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Reciprocal expression of trefoil factor-1 and thyroid transcription factor-1 in lung adenocarcinomas

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Abstract

Molecular targeted therapies against *EGFR* and *ALK* have improved the quality of life of lung adenocarcinoma patients. However, targetable driver mutations are mainly found in thyroid transcription factor-1 (TTF-1)/NK2 homeobox 1 (NKX2-1)-positive terminal respiratory unit (TRU) types and rarely in non-TRU types. To elucidate the molecular characteristics of the major subtypes of non-TRU-type adenocarcinomas, we analyzed 19 lung adenocarcinoma cell lines (11 TRU types and 8 non-TRU types). A characteristic of non-TRU-type cell lines was the strong expression of *TFF-1* (trefoil factor-1), a gastric mucosal protective factor. An immunohistochemical analysis of 238 primary lung adenocarcinomas resected at Jichi Medical University Hospital revealed that TFF-1 was positive in 31 cases (13%). Expression of TFF-1 was frequently detected in invasive mucinous (14/15, 93%), enteric (2/2, 100%), and colloid (1/1, 100%) adenocarcinomas, less frequent in acinar (5/24, 21%), papillary (7/120, 6%), and solid (2/43, 5%) adenocarcinomas, and negative in micropapillary (0/1, 0%), lepidic (0/23, 0%), and microinvasive adenocarcinomas or adenocarcinoma in situ (0/9, 0%). Expression of TFF-1 correlated with the expression of HNF4-α and MUC5AC ($P < .0001$, $P < .0001$, respectively) and inversely correlated with that of TTF-1/NKX2-1 ($P < .0001$). These results indicate that TFF-1 is characteristically expressed in non-TRU-type adenocarcinomas with gastrointestinal features. The TFF-1-positive cases harbored *KRAS* mutations at a high frequency, but no *EGFR* or *ALK* mutations. Expression of TFF-1 correlated with tumor spread through air spaces, and a poor prognosis in advanced stages. Moreover, the knockdown of TFF-1 inhibited cell proliferation and soft-agar colony formation and induced apoptosis in a TFF-1-high and *KRAS*-mutated lung adenocarcinoma cell line. These results indicate that TFF-1 is not only a biomarker, but also a potential molecular target for non-TRU-type lung adenocarcinomas.

KEYWORDS

lung adenocarcinoma, mucinous adenocarcinoma, non-TRU type, TFF-1, TTF-1

1 | INTRODUCTION

Lung cancer is the leading cause of cancer death in many developed countries, including the United States and Japan,[1,2] and adenocarcinoma is the most common histological subtype of primary lung cancer.

The existence of a distinct subset of lung adenocarcinomas arising from a terminal respiratory unit (TRU) was previously proposed by Yatabe et al.[3-5] The TRU-type lung adenocarcinomas show histologically nonmucinous lepidic growth or papillary components, and frequently express thyroid transcription factor-1 (TTF-1)/NK2 homeobox 1 (NKX2-1) at high levels.[3-5] The genetic backgrounds of TRU types have been investigated in detail, and epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (*ALK*) fusions were found to be specific to TRU types.[4-6] However, limited information is currently available on non-TRU-type lung adenocarcinomas.

Non-TRU-type lung adenocarcinomas are not a single entity and could encompass distinct histological and molecular subtypes.[7-9] Our previous analysis of cell lines and primary tumors revealed at least 2 distinct subtypes of non-TRU lung adenocarcinomas: one type with an EMT phenotype and another that sustained an epithelial phenotype.[7,8] Each subtype may have its own histological and molecular characteristics. For example, the loss of the chromatin remodeling factors, BRG-1 and BRM, is prominent in the former subtype and could be a cause for the loss of epithelial features in this subtype.[10]

In the present study, we compared *TTF-1/NKX2-1*-high and *CDH1*-high adenocarcinoma cell lines ("TRU-type" cell lines) (n = 11) and *TTF-1/NKX2-1*-low and *CDH1*-high adenocarcinoma cell lines ("non-TRU-type" cell lines) (n = 8) in order to elucidate the molecular characteristics of one major subtype of non-TRU-type adenocarcinomas retaining the epithelial phenotype. The results obtained identified the strong expression of trefoil factor-1 (*TFF-1*), a gastric mucosal protective factor, as a characteristic of "non-TRU-type" cell lines.

The TFF-1 peptide is a mucin-correlated molecule produced by gastric surface mucous cells together with the MUC5AC mucin.[11] Trefoil factor-1 participates in mucosal maintenance/repair and gastrointestinal cell differentiation.[12-14] Trefoil factor-1 knockout mice developed gastric tumors, suggesting a tumor suppressor role for TFF-1 in the stomach,[15] and *TFF-1* has been reported to function as an oncogene in the breast, kidney, and colon.[16-18] However, there is currently no information on the role of TFF-1 in lung tumors.

This is the first study to show the detailed expression pattern of TFF-1 in lung adenocarcinomas. Furthermore, we show that TFF-1 is a potential molecular target for *KRAS*-mutated non-TRU-type adenocarcinomas with gastrointestinal features.

2 | MATERIALS AND METHODS

2.1 | Cell lines and media

We used 19 lung cancer cell lines. The sources and histological types of these cell lines are shown in Table S1. All cell lines were maintained in RPMI-1640 supplemented with 10% FCS, glutamine, and antibiotics in a humidified atmosphere with 5% CO_2 and 95% air.

2.2 | Gene expression profile and single nucleotide polymorphism array analyses of 19 lung adenocarcinoma cell lines

A comprehensive gene expression analysis was undertaken using an oligonucleotide microarray (GeneChip Human Genome U133A; Affymetrix) as described previously.[19-21] A single nucleotide polymorphism array (Human Mapping 50K *Xba*I array; Affymetrix) analysis was carried out using the Genome Imbalance Map algorithm as previously described.[22]

2.3 | Cohort descriptions

In the present study, we analyzed 2 cohorts of primary lung adenocarcinomas resected at Jichi Medical University Hospital.

The first cohort consisted of 238 unselected primary lung adenocarcinomas patients who underwent surgical resection of the tumor at Jichi Medical University Hospital between 2010 and 2013. Patients included 132 men and 106 women, ranging in age between 36 and 84 years (average, 67.6 years). Each case was reassigned based on the TNM classification and pathological stage according to the International Association for the Study of Lung Cancer 8th edition lung cancer staging system[23]; one case was stage 0, 146 were stage I (97 stage IA, 49 stage IB), 31 were stage II (5 stage IIA, 26 stage IIB), 49 were stage III (32 stage IIIA, 13 stage IIIB, 13 stage IIIC), and 5 were stage IV (4 stage IVA, 1 stage IVB). The stages of 6 cases were unknown. Each case was classified by 2 pathologists (DM and TY) as follows: nonmucinous adenocarcinoma in situ (n = 1), minimally invasive adenocarcinoma (n = 8), lepidic adenocarcinoma (n = 23), papillary adenocarcinoma (n = 120), acinar adenocarcinoma (n = 24), solid adenocarcinoma (n = 43), micropapillary adenocarcinoma (n = 1), invasive mucinous adenocarcinoma (n = 15), pulmonary enteric adenocarcinoma (n = 2), and colloid adenocarcinoma (n = 1).

The second cohort consisted of 43 lung adenocarcinoma patients who underwent lung cancer surgery at Jichi Medical University Hospital between August 2010 and June 2015, and whose tumors showed a non-TRU-type histology. The details of this cohort were described in our previous study.[8] Among the 43 cases, 28 were duplicated in the first cohort (238 unselected primary lung adenocarcinoma cases).

Informed consent was obtained from all patients, and the study was approved by the Institutional Ethics Review Committee.

2.4 | Publicly available data on primary lung adenocarcinomas

Data on the gene expression levels, histological differentiation, and overall survival of 442 primary lung adenocarcinoma cases were

obtained from Shedden et al's data.[24] Data on the gene expression levels, genetic mutations, and overall survival of 230 primary lung adenocarcinoma cases were obtained from The Cancer Genome Atlas (TCGA) data.[25]

2.5 | Immunohistochemistry and evaluation

Formalin-fixed, paraffin-embedded tumor specimens were analyzed by immunohistochemistry for the expression of TTF-1/NKX2-1, hepatocyte nuclear factor -4α (HNF4-α), MUC5AC, and TFF-1. Tissue sections were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Tissue sections for TTF-1/NKX2-1, HNF4-α, and TFF-1 were then autoclaved in 10 mmol/L citrate buffer (pH 6.0) at 120°C for 10 minutes, while those for MUC5AC were heated in 10 mmol/L citrate buffer (pH 6.0) at 95°C for 10 minutes in a microwave oven. Sections were then preincubated with 10% normal horse serum in PBS, incubated with a mouse monoclonal anti-human TTF-1/NKX2-1 Ab (M3575, clone 8G7G3/1) from Dako at a dilution of 1:100, a mouse monoclonal anti-human HNF4-α (H1415) Ab from Perseus Proteomics at a dilution of 1:60, a mouse monoclonal anti-human MUC5AC Ab (NCL-MUC-5AC) from Leica Biosystems at a dilution of 1:100, and a rabbit monoclonal anti-human TFF-1 Ab (GTX121461) from GeneTex at a dilution of 1:100 at 4°C overnight. All Abs were detected with a streptavidin-HRP conjugate according to the manufacturer's instructions. 3,3′-Diaminobenzidine tetrahydrochloride was used as a chromogen, and hematoxylin was used as a light counterstain. When staining was undertaken on cancer tissue sections, a positive control section was also stained, and we always confirmed that positive control cells were correctly stained. Immunohistochemical staining was evaluated independently by 2 pathologists (DM and TY). Immunoreactivity was scored based on the percentage of cells that stained positively: 0, negative; 1+, less than 10%; 2+, 10%-50%; 3+, more than 50%. The expression of each marker Ab (TTF-1/NKX2-1, HNF4-α, MUC5AC, and TFF-1) in a tumor was defined as positive when at least 10% of tumor cells were stained (scores 2+ and 3+) and negative when less than 10% were stained (scores 0 and 1+).

2.6 | Definition of tumor spread through air spaces

Spread through air spaces (STAS) was defined as the detachment of tumor cells observed within air spaces in the surrounding lung parenchyma beyond the edge of the main tumor.[26] Two pathologists (DM and TY) graded STAS into 3 grades as follows: grade 1, no definite STAS found; grade 2, tumor cell nests less than 3 alveolae away from the main tumor mass or tumor cell nests surrounding less than 50% of the circumference of the main tumor mass; and grade 3, tumor cell nests more than 3 alveolae away from the main tumor mass, and surrounding more than 50% of the circumference of the main tumor mass.

2.7 | Generation of TFF-1-deficient cell lines

We knocked down TFF-1 expression using TRC lentiviral shRNA vectors. We selected 2 plasmids of TFF-1 shRNA: TFF-1-A (TRCN0000033617; mature antisense sequence 5′-TGAAAGACAGAATTGTGGTTT-3′) and TFF-1-B (TRCN0000033618; mature antisense sequence 5′-GCCCTCCCAGTGTGCAAATAA-3′). The frameworks of these shRNAs were formed by the pLKO.1 cloning vector (#10878; Addgene). 293T packaging cells (Riken) were seeded on 60-mm dishes, and were cotransfected with shRNA plasmids, the pCMV-VSV-G and pCMV-dR8.2 dvpr vectors (Addgene), using Lipofectamine 2000 (Invitrogen) to produce functional lentiviral particles. The vector containing a random DNA sequence was used as a negative control (shScramble). A549, HCC827, L27, H1993, and H441 cells were transduced with shTFF-1-A, shTFF-1-B, and shScramble. After a brief selection with puromycin, transduced cells were used in experiments without cloning.

2.8 | Cell growth assay

Cell growth was analyzed using CCK-8 (Dojindo Molecular Technology). Briefly, 5×10^2 cells (A549, L27, and H1993) and 2×10^3 cells (H441 and HCC827) in RPMI with 10% FBS were seeded on a 96-well microtiter plate with 10 replicates for each condition. After the indicated time for the incubation, CCK-8 reagent was added to each well and incubated for 4 hours. The formazan dye formed by viable cells was measured at 450 nm for absorbance values with a reference at 630 nm using a 680XR microplate reader (Bio-Rad).

2.9 | Western blot analysis

Cells were lysed in lysis buffer consisting of 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 50 mmol/L NaF, and 1 mmol/L Na_3VO_4 with a cocktail of proteinase inhibitors. After sonication, lysates were boiled at 98°C for 5 minutes and cleared by centrifugation. Protein concentrations were measured by the DC Protein Assay Kit (Bio-Rad). Regarding western blotting, equal amounts of protein samples were size-separated on 8% polyacrylamide gels and electroblotted onto nitrocellulose membranes. Nonspecific binding was blocked by immersing membranes for 20 minutes in 5% skim milk in TBS at room temperature. The membranes were washed with TBS buffer containing 0.1% Tween-20, incubated at room temperature for 1 hour with primary Abs, washed, and then reacted with peroxidase-conjugated secondary Abs. The antigen was detected using ECL Western Blotting Detection Reagents (Amersham) following the manufacturer's instructions. The sources of the Abs used in the present study are summarized in Table S2.

2.10 | Colony formation assay

A549 cells (A549-shScramble and A549-shTFF-1) (1×10^3 cells/well), HCC827 cells (HCC827-shScramble and HCC827-shTFF-1)

(1 × 10^3 cells/well), and H441 cells (H441-shSramble and H441-shTFF-1) (4 × 10^3 cells/well) in RPMI with 10% FBS were seeded on 6-well plates. Plates were incubated for 9 days until colonies had formed. The wells of these plates were rinsed out with PBS, fixed with methanol, stained with Giemsa stain solution, confirmed under an inverted light microscope, and photographed.

2.11 | Soft-agar assay

To measure colony formation, 1000 cells were suspended in 0.33% Noble agar (BD Biosciences) mixed with cell culture medium, and immediately plated onto 6-well plates precast with 0.5% Noble agar mixed with cell culture medium. Cells were incubated at 37°C in a CO_2 incubator. Two weeks after plating, the total numbers of colonies with a diameter larger than 50 μm were counted under the stereomicroscope. The mean and SD of colony numbers per dish were calculated from triplicate cultures for each experimental group.

2.12 | Immunofluorescence staining of cleaved caspase-3

A549 cells (A549-shScramble and A549-shTFF-1-A) (1 × 10^3 cells per chamber) were seeded into 4-chamber culture slides (BD Falcon). After 24 hours, culture medium was removed and cells were fixed with 100% methanol for 15 minutes at 4°C. Cells were washed with PBS 3 times for 5 minutes each, followed by a blocking step with blocking buffer (1× PBS/5% normal goat serum/0.3% Triton X-100) for 60 minutes. The cells were subjected to immunofluorescence staining with cleaved caspase-3 (Asp175) rabbit mAb (1:400; Cell Signaling Technology) at 4°C overnight. The cells were then washed with PBS 3 times for 3 minutes each, and incubated with goat anti-rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11008) (Invitrogen) (1:400 dilution) for 2 hours at room temperature, then the cells were washed with PBS 3 times for 5 minutes each. The cells were exposed to DAPI for 10 minutes at room temperature in the dark, then were washed with PBS 3 times for 5 minutes each. The cover slips were mounted on slides with glycerol/PBS. The mean and SD of cleaved caspase-3-positive ratio (%) were calculated, based on the numbers of the cells positive for cleaved caspase-3 and DAPI, respectively, counted by BZ-X800 Analyzer (Keyence) from 8 independent rectangular areas (×100 field of view) of the cultures for each experimental group.

2.13 | Propidium iodide flow cytometry assay

Late apoptosis/necrosis was evaluated by staining for propidium iodide (Wako). In brief, A549 cells (A549-shScramble and A549-shTFF-1-A) (5 × 10^5 cells) were seeded on a 6-cm dish; after 4 days, the cells were trypsinized and resuspended at 1 × 10^6 cells/100 μL in RPMI. Thereafter, propidium iodide at a concentration of 50 μg/mL was added and incubated at room temperature for 5 minutes. Subsequent analysis was carried out using FACSVerse (Becton Dickinson) and FlowJo version 10.6.1

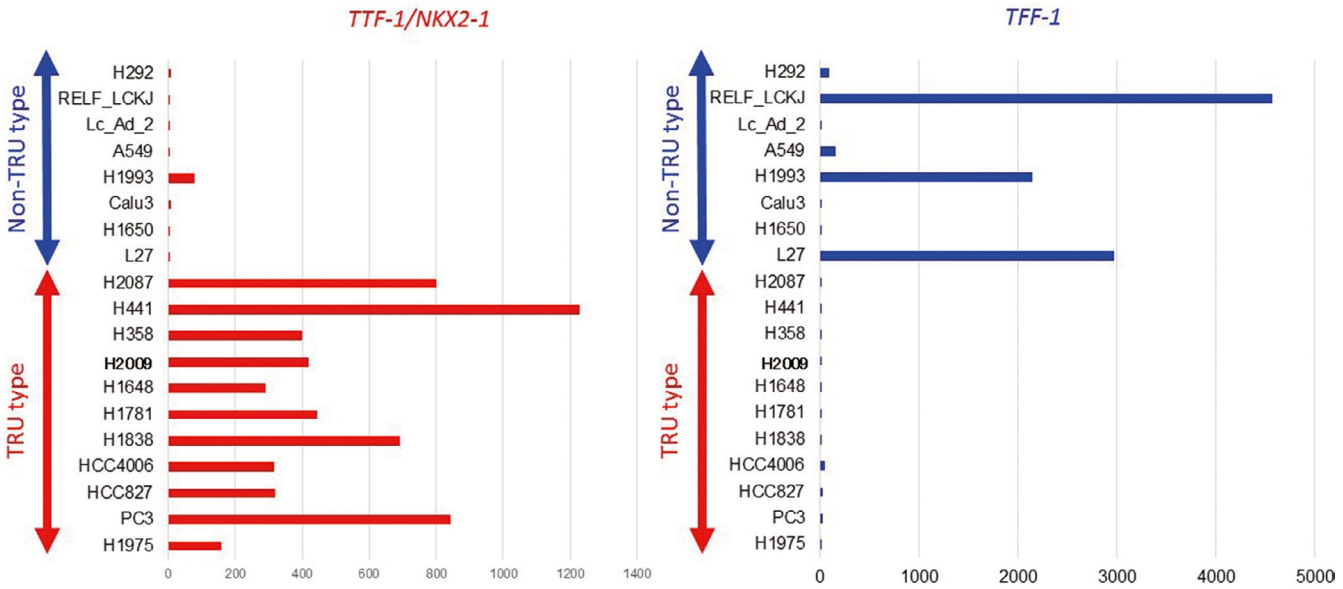

FIGURE 1 Gene expression levels of *TTF/NKX2-1* (left panel) and trefoil factor-1 (*TFF-1*) (right panel) in 19 adenocarcinoma cell lines. H292, RERF-LC-KJ, RELF-LC-Ad2, A549, H1993, Calu3, H1650, and L27 were *CDH1*-high but *TTF-1/NKX2-1*-low adenocarcinoma cell lines ("non-TRU-type" cell lines). H2087, H441, H358, H2009, H1648, H1781, H1838, HCC4006, HCC827, PC3, and H1975 were *CDH1*-high and *TTF-1/NKX2-1*-high adenocarcinoma cell lines ("TRU-type" cell lines). Strong expression of *TFF-1* was frequently detected in "non-TRU-type" cell lines. Expression data were normalized with the Affymetrix MAS5.0 algorithm with target intensity of 100

TABLE 1 Relationships between trefoil factor-1 (TFF-1) expression levels and histological subtypes of lung adenocarcinomas

Histological subtypes	TFF-1 expression		
	Positive, n (%)	Negative, n (%)	Total, n (%)
Invasive mucinous adenocarcinoma	14 (93)	1 (7)	15 (100)
Enteric adenocarcinoma	2 (100)	0 (0)	2 (100)
Colloid adenocarcinoma	1 (100)	0 (0)	1 (100)
Acinar adenocarcinoma	5 (21)	19 (79)	24 (100)
Papillary adenocarcinoma	7 (6)	113 (94)	120 (100)
Solid adenocarcinoma	2 (5)	41 (95)	43 (100)
Micropapillary adenocarcinoma	0 (0)	1 (100)	1 (100)
Lepidic adenocarcinoma	0 (0)	23 (100)	23 (100)
Minimally invasive adenocarcinoma	0 (0)	8 (100)	8 (100)
Non-mucinous adenocarcinoma in situ	0 (0)	1 (100)	1 (100)
Total	31	207	238

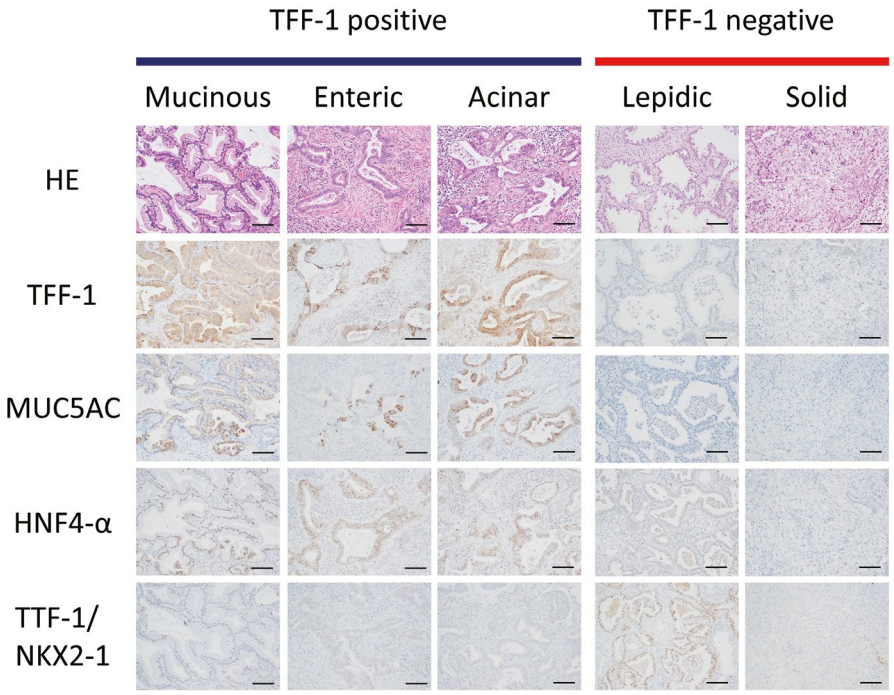

FIGURE 2 Staining of sections from representative cases of trefoil factor-1 (TFF-1)-positive adenocarcinomas (mucinous, enteric, and acinar adenocarcinomas) (left panels) and TFF-1-negative adenocarcinomas (lepidic and solid adenocarcinomas) (right panels) for H&E, TFF-1, mucin 5AC (MUC5AC), hepatocyte nuclear factor-4α (HNF4-α), and TTF-1/NK2 homeobox 1 (NKX2-1). Scale bar = 100 μm. TFF-1-positive cases were frequently positive for MUC5AC and HNF4-α and negative for TTF-1/NKX2-1. Lepidic adenocarcinomas were frequently negative for TFF-1, MUC5AC, and HNF4-α and positive for TTF-1/NKX2-1. Solid adenocarcinoma was more likely to be negative for TFF-1, MUC5AC, HNF4-α, and TTF-1/NKX2-1

software. Results are calculated as mean ± SD of values from 3 individual experiments.

2.14 | Bioinformatic analyses

We used the Cluster program (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) for a cluster analysis of the gene expression data of lung adenocarcinoma cases. In brief, we undertook average linkage hierarchical clustering on 442 lung adenocarcinoma cases using the mean centering of genes. We then displayed the results obtained with the aid of TreeView software (http://jtreeview.sourceforge.net/). The image used a color code to represent relative expression levels. Red represents expression levels greater than the mean for a given gene across all samples. Green represents expression levels less than the mean across samples.

2.15 | Whole exome sequencing and RNA sequencing

Details were shown in our previous study.[8]

2.16 | Statistical analysis

The χ^2-test was used to evaluate clinicopathological correlations. Multivariate logistic regression analysis was applied to identify the independent clinical factors associated with TFF-1 expression. The Mann-Whitney *U* test was used to evaluate cell growth. The *t* test was used to evaluate the cleaved caspase-3 positive ratio by immunofluorescence staining, growing colonies of soft-agar colony formation assay, and propidium iodide positive ratio by flow cytometry. Survival curves were generated using the Kaplan-Meyer method and

TABLE 2 Relationships between trefoil factor-1 (TFF-1) expression levels and clinicopathologic factors, and expression levels of thyroid transcription factor-1 (TTF-1)/NK2 homeobox 1 (NKX2-1), hepatocyte nuclear factor-4α (HNF4-α), and mucin 5AC (MUC5AC) in 238 primary lung adenocarcinomas

	TFF-1 expression		
	Positive	Negative	*P* value[g]
Age, y			
<60	2	37	.1091
≥60	29	170	
Sex			
Male	19	113	.4839
Female	12	94	
Pathological stage[a]			
0-II	26	152	.3117
III-IV	5	49	
Pathological T stage			
Tis, T1mi, T1	8	105	.0096
T2, T3, T4	23	102	
Nodal involvement[b]			
Positive	9	55	.8592
Negative	22	145	
Lymphatic invasion			
Positive	15	77	.2328
Negative	16	130	
Vessel invasion			
Positive	16	98	.6572
Negative	15	109	
Pleural invasion			
Positive	15	79	.2775
Negative	16	128	
Tumor invasion size, cm[c]			
<3	12	144	.0014
≥3	18	63	
Pulmonary metastasis[d]			
Positive	0	14	.1346
Negative	31	192	
Spread through airspaces[e]			
G3	5	19	.0098
G1 or G2	12	187	
Nonmucinous lepidic predominant[f]			
Yes	0	32	.0186
No	31	175	
Papillary predominant			
Yes	10	116	.0134
No	21	91	
Acinar predominant			
Yes	5	14	.0728
No	26	193	
Solid predominant			
Yes	2	42	.0642
No	29	165	
TTF-1/NKX2-1 expression			
Positive	8	183	<.0001
Negative	23	24	
HNF4-α expression			
Positive	23	5	<.0001
Negative	8	202	
MUC5AC expression			
Positive	22	10	<.0001
Negative	9	197	

[a]Stages of 6 cases were unknown because pathological examination of the lymph nodes and metastasis was not undertaken.
[b]Presence or absence of nodal involvement in 7 cases was unknown.
[c]Tumor invasion size of 1 case was unknown.
[d]Presence or absence of pulmonary metastasis in one case was unknown.
[e]Invasive mucinous adenocarcinoma cases (n = 15) were excluded.
[f]Adenocarcinoma in situ, minimally invasive adenocarcinoma, and lepidic adenocarcinoma.
[g]Underlined values indicate <.05.

differences in survival were analyzed by the log-rank test. Univariate Cox regression analysis was used to identify the univariate predictors. Variables that showed significant difference in survival in the univariate analysis were included in a multivariate Cox regression analysis. The results obtained were considered to be significant if the *P* value was less than .05. All statistical calculations were carried out using the StatView computer program (Abacus Concepts).

3 | RESULTS

3.1 | Reciprocal expression of TTF-1 and TFF-1 in lung adenocarcinoma cell lines

Our previous study revealed that non-TRU-type lung adenocarcinomas are largely divided into 2 subtypes: one that sustains the epithelial phenotype and another with the EMT phenotype.[8] Comparisons between TRU types vs non-TRU types might be confounded by the heterogeneity of non-TRU-type lung adenocarcinomas. Therefore, in order to elucidate the characteristics of the former subtype of non-TRU-type adenocarcinomas, we collected *CDH1*-high adenocarcinoma cell lines sustaining the epithelial phenotype (n = 19) (shown in Table S1), and then compared gene expression data on *TTF-1/*

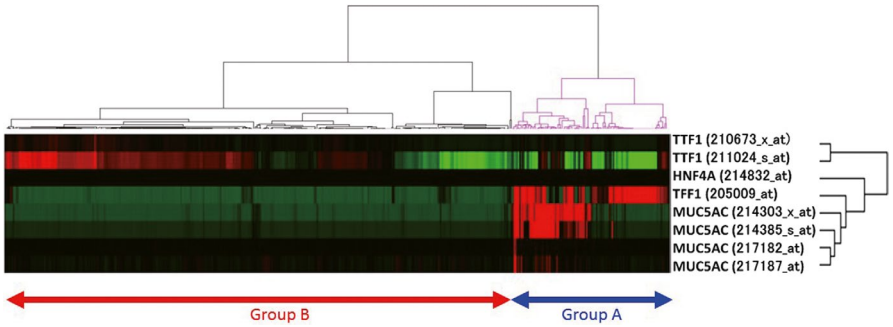

FIGURE 3 Analysis of publicly available data on 442 primary lung adenocarcinoma cases.[24] A hierarchical cluster analysis using the gene expression levels of *TTF-1/NKX2-1*, *HNF4A*, *TFF-1*, and *MUC5AC* classified 442 cases into 2 groups: Group A with high expression levels of *TFF-1*, and Group B with low expression levels of *TFF-1*

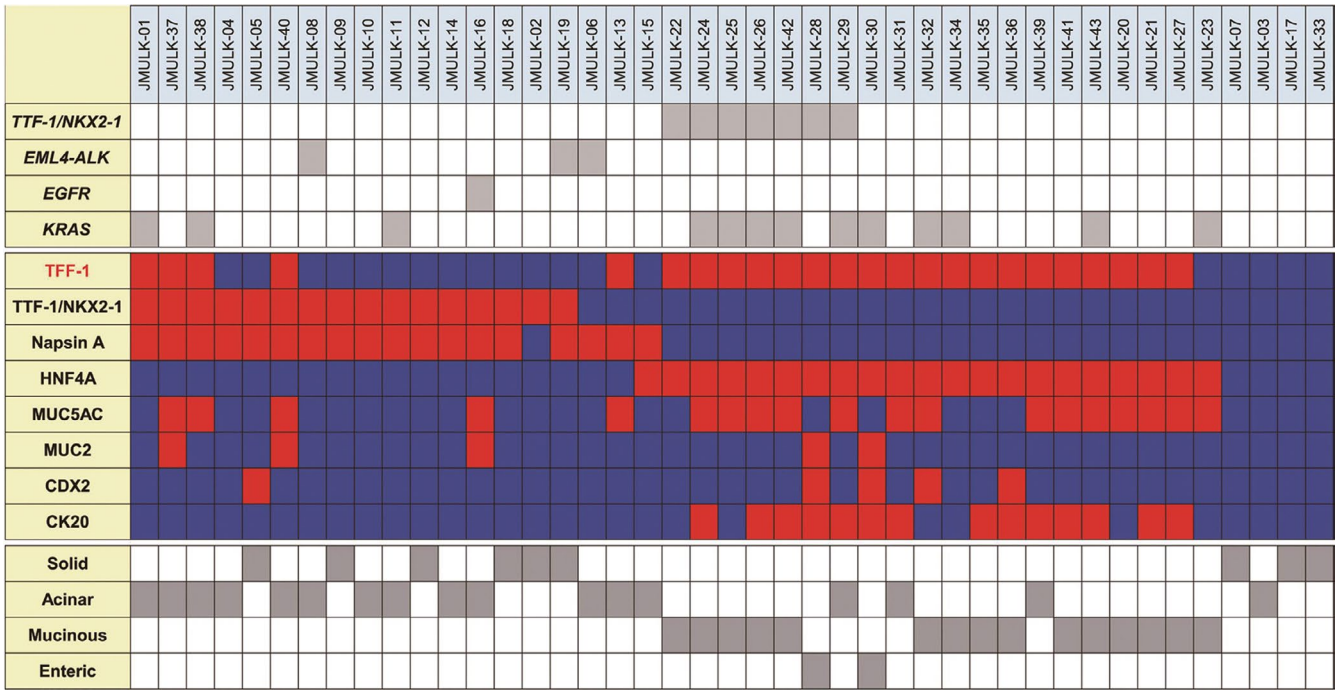

FIGURE 4 Top panel, Genetic status of *TTF-1/NKX2-1*, *KRAS*, *EGFR*, and *EML4-ALK* in 43 cases of lung adenocarcinoma with the nonterminal respiratory unit-type morphology. Gray filled box indicates the presence of genetic abnormalities; white box indicates WT. Middle panel, Immunohistochemical expression of trefoil factor-1 (TFF-1), thyroid transcription factor-1 (TTF-1)/NK2 homeobox 1 (NKX2-1), Napsin A, hepatocyte nuclear factor-4α (HNF4-α), mucin 5AC (MUC5AC), MUC2, caudal-type homeobox 2 (CDX2), and cytokeratin 20 (CK20) in 43 cases. Red filled box indicates positive; blue indicates negative. Lower panel, Histological subtypes of 43 cases

NKX2-1-high cell lines (n = 11) and *TTF-1/NKX2-1*-low cell lines (n = 9) (shown in Figure 1). We extracted genes that were characteristically expressed at high levels in *TTF-1/NKX2-1*-low cell lines according to correlation coefficients (shown in Table S3), and found that *TFF-1*, a gastric mucosal protective factor, was uniquely expressed in *TTF-1/NKX2-1* negative, *CDH1*-high cell lines (shown in Figure 1).

3.2 | Strong TFF-1 expression frequently detected in non-TRU-type primary lung adenocarcinomas and correlates with gastrointestinal features

In order to confirm whether TFF-1 is frequently positive in primary non-TRU-type lung adenocarcinomas, we undertook an immunohistochemical analysis of TFF-1 using 238 primary lung adenocarcinoma cases surgically resected at Jichi Medical University Hospital. The results obtained revealed that 31 cases (13%) were positive for TFF-1. Trefoil factor-1 was frequently positive in adenocarcinomas with gastrointestinal features; ie, invasive mucinous (14/15 cases; 93%), enteric (2/2 cases; 100%), and colloid (1/1 case; 100%) adenocarcinomas. Expression of TFF-1 was detected in a proportion of acinar (5/24 cases; 21%), papillary (7/120 cases; 6%), and solid adenocarcinomas (2/43 cases; 5%). Trefoil factor-1 was negative in micropapillary (0/1 case, 0%), lepidic (0/23 cases, 0%), and microinvasive adenocarcinomas or adenocarcinoma in situ (0/9 cases, 0%) (shown in Table 1 and Figure 2). Statistical analyses showed that TFF-1 expression was inversely associated with a lepidic or papillary predominant pattern (P = .0186 and P = .0134, respectively) (Table 2). Solid adenocarcinomas, possible representatives of non-TRU-type adenocarcinomas,[3] were also more likely to be negative for TFF-1

TABLE 3 Relationship between *TFF-1* gene expression levels and genetic mutations in 230 primary lung adenocarcinoma cases (The Cancer Genome Atlas Research Network[25])

	TFF-1 expression		
	High	Low	*P* value[a]
KRAS mutations			
Positive	12	63	.0028
Negative	6	149	
EGFR mutations			
Positive	0	33	.0833
Negative	18	179	
ALK fusions			
Positive	0	3	>.999
Negative	18	209	
RET fusions			
Positive	0	2	>.999
Negative	18	210	
ROS1 fusions			
Positive	0	4	>.999
Negative	18	208	
HER2 mutations			
Positive	0	6	>.999
Negative	18	206	
BRAF mutations			
Positive	0	22	>.999
Negative	18	190	
TTF-1/NKX2-1 mutations			
Positive	0	1	>.999
Negative	18	211	

[a]Underlined values indicate <.05.

(*P* = .0642) (Table 2). Expression of TFF-1 correlated with the expression of the gastrointestinal markers HNF4-α and MUC5AC (*P* < .0001 and *P* < .0001, respectively) and inversely correlated with TTF-1/NKX2-1 expression (*P* < .0001) (Table 2). These results indicate that TFF-1 is characteristically expressed in relatively differentiated non-TRU-type primary lung adenocarcinomas with gastrointestinal features.

Table 2 also shows the relationships between TFF-1 expression levels and clinicopathologic factors in 238 cases. Trefoil factor-1 expression correlated with an advanced pT stage and invasion size (*P* = .0096 and *P* = .0014, respectively). Extensive STAS was also significantly frequent in TFF-1-positive cases, even excluding invasive mucinous adenocarcinoma cases (*P* = .0098) (Table 2 and Figure S1). Multivariate logistic regression analysis revealed that extensive STAS is the only independent factor correlated with TFF-1 expression, among pT stage, invasion size, and STAS (Table S4).

3.3 | Hierarchical cluster analysis of 442 primary lung adenocarcinoma cases

We undertook a hierarchical cluster analysis on the publicly available data of 442 primary lung adenocarcinoma cases (Shedden et al's data)[24] using the gene expression of *TTF-1/NKX2-1*, *TFF-1*, *MUC5AC*, and *HNF4A*. As shown in Figure 3, 442 lung adenocarcinoma cases were mainly classified into 2 groups: Group A (*TFF-1*-high cases) (n = 91) and Group B (*TFF-1*-low cases) (n = 351). Group A (*TFF-1*-high cases) frequently showed strong expression of *MUC5AC* and weak expression of *TTF-1/NKX2-1*. Based on histological data, Group A (*TFF-1*-high cases) significantly showed a histologically more differentiated morphology than Group B (*TFF-1*-low cases) (*P* = .0392) (Table S5). These results are consistent with our findings from an immunohistochemical analysis of 238 cases (our Jichi cohort); TFF-1 was positive in morphologically well differentiated subtypes, such as mucinous and acinar adenocarcinomas, but tended to be negative in solid adenocarcinomas, a morphologically poorly differentiated subtype.

3.4 | Trefoil factor-1-positive cases frequently harbor *KRAS* mutations, but no other common driver mutations

We also carried out immunostaining for TFF-1 using 43 lung adenocarcinoma cases with the non-TRU-type morphology, which we analyzed by next generation sequencing and immunohistochemistry in our previous study.[8] The results obtained are shown in Figure 4. Twenty-three out of 43 cases (53%) were positive for TFF-1 among non-TRU-type morphology cases, significantly showing gastrointestinal features with strong expression of HNF4-α (*P* < .0001, χ^2 test) and MUC5AC (*P* < .0001, χ^2 test) and weak expression of TTF-1/NXK2-1 (*P* = .0017, χ^2 test) (Figure 4). Figure 4 also shows that *KRAS* mutations and inactivating mutations in *TTF-1/NKX2-1* were frequent in TFF-1-positive cases (*P* = .0123 and *P* = .0436, respectively, χ^2 test), whereas targetable driver mutations, such as *EGFR* mutations and *ALK* fusions, were not detected.

Using publicly available data on gene expression and genetic mutations in 230 primary lung adenocarcinoma cases in TCGA data,[25] we examined the relationships between *TFF-1* gene expression levels and major driver mutations. Among the 230 cases, most showed low *TFF-1* expression levels; however, a small number of cases showed high *TFF-1* expression levels (Figure S2). The frequency of *KRAS* mutations was significantly high in *TFF-1*-high cases (n = 18) (*P* = .0028), whereas no genetic abnormalities were detected in *EGFR*, *ALK*, *RET*, or *ROS1* (Table 3).

These results are consistent with our previous findings from an analysis of 43 cases with the non-TRU-type morphology (the second Jichi cohort in this study) (Figure 4), except that the *TTF-1/NKX2-1* mutation was only found in 1 case with low *TFF-1* level expression among the 230 cases examined (TCGA data).

3.5 | Overall survival analysis of lung adenocarcinoma cases in datasets

We undertook an overall survival analysis of: (i) 238 cases in Jichi Medical University Hospital, depending on the immunohistochemical expression levels of TFF-1; (ii) 442 cases in Shedden et al's data[24] between Group A (*TFF-1*-high) and Group B (*TFF-1*-low); and (iii) 230 cases in TCGA data,[25] depending on the gene expression levels of *TFF-1*. The results obtained are shown in Figure 5. The survival analysis revealed that the prognosis of TFF-1-high cases was significantly worse among those with advanced stages (stage III or IV) (Figure 5A,B). Figure 5C also showed that *TFF-1*-high cases had a worse prognosis than *TFF-1*-low cases in the advanced stages, but the difference did not achieve significance.

Based on these results, we speculate that TFF-1 is not only a marker for abnormal gastrointestinal differentiation, but also plays an oncogenic role in lung adenocarcinomas.

3.6 | Multivariate analysis of overall survival among clinicopathologic factors

For confirmation, we undertook the univariate Cox regression analysis of the clinicopathologic factors listed in Table 2 in all cases in the Jichi cohort study. The results are shown in Table 4a. In this analysis, various clinicopathologic factors such as invasion size, pleural invasion, pulmonary metastasis, pT stage, lymphatic invasion, vessel invasion, lymph node metastasis, stage, STAS, solid predominant morphology, TTF-1/NKX2-1 expression, were statistically correlated with patient prognosis.

Next, we undertook univariate and multivariate Cox regression analyses of the clinicopathologic factors listed in Table 2, in the cases with advanced stage, in the Jichi cohort study. Interestingly, in the univariate analysis, the biological markers and histomorphology, such as TTF-1/NKX2-1, HNF4-α, MUC5AC, and TFF-1 expression and solid predominant morphology, were statistically correlated with patient survival in the advanced stage (Table 4b).

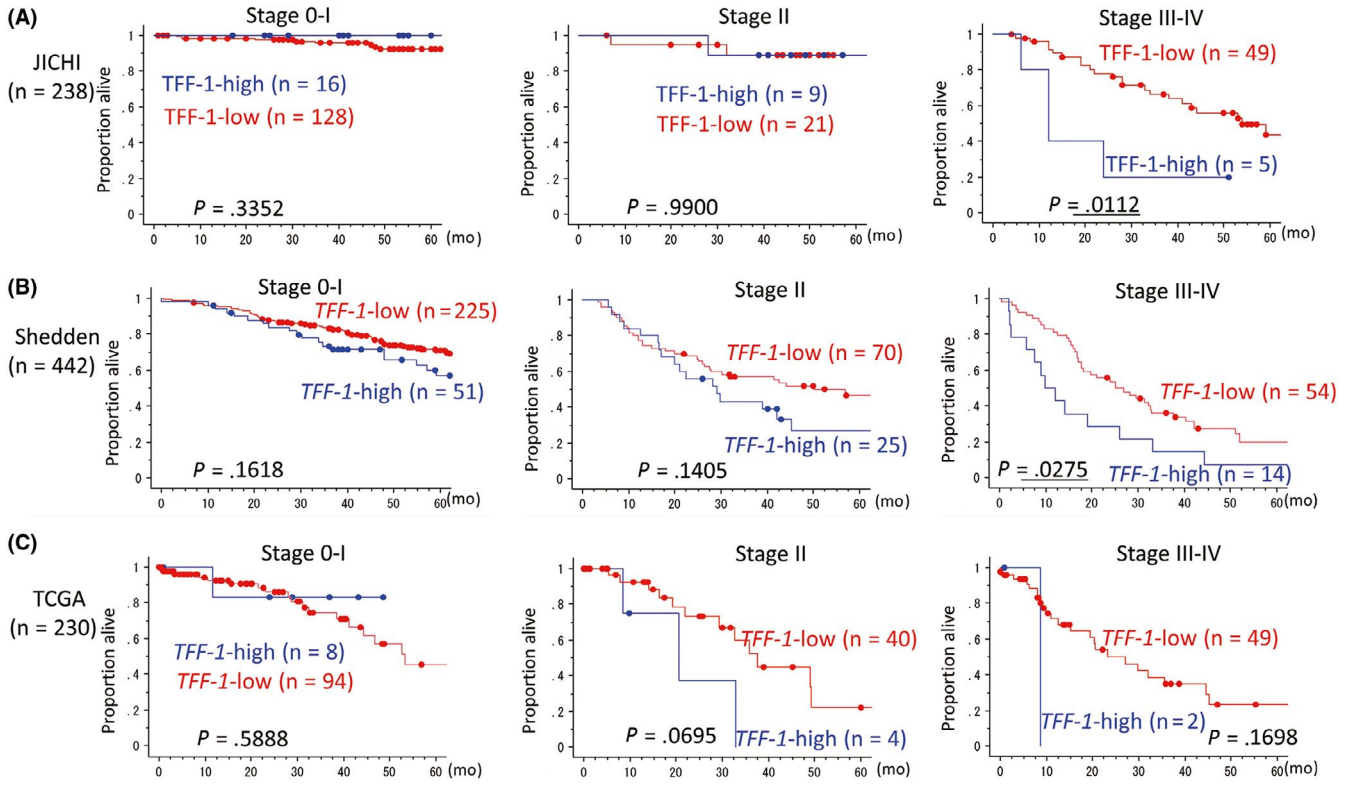

FIGURE 5 A, Patient overall survival according to immunohistochemical expression levels of trefoil factor-1 (TFF-1) among 238 cases of lung adenocarcinoma at Jichi Medical University Hospital. Patients were separated according to pathological stage (0-I, II, and III-IV). TFF-1-high cases showed a significantly worse prognosis than TFF-1-low cases among those with advanced stage disease (III-IV). B, Patient overall survival in Group A (*TFF-1*-high cases) and Group B (*TFF-1*-low cases) among 442 cases in Shedden et al's data[24]. Group A (*TFF-1*-high cases) and Group B (*TFF-1*-low cases) were based on a hierarchical cluster analysis using the gene expression levels of *TTF-1/NKX2-1*, *HNF4A*, *TFF-1*, *and MUC5AC* in 442 cases. Patients were separated according to pathological stage (0-I, II, and III-IV). C, Patient overall survival according to *TFF-1* gene expression levels among 230 cases in The Cancer Genome Atlas (TCGA) data.[25] Patients were separated according to pathological stage (0-I, II, and III-IV)

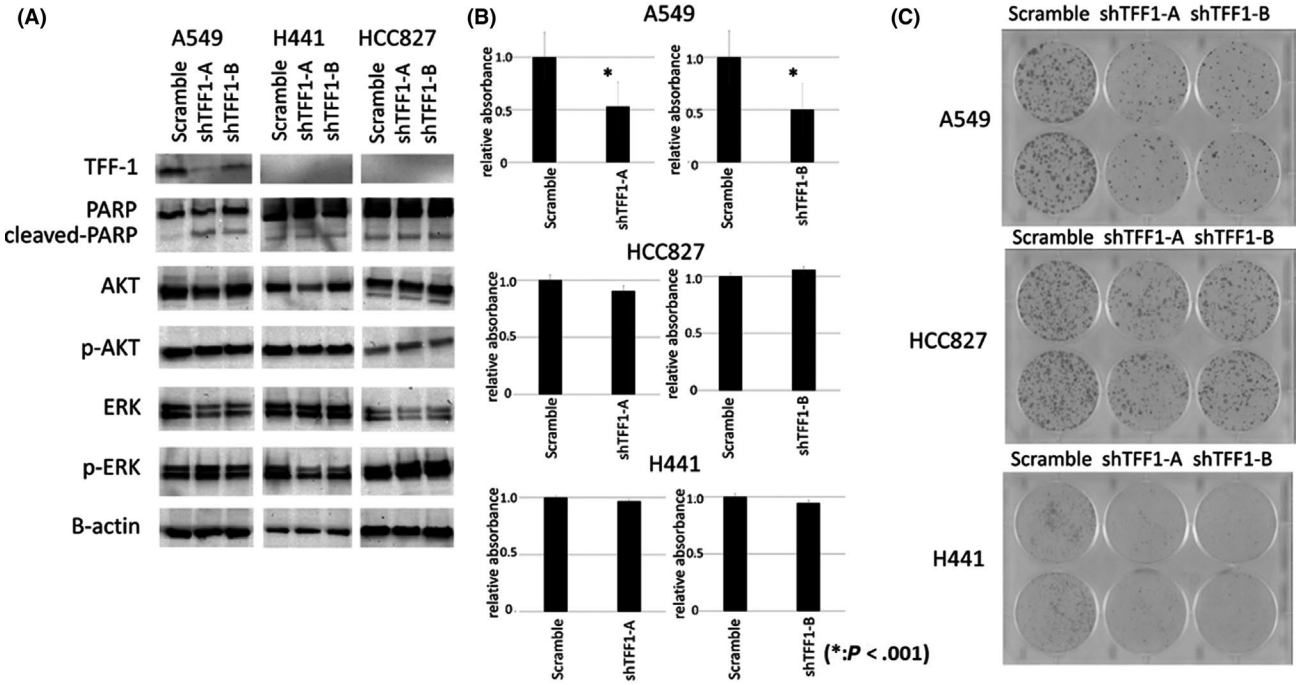

FIGURE 6 In vitro analysis of A549 cell lines (A549-shScramble, A549-shTFF-1-A, and A549-shTFF-1-B), H441 cell lines (H441-shScramble, H441-shTFF-1-A, and H441-shTFF-1-B), and HCC827 cell lines (HCC827-shScramble, HCC827-shTFF-1-A, and HCC827-shTFF-1-B). A, Protein expression levels of trefoil factor-1 (TFF-1), poly ADP-ribose polymerase (PARP), cleaved PARP, AKT, phosphorylated (p-) AKT, ERK, p-ERK, and β-actin by western blot analysis. B, In vitro growth assay. Cell growth was assessed after 4 days of incubation. Means ± SD of absorbance are shown (n = 10, **P* < .001, Mann-Whitney *U* test). C, Representative images of a macroscopic colony formation assay on 4 replicates of cell cultures. Knockdown of TFF-1 markedly inhibited colony formation by A549 cells, but had no effect on colony formation by HCC827 cells. No colony formation was observed in H441 or H441-shTFF-1 cells

We undertook the multivariate analysis, excluding TTF-1/NKX2-1, HNF4-α, and MUC5AC expressions which were significantly correlated with TFF-1 expression, and TFF-1 expression and solid predominant morphology remained significant in the multivariate analysis (Table 4c).

3.7 | Knockdown of TFF-1 inhibits proliferation of *TFF-1*-high and *KRAS*-mutated cells

The TFF-1-positive cases frequently harbored *KRAS* mutations, but targeting KRAS has yet to achieve clinical success. We speculated that TFF-1 itself is a candidate for the molecular target of TFF-1-positive and *KRAS*-mutated non-TRU-type adenocarcinomas.

In order to elucidate the biological functions of TFF-1 in lung adenocarcinomas, we undertook knockdown experiments using a *TFF-1*-high and *KRAS*-mutated lung adenocarcinoma cell line (A549) and 2 *TFF-1*-low lung adenocarcinoma cell lines (H441 and HCC827). As shown in Figure 6A, the knockdown of TFF-1 did not suppress the phosphorylation of ERK or AKT, but induced apoptosis, as indicated by increased cleaved poly ADP-ribose polymerase, in *TFF-1*-high and *KRAS*-mutated cell lines (A549), but not in *TFF-1*-low cell lines (HCC827 and H441) (Figure 6A). We applied propidium iodide staining-based FACS analysis on A549-shScramble cells and A549-shTFF-1-A cells. The results showed that the percentage of late apoptotic/necrotic cells in the A549-shTFF-1-A cells was higher than that in the A549-shScramble cells (Figure S3). We also confirmed the percentage of the cleaved caspase-3 positive cells was significantly higher in A549-shTFF-1-A cells compared with A549-shScramble cells by immunofluorescence staining (Figure S4). Thus, knockdown of TFF-1 increased the apoptosis of A549 cells.

In the cell growth assay, the knockdown of TFF-1 significantly inhibited the proliferation of a *TFF-1*-high and *KRAS*-mutated cell line (A549), but not of the *TFF-1*-low cell lines (HCC827 and H441) (Figure 6B). We also confirmed that the knockdown of TFF-1 markedly inhibited colony formation by A549 in the colony formation assay (Figure 6C). Next, we undertook the soft-agar assay, which revealed that the colony number was significantly decreased by the knockdown of TFF-1 (Figure S5), suggesting that TFF-1 could promote the anchorage-independent growth of A549 cells in soft agar.

We also undertook knockdown experiments using *TFF-1*-high and *KRAS*-WT lung adenocarcinoma cell lines, L27 and H1993; however, knockdown of TFF-1 did not inhibit the proliferation of L27 or H1993 cells (Figure S6). We have previously reported that L27 and H1993 harbored *MET* amplification and were specifically sensitive to MET inhibitor, PHA665752.[27] We speculated that *MET* amplification was such a strong growth driver that the knockdown of TFF-1 had no effect on the cell proliferation.

TABLE 4 Univariate and multivariate Cox regression analysis of clinicopathologic factors in our cohort of patients with lung adenocarcinomas resected at Jichi Medical University Hospital

Pathological factors	Hazard ratio	95% confidence interval	*P* value[a]
(a) Univariate Cox regression analysis, all disease stages (n = 238)			
Age, y (≥60 vs <60)	1.556	0.606-3.994	.3573
Sex (male vs female)	1.874	0.946-3.714	.0721
Pathological stage (III-IV vs 0-II)	10.095	4.975-20.482	<.0001
Pathological T stage (T2-T4 vs Tis-T1)	6.807	2.657-17.441	<.0001
Nodal involvement	7.360	3.627-14.933	<.0001
Lymphatic invasion	7.199	3.294-15.737	<.0001
Vessel invasion	4.216	1.994-8.915	.0002
Pleural invasion	4.250	2.107-8.574	<.0001
Tumor invasion size, cm (≥3 vs <3)	2.892	1.524-5.490	.0012
Pulmonary metastasis	3.508	1.464-8.408	.0049
STAS (G3 vs G1 or G2)	2.413	1.104-5.275	.0273
Papillary predominant	0.831	0.440-1.571	.5697
Acinar predominant	0.951	0.292-8.058	.9339
Solid predominant	4.187	2.176-8.915	<.0001
TTF-1/NKX2-1 expression	0.452	0.228-0.895	.0228
HNF4-α expression	1.283	0.501-3.286	.6044
MUC5AC expression	1.706	0.750-3.878	.2024
TFF-1 expression	1.095	0.428-2.806	.8504
(b) Univariate Cox regression analysis, advanced disease stages (n = 54)			
Age, y (≥60 vs <60)	0.579	0.217-1.542	.2744
Sex (male vs female)	1.654	0.736-3.715	.2234
Pathological T stage (T2-T4 vs Tis-T1)	3.622	0.854-15.356	.081
Nodal involvement	1.132	0.266-4.818	.867
Lymphatic invasion	0.926	0.123-6.944	.9402
Vessel invasion	1.478	0.557-3.924	.4323
Pleural invasion	2.210	0.831-5.877	.1119
Tumor invasion size, cm (≥3 vs <3)	0.724	0.332-1.579	.4162
Pulmonary metastasis	0.980	0.393-2.443	.9653
STAS (G3 vs G1 or G2)	1.236	0.550-2.777	.6184
Papillary predominant	0.558	0.256-1.215	.1418
Acinar predominant	0.618	0.145-2.631	.5155
Solid predominant	2.918	1.256-6.779	.0127
TTF-1/NKX2-1 expression	0.204	0.081-0.516	.0008
HNF4-α expression	8.534	2.607-27.933	.0004
MUC5AC expression	4.446	1.722-11.480	.002
TFF-1 expression	3.691	1.239-10.998	.0191
(c) Multivariate Cox regression analysis, advanced disease stages (n = 54)			
Solid predominant	3.611	1.501-8.689	.0042
TFF-1 expression	5.244	1.666-16.504	.0046

Abbreviations HNF4-α, hepatocyte nuclear factor-4α; MUC5AC, mucin 5AC; NKX2-1, NK2 homeobox 1; STAS, spread through air spaces; TTF-1, thyroid transcription factor-1.

[a]Underlined values indicate <.05.

4 | DISCUSSION

The present results identified the strong expression of TFF-1 as a characteristic of non-TRU-type adenocarcinomas with abnormal gastrointestinal differentiation, and also that it correlated with a poorer prognosis in patients with advanced stage disease. The knockdown of TFF-1 inhibited the proliferation of A549, a *TFF-1*-high and *KRAS*-mutated lung adenocarcinoma cell line. To the best of our knowledge, this is the first study to show the histological expression pattern and oncogenic role of TFF-1 in lung adenocarcinomas.

Thyroid transcription factor-1/NKX2-1 has been shown to repress the gastric differentiation program in the lungs of mice, and its loss was found to reactivate the intrinsic gastric differentiation program, resulting in the induction of mucinous adenocarcinomas.[28,29] It is one of the regulators of gastrointestinal differentiation.[14] As TFF-1 is mainly expressed in well to moderately differentiated carcinomas, as shown in the present study, the induction of TFF-1 expression might occur in the early stages of the development of non-TRU-type lung adenocarcinomas, following the silencing of TTF-1/NKX2-1, and could play a role in abnormal gastrointestinal differentiation.

Trefoil factor-1 was previously reported to transactivate EGFR, which, in turn, might activate PI3K/AKT and ERK signaling pathways.[30] However, in the present study, the knockdown of TFF-1 did not suppress the phosphorylation of ERK or AKT. The knockdown of TFF-1 was found to induce apoptosis, which is consistent with previous findings showing that TFF-1 decreased caspase-3, -6, -8, and -9 activities and protected cells from chemical-, anchorage-free-, or Bad-induced apoptosis.[31] Our immunohistochemical analysis of 238 cases of primary adenocarcinomas revealed that the strong expression of TFF-1 correlated with extensive STAS. We also found that knockdown of TFF-1 significantly inhibited colony formation in the soft-agar assay. Based on these findings, we speculate that TFF-1 contributes to inhibiting detachment-induced apoptosis and the formation of STAS.

In the present study, TFF-1 expression correlated with a worse prognosis in the advanced stages only. We also found that mucinous adenocarcinomas, the main TFF-1-positive cases, had a better prognosis than the other types in the early stages in the Jichi Medical University Hospital cohort (not shown). These characteristics of TFF-1-positive tumors could be explained as follows. In our analysis of 238 cases, TFF-1 expression correlated with STAS, but not with vessel, lymphatic, or pleural invasion. These results suggest that TFF-1 does not induce destructive invasion or metastasis through the lymphovascular system, but might be important for tumor survival, proliferation, and metastasis through air spaces. Therefore, if tumors are completely resected in the early stages, TFF-1 will lose its opportunity to play an oncogenic role in later stages.

In summary, TFF-1 was strongly expressed in non-TRU-type lung adenocarcinomas with gastrointestinal features and correlated with a worse prognosis at advanced stages. The knockdown of TFF-1 inhibited the proliferation of *TFF-1*-high cell lines, suggesting that TFF-1 itself is a potential target of cancer therapy for non-TRU-type lung adenocarcinomas.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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