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Trefoil Factor 3 as a Novel Biomarker to Distinguish Between Adenocarcinoma and Squamous Cell Carcinoma

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Abstract: In carcinoma, such as of the lung, the histological subtype is important to select an appropriate therapeutic strategy for patients. However, carcinomas with poor differentiation cannot always be distinguished on the basis of morphology alone nor on clinical findings. Hence, delineation of poorly differentiated adenocarcinoma and squamous cell carcinoma, the 2 most common epithelial-origin carcinomas, is pivotal for selection of optimum therapy. Herein, we explored the potential utility of trefoil factor 3 (TFF3) as a biomarker for primary lung adenocarcinoma and extrapulmonary adenocarcinomas derived from different organs. We observed that 90.9% of lung adenocarcinomas were TFF3-positive, whereas no expression of TFF3 was observed in squamous cell carcinomas. The subtype of lung carcinoma was confirmed by four established biomarkers, cytokeratin 7 and thyroid transcription factor 1 for adenocarcinoma and P63 and cytokeratin 5/6 for squamous cell carcinoma. Furthermore, expression of TFF3 mRNA was observed by quantitative PCR in all of 11 human lung adenocarcinoma cell lines and highly correlated with markers of the adenocarcinomatous lineage. In contrast, little or no expression of TFF3 was observed in 4 lung squamous cell carcinoma cell lines. By use of forced expression, or siRNA-mediated depletion of TFF3, we determined that TFF3 appeared to maintain rather than promote glandular differentiation of lung carcinoma cells. In addition, TFF3 expression was also determined in adenocarcinomas from colorectum, stomach, cervix, esophagus, and larynx. Among all these extrapulmonary carcinomas, 93.7% of adenocarcinomas exhibited TFF3 positivity, whereas only 2.9% of squamous cell carcinomas were TFF3-positive. Totally, 92.9% of both pulmonary and extrapulmonary adenocarcinomas exhibited TFF3 positivity, whereas only 1.5% of squamous cell carcinomas were TFF3-positive. In conclusion, TFF3 is preferentially expressed in adenocarcinoma and may function as an additional biomarker for distinguishing adenocarcinoma from squamous cell carcinoma.

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Abbreviations: ADC = adenocarcinoma, ALK = anaplastic lymphoma kinase, ASC = adenosquamous carcinoma, ATCC = the American Type Culture Collection, CgA = chromogranin A, CK = cytokeratin, EGFR = epidermal growth factor receptor, IHC = immunohistochemistry, NSCLC = non-small cell lung cancer, NUS = National University of Singapore, PBS = phosphate-buffered saline, qPCR = quantitative PCR, SCC = squamous cell carcinoma, Syn = synaptophysin, TFF3 = trefoil factor 3, TTF-1 = thyroid transcription factor 1, WHO = World Health Organization.

INTRODUCTION

Lung cancer is among the most common contributors to cancer-related deaths, being reported to account for approximately 1.4 million deaths per year worldwide.[1] Lung cancer is generally divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (approximately 85% and 15% of all lung cancer, respectively).[2] Lung adenocarcinoma (ADC) and squamous cell carcinoma (SCC) are the 2 main histological types of NSCLC confronted in the clinic.[3] Patients with lung ADC experience a worse prognosis than those with SCC.[3] In addition, different therapeutic strategies are applied for patients with diagnoses differentiating lung ADC and SCC.[4–6] Hence, accurate diagnosis not only improves therapeutic efficacy, but also avoids severe, unwarranted and/or unnecessary side effects. For example, patients with lung SCC do not receive Avastin (Bevacizumab) because of a 30% mortality rate in these patients consequent to fatal hemoptysis.[4–6] Furthermore, targeted therapies, such as epidermal growth factor receptor (EGFR or HER1) or anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors, are more effective in lung ADC than lung SCC.[5] However, distinguishing poorly differentiated lung ADC and SCC may present a challenge to the pathologist when diagnosis is solely dependent on morphological features. In advanced cases of lung carcinoma, patients do not benefit from surgical removal of the primary tumor, and a biopsy specimen is usually the only tissue available for diagnosis. Thus, the distinction between lung ADC and SCC becomes important for diagnosis, prognosis, and therapy. Currently, several biomarkers have been found to distinguish lung ADC from SCC, such as cytokeratin 7 (CK 7) and thyroid transcription factor 1 (TTF-1) for ADC and P63

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and CK5/6 for SCC.[7,8] A combination of these markers has been demonstrated to result in better differentiation of ADC and SCC for diagnosis.

The trefoil factor family of peptides contains 3 small secreted proteins as members.[9] Trefoil factor 3 (TFF3), previously designated as intestinal trefoil factor (ITF) or hP1.B, contains 59 amino acid residues with 1 trefoil domain and a molecular weight approximating 7 kDa (monomer) or 14 kDa (dimer).[10,11] TFF3 is normally localized to goblet cells of the small and large intestine[12] and to serous cells of the submandibular gland.[13] Additionally, TFF3 protein has been reported to be expressed in normal lung, colon, stomach, pancreas, trachea, spleen, liver, and uterus among other organs.[14] Recent studies have also shown that TFF3 expression is increased in carcinoma[15–18] and is involved in tumor cell growth, scattering, invasion,[15,19,20] and metastasis.[17,18]

Herein, we determined the expression of TFF3 by immunohistochemistry (IHC) in primary lung ADC, SCC, adenosquamous carcinoma (ASC), and large cell neuroendocrine carcinoma, in comparison with established biomarkers. The results were verified on human lung ADC and SCC cell lines. Moreover, TFF3 expression was also determined in other common extrapulmonary ADC and SCC. Our data demonstrate that TFF3 may be useful as a biomarker for differential diagnoses between ADC and SCC.

MATERIALS AND METHODS

Cell Culture and Reagents

Lung ADC cell lines, HCC-460, HCC-827, NCI-H1975, HCC-2935, HCC-4006, HCC-2279, A549, PC-14, NCI-H1299, NCI-H820, and NCI-H23, were obtained from Prof. H. Phillip Koeffler's laboratory at The Cancer Science Institute of Singapore, National University of Singapore (NUS). The ASC cell line H596 and SCC cell lines, H697 and H1270, were obtained from Dr. Richie Soong's Laboratory at The Cancer Science Institute of Singapore, NUS. The SCC cell line LC-1/sq and LC-1F were obtained from Dr. Md. Zakir Hossain's laboratory at The Cancer Science Institute of Singapore, NUS. Cell lines (ADC or SCC cell lines) not listed in the American Type Culture Collection (ATCC) were cultured in RPMI 1640 medium supplemented with 10% FBS, streptomycin (100 mg/mL), and penicillin (100 U/mL) at 37°C in a 5% CO_2 incubator. All other cell lines were cultured using ATCC-recommended conditions.[21]

Human TFF3 cDNA and siRNA plasmid constructs have been previously described.[18] Cells were transiently transfected with 1 μg of plasmid or their respective vector control using FuGENE HD (Promega, Madison, WI) for 24 hours and further assays performed.[18]

Specimens

In this study, we collected several different cohorts of human tissue specimens. For IHC, we obtained the primary tumors of 44 lung ADC, 67 lung SCC, 3 lung ASC, 3 large cell neuroendocrine carcinoma of the lung, 35 cervical ADC, 20 esophageal ADC, 29 colorectal ADC, 27 gastric ADC, 33 cervical SCC, 19 esophageal SCC, and 16 laryngeal SCC specimens from patients who underwent surgery at The Affiliated Hospitals of Anhui Medical University (Hefei, China) between 2001 and 2013. All tissues were formalin-fixed and embedded in paraffin. Patients who had been administered either chemotherapy or radiation therapy before surgery were excluded. Tumor diagnosis was defined according to the 2003 World Health Organization (WHO) classification of tumors. All tissue diagnoses were confirmed by permanent histology. The use of patient samples was approved by The Biomedical Ethics Committee of Anhui Medical University and included written informed consent from each patient.

Immunohistochemistry

Immunohistochemistry was performed on 4-μm sections using a 2-step histostaining kit (Maixin, Fuzhou, China) with a polyclonal antibody against human TFF3 (1:100 dilution) or a monoclonal antibody against human TFF3 (1:100, clone 15C6, Santa Cruz, CA) and similarly for CK7 (working solution, clone OV-TL 12/30, Maixin), TTF-1 (working solution, clone SPT24, Maixin), CK5/6 (working solution, clone D5/16B4, Maixin), P63 (working solution, clone 4A4, Maixin). The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solution. For antigen retrieval, slides were heated in a microwave oven in 0.01 mol/L sodium citrate buffer (pH 6.0) for 10 min. Slides were cooled in the same buffer and subsequently immersed in 3% hydrogen peroxide in methanol for 10 min to inhibit endogenous peroxidase activity. Slides were rinsed with phosphate-buffered saline (PBS, 3 min, 3 times), and incubated with primary antibody at 4°C overnight. The slides were subsequently rinsed in PBS as before, incubated for 20 min at 37°C with universal horseradish peroxidase secondary antibody (Maixin), rinsed in PBS as above, incubated with 3,3'-diaminobenzidine tetrahydrochloride, and counterstained with hematoxylin. Samples known to be positive for the antigen were used as controls. For negative controls, the primary antibody was replaced with PBS. Each slide was scored in a blinded manner by 2 pathologists. The overall percentage of immunostained cells on each specimen was determined according to the pattern of intracellular localization. The extent of the TFF3-specific immunostaining was determined by the percentage of cells with cytoplasmic staining. The sections were scored in a semiquantitative manner, which divided the specimens into 2 grades: tumors were defined as TFF3-positive if $>10\%$ of tumour cells were TFF3 immunoreactive, and the remainders were designated as negative.[22]

Quantitative PCR Analysis

Total cellular RNA was extracted and converted to cDNA by use of SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Singapore) as per manufacturer's instructions. Quantitative PCR (qPCR) was performed using an ABI 7700 real-time PCR system (Applied Biosystems, Foster City, CA) as previously described.[23] Briefly, multiple gene markers and an endogenous housekeeping gene (*β-ACTIN*) were used for real-time PCR analysis using the SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen). Each marker was determined in triplicate in a 384-well plate using a 2-step amplification program of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 20 s and 60°C for 30 s. At the end of the amplification, a melting curve analysis was performed, consisting of denaturation at 95°C for 1 min and re-annealing at 55°C for 1 min. Standard curves were generated from each experimental plate using serial 5-fold dilutions of untreated cDNA. The geometric mean of the cycle threshold (Ct) value for each reaction was calculated. The changes of gene expression were expressed as fold change and represented by the average of 3 experiments ($P < 0.05$). To compensate for potential differences between markers, the relative expression

of each was computed as previously described,[23] >1.5-fold change in relative expression was considered as significant. We also detected other biomarkers, to supplement the IHC on lung ADC and SCC. CK5/6 (divided into KRT5, KRT6A, and KRT6B mRNA), P63 (TP63 mRNA), CK7 (KRT7 mRNA), and TTF-1 (TTF-1 mRNA) were determined in the cell lines as described above. A list of the genes analyzed by qPCR and primer sequences is tabulated in Supplemental Digital Content 1, http://links.lww.com/MD/A268.

Statistical Analyses

All statistical analyses were performed using SPSS software system for Windows (version 19.0; SPSS, Chicago, IL). The chi-squared (χ^2) test was used to analyze the difference in the expression between ADC and SCC of lung, cervix, esophagus, and all other organs. The statistical differences among subgroups in qPCR analysis were compared using an unpaired 2-tailed Student *t* test. Spearman co-efficient was calculated to determine the correlation between the expression of TFF3 and established biomarkers. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of TFF3 Protein in Primary Lung Adeno- and Squamous Cell Carcinoma

IHC for TFF3 protein was performed on 117 human primary lung carcinomas comprised of 44 ADCs, 67 SCCs, 3 ASCs, and 3 large cell neuroendocrine carcinomas. In normal lung tissue, expression of TFF3 was observed in the epithelial ciliated cells, goblet cells, and submucosal gland cells of the respiratory airways, an observation concordant with previously published studies.[14,24] TFF3 immunoreactivity was predominantly located in the cytoplasm of these cell types. In contrast, plasma cells and other inflammatory cells (macrophage, lymphocytes, and granulocytes) were completely negative for TFF3 expression. Interestingly, TFF3 exhibited prominent immunoreactivity in the tumor cells of lung ADC. Of the 44 primary lung ADCs, 40 (90.9%) were positive for TFF3 expression, whereas TFF3 immunoreactivity was not detected in any of the 67 primary lung SCCs (Figure 1, Table 1). χ^2 Test was used to evaluate the relationships between histological types (ADC and SCC) (Table 1). Hence, a highly significant difference was observed between the expression of TFF3 in lung ADC and lung SCC ($P < 0.001$). To verify the accuracy of the histopathological diagnosis of primary lung ADC and SCC, the differentiation subtypes of all cases were confirmed by IHC with following biomarkers: CK7 and TTF-1 for ADC[8] and P63 and CK5/6 for SCC[7,8] (Figure 1). As shown in Table 1, expression of TFF3 was observed in 90.9% of ADC, which was equal to or higher in percentage than the expression of other established biomarkers (90.9% for CK7 and 81.8% for TTF-1) in ADC. Moreover, we examined TFF3 expression in 3 lung ASC samples. The tumor cells of the ADC portion were positive for TFF3, whereas cells of the SCC portion of the tumor were negative (Figure 1). As shown in Table 2, the expression of TFF3 was positively and significantly correlated to CK7 and TTF-1, the established biomarkers of lung ADC ($P < 0.001$, $r_s = 0.518$ and 0.334). In contrast, TFF3 expression was negatively correlated to P63 and CK5/6, markers of SCC ($P < 0.001$, $r_s = -0.416$ and -0.756). These results were obtained with a highly specific affinity-purified polyclonal antibody to human TFF3 and confirmed by use of a specific commercially available monoclonal antibody. The pattern of immunoreactivity for both the polyclonal and monoclonal antibodies to TFF3 was similar, but with more intense immunoreactivity observed with the polyclonal antibody as could be expected (Supplemental Digital Content 2, http://links.lww.com/MD/A269).

In addition to ADC and SCC, we examined the expression of TFF3 in 3 large cell neuroendocrine carcinomas of the lung. We observed no expression of TFF3 in large cell neuroendocrine carcinomas of the lung (Table 1).

mRNA Levels of TFF3 in Lung Adeno- and Squamous Cell Carcinoma Cell Lines

We next examined the mRNA levels of TFF3 by qPCR in 16 NSCLC cell lines, 11 of which have been reported to be derived from lung ADC,[23] 4 of which have been reported to be derived from lung SCC[21,25] and 1 of which has been reported to be derived from lung ASC.[21] To first verify the differentiation lineage of the different cell lines, we utilized a number of markers for either glandular (KRT7 and TTF-1) or squamous (KRT5, KRT6A, KRT6B, and TP63) lineages. High expression of KRT7 and TTF-1 mRNA was clustered in the 11 NSCLC cell lines identified as adenocarcinomatous and was largely not expressed in the cell lines reported to be of squamous differentiation (Figure 2A, Supplemental Digital Content 3, http://links.lww.com/MD/A268). The mRNAs of the 4 markers of the squamous lineage (KRT5, KRT6A, KRT6B, and TP63) were all highly expressed in the NSCLC lines identified to be of squamous origin (H697, H1270, LC-1/sq, and LC-1F) and adenosquamous origin (H596) (Figure 2A, Supplemental Digital Content 3, http://links.lww.com/MD/A268). KRT7 was expressed at low levels in a number of the cell lines of adenocarcinomatous origin (HCC-460, HCC-827, HCC-2279, A549, and PC-14), but all of these cell lines expressed TTF-1 (Figure 2A, Supplemental Digital Content 3, http://links.lww.com/MD/A268). Determination of TFF3 mRNA levels by qPCR revealed TFF3 mRNA expression to varying degrees in all ADC cell lines examined and in contrast to the lung SCC lines, which were negative for, or expressed extremely low levels of, TFF3 mRNA (Figure 2B). The differences in TFF3 mRNA levels between lung ADC cells and lung SCC cells were highly significant ($P < 0.001$). The mRNA levels of TFF3 were positively and significantly correlated with expression of KRT7 and TTF-1, established biomarkers of lung ADC[8] (Table 3). Concordantly, the mRNA levels of TFF3 demonstrated a highly significant negative correlation with expression of KRT5, KRT6A, KRT6B, and TP63, biomarkers for lung SCC[7,8] (Table 3).

Effects of Modulation of TFF3 Expression on Lineage-Specific Markers

We next either depleted the expression of TFF3 in NSCLC cell lines of adenocarcinomatous origin (NCI-1975 and HCC-2935) or forced the expression of TFF3 in NSCLC cell lines of squamous lineage (H1270) or adenosquamous lineage (H596) and determined the expression of the markers of glandular and squamous differentiation (Figure 3).

siRNA-mediated depletion of TFF3 expression in NCI-H1975 or HCC-2935 cells resulted in decreased mRNA levels of TTF-1 relative to their vector control cells. mRNA levels of KRT7 were slightly increased in NCI-H1975 and slightly decreased in HCC-2935 cells upon siRNA-mediated depletion of TFF3 (Figure 3A). Concomitantly, mRNA levels of the squamous markers TP63, KRT5, and KRT6B were significantly

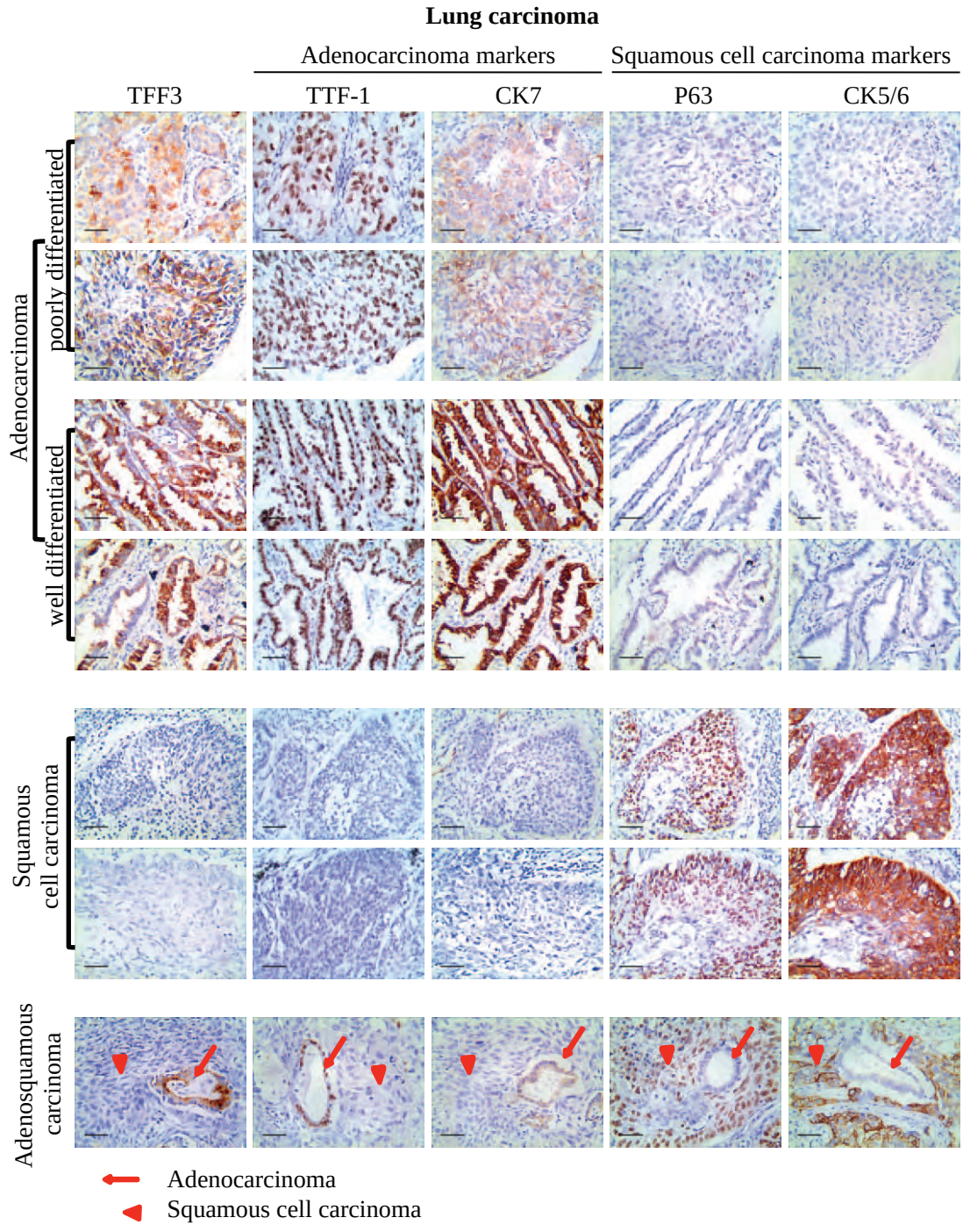

FIGURE 1. High expression of TFF3 in primary lung adenocarcinoma and no (or low) expression in primary lung squamous cell carcinoma, as determined by immunohistochemistry. Top panels: Positive expression of TFF3, TTF-1, and CK7 is observed in poorly- or well-differentiated primary lung adenocarcinoma, whereas expression of P63 and CK5/6 was not detected. IHC, magnification ×200, bars: 50 μm. Center panels: Expression of P63 and CK5/6 and lack of expression of TFF3, TTF-1, and CK7 in poorly- or well-differentiated primary lung squamous cell carcinoma. IHC, magnification ×200, bars: 50 μm. Bottom panels: primary lung adenosquamous carcinoma, which contains both adenocarcinoma (arrows) and squamous cell carcinoma (arrowheads). Note that the adenocarcinoma portion is positive for TFF3, TTF-1, and CK7, but negative for P63 and CK5/6. The squamous cell carcinoma portion is negative for TFF3, TTF-1, and CK7, but positive for P63 and CK5/6. IHC, magnification ×200, bars: 50 μm. TFF3 = trefoil factor 3, TTF-1 = thyroid transcription factor 1.

TABLE 1. Expression of TFF3, TTF-1, and CK7 in Primary Adenocarcinoma, Squamous Cell Carcinoma, and Large Cell Neuroendocrine Carcinoma of the Lung

			TFF3 Expression		TTF-1 Expression		CK7 Expression	
Tumor types	**Differentiation**	**n**	**Negative, n (%)**	**Positive, n (%)**	**Negative, n (%)**	**Positive, n (%)**	**Negative, n (%)**	**Positive, n (%)**
Adenocarcinoma	Well	2	0 (0)	2 (100)	0 (0)	2 (100)	0 (0)	2 (100)
	Moderate	32	1 (3.1)	31 (96.9)	8 (25)	24 (75)	3 (9.4)	29 (90.6)
	Poor	10	3 (30)	7 (70)	0 (0)	10 (100)	1 (10)	9 (90)
	Total	44	4 (9.1)	40 (90.9)*	8 (18.2)	36 (81.8)	4 (9.1)	40 (90.9)
Squamous cell carcinoma	Well	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	37	37 (100)	0 (0)	37 (100)	0 (0)	34 (91.9)	3 (8.1)
	Poor	30	30 (100)	0 (0)	26 (100)	4 (100)	21 (70)	9 (30)
	Total	67	67 (100)	0 (0)	63 (94)	4 (6)	55 (82.1)	12 (17.9)
Large cell neuroendocrine carcinoma		3	3 (100)	0 (0)	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)

TFF3 = trefoil factor 3, TTF-1 = thyroid transcription factor 1.
* Compared with squamous cell carcinoma, $P < 0.001$.

increased in NCI-H1975 with depleted expression of TFF3. Depleted expression of TFF3 in HCC-2935 cells increased KRT6A and KRT6B mRNA levels but not TP63 or KRT5 (Figure 3A). Conversely, there was no consistent pattern of alteration in the mRNA expression of markers of either squamous or adenocarcinomatous lineages upon forced expression of TFF3 in the squamous carcinoma cell line H1270 (Figure 3B) or the adenosquamous carcinoma cell line H596 (Figure 3C).

Expression of TFF3 Protein in Extrapulmonary Adenocarcinoma

We further wished to determine whether our observations on TFF3 expression from NSCLC were able to be applied to differentiate ADC from SCC in cancers of extrapulmonary origins. We therefore examined the expression of TFF3 in 35 cervical ADCs, 20 esophageal ADCs, 29 colorectal ADCs, 27 gastric ADCs, 33 cervical SCCs, 19 esophageal SCCs, and 16 laryngeal SCCs. As shown in Table 4, the vast majority of ADCs of extrapulmonary origins were positive for TFF3 (104/111, 93.7%). In contrast, the vast majority of the SCCs of extrapulmonary origins were negative for TFF3 (66/68, 97.1%). The differences in expression of TFF3 between ADC and SCC were highly significant ($P < 0.001$). In addition to the ADC component, the normal glandular epithelium in the esophagus, stomach, colorectum, cervix, and larynx was also positive for TFF3 (Figure 4). TFF3 expression was observed in the adjacent normal human gastrointestinal tract in mucous-secreting cells of the superficial gastric mucosa and to goblet cells of the colorectum.[26–28] TFF3 was also expressed in gland-like structures of the cervical epithelium and in the surface epithelium of the endocervix. The serous cells of the small submucosal glands in the esophagus and larynx also displayed immunoreactivity for TFF3. These results are in accordance with previously published results reporting the localization of TFF3 in normal human tissue.[14,29–32] No immunoreactivity for TFF3 protein was observed in the stratified squamous epithelium nor in squamous cell carcinoma of the cervix, esophagus, or larynx. Expression of TFF3 was observed in 93.7% of extrapulmonary ADCs examined (esophageal, gastric, colorectal, and cervical), whereas no expression of TTF-1 was observed in extrapulmonary ADC, and the expression of CK7 was observed in 51.4% of extrapulmonary ADCs (Table 4).

TABLE 2. The Correlation Between the Expression of TFF3 and CK7, TTF-1, P63, or CK5/6 in Primary Lung Carcinoma

				CK7		TTF-1		P63		CK5/6	
		n		+	−	+	−	+	−	+	−
TFF3	Adenocarcinoma	44	+	38	4*	34	8†	6	36‡	1	41§
			−	2	0	2	0	0	2	0	2
	Squamous cell carcinoma	67	+	0	0	0	0	0	0	0	0
			−	12	55	4	63	15	54	20	47

TFF3 = trefoil factor 3, TTF-1 = thyroid transcription factor 1.
* Correlated to TFF3, $P < 0.001$, $r_s = 0.518$.
† Correlated to TFF3, $P < 0.001$, $r_s = 0.334$.
‡ Correlated to TFF3, $P < 0.001$, $r_s = -0.416$.
§ Correlated to TFF3, $P < 0.001$, $r_s = -0.756$.

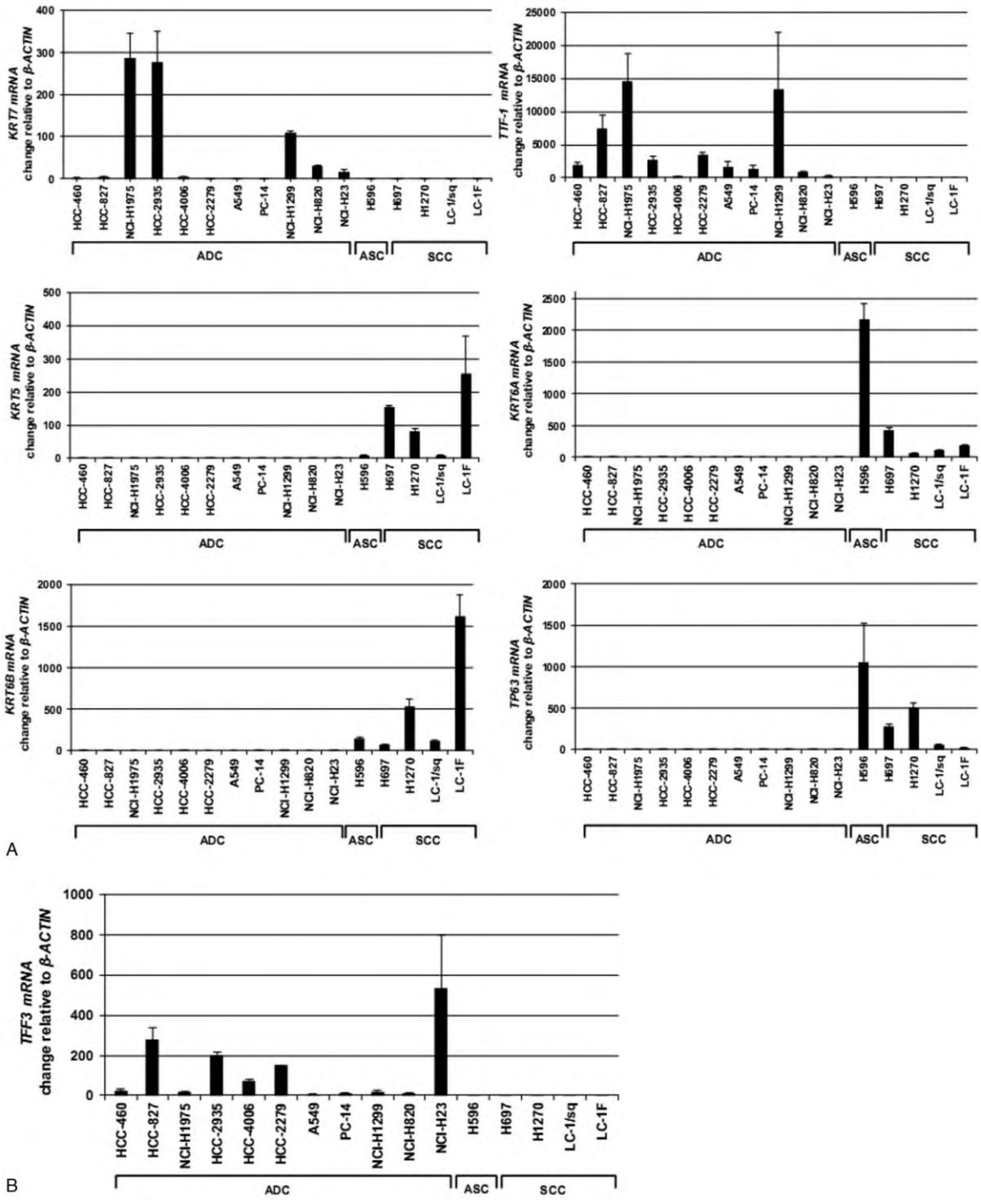

FIGURE 2. A, mRNA levels of KRT5, KRT6A, KRT6B, KRT7, TP63, and TTF-1 in lung adenocarcinoma (ADC), squamous cell carcinoma (SCC), and adenosquamous carcinoma (ASC) cell lines. B, Quantitative PCR analysis was used to evaluate the mRNA levels of TFF3 in lung carcinoma cell lines. High levels of TFF3 mRNA were observed in adenocarcinoma cell lines and low levels of TFF3 mRNA were observed in lung squamous cell carcinoma and adenosquamous cell carcinoma cell lines. Change in gene expression is expressed relative to β-ACTIN. Values are representative of 3 independent biological experiments. Columns are mean of triplicate experiments; bars ± SD.

TABLE 3. Correlation Co-efficient Analysis Between the mRNA Levels of TFF3 With Various Markers of Lung Glandular or Squamous Lineage in 11 Lung Adenocarcinoma Cell Lines

		TFF3	
	Gene	r_s	***P***
Squamous	KRT5	−0.599	0.014
	KRT6A	−0.506	0.046
	KRT6B	−0.564	0.023
	TP63	−0.478	0.063
Glandular	KRT7	0.724	0.002
	TTF-1	0.663	0.001

DISCUSSION AND CONCLUSIONS

Identification of previously unutilized, sensitive biomarkers is still a priority for improved differential diagnosis of lung ADC and SCC. Although TTF-1 and CK7 are frequently expressed in lung ADC,[8,33] and P63 and CK5/6 are frequently expressed in lung SCC,[7,8,34,35] the sensitivity of these markers remains limited. For example, the expression of TTF-1 has been reported in 73% to 84.4%, and CK7 in 90% to 97% of lung ADCs, whereas the expression of P63 was reported in 75% to 95% and CK5/6 in 75% to 100% of lung SCCs, respectively.[7,8,35–38] Herein, we evaluated the value of TFF3 expression in formalin-fixed paraffin-embedded samples for the common primary lung cancers including ADC, SCC, ASC, and large cell neuroendocrine carcinoma. Our data demonstrated that the expression of TFF3 was observed in 90.9% of lung ADCs (40/44). In comparison with established biomarkers for lung ADC, the expression rate of TFF3 was higher than that observed for TTF-1 and equal to that observed for CK7. To confirm the IHC results on clinical samples, we also determined TFF3 expression by qPCR using 11 lung ADC and 4 lung SCC cell lines. All ADC cell lines expressed TFF3, whereas almost all the SCC cell lines exhibited no or extremely low expression of TFF3. In addition, we examined the potential functional role of TFF3 in adenocarcinomatous and squamous differentiation of NSCLC by forced or depleted expression of TFF3 in either lung SCC or lung ADC cell lines, respectively. We speculate that the expression of TFF3 may be required to maintain the adenocarcinomatous lineage in NSCLC, rather than drive the de-differentiation of the squamous lineage. However, further detailed molecular and mechanistic studies of the effect of TFF3 on lineage differentiation would be required to support this notion. It should be noted that the lack of TFF3 expression in SCC does not exclude the possibility that TFF3 may participate in clinical progression of SCC. TFF3 is a secreted protein[17] and may act in an endocrine or paracrine manner distal to its site of expression. Indeed, Storesund et al[39] have demonstrated that oral keratinocytes respond to TFF3 stimulation with enhanced migration. Hence, squamous cell carcinoma cells may possess an oncogenic response to paracrine or endocrine TFF3. We also examined the expression of TFF3 in large cell neuroendocrine carcinomas of the lung and

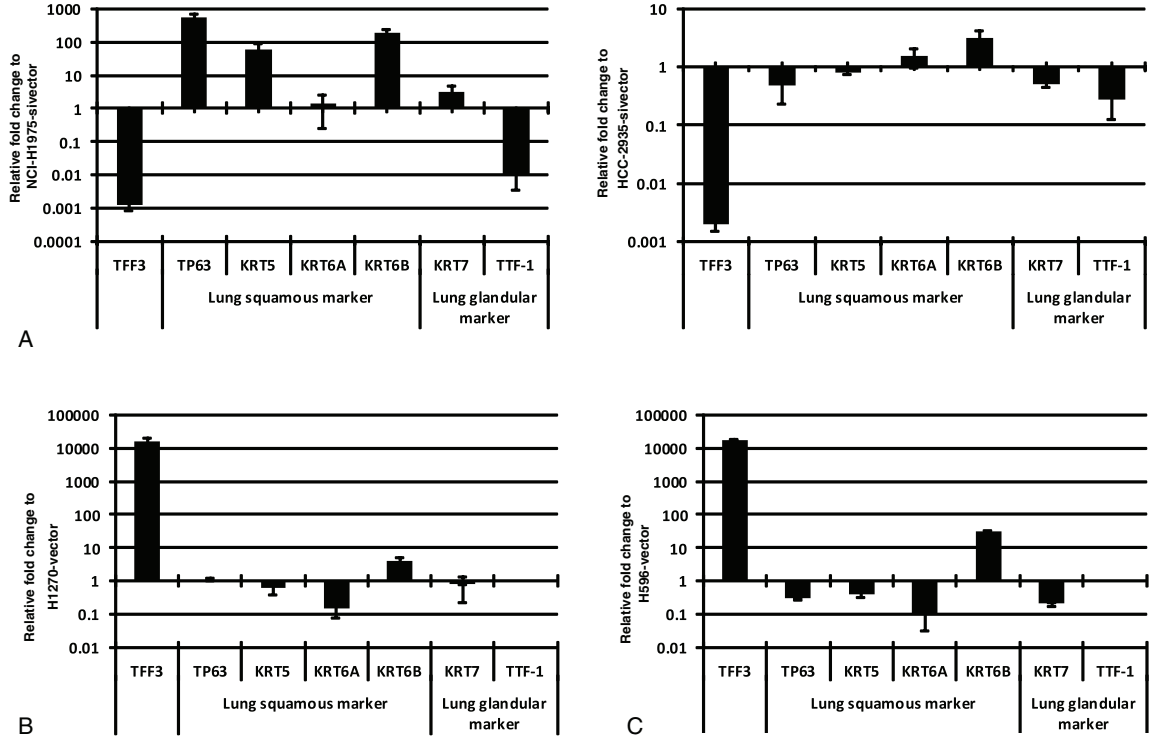

FIGURE 3. Effect of modulation of TFF3 expression on lineage-specific markers in lung adenocarcinoma (ADC), squamous cell carcinoma (SCC), and adenosquamous carcinoma (ASC) cell lines. (A) siRNA-mediated depletion of TFF3 in non-small cell lung cancer (NSCLC) cell lines, NCI-1975 and HCC-2935, of glandular differentiation or (B) forced expression of TFF3 in NSCLC cell lines, H1270, of squamous lineage; and (C) H596, of adenosquamous lineage. Change in mRNA levels of lineage-specific markers, KRT7 and TTF-1 for glandular; and KRT5, KRT6A, KRT6B, and TP63 for squamous are presented. Fold change values are representative of 3 independent biological experiments. Columns are mean of triplicate experiments; bars ± SD.

TABLE 4. Expression of TFF3, TTF-1, and CK7 in Extrapulmonary Adenocarcinoma and Squamous Cell Carcinoma

			TFF3 Expression		TTF-1 Expression		CK7 Expression	
Tumor types	**Organs**	**n**	**Negative, n (%)**	**Positive, n (%)**	**Negative, n (%)**	**Positive, n (%)**	**Negative, n (%)**	**Positive, n (%)**
Adenocarcinoma	Colorectum	29	0 (0)	29 (100)	29 (100)	0 (0)	27 (93.1)	2 (6.9)
	Stomach	27	2 (7.4)	25 (92.6)	27 (100)	0 (0)	27 (100)	0 (0)
	Esophagus	20	1 (5)	19 (95)*	20 (100)	0 (0)	0 (0)	20 (100)
	Cervix	35	4 (11.4)	31 (88.6)†	35 (100)	0 (0)	0 (0)	35 (100)
	Total	111	7 (6.3)	104 (93.7)‡	111 (100)	0 (0)	54 (48.6)	57 (51.4)
Squamous cell carcinoma	Cervix	33	32 (97)	1 (3.0)	33 (100)	0 (0)	32 (97)	1 (3)
	Esophagus	19	18 (94.7)	1 (5.3)	19 (100)	0 (0)	19 (100)	0 (0)
	Larynx	16	16 (100)	0 (0)	16 (100)	0 (0)	16 (100)	0 (0)
	TOTAL	68	66 (97.1)	2 (2.9)	68 (100)	0 (0)	67 (98.5)	1 (1.5)

* Compared with esophageal SCC, $P < 0.001$.
† Compared with cervical SCC, $P < 0.001$.
‡ Compared with total SCC, $P < 0.001$.

observed that none of the 3 large cell neuroendocrine carcinomas expressed TFF3. Hence, use of TFF3 expression should be restricted to differentiate between lung ADC and SCC. In any case, further detailed functional studies are required to understand the role and mechanism of TFF3 in NSCLC differentiation.

To extend our findings, IHC detection of TFF3 was also performed in 7 more epithelial neoplasms of 5 different extrapulmonary origins. We observed a high frequency of TFF3 positivity in cervical ADC (31/35, 88.6%), esophageal ADC (19/20, 95%), colorectal ADC (29/29, 100%), and gastric ADC (25/27, 92.6%). In contrast, the combined expression rate of TFF3 in 68 SCCs from the esophagus, cervix, and larynx was extremely low (2/68, 2.9%). Similar to the observation of TFF3 expression in primary lung ADC, the frequency of positive expression of TFF3 was significantly higher than that observed for CK7 in extrapulmonary ADC (93.7% vs 51.4% respectively). TTF-1, as a marker specifically for lung ADC, is not commonly expressed in extrapulmonary ADC[40,41] and these observations were reiterated herein. It should also be noted that although expression of CK7 was observed in cervical ADC and esophageal ADC, CK7 was only expressed in a small percentage of colorectal ADC and not in gastric ADC as previously reported.[42] Therefore, expression of TFF3 is a more sensitive marker of ADC of ''all organs'' origin than either TTF-1 or CK7.

Previous studies have demonstrated that TFF3 is often focally expressed in specific cell types of specific tissues and organs.[9,12] Herein, we have confirmed the localization of TFF3 in specific cell types (eg, goblet cells) of normal tissues and organs as reported previously. In addition, we have confirmed the lack of TFF3 expression in normal stratified squamous epithelium reported by other investigators. For example, Kouznetsova et al[26] reported that the stratified squamous epithelium of the esophagus is completely devoid of TFF3 expression.[26] In terms of cancer, Huang et al[37] have previously reported that of 56 colorectal ADCs, 96% of the primary tumors were positive for TFF3 expression.[37] These results are concordant with the data presented herein. However, the positivity rate of TFF3 expression in gastric ADC has been reported to range from 46.8% to 62%,[27,28,43–45] which is in apparent deviance to our results herein (92.6%). The reason for this discrepancy appears simply because of use of a different evaluation system for TFF3 positivity. We considered the cytoplasmic immunoreactivity for TFF3 in >10% of cells sufficient for the sample to be designated as TFF3-positive. In contrast, the other reports assessed TFF3 expression semi-quantitatively by multiplication of the scores for the intensity (0, 1, 2, or 3) and extent of staining (0, 0%; 1, 0%–10%; 2, 10%–50%; 3, 50%–100%), and then grouped the specimens into 2 categories where scores of 0 to 3 were considered negative and 4 to 9 positive.[27,28,43–45] Technically, the 2 categories should have been correctly designated as either low or high expression of TFF3 and not as ''negative'' or ''positive.'' We ourselves have also used this semi-quantitative approach for evaluation of the association of TFF3 expression with survival outcomes in breast cancer.[18]

TFF3 is a secreted protein and can be detected in the serum of patients with prostate,[46] endometrial,[47] gastric,[48] metastatic colorectal,[49] liver,[50] and lung cancer.[51] Qu et al[52] observed increased serum TFF3 in lung cancer patients compared with a control cohort, with no discriminatory value of the levels of serum TFF3 for either ADC or SCC. Although the investigators also examined TFF3 expression in tumor samples by western blot analysis, this experimental approach would clearly not allow distinction between TFF3 expressed in focal, but high levels, in normal tissue and/or the tumor, nor allow an accurate assessment of the tumor content in any specimen used. In contrast, IHC, as used herein, allows an accurate determination of TFF3 expression specifically in carcinoma cells. Concordant with our work herein, TFF3 has been reported to be dramatically downregulated in oral SCC compared with normal healthy controls, as observed by IHC, but without change in salivary TFF3 levels.[51] In any case, serum levels of TFF3 are observed to be elevated not only in carcinomas of multiple organ origins[46–50] but also in non-malignant conditions, such as chronic obstructive pulmonary disease,[53] chronic kidney disease,[54] and inflammatory bowel disease.[55] Increased serum levels of TFF3 may therefore increase non-specifically in morbidity, and potentially in morbidities that are associated with an inflammatory response. It is therefore apparent that the discriminatory value of TFF3 for ADC only pertains to IHC determination of TFF3 expression.

There were several limitations in this study. The study was performed in a single institution using a homogenous Han

Chinese ethnic group. Therefore, a multicenter trial based on various ethnic groups may be valuable for determining the generalizability of our results. Furthermore, we only determined TFF3 expression in primary lung ADC, SCC, and large cell neuroendocrine carcinoma. Further studies with larger and ethnically diverse cohorts, and more varied histological types, are needed to confirm our results.

In conclusion, our results demonstrate that TFF3 is a sensitive and specific IHC biomarker for ADC, which could prove valuable for differential diagnosis of certain tumor subtypes where clinically indicated, or for research purposes.

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