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Tranilast Inhibits TGF-β1–induced Epithelial-mesenchymal Transition and Invasion/Metastasis *via* the Suppression of Smad4 in Human Lung Cancer Cell Lines

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Abstract. *Background/Aim: Transforming growth factor β1 (TGF-β1) is an important epithelial-mesenchymal transition (EMT) activator that regulates the expression of E-cadherin and vimentin through Smad signalling. Tranilast is an anti-allergic drug that inhibits TGF-β1, and is used in the treatment of keloids and hypertrophic scars. We investigated whether tranilast inhibits TGF-β1-induced EMT and invasiveness in human non-small cell lung cancer cell lines. Materials and Methods: We examined the effects of tranilast treatment on EMT markers, TGF-β1/Smad signalling, and cell invasiveness in A549 and PC14 cells. Tumours from a mouse orthotopic lung cancer model with or without tranilast treatment were also immunohistochemically evaluated. Results: Tranilast increased E-cadherin expression via Smad4 suppression and inhibited cell invasion in TGF-β1-stimulated cells. Tranilast treatment of the in vivo mouse model reduced the pleural dissemination of cancer cells and suppressed vimentin and Smad4 expression. Conclusion: Tranilast inhibited TGF-β1-induced EMT and cellular invasion/metastasis by suppressing Smad4 expression in cancer cells.*

Lung cancer is one of the most lethal malignancies, and is a leading cause of cancer-related death worldwide (1, 2). Although surgical resection is the preferred option for early-stage non-small cell lung cancer (NSCLC), almost 75% of cases present with unresectable cancer with distant metastases at the initial diagnosis (3). Thus, the prevention of metastasis is a crucial strategic target for improving prognoses. The shift of a cancer phenotype from an early localized phase into invasive and metastatic malignancy is facilitated by epithelial-mesenchymal transition (EMT) (4). EMT is thought to be an essential step in the metastatic process (5), and results from the interplay among various growth factors and polypeptides (6). Transforming growth factor β1 (TGF-β1), which has been identified to be the main activator of EMT, can effectively induce this transition in NSCLC cells (7). In the tumour microenvironment, TGF-β1 has been reported to be produced and secreted by various cell types including cancer cells, fibroblasts, macrophages, leukocytes, and endothelial cells (8).

Tranilast was initially developed as an anti-allergic agent that inhibits chemical mediators secreted by mast cells, and was approved in 1982 for the clinical treatment of bronchial asthma. In the late 1980s, this drug was found to have anti-fibrotic properties (9), and its clinical use for the treatment of keloids and hypertrophic scars was subsequently approved in 1993. Studies have reported that tranilast inhibits collagen synthesis by fibroblasts derived from keloids and hypertrophic scars, and one of its anti-fibrotic effects is thought to be caused by the inhibition of TGF-β1, which enhances collagen synthesis (10, 11). However, little is known about the effects of tranilast on TGF-β1–induced EMT and invasiveness in cancer cells.

In this study, we investigated whether tranilast inhibits TGF-β1–induced EMT and cell invasiveness in NSCLC cells and examined the phase at which TGF-β1 signalling is inhibited. We also evaluated the *in vivo* effects of tranilast on tumour characteristics using a mouse orthotopic lung cancer model.

Materials and Methods

Cell culture. Two human NSCLC cell lines, A549 and PC14, were maintained in Dulbecco's modified Eagle's medium or Roswell Park

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Key Words: EMT, tranilast, Smad4, TGF-β1.

Memorial Institute 1640 medium (Sigma-Aldrich, MO, USA) supplemented with 10% foetal bovine serum (FBS; HyClone, Thermo Fisher Scientific K.K., Kanagawa, Japan) and 100 U/ml penicillin/streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a humidified incubator with 5% CO_2 at 37˚C. The cells were used for experiments within 15 passages.

Western blot assays. Total cells were lysed using radioimmunoprecipitation assay buffer containing protease inhibitors (25955-24, Nacalai Tesque, Kyoto, Japan) and 0.1% sodium dodecyl sulphate (#08714-04, Nacalai Tesque) and a phosphatase inhibitor cocktail (1:100, #07575-51, Nacalai Tesque). Cell lysates were resolved by 20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The following primary antibodies were used: anti-E-cadherin (M106 TaKaRa, Shiga, Japan); anti-N-cadherin (#610921 BD Transduction Laboratories, NJ, USA); anti-β1-actin (#A5441, Sigma-Aldrich, St Louis, MO, USA); anti-vimentin, and anti-Smad2/3, anti-phospho-Smad2, anti-phospho-Smad3, and anti-Smad4 (D21H3, #9963 Cell Signaling Technology MA, USA). The membranes were blocked with 5% FBS at room temperature for 1 h. Following overnight incubation with the primary antibodies, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, PA, USA). The proteins were visualized using the EzWestLumi Plus detection kit (ATTO, Tokyo, Japan), and luminescence was detected using the LuminoGraph II imaging system (ATTO).

Enzyme-linked immunosorbent assay (ELISA). A549 cells were initially cultured in the media containing 1ng/ml of TGF-β1. Then, after the time of interest with or without tranilast, the conditioned media were applied to ELISA assay. The concentration of TGF-β1 in cell culture medium under various conditions was measured using ELISA according to the manufacturer's instructions (DR100B, R&D Systems, MN, USA).

Cell invasion assay. A549 and PC14 cells were cultured in media containing 1 ng/ml of TGF-β1. After a 48-h incubation with 0 or 50 μM tranilast, the cells were harvested for use in the subsequent experiments. The cells (100,000 cells per well) were suspended in 0.1% FBS containing medium and seeded on Matrigel-coated membranes with 8-μm pores at the bottom of the upper chamber of a Matrigel Invasion Chamber (#354480, Corning, MA, USA). Media containing 10% FBS was added to the lower chamber as a chemoattractant. Tranilast was added to each chamber at the indicated concentrations. After a 14-h incubation, the invading cells that were attached to the lower surface of the membranes were fixed and stained. The invasiveness of cancer cells was assessed using a BZ-9000 microscope (Keyence, Osaka, Japan). The numbers of invading cells were counted in five random fields at 100× magnification.

Gene knockdown of Smad4. MISSION short interfering RNAs (siRNAs) for human Smad family member 4 (Smad4) were purchased from Sigma-Aldrich (si-Smad4: #1 SASI_Hs01_00207793, #2 SASI_Hs_00207794, and #3 SASI_Hs_00207795). The siRNAs were transfected to cancer cell lines using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). To evaluate the efficiency of Smad4 knockdown, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot assays were performed.

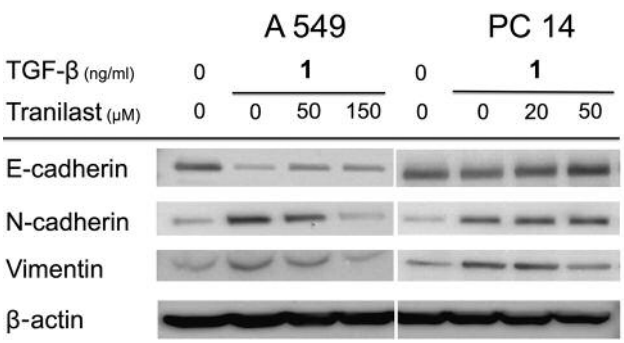

Figure 1. *Western blot analysis of epithelial-mesenchymal transition markers and β-actin in A549 and PC14 cells treated with or without TGF-β1 and different concentrations of tranilast. TGF-β1: Transforming growth factor β1.*

qRT-PCR. Total RNAs from A549 and PC14 cells with or without TGF-β1 and with or without tranilast were extracted and reverse transcribed to cDNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences, PA, USA) according to the manufacturer's instructions. Each cDNA sample was mixed with THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan) and TaqMan Gene Expression Assay probe/primer set (Thermo Fisher Scientific). These reactions were run using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The comparative Ct (ΔΔCt) method was used to determine relative expression levels using β1-actin as the internal control.

Mouse orthotopic lung cancer model. Six- to seven-week-old male athymic nude mice (BALB nu/nu) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained in a pathogen-free environment on a 12-h light/12-h dark cycle with sterile food and water. To establish an orthotopic lung cancer model, a BD Matrigel basement matrix (BD Biosciences, MA, USA) was used to settle cancer cells that were then injected into the lungs (12). Twenty-four mice were anaesthetized with intraperitoneal injection of sodium pentobarbital (4.5 mg/kg) and inhalation of 2% isoflurane, and a 1-cm incision was made on the left thorax. A total of 1.5×10^6 A549 cells in 15 μl phosphate-buffered saline were mixed with 15 μl of Matrigel on ice. The mixture was immediately injected using a 30-gauge needle into the left lung parenchyma through the intercostal muscle. The skin incision was sutured with 3-0 monofilament, and the mice were observed for 30 min until fully recovered.

Five days later, the mice were randomly divided into two groups (tranilast group and control group). The tranilast group was administered 200 mg/kg/day of tranilast suspended in 200 μl of 1% $NaHCO_3$ by oral gavage for 35 days, and the control group was administered only 200 μl of 1% $NaHCO_3$ daily for the same duration. On the 36th day, the mice were sacrificed, and the surgical outcomes were evaluated (lung tumour development, effusion, dissemination, lymph node metastasis, and metastasis to other organs). Lung tissue with orthotopic tumours was removed for histological and immunohistochemical analysis.

Immunohistochemical analysis of EMT markers and Smad4. Immunohistochemical analysis of E-cadherin, vimentin, and Smad4

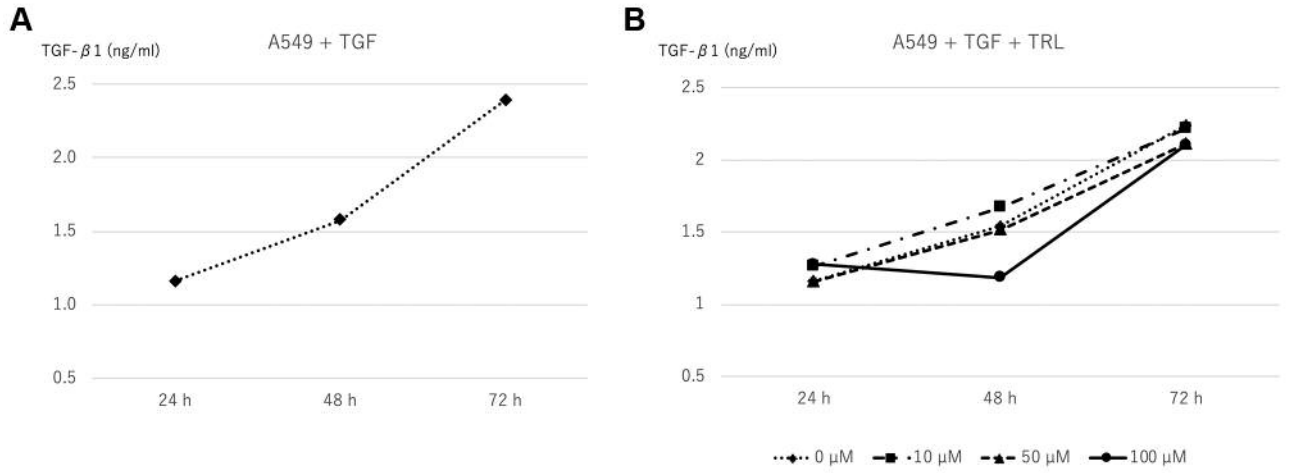

Figure 2. *Changes in TGF-β1 levels in cell culture medium during the 72-h period after cell seeding or treatment. (A) The levels of TGF-β1 gradually increased in the medium of A549 cells treated with TGF-β1. (B) The levels of TGF-β1 gradually increased in the medium of A549 cells treated with TGF-β1 and tranilast at all concentrations of tranilast. TGF-β1: Transforming growth factor β1; TRL: tranilast.*

was performed manually using the standard avidin-biotin-peroxidase complex method with the following rabbit monoclonal antibodies: anti-E-cadherin (#3195 24E10 diluted 1:400, Cell Signaling Technology), anti-vimentin (#5741 D21H3 diluted 1:100, Cell Signaling Technology), and anti-Smad4 (#46535 03R4N diluted 1:400, Cell Signaling Technology). Staining was performed with 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) and haematoxylin. Three investigators (KT, SN, and RM) independently scored the immunostained sections using H-scores in a blinded fashion, and the expression of E-cadherin, vimentin, and Smad4 was categorized as either positive or negative.

Statistical analysis. Statistical analyses were performed using JMP Pro 13 software (SAS Institute, NC, USA). Data from the cell invasion assay and qRT-PCR were analyzed using one-way analysis of variance. The chi-squared test was used for inter-group comparisons in the immunohistochemical analysis. *p*-Values (two-tailed) below 0.05 were considered statistically significant.

Results

Tranilast inhibited TGF-β1–induced EMT in A549 and PC14 cells. Treatment of A549 and PC14 cell lines with 1 ng/ml of TGF-β1 for 48 h, which have an epithelial phenotype when cultured under normal conditions, resulted in increased expression of mesenchymal phenotypic markers and decreased expression of epithelial phenotypic markers. Following a 24-h exposure to TGF-β1, tranilast was added to the cell cultures at various concentrations (A549: 50, 100, and 150 μM; PC14: 20, 50, and 100 μM), and incubated for an additional 24 h. Tranilast treatment restored the expression of TGF-β1–induced EMT markers (E-cadherin, N-cadherin, and vimentin), to the levels of the epithelial stage in A549 cells (Figure 1). In PC14 cells, tranilast reinstated only the levels of vimentin.

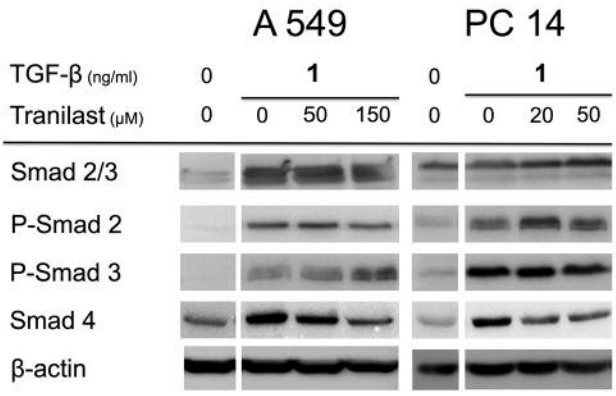

Figure 3. *Western blot assays of Smad and β-actin in A549 and PC14 cells treated with or without TGF-β1 and different concentrations of tranilast. TGF-β1 induced the phosphorylation of Smad2 and 3 and increased the expression of Smad4. The addition of tranilast decreased the expression of Smad4 in both cell lines. TGF-β1: Transforming growth factor β1.*

Tranilast did not inhibit the release of TGF-β1 from A549 cells. To elucidate the mechanisms involved in the reversal of TGF-β1–induce0d EMT by tranilast, the concentration of TGF-β1 in the culture medium of tranilast-treated cells was measured using ELISA. The A549 cells were treated with 1 ng/ml TGF-β1 for 24 h and then treated with different concentrations of tranilast for an additional 24, 48 or 72 h. The concentrations of TGF-β1 gradually increased in the culture medium with A549 cells treated by TGF-β1 (Figure 2A). However, the release of TGF-β1 by A549 cells was not suppressed by tranilast treatment (Figure 2B) after the initial

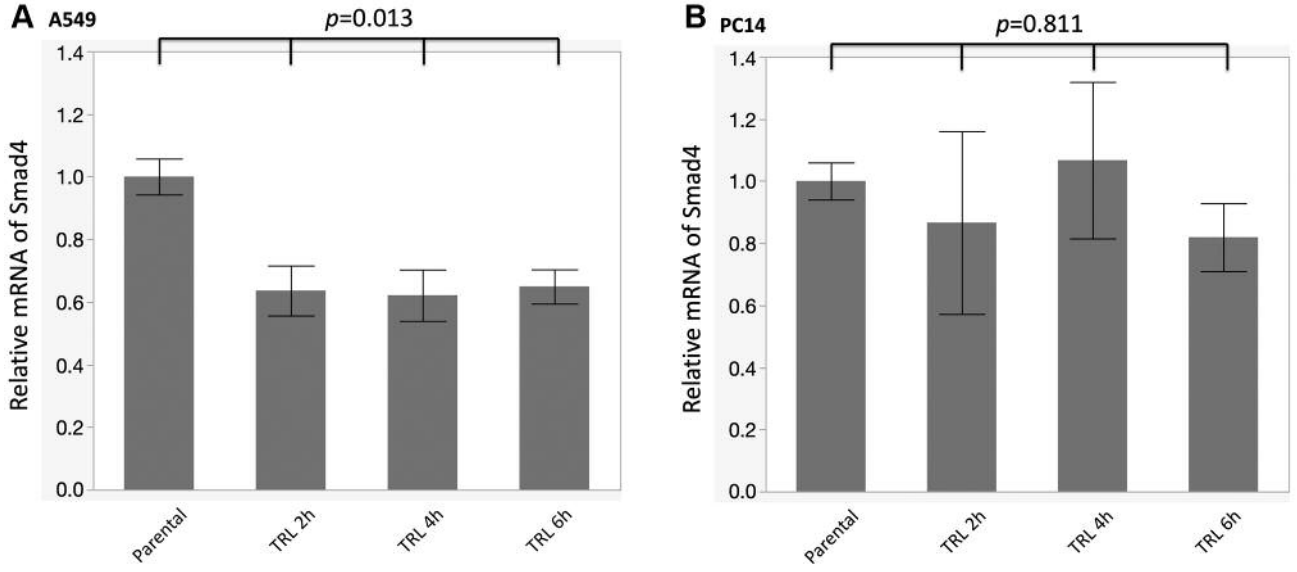

Figure 4. *Time-dependent relative mRNA expression of Smad4 following tranilast treatment of A549 and PC14 cells. (A) Smad4 transcription was significantly suppressed in A549 cells after tranilast treatment. (B) Smad4 transcription was not significantly suppressed in PC14 cells after tranilast treatment. TRL: Tranilast.*

addition of TGF-β1. These results suggested that tranilast did not inhibit the release of TGF-β1 from A549 cells.

Tranilast inhibited the TGF-β1 signalling pathway via the suppression of Smad4. Smad proteins consitute the major downstream mediators of TGF-β1 signaling, and the TGF-β1/Smad signalling pathway plays multiple roles in regulating cell growth, differentiation, invasion, and metastasis (13). Western blot assays showed that TGF-β1 induced the phosphorylation of Smad2 and Smad3, and increased Smad4 expression. Although tranilast did not affect the TGF-β1–induced phosphorylation of Smad2/3, it decreased the expression of Smad4 (Figure 3). We also assessed the mRNA levels of Smad4 in the presence or absence of tranilast. The results showed that tranilast significantly suppressed the transcription of Smad4 in A549 cells (Figure 4A), but had no similar effect in PC14 cells (Figure 4B).

Vimentin expression was suppressed by Smad4 knockdown in TGF-β1–stimulated A549 and PC14 cells. Following the findings that tranilast suppressed Smad4 expression, we evaluated the effect of Smad4 knockdown on the expression of EMT markers and the invasiveness of TGF-β1–stimulated A549 and PC14 cells. In these cell lines, Smad4 was successfully knocked down *via* specific siRNAs. The efficacy of Smad4 knockdown was verified using qRT-PCR and western blot assays (Figure 5A). Smad4 knockdown under TGF-β1 stimulation suppressed vimentin expression but showed no difference on the expression E- and N-cadherin in both cell lines (Figure 5B).

Tranilast inhibited TGF-β1–mediated cell invasion in A549 and PC14 cells. Matrigel invasion assays were performed to evaluate the inhibitory effect of tranilast on TGF-β1–mediated invasion. In addition, Smad4-knockdown cancer cells were compared with the control group, TGF-β1 group, and TGF-β1 + tranilast group. After being cultured in the presence or absence of TGF-β1, tranilast was added at various concentrations and siRNA was transfected into cells in the TGF-β1 group. After a 24-h incubation, the cells were collected and seeded onto the upper chambers pre-coated with Matrigel, and cultured for an additional 14 h. Cell invasion was apparently promoted under TGF-β1 stimulation. Furthermore, invasion was significantly suppressed in the tranilast and Smad4-knockdown groups when compared with the TGF-β1 group (Figure 6). These results suggest that Smad4 is a key factor in the TGF-β1-induced Smad-mediated cancer cell invasion, and that tranilast reduced the invasiveness of TGF-β1–stimulated A549 and PC14 cells *via* Smad4 suppression.

Tranilast suppressed the pleural dissemination of cancer cells and vimentin expression in the mouse orthotopic lung cancer model. All mice were sacrified after 36 days of treatment, and we evaluated the presence of orthotopic lung tumours in their left lungs. The surgical outcomes (effusion, dissemination, mediastinal lymph nodes, and abdominal

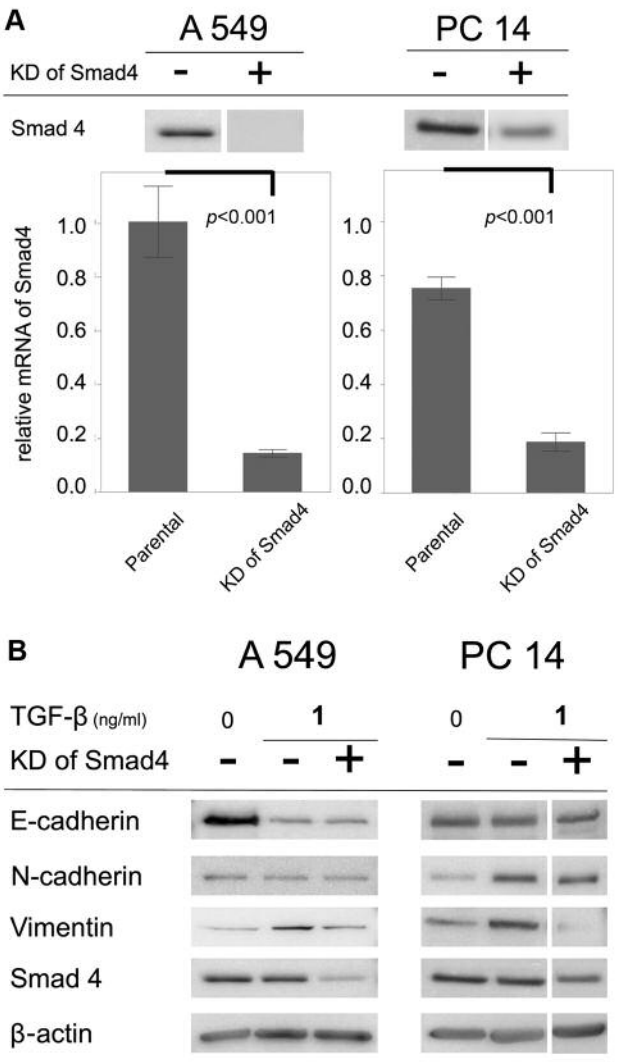

Figure 5. *(A) The efficacy of Smad4 knockdown was verified using qRT-PCR and western blot assays. (B) Western blot assays of epithelial-mesenchymal transition markers and β-actin in A549 and PC14 cells treated with or without TGF-β1 and with or without Smad4 knockdown. Smad4 knockdown suppressed vimentin expression in both cell lines but had no effects on E-cadherin or N-cadherin levels. KD: Knockdown; TGF-β1: transforming growth factor β1.*

findings) were also examined. Orthotopic lung cancer was confirmed in six mice in the tranilast group and nine mice in the control group. Upper and/or lower mediastinal lymph node metastasis was detected in all cases. Although there was no significant difference in pleural effusion, the dissemination of cancer cells in the left thorax was significantly suppressed ($p<0.05$) in the tranilast group (1 case, 16.7%) when compared with the control group (7 cases; 77.8%) (Table I, Figures 7 and 8). Furthermore, pleural dissemination of cancer cells was significantly suppressed in the tranilast group not only in the implanted side, but also in the non-implanted side (Figure 8). Although the brain, liver, abdominal lymph nodes, and adrenal grands were also screened, metastasis to these areas was not detected in any of the mice.

Immunohistochemical analysis was performed to examine the expression of EMT markers and Smad4 (Table I, Figure 7). Vimentin was significantly suppressed ($p=0.005$) in the tranilast group (vimentin-positive: 16.7%) when compared with the control group (vimentin-positive: 88.9%). Similarly, Smad4 was significantly suppressed ($p=0.02$) in the tranilast group (Smad4-positive: 66.7%) when compared with the control group (Smad4-positive: 100%). There was no difference in the expression of E-cadherin between the groups.

Discussion

This study demonstrated that tranilast inhibited TGF-β1–induced EMT and cell invasion in A549 and PC14 cancer cells, and suppressed the pleural dissemination of tumour cells in mice. Our *in vitro* and *in vivo* experiments indicated that these effects were achieved through the suppression of Smad4 in TGF-β1/Smad signalling.

Tranilast has been reported to inhibit the release of TGF-β1 from mast cells and fibroblasts (10), but its mechanism in cancer cells remains unclear. Our experiments showed that tranilast did not inhibit TGF-β1 release from a lung cancer cell line. Previous studies have reported that pre-treatment with tranilast inhibits TGF-β1–induced phosphorylation of Smad2 and Smad2/3 in human peritoneal mesothelial cells and homozygous corneal fibroblasts (14, 15), but these studies did not examine the effects of tranilast on Smad4. EMT is accompanied by a cell junction switch from E-cadherin to N-cadherin (16, 17), and TGF-β1 signalling is known to be an important pathway to induce EMT in a Smad4-dependent manner by inducing the translocation of the Smad2/3/4 complex to the nucleus, which leads to the expression of mesenchymal markers (18). Wang *et al*. have proposed that Smad4 acts as a central mediator of the TGF-β1 signalling pathway (19). In this study, we found that tranilast suppressed TGF-β1induced Smad4 levels in A549 and PC14 cells. Furthermore, tranilast was able to significantly suppress Smad4 mRNA expression in TGF-β1–stimulated A549 cells, but this effect was not observed in PC14 cells. The mechanisms by which tranilast suppresses TGF-β1 signalling have yet to be elucidated as this drug is seems to have several targets and exerts effects on mast cells, fibroblasts, tumour microenvironments, and cancer cells (20). Because EMT can be induced by various signaling factors, the specific inhibitory effects of tranilast on EMT remain unclear due to possible crosstalk with other pathways. Nonetheless, our experiments showed that tranilast reduced cancer cell invasion in TGF-β1–

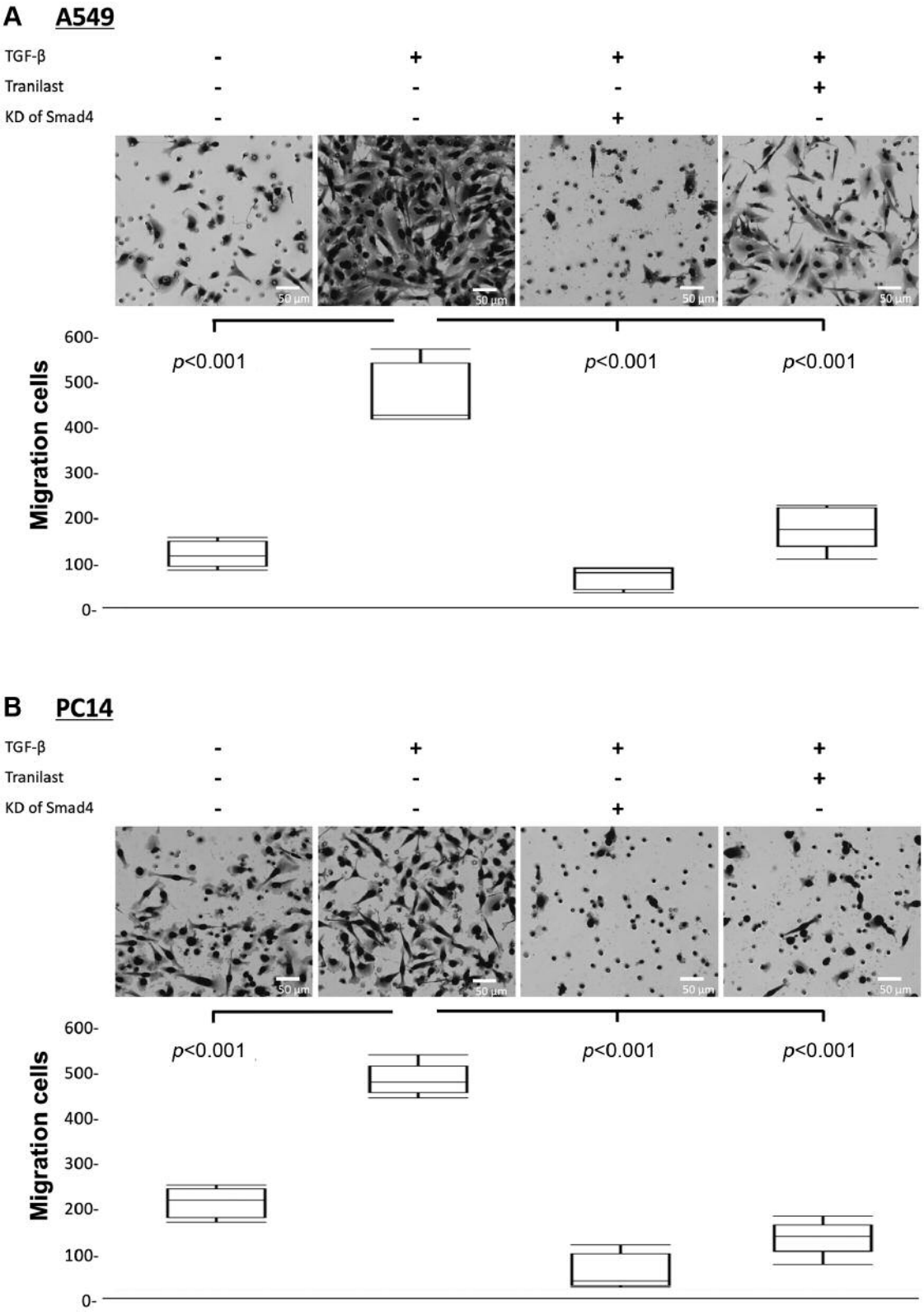

Figure 6. *Matrigel invasion assays to examine the effects of tranilast on TGF-β1–mediated cell invasion with or without Smad4 knockdown. Smad4 knockdown and tranilast treatment following exposure to TGF-β1 (1 ng/ml) significantly suppressed cell invasion in (A) A549 cells and (B) PC14 cells. TGF-β1: Transforming growth factor β1.*

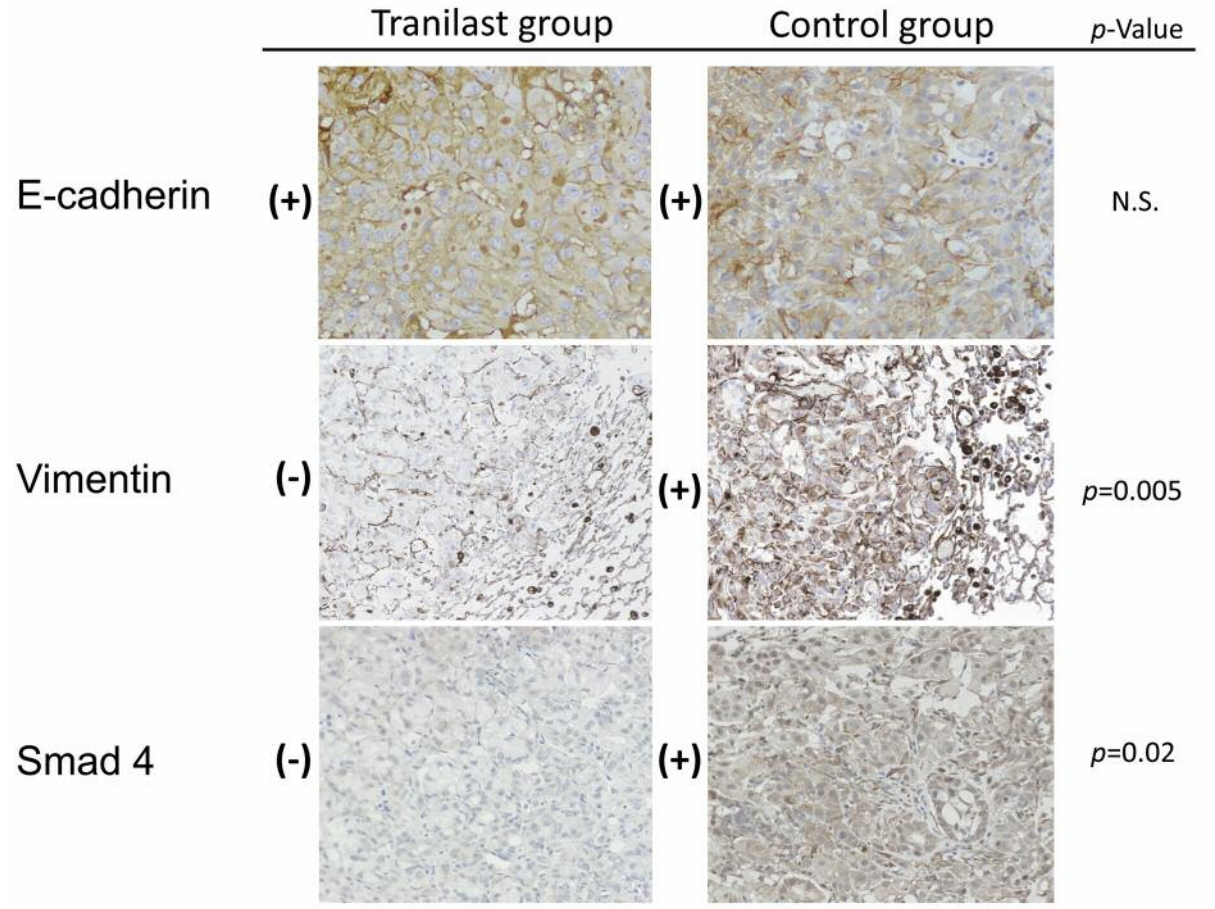

Figure 7. *Immunohistochemical staining of E-cadherin, vimentin, and Smad4 of orthotopic lung tumours from the tranilast and control mouse groups. Staining was performed using diaminobenzidine tetrahydrochloride and haematoxylin. Tranilast treatment significantly suppressed vimentin and Smad4 levels. However, no significant difference was observed in E-cadherin levels between the groups. N.S.: not significant.*

Table I. *Surgical and immunohistochemical findings of the effects of tranilast in a mouse orthotopic lung cancer model.*

	Tranilast group (200 mg/kg/day) n=6		Control group n=9		
Surgical findings	n	%	n	%	*p*-Value
Upper mediastinal lymph node metastasis	5	83.3%	7	77.8%	N.S.
Lower mediastinal lymph node metastasis	6	100%	8	88.9%	N.S.
Dissemination					
Left thorax (+)	1	16.7%	7	77.8%	0.02
Right thorax (+)	0	0%	5	55.6%	0.02
Effusion					
Left thorax (+)	2	33.3%	5	55.6%	N.S.
Right thorax (+)	2	33.3%	3	33.3%	N.S.
Immunohistochemical findings					
E-cadherin (+)	5	83.3%	7	77.8%	N.S.
Vimentin (+)	1	16.7%	8	88.9%	0.005
Smad4 (+)	2	33.3%	8	88.9%	0.02

N.S.: Not significant.

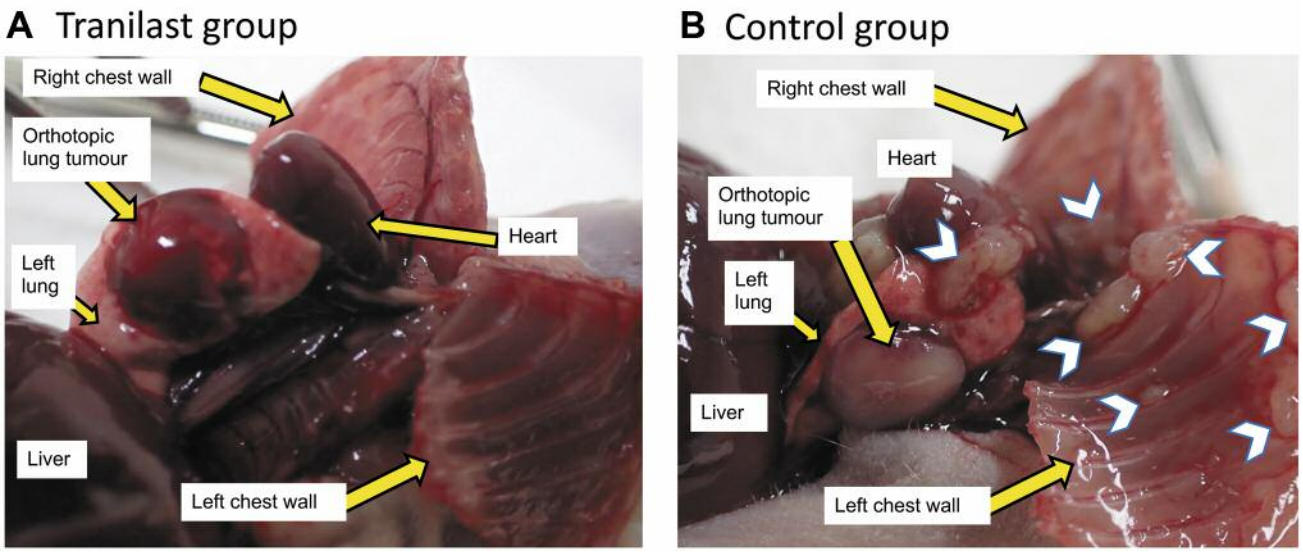

Figure 8. *Dissemination of cancer cells in the thoracic cavity of (A) a representative image of thoracic findings from the tranilast group and (B) a representative image of thoracic findings from the control group. In the control group mouse, widespread dissemination of cancer cells is observed in both sides of the thoracic cavity (arrowheads), but no nodules are observed in the tranilast group mouse.*

stimulated A549 and PC14 cells. Similarly, the knockdown of Smad4 in these cells also reduced invasiveness. This suggests that tranilast plays a critical role in inhibiting Smad4, which is an important EMT mediator.

In this study, we used an orthotopic lung cancer model as cancer cells grow better in their tissue of origin, and more clinically relevant studies can be performed using the orthotopic site of tumour growth (12). We conducted these experiments because few studies have reported the effects of tranilast in a mouse lung orthotopic model. Tranilast was observed to significantly suppress the pleural dissemination of cancer cells when compared with the control group. According to the 8th edition of the TNM classification for NSCLC (2), lung cancer with pleural dissemination is categorized as stage IV and is therefore associated with poor prognosis. Our findings imply that tranilast may be able to improve the prognosis of mice with lung cancer.

A previous study has reported that A549 cells exhibit an EMT phenotype,E-cadherin-negative and vimentin-positive, 44 days after implantation in a mouse orthotopic lung cancer model (21). Our experiments shoed that tranilast suppressed vimentin expression, but did not have any effect on E-cadherin expression. We have reported that vimentin-positive lung adenocarcinoma patients who had undergone surgery at our institution had significantly poorer prognoses than the vimentin-negative ones (22). Other studies have also noted that the expression of vimentin is a negative prognostic indicator (23, 24). Our results suggest that tranilast has the potential to suppress the induction of vimentin expression in EMT, which may in turn contribute to better prognoses.

Furthermore, tranilast has been clinically used in the treatment of allergic and hypertrophic diseases for several decades and adverse effect rate is only 2.36% (25). Accordingly, it has the potential to be safely used in clinical applications as a theraputic agent against cancer invasion and metastasis.

The study has several limitations. First, TGF-β1 signalling involves other pathways apart from the TGF-β1/Smad pathway, but the influence of tranilast on these pathways was not examined. Second, although the behaviour of Smad4 expression in response to tranilast treatment was similar in both cell lines, this drug did not suppress the transcription of TGF-β1–stimulated Smad4 in PC14 cells. The reason for the differential effect on Smad4 transcription is unclear. Third, we did not examine the correlation between EMT and Smad family protein expression in clinical specimens because tranilast is not used in lung cancer patients who have undergone surgery.

In conclusion, tranilast was found to suppress TGF-β1/Smad signalling by inhibiting the expression of Smad4 in NSCLC cell lines treated with TGF-β1. In addition, tranilast decreased pleural dissemination and the expression of both vimentin and Smad4 in a mouse orthotopic lung cancer model. These findings indicate that tranilast has potential applications in the treatment of cancer invasion and metastasis through the inhibition of TGF-β1–induced EMT.

Conflicts of Interest

The Authors declare that there are no potential conflicts of interest. Although tranilast was provided by Kissei Pharmaceutical Co. Ltd.,

the company had no involvement in the study design, data analysis and interpretation, preparation of the manuscript, or decision to submit the manuscript for publication.

Authors' Contributions

Koji Takahashi and Toshi Menju conceived and designed this study. Ryo Miyata, Shigeto Nishikawa, and Koji Takahashi performed the animal experiments and evaluated the data. Koji Takahashi wrote the initial draft of the manuscript and produced the figures and table. All authors contributed to the design of research, discussion of the results, and review of the manuscript prior to submission. Toshi Menju, Makoto Sonobe, and Hiroshi Date supervised this study and critically reviewed the manuscript.

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