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Original Paper

TTF1 expression in non-small cell lung carcinoma: association with *TTF1* gene amplification and improved survival

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Abstract

Acquired chromosomal aberrations play an important role in tumour development and progression. Such genetic alterations occur in a significant proportion of non-small cell lung carcinomas (NSCLCs) and include amplification of 14q13.3, which contains the *TTF1* gene. We asked whether *TTF1* amplification is associated with increased TTF1 protein expression in NSCLCs, and whether TTF1 is associated with clinicopathological features, including patient survival. We used a FISH assay and quantitative immunohistochemical staining to interrogate a population-based cohort of 538 NSCLCs from Swiss patients for *TTF1* amplification and protein expression. We found *TTF1* amplification in ∼13% of adenocarcinomas (ACs) and in ∼9% of squamous cell carcinomas (SCCs) and *TTF1* amplification was associated with increased TTF1 protein expression. High-level TTF1 expression was significantly associated with smaller tumour size, female gender and longer overall survival only among ACs (median survival 82 versus 28 months; $p = 0.002$). On multivariate analysis, high TTF1 expression was an independent predictor of favourable prognosis in patients with AC [hazard ratio, 0.56 (95% CI 0.38–0.83); $p = 0.008$]. We conclude that *TTF1* amplification is a mechanism of high-level TTF1 expression in a subset of NSCLCs. When expressed at high levels, this routinely used diagnostic marker is also an independent biomarker of favourable prognosis in AC.

Keywords: ***TTF1*; amplification; non-small cell lung cancer; biomarker; thyroid cancer**

Introduction

Lung cancer is a leading cause of cancer death worldwide, and recent work has focused on identifying recurrent genetic alterations associated with the development and progression of this malignancy. In a recent study characterizing genome-wide copy-number alterations in 528 primary lung adenocarcinomas, Weir *et al* described 26 large-scale and 31 focal, recurrent copy-number alterations [1]. The commonest recurrent large-scale genetic alteration is amplification of 5p; few such large-scale events are linked to defined functional effects involving specific genes. By contrast, smaller-scale focal events are more likely to include tumour suppressor or oncogenes. The most prevalent focal event found in lung adenocarcinoma is amplification of 14q13.3, which includes the *TTF1* gene.

TTF1 encodes a transcription factor (thyroid transcription factor 1) expressed in the thyroid, the lung and the developing central nervous system. Within the lung, expression is confined mainly to the peripheral airways and small bronchioles [2]. TTF1 is also expressed in the majority of pulmonary adenocarcinomas [3–9], a property that is diagnostically exploited in the distinction of primary pulmonary adenocarcinomas from metastases originating in other extrathyroidal organs [10]. The regulation of TTF1 expression in normal lung and pulmonary adenocarcinoma is poorly understood, and the relationship between *TTF1* amplification and protein expression has not been established. In contrast to adenocarcinoma, TTF1

is rarely expressed in squamous cell carcinoma of the lung [3–9], and the prevalence of *TTF1* amplification has not been studied in this subset of non-small cell lung carcinomas (NSCLCs).

Also unknown is the prognostic relevance of TTF1 expression. Various series have reported poor, equivalent or improved survival associated with TTF1 expression in lung carcinoma, although the results of a recent meta-analysis suggest that TTF1 expression predicts better survival, at least among patients with adenocarcinoma [3–9,11–14]. In this study, we assess *TTF1* amplification status and TTF1 protein expression levels in a large series of NSCLCs to determine whether these parameters are correlated with one another, and to investigate their association with clinicopathological features, including patient survival.

Materials and methods

NSCLC patient cohort and tissue microarray (TMA) construction

We retrospectively examined a cohort of 538 consecutive patients with NSCLC who underwent surgery with curative intent for lung cancer between January 1993 and December 2002 in Zurich, Switzerland; 497 patients were treatment-naïve and 41 patients received neoadjuvant chemotherapy; 490 patients who had complete clinical datasets and sufficient tissue to evaluate by FISH were included. In three of these included cases, immunohistochemical data was not available. Only non-small cell carcinomas [adenocarcinoma (AC), squamous cell carcinoma (SCC) and adeno-squamous carcinoma (ASC)] were included. Surgical lung specimens were processed according to the guidelines of the Swiss Society of Pathology. The study was approved by the institutional ethical review boards of the University Hospital Zürich and the Weill Cornell Medical Center. Overall survival time was obtained from the Cancer Registry of the Kanton Zürich and was defined as the interval between the date of surgery and the date of death. The end date of follow-up was 31 July 2007, and the median duration of follow-up was 45 (range 1–169) months.

TMAs were constructed using a custom-made, semi-automatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA), as previously described. Two representative 0.6 mm cores of viable tissue from each tumour were included in the TMA [15]. Normal lung tissue samples were also incorporated into the TMA.

Thyroid tumour cohort and TMA construction

Thyroid TMAs collectively containing the following cases ($n = 216$) were constructed from selected cases from the paraffin blocks from the Pathology Archives of the University of Michigan: primary papillary carcinoma (PC), 109; metastatic PC, 34; follicular adenoma, 10; follicular carcinoma (total), 13; follicular carcinoma with *PAX8–PPARγ* rearrangement, 4; oncocytic adenoma, 1; oncocytic carcinoma, 9; anaplastic carcinoma, 11; nodular hyperplasia, 9; and medullary carcinoma, 16. Most of the cases ($n = 175$) were arrayed in triplicate, using 0.6 mm diameter cores, while the remaining cases ($n = 41$) were arrayed in duplicate, using 1.0 mm cores. The University of Michigan IRB approved the studies.

TTF1 immunohistochemistry and evaluation

Sections (4.5 μm) of the TMA were de-paraffinized and stained using an automated immunostaining device (Ventana Medical Systems, Tucson, AZ), applying the monoclonal mouse anti-thyroid transcription factor 1 (TTF1, 1 : 50, clone 8G7G3/1; Dako, Carpinteria, CA, USA). Detection with a secondary anti-mouse antibody was performed using Ultraview Amp (Ventana). The intensity of immunohistochemical nuclear staining was quantified for each case, using a semi-automated quantitative image analysis system (ACIS II, Clarient Chromavision Medical Systems, San Juan Capistrano, CA, USA) that has been previously validated [16]. The output data of TTF1 expression were represented by a arbitrary, continuous, unitless scale of average staining intensity for each case, range 0–255. Along this spectrum, samples scored in the range 42–201.

Assessment of *TTF1* amplification status using a two-colour interphase FISH assay

For assessing *TTF1* amplification, a probe spanning the *TTF1* gene locus (chr14q13.3) and a reference probe spanning a stable region in adenocarcinomas of the lung (chr14q24.1) were used. For the TTF1 target probe, the Biotin-14-dCTP-labelled BAC clone RP11-1083E2 (eventually conjugated to produce a red signal) and for the reference probe the Digoxin-dUTP labelled BAC clone RP11-72J8 (eventually conjugated to produce a green signal) were applied as probes. The BAC clones were obtained from the BACPAC Resource Center, Children's Hospital Oakland Research Institute (CHORI) (Oakland, CA, USA). Tissue hybridization, washing and colour detection were performed as described previously [17].

At least one TMA core could be evaluated on 490 cases. The samples were analysed under a ×60 oil immersion objective, using an Olympus BX-51 fluorescence microscope equipped with appropriate filters, a CCD (charge-coupled device) camera and the CytoVision FISH imaging and capturing software (Applied Imaging, San Jose, CA, USA). Semi-quantitative evaluation of the tests was independently performed by two evaluators (SP and PW). For each case, an attempt was made to analyse at least 100 nuclei. *TTF1* amplification status was assessed by comparing the number of (red) *TTF1* probe signals with the number of (green) reference signals. Normal lung tissue cells exhibited two copies of each signal. Cases containing more than two signals per cell nucleus for

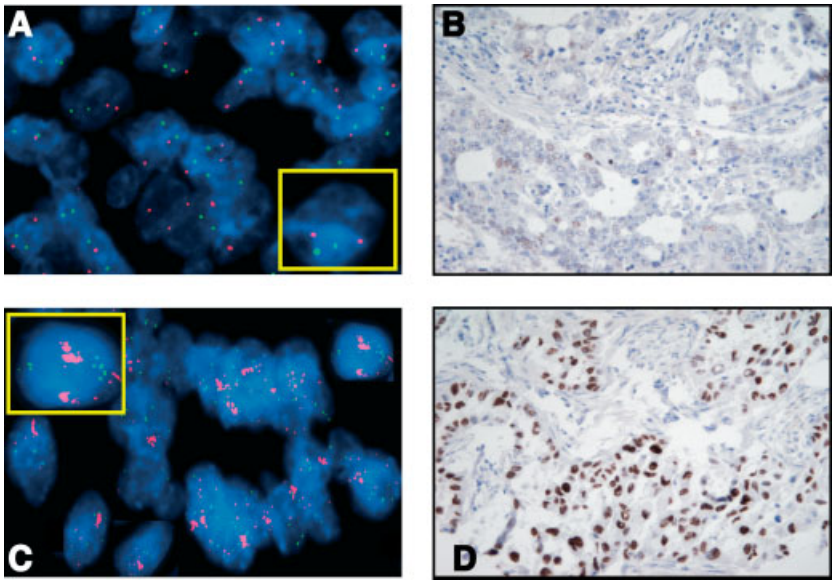

Figure 1. (A) FISH image of a lung adenocarcinoma without *TTF1* amplification, displaying a 2 : 2 ratio of the TTF1 probe (red) to reference probe (green). The yellow boxed area shows a magnified representative nucleus. (B) Anti-TTF1 immunohistochemistry image corresponding to (A), showing low (49 on the intensity scale) expression of TTF1. (C) FISH image of a lung adenocarcinoma, displaying a high-level amplification of the TTF1 probe. The yellow boxed area shows a magnified representative nucleus. (D) Anti-TTF1 immunohistochemistry image corresponding to (C), showing high (192 on the intensity scale) expression of TTF1

both the *TTF1* and reference probes were categorized as polysomic. Cases with only two reference probe signals/cell but more than two *TTF1* probe signals were categorized as harbouring *TTF1* amplification (Figure 1), and were divided into low-level amplification (three to nine additional *TTF1* signals relative to the reference probe) or high-level amplification (>10 additional *TTF1* signals). Since performing interphase FISH on tissue sections can result in misclassification on the basis of overlapping nuclei, section artifacts and nuclear size exceeding the thickness of the tissue selection, we used a rather broad range to define low-level amplification (three to nine additional copies) in order to better differentiate true high-level amplification from non-amplified tumours. Rare cases demonstrating heterogeneity in amplification status were classified according the area with the greatest degree of amplification [18].

Statistics

Continuous variables (ie age, tumour size and TTF1 immunohistochemical staining intensity) were compared among subgroups using Student's *t*-test. Categorical variables (ie gender, histological subtype, staging parameters, differentiation and TTF1 amplification status) were compared among subgroups using χ^2 or Fisher's exact test, as appropriate. Disease-free survival curves were generated using the Kaplan–Meier method (Prism 5, GraphPad Software Inc., San Diego, CA, USA) and compared using the log-rank and Wilcoxon tests. A multivariate Cox proportional hazards regression model of independent prognostic factors for overall survival was performed (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

Results

TTF1 amplification status in NSCLCs

We observed examples of both high-level amplification (>10 *TTF1* signals relative to the reference signals and most often too numerous to count; Figure 1C) and low-level amplification (3–10 additional *TTF1* signals relative to the reference signals) of *TTF1* among NSCLCs. Amplifications were observed in a total of 29 (12.9%) adenocarcinomas (10 low-level and nine high-level amplifications); 23 (9.4%) squamous cell carcinomas (nine low-level and 14 high-level amplifications); and one high-level amplification out of 20 adenosquamous carcinomas. Due to the relative paucity of adenosquamous carcinomas in our cohort, these were excluded from further analysis. No amplification was detected in adjacent normal lung tissue. None of the patient or tumour characteristics studied showed a significant association with high-level *TTF1* amplification, although tumours with low-level amplification had a greater mean size than lesions with no amplification ($p = 0.04$). The clinical and pathological features and associated *TTF1* amplification and expression data are summarized in Table 1.

TTF1 expression status in NSCLCs

Using a quantitative assessment of immunohistochemical staining intensity, we identified a broad range of TTF1 expression levels (Figure 2). Overall, mean expression of TTF1 was significantly greater in adenocarcinomas than in squamous cell carcinomas ($p = 0.0001$; Tables 1, 2, and Figure 2). ACs in the top quartile with respect to TTF1 protein expression were associated with female gender, smaller tumour size and

Table 1. Association of clinicopathological parameters, *TTF1* amplification status and TTF1 protein expression levels for patients with adenocarcinomas of the lung

		TTF1 amplification status				TTF1 protein expression level		
Adenocarcinoma	**Total (Σ = 225)**	**None (Σ = 196)**	**Low-level (Σ = 10)**	**High-level (Σ = 19)**	***p****	**Quartiles 1–3 (Σ = 166)**	**Quartile 4 (Σ = 56)**	***p*****
Gender								
Male	140	123	8	9	0.22	110	28	0.04
Female	85	73	2	10		56	28	
Age (years)	61.4 ± 10.1	61.8 ± 10.1	59.6 ± 10.5	58.0 ± 9.7	0.1	61.5 ± 10.1	61.3 ± 10.2	0.9
Tumour size (cm)	4.0 ± 2.2	4.0 ± 2.2	5.5 ± 2.1	3.6 ± 1.6	0.4	4.2 ± 2.3	3.5 ± 1.7	0.04
Stage								
I	91	82	4	7	1.0	65	27	0.6
II	61	51	4	6		46	14	
III	53	47	1	5		40	12	
IV	18	16	1	1		15	3	
T score								
pT1	50	43	2	5	0.1	30	19	0.05
pT2	145	129	7	9		112	32	
pT3	10	7	1	2		9	1	
pT4	20	17	0	3		15	4	
Node status								
Negative	112	97	6	9	1.0	80	30	0.5
Positive	113	99	4	10		86	26	
Grade								
Low–intermediate	134	120	3	11	1.0	102	30	0.3
High	91	76	7	8		64	26	
TTF1 amplification								
None	196	—	—	—	—	150	43	0.02
Low-level	10	—	—	—		6	4	
High-level	19	—	—	—		10	9	
TTF1 protein expression	129.0 ± 38.0	125.8 ± 38.2	145.6 ± 24.6	153.1 ± 29.9	0.003	—	—	—
Median survival (months)	42	43	19	63	0.15	28	82	0.002

p Values listed are derived from χ^2 test (categorical data) or Student's *t*-test (continuous variables), comparing cases with high-level amplification versus those with no amplification (*), or comparing cases from the lower three quartiles based on quartile of TTF1 protein expression versus those in the upper quartile (**); Fisher's exact test in comparing categories in which the expected frequency was <5. The log rank test was used to obtain *p* values for comparing median survival.

lower pathological tumour stage (pT status). Moreover, we found an association between TTF1 protein expression and gene amplification, in that ACs harbouring high-level *TTF1* amplifications showed significantly greater TTF1 protein expression than those without amplification ($p = 0.003$) (Figure 1, Table 1). There was no difference between low-level amplification and TTF1 protein expression. Among SCCs, high-level *TTF1* amplification was again associated with higher protein expression levels; however, we found no significant association between any of the clinicopathological parameters of SCC and *TTF1* gene or protein levels (Table 2).

TTF1 and NSCLC patient survival

The prognostic significance of *TTF1* amplification and protein expression was assessed using the Kaplan–Meier method and Cox proportional hazard multivariate regression analysis. When ACs were stratified by quartiles based on the level of TTF1 protein expression, patients with tumours in the highest quartile showed a significantly improved median overall survival in univariate analysis (82 versus 28 months; $p = 0.002$; Tables 1, 2 and Figure 3A). No difference in median survival was noted comparing quartiles 1, 2 and 3 (27.5 months, 27.5 months and 32 months, respectively; $p > 0.9$). Furthermore, on multivariate analysis, high-level TTF1 expression proved to be an independent predictor of favourable prognosis among ACs [hazard ratio (HR) 0.56; 95% CI 0.38–0.83; $p = 0.008$]. A trend toward increased overall survival among patients with ACs harbouring high-level *TTF1* amplification was also observed in univariate analysis (median survival of 63 versus 43 months; $p = 0.15$). No association between *TTF1* amplification or protein expression level and outcome among patients with SCC was seen (Figure 3B).

TTF1 amplification status in thyroid tumours

Since TTF1 is also highly expressed in thyroid tumours, we evaluated a cohort of 216 primary and

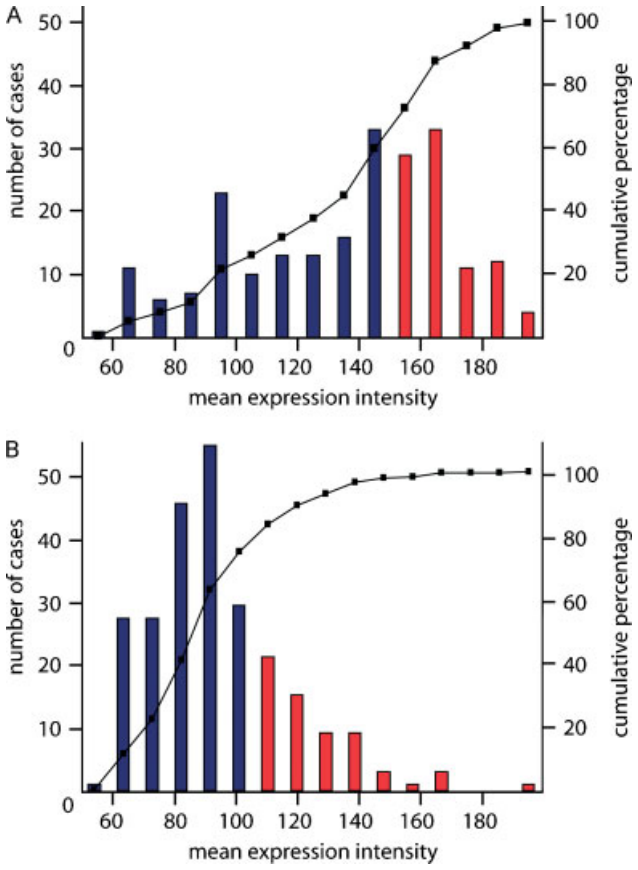

Figure 2. Histogram of the mean TTF1 expression intensity distribution for adenocarcinomas (A) and squamous cell cancers (B). The left *y* axis indicates the total number of cases, while the right *y* axis indicates the cumulative percentage. The blue-coloured bars represent quartiles 1–3; the red-coloured bars represent the top quartile of TTF1 protein expression levels

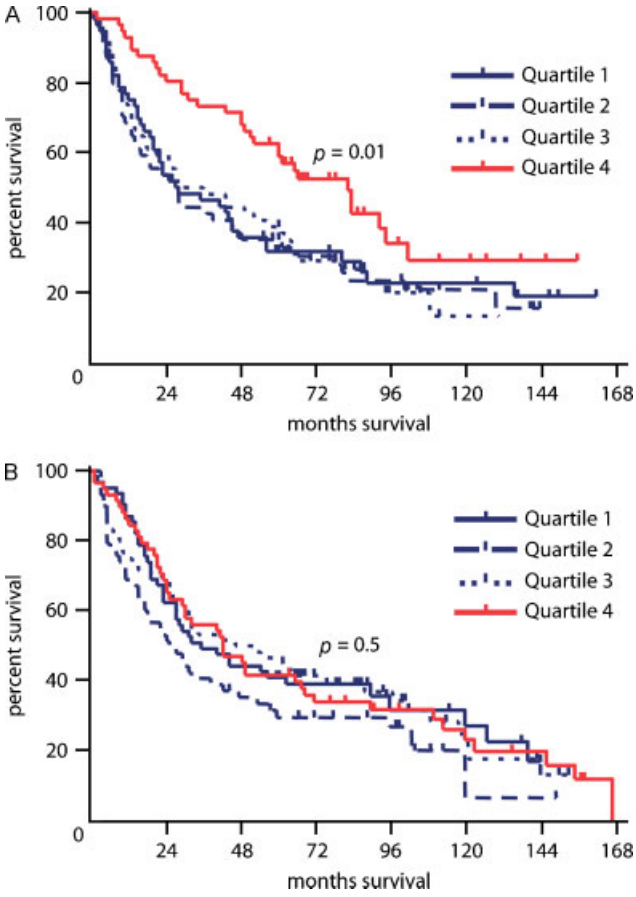

Figure 3. Overall survival for patients with adenocarcinomas (A) and squamous cell carcinomas (B) dependent on TTF1 protein expression levels. The blue-coloured curves represent the cases within quartiles 1–3 of TTF1 protein expression, while the red-coloured curves represent the cases with top quartile of TTF1 protein expression. Patient with adenocarcinoma expressing TTF1 in the top quartile have a significant survival benefit over the patients expressing TTF1 in the lower three quartiles. Patients with squamous cell carcinomas did not show any survival benefit with regard to their TTF1 expression levels. The *p* values refer to comparison of top quartile versus lower three quartiles (log rank test)

metastatic thyroid tumours for *TTF1* amplification. None of these tumours showed evidence of *TTF1* amplification.

Discussion

Recent discoveries have drawn attention to the role of acquired, recurrent chromosomal alterations in common epithelial malignancies, such as adenocarcinoma of the lung, breast and prostate [1,19–21]. These events, including amplifications, deletions, gene rearrangements and mutations, are implicated as causal events in tumour origin and progression, and often carry great prognostic and predictive significance. One such alteration in lung cancer is amplification of the *TTF1* gene, which occurs in ~12% of adenocarcinomas [1,22,23] but not in adjacent normal lung tissue. In this study, we investigated the relationship between *TTF1* amplification and protein expression, and the association of these findings with clinicopathological features and overall survival in a large series of NSCLCs. We found high-level *TTF1* amplification in 12.9% of ACs, and tumours harbouring this genetic alteration expressed significantly higher TTF1 protein levels than tumours without *TTF1* amplification, which is in agreement with Kwei *et al* [23]. High-level TTF1 expression was, in turn, associated with female gender, smaller tumour size, lower pathological tumour stage and improved overall survival among patients with ACs.

We also found high-level TTF1 amplification in 9.4% of SCCs, a subset of NSCLC only anecdotally described to have this amplification [23]. Overall, SCCs showed a much lower level of TTF1 expression than ACs, and exhibited no significant association between TTF1 expression status and any clinicopathological parameter, including overall survival. These results are consistent with prior studies demonstrating a low prevalence of TTF1 expression among SCCs of the lung [3–9].

Our findings regarding the frequency of *TTF1* amplification in ACs are in keeping with previous estimates. Weir *et al* and Kwei *et al* found high-level copy number gains at this locus in 12% and 11% of ACs,

Table 2. Association of clinicopathological parameters, *TTF1* amplification status and TTF1 protein expression levels for patients with squamous cell carcinomas of the lung

		TTF1 amplification status				TTF1 protein expression level		
Squamous cell carcinoma	**Total (Σ = 245)**	**None (Σ = 222)**	**Low-level (Σ = 9)**	**High-level (Σ = 14)**	***p****	**Quartiles 1–3 (Σ = 184)**	**Quartile 4 (Σ = 61)**	***p*** **
Gender								
Male	198	184	5	9	0.14	152	46	0.3
Female	47	38	4	5		32	15	
Age (years)	64.9 ± 9.2	65 ± 9.3	60.3 ± 8	67.1 ± 6.6	0.4	64.6 ± 9.2	66 ± 9.1	0.3
Tumour size (cm)	4.2 ± 2.1	4.1 ± 2.1	3.9 ± 2.2	5.1 ± 2.5	0.09	4.1 ± 2.1	4.5 ± 2.2	0.2
Stage								
I	94	86	4	4	1.0	68	26	0.09
II	90	82	1	7		65	25	
III	53	47	3	3		44	9	
IV	8	7	1	0		7	1	
T status								
pT1	53	47	3	3	1.0	44	9	0.5
pT2	139	128	3	8		98	41	
pT3	32	29	1	2		24	8	
pT4	21	18	2	1		18	3	
Node status								
Negative	124	113	6	5	0.4	89	35	0.2
Positive	121	109	3	9		95	26	
Grade								
Low–intermediate	136	121	6	9	0.6	98	38	0.2
High	109	101	3	5		86	23	
TTF1 amplification								
None	222	—	—	—	—	169	53	0.12
Low-level	9	—	—	—		7	2	
High-level	14	—	—	—		8	6	
TTF1 protein expression	80.4 ± 4.5	80 ± 23.5	75.5 ± 6.8	93.6 ± 35.1	<0.05	—	—	—
Median survival (months)	34	32	53	42	0.8	32	42	0.7

p Values listed are derived from χ^2 test (categorical data) or Student's *t*-test (continuous variables), comparing cases with high-level amplification versus those with no amplification (*), or comparing cases from the lower three quartiles based on quartile of TTF1 protein expression versus those in the upper quartile (**); Fisher's exact test in comparing categories in which the expected frequency was <5. The log rank test was used to obtain *p* values for comparing median survival.

making it the most common recurrent focal genetic alteration in this subset of NSCLC [1,23]. Kendall *et al* found amplification of 14q13.3 — a region encoding *TTF1* as well as two additional transcriptional factors (*NKX2-8* and *PAX9*) — in 4.2% and 8.8% of two independent AC cohorts. The variation in observed frequencies could be due in part to different detection methods and definitions of high-level versus low-level *TTF1* amplification. Using FISH, we found low-level (two to nine additional copies of *TTF1*) amplification in 4.4% of ACs, while a frequency of such events of 10–15% may be inferred from the data of Kendall *et al*. Weir *et al* used high-density SNP arrays as a discovery tool and a FISH assay as a gold standard to confirm the amplification frequency. In contrast, Kendall *et al* used only array CGH to study copy number variation without *in situ* confirmation, a technique that could lead to errors in estimating the degree of amplification due to known limitations of aCGH and SNP, including saturation and dilution artifacts. In agreement with Kwei *et al*, thus, we feel that ~12% represents an accurate estimate of the true frequency of high-level *TTF1* amplification in ACs.

Our data demonstrate a clear association between TTF1 protein expression and improved prognosis, as well as a trend toward prolonged overall survival among patients harboring *TTF1* amplification. The prognostic significance of TTF1 protein expression has been extensively studied, and prior studies have reported shorter, equivalent or longer median survival associated with TTF1 in NSCLC, although many of these studies were limited by non-standardized, semi-quantitative criteria for stratifying lesions on the basis of TTF1 expression [4–9,11–14]. To overcome these limitations, we quantitatively assessed TTF1 protein expression using a continuous measure of IHC staining intensity [16], and found that a prognostic benefit is indeed present among the highest quartile of lesions with respect to TTF1 expression. Our finding that the survival benefit is limited to the subgroup of patients with the highest TTF1 expression levels may explain why earlier studies using less quantitative methods

yielded contradictory results. Further, our data agree with a recent meta-analysis in which a clear survival benefit is predicted among ACs expressing high levels of TTF1 [3]. Finally, multivariate analysis in our cohort establishes high-level TTF1 expression as an independent prognostic biomarker in lung AC.

Although little doubt remains that TTF1 expression portends a favourable prognosis in AC, the mechanism accounting for this association is unknown. Since TTF1 is normally expressed in the terminal respiratory unit, its presence within tumours could constitute a marker of retained differentiation and, consequently, a less aggressive phenotype [2]. Alternatively, the predictive value of TTF1 expression could reflect underlying associated pathways that carry their own prognostic implications, based on less aggressive disease or improved response to available treatment regimens. For instance, TTF1 expression has been shown to positively correlate with the presence of EGFR mutations and response to tyrosine kinase inhibitors in lung ACs [24]. Conversely, we previously found that *TTF1* amplification is mutually exclusive with rearrangements involving *ALK* and *EML4*, an event that occurs in a minority of NSCLCs and results in the formation of an oncogenic fusion gene [17,19]. Further work will be necessary to determine whether TTF1 expression directly and independently accounts for the observed benefit in overall survival in AC, or whether TTF1 is merely a co-phenomenon of underlying, less aggressive tumourigenic pathways.

The question of whether *TTF1* constitutes a proto-oncogene remains unsettled. Weir *et al* demonstrated a loss of anchorage-independent growth upon knockdown of TTF1 expression in a NSCLC cell line. Kwei showed a reduced cell proliferation when knocking down TTF1 in lung cancer cells with amplification. Kendall *et al* reported a higher growth rate in cells transfected with *TTF1* and nearby transcription factor genes on 14q13.3 [1,22,23]. While these cell line-based findings suggest that TTF1 expression can lead to accelerated proliferation, rigorous functional studies will be necessary to establish whether TTF1 plays a causative role in malignant transformation in lung AC. Since not all tumours expressing high TTF1 levels harbour a gene amplification, TTF1 expression in such cases must be driven by mechanisms other than a gain in *TTF1* copy numbers. Such alternative mechanisms may include altered transcriptional regulation, mutation, gene rearrangements or epigenetic events, and further studies will be necessary to distinguish among these and other possibilities. Interestingly, a cohort of thyroid tumours showed no *TTF1* amplification, suggesting that in these tumours amplification of *TTF1* does not drive TTF1 expression, neither does it play a significant role in the pathogenesis of thyroid cancer.

In summary, recurrent genetic rearrangements occur in a significant number of NSCLCs. *TTF1* amplification is found in ~12% of lung ACs, and is associated with increased TTF1 protein expression. High level of TTF1 expression independently predicts improved survival among patients with AC, and future experiments will be required in order to define the mechanisms underlying this association.

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