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Negative Thyroid Transcription Factor 1 Expression Defines an Unfavorable Subgroup of Lung Adenocarcinomas

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Introduction: Thyroid transcription factor 1 (TTF1) is a master regulator of pulmonary differentiation that is downregulated in a subset of lung adenocarcinoma, of which the clinicopathologic characteristics were not fully clarified.

Methods: One thousand forty-two lung adenocarcinoma patients who underwent surgery were investigated for clinic characteristics, histologic subtyping, and spectrum of well-identified driver mutations. TTF1 expression was correlated with these clinicopathologic factors and survival.

Results: Compared with TTF1 positive (TTF1+) patients, the 133 negative individuals (12.8%, TTF1−) were more likely to be male ($p = 0.006$) and heavy smokers ($p = 0.002$) who had larger tumor size ($p < 0.001$) and more advanced disease stage ($p < 0.001$). TTF1− presented more in solid and invasive mucinous-predominant carcinomas (both $p < 0.001$), whereas TTF1+ was identified in 100% patients with adenocarcinoma in situ, minimally invasive and lepidic-predominant adenocarcinomas. The TTF1− tumors harbored the known driver mutations in significantly low frequency compared with TTF1+ adenocarcinomas (57.8% versus 78.1%, $p < 0.001$), especially in epidermal growth factor receptor (*EGFR*) mutations (37.6% versus 60.7%, $p < 0.001$). There was no significant difference in recurrence-free survival between the TTF1− and TTF1+ patients, either for the whole cohort or stratified by pathologic stage, or among the driver mutation-defined subsets. However, recurrence of multiple metastases was more likely to occur in patients with TTF1− adenocarcinomas (88.1% versus 32.4%, $p < 0.001$). Multivariate analysis revealed that TTF1− independently predicted both poor postrecurrence survival (hazard ratio = 1.664; 95% confidence interval , 1.097–2.524; $p = 0.017$) and unfavorable overall survival (hazard ratio = 1.553; 95% confidence interval , 1.013–2.381; $p = 0.043$).

Conclusions: TTF1− correlated with solid and invasive mucinous subtypes of lung adenocarcinoma and lower frequency of *EGFR* mutations. It defines a subgroup of lung adenocarcinomas with unfavorable outcomes.

Key Words: Thyroid transcription factor 1, Lung adenocarcinoma, Subtype, Driver mutation, Survival

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Thyroid transcription factor 1 (TTF1), a homeodomain-containing nuclear transcriptional protein of the *Nkx2* gene family, is a transcription factor that regulates the expression of multiple genes involved in lung development.[1] In normal lung, TTF1 plays a decisive role in the maintenance of the function of terminal respiratory unit cells.[2] Positive TTF1 (TTF1+) staining by immunohistochemistry (IHC) has been detected in primary lung adenocarcinomas and was used as a diagnostic marker.[3,4] TTF1 controls tumor differentiation and limits metastatic potential in vivo. Upregulation of TTF1 was correlated with favorable survival and downregulation linked to loss of differentiation, enhanced tumor seeding ability, and increased metastatic proclivity.[5] Moreover, a recent investigation using a genetically engineering mouse model indicated that lung adenocarcinomas with *KRAS* and *TP53* mutations required additional alterations, such as loss of TTF1 expression, to initiate the metastatic cascade.[6] Also, other major oncogenic mutations in lung adenocarcinoma have been found to occur in *EGFR, BRAF, HER2, ALK, ROS1,* and *RET*. Those gene mutations defined subsets of lung cancers that could be amenable to treatment with specific kinase inhibitors.[1] However, whether the patients with negative TTF1 (TTF1−) expression lung adenocarcinoma harbor those targetable mutations are still largely unknown.

The International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society has provided a new classification for lung adenocarcinoma, which has been reported to be associated with prognosis.[7] Previous studies have been demonstrated that TTF1+ expression was associated with lepidic, acinar, papillary, and micropapillary predominant adenocarcinoma but did not draw a definite conclusion at the association of TTF1− expression

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with this novel classification system in lung adenocarcinoma because of the limited number of cases.[8–16]

Here, we analyzed 1042 resected pulmonary adenocarcinomas for TTF1 expression and described the clinicopathologic and mutational characteristics in patients with TTF1− tumors. These investigations allowed us to define the specific characteristics associated with TTF1− lung adenocarcinomas.

PATIENTS AND METHODS

Between 2008 and 2013, consecutive patients with pulmonary tumors were prospectively included. After preoperative work-up (enhanced thoracic computed tomography, abdominal ultrasonography, brain magnetic resonance imaging, and bone scan for all patients and positron emission tomography/computed tomography for some) to exclude regional and systemic disease, patients underwent surgery with curative intent at Fudan University Shanghai Cancer Center. Lymphadenectomy was routinely done for all patients, and adjuvant chemotherapy was suggested for those who had nodal metastases. Inclusion criteria for this study included (1) patients with pulmonary tumors underwent complete resection with curative intent; (2) pathologically confirmed lung adenocarcinomas and sufficient tissue for comprehensive mutational analyses; (3) those with history of adenocarcinomas from other organs were excluded from this study. Clinical data were collected from the prospectively maintained database. Disease stage was based on the 7th edition of the American Joint Committee on Cancer staging manual.[17] Recurrence-free survival (RFS) and overall survival (OS) were recorded based on follow-up clinic or telephone. The Institutional Review Board of Fudan University Shanghai Cancer Center approved this study. All patients gave written informed consent.

Histologic Evaluation and Immunohistochemical Analysis of TTF1

IHC was performed on 4-μm thick formalin-fixed, paraffin-embedded sections. Slides were deparaffinized and pretreated with 1 mmol/liter ethylenediaminetetraacetic acid and heat-mediated antigen retrieval solution in a microwave oven. Further steps were done at room temperature in a hydrated chamber. Slides were preincubated in 20% normal goat serum. TTF1 (1:100, 8G7G3/1, DAKO, Glostrop, Denmark) were applied. The slides were then washed in Tris–HCl and detected with horseradish peroxidase-conjugated anti-rabbit EnVision+ kit (DAKO). All slides were counterstained with hematoxylin. Adenocarcinoma was further confirmed using IHC method. Solid-predominant tumors were diagnosed based on some amount (≥5%) of other histologic patterns (lepidic, acinar, papillary, or micropapillary). Two pathologists (L.S. and Y.L.) blindly reviewed the slides. We used whole tissue blocks to identify TTF1 status. Nuclear staining of tumor cells was considered TTF1+. Tumors with completely no TTF1 expression in nuclei were defined as TTF1−. All tumors were classified according to the new classification system.[7]

Mutational Analysis

EGFR (exons 18–22), *HER2* (exons 18–21), *KRAS* (exons 2–3), and *BRAF* (exons 11–15) were amplified by polymerase chain reaction (PCR) using cDNA from each tumor specimen. Sanger sequencing was then performed to analyze the amplified products. A combination strategy of quantitative real-time PCR and reverse transcriptase PCR was used to detect *ALK*, *ROS1*, and *RET* fusions, with validation using fluorescent in situ hybridization.[18–21]

Statistical Analysis

Pearson's χ^2 test or Fisher's exact test was used to investigate the associations between the categorical variables. Comparison of continuous variables was examined by independent Student's *t* test and Mann–Whitney *U* test. The survival distribution was analyzed using the Kaplan–Meier method, and log-rank tests were employed for comparisons between two categories in univariate analysis. Multivariate survival analysis was conducted using the Cox proportional hazards regression (forward likelihood ratio model) to identify independent prognostic factors. All statistical analyses were two-sided, with *p* value less than or equal to 0.05 indicative of statistical significance, and performed using SPSS (version 19.0 IBM Corporation, Armonk, NY).

RESULTS

Clinicopathologic Data and Correlation With TTF1 Expression

A total of 1042 consecutive patients with lung adenocarcinoma were included in this study, of which 133 (12.8%) cases with completely negative TTF1 expression in nuclei. Clinicopathologic parameters analyzed included age, sex, smoking history, tumor size, lymph nodal status, and pathologic stage. Compared with TTF1+ adenocarcinoma, negative patients are more likely to be male ($p = 0.006$) and heavy smokers ($p = 0.002$). They also had larger tumor size ($p < 0.001$) and more advanced disease stage ($p < 0.001$). In addition, TTF1 expression was not significantly associated with age ($p = 0.219$) and lymph node metastasis ($p = 0.753$; Table 1).

Association Between TTF1 and Histologic Subtypes

Among TTF1− group, the major subtype was acinar-predominant adenocarcinomas (36.8%), followed by solid (32.3%) and invasive-mucinous (18.1%) subtypes. Of the TTF1+ tumors, acinar (48.6%), solid (13.1%), and papillary (12.5%) were in the majority. There were no adenocarcinoma in situ, minimally invasive adenocarcinomas, or lepidic-predominant tumors in TTF1− group. However, compared with the TTF1+ adenocarcinomas, TTF1− tumors presented more as solid and invasive mucinous subtypes (both $p < 0.001$), whereas less in acinar component ($p = 0.007$). Among the 86 invasive-mucinous tumors, there were 18 (20.9%) cases with pure-mucinous component, which were all negative for the TTF1 staining. In addition, the two enteric cases showed no TTF1 staining (Table 1).

Status of Common Oncogenic Mutations

The prevalence of the driver mutations was similar as previously reported for the study population.[2,19–21] Compared with TTF1+ tumors, TTF1− adenocarcinomas harbored

