

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

Junko Hamamoto,¹ Kenzo Soejima,¹ Katsuhiko Naoki,¹ Hiroyuki Yasuda,¹ Yuichiro Hayashi,² Satoshi Yoda,¹ Sohei Nakayama,¹ Ryosuke Satomi,¹ Hideki Terai,¹ Shinnosuke Ikemura,¹ Takashi Sato,¹ Daisuke Arai,¹ Kota Ishioka,¹ Keiko Ohgino¹ and Tomoko Betsuyaku¹

Departments of ¹Pulmonary Medicine; ²Pathology, School of Medicine, Keio University, Tokyo, Japan

Key words

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Correspondence

Kenzo Soejima, Department of Pulmonary Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.

Tel: +81-3-3353-1211 (ext. 61430); Fax: +81-3-3353-2502; E-mail: ksoejima@cpnet.med.keio.ac.jp

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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.

Lung cancer is the leading cause of cancer deaths worldwide.⁽¹⁾ Although standard treatments such as chemotherapy and radiotherapy have apparently reached a plateau of effectiveness in improving the survival of advanced non-small cell lung cancer (NSCLC) patients, new, targeted treatments offer a potential improvement for treating NSCLC. Currently, two types of targets are being successfully developed for the treatment of advanced NSCLC: tumor angiogenesis and growth factor receptors. These therapeutic drugs are small molecule inhibitors and monoclonal antibodies.⁽²⁾ Hence, considerable efforts have been initiated to identify novel targets for new biological agents that may safely and effectively be administered to advanced NSCLC patients.

“Druggable” targets include a subset of 3051 genes in the human genome that express proteins that can bind to drug-like molecules, and that have oral bioavailability. Hopkins and Groom showed that nearly half of these drug targets fall into just six gene families: G-protein-coupled receptors, serine/threonine and tyrosine kinases, zinc metalloproteases, serine proteases, nuclear hormone receptors, and phosphodiesterases.^(3,4)

In this context, we have identified *TMPRSS4* as a putative, novel, druggable target by screening surgically resected sam-

ples from 90 Japanese patients with non-small-cell lung carcinoma (NSCLC) using cDNA microarray. Transmembrane protease, serine 4 (*TMPRSS4*) belongs to the type II transmembrane serine protease family and this putative, serine-type endopeptidase may play a role in proteolysis.⁽⁵⁾ Mutations in this gene are associated with autosomal recessive postlingual hearing loss.⁽⁶⁾ Overexpression of *TMPRSS4* in squamous NSCLC patients is reported to be associated with poor prognosis.⁽⁷⁾ We have also identified tissue factor pathway inhibitor 2 (*TFPI-2*) as a gene that directly regulates the expression of *TMPRSS4*. *TFPI-2* is a serine protease inhibitor, showing a strong inhibitory effect towards the amidolytic activities of trypsin, chymotrypsin, plasmin, plasma kallikrein, and factor Xia.^(8–14) Known as a tumor suppressor, aberrant methylation of the *TFPI-2* gene was detected in various carcinomas.^(15–22)

In this report, we show that the overexpression of *TMPRSS4* and the downregulation of *TFPI-2* through DNA methylation are observed not only in lung cancer cell lines but also in tumor specimens from NSCLC patients. We further show that the cancer chemotherapeutic agent 5-aza-2'-deoxycytidine or trichostatin A (TSA) inhibits *TFPI-2* methylation, leading to the upregulation of *TFPI-2* and downregulation of *TMPRSS4*

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 RMA 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 ΔC_t method (cycle threshold), in which the ΔC_t value was calculated by subtracting the C_t 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 non-specific staining and were then incubated with anti-human *TMPRSS4* 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 the detection of proteins, 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'-GCCACCAGCCATCAGTAAG-GAAGG-3' and 5'-AGCAGAGGGCCCCCACTCACCAT-3'. Nested PCR products amplified with the primers 5'-GGTACCCTGCAGGCCAGCTTCAGATCTCTG-3' and 5'-GCTAGCGCTGGCTCTGTGATCCTCCCG-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, Schweiz) 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 Cell Titer 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 s15514 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 μ g 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 μ L containing 300 nM forward and reverse primers and a 100 nM probe with bisulfite-converted genomic DNA in 1 \times TaqMan Universal PCR master mix. The primer and probe sequences were as follows: forward (5'-GGCGAAGTTGTTATTAGTCGTC) and reverse (5'-TAAAAAATAAACCTACGCGCA) primers and the probe (5'-6FAM-AAACTCTCGCTCCTTACGCCG-BHQ1) for methylated *TFPI-2*; or forward (5'-TGGTGATGGAG-GAGGTTTAGTAAGT) and reverse (5'-AACCAATAAAACC-TACTCCTCCCTTAA) primers and the probe (5'-6FAM-ACCACCACCAACACACAATAACAAACACA-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 ΔC_t method (cycle threshold), in which the ΔC_t value was calculated by subtracting the C_t 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 SPSS 19.0 software (SPSS; IBM, Chicago, IL, USA).

Results

Upregulation 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 twofold 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.

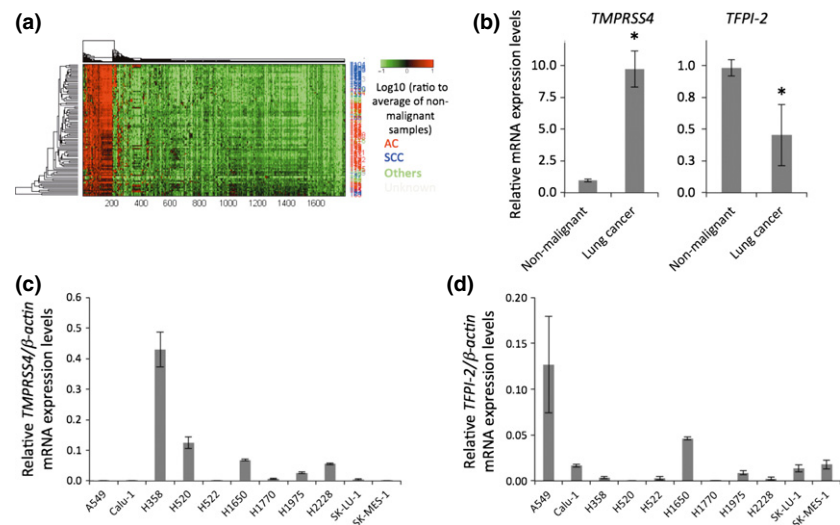
The expression of *TMPRSS4* and *TFPI-2* was analyzed by immunohistochemistry in six cases (Fig. 2). These cases included three ACs and three SCCs. *TMPRSS4* was expressed mainly in the cell membrane and also in the cytoplasm of cancer cells, and *TFPI-2* was expressed in the cytoplasm of cancer cells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

lase 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
<i>TMPRSS4</i>	Transmembrane protease, serine 4	Peptidase S1 and S6, chymotrypsin/Hap Speract/scavenger receptor
<i>ST14</i>	Suppression of tumorigenicity 14	Peptidase S1 and S6, chymotrypsin/Hap
<i>SLC12A8</i>	Solute carrier family 12, member 8	Amino acid permease-associated region
<i>BDH1</i>	3 hydroxybutyrate dehydrogenase type 1	Short-chain dehydrogenase/reductase SDR
<i>BUB1</i>	Budding uninhibited by benzimidazoles 1 homolog (yeast)	Serine/threonine protein kinase
<i>CDC2</i>	Cell division cycle 2	Serine/threonine protein kinase
<i>MELK</i>	Maternal embryonic leucine zipper kinase	Tyrosine protein kinase
<i>MMP11</i>	Matrix metalloproteinase 11	Serine/threonine protein kinase
<i>MMP12</i>	Matrix metalloproteinase 12	Peptidase, metalloproteinases
<i>NEK2</i>	NIMA (never in mitosis gene a)-related kinase 2	Peptidase, metalloproteinases
<i>TOP2A</i>	Topoisomerase (DNA) II alpha	Serine/threonine protein kinase
<i>TTK</i>	TTK protein kinase	Tyrosine protein kinase
<i>COL11A1</i>	Collagen, type XI, alpha 1	DNA topoisomerase, type IIA, subunit B or N-terminal
<i>TK1</i>	Thymidine kinase 1, soluble	DNA topoisomerase, type IIA, subunit A or C-terminal
<i>XDH</i>	Xanthine dehydrogenase	ATP-binding region, ATPase-like
		Serine/threonine protein kinase
		Fibrinogen, alpha/beta/gamma chain, C-terminal globular
		Thymidine kinase
		Oxidoreductase, molybdopterin binding

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.

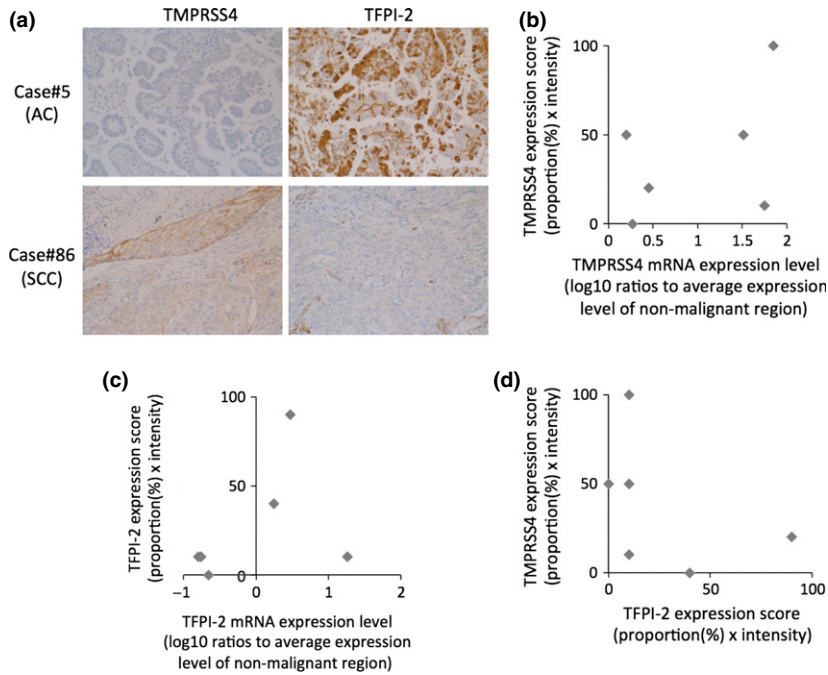


Fig. 2. Immunohistochemical TMPRSS4/TFPI-2 staining of non-small-cell lung carcinoma (NSCLC) specimens. (a) Representative images of TMPRSS4 and TFPI-2 staining. (b) Scatter diagram represents the relationship between the expression score of TMPRSS4 (y-axis) and *TMPRSS4* mRNA expression level (x-axis) 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.

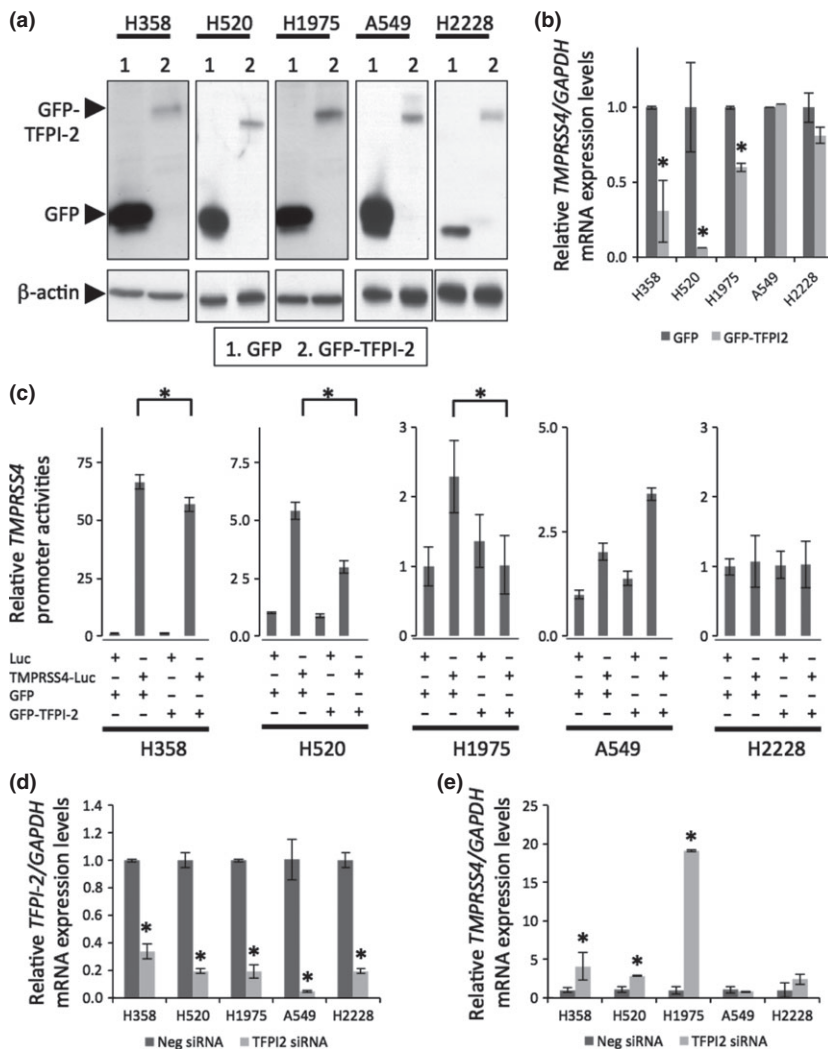


Fig. 3. *TFPI-2* downregulates the expression of *TMPRSS4* in lung cancer cells. (a) NCI-H358, NCI-H520, NCI-H1975, A549, and NCI-H2228 cells were transfected with GFP plasmid alone (lane 1) or with GFP-*TFPI-2* (lane 2) for 48 h, and protein levels of *TFPI-2* were detected with α -GFP antibody. (b) 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).

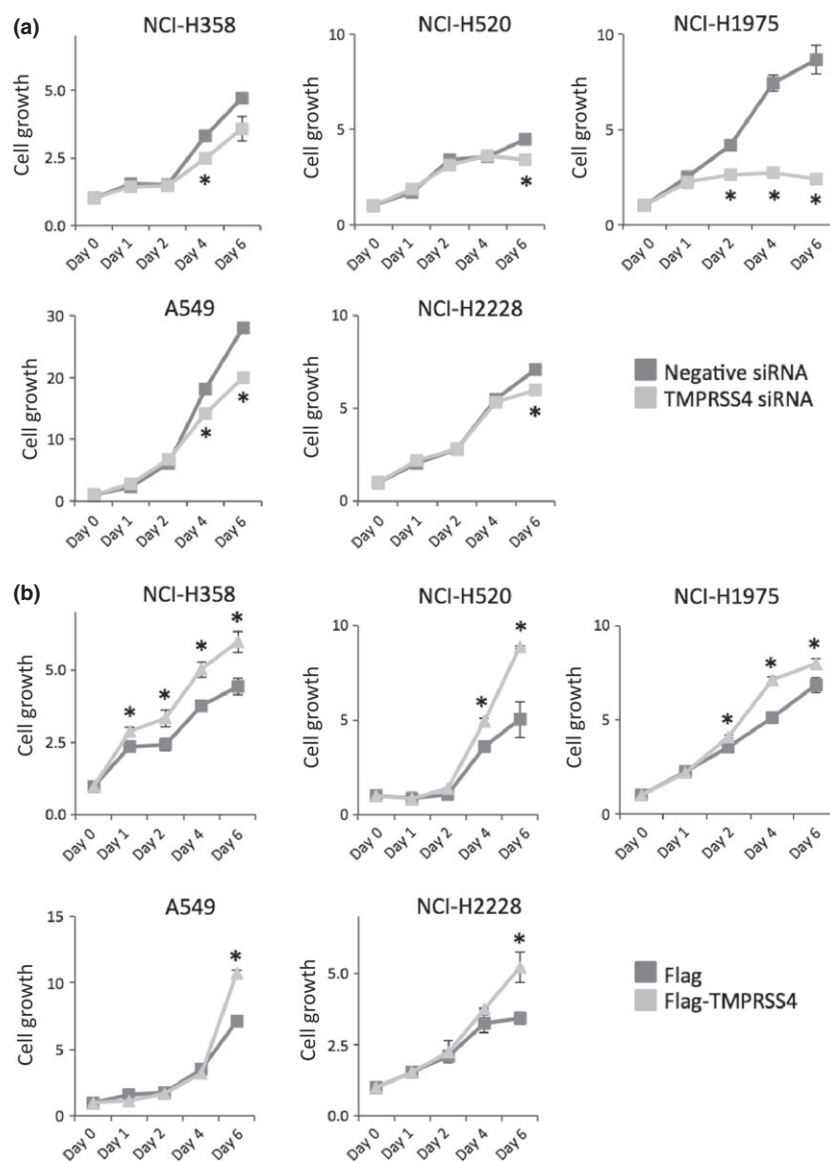


Fig. 4. Effect of knockdown or overexpression of *TMPRSS4* on lung cancer cell growth. NCI-H358, NCI-H520, NCI-H1975, A549, and NCI-H2228 cells were seeded at 5000 cells/well for NCI-H358 and 1000 cells/well for other cell lines. *TMPRSS4* siRNA (a) or a *TMPRSS4* overexpression plasmid (b) was transfected the next day. Cell growth was measured using a proliferation assay from day 0 to day 6 and calculated as fold change from day 0. * $P < 0.05$ compared to negative siRNA or Flag plasmid alone. All experiments were carried out in triplicate. Data are representative of three independent experiments.

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.^(5,7,23) Overexpression of *TMPRSS4* and its association with poor prognosis in squamous cell lung cancer patients have been reported.⁽⁷⁾ Indeed, we also found the tendency of higher expression of *TMPRSS4* in SCC. We could not show the association of *TMPRSS4* expression and 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.⁽²⁶⁾ Differences in detection

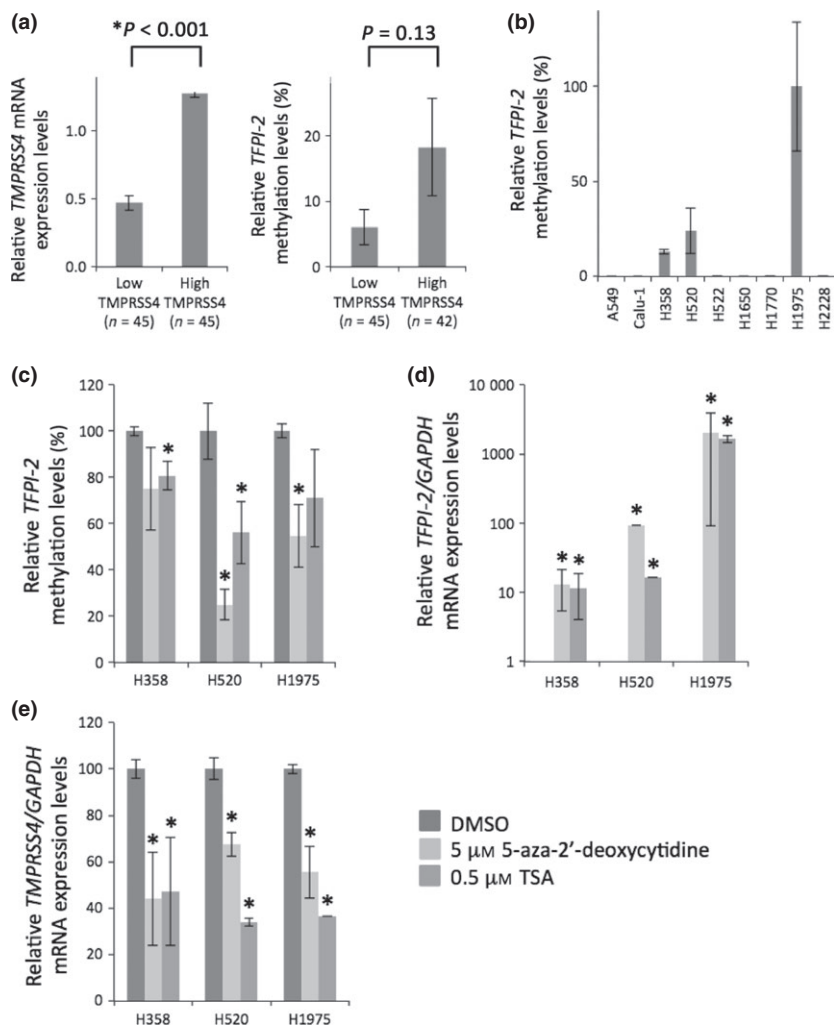


Fig. 5. *TFPI-2* is methylated in lung cancer specimens and cell lines. (a) Methylation levels of *TFPI-2* were measured using MethyLight analysis in 87 clinical lung cancer samples. Methylation levels of β -actin were used as a control. *TFPI-2*/ β -actin methylation levels in H1975 cells (which had 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 μ M 5-aza-2'-deoxycytidine for 72 h or 0.5 μ M 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.

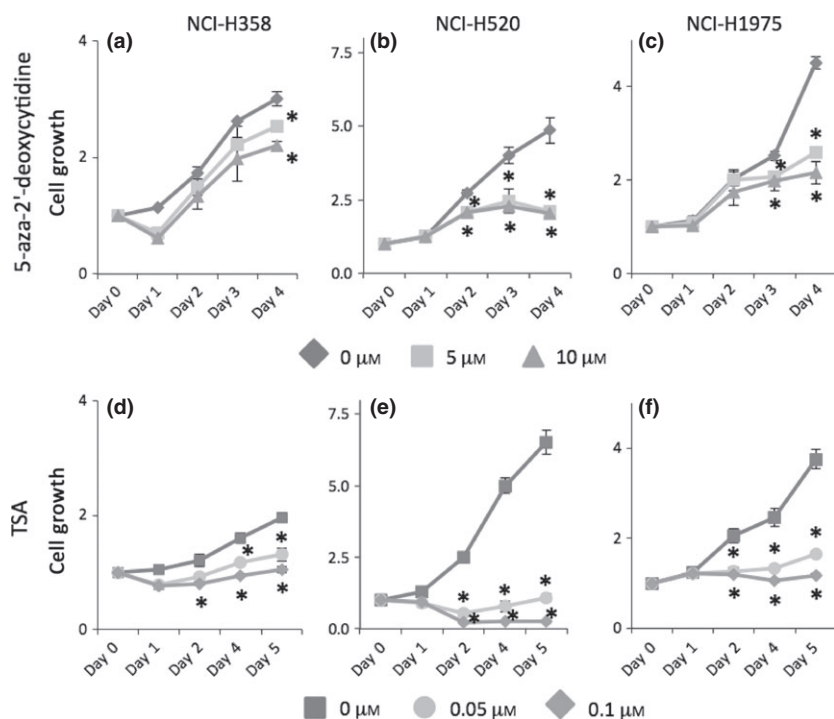


Fig. 6. Growth inhibition in lung cancer cells by 5-aza-2'-deoxycytidine or trichostatin A (TSA). (a–d) Cell growth was measured by a proliferation assay from day 0 to day 4 for 5-aza-2'-deoxycytidine or day 5 for TSA and calculated as fold change from day 0. NCI-H358 (a, d), NCI-H520 (b, e) and NCI-H1975 (c, f) cells were seeded at 5000 cells/well for NCI-H358 and 1000 cells/well for others, and treatment with 5 or 10 μ M 5-aza-2'-deoxycytidine (a–c) or 0.05 or 0.1 μ M 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.^(27,28) 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.^(29,30) The development of small molecule inhibitors of *TMPRSS4* may improve the current results for the treatment of NSCLC. Recently, 2-hydroxydiarylamide derivatives were reported as potential *TMPRSS4* inhibitors, and their inhibitory activity against *TMPRSS4* was correlated with anti-invasive activity in SW480 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 in 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 obtained growth advantage. Moreover, siRNA 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 4 (Fig. 4a), suggesting that *TMPRSS4* isoform 4 as well as isoform 1 would be important for cell growth in NSCLC.

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 MethyLight 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,6). 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* transcription 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 *Lyf-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.cbrc.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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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Differential expression/methylation pattern of *TMPRSS4/TFPI-2* between adenocarcinoma (AC) and squamous cell carcinoma (SCC) of clinical lung cancer samples.

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.