Methylation-induced downregulation of TFPI-2 causes TMPRSS4 overexpression and contributes to oncogenesis in a subset of non-small-cell lung carcinoma

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We identified transmembrane protease, serine 4 (TMPRSS4) as a putative, druggable target by screening surgically resected samples from 90 Japanese non-small-cell lung cancer (NSCLC) patients using cDNA microarray. TMPRSS4 has two druggable domains and was upregulated in 94.5% of the lung cancer specimens. Interestingly, we found that TMPRSS4 expression was associated with tissue factor pathway inhibitor 2 (TFPI-2) expression in these clinical samples. In contrast to TMPRSS4, TFPI-2 expression was downregulated in NSCLC samples. The in vitro induction of TFPI-2 in lung cancer cell lines decreased the expression of TMPRSS4 mRNA levels. Reporter assay showed that TFPI-2 inhibited transcription of TMPRSS4, although partially. Knockdown of TMPRSS4 reduced the proliferation rate in several lung cancer cell lines. When lung cancer cell lines were treated with 5-aza-2′-deoxycytidine or trichostatin A, their proliferation rate and TMPRSS4 mRNA expression levels were also reduced through the upregulation of TFPI-2 by decreasing its methylation in vitro. The TFPI-2 methylation level in the low TMPRSS4 group appeared to be significantly low in NSCLC samples (P = 0.02). We found a novel molecular mechanism that TFPI-2 negatively regulates cell growth by inhibiting transcription of TMPRSS4. We suggest that TMPRSS4 is upregulated by silencing of TFPI-2 through aberrant DNA methylation and contributes to oncogenesis in NSCLC.
in lung cancer cell lines, resulting in the inhibition of their growth.

Materials and Methods

Patients and tissue specimens. Ninety anonymous, surgically resected NSCLC samples obtained at Keio University Hospital (Tokyo, Japan) were collected for this study. All the samples were obtained in accordance with the institutional review board of our institute (Institutional Review Board #16-90-1). Tumor tissues were intraoperatively dissected along with surrounding non-malignant tissues; paired non-malignant lung tissues were also obtained from the same patients from an area adjacent to their tumors.

Microarray. GeneChip Human Genome 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) were used to monitor the expression profiles of the samples. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) after treatment with TRIzol (Invitrogen, Carlsbad, CA, USA), and labeled cRNA was prepared using standard Affymetrix protocols. The signal intensities of the probe sets were normalized by the Affymetrix Power Tools RNA method using Resolver software (Rosetta Inpharmatics, Seattle, WA, USA), and log ratio values to the average of the non-malignant samples were calculated for each sample using Resolver software.

Cell lines and materials. All tested cells were obtained from ATCC (Manassas, VA, USA) and cultured according to the supplier’s instructions. Both 5-aza-2’-deoxycytidine and TSA and were purchased from Wako (Osaka, Japan).

Quantitative RT-PCR. cDNA was synthesized from 1 μg total RNA using TaqMan reverse transcription reagents (Invitrogen). All tumor samples were assessed histopathologically to ensure that more than 70% of the samples contained malignant tissue before RNA extraction. Quantitative real-time PCR assays for human TFPI-2 and TMPRSS4 were carried out in triplicate for cDNA samples in 96-well plates. Polymerase chain reaction was carried out in a single plate when gene expression was compared among different cell lines. Data were collected and analyzed using the ABI 7000 sequence detector system (Applied Biosystems, Foster City, CA, USA). Pre-designed TaqMan probes and primers for TFPI-2 (Hs00197918_m1), TMPRSS4 (Hs00212669_m1), ACTB (β-actin) (4326315E), and GAPDH (4310884E) genes were purchased from Applied Biosystems. Samples were analyzed in triplicate. Relative quantification values were calculated using the ΔCt method (cycle threshold), in which the ΔCt value was calculated by subtracting the Ct value of TFPI-2 from that of β-actin or GAPDH. β-actin was used for the experiments comparing gene expression among different cell lines.

Immunohistochemistry. Immunohistochemical analysis was carried out for six NSCLC specimens. We randomly selected three samples each from those specimens with relatively low TFPI-2 and high TMPRSS4 expression, or from those with relatively high TFPI-2 and low TMPRSS4 expression, estimated by cDNA microarray. All tumor tissues as well as the surrounding lung tissues were removed and embedded in paraffin and cut into 4-μm-thick sections. These sections were deparaffinized, rehydrated, and incubated in 0.03% H2O2 in 95% methanol at room temperature for 20 min to block endogenous peroxidase activity. Antigen retrieval was carried out using a microwave oven in 10 mM citrate buffer (pH 6.0). All sections were incubated for 20 min with normal horse serum to eliminate nonspecific staining and were then incubated with anti-human TFPI-2 antibody (1:50, #11283-1-AP; ProteinTech, Chicago, IL, USA) or anti-human TFPI-2 antibody (1:100, #sc-28864; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. This was followed by incubation with the secondary antibody (ImmPRESS Reagent Kit; Vector Laboratories, Burlingame, CA, USA) for 30 min. Slides were then incubated in diaminobenzidine/Tris solution (3 diaminobenzidine/Tris tablets diluted in 150 mL distilled water; Muto Pure Chemicals, Tokyo, Japan) supplemented with 15 μL of 30% H2O2. Finally, the slides were counterstained with hematoxylin. The proportion of cells stained and the staining intensity were assessed by the pathologist as follows: intensity score 0, absence of staining; 1, weakly stained; 2, moderately stained; and 3, strongly stained. The total score was calculated by multiplying the proportion score with the intensity score.

Western blot analysis. Protein was lysed with cell lysis buffer (Cell Signaling Technologies, Danvers, MA, USA) and protein concentrations were calculated by BCA protein assay (Thermo Scientific, Waltham, Massachusetts, USA) and denatured and reduced with sample buffer. Equal amounts of protein were subjected to electrophoresis. The fractionated proteins were transferred to PVDF membranes. Next, the membrane was subjected to the diluted primary antibodies (α-GFP [Evrogen, Moscow, Russia], α-β-actin [Sigma-Aldrich, St. Louis, MO, USA]) followed by incubation with secondary antibodies. For detection of protein, the membrane was incubated with agitation in LumiGLO reagent and peroxide (Cell Signaling Technologies) and exposed to X-ray film.

Reporter assay. The 2000-bp promoter region of TMPRSS4 was cloned from human genomic DNA (TaKaRa, Shiga, Japan) with the primers 5'-GCCACCAAGCCATCAGTAAAG-AGG-3' and 5'-AGCAGAGGGCCCCCCACACTCACC-3'. Nested PCR products amplified with the primers 5'-GTACCTGCAGCCAGCTCAGTCTGTGAGAG-3' and 5'-GCTAGCCGTTGCTTGATCCTCCTCCG-3' were digested with the restriction enzymes KpnI and NheI (New England Biolabs, Beverly, MA, USA) and cloned into a pGL4.10 firefly luciferase plasmid (Promega, Madison, WI, USA). Cells were transfected with pGL4.10 (Promega) or pGL4.10-TMPRSS4 promoter and pCMV6-AC-GFP ( OriGene, Rockville, MD, USA) or pCMV6-AC-GFP-tagged TFPI-2 using the X-tremeGENE 9 DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer’s protocol. All cells were also transfected with the same amount of pRL-TK Renilla luciferase plasmid (Promega) for normalization. Cells were lysed in Passive Lysis Buffer (Promega) by incubation for 15 min at room temperature. After centrifugation at 14,000 g for 5 min, the supernatant was used for the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were measured with the Synergy 4 multimode plate reader (BioTek, Winooski, VT, USA). The ratios of firefly to Renilla activity in cells transfected with pGL4.10 and pCMV6-AC-GFP was normalized to 1.

Proliferation assay and knockdown/overexpression of TMPRSS4 and/or TFPI-2. NCI-H358, NCI-H520, NCI-H1975, A549, and NCI-H2228 cells were seeded in 96-well plates at a density of 5000 cells/well for NCI-H358 and 1000 cells/well for other cell lines. The following day, an equal volume of medium containing TSA or 5-aza-2’-deoxycytidine (adjusted to 2× the final concentrations) was added to each well. The cell population density was measured on days 0–4 with a CellTiter 96 Aqueous One kit (Promega) using a microplate reader (Model680; Bio-Rad, Hercules, CA, USA).

For the knockdown assay, siRNA against TFPI-2 (mixture of sI5514 and s224834; Dharmacon, Lafayette, CO, USA) or
TMPRSS4 (mixture of s32202, s32203, and s32204; Dharmacon) was mixed with siLentFect (Bio-Rad), and the assay was carried out the next day. As a negative control, each non-targeting siRNA (Dharmacon) was used. For the overexpression assay, pCMV-Tag2B (Stratagene, La Jolla, CA, USA), FLAG-tagged TMPRSS4, GFP-tagged TFPI-2, or pCMV6-AC-GFP (OriGene) mixed with X-tremeGENE 9 DNA Transfection Reagent (Roche) were transfected into the cells according to the manufacturer’s protocol.

**Bisulfite treatment and MethyLight analysis.** Genomic DNA was prepared using the DNeasy kit (Qiagen) according to the manufacturer’s protocol. Approximately 1 μg genomic DNA was bisulfite-treated with the EZ DNA Methylation-GOLD kit (ZYMO Research, Orange, CA, USA).

MethyLight analysis was carried out using the ABI 7000 sequence detector system. In brief, the PCR assay was carried out in a final reaction volume of 30 μL containing 300 nM forward and reverse primers and a 100 nM probe with bisulfito-converted genomic DNA in 1× TaqMan Universal PCR master mix. The primer and probe sequences were as follows: forward (5'-GGCGAAGTTGTTATTAGTCGTC) and reverse (5'-GGCGAAGTTGTTATTAGTCGTC) primers and the probe (5'-6FAM-AAACTCTCGCGCTCCTTACGCCG-BHQ1) for methylated TFPI-2; or forward (5'-TGTTGTAGGAGGAGGTATGTAAG) and reverse (5'-ACCAATATAACC-TACTCCTCTCTTATA) primers and the probe (5'-6FAM-ACCCACACCAACACATAAACACAC-TAMRA) for methylated β-actin. Polymerase chain reaction was carried out under the following cycling conditions: 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Amplification of β-actin was used as an internal reference. Samples were analyzed in triplicate. A relative quantification value was calculated using the ΔCt method (cycle threshold), in which the ΔCt value was calculated by subtracting the Ct value of TFPI-2 from that of β-actin.

**Statistical analysis.** The hypergeometric test for gene ontology enrichment was carried out using the Gene Set Annotator developed by Rosetta Inpharmatics. Statistical analyses of the proliferation assay or mRNA expression levels were carried out using Student’s t-tests. All P-values were two sided and P < 0.05 was considered statistically significant. Kaplan–Meier curves and Cox regression analysis were carried out using srsr 19.0 software (SPSS; IBM, Chicago, IL, USA).

**Results**

Uproegulation of TMPRSS4 and downregulation of TFPI-2 in clinical lung cancer samples. We initially carried out mRNA profiling of 90 Japanese NSCLC patients (54 adenocarcinomas [AC], 24 squamous cell carcinomas [SCC], and 12 other lung cancers; patient characteristics are shown in Table S1), and identified 120 genes that were commonly upregulated more than twofold with a ratio P-value <0.001 in >75% of the samples (Fig. 1a). With these criteria, approximately 1700 probes had a greater than two-fold change, and of these, 163 probes were upregulated. Eliminating overlapping genes and expressed sequence tags, we obtained 120 genes as unique, upregulated genes. Among these 120 genes, 15 genes were found to have druggable domain(s) (Table 1), as determined by the Gene Set Annotator (Rosetta Inpharmatics). We prioritized these 15 genes in terms of cancer relevance and unknown mechanism for tumorigenesis.

TMPRSS4 overexpression has been reported in various cancers including lung cancer (5,7,23) We also confirmed that TMPRSS4 was overexpressed not only in clinical lung cancer samples but also in several lung cancer cell lines (Fig. 1b,c). It is reported that TMPRSS4 promotes tumor growth, invasion, metastasis, and the epithelial–mesenchymal transition process and regulates *in vitro* cell growth (24,25); however, only limited mechanisms for tumorigenesis by TMPRSS4 have been clarified.

In contrast, TFPI-2 was downregulated in both clinical lung cancer samples and many of the cell lines (Fig. 1b,d). TMPRSS4 was overexpressed in 94.5% of the lung cancer patient specimens compared to their corresponding non-malignant samples, whereas TFPI-2 was downregulated in 82.4% of the specimens. Regarding the difference in histology, higher mRNA expression level of TMPRSS4 was observed in SCC compared to AC, although it was not significant (Fig. S1a). The expression level of TFPI-2 mRNA was significantly lower in SCC compared to AC (Fig. S1b). TMPRSS4 was upregulated in 96.3% of AC and 95.8% of SCC. TFPI-2 was downregulated in 77.8% of AC and 100% of SCC.

The expression of TMPRSS4 and TFPI-2 was analyzed by immunohistochemistry in six cases (Fig. 2). These cases included three ACs and three SCCs. TMPRSS4 was expressed mainly in the cell membrane and also in the cytoplasm of cancer cells, and TFPI-2 was expressed in the cytoplasm of cancer cells (Fig. 2a). TMPRSS4 was not expressed in stromal cells, whereas TFPI-2 was expressed not only in cancer cells but also in immune cells and stromal cells. Although the sample size was small and might not be representative of all cases, protein and mRNA expressions showed a weak correlation for both genes (r = 0.348 for TMPRSS4; r = 0.395 for TFPI-2) (Fig. 2b,c), and the protein expressions of these genes seemed to be negatively correlated (r = −0.524) (Fig. 2d).

**Downregulation of TMPRSS4 by TFPI-2.** Because TMPRSS4 is a serine protease and TFPI-2 encodes a broad-spectrum serine protease inhibitor, we speculated that there is some relationship between TMPRSS4 and TFPI-2. Although we first hypothesized that TFPI-2 inhibits the protease activity of TMPRSS4, surprisingly, we found that the mRNA expression level of TMPRSS4 was reduced when TFPI-2 was overexpressed by plasmid transfection in NCI-H358, NCI-H520, and NCI-H1975 cells (Fig. 3a, b). We checked whether the reporter activity of endogenous TMPRSS4 is inhibited by the overexpression of TFPI-2. The reporter activity of the TMPRSS4 promoter region was partially inhibited by TFPI-2 in three cell lines (Fig. 3c). Conversely, the knockdown of TFPI-2 by siRNA induced an increase in TMPRSS4 mRNA expression (Fig. 3d,e).

**Effect on cell growth by expression level of TMPRSS4.** Next, a proliferation assay was carried out to assess the effect of TMPRSS4 on cell growth by knockdown or overexpression of the gene. The knockdown of TMPRSS4 expression by siRNA treatment inhibited growth in all of five tested cell lines, NCI-H358, NCI-H520, NCI-H1975, A549, and NCI-H2228 (Fig. 4a); conversely, the overexpression of TMPRSS4 augmented the growth of these cell lines (Fig. 4b).

TFPI-2 methylation in NSCLC regulates the expression of TMPRSS4. As aberrant methylation of the TFPI-2 gene in various cancers has been reported, we carried out MethyLight analysis of 87 clinical lung cancer samples, with the exception of three samples that were omitted from the analysis because their β-actin methylation status could not be detected. We observed that TFPI-2 was methylated in lung cancer specimens. Because we did not see an apparent correlation between the level of TMPRSS4 mRNA and that of TFPI-2 methylation, we divided the lung cancer patients into two groups based on their TMPRSS4 expression levels. We found that the average expression level of TMPRSS4 in the low TMPRSS4 group...
Fig. 1. Upregulation of TMPRSS4 and downregulation of TFPI-2 in lung cancer samples. (a) Pseudocolor image showing log10 expression ratios to the average expression level of the control lung region of 90 specimens for each of approximately 1700 probes (x-axis) across the 90 specimens (y-axis) tested by microarray. Red indicates upregulation; green indicates downregulation. AC, adenocarcinoma; SCC, squamous cell carcinoma. (b) Average relative mRNA expression levels of TMPRSS4 or TFPI-2 in 90 clinical lung cancer samples. Results are expressed as fold change to the average expression level of non-malignant regions, mean ± SEM. *P < 0.05 compared to non-malignant control. (c) Expression levels of TMPRSS4, β-actin mRNA in lung cancer cell lines were measured by RT-PCR. Relative TMPRSS4 expression mRNA level to β-actin was calculated by the ΔΔCt method. Results are expressed as the mean ± SD. (d) Expression levels of TFPI-2/β-actin mRNA in lung cancer cell lines were measured by RT-PCR as in (c). Experiments were carried out in technically triplicate (c, d).

(n = 45) was 0.5 (log10 ratios to average expression level of non-malignant region) and in the high TMPRSS4 group (n = 45), it was 1.3 (Fig. 5a, P < 0.001). The average methylation level of TFPI-2 in the low TMPRSS4 group was 6.0% in H1975 cells (cells in which TFPI-2 was most methylated among the tested lung cancer cell lines), and in the high TMPRSS4 group it was 18.3% (Fig. 5a, P = 0.13). We also found that the methylation level of TFPI-2 was significantly higher in SCC patients compared to those with AC, corresponding to the inverse expression level of mRNA in these histological subtypes (Fig. S1b,c).

The TFPI-2 gene was methylated in several lung cancer cell lines, including NCI-H358, NCI-H520, and NCI-H1975 (Fig. 5b). When these cells were treated with the methylation inhibitor 5-aza-2′-deoxycytidine (5 μM) or the histone deacetylase inhibitor TSA (0.5 μM), the methylation level of TFPI-2 decreased and the mRNA expression level of TFPI-2 dramatically increased (Fig. 5c,d). Conversely, these inhibitors reduced TMPRSS4 expression (Fig. 5e). Finally, these agents inhibited the growth of these cell lines (Fig. 6).

Discussion

We have identified TMPRSS4 as a potential therapeutic target in 90 NSCLC patients using comprehensive mRNA expression analysis. Through further analysis, we have found a novel molecular mechanism that TFPI-2 negatively regulates cell growth by inhibiting transcription of TMPRSS4.

We showed that mRNA expression of TMPRSS4 was upregulated and that of TFPI-2 was downregulated in tumor

Table 1. Fifteen candidate genes assessed as druggable targets in non-small-cell lung cancer patients

| Gene     | Description                                      | Druggable domain                        |
|----------|--------------------------------------------------|-----------------------------------------|
| TMPRSS4  | Transmembrane protease, serine 4                 | Peptidase S1 and S6, chymotrypsin/Hap   |
|          |                                                  | Speract/s scavenger receptor            |
| ST14     | Suppression of tumorigenicity 14                 | Peptidase S1 and S6, chymotrypsin/Hap   |
| SLC12A8  | Solute carrier family 12, member 8              | Amino acid permease-associated region    |
| BDH1     | 3-hydroxybutyrate dehydrogenase type 1           | Short-chain dehydrogenase/reductase SDR |
| BUB1     | Budding uninhibited by benzimidazoles 1 homolog (yeast) | Serine/threonine protein kinase |
| CDC2     | Cell division cycle 2                            | Serine/threonine protein kinase         |
| MELK     | Maternal embryonic leucine zipper kinase         | Tyrosine protein kinase                  |
| MMP11    | Matrix metalloproteinase 11                     | Serine/threonine protein kinase         |
| MMP12    | Matrix metalloproteinase 12                     | Peptidase, metalloproteinases           |
| NEK2     | NIMA (never in mitosis gene a)-related kinase 2 | Peptidase, metalloproteinases           |
| TOP2A    | Topoisomerase (DNA) II alpha                     | Serine/threonine protein kinase         |
| TTK      | TTK protein kinase                               | Tyrosine protein kinase                 |
| COL11A1  | Collagen, type XI, alpha 1                       | DNA topoisomerase, type IIA, subunit B or N-terminal |
| XK1      | Thymidine kinase 1, soluble                      | DNA topoisomerase, type IIA, subunit A or C-terminal |
| XDH      | Xanthine dehydrogenase                           | ATP-binding region, ATPase-like         |

Microarray analysis of 90 non-small-cell lung cancer samples and paired normal samples showed 120 genes that were commonly upregulated more than twofold with a ratio P-value < 0.001 in more than 70 samples. Of these, 15 genes were found to have a druggable domain and were considered candidate genes.
Fig. 2. Immunohistochemical TMPRSS4/TFPI-2 staining of non-small-cell lung carcinoma (NSCLC) specimens. (a) Representative images of TMPRSS4 and TFPI-2 staining. (b) Scatter diagram represents the relationship between the expression score of TMPRSS4 (y-axis) and TFPI-2 mRNA expression level (x-axis) in six NSCLC specimens. The expression score was calculated by multiplying the proportion (%) with the intensity score of immunohistochemical analysis. The mRNA expression level was calculated as log10 ratios to average expression level of non-malignant region according to cDNA microarray data. (c) Same scatter diagram for TFPI-2 as for TMPRSS4 in (b). (d) Scatter diagram represents the relationship between the expression score of TMPRSS4 (y-axis) and TFPI-2 (x-axis) in six NSCLC specimens.

Fig. 3. TFPI-2 downregulates the expression of TMPRSS4 in lung cancer cells. (a) NCI-H358, NCI-H520, NCI-H1975, A549, and NCI-H2228 cells were transfected with GFP plasmid alone (lane 1) or with GFP-TFPI-2 (lane 2) for 48 h, and protein levels of TFPI-2 were detected with α-GFP antibody. (b) mRNA expression levels of TMPRSS4/GAPDH in the same experiments as (a) were detected with RT-PCR. Cells treated with GFP plasmid were calculated as 1 in (b). (c) Five cell lines were co-transfected with a GFP or GFP-TFPI-2 expression plasmid and a Luc or TMPRSS4-Luc reporter plasmid for 24 h together with an internal control RL-TK plasmid, and the effect on the reporter activity of TMPRSS4 by TFPI-2 was evaluated by the luciferase assay. (d, e) NCI-H358, NCI-H520, and NCI-H1975 cells were transfected with negative control or TFPI-2 siRNAs for 48 h, and mRNA expression levels of TFPI-2/GAPDH (d) and TMPRSS4/GAPDH (e) were evaluated by RT-PCR. Each value from cells treated with negative siRNA was calculated as 1. *P < 0.05 compared to GFP alone or to negative control siRNA. All experiments were carried out three times independently. Representative data are shown for Western blotting (a).
specimens from NSCLC patients as well as in several lung cancer cell lines. The results were also partially supported by immunohistochemical analysis in six patients, although the number of samples might be insufficient. It should be noted that a set of clinical samples has diversity that includes smoking status, sex, various histology, and mutation status. To minimize discrepancy between clinical samples and cell lines in terms of sample diversity as much as possible, we tested various cell lines in histology and mutation status, such as A549 (AC, KRAS mutation positive), NCI-H520 (SCC), and NCI-H2228 (AC, ALK fusion positive) in addition to NCI-H358 (bronchioloalveolar cell carcinoma, KRAS mutation positive) and NCI-H1975 (AC, EGFR mutation positive, T790M+L858R) for a series of in vitro experiments. We found that TMPRSS4 augmented cell growth in all cell lines tested, however, the expression of TMPRSS4 was regulated by TFPI-2 only in NCI-H358, NCI-H520, and NCI-H1975 cells. Moreover, aberrant methylation of TFPI-2 was only observed in these cell lines. These results indicate that the proposed mechanism for regulation of TMPRSS4 expression through the methylation status of TFPI-2 cannot be applied to all cases, but is evident in a subset of cases. Further study should be undertaken to clarify what context of NSCLC is related to this mechanism.

Overexpression of TMPRSS4 has been reported in lung, liver, ovarian, pancreatic, prostate, gastric, colorectal, prostate, and thyroid carcinomas. Overexpression of TMPRSS4 and its association with poor prognosis in squamous cell lung cancer patients have been reported. Indeed, we also found the tendency of higher expression of TMPRSS4 in SCC. We could not show the association of TMPRSS4 expression with prognosis in SCC as well as in AC and NSCLC, although there was a tendency for poor prognosis in the high TMPRSS4 group of SCC patients (P = 0.300) (Fig. S2). Median survival time was 2677 days for the TMPRSS4 high group in SCC patients but it was not reached for the TMPRSS4 low group in SCC patients. The hazard ratio of median survival time for the TMPRSS4 high group in SCC patients was 2.977, estimated by Cox regression analysis (95% confidence interval, 0.342 – 25.896; P = 0.323). The number of patients might not be sufficient to show significant difference. However, there was another study using immunohistochemistry that indicated relatively higher TMPRSS4 expression in adenocarcinoma. Differences in detection
Fig. 5. TFPI-2 is methylated in lung cancer specimens and cell lines. (a) Methylation levels of TFPI-2 were measured using MethyLight analysis in 87 clinical lung cancer samples. Methylation levels of β-actin were used as a control. TFPI-2/β-actin methylation levels in H1975 cells (which had the most TFPI-2 methylation of the lung cancer cell lines tested) were calculated as 100. We dichotomized the lung cancer patients by their TMPRSS4 expression levels, and we calculated the average expression levels of TMPRSS4 and methylation levels of TFPI-2 in each group. (b) Methylation levels of TFPI-2 in lung cancer cell lines were measured using MethyLight analysis. The experiment was carried out in technically triplicate. (c–e) NCI-H358, NCI-H520, and NCI-H1975 cells were treated with 5 μM 5-aza-2′-deoxycytidine for 72 h or 0.5 μM trichostatin A (TSA) for 24 h. Changes in the methylation level induced by these treatments were measured using MethyLight analysis (c) and changes in the mRNA levels of TFPI-2/GAPDH (d) and TMPRSS4/GAPDH (e) were measured by RT-PCR. *P < 0.05 compared to DMSO alone. Experiments were carried out in triplicate.

Fig. 6. Growth inhibition in lung cancer cells by 5-aza-2′-deoxycytidine or trichostatin A (TSA). (a–d) Cell growth was measured by a proliferation assay from day 0 to day 4 for 5-aza-2′-deoxycytidine or day 5 for TSA and calculated as fold change from day 0. NCI-H358 (a, d), NCI-H520 (b, e) and NCI-H1975 (c, f) cells were seeded at 5000 cells/well for NCI-H358 and 1000 cells/well for others, and treatment with 5 or 10 μM 5-aza-2′-deoxycytidine (a–c) or 0.05 or 0.1 μM TSA (d–f) was given on day 1. *P < 0.05 compared to 0 (DMSO alone). Data are representative of three independent experiments.
methods might cause this variance. Considering the therapeutic potential of targeting TMPRSS4, it consists of four domains: transmembrane, LDL receptor class A, group A scavenger receptor, and serine protease. Among these, the scavenger receptor and serine protease domains (peptidase S1 and S6, chymotrypsin/Hap domain) are supposed to be druggable. Several small molecule inhibitors targeting serine proteases or scavenger receptors have been developed. The development of small molecule inhibitors of TMPRSS4 may improve the current results for the treatment of NSCLC. Recently, 2-hydroxydiarylilide derivatives were reported as potential TMPRSS4 inhibitors, and their inhibitory activity against TMPRSS4 was correlated with anti-invasive activity in SW-480 colon cancer cells. (31)

As TMPRSS4 has six isoforms (isoform 1, 3, 4, 5, 6, and 7), including isoform 3 and 7 deleted in the scavenger receptor druggable domain, we sequenced TMPRSS4 cDNA amplified from cell lines we used in this study. As a result, we found that isoform 1 was expressed in NCI-H358 and A549, isoform 4 was expressed by NCI-H2228, and both isoforms 1 and 4 were expressed in NCI-H1975 and NCI-H520 (Fig. S3). Isoform 1 is the longest splicing variant and isoform 4 is only two amino acids shorter than isoform 1; these two variants both have two druggable domains. The expression plasmid we used contained TMPRSS4 isoform 1 and all the cell lines that overexpressed isoform 1 were generated against METHO muscle-derived MyoD as transfected mRNA for TMPRSS4 (#s32202 and #s32204 target all isoforms, #s32203 targets isoform 1, 3, 4, and 7) suppressed proliferation of all tested cell lines including NCI-H2228 that expressed only isoform 1. Moreover, siRNA for TMPRSS4 isoform 1 obtained growth advantage. As a result, we found that the longest splicing variant and isoform 4 is only two amino acids shorter than isoform 1; these two variants both have two druggable domains.

Downregulation of TFPI-2 has also been reported to contribute to tumor invasion in various cancers, (15–22) although we could not detect any correlation between TFPI-2 expression levels and pathological T factor, pathological N factor, or pathological stage in this study, probably due to the small sample size. The silencing of TFPI-2 mediated by aberrant DNA methylation has been observed in gastric, colon, cervical, prostate, esophageal squamous cell, and lung carcinomas (15,16,19–21). Wu et al. (17) reported that TFPI-2 methylation predicts poor prognosis in NSCLC. Although we did not observe a significant correlation between TMPRSS4 mRNA expression and TFPI-2 methylation levels, we found that TFPI-2 methylation status was related to the expression level of TMPRSS4 (Fig. 5a). Elevated TMPRSS4 expression may be one of the explanations for poor prognosis in NSCLC patients with TFPI-2 methylation.

As TFPI-2 was methylated in several lung cancer cell lines, as determined by MethylLight analysis (Fig. 5b), we evaluated whether the cancer chemotherapeutic agents 5-aza-2'-deoxycytidine and TSA could inhibit cell growth. We found that these agents inhibit cell growth by downregulating TMPRSS4 by demethylating and re-expressing the TFPI-2 gene (Figs 5c–e). Overexpression of TFPI-2 induced the reduction of TMPRSS4 mRNA directly, in part, by inhibiting its reporter activity (Fig. 3c). However, the precise mechanism of TMPRSS4 inhibition by TFPI-2 remains unclear. As TFPI-2 is a protease inhibitor, but not a transcription factor, this regulation is not considered to be a direct effect. There is a possibility that TFPI-2 regulates a miRNA targeting TMPRSS4 or a transcription factor of TMPRSS4. Although there are no published reports of miRNAs regulating expression of TMPRSS4 or transcription factors, several miRNAs including hsa-miR-345 are predicted to regulate TMPRSS4 expression by the Target Scan Human 6.1 database in silico. We previously carried out miRNA profiling with the same Japanese lung cancer sample sets using an RT-PCR based assay, (32) and found there was no correlation between the expression levels of TMPRSS4 and miR-345 (r = 0.12). There was also no miRNA showing a high inverse relationship (r = −0.5) with TMPRSS4 in 380 tested miRNAs. However, regulatory transcription factor binding sites of Lfy-1, AML-1a, SRY, AP-1, c-Ets-1, c-Rel, NF-κ, HSF2, MyoD, and GATA-2 were observed in the TMPRSS4 gene promoter in silico (1000 bp upstream of 5'-TMPRSS4; threshold: 90.0 point; taxonomy; vertebrate; TFSEARCH version 1.3 (http://www.cbrj.jp/research/db/TFSEARCH.html). Reporter assays for each candidate and subsequent chromatin immunoprecipitation may reveal transcription factor's and clarify the precise pathway/s in a future study.

In summary, we have shown that TMPRSS4 is upregulated by the silencing of TFPI-2 through aberrant DNA methylation in NSCLC. Originally, each factor was reported to be independently associated with a poor prognosis in NSCLC patients. (7,17) Here, we have shown the apparent linkage of both factors and identified a therapeutic potential for NSCLC patients by downregulating TMPRSS4 directly or indirectly through the demethylation of TFPI-2. However, more precise roles of TMPRSS4 and TFPI-2 as novel therapeutic targets for NSCLC should be further explored.

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Disclosure Statement

The authors have no conflict of interest.

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Clinical characteristics of test set of 90 non-small-cell lung carcinoma patients.

Fig. S2. Kaplan–Meier curves of non-small-cell lung carcinoma patients according to TMPRSS4 mRNA expression levels.

Fig. S3. Expression of TMPRSS4 isoforms in non-small-cell lung cancer cell lines.

Table S1. Clinical characteristics of test set of 90 non-small-cell lung carcinoma patients.