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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Thyroid transcription factor 1 (TITF-1) is a transactivating factor with important functions in the normal peripheral lung and that exhibits dysregulated expression in non-small cell lung cancers (NSCLC) and amplification in a significant fraction of lung tumors typical of a lineage-specific oncogene. We tested the hypothesis that the abnormal expression of TITF-1 at the protein and DNA levels may occur in different subsets of patients with distinct clinicopathological and unique molecular features. We demonstrated significant association of TITF-1 protein expression and copy number gain with favorable and poor prognosis, respectively, in NSCLC patients. Importantly, we unraveled distinct correlations between TITF-1 protein expression and copy number gain of the gene with mutations in the EGFR and KRAS oncogenes, respectively, in lung adenocarcinoma. Our findings suggest that lung adenocarcinomas exhibiting elevated protein or copy gain of TITF-1 may be of different cell lineage populations with distinct oncogene mutation patterns, and therefore subject to dissimilar anticancer therapies.

**Translational Relevance**

Thyroid transcription factor 1 (TITF-1) is a transactivating factor with important functions in the normal peripheral lung and that exhibits dysregulated expression in non-small cell lung cancers (NSCLC) and amplification in a significant fraction of lung tumors typical of a lineage-specific oncogene. We tested the hypothesis that the abnormal expression of TITF-1 at the protein and DNA levels may occur in different subsets of patients with distinct clinicopathological and unique molecular features. We demonstrated significant association of TITF-1 protein expression and copy number gain with favorable and poor prognosis, respectively, in NSCLC patients. Importantly, we unraveled distinct correlations between TITF-1 protein expression and copy number gain of the gene with mutations in the EGFR and KRAS oncogenes, respectively, in lung adenocarcinoma. Our findings suggest that lung adenocarcinomas exhibiting elevated protein or copy gain of TITF-1 may be of different cell lineage populations with distinct oncogene mutation patterns, and therefore subject to dissimilar anticancer therapies.

The epidermal growth factor receptor (EGFR) in lung adenocarcinomas (10–12).

NK2 homeobox 1 (NKX2.1) otherwise known as thyroid transcription factor 1 (TITF-1) is a homeodomain-containing transactivating factor predominantly expressed in the terminal lung bronchioles and lung periphery in the developing and adult mouse (13–15). In addition, TITF-1 is crucial for branching morphogenesis during normal lung development (14–17) and transactivates the expression of the surfactant proteins (SP) such as SPs-A, -B, and -C which are in turn typically expressed in the Clara cells and are important for the differentiation of alveolar type II pneumocyte cells in the peripheral lung (18–20). More recently, TITF-1 expression and function have been shown to be important in the etiology of congenital pulmonary disease (21) and NSCLC (22–24). TITF-1 is part of the 14q13.3 cytoband locus which is amplified in a significant fraction of lung tumors (22, 24). In addition, knockdown of TITF-1 expression by RNA interference results in lung adenocarcinoma cell growth inhibition and apoptosis demonstrating a lineage-specific dependency of lung adenocarcinomas on TITF-1 (22–24). In contrast to the expected prosurvival properties of a cell lineage oncogene, TITF-1 protein expression by immunohistochemistry is a marker of favorable prognosis in NSCLC patients (25–28) and is associated with good prognosis in early-stage (stage-I) lung adenocarcinoma patients (29). More recently, amplification of the TITF-1 gene has been identified in lung SCCs (28) despite lack of protein expression; however, the relevance of this copy number gain to the clinical outcome of NSCLC patients is still unclear, although Weir and colleagues reported no significant difference in patient survival between adenocarcinoma tumors with or without amplification of TITF-1 (24).

In this study, we sought to investigate protein and copy number levels of the TITF-1 gene in NSCLC patients and correlate, in parallel, both levels of analyses with the clinicopathological and molecular features of the patients. We demonstrate the significant and close association of TITF-1 protein with the protein expression of all 3 SPs tested. In addition, we find a significant association of TITF-1 protein expression and copy gain with favorable and poor prognosis, respectively, in lung adenocarcinoma patients, despite a significant positive correlation between the gene’s copy number and protein expression. Lastly, we unravel distinct significant correlations between TITF-1 protein expression and DNA copy gain with mutations in the lung adenocarcinoma-prevalent EGFR and KRAS oncogenes.

**Methods**

**Human lung tissues and tissue microarray**

All human tissues were obtained from the Lung Cancer Specialized Program of Research Excellence (SPORE) Tissue Bank at the MD Anderson Cancer Center. For each tissue sample, the percentage of malignant tissue was calculated and the cellular composition of specimens was determined by histological examination (I.I.W.) following Hematoxylin-Eosin (H&E) staining.

Specimens resected from NSCLC patients with stages I to IV disease according to the revised International System for Staging Lung Cancer (30) who had no prior chemotherapy or radiotherapy were used for tissue microarray (TMA) analysis by immunohistochemistry. Clinicopathological characteristics of the patients are summarized in Supplementary Table S1. Patients who had smoked at least 100 cigarettes in their lifetime were defined as smokers. Samples were fixed in formalin, embedded in paraffin, stained with H&E, and reviewed by an experienced pathologist (I.I.W.). The 321 tissue specimens collected from 321 patients included 117 SCCs and 204 adenocarcinomas. All tumors and lesions were classified according to the World Health Organization (WHO) 2004 criteria as previously described (31). The TMAs were prepared with a manual tissue arrayer (Advanced Tissue Arrayer ATA100, Chemicon International) using 1-mm-diameter cores in triplicate for tumors. Sections were then determined if they were suitable for analysis of TITF-1 protein expression by immunohistochemistry and TITF-1 amplification by FISH analysis. Of the entire TMA set, 179 and 170 adenocarcinomas were used for immunohistochemistry and FISH analyses, respectively. In addition, 117 and 99 lung SCC sections were used for immunohistochemistry and FISH analyses, respectively. Moreover, 151 lung adenocarcinomas were subsequently analyzed for correlation of TITF-1 expression by immunohistochemistry and FISH analyses. Furthermore, data on EGFR and KRAS mutational status were available and conducted on 178 and 120 lung adenocarcinoma sections, respectively.
Immunohistochemistry analysis

Tissue section slides were baked at 56°C overnight, then deparaffinized in xylene and rehydrated through a graded series of ethanol concentrations. Antigen retrieval was carried out using a decloaker for analysis of SP-B expression and a steamer (PH = 9, 20 minutes) for assessment of TITF-1 protein expression. Intrinsic peroxidase activity was blocked by 3% hydrogen peroxide in H2O2 for 15 minutes. 1 protein expression. Intrinsic peroxidase activity was blocked by 3% hydrogen peroxide in H2O2 for 15 minutes and 5% goat serum (Sigma) solution was used for blocking nonspecific antibody binding by incubating at room temperature for 60 minutes. Slides were then incubated at room temperature for 1 hour with primary antibodies raised against TITF-1 (dilution 1:100, clone 8G7G3/1, Cell Marque), SP-A (dilution 1:150, clone PE10, Thermo Scientific), SP-B (dilution 1:150, clone SPB02, Thermo Scientific), and SP-C (dilution 1:300, Chemicon). After 3 washes in Tris-buffered saline with Tween-20 (TBST) for 5 minutes each, the slides were incubated with Dako Envision + Dual Link for 30 minutes at room temperature. Following 3 additional washes in TBST, the slides were incubated with Dako chromogen substrate for 5 minutes and were counterstained with hematoxylin for 5 minutes. The slides were read under microscope. Two pathologists (X.T. and I.W.) examined both the intensity and extent of immunostaining by light microscopy using a x 20 magnification objective. Cytoplasmic and nuclear expression were quantified using a 4-value intensity score (0, none; 1+, weak; 2+, moderate; and 3+, strong) and the percentage (0–100%) of the extent of reactivity. A final expression score was obtained by multiplying the intensity and reactivity extension values (range, 0–300).

FISH analysis

TITF-1 DNA was originally provided by Dr. Wam Lam (British Columbia Cancer Research Center, Vancouver, British Columbia, Canada). The DNA was labeled with Spectrum Red conjugated diUTP (Abbott Laboratories) using the Vysis Nick translation kit, according to the manufacturer’s instructions. A chr14 control probe (14qII) was prepared using the BAC clone RP11–324B11 and labeled with Spectrum Green-conjugated diUTP using the same procedure as described above for the TITF-1 probe. The FISH assay was conducted on the TMAs using a standard protocol (32). Briefly, slides were incubated for 4 hours at 56°C, deparaffinized in Citri-Solv (Thermo Fisher Scientific), and were hydrated. The slides were then incubated in 2× saline–sodium citrate (SSC) buffer at 75°C for 10 to 13 minutes, digested in 0.25 mg/mL proteinase K/2× SSC at 45°C for 10 to 13 minutes, washed in 2× SSC for 5 minutes, and dehydrated. Following application of the probe mixtures, samples were denatured for 15 minutes at 80°C and incubated at 37°C for 48 hours, after which post-hybridization washes were carried out with 2× SSC/0.3% NP40 (pH 7.0–7.5) at 72°C for 2 minutes. Slides were then washed in 2× SSC for 2 min, dehydrated and chromatin was counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.3 μg/mL in Vectashield mounting medium, Vector Laboratories).

Analysis was carried out in epifluorescence microscope using single interference filters sets for green (fluorescein isothiocyanate, FITC), red (Texas Red), and blue (DAPI) as well as dual (red/green) band pass filters. Monochromatic images were captured and merged using the CytoVision workstation (Genetix). The quality of the preparation and the intensity of the fluorescence signal were variable per slide and per tissue core. The assay was repeated once for each array to obtain results in higher number of cores. Specimens displaying a gene signal number per cell of 4 or greater were considered to exhibit copy number gain (4–10 signals, low copy gain; greater than 10 signals, high level of copy number gain). The maximum value among the 3 cores was considered to represent each case.

DNA extraction and quantitative PCR

NSCLC tissues (n = 82, 53 adenocarcinomas and 29 SCCs) were dissected from formalin-fixed paraffin-embedded (FFPE) Hematoxylin-stained tissue sections using manual microdissection under stereomicroscope to ensure that tumor cell proportions are greater than 70% for subsequent DNA extraction. Tumor DNA was extracted using the PicoPure DNA extraction Kit (Arcturus) according to the manufacturer’s instructions. Two μL of DNA was added to a 20 μL of final volume reaction mixture consisting of 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems) and 0.5 μmol/L of each of forward and reverse primers spanning the TITF-1 gene (forward, 5′-gtcggcgttggagctc-3′; reverse, 5′-cactgcctcctagtc-3′). β-actin gene was used as an endogenous reference gene. TaqMan Control Human Genomic DNA (Applied Biosystems) was amplified as a standard control for calibration. All samples and standard DNA reactions were carried out in triplicates. Quantitative PCR (qPCR) was carried out using an ABI 7300 Real-Time PCR System Sequence (Applied Biosystems) at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The quantity of the target genes were normalized using the level of the β-actin gene, and expressed as relative quantities (RQ) compared with the value of the Human Genomic DNA. RQ equal or larger than 2 was considered as gene copy gain.

EGFR and KRAS mutational status

Exons 18 and 21 of EGFR and exons 1 and 2 of KRAS were PCR-amplified using DNA extracted from microdissected tumor cells, as previously described (33). Each PCR was carried out using HotStarTaq Master Mix (Qiagen) for 40 cycles at 94°C for 30 seconds, at annealing temperature for 30 seconds, and 72°C for 30 seconds, followed by a 7-minute extension at 72°C. All primer sequence and annealing temperatures are listed in Supplementary Table S2. PCR products were directly sequenced using the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp.). Sequence variants were confirmed by independent PCR amplifications from at least 2 independent microdissections and in both directions.
Statistical analyses
Data were summarized using descriptive statistics and frequency tabulation. Associations between categorical variables were assessed via cross-tabulation, chi-squared tests, and Fisher’s exact tests. Differences in continuous markers between groups were tested using the rank-based nonparametric Wilcoxon-rank sum test or Kruskal–Wallis tests. Survival rates were calculated using the Kaplan–Meier method for estimation of survival probability. Univariate and multivariate Cox proportional hazard models were applied to assess the effects of covariates on overall and recurrence-free survival. All computations were done in Statistical Analysis Software (SAS; Cary) and S-plus 7.0 (TIBCO software Inc.).

Results
Immunohistochemical expression of TITF-1, SP-A, SP-B, and SP-C proteins in NSCLC
We assessed the protein expression of TITF-1 and the SPs, SP-A, SP-B, and SP-C which are typically transactivated by TITF-1 (18–20), in a series of lung adenocarcinomas (n = 179) and SCCs (n = 117) by immunohistochemistry. TITF-1 protein expression was mainly nuclear (Fig. 1A), prevalent in lung adenocarcinomas (68.7% with TITF-1 protein greater than 0) and absent in SCCs (all zero TITF-1 protein; P < 0.0001; Supplementary Table S3). Similarly, the immunohistochemical protein expression (greater than a score of zero) of SP-A and SP-B was mainly cytoplasmic (Fig. 1A) and only evident in lung adenocarcinomas (19.8% and 16.4%, respectively) and was absent in SCC (P < 0.0001; Supplementary Table S3). In contrast, SP-C protein was expressed in both NSCLC subtypes as 84.5% of lung adenocarcinomas and 93.6% of SCCs exhibited an SP-C expression score of greater than zero (Supplementary Table S3). A significant positive correlation was found between the expression of any of the 3 SPs analyzed and TITF-1 expression when lung adenocarcinoma patients were stratified by the absence or presence of TITF-1 immunoreactivity (Table 1).

We then correlated TITF-1 protein expression with other clinicopathological features besides histology (Table 2). TITF-1 protein expression was significantly associated with female gender (P = 0.0001), never-compared with ever-smokers (P < 0.001), and never-compared with former- or current-smokers (P = 0.0004; Table 2).

We next asked whether TITF-1 protein expression exhibits prognostic properties in lung adenocarcinomas, the NSCLC subtype where it is predominantly expressed. Lung adenocarcinoma patients with higher than the median expression of TITF-1 protein (≥125) exhibited favorable survival compared with patients with lower expression (less than total score of 125; P = 0.0478 of the log-rank test) with a HR of 0.639 (Fig. 1B). Similar results were obtained when stages I and II lung adenocarcinoma patients (n = 140) were analyzed alone using the same cut-off for TITF-1 protein expression (P = 0.01 of the log-rank test, HR = 0.485; Fig. 1C). In contrast to those findings, no significant association was found between TITF-1 protein expression and recurrence-free survival in lung adenocarcinoma patients (data not shown). In addition, multivariate Cox proportional hazard regression analysis revealed that TITF-1 protein expression was not an independent predictor of overall or recurrence-free survival in lung adenocarcinoma (data not shown).

TITF-1 gene amplification in NSCLC and association with clinicopathological features
We next assessed the gene dosage levels of the TITF-1 gene in NSCLC patients by 2 different methodologies, copy

Figure 1. TITF-1 immunohistochemical expression in NSCLC. A, representative photomicrographs of the immunohistochemical expression of TITF-1, SP-A, SP-B, and SP-C. Kaplan–Meier survival probability plots of all stages (B) and stages I and II only (C) lung adenocarcinoma patients stratified by TITF-1 protein expression. P values were obtained by the log-rank test. EN/NE, censored events/total number of cases.
number gain and amplification by FISH (170 lung adenocarcinomas and 99 SCCs) and copy gain by qPCR in 46 lung adenocarcinomas and 36 SCCs. TITF-1 copy number gain by FISH was found in 18.9% of lung adenocarcinomas and in 20.2% of lung SCCs (Fig. 2A and Supplementary Table S4). A high level of TITF-1 copy number gain (>10 copies) was found in 8.3% and 3.0% of lung adenocarcinomas and SCCs, respectively (Supplementary Table S4). Two lung adenocarcinoma cases and 1 case of lung SCC were found to have greater than 20 signals per cell; the high level of TITF-1 copy gain by FISH detected in the SCC case is depicted in Supplementary Figure 1. We then determined to correlate TITF-1 protein with its DNA copy gain in lung adenocarcinomas as both variables were assessed and found in these set of patients. A significant positive correlation was found between the TITF-1 protein expression (as a continuous score variable) with the presence of TITF-1 copy gain assessed by FISH (P = 0.004; Fig. 2B) in lung adenocarcinomas. The mean and median of the TITF-1 protein score, assessed by immunohistochemical analysis, were 200.69 ± 115.73 and 240 (min, 0; max, 300), respectively, in TITF-1 FISH-positive lung adenocarcinoma patients (n = 29) and 127.1 ± 122.61 and 93 (min, 0; max, 300), respectively, in TITF-1 FISH-negative adenocarcinoma specimens (n = 122).

We also assessed TITF-1 copy number gain by qPCR in a subset of patients that we had analyzed by FISH (n = 82, 46 lung adenocarcinomas and 36 SCCs). A similar percentage of lung adenocarcinomas (18.4%) and SCCs (18.2%) exhibited greater than 3 copies of TITF-1 analyzed by qPCR (Supplementary Table S5). Similar to the FISH analysis, a slightly higher percentage of lung adenocarcinomas (10.2%) displayed a higher ratio (≥5) of TITF-1 copy gain than that of SCCs (4.6%, Supplementary Table S5). Moreover, we found a significant correlation between levels of TITF-1 copy gain by FISH and qPCR analyses in both lung adenocarcinomas (P = 0.0037) and lung SCCs (P = 0.048). The mean and median levels of relative TITF-1 RQ by qPCR in TITF-1 FISH-positive lung adenocarcinomas (n = 11) were 5.12 ± 5.55 and 3.41 (min, 0.53; max, 24.22), compared with 2.49 ± 4.31 and 1.41 (min, 0.48; max, 74.93) in TITF-1 FISH-negative adenocarcinomas (n = 35). In addition, the mean and median levels of TITF-1 RQ assessed by qPCR in TITF-1 FISH-positive SCCs (n = 9) were 13.61 ± 24.16 and 2.23 (min, 0.82; max, 74.93), compared with 1.64 ± 1.18 and 1.28 (min, 0.56; max, 4.8) in TITF-1 FISH-negative SCCs.

We next investigated the role of TITF-1 copy number gain by FISH in prognosis of both lung adenocarcinoma and SCC patients because we detected TITF-1 gain in both

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Covariate type (n)</th>
<th>TITF-1 negative, n (%)</th>
<th>TITF-1 positive, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Female (148)</td>
<td>65 (43.9)</td>
<td>83 (56.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Male (135)</td>
<td>95 (70.4)</td>
<td>40 (29.6)</td>
<td></td>
</tr>
<tr>
<td>Tobacco history</td>
<td>Never (53)</td>
<td>17 (32.1)</td>
<td>36 (67.9)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Ever (229)</td>
<td>142 (62)</td>
<td>87 (38)</td>
<td></td>
</tr>
<tr>
<td>Smoking habit</td>
<td>Never (53)</td>
<td>17 (32.1)</td>
<td>36 (67.9)</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Former (140)</td>
<td>86 (61.4)</td>
<td>54 (38.6)</td>
<td></td>
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<tr>
<td></td>
<td>Current (89)</td>
<td>56 (62.9)</td>
<td>33 (37.1)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>Adenocarcinoma (179)</td>
<td>56 (31.3)</td>
<td>123 (68.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>SCC (104)</td>
<td>104 (100)</td>
<td>0 (0)</td>
<td></td>
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</tbody>
</table>

P values were obtained by the Fisher’s exact test.

Table 1. Correlation of the immunohistochemical protein expression of TITF-1 and the SP-A, SP-B, and SP-C SPs in lung adenocarcinomas

<table>
<thead>
<tr>
<th>Covariate (n)</th>
<th>Covariate expression (n)</th>
<th>TITF-1 negative, n (%)</th>
<th>TITF-1 positive, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-A (171)</td>
<td>Negative (135)</td>
<td>49 (36)</td>
<td>86 (64)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Positive (36)</td>
<td>4 (11)</td>
<td>32 (89)</td>
<td></td>
</tr>
<tr>
<td>SP-B (168)</td>
<td>Negative (137)</td>
<td>47 (34)</td>
<td>90 (66)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Positive (31)</td>
<td>2 (7)</td>
<td>29 (94)</td>
<td></td>
</tr>
<tr>
<td>SP-C (171)</td>
<td>Negative (23)</td>
<td>14 (61)</td>
<td>9 (39)</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>Positive (148)</td>
<td>37 (25)</td>
<td>111 (75)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: P values were obtained by the Fisher’s exact test. TITF-1 immunoreactivity greater than zero was considered positive.

Table 2. Association of TITF-1 protein expression with NSCLC histology, gender, tobacco history, and smoking habits
subtypes of NSCLC. NSCLC patients with TITF-1 copy number gain (low and high) by FISH (n = 51, 33 adenocarcinomas and 18 SCCs) displayed a trend for poorer survival (P = 0.08) compared with NSCLC patients lacking TITF-1 copy number gain (n = 218, 137 adenocarcinomas and 81 SCCs). However, multivariate Cox proportional hazard regression analysis revealed that copy number gain of the TITF-1 gene by FISH was an independent predictor of poor survival in NSCLC alone with age (P < 0.0001), and stage-I disease (P < 0.0001; Fig. 2D). Similar results were obtained when NSCLC patients were dichotomized on the basis of the presence or absence of high level of TITF-1 copy gain (>10 signals; data not shown).

Association of TITF-1 protein and copy number gain with EGFR and KRAS mutations in lung adenocarcinoma

Molecular abnormalities in the expression or function of KRAS and EGFR contribute to tumor development and progression and therefore serve as crucial targets for molecularly driven target-specific therapies (2, 34). We had previously investigated the prevalence of EGFR and KRAS mutations in our clinical sample sets and correlated the extent of the mutations of those 2 oncogenes with patients’ clinicopathological features (33, 35). However, the relationship of TITF-1 abnormalities with the mutational pattern of oncogenes prevalent in lung adenocarcinoma, for example, EGFR and KRAS, is not well understood. Therefore, we sought to correlate the presence of mutations in the KRAS and EGFR oncogenes with DNA copy gain and protein levels of the TITF-1 gene. Interestingly, the protein levels and gene dosage extent of TITF-1 exhibited different correlation patterns. TITF-1 protein expression as a continuous variable displayed border-line significant positive correlation with the presence of EGFR mutations (P = 0.05; Supplementary Fig. 2). The mean and median levels of TITF-1 immunohistochemical protein in EGFR mutant lung adenocarcinomas (n = 22) were 183.94 ± 124.58 and 225 (min, 0; max, 300), compared with 124.62 ± 121.06 and 100 (min, 0; max, 300) in EGFR wild-type adenocarcinomas (n = 137). No significant differences were found in TITF-1 protein expression between KRAS mutant (n = 10; mean, 126.67 ± 127.57; median, 75) and wild type (n = 94; mean, 112.78 ± 117.68; median, 66.67) lung adenocarcinomas. On the other hand, TITF-1 copy gain by FISH displayed a significant positive correlation with the presence of KRAS mutations as 55% or 6 of 11 KRAS mutant patients exhibited TITF-1 copy number gain compared with 15.9% of patients with wild-type KRAS (14 of 88 patients; P = 0.008 of the Fisher’s exact test; Table 3). Moreover and in accordance with the analysis of TITF-1 protein expression as a continuous variable, 64% of lung adenocarcinoma patients with mutant EGFR exhibited a TITF-1 protein score greater than or equal to 200 compared with 33% of patients with wild-type EGFR (P = 0.006; Table 3). These new findings further demonstrate the dissimilarities between the extent of protein and DNA of TITF-1 in lung adenocarcinomas with EGFR versus KRAS mutations.

Discussion

In this study, we investigated the protein and DNA copy number gain of the TITF-1 cell lineage gene in a TMA comprised mainly of lung adenocarcinomas and SCCs and in correlation with molecular and
clinico-pathological features. We found that immunohistochemical protein expression of TITF-1 is prevalent in lung adenocarcinomas and absent in SCCs, correlates positively and significantly with the protein expression of the SP-A, SP-B, and SP-C SPs and with female gender and never smoking status. Moreover, copy gain of TITF-1 DNA assessed by FISH, was observed in both adenocarcinomas and SCCs and correlated positively and significantly with TITF-1 protein in lung adenocarcinomas as well as copy gain in NSCLC assessed by qPCR. Interestingly, while higher expression of TITF-1 protein was predictive of favorable prognosis in lung adenocarcinomas, TITF-1 copy number gain was an indicator of poor survival in NSCLC. Lastly, TITF-1 protein and DNA copy gain exhibited distinct correlations with oncogene mutation status in lung adenocarcinomas as the former correlated significantly with EGFR mutations, whereas the latter was associated with mutations in KRAS. The opposing prognostic, clinicopathological and molecular characteristics of TITF-1 protein expression and copy number gain suggest that different subsets of NSCLC patients may express TITF-1 through different cellular mechanisms.

Our findings on the association of higher levels of TITF-1 protein expression with favorable prognosis and overall survival are in accordance with previous findings by independent groups (25–29). In this study, we further investigated TITF-1 protein expression in clinical samples of NSCLC by correlating its expression with that of 3 SPs, SP-A, SP-B, and SP-C. We unraveled significant associations of TITF-1 protein expression with that of the 3 SPs. It is noteworthy that staining patterns of TITF-1 in the developing and adult lung are very similar to those observed for the 3 SPs (18–20, 36). Moreover, TITF-1 is known to facilitate the differentiation of alveolar type II cells, in part, by transactivating the expression of the 3 surfactant genes following direct binding on their promoter regions (18–20, 36). It is worthy to mention that the combined use of Napsin A, an aspartic proteinase involved in the maturation of SP-B, and TITF-1 results in improved sensitivity and specificity for identifying pulmonary adenocarcinoma in primary lung tumors and in metastatic settings (37). Here, we provide new evidence of positive association of TITF-1 protein and that of the 3 major SPs in clinical specimens of human lung adenocarcinoma. These findings raise the possibility of the combined use of TITF-1 and SP expression in the diagnosis of pulmonary adenocarcinomas as the usage of TITF-1 with any of the SPs may increase the number of adenocarcinoma cases with positive expression of either marker.

We tested the association of TITF-1 protein expression with prognosis in lung adenocarcinomas because we only noted the protein in this subtype of NSCLC. In contrast, we analyzed the prognostic capacity of TITF-1 copy number gain in lung adenocarcinomas and SCCs as we detected increased TITF-1 gene dosage in both subtypes of NSCLC. It is worthwhile to note that Kwei and colleagues previously demonstrated amplification of TITF-1 assessed by comparative genomic hybridization (CGH) in both lung adenocarcinomas and SCCs, albeit at a lower frequency (11% and 3%, respectively; ref 22). In the present study, we detected TITF-1 both low and high copy number gain in 20.1% of lung SCCs assessed. Specifically, 17.2% of lung SCCs exhibited low level of amplification (4–10 copies, data not shown) and 3% (3 cases) displayed high level of TITF-1 copy number gain (>10 copies, Supplementary Table S4), which is similar to the rate of amplification found in lung SCC in the previously reported CGH study by Kwei and colleagues (22). Interestingly, we also found that 1 case of lung SCC harbored greater than 20 copies of TITF-1 (Supplementary Fig. 1). It is worthwhile to note that a recent study by Bass and colleagues using single nucleotide polymorphism (SNP) arrays showed the absence of TITF-1 amplification in a set of lung SCCs (n = 47; ref 5). In our study and as mentioned before, only 3 of 99 lung SCCs were found to display more than 10 signals of TITF-1 per cell that we considered to harbor high copy gain of the gene rather than amplification. Due to the lack normalization to DNA content, a shortcoming in our FISH analysis, it is not clear whether specimens displaying high level of TITF-1 copy number gain actually harbor amplified TITF-1.

We found a significant positive correlation between TITF-1 protein expression and DNA copy number levels assessed by FISH analysis in lung adenocarcinomas. Previously, Perner and colleagues demonstrated a similar positive

<table>
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<tr>
<th>Covariate</th>
<th>Covariate type</th>
<th>TITF-1 FISH</th>
<th>TITF-1 IHC ≥ 200a</th>
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<td>EGFR</td>
<td>Mutant</td>
<td>5/16 (31)</td>
<td>14/22 (64)</td>
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<tr>
<td></td>
<td>wild type</td>
<td>27/133 (20)</td>
<td>45/137 (33)</td>
</tr>
<tr>
<td>KRAS</td>
<td>Mutant</td>
<td>6/11 (55)</td>
<td>3/10 (30)</td>
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<tr>
<td></td>
<td>wild type</td>
<td>14/88 (16)</td>
<td>27/94 (29)</td>
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</table>

P values were obtained by the Fisher’s exact test.

aTITF-1 immunoreactivity greater than or equal to 200 was considered positive.
correlation between the protein products and the DNA copy gain levels of the \textit{TITF-1} gene (28). Moreover, we further demonstrated that, unlike the association of \textit{TITF-1} protein with overall survival of lung adenocarcinoma patients, \textit{TITF-1} copy gain by FISH analysis appears to be a marker of poor prognosis in NSCLC. We also attempted to investigate the survival of lung adenocarcinoma patients alone with \textit{TITF-1} copy number gain stratified by the presence or absence of \textit{TITF-1} protein expression. Lung adenocarcinoma patients with copy number gain of \textit{TITF-1} displayed worse survival than adenocarcinoma patients with both copy gain and protein expression (data not shown), demonstrating the association of \textit{TITF-1} copy number gain and \textit{TITF-1} protein expression with poor and good prognosis, respectively, in the lung adenocarcinoma population alone. The better survival correlations with \textit{TITF-1} protein and worse outcomes associated with \textit{TITF-1} copy gain are in close agreement with those reported earlier by Barletta and colleagues (38). It is uncertain why protein levels and DNA copy gain of \textit{TITF-1} have opposing associations with prognosis of NSCLC patients including lung adenocarcinomas alone. However, it is worthwhile to mention that oncogene copy number gain is a feature of cell lineage genes that elicit prosurvival functions important for the population of cells expressing the oncogene or for the pathological condition, that is, cancer, whose cells are driven by amplification of the oncogene (5, 6). In this context, it is not surprising that NSCLC patients exhibiting copy number gain of the \textit{TITF-1} gene display poor survival and these findings no longer become counterintuitive. Moreover, it is plausible to suggest that \textit{TITF-1} copy number gain may be more reliable than protein expression for studying cell lineage patterns of expression in NSCLC. It is noteworthy that \textit{TITF-1} protein was found to be elevated in primary lung adenocarcinoma compared with matched metastatic lesions in the brain (39). This is in accordance with our findings and the results of previous reports by other groups (25–29) on the utility of \textit{TITF-1} protein expression as a marker of favorable prognosis in lung cancer. Interestingly, Tanaka and colleagues reported that \textit{TITF-1} gene dosage, which we demonstrated in this study to be a marker of dismal prognosis in NSCLC, was higher in metastatic sites compared with primary lung tumors (23). Therefore, our current findings and previous reports by others suggest that lung adenocarcinoma cells with either elevated \textit{TITF-1} protein expression or copy gain of the \textit{TITF-1} gene may originate from different cell populations with dissimilar biological properties and effects on patient clinical outcome. Congruent with this probable thought, is our novel finding that \textit{TITF-1} protein expression and \textit{TITF-1} gene copy number were associated with mutations in different oncogenes in lung adenocarcinomas. Our current study shed light on positive associations between \textit{TITF-1} gene dosage and protein expression level with mutations in the \textit{KRAS} and \textit{EGFR} oncogenes, respectively. It is noteworthy, that overexpression of \textit{HRAS} inhibits the mRNA and protein levels of \textit{TITF-1} as well as its transcriptional factor function (40). Thus, it is plausible to suggest that \textit{TITF-1} copy number gain is favored in lung adenocarcinomas with mutant \textit{KRAS}. Since, mutations in \textit{EGFR} and \textit{KRAS} occur almost mutually exclusively in lung adenocarcinomas (2), our results suggest that elevated expression of \textit{TITF-1} may be variably controlled (copy gain versus transcription) in distinct subsets of lung adenocarcinoma patients. Moreover, the association of \textit{TITF-1} protein, but not copy gain, with \textit{EGFR} mutations is in accordance with our finding on the increased levels of \textit{TITF-1} protein in never-smoker compared with ever-smoker lung cancer patients.

It has been suggested that other genes within the \textit{TITF-1} ampiclon (14q13.3) may facilitate, cooperate with, or even enhance the prosurvival biological properties of \textit{TITF-1} (22, 24, 41, 42). Kwei and colleagues reported the location of several genes within the \textit{TITF-1} ampiclon including the paired box transcriptional factor member \textit{PAX9} and \textit{NKK2.8} (22, 24). It is worthwhile to mention that Kendal and colleagues demonstrated that coamplified \textit{TITF-1}, \textit{PAX9}, and \textit{NKK2.8} exhibit oncogenic cooperation and cell prosurvival and proliferative properties (42). Overexpression of both \textit{TITF-1} and \textit{NKK2.8} simultaneously in BEAS-2B immortalized human bronchial epithelial cells elicited the highest increase in cell colony growth compared with single-gene transfected cells (42). Moreover, pathway signatures that overlap downstream of both \textit{TITF-1} and \textit{NKK2.8} defined lung adenocarcinoma patients with most dismal prognosis compared with signatures downstream of either transcriptional factor alone (41). It is therefore tempting to speculate that co-amplification or copy gain of \textit{NKK2.8} and/or \textit{PAX9} together with \textit{TITF-1} may contribute to the observed poor survival patterns observed in NSCLC patients exhibiting copy gain of the \textit{TITF-1} gene compared with patients with exhibiting only elevated expression of the protein product of this cell lineage gene. It is also plausible that \textit{TITF-1} copy gain and its correlation with important biological outcomes in NSCLC may only be a surrogate marker, for example, in SCCs, of another molecular defect in a gene nearby or within the 14q13.3 ampiclon, for example \textit{NKK2.8} or \textit{PAX9}. It is worthwhile to mention that \textit{NKK2.8} displays dissimilar patterns of expression relative to those of \textit{TITF-1} in the developing and adult mouse lung (43). Therefore, it is possible that expression of the \textit{NKK2.8} protein or its copy number gain may subdivide patients exhibiting positive expression of \textit{TITF-1} protein into subsets with different clinical outcomes. Moreover, lung SCC patients with copy number gain of the \textit{TITF-1} gene, as shown in this study and previously (28, 38), may display positive expression of the \textit{NKK2.8} protein and that transactivation of \textit{TITF-1} may be inhibited in lung SCCs and not in adenocarcinomas by yet unknown mechanisms.

In summary and herein, we further highlight the cell lineage pattern of \textit{TITF-1} in human NSCLC and its correlation at the protein level with that of SPs in clinical specimens of lung adenocarcinoma. Moreover, we demonstrate that \textit{TITF-1} protein and gene dosage are associated with discordant clinical outcomes. We also noted in lung SCCs...
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Abnormalities of the TITF-1 Lineage-Specific Oncogene in NSCLC: Implications in Lung Cancer Pathogenesis and Prognosis

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