fab2 Alexa Fluor® 488 molecular probes (cat. no. 4408S) and anti-rabbit IgG Fab2 Alexa Fluor 555 molecular probes (cat. no. 4413S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

In vitro induction and reversion of EMT. EMT was induced by treatment with recombinant human TGF- β (PeproTech, Inc., Rock Hill, NJ, USA) and FGF-2 (Cell Signaling Technology, Inc.). Based on preliminary experiments (data not shown), a combination of TGF- β (10 ng/ml) and FGF-2 (10 ng/ml) was admixed into the complete medium for induction of EMT following 24 h of serum starvation. To evaluate the EMT phenotypes, cells harvested at 48 h after the admixture of the agents were used. Pirfenidone (0.2 and 2.0 mM) or nintedanib (0.1 and 1.0 μ M) was then added to cells in which the EMT had already been induced, with TGF- β /FGF-2 being supplemented continuously. The phenotypic alterations were evaluated after 72 h of culture with these agents.

Evaluation of cell viability. Cell viability was determined using an MTT assay according to the manufacturer's protocol

(Promega Corp., Madison, WI, USA). Cells were seeded into 96-well culture plates at a density of 1x10³ cells/well. Cells were allowed to attach for 24 h prior to drug treatment. Cells were treated with various concentrations of pirfenidone (0, 0.02, 0.2, 2.0 and 10 mM) or nintedanib (0, 0.01, 0.1, 1.0 and 10 μ M) for 48 h of culture at a 37°C. The absorbance at 570 nm in the resulting solution was measured using the Infinite® 200 PRO microplate reader (Tecan Schweiz AG, Seestrasse, Switzerland).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were cultured until 80% confluence was achieved in 6-well culture plates, the total RNA was extracted using the RNeasy® Mini kit (cat. no. 74104; Qiagen GmbH, Hilden, Germany) and the cDNA was synthesized using the SuperScript® First-Strand Synthesis kit for RT-PCR (cat. no. 11904-018; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The expression levels of various mRNAs were quantified using RT-qPCR with the TaqMan® Gene Expression Assay kit, TaqMan Fast Advanced Master mix (cat. no. 4444557) and StepOnePlus Real-Time PCR system (all Thermo Fisher Scientific, Inc.). A total of 25 ng synthesized cDNA was used for each qPCR reaction. Each sample was measured in triplicate. GAPDH was used for normalization. Relative expression levels were calculated as using the 2^{- $\Delta\Delta C_q$} method (23). TaqMan probes for GAPDH, E-cadherin, vimentin, fibronectin, Slug and programmed death-ligand 1 (PD-L1) were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.; assay identification nos. Hs02758991_g1, Hs01023894_m1, Hs00185584_m1, Hs00365052_m1, Hs00950344_m1 and Hs01125301_m1, respectively). The following thermocycling conditions were maintained: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Fluorescent immunohistochemistry. For fluorescent immunohistochemical evaluation, cells were seeded into a 4-well chamber slide (cat. no. 177399; Thermo Fisher Scientific, Inc.) at density of 1x10⁴ cells/well and cultured until 80% confluence was achieved. Cells grown on the chamber slide were fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature and with acetone (both Sigma-Aldrich; Merck KGaA) for 10 min at -20°C. Non-specific binding was blocked using 1% bovine serum albumin with 0.2% Triton X-100 (both Sigma-Aldrich; Merck KGaA) in PBS for 1 h at room temperature. Then, the cells were incubated with primary antibodies for 2 h at room temperature. Subsequently, the cells were incubated with the corresponding secondary antibodies and counterstained with DAPI (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at room temperature for 30 min prior to observation using the EVOS FL Imaging system (Thermo Fisher Scientific, Inc.).

Wound healing assay. Cell motility was assessed using a wound healing assay. Confluent cells were scratched with micropipette tips (Thermo Fisher Scientific, Inc.). Following washing with PBS, the cells were incubated for a further 9 h at 37°C. Scratch areas were viewed using an inverted microscope (magnification, x10; Nikon Corporation, Tokyo, Japan) and

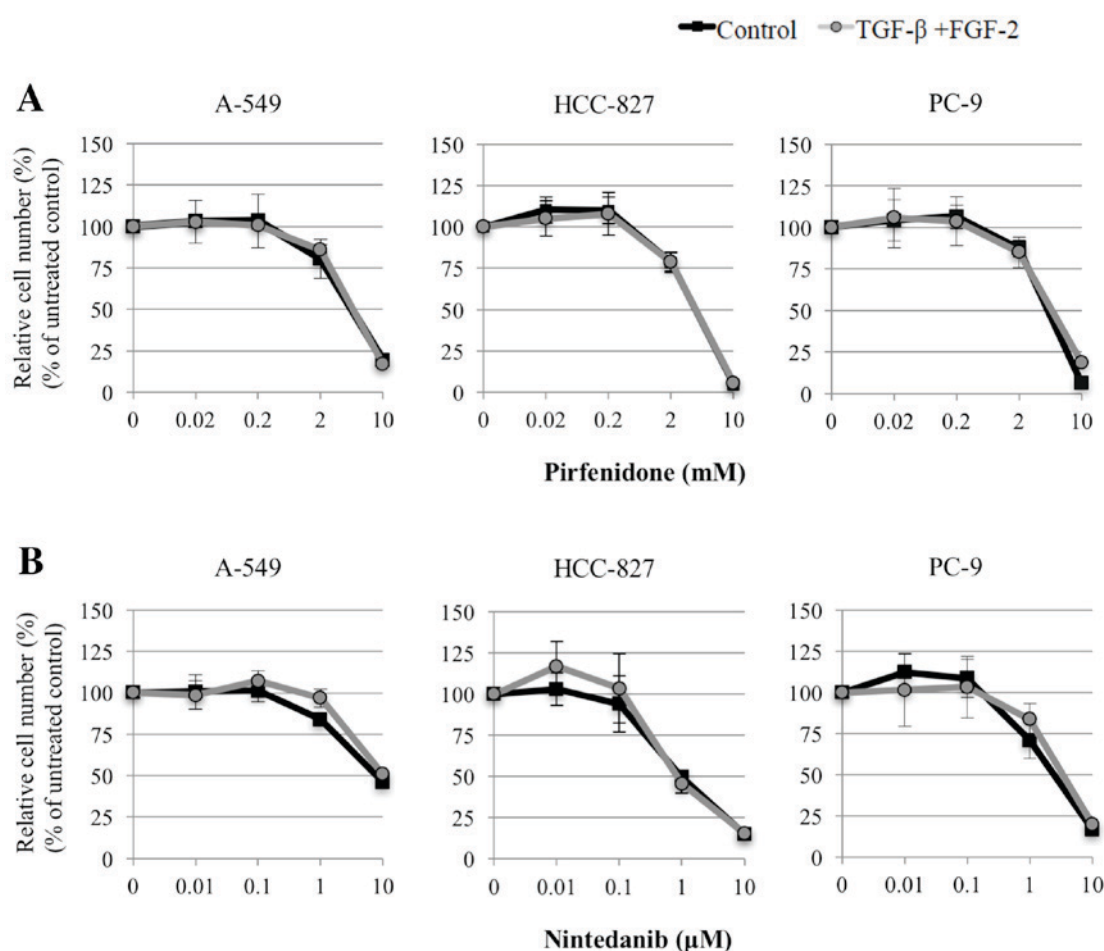


Figure 1. (A and B) Inhibition of cell growth in A-549, HCC-827 and PC-9 cells by pirfenidone and nintedanib, as assessed using an MTT assay. Based on the results, doses of 0.2 and 2.0 mM of pirfenidone, and 0.1 and 1.0 μ M of nintedanib were selected for subsequent experiments. Data are presented as the mean \pm standard error of the mean (n=3, in triplicate). TGF- β , transforming growth factor- β ; FGF-2, fibroblast growth factor-2.

were quantified using ImageJ software (version 1.46v; National Institutes of Health, Bethesda, MD, USA). The wound closure rates were determined as a percentage of the total repaired area per hour, and were normalized to the control.

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Statistical analysis was performed using Microsoft Excel software (version 2010; Microsoft Corporation, Redmond, WA, USA). Differences between groups were analyzed using the two-tailed Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were repeated ≥ 3 times, in triplicate.

Results

Cytotoxicity of pirfenidone and nintedanib. Fig. 1 shows the cytotoxicity of the agents, as assessed using the MTT assay. Based on these results, the borderline sublethal dose points, at which cell viability began to decline, and the adjacent lower dose point for each agent were selected for use in subsequent experiments (0.2 and 2.0 mM for pirfenidone; 0.1 and 1.0 μ M for nintedanib).

In vitro induction of EMT. RT-qPCR in the three cell lines revealed that, compared with the untreated control

group, the combination of TGF- β and FGF-2 significantly downregulated E-cadherin and upregulated vimentin, fibronectin, and Slug, resulting in an EMT phenotype, with the exception of E-cadherin expression in PC-9 cells where no significant differences were observed (Fig. 2). Fluorescent immunohistochemical analysis of E-cadherin at the cell junction and fibronectin in the cytoplasm supported the results of the RT-qPCR analyses (Fig. 3). The wound-healing assay showed significantly increased cell motility associated with the EMT phenotype in A-549 and PC-9 cells (both $P < 0.05$), and a degree of increased cell motility in HCC-827 cells compared with the untreated control groups (Fig. 4A). The expression of PD-L1 assessed using RT-qPCR was significantly increased following EMT induction in all cell lines compared with the untreated cells (Fig. 4B). These findings appear to support the successful induction of EMT in all three cell lines.

Effects of pirfenidone and nintedanib on EMT-induced cells.

Although no significant or consistent effects on the expression levels of E-cadherin and vimentin were observed, pirfenidone or nintedanib treatment at various doses significantly suppressed fibronectin and Slug expression levels compared with the EMT-phenotype control groups (Figs. 2 and 3). Furthermore, the wound healing assay results demonstrated that both of the agents significantly reverted the enhanced cell

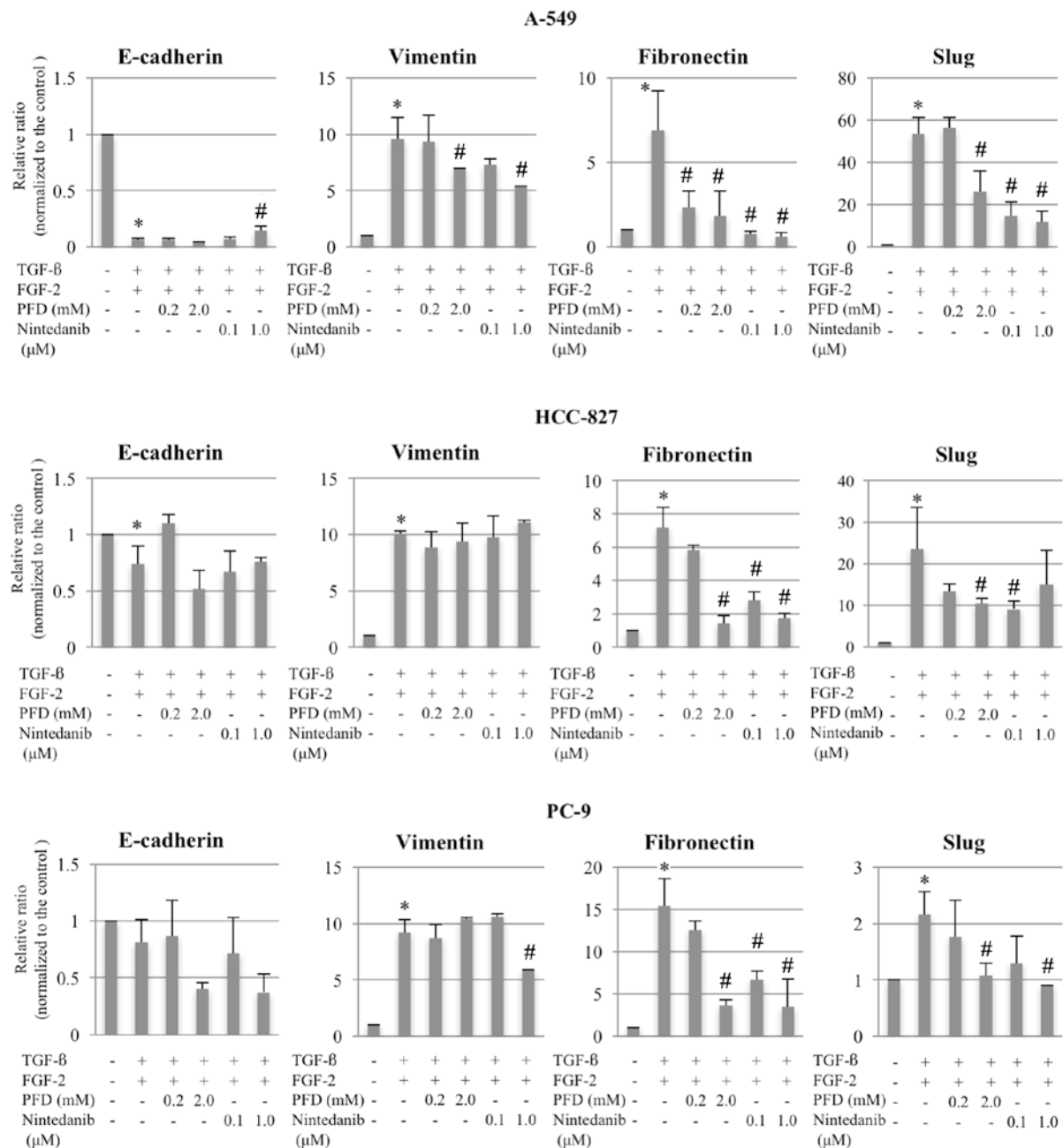


Figure 2. Changes in mRNA expression levels of E-cadherin, vimentin, fibronectin and Slug following EMT induction, and treatment with pirfenidone or nintedanib in A-549, HCC-827 and PC-9 cells. The relative ratios of mRNA in the treatment groups vs. the controls were evaluated using reverse transcription-quantitative polymerase chain reaction for cells treated with TGF- β (10 ng/ml) and FGF-2 (10 ng/ml). Following EMT induction, pirfenidone (0.2 or 2.0 mM) or nintedanib (0.1 or 1.0 μ M) were added. Data are presented as the mean \pm standard error of the mean (n=6, in triplicate) of the relative ratios of expression compared with the levels of the respective controls. GAPDH was used for normalization. *P<0.05 compared with untreated control (no TGF- β /FGF-2); #P<0.05 compared with EMT-phenotype control (TGF- β /FGF-2-treated cells). PFD, pirfenidone; TGF- β , transforming growth factor- β ; FGF-2, fibroblast growth factor-2; EMT, epithelial-to-mesenchymal transition.

motility in all three cell lines compared with the EMT-phenotype control groups (Fig. 4A). Furthermore, following treatment with pirfenidone or nintedanib at various doses, PD-L1 expression was significantly downregulated compared with the EMT-induced cells, except in response to nintedanib in PC-9 cells (Fig. 4B).

Discussion

The present study demonstrated that a borderline sublethal dose of pirfenidone or nintedanib could revert the EMT phenotype

that had been induced by a combination of TGF- β and FGF-2 in three different human lung adenocarcinoma cell lines. The altered expression of mesenchymal markers and Slug, together with the alteration to cell motility, support this observation. The present results are consistent with a previous report indicating that nintedanib suppressed the EMT in ovarian cancer cells (22). The same authors also reported that the promotion of E-cadherin expression in A-549 cells occurred through ZEB1 downregulation (22). Furthermore, nintedanib was demonstrated to inhibit TGF- β -induced myofibroblast differentiation through the inhibition of early events in TGF- β signaling and

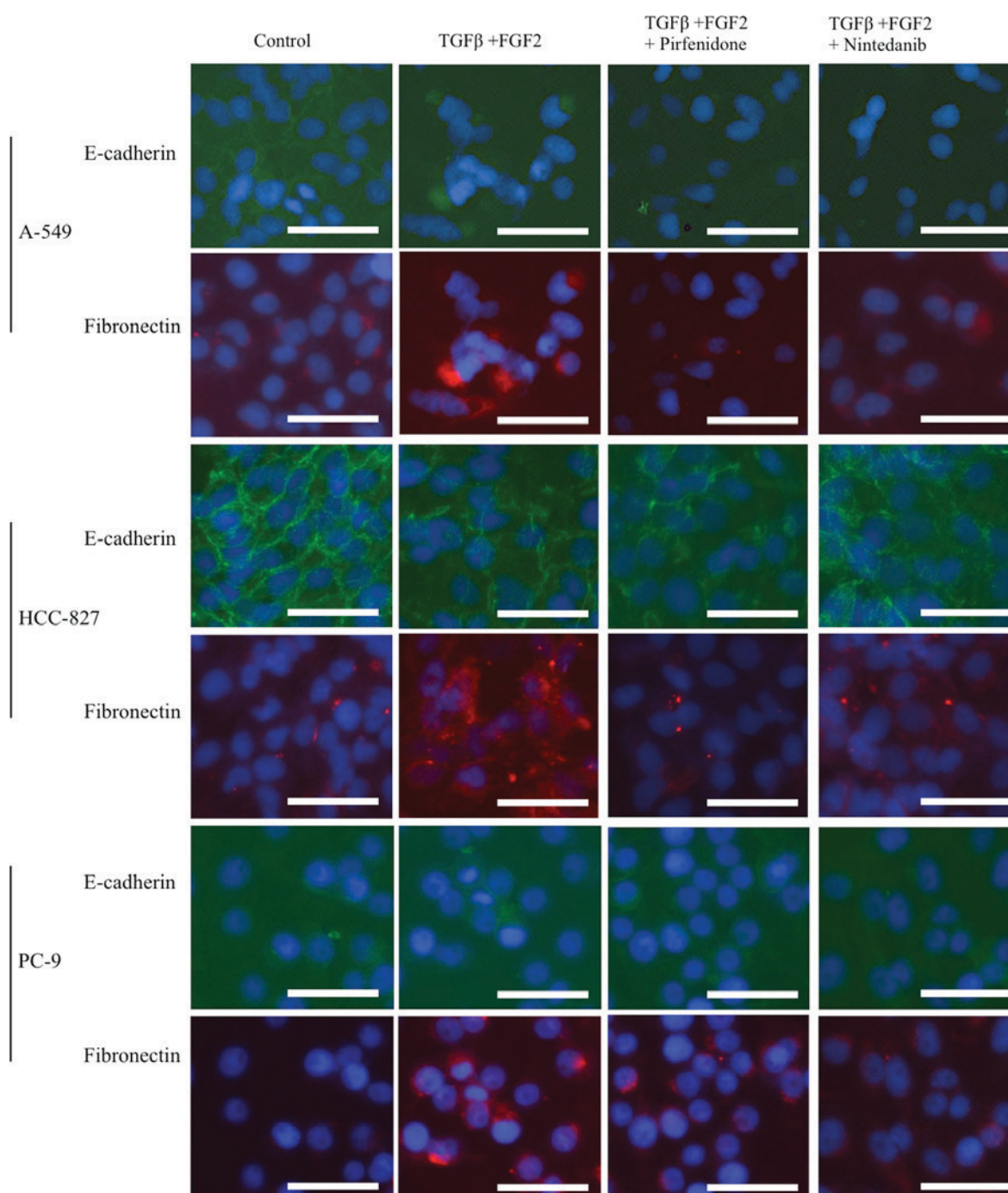


Figure 3. Fluorescent immunohistochemical staining for E-cadherin and fibronectin in A-549, HCC-827 and PC-9 cells shows the effects of epithelial-to-mesenchymal transition induction and its reversion by treatment with pirfenidone at a dose of 2.0 mM or nintedanib at a dose of 1.0 μ M. The changes in E-cadherin expression on the cell surfaces and the changes in fibronectin expression in the cytoplasm were confirmed. Cells were counterstained with DAPI. Scale bar, 50 μ m. TGF- β , transforming growth factor- β ; FGF-2, fibroblast growth factor-2 (n=3).

the activation of SMAD family member 3 (Smad3) in lung fibroblast cells (18). Therefore, the present study has provided novel evidence that pirfenidone possesses a similar ability to revert the EMT in human lung adenocarcinoma cells.

Previous studies have demonstrated that pirfenidone is able to suppress the differentiation of several cell types (24-28). Conte *et al* (24) reported that pirfenidone reduced fibroblast proliferation, attenuated TGF- β -induced α -smooth muscle actin and inhibited the TGF- β -induced phosphorylation of Smad3 in human lung fibroblast cells. In addition, pirfenidone has been reported to suppress the differentiation of nasal

polyp-derived fibroblasts (25), retinal pigment epithelial cells (26,27) and cardiac fibroblasts (28). Kozono *et al* (29) reported that pirfenidone-treated pancreatic stellate cells suppressed the invasiveness and migration of pancreatic cancer cells. Pirfenidone's ability to induce EMT reversion in cancer cells may be similar to the previously reported findings. Furthermore, the present study confirmed the previously reported observation that PD-L1 expression was enhanced and suppressed according to the induction and reversion of EMT, respectively (30), suggesting the involvement of the EMT phenotype in the process of immune evasion in cancer.

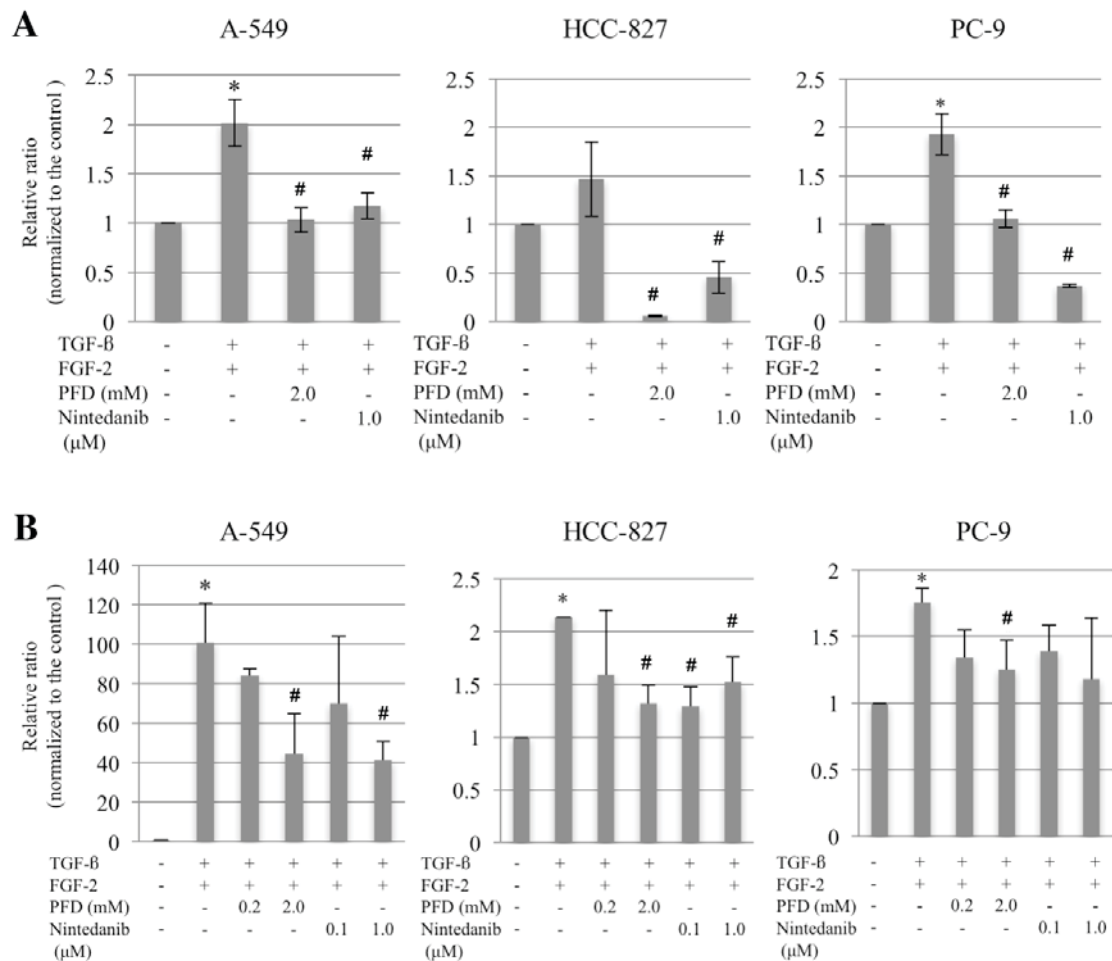


Figure 4. (A) A wound-healing assay showed enhanced cell motility following treatment with TGF- β /FGF-2 in all three cell lines, whereas pirfenidone and nintedanib suppressed the effect. For quantification, the cell migration distance was measured after 9 h (n=9, in triplicate) for each group. (B) Alteration of PD-L1 expression according to the induction and reversion of EMT. The relative mRNA expression of PD-L1 was normalized to that in the control. Data are presented as the mean \pm standard error of the mean (n=3, in triplicate) of the relative ratio compared with the control. *P<0.05 compared with untreated control (no. TGF- β /FGF-2); #P<0.05 compared with EMT-phenotype control (TGF- β /FGF-2-treated cells). PFD, pirfenidone; TGF- β , transforming growth factor- β ; FGF-2, fibroblast growth factor-2; EMT, epithelial-to-mesenchymal transition; PD-L1, programmed death-ligand 1.

The limitations of the present study should be considered when interpreting these results. Firstly, only the combination of TGF- β and FGF-2 was used to induce EMT, and other known EMT-inducing factors were not examined. Secondly, the important signaling pathways underlying EMT induction and reversion were not identified. Lastly, the study only included *in vitro* experiments, and thus may not reflect *in vivo* effects. Further studies associating the phenomena observed in the present study and investigations into their clinical relevance are warranted.

In conclusion, the results of the present study demonstrated that pirfenidone and nintedanib could each revert EMT induction and downregulate the expression of PD-L1 in human lung adenocarcinoma cells. Pirfenidone, thereby, may modify tumor progression and responsiveness to chemotherapy and/or immunotherapy for cancer.

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