Diagnostic utility of trefoil factor families for the early detection of lung cancer and their correlation with tissue expression

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Abstract. Trefoil factors (TFFs) are upregulated in numerous types of cancer, including those of the breast, the colon, the lung and the pancreas, suggesting their potential utility as biomarkers for screening. In the present study, the clinical relevance of serum or urinary TFFs as biomarkers were comprehensively evaluated and the correlation with TFF expression levels in lung cancer tissue was examined. Serum and urine were collected from 199 patients with lung cancer and 198 healthy individuals. Concentrations of serum and urinary TFF1, TFF2 and TFF3 were measured using ELISA and the potential of TFF levels to discriminate between cancer and non-cancer samples was evaluated. In 100 of the cancer cases, expression of TFF1-3 was analyzed using immunohistochemical staining of paraffin sections. Furthermore, the relationship between TFF levels and clinicopathological factors among these cancer cases was analyzed using immunohistochemistry of tissue specimens, quantified and statistically analyzed. While serum levels of all TFFs measured using ELISA were significantly higher in patients with lung cancer compared with those in healthy individuals, urinary TFFs were lower. Areas under the curve (AUC) of the receiver operating characteristic curves for serum/urinary TFF1, TFF2 and TFF3 were 0.709/0.594, 0.722/0.501 and 0.663/0.665, respectively. Furthermore, the combination of serum TFF1, TFF2, TFF3 and urinary TFF1 and TFF3 demonstrated the highest AUC (0.826). In the clinicopathological analysis, serum TFF1 was higher in the early pathological T-stage (pTis/1/2) compared with the later stage (pT3/4) and TFF2 was higher in the pN0/1 than the pN2 group. With regards to the histological types, urinary TFF1 was higher in squamous cell carcinoma than adenocarcinoma (AC), but TFF2 tended to be higher in AC. Using immunohistochemical analysis, although TFF1 and TFF3 expression showed positive correlation with serum concentrations, TFF2 was inversely correlated. In conclusion, serum and urinary TFF levels are promising predictive biomarkers, and their measurements provide a useful *in vivo* and non-invasive diagnostic screening tool. In particular, TFF1 and TFF3 could be surrogate markers of clinicopathological profiles of human lung cancer.

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Abbreviations: TFF, trefoil factor; AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; SmCC, small cell carcinoma

Key words: TFF, lung carcinoma, ELISA, biomarker, immunohistochemistry

Introduction

Lung cancer is one of the most common malignancies worldwide and remains the leading cause of cancer-related death (1). The incidence of lung cancer is still increasing in the world, and screening by chest X-ray and computed tomography (CT) has shown a high success rate in diagnosis. Recent advances in targeted therapy for lung cancer have shown benefits for patients with adenocarcinoma (AC) who harbor mutations in driver genes. However, less satisfactory results have been obtained for more complex cases, such as drug-resistant cases, and treatment options for non-AC cases are limited (2,3). Therefore, the overall cure and survival rates in patients with advanced-stage lung cancer remain low (22% 5-year overall

survival rate) (4). This compels researchers to find more effective and useful biomarkers for diagnosis, prognosis and further clarification of the unknown pathological aspects of lung cancer. Ideally, the identification and better characterization of more easily accessible and cost-effective biomarkers could revolutionize the diagnoses and therapies for these types of cancer.

Proteins secreted by cancer cells are potentially useful candidates for circulating tumor markers. Although assays, predominantly ELISA, evaluating a number of clinically relevant proteins in serum have been established, several of which have been reported to be useful in the diagnosis and monitoring of advanced cancers, such as CEA, NSE and CYFRA21-1 (5-7), these assays have not been suited to use for mass screening or diagnosis in the early stages of cancer, due to their relatively low sensitivity/specificity and high costs.

The trefoil factor (TFF) family, which comprises gastric peptide pS2/TFF1, spasmolytic peptide/TFF2 and intestinal trefoil factor/TFF3, are expressed and secreted in almost all mucus-secreting cells in mucosal surfaces, predominantly those of the gastrointestinal tract (8,9). TFFs are small (7-12 kDa) protease-resistant proteins with a conserved 'trefoil' domain, which contains 3 disulfide bonds that provide functional stability (8,10). TFFs can be detected as monomeric, homo-dimeric or hetero-dimeric forms in secretions and serve an essential role in epithelial restitution and mucosal protection (8,10).

Individual TFFs have been characterized as follows. *In vitro* experiments using cultured cells have reported that TFF1 serves a pivotal role in enhanced growth, survival, migration and invasion of pancreatic and colonic cancer cells (11-13). Similarly, overexpression of TFF3 has been reported to drive proliferation, migration and invasion, and the angiogenic and antiapoptotic capacities of breast and colonic cancer cells (13-16). Notably, high TFF expression levels have been reported clinically in breast (TFF1 and 3) (17), lung (TFF1 and 3) (18,19), pancreas (TFF2) (20) and colon cancers (TFF3) (21). Moreover, expression of TFF2 has been reported to be predictive of a more aggressive phenotype in gastric cancer (22,23) and TFF3 in gastric (23-25), colorectal (25,26) and breast cancer (25,27). However, loss of TFF1 is carcinogenic in mice (10,28), and the region of chromosome 21q22.3 in which the 3 TFF genes are clustered is frequently deleted in human gastric cancer (10). In cancer cell lines, exogenous TFF1 consistently reduced cell proliferation by delaying the G1 to S phase transition in human colonic and gastric carcinoma cells (29). Similarly, in a previous study, it was shown that TFF1 can suppress cell proliferation, survival, migration and invasion in lung carcinoma cells (30). Notably, the expression of TFF1 is also predictive of improved survival time for patients with breast cancer (27,31). Moreover, TFF3 was also reported to inhibit the growth of colorectal cancer cells, in a cell-type dependent manner (32). In clinical specimens, TFF3 expression was reported to be lower in colorectal cancer compared with normal tissue (33). These observations indicate that TFF1 and TFF3 can also function as tumor suppressor-like proteins.

Serum TFFs (sTFFs) have been sporadically reported as possible biomarkers in cancer screening: TFF1 and TFF3 for breast (17), gastric (34,35), colorectal (36) and endometrial cancer (37), and all TFFs for prostate cancer (38). Moreover, sTFF1 and sTFF3 have been described as possible prognostic markers in endometrial (37), gastric (39) and colorectal cancer (40,41). However, the utility of TFF3, as suggested by its high serum concentration in patients with lung cancer regardless of histological type (42), has yet to be established as a clinical diagnostic tool. Moreover, the potential correlation between serum and/or urinary TFFs (uTFFs) and tissue expression has yet to be assessed.

To extend previously reported *in vitro* analysis (30) into potential *in vivo* clinical analyses, a comprehensive survey of all TFFs in both serum and urine was conducted in the present study. uTFF analysis was of particular interest as this is a completely non-invasive diagnostic method and is potentially suitable for mass screening. Overall data were evaluated clinicopathologically and compared to protein expression levels of TFFs in cancer tissues.

Materials and methods

Patients. Patients' clinicopathological profiles, including tumor stage by TNM classification (43) were presented in Table I and further details were presented in Table SI. The study population consisted of 199 patients according to the following inclusion criteria; those with lung cancer, without history of malignant tumor nor history of other malignant tumor, who had undergone surgery at the Saitama Medical Center at Jichi Medical University (Saitama, Japan) from January 2017 to Jul 2019. These patients included of 142 AC, 45 squamous cell carcinoma (SCC), 4 large cell carcinoma (LCC), 4 small cell carcinoma (SmCC) and 4 pleomorphic carcinoma cases. Serum and urine were collected from 198 healthy individuals (Table SII), whose ages (range, 38-75 years old; mean, 67.5 years old) and sex (127 males and 71 females) were matched as closely as possible with the patients with lung cancer (range, 32-93 years old; mean, 69.3 years old; 128 males and 71 females). Patients or healthy individuals were excluded from the study if they had a history of malignancy, pre-operative chemotherapy and/or diabetes mellitus. Individuals with a *Helicobacter pylori* infection were also excluded from the study as this infection has been reported to enhance sTFF levels (35).

All patients and healthy individuals were confirmed to have normal urinary creatinine levels, and all uTFFs were normalized to urinary creatinine to avoid variations in urinary protein concentration.

ELISA. A total of 0.5 ml of serum and 2 ml of morning urine were collected from each patient after overnight fasting. The samples were centrifuged at 5,000 x g for 15 min at 0˚C and the supernatant collected. All serum and urine samples were frozen at -80˚C before use. Polyclonal anti-sera were raised in our laboratory, in rabbits immunized with recombinant human TFF1, TFF2 and TFF3 which had also been prepared in our laboratory as previously described (32). The IgG fraction was affinity purified using protein A into a concentrated solution of 1 mg/ml. Each antibody solution was diluted with PBS to a 1/200 working solution and was coated onto 96-well microtiter plates. Concentrations of serum or urine TFF1, TFF2 and TFF3 were measured using these in-house ELISA plates

Table I. Patients and tumor characteristics.

Characteristics	ELISA Patients (n=199)	ELISA Control (n=198)	IHC Patients (n=100)
Sex, n			
Male	128	127	65
Female	71	71	35
Age, years			
<70, n	95	95	47
>70, n	104	103	53
Range	32-93	38-75	32-93
Mean, median	69.3, 71	67.5, 71	68.6, 70
Histology, n			
AC	142		73
SCC	45		23
LCC	4		1
SmCC	4		2
Pleomorphic	4		1
pT factor, n			
Tis	6		2
T1mi	19		12
T1a	30		15
T1b	37		18
T1c	16		6
T2a	42		22
T2b	18		9
T3	20		10
T4	11		6
pN factor, n			
N0	148		70
N1	23		12
N2	28		18
pStage, n			
I	125		63
II	41		14
IIIa	33		23

AC, Adenocarcinoma; LCC, large cell carcinoma; IHC, immunohistochemistry; SCC, squamous cell carcinoma; SmCC, small cell carcinoma; pT, tumor stage; pN, node stage.

generated by coating with antibodies against TFF1, TFF2 or TFF3, as previously reported (17,35). Detection sensitivities were 7.0 pg/ml for TFF1, 30.0 pg/ml for TFF2 and 30.0 pg/ml for TFF3. It has been previously reported that each TFF antibody reacted specifically and no cross reactivity with other TFFs was reported (17,35). Appropriate sample dilutions were determined to place samples within the linear range of the calibration curve, generated using recombinant human TFF1, 2 or 3 as previously described (35). Absorbance at 450 nm was measured on a DTX-880 plate reader (Beckman Coulter, Inc.) and sample TFF concentrations were calculated from the calibration curves. There was no difference in the detection quality between serum and urine TFFs.

Tissues and immunohistochemistry (IHC). Of the 199 samples of primary lung carcinoma obtained by surgery in the Saitama Medical Center, 100 samples which had been preserved and fixed at a high quality, and demonstrated no necrosis or degeneration and positive staining in bronchial gland cells, were prepared for IHC by paraffin section. These 100 samples consisted of 73 AC, 23 SCC, 1 LCC, 2 SmCC and 1 pleomorphic carcinoma case (Table I). Tissues from pathological lesions, together with adjacent normal tissues were fixed in 10% formaldehyde at room temperature for 24 h. Tissues were sliced into small pieces, embedded in paraffin and cut into 3.5 μm sections. Heat-induced epitope was performed using PT Link (cat. no. PT100/PT101; Agilent Technologies, Inc.) and EnVision^{TM} FLEX Target Retrieval Solution, High pH (pH 9.0; cat. no. S2367; Agilent Technologies, Inc.) for TFF1 and TFF3, and Low pH (pH 6.0; cat. no. #S2031; Agilent Technologies, Inc.) for TFF-2 at 95˚C for 30 min. As the antibodies used for ELISA did not react in paraffin-embedded tissues, commercialized primary antibodies as follows were used: Anti-TFF1 rabbit polyclonal (1:350; cat. no. PA5-31863; Invitrogen; Thermo Fisher Scientific, Inc.), anti-TFF2, rabbit polyclonal (1:50; cat. no. ab131147; Abcam) and anti-TFF3 rabbit monoclonal (1:300; cat. no. ab108599; Abcam). Sections were incubated with the blocking solution included in the kit for 20 min at room temperature, with primary antibodies at 4˚C overnight and with secondary antibodies for 20 min at room temperature and visualization was performed with a Catalyzed Signal Amplification System II kit which included secondary antibodies (cat. no. K1497 and K1501; Agilent Technologies, Inc.). A total of 5 high magnification fields of view were randomly selected from each section and 200 cells were counted per field. Images were digitally captured using a BX51 bright-field microscope (Olympus Corporation) equipped with a DP-72 digital camera in conjunction with the cellSens imaging software (version 1.18, Olympus Corporation). Adobe Photoshop was used for image processing (CS4 Extended; Adobe Systems Europe, Ltd.)

IHC score was determined semi-quantitatively by multiplication of the 'positive fraction' with the 'intensity-score' according to the following system. Firstly, the positive fraction was sub-divided as follows: i) 0, no staining; ii) 1, <10% staining; iii) 2, 10-50% staining; and iv) 3, >50% staining of cells with intensity score >0. Secondly, intensity-score was determined as follows: i) 0 if there was no staining or staining was weaker than that in non-neoplastic cells; ii) 1 if the staining was the same as in non-neoplastic cells; and iii) 2 if there was more intense staining than in the non-neoplastic cells. An IHC score >0 was defined as 'positive' (44). Staining was evaluated by two observers and discordance was resolved by discussion.

Statistical analysis. Statistical analyses were performed using the JMP software package (version 11; SAS Institute, Inc.) and StatMate IV (GraphPad Software; Dotmatics). Sufficient sample sizes were confirmed and statistics were calculated with a 0.80 power at the 0.05α level using EZR 1.54 (https://www.softpedia.com/get/Science-CAD/EZR.shtml).

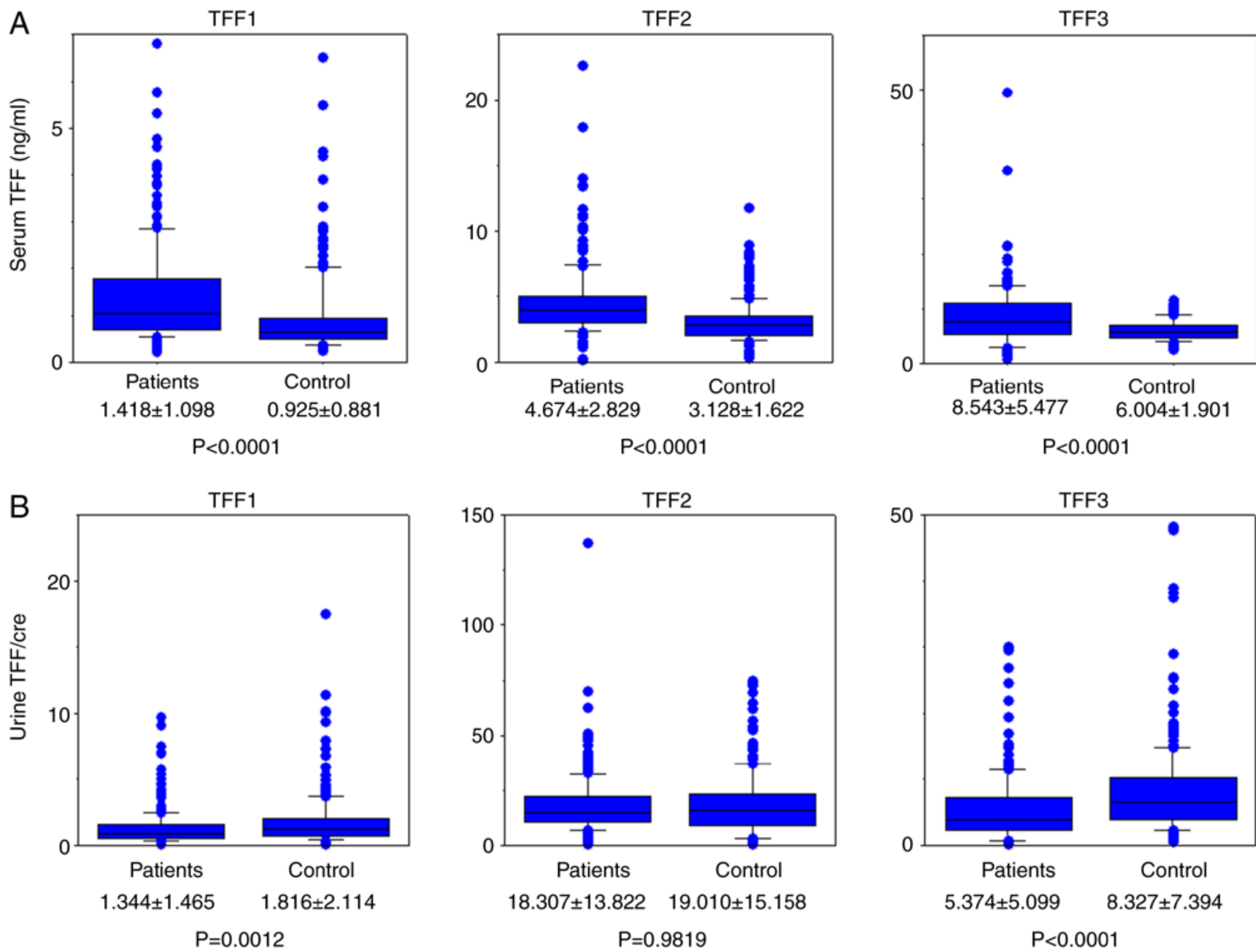


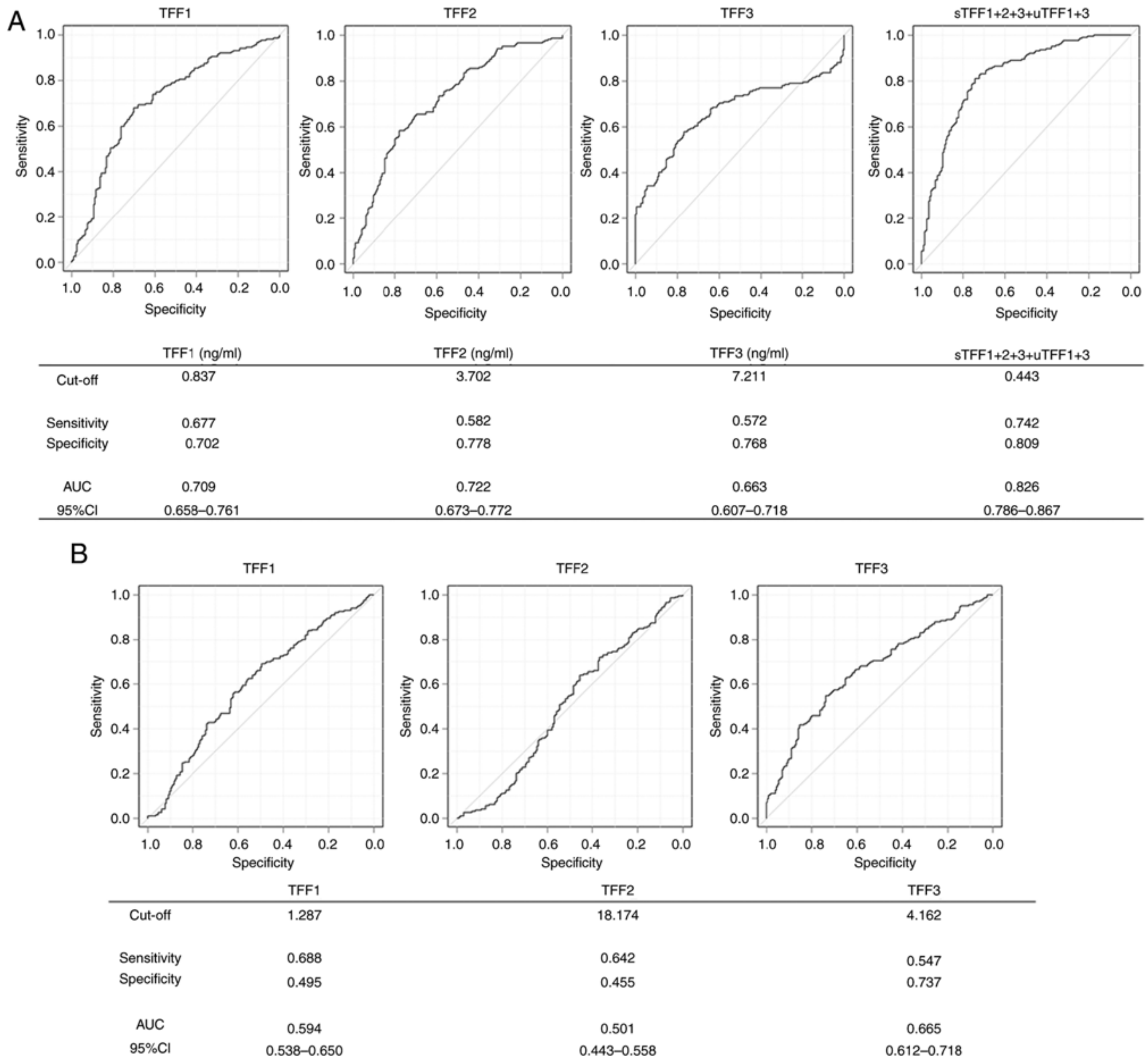
Figure 1. Box-and-whisker plots of serum and urinary levels of TFFs in patients with lung cancer and healthy individuals. Samples were collected from 199 patients with lung cancer and 198 healthy individuals. (A) Mean concentration of all serum TFFs was elevated in patients with carcinomas compared with healthy individuals (control) (P<0.0001). (B) In patients with carcinomas, mean urinary TFF1 and TFF3 values normalized to urinary creatinine were significantly lower compared to healthy individuals (control), while that for TFF2 was not. Data are presented as mean ± standard deviation. cre, creatinine; TFF, trefoil factor.

The Receiver operating characteristics (ROC) sensitivity and specificity of serum or urine TFFs were plotted and the figures were generated using EZR 1.54 (https://www.softpedia.com/get/Science-CAD/EZR.shtml). Area under the curve (AUC) was calculated to determine the marker cut-off points and the discriminatory potential of each TFF, as well as each combination of TFFs. Logistic regression modeling was used to estimate the odds ratio with 95% confidence interval (CI) to construct a final composite score. Validation of analysis was performed using Fisher's exact test in all sample groups, including those from patients with lung cancer and from healthy individuals, with the cut-off value (threshold) showing the highest AUC (combination of sTFF1/2/3 + uTFF1/3). Observers' agreement in the evaluation of IHC results was analyzed using κ statistics as follows: i) 0, poor agreement; ii) 0<κ<0.2, slight agreement; iii) 0.2<κ<0.4, fair agreement; iv) 0.4<κ<0.6, moderate agreement; v) 0.6<κ<0.8, substantial agreement; and vi) 0.8<κ<1.0, almost perfect agreement. The Spearman's rank correlation coefficient (ρ) was used as a measure of correlation between levels of each TFF assessed using ELISA or between IHC score and TFFs levels. Results were categorized as follows: i) no correlation, ρ=0; ii) equivocal, ρ<0.2; iii) low, 0.2<ρ<0.4; iv) substantial, 0.4<ρ<0.7; v) high, 0.7<ρ<1.0; and vi) complete, ρ=1.0. The difference in TFF levels among clinicopathological variables were analyzed using Mann-Whitney U test between two groups or Kruskal-Wallis test followed by Dunn's test among multiple groups. All tests were two-sided and P<0.05 was considered to indicate a statistically significant difference.

Results

Serum and urine TFFs. Concentrations of all TFFs in serum and urine were measured using ELISA. All raw results from patients with lung cancer and from healthy individuals are presented in Tables SI and SII, respectively. sTFF1, sTFF2 and sTFF3 concentrations in patients with lung cancer were all significantly higher compared with those in healthy individuals (P<0.0001; Fig. 1A). uTFF1 and uTFF3 values normalized using the creatinine level were lower in patients with lung cancer (P=0.0012 and P<0.0001, respectively; Fig. 1B).

Based on the results of upregulated sTFFs and downregulated uTFFs in patients with lung cancer, the precise diagnostic potential of either source alone or in combination was explored



	TFF1 (ng/ml)	TFF2 (ng/ml)	TFF3 (ng/ml)	sTFF1+2+3+uTFF1+3
Cut-off	0.837	3.702	7.211	0.443
Sensitivity	0.677	0.582	0.572	0.742
Specificity	0.702	0.778	0.768	0.809
AUC	0.709	0.722	0.663	0.826
95%CI	0.658–0.761	0.673–0.772	0.607–0.718	0.786–0.867

	TFF1	TFF2	TFF3
Cut-off	1.287	18.174	4.162
Sensitivity	0.688	0.642	0.547
Specificity	0.495	0.455	0.737
AUC	0.594	0.501	0.665
95%CI	0.538–0.650	0.443–0.558	0.612–0.718

Figure 2. ROC curves. ROC curves were generated for each TFF and the sTFF1 + 2 + 3 + uTFF1 + 3 combination. Cut-off values and their sensitivity/specificity, as well as AUC and 95% CI are indicated in tables below the curves. (A) sTFFs and the combination of sTFF1 + 2 + 3 and uTFF1 + 3. (B) uTFFs. AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic; sTFF, serum trefoil factor; uTFF, urine TFF.

to evaluate how well these levels in patients with lung cancer or healthy individuals could be differentiated. The ability to detect and segregate patients with lung cancer by ROC analysis was assessed, which generated a graphical plot of the sensitivity vs. specificity of TFF levels. AUC and sensitivity or specificity at the optimal cut-offs are presented in Fig. 2. For serum, the AUC/cut-off values for TFF1, TFF2 and TFF3 were 0.709/0.837, 0.722/3.702 and 0.663/7.211 ng/ml, respectively (Fig. 2A). With these cut-off values, the sensitivity/specificity for discriminating between patients with lung cancer and healthy individuals were calculated as 0.677/0.702 in TFF1, 0.582/0.778 in TFF2 and 0.572/0.768 in TFF3. For urine, the AUC/cut-off values for TFF1, TFF2 and TFF3 were 0.594/1.287, 0.501/18.174 and 0.665/4.162, respectively (Fig. 2B). The resultant sensitivity/specificity values were 0.688/0.495 for TFF1, 0.642/0.455 for TFF2 and 0.547/0.737 for TFF3.

The diagnostic potential of TFF data in combination was also evaluated. Among the AUCs calculated, the highest value was for the combination of sTFF1 + 2 + 3 with uTFF1 + 3, which was 0.826 with a cut-off value of 0.443 (Fig. 2A). Therefore, while any single TFF discriminated patients with lung cancer, this combination of TFFs demonstrated an improved diagnostic performance.

Validation of TFFs for their potential in diagnostic screening. Instead of dividing the clinical sample groups into training and validation sets, the utility of TFFs as a biomarker for baseline screening was evaluated in all 397 cases of both patients with lung cancer and healthy individuals (Tables SI and SII). A cut-off value of 0.443 was used, which showed the highest AUC by the combination of sTFF1 + 2 + 3 and uTFF1 + 3. Cases were divided into 4 categories as shown in the cross-tabulation table (Table II) and the differences were statistically analyzed.

Table II. Case-distribution of all participants separated with the cut-off (0.443) by the combination of sTFF1 + 2 + 3 with uTFF1 + 3 (n=397).

Patients	>cut-off	<cut-off	Statistical result
Patients with lung cancer (n=199)	160	39	Sensitivity=0.804
Healthy individuals (n=198)	51	147	Specificity=0.742
Statistical result	Positive predictive value=0.758	Negative predictive value=0.790	P=7.09x10^{-29}

Statistical analysis was performed using Fisher's exact test.

With this cut-off value, the sensitivity and the specificity of patients with lung cancer and healthy individuals were 0.804 and 0.742, respectively, and the positive and negative predictive values were 0.758 and 0.790, respectively. The difference was determined to be statistically significant using Fisher's exact test (P<0.0001).

Expression of TFFs by IHC. In parallel to the ELISA, TFF expression by IHC was also analyzed. Inter-observer agreement in the evaluation of staining results ranged from 'substantial agreement' to 'almost perfect agreement' (TFF1, κ=0.837; TFF2, κ=0.791; TFF3, κ=0.830). Representative IHC results are presented in Fig. 3, with staining results detailed in Table III, and the results of individual patients presented in Table SIII. All TFFs were expressed in the cytoplasm of bronchial glands and occasionally in bronchial epithelial cells, but not in the alveolar epithelial cells or interstitial cells in normal tissue. Among the 100 cancer cases analyzed, TFF1, TFF2 and TFF3 were positively stained in 41 (41.0%), 37 (37.0%) and 50 cases (50.0%), respectively. TFF1 was expressed in the cytoplasm of 29/73 (39.7%) and 12/23 (52.2%) cases of AC and SCC, respectively, but in no cases of SmCC, LCC or pleomorphic carcinoma. IHC scores of TFF1 staining were categorized into the following results: score 6, 3 cases; score 4, 8 cases; score 3, 10 cases; score 2, 13 cases; score 1, 7 cases; score 0, 59 cases. TFF2 was expressed in the cytoplasm of 25/73 (34.2%), 10/23 (43.5%), 2/2 (100%) and 0/2 cases (0%) of AC, SCC, SmCC and others (pleomorphic carcinoma and large cell carcinoma), respectively. TFF3 was expressed in the cytoplasm of 38/73 (52.1%), 12/23 (52.2%) cases of AC and SCC, respectively and none (0/4) of others (Table III). Overall, 83 out of 100 (83.0%) cases were positive for at least one of TFF1, TFF2 and TFF3 when assessed by IHC staining. There were no specific microscopic staining patterns, such as co-expression or reciprocal expression, among these three proteins as a whole.

Correlation between sTFFs and uTFFs. Correlations between serum and urine TFFs levels were determined using Spearman's rank correlation test. As shown in Table IV, the correlation coefficient (ρ) was generally higher between sTFF1 and sTFF2 in both the patients with lung cancer and the healthy individuals. The ρ between sTFF1 and sTFF2 was 0.494 in patients and 0.473 in healthy individuals (deemed 'substantial correlation'; both P<0.001). Furthermore, there was a significant positive correlation between sTFF1 and sTFF2 in all groups of pT pathological tumor stage (pT) or pathological nodal stage (pN) categories (except pT4) as well as in AC, which demonstrated ρ-values ranging from 0.448-0.717. Most of the ρ-values were within the range of 'substantial correlation', while the pN1 group had a score of 0.717, which was categorized as a 'high correlation'. In healthy individuals, all uTFF1, uTFF2 and uTFF3 values were correlated with each other in contrast to the patients with lung cancer. In the patients with lung cancer, the positive correlation between TFF1 and TFF2 was weaker in urine and the ρ-values between uTFF1 and uTFF2 were within the range of 'substantial correlation' in pT2, pT4 (marginally) and pN1-2 groups. uTFF1 and uTFF3 showed 'high correlation' in pT4 and 'substantial correlation' in pN1/2 groups of lung cancer cases, but uTFF2 and uTFF3 correlated only in pT4 with ρ=0.736, although it indicated a 'high correlation'. Between sTFF and uTFF, a 'substantial correlation' was observed for all TFFs (TFF1, 0.516; TFF2, 0.463; TFF3, 0.560; P<0.0001 for all; Table V). No inverse correlation was found between each sTFF or uTFF.

Correlation of sTFFs and uTFFs with IHC scores. The correlations between sTFF and uTFF levels and IHC-determined TFF expression levels in lung cancer tissues were examined, the results of which are presented in Table V. A statistically significant correlation between sTFF1-3 levels and IHC scores was demonstrated. Using Spearman's correlation test, sTFF1 and sTFF3 showed ρ-values of 0.563 and 0.813 with the IHC scores, respectively ('substantial' or 'high correlation', respectively; P<0.0001), whereas TFF2 had a ρ-value of -0.657 ('substantial inverse correlation'; P<0.0001). Moreover, analysis using Kruskal-Wallis test followed by Dunn's test, groups with a higher IHC score generally showed higher sTFF1 and sTFF3 at statistically significant levels (P<0.0001; Table VI). However, a significant inverse association was confirmed for sTFF2. Analysis of urine data using Spearman's test, demonstrated no correlation with IHC score (TFF1, ρ=0.197; TFF2, ρ=-0.281; TFF3, ρ=0.342; Table V).

Association of sTFFs and uTFFs with clinicopathological features. Next, sTFF and uTFF levels were compared with clinicopathological variables (Tables VI and VII, respectively). In the pT category, the pTis/1/2 group showed a higher sTFF2 mean value than the pT3/4 group, at a statistically significant level (P=0.018). Furthermore, the pN0/1 group had a higher mean sTFF1 value than the pN2 group, at a statistically significant level (P=0.033). Histologically, although SCC cases had a significantly higher mean uTFF1 value than AC (P=0.027), AC had a higher mean level of uTFF2,

Table III. Results of immunohistochemical staining (n=100).

	TFF1						TFF2						TFF3					
IHC score	0	1	2	3	4	6	0	1	2	3	4	6	0	1	2	3	4	6
Total number of cases	59	7	13	10	8	3	63	5	16	7	6	3	50	11	20	4	6	9
Histological type (cases), n																		
AC (73)	44	5	11	6	5	2	48	4	9	6	5	1	35	9	15	3	6	5
SCC (23)	11	2	2	4	3	1	13	1	5	1	1	2	11	2	5	1	0	4
LCC (1)	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
SmCC (2)	2	0	0	0	0	0	0	0	2	0	0	0	2	0	0	0	0	0
Pleomorphic (1)	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0

TFF, trefoil factor; IHC score, immunohistochemical score; AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; SmCC, small cell carcinoma.

but not at the level of statistical significance (P=0.062). No significant difference in TFF levels was demonstrated for any other factors.

Discussion

The identification of novel diagnostic markers is valuable not only because it enables the early detection of disease, but also as it may allow post-operative or post-chemotherapeutic monitoring of patients.

The involvement of TFFs in cancer has been previously described, and their normal biological functions have been shown to be quite diverse. Aberrant expression of TFFs, in particular, of TFF1 and TFF3, has been observed in numerous types of clinical cancer, including those of the breast and the lung (8,45). Furthermore, higher expression of TFF1 and/or TFF3 has been reported to be associated with chemoresistance (46,47), lymph node/distant metastasis, high Tumor-Node-Metastasis stage and a poor prognosis in colorectal (13,48), gastric (24) and endometrial cancer (37).

sTFFs have been sporadically reported as cancer biomarkers in the literature (17,34,36-38,49,50). In particular, sTFF1 and sTFF3 have been described as predictive markers for lymph node metastasis and for worse prognosis in prostatic (38), gastric (39) and colorectal cancer (46,48,51). Consistently, decreased sTFF3 levels were significantly associated with positive responses to chemotherapy in both gastric and colorectal cancer (52).

In lung cancer, sTFF1 has been described as a marker of AC, and the level of sTFF1 was reported to have normalized following the removal of tumors in a small sample group, although these values did not reach levels of significance as a screening tool for tumors (53). However, sTFF3 has been reported to be elevated in all histological types of lung cancer (42). Overall, as there have not been many reports on the involvement of TFFs in lung cancer, conclusions are somewhat equivocal. In particular, changes in sTFF and uTFF levels have not previously been investigated in precise detail. Therefore, the utility of TFFs in diagnosing lung cancer is still controversial. In the present study, the utility of TFFs as serum or urinary biomarkers were evaluated with additional analyses of the relationship between TFF expression in lung cancer tissue and clinicopathological factors.

Firstly, all sTFF levels were significantly higher in patients with lung cancer (P<0.0001), while uTFFs were lower. In the latter, the results were statistically significant for uTFF1 and 3, but not for uTFF2. Using ROC analysis, TFFs successfully discriminated patients with lung cancer from healthy individuals and an analysis of combined sTFFs and uTFFs gave the highest AUC of 0.826. Currently established markers for lung cancer have shown satisfactorily high AUCs, with carcinoembryonic antigen (CEA) providing an AUC up to 0.850 in AC (5), CYFRA21-1 and 'SCC-antigen' providing AUCs up to 0.930 and 0.780, respectively in SCC (5), and neuron-specific enolase (NSE) and pro-gastrin-releasing peptide providing AUCs up to 0.890 and 0.950, respectively in SmCC (7). However, the AUCs for these markers evaluated against all histological types as a whole were far lower. The combination of CYFRA/CEA/NSE/'SCC-antigen' for combined lung cancer cases demonstrated an AUC of 0.854 compared with non-cancer cases (6). the AUC obtained in the present study by combining 5 values from 3 proteins, 2 of which were from urine, was equivalent to that value regardless of histological type. Indeed, the screening and diagnostic potential of sTFF1 + 2 + 3 with uTFF1 + 3 was verified in this sample group, when assessed as a whole (Table II).

As another detection tool, low-dose helical CT (LDCT) is a sensitive screening method and demonstrates high performance in the detection of pulmonary nodules and could therefore detect a high proportion of small cancers at an early stage in baseline screening. For the detection of cancer, the highest reported sensitivity was 96%, but with the lowest sensitivity at 49% (54). However, the highest reported specificity was 95%, but with the lowest sensitivity at 55% (55) for the detection of cancer among all pulmonary nodules, including non-neoplastic lesions. The sensitivity/specificity in the ELISA system used in the present study was 74.2/80.9%; both values being in the middle of those previously reported highest and lowest scores. Moreover, LCDT has a consistently high false-positive rate (≤98%) in single baseline examination, depending on the entry and imaging diagnostic criteria (56). For example, inflammatory scarring,

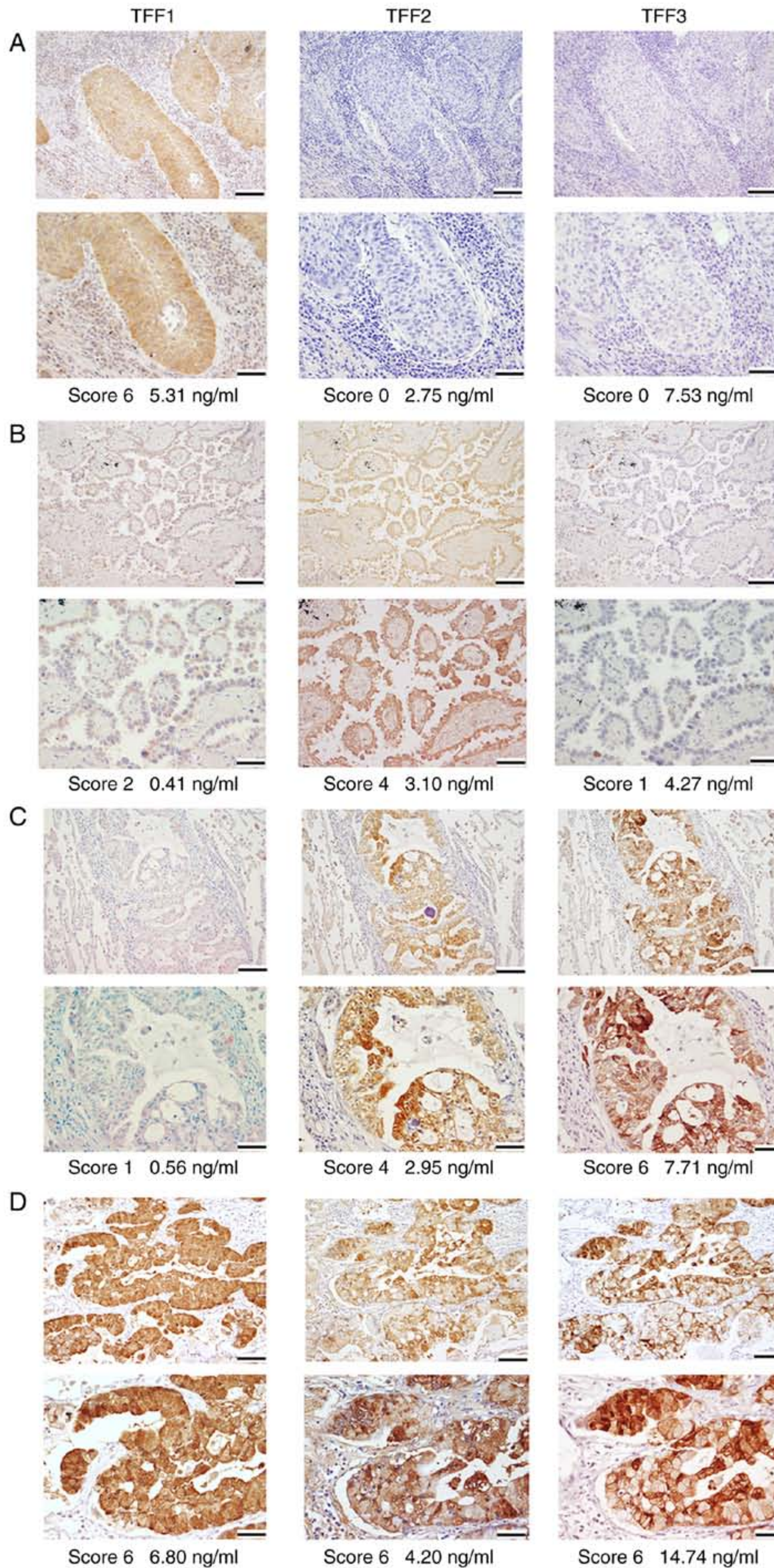


Figure 3. TFF immunohistochemical staining of patient tissues. Representative TFF1, TFF2 and TFF3 staining, immunohistochemical score and mean concentrations of serum TFF. (A) Squamous cell carcinoma, (B-D) Adenocarcinoma. In each panel, the upper row presents the lower power view, scale bar=200 mm, and the lower row presents the higher power view of a portion of the lower power view, scale bar=100 mm. TFF, trefoil factor.

Table IV. Correlation coefficients (ρ) between each TFF and P-values analyzed by Spearman's rank correlation test.

	Serum						Urine					
	TFF1 vs. TFF2		TFF1 vs. TFF3		TFF2 vs. TFF3		TFF1 vs. TFF2		TFF1 vs. TFF3		TFF2 vs. TFF3	
Characteristic	ρ	P-value	ρ	P-value	ρ	P-value	ρ	P-value	ρ	P-value	ρ	P-value
Healthy individuals (n=198)	0.473*	<0.001	0.294	-	0.352	-	0.528*	<0.001	0.756**	<0.001	0.545*	<0.001
Patients (n=199)	0.494*	<0.001	0.348	-	0.349	-	0.345	-	0.339	-	0.162	-
pTis/T1	0.497*	<0.001	0.326	-	0.443*	<0.001	0.265	-	0.304	-	0.108	-
pT2	0.486*	<0.001	0.458*	<0.001	0.230	-	0.471*	<0.001	0.352	-	0.172	-
pT3	0.567*	0.0091	0.322	-	0.316	-	0.393	-	0.322	-	0.319	-
pT4	0.582	0.066	0.418	0.203	0.109	-	0.609*	0.052	0.718**	0.016	0.736**	0.013
pN0	0.448*	<0.001	0.289	-	0.344	-	0.283	-	0.286	-	0.120	-
pN1	0.717**	<0.001	0.707**	<0.001	0.743**	<0.001	0.592*	0.0029	0.420*	0.046	0.356	-
pN2	0.602*	<0.001	0.459*	0.014	0.273	-	0.535*	0.0033	0.484*	0.009	0.270	-
Adenocarcinoma	0.570*	<0.001	0.345	-	0.353	-	0.374	-	0.312	-	0.154	-
Squamous cell carcinoma	0.334	-	0.187	-	0.396	-	0.392	-	0.269	-	0.096	-

P-values for all groups were only indicated for categories showing ρ>0.4. *substantial correlation, **high correlation. pN, node stage; pT, tumor stage; TFF, trefoil factor.

Table V. Correlation Coefficients (ρ) between sTFF, uTFF and IHC score by Spearman's rank correlation test in patients with lung cancer.

Factor	sTFF1	sTFF2	sTFF3	IHC score
uTFF (n=199)				
ρ	0.516*	0.195	0.139	0.197
P-value[a]	<0.000	-	-	-
uTFF2 (n=199)				
ρ	0.128	0.463*	-0.008	-0.281
P-value[a]	-	<0.0001	-	-
uTFF3 (n=199)				
ρ	0.169	0.234	0.560*	0.342
P-value[a]	-	-	<0.0001	-
IHC score (n=100)				
ρ	0.563*	-0.657*	0.813**	-
P-value[a]	<0.0001	<0.0001	<0.0001	-

[a]P-values are indicated only for categories showing ρ>0.4. *substantial correlation, **high correlation. sTFF, serum trefoil factor; uTFF, urinary TFF; IHC, immunohistochemistry.

hamartoma and fungal granuloma are major factors that could contribute to a high false-positive rate (56,57). This is a major limitation of LDCT as a method for screening. Accordingly, repeated examinations are necessary with this method and in diagnoses, baseline examination must be re-evaluated by further investigation, such as follow-up with detailed (high resolution, thin section) CT, usually repeated 3-4 times over 2-4 years (54,57). Furthermore, results of randomized trials have not yet confirmed that LDCT screening has a measurable effect in decreasing mortality (56,58). Overall, the ELISA screening method utilized in the present study is more convenient and thus, has the potential to be more prognostic at baseline screening than results reported using LDCT, to date.

In the patients with lung cancer analyzed in the present study, TFF levels were higher in serum but lower in urine. It was hypothesized that TFFs may be present in a modified form in serum, including as homo- or heterodimers, and thus, are not rapidly excreted into the urine through normal turnover in

Table VI. Association between sTFF levels and clinicopathological variables.

Clinicopathological factors (n=199)	Number of cases	sTFF1		sTFF2		sTFF3	
		Mean + SD	P-value	Mean + SD	P-value	Mean + SD	P-value
Age, years							
<70, n	95	1.351±1.192	0.0764	4.472±3.058	0.0728	8.243±5.923	0.1932
>70, n	104	1.479±1.007		4.859±2.604		8.817±5.049	
Sex							
Male	128	1.396±1.003	0.7101	4.579±2.570	0.738	8.959±6.051	0.216
Female	71	1.458±1.259		4.846±3.258		7.793±5.419	
pT factor							
pTis/T1/T2	168	1.408±1.108	0.928	4.801±2.862	0.018	8.311±4.662	0.729
pT3/T4	31	1.469±1.060		3.987±2.582		9.803±8.662	
pN factor							
pN0/1	171	1.473±1.129	0.033	4.770±2.912	0.2521	8.227±4.690	0.3174
pN2	28	1.082±0.825		4.087±2.216		10.215±8.835	
pStage							
I	125	1.481±1.167	0.085	4.814±2.933	0.093	8.419±4.883	0.618
II	41	1.492±1.064		4.145±2.390		7.870±4.182	
IIIa	33	1.086±0.794		3.971±2.134		9.850±8.303	
Histological type							
Adenocarcinoma	142	1.448±1.160	0.5596	4.880±3.007	0.154	8.644±5.814	0.3939
Squamous cell carcinoma	45	1.418±0.9820		4.380±2.500		8.764±4.558	
Small cell carcinoma	4	1.241±1.043		2.996±0.906		5.809±3.558	
Others[a]	8	1.004±0.493		3.683±0.885		7.251±4.986	
IHC score	100						
0		38.746	<0.0001	63.873	<0.0001	29.460	<0.0001
1		47.000		67.600		46.727	
2		56.385		26.125		70.250	
3		75.700		22.000		77.500	
4		81.125		11.000		91.333	
6		98.667		16.667		88.889	

[a]Others are large cell carcinoma and pleomorphic carcinoma. P-values were obtained by Mann-Whitney U test for two groups and by Kruskal-Wallis test for the comparison of three or more groups. sTFF, serum trefoil factor; IHC, immunohistochemistry, pN, node stage; pT, tumor stage.

patients with lung cancer. In healthy individuals, all TFFs are present in un-modified form in serum and are rapidly excreted into the urine through normal turnover. Supporting the aforementioned hypothesis, all uTFF levels showed 'substantial' to 'high' correlation with each other in healthy individuals, i.e., all TFF were coordinately excreted. However, such a correlation was not found among patients with lung cancer as a whole (Table IV).

Secondly, the observation that sTFF2 levels were higher in the pTis/1/2 group compared with the pT3/4 group and sTFF1 was higher in the pN0/1 group compared with the pN2 group suggested that upregulation of TFF1and TFF2 was an early and transient phenomenon during tumor growth and nodal metastasis in lung carcinoma. Moreover, patients with SCC showed higher uTFF1 levels, but patients with AC showed higher uTFF2 levels. However, the significance and the mechanism of such differences depending on the histological type are unclear.

Thirdly, TFF1, TFF3 and, less intensely, TFF2 demonstrated positive IHC staining in normal bronchial epithelial cells and bronchial glands in the lung in the present study, as has been previously described (59,60). All TFFs were detected in cancer tissues of AC, SCC and SmCC, with a slightly higher frequency in SCC: i.e., 43.5-52.2% in SCC vs. 34.2-52.1% in AC and none in the other subtypes, except for SmCC in which both cases expressed TFF2 (IHC score of 2). Although AC, SCC and a minor fraction of SmCC have been reported to express TFF1 (18,61), the present study was, to the best of our knowledge, the first report of IHC expression of all TFFs in all histological types.

Fourthly, in a previous report on breast carcinoma, TFF1, but not TFF3, was reported to show a positive correlation between serum concentration and IHC staining (17). sTFF2 was lower in patients with cancer in that study, however, a statistical analysis could not be performed due to the absence

Table VII. Association between uTFF levels and clinicopathological variables.

Clinicopathological) factors (n=199	Number of cases	uTFF1		uTFF2		uTFF3	
		Mean + SD	P-value	Mean + SD	P-value	Mean + SD	P-value
Age, years							
<70	95	1.397±1.696	0.620	19.522±16.988	0.603	5.411±5.452	0.813
>70	104	1.295±1.223		17.198±10.064		5.341±4.780	
Sex							
Male	128	1.366±1.448	0.281	16.538±9.463	0.126	5.454±5.194	0.734
Female	71	1.304±1.505		21.460±19.023		5.230±4.956	
pT factor							
pTis/T1/T2	168	1.334±1.500	0.760	18.647±14.232	0.174	5.394±5.138	0.841
pT3/T4	31	1.397±1.279		16.466±11.366		5.266±4.960	
pN factor							
pN0/1	171	1.397±1.542	0.309	18.531±14.202	0.623	5.389±5.107	0.943
pN2	28	1.018±0.797		16.958±11.346		5.285±5.139	
pStage							
I	125	1.376±1.240	0.399	16.355±9.641	0.243	5.277±4.886	0.98
II	41	1.558±1.510		18.561±21.203		4.958±3.929	
IIIa	33	1.171±1.035		14.864±8.759		4.793±4.437	
Histological type							
Adenocarcinoma	142	1.273±1.503	0.027	19.512±14.974	0.062	5.483±5.172	0.444
Squamous cell carcinoma	45	1.704±1.467		16.094±10.861		5.343±5.158	
Small cell carcinoma	4	0.780±0.383		9.874±5.352		2.347±1.978	
Others[a]	8	0.971±0.786		14.395±4.235		5.509±4.914	
IHC score (n=100)							
0		45.169	-	56.302	-	41.120	-
1		65.571		48.400		55.727	
2		49.692		42.563		57.750	
3		74.000		44.857		42.750	
4		54.500		25.833		75.333	
6		34.667		37.000		67.000	

[a]Others are large cell carcinoma and pleomorphic carcinoma. P-values were obtained by Mann-Whitney U test for two groups and by Kruskal-Wallis test for the comparison of three or more groups uTFF, urinary trefoil factor; IHC, immunohistochemistry.

of TFF2-positive cases as assessed using IHC (17). However, in the present study, TFF1 and TFF3 were demonstrated to show a positive correlation between serum concentrations and IHC score, and a 'substantial' inverse correlation for TFF2 by Spearman's test. These results for TFF1 and TFF3 were also demonstrated to be statistically significant, ie, the concentration of sTFF1 and sTFF3 was higher in groups having a higher IHC score. There are numerous possible mechanisms to explain the inverse correlation between high sTFF2 and low IHC score. Aberrantly overexpressed TFF2 may be secreted into the bronchial lumen, but not into serum. For example, in well differentiated breast cancer, polarized TFF3 expression leads to secretion into the ductal lumen, but not into serum (27). Alternatively, a major fraction of elevated sTFF2 may not derive directly from cancer, but from the mucus-secreting cells of bronchial glands. Lastly, TFF2 may be exhausted in cancer cells after increased secretion into the serum even if it is aberrantly overexpressed. Collectively, sTFFs may be regulated at numerous levels in a complex manner, such as expression in the cells, secretion into the lumen or the serum and excretion into the urine.

Functionally, TFF1 in the stomach has been described as being involved in the formation of a protective mucous layer (62) and stabilization of cell junctions by binding to MUC2, MUC5AC (8,63) and gastrokine-2 (GKN2) (64). TFF2 also binds to GKN2 (64) and MUC6 (65), and maintains the inner layer of the gastric mucus (66). TFF3 is a constituent of goblet cells in both the small and large intestine, but not in the stomach, and co-localizes with MUC5B and MUC8 in bronchial gland mucous cells (10,60), and with MUC5AC in respiratory goblet cells (60,67). Therefore, in the lungs, TFFs bind with mucin from the bronchial gland, in a manner similar to the stomach, and thereby may maintain cell adhesion and support restitution. In cancer, it is not surprising that TFFs are

secreted from the bronchial glands and protect the adjacent bronchial mucosa from deleterious damage as a safe-guard mechanism. Indeed, the induction of mucinous metaplasia was reported in transgenic mice with high TFF3 expression (68). Therefore, by physical and/or chemical stimuli, TFF actively serves a role in enhancing the production of mucin, which contains its binding partners.

Previous studies suggested mutual regulation among TFFs. *TFF2*^{KO} mice were reported to have shown high TFF3 expression in the gastric antrum (69). In a rat model of colitis, TFF1 expression was elevated and TFF3 was decreased in the colon during the acute phase of disease (70), whereas the opposite pattern of expression was demonstrated in the restitution phase (70,71). Hence, a coordinated regulation of TFF expression to ensure mucosal protection and restitution depending on the stage of the disease may exist. In the present study, although sTFF1 and sTFF2 levels showed 'substantial' to 'high' correlation in patients with lung cancer as a whole or in each pTN category, concurrent relative suppression, i.e., inverse correlation, of sTFF3 was not found. Moreover, although the staining frequencies and intensities of TFF1 and TFF3 were predominant compared with TFF2, no specific pattern of staining, such as exact overlapping or reciprocal staining of each TFF, was observed.

In contrast with other cancer-specific markers, TFFs are produced not only by cancer cells, but also by non-neoplastic cells as components of tissue-protective mucin (8) or regulators of inflammation (72). Therefore, although the clinical utility of circulating and excreting TFFs as cancer biomarkers for the diagnostic screening of lung cancer may be cautiously evaluated, increased TFFs levels also reflects epithelial damage. Regardless of the underlying mechanism, results from the present study demonstrated that discrimination between patients with lung cancer and healthy individuals using TFFs was possible, and that all TFFs assessed provided unique biomarker information that could contribute to a panel of markers having significant diagnostic potential. However, a limitation of the present study was the short period of patient follow-up, and thus the long-term prognostic power of TFFs could not be precisely evaluated.

Based on the results of the present study, it is proposed that this combined serum/urine assay of TFFs represents a novel, easily monitored tool for accurately diagnosing lung cancer, with TFF1 and TFF2 levels in particular showing good correlation with clinicopathological factors. This assay may permit larger-scale cancer screening, particularly in the early stages of disease. Considering the limitations of the present work, further studies to clarify how sTFF and uTFF levels change after surgery and to clarify any correlation with relapse and metastases, as well as detailed analysis in relation to smoking history are required. If these efforts are successful, this s/uTFFs analysis may further contribute to cancer management and provide a tool to monitor the course of the disease after surgery and to evaluate the effects of adjuvant therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study could be available from the corresponding author on reasonable request.

Authors' contributions

YD and SN designed the study. KM and YD performed immunohistochemical staining and evaluated the results. KM, YI, MF and TY performed sample preparation and ELISA and contributed to the quantification. TK, HT, YO and SN collected the clinical samples. KM, TK and HT statistically analyzed the data and undertook its interpretation. YI, MF and YO established the ELISA experimental system and provided technical support. KM, YD, TK, HT and SN drafted and completed the manuscript. KM, YD and TK confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study protocol adhered to the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee in Saitama Medical Center, Jichi Medial University (Saitama, Japan; approval no. S17-035, S20-137), the University of Tokyo (Tokyo, Japan; approval no. 11414-[1,2]) and Kyoto Prefectural University of Medicine (Kyoto, Japan; approval no. ERB-C-1239-1,2). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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