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
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)/NKX2 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.

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% CO2 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 XbaI 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 system23; 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 IIC), 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. 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.

### 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 -α (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. After the indicated time for the incubation, CCK-8 reagent was added on a 96-well microtiter plate with 10 replicates for each condition. Nonspecific binding was blocked by immersing membranes for 20 minutes in 5% skim milk in TBS at room temperature. The membranes were blotted, equal amounts of protein samples were size-separated on 8% polyacrylamide gels and electroblotted onto nitrocellulose membranes.

**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′-GCCCCTCCAGTGTGCAAATAA-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 × 10^4 cells (A549, L27, and H1993) and 2 × 10^5 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_2VO_4 with a cocktail of protease 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)
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 CO₂ 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 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.

2.12 | Immunofluorescence staining of cleaved caspase-3

A549 cells (A549-shScramble and A549-shTFF-1-A) (1 × 10³ 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 (1:1000; 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 × 10⁵ cells) were seeded on a 6-cm dish; after 4 days, the cells were trypsinized and resuspended at 1 × 10⁶ 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, RERF-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 TFF-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.
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 \(\chi^2\)-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 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 TFF-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 TFF-1/NKX2-1. Lepidic adenocarcinomas were frequently negative for TFF-1, MUC5AC, and HNF4-α and positive for TFF-1/NKX2-1. Solid adenocarcinoma was more likely to be negative for TFF-1, MUC5AC, HNF4-α, and TFF-1/NKX2-1.
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).

### 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. 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/
NKX2-1-high cell lines (n = 11) and TFF-1/NKX2-1-low cell lines (n = 9) (shown in Figure 1). We extracted genes that were characteristically expressed at high levels in TFF-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%), lepider (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 lepideric or papillary predominant pattern (.0186 and .0134, respectively) (Table 2). Solid adenocarcinomas, possible representatives of non-TRU-type adenocarcinomas, 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 | \(p\) value\(^a\) |       |       |
|---------------------|------------------|-----------------|-------|-------|
|                     | High  | Low   |       |       |
| 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   |       |       |
| TFF-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-\(\alpha\) and MUC5AC \((P < .0001\) and \(P < .0001\), respectively) and inversely correlated with TFF-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 TFF-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 TFF-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-\(\alpha\) \((P < .0001, \chi^2\) test\) and MUC5AC \((P < .0001, \chi^2\) test\) and weak expression of TFF-1/NKX2-1 \((P = .0017, \chi^2\) test\) (Figure 4). Figure 4 also shows that KRAS mutations and inactivating mutations in TFF-1/NKX2-1 were frequent in TFF-1-positive cases \((P = .0123\) and \(P = .0436\), respectively, \(\chi^2\) 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 TFF-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-\(\alpha\), MUC5AC, and TFF-1 expression and solid predominant morphology, were statistically correlated with patient survival in the advanced stage (Table 4b).

![Figure 5](image-url)

**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).
We undertook the multivariate analysis, excluding TFF-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<sup>a</sup> |
|-----------------------------------------------------------|--------------|-------------------------|---------------------|
| (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.

<sup>a</sup>Underlined values indicate <.05.
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CONFLICT OF INTEREST

The authors have no conflict of interest.

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REFERENCES

1. Statistics and Information Department. Vital Statistics. 2000. Tokyo: Ministry of Health, Labor and Welfare; 2001.
2. JemalA, SiegelR, WardE, et al. Cancer statistics, 2006. CA Cancer J Clin. 2006;56:106-130.
3. YatabeY, MitsudomiT, TakahashiT, MitsudomiT. EGFR mutation is specific for terminal respiratory unit-type adenocarcinomas. Am J Surg Pathol. 2002;26:767-773.
4. YatabeY, MitsudomiT. Epidermal growth factor receptor mutations in lung cancers. Pathol Int. 2007;57:233-244.
5. YatabeY, KosakaT, TakahashiT, MitsudomiT. EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. Am J Surg Pathol. 2005;29:633-639.
6. InamuraK, TakeuchiK, TogashiY, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. Mod Pathol. 2009;22:508-515.
7. MatsubaraD, IshikawaS, SachikoO, et al. Co-activation of epidermal growth factor receptor and c-MET defines a distinct subset of lung adenocarcinomas. Am J Pathol. 2010;177:2191-2204.
8. MatsubaraD, SodaM, YoshimotoT, et al. Inactivating mutations and hypermethylation of the NKX2-1/TTF-1 gene in non-terminal respiratory unit-type lung adenocarcinomas. Cancer Sci. 2017;108:1888-1896.
9. SumiyoshiS, YoshizawaA, SonobeM, et al. Non-terminal respiratory unit type lung adenocarcinoma has three distinct subtypes and is associated with poor prognosis. Lung Cancer. 2014;84:281-288.
10. MatsubaraD, KishabaY, IshikawaS, et al. Lung cancer with loss of BRG1/BRM, shows epithelial mesenchymal transition phenotype and distinct histologic and genetic features. Cancer Sci. 2013;104:266-273.
11. PoulsomR, BegosDE, ModlinIM. Molecular aspects of restitution: functions of trefoil peptides. Yale J Biol Med. 1996;69:137-146.
12. RioM, BellocqJ, DanielJ, et al. Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. Science. 1988;241:705-708.
13. KjellevS. The trefoil factor family - small peptides with multiple functionalities. Cell Mol Life Sci. 2009;66:1350-1369.
14. Bossenmeyer-PouriéC, KannanR, RiberaS, et al. The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis. J Cell Biol. 2002;157:761-770.
15. LefebvreO, ChenardM-P, MassonR, et al. Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. Science. 1996;274:259-262.
16. Perry JK, Kannan N, Grandison PM, Mitchell MD, Lobo PE. Are trefoil factors oncogenic? *Trends Endocrinol Metab*. 2008;19:74-81.

17. Emami F, Floch NL, Bruyneel E, et al. Induction of scattering and cellular invasion by trefoil peptides in src- and RhoA-transformed kidney and colonic epithelial cells. *Faseb J*. 2001;15:351-361.

18. Rodrigues S, Rodrigue CM, Attoub S, et al. Induction of the adenoma-carcinoma progression and Cdc25A-B phosphatases by the trefoil factor TFF1 in human colon epithelial cells. *Oncogene*. 2006;25:6628-6636.

19. Ishii M, Hashimoto S-I, Tsutsumi S, et al. Direct comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis. *Genomics*. 2000;68:136-143.

20. Midorikawa Y, Yamamoto S, Ishikawa S, et al. Molecular karyotyping of human hepatocellular carcinoma using single-nucleotide polymorphism arrays. *Oncogene*. 2006;25:5581-5590.

21. Wang T, Niki T, Goto A, et al. Hypoxia increases the motility of lung adenocarcinoma cells A549 via activation of the epidermal growth factor receptor pathway. *Cancer Sci*. 2007;98:506-511.

22. Ishikawa S, Komura D, Tsuji S, et al. Allelic dosage analysis with genotyping microarrays. *Biochem Biophys Res Commun*. 2005;333:1309-1314.

23. Dettetbeck F, Bofaa DJ, Kim AW, Tanoue LT. The eighth edition lung cancer stage classification. *Chest*. 2017;151:193-203.

24. Shedden K, Taylor JM, Enkemann SA, et al. Gene expression–based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. *Nat Med*. 2008;14:822-827.

25. Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma. *Nature*. 2014;511:543-550.

26. Kadota K, Nitadori J-I, Sina CS, et al. Tumor spread through air spaces is an important pattern of invasion and impacts the frequency and location of recurrences after limited resection for small stage I lung adenocarcinomas. *J Thorac Oncol*. 2015;10:806-814.

27. Matsubara D, Ishikawa S, Oguni S, et al. Molecular predictors of sensitivity to the MET inhibitor PHA665752 in lung carcinoma cells. *J Thorac Oncol*. 2010;5:1317-1324.

28. Maeda Y, Tsuchiya T, Hao H, et al. Kras(G12D) and Nk2-1 haploinsufficiency induce mucinous adenocarcinoma of the lung. *J Clin Invest*. 2012;122:4388-4400.

29. Snyder EL, Watanabe H, Magendantz M, et al. Nk2-1 represses a latent gastric differentiation program in lung adenocarcinoma. *Mol Cell*. 2013;50:185-199.

30. Radilloff DR, Watanabe TP, Feng J, Schilling S, Seto E, Wang XF. Trefoil factor 1 acts to suppress senescence induced by oncogene activation during the cellular transformation process. *Proc Natl Acad Sci U S A*. 2011;108:6591-6596.

31. Buache E, Etique N, Alpy F, et al. Deficiency in trefoil factor 1 (TFF1) increases tumorigenicity of human breast cancer cells and mammary tumor development in TFF1-knockout mice. *Oncogene*. 2011;30:3261-3273.

**SUPPORTING INFORMATION**

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

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