An Integrated Molecular Analysis of Lung Adenocarcinomas Identifies Potential Therapeutic Targets among TTF1-Negative Tumors, Including DNA Repair Proteins and Nrf2

Robert J.G. Cardnell1, Carmen Behrens1, Lixia Diao2, YouHong Fan1, Ximing Tang3, Pan Tong2, John D. Minna4, Gordon B. Mills5, John V. Heymach1, Ignacio I. Wistuba3, Jing Wang2, and Lauren A. Byers1

Abstract

Purpose: Thyroid transcription factor-1 (TTF1) immunohistochemistry (IHC) is used clinically to differentiate primary lung adenocarcinomas (LUAD) from squamous lung cancers and metastatic adenocarcinomas from other primary sites. However, a subset of LUAD (15%–20%) does not express TTF1, and TTF1-negative patients have worse clinical outcomes. As there are no established targeted agents with activity in TTF1-negative LUAD, we performed an integrated molecular analysis to identify potential therapeutic targets.

Experimental Design: Using two clinical LUAD cohorts (274 tumors), one from our institution (PROSPECT) and The Cancer Genome Atlas, we interrogated proteomic profiles (by reverse phase protein array, RPPA), gene expression, and mutational data. Drug response data from 74 cell lines were used to validate potential therapeutic agents.

Results: Strong correlations were observed between TTF1 IHC and TTF1 measurements by RPPA (Rho = 0.57, P < 0.001) and gene expression (NKK2-1, Rho = 0.61, P < 0.001). Established driver mutations (e.g., BRAF and EGFR) were associated with high TTF1 expression. In contrast, TTF1-negative LUAD had a higher frequency of inactivating KEAP1 mutations (P = 0.001). Proteomic profiling identified increased expression of DNA repair proteins (e.g., Chk1 and the DNA repair score) and suppressed PI3k/mTOR signaling among TTF1-negative tumors, with differences in total proteins confirmed at the mRNA level. Cell line analysis showed drugs targeting DNA repair to be more active in TTF1-low cell lines.

Conclusions: Combined genomic and proteomic analyses demonstrated infrequent alteration of validated lung cancer targets (including the absence of BRAF mutations in TTF1-negative LUAD), but identified novel potential targets for TTF1-negative LUAD, including KEAP1/Nrf2 and DNA repair pathways.

Clinical Cancer Research

Introduction

Thyroid transcription factor-1 (TTF1, gene name NKX2-1) is a homeodomain-containing transcription factor expressed in the thyroid, lungs, and diaphragm during embryogenesis. In normal development, TTF1 is essential for lung morphogenesis and differentiation (1), but the function of TTF1 in cancer biology is complicated with implications for both oncogenic and tumor-suppressive signaling (reviewed in ref. 2).

Clinically, TTF1 is used as an immunohistochemical marker (IHC) for the differential diagnosis of lung adenocarcinoma (LUAD) and thyroid cancer. TTF1 plays an important role in: 1) distinguishing primary LUADs from metastatic adenocarcinomas originating from other sites (e.g., breast or colon cancer) and 2) distinguishing LUAD from squamous lung cancer. Nevertheless, a subset of LUAD (~15%–20%) does not express TTF1 (3, 4), and these TTF1-negative LUADs are associated with worse clinical outcomes (4).

Over the past ten years, major advances have been made in the treatment of LUAD, including the identification of druggable driver mutations and fusions in subsets of patients (~15%–20%), that respond to specific targeted therapies (e.g., EGFR mutations and ALK, RET, and ROS1 fusions). However, the most common of these—EGFR mutations and ALK fusions—occur rarely in TTF1-negative tumors (5–7). In fact, due to the low incidence of established driver genes in TTF1-negative tumors, TTF1 status is used in some parts of the world to determine whether a patient is likely to benefit from EGFR mutation testing (8, 9). Given the low rates of established targetable alterations in TTF1-negative LUAD, chemotherapy remains the current standard of care for the vast majority of these patients.
**Translational Relevance**

Thyroid transcription factor-1 (TTF1) is an immunohistochemical marker routinely used in the diagnosis of lung adenocarcinoma (LUAD). However, approximately 15% to 20% of LUADs do not express TTF1 and these cancers are associated with a worse prognosis. Currently, there are no targeted therapies, with proven efficacy in these patients, particularly as TTF1-negative tumors rarely harbor established druggable mutations such as EGFR. Here, we report distinct proteomic profiles in TTF1-negative tumors, including higher expression of several potential drug targets, suggesting a distinct biology. To explore translational applications of these findings, we analyzed preclinical models and found that inhibitors of these proteins (especially DNA repair proteins) had relatively greater activity in TTF1-negative cell lines, supporting their further investigation as candidate therapeutic targets. Despite its negative prognostic associations, the routine use of TTF1 IHC provides a key opportunity to leverage TTF1 as a predictive biomarker to guide treatment selection of drugs that work preferentially in this population.

We have previously shown the potential for proteomic profiling to identify novel therapeutic targets and predictive markers for lung cancer (10, 11). Proteomic profiling is complementary to other molecular profiling approaches (e.g., DNA sequencing) because it can identify highly expressed protein targets or pathway activation independent of a mutation, fusion, or gene amplification. Moreover, protein profiling, which includes analysis of post-translational modifications, directly measures the targets of small-molecule inhibitors or other targeted therapies providing a readout of pathway activation, which cannot be ascertained by nucleic acid sequencing. Consequently, proteomic profiling has the potential to identify therapeutic vulnerabilities in otherwise "oncogene-negative" cancers.

Given the poor prognosis of patients with TTF1-negative LUAD and the absence of a validated targeted therapy for this population, there is a critical need to better understand the pathophysiology of TTF1-negative LUADs with the goal of identifying more active treatments for this subset of lung cancers. Despite its negative prognostic associations, the routine use of TTF1 IHC in the diagnosis of LUAD provides a key opportunity to leverage TTF1 as a predictive biomarker to guide treatment selection of drugs that work preferentially in this population. Toward this goal, we performed an integrated molecular and drug analysis to identify pathways or targets enriched in TTF1-negative LUAD.

In this study, we investigated the unique clinical, genomic, and proteomic profiles of TTF1-negative tumors in two large, independent LUAD cohorts (274 tumors total). Using reverse phase protein array (RPPA) profiling, >127 total and/or phosphorylated proteins representing key oncogenic pathways were quantified in each tumor to identify potential therapeutic targets overexpressed in TTF1-negative tumors. We then identified drugs with preferential in vitro activity in TTF1-negative cell lines using a large panel of targeted drugs and chemotherapies, including drugs inhibiting candidate targets identified by RPPA profiling.  

**Materials and Methods**

**PROSPECT and TCGA cohorts**

The MD Anderson cohort was obtained from the Profiling of Resistance Patterns and Oncogenic Signaling Pathways in Evaluation of Cancers of the Thorax (PROSPECT) study, developed in 2006 to investigate novel molecular mechanisms of therapeutic resistance and generate rational therapeutic strategies for overcoming resistance. Surgically resected tumors, collected between 2006 and 2010, from 189 patients were included in PROSPECT, and of these, 152 were LUADs. RPPA profiling was performed on 93 of these samples. Clinical characteristics of these 93 patients are presented in Table 1.

The Cancer Genome Atlas (TCGA) LUAD cohort (12) included material from 279 previously untreated LUAD patients. This analysis uses the molecular profile of a 181 patient subset for which RPPA was performed. Clinical characteristics of these samples are described in Table 1.

**RPPA, gene-expression, and sequence analysis**

**PROSPECT.** RPPA analysis of PROSPECT samples was conducted at MDACC as previously reported (10). Briefly, the slide images were quantified using MicroVigene 4.0 (VigeneFech). The spot level raw data were processed using the R package SuperCurve (13, 14), which returns the estimated protein concentrations (raw concentration) and a QC score for each slide. Only slides with QC scores >0.8 were used for downstream analysis. Raw concentration data were normalized by median centering each sample across all the proteins to correct loading bias. Gene-expression analysis and sequencing of select genes were conducted as reported elsewhere (15–18).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PROSPECT (N = 93), n (%)</th>
<th>TCGA (N = 181), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>38 (41)</td>
<td>105 (58)</td>
</tr>
<tr>
<td>Male</td>
<td>55 (59)</td>
<td>76 (42)</td>
</tr>
<tr>
<td>Stage I</td>
<td>48 (52)</td>
<td>95 (52)</td>
</tr>
<tr>
<td>Stage II</td>
<td>26 (28)</td>
<td>39 (22)</td>
</tr>
<tr>
<td>Stage III</td>
<td>19 (20)</td>
<td>40 (22)</td>
</tr>
<tr>
<td>Stage IV</td>
<td></td>
<td>7 (4)</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td>40 (43)</td>
<td></td>
</tr>
<tr>
<td>No adjuvant chemotherapy</td>
<td>53 (57)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>8 (9)</td>
<td>27 (15)</td>
</tr>
<tr>
<td>Former</td>
<td>49 (52)</td>
<td>111 (62)</td>
</tr>
<tr>
<td>Current</td>
<td>36 (39)</td>
<td>35 (19)</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>8(4)</td>
</tr>
<tr>
<td>K-Ras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>26 (28)</td>
<td>47 (26)</td>
</tr>
<tr>
<td>Nonmutant</td>
<td>64 (69)</td>
<td>134 (74)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (3)</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>5 (5)</td>
<td>27 (15)</td>
</tr>
<tr>
<td>Nonmutant</td>
<td>50 (54)</td>
<td>154 (85)</td>
</tr>
<tr>
<td>Unknown</td>
<td>38 (41)</td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>0 (0)</td>
<td>13 (7)</td>
</tr>
<tr>
<td>Nonmutant</td>
<td>77 (82)</td>
<td>168 (93)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (10)</td>
<td></td>
</tr>
<tr>
<td>PI3KCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>7 (8)</td>
<td>13 (7)</td>
</tr>
<tr>
<td>Nonmutant</td>
<td>77 (82)</td>
<td>168 (93)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (10)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** LUAD clinical cohort characteristics

Published OnlineFirst April 15, 2015; DOI: 10.1158/1078-0432.CCR-14-3286
TCGA. Protein expression (RPPA) was measured at MDACC as previously described (12); gene expression and whole-exome sequencing data generated as described elsewhere (12) were downloaded from the TCGA website (19).

Cell lines. Cell lines were established by J.D. Minna and A. Gazdar at the National Cancer Institute and the University of Texas Southwestern Medical Center (Dallas, TX) or obtained from the ATCC, cells were grown in RPMI-1640 medium supplemented with 10% FBS unless specified by the ATCC. RPPA and gene-expression analysis of LUAD cell lines and cell line authentication (by DNA fingerprinting) were performed as described previously (11). Fingerprinting results were compared with reference fingerprints maintained by the primary source of the cell line. NKX2-1 gene expression was used as a surrogate for TTF1 expression as TTF1 RPPA data were available only for a subset of cell lines.

DNA repair score

The DNA repair score is a proteomic signature measuring the degree of expression of DNA repair proteins (20) derived by taking the sum expression levels of 17 DNA repair proteins that are coordinately expressed in lung cancer as measured by RPPA. Proteins in the DNA repair score are: pChk1, FANCD2, MRE11, pChk2, Chk2, ATRIP, pATM, XRCC1, 53BP1, DNA-PKcs, ATM, RAD50, NBS1, MSH2, PARP1, RAD51, and BRCA1.

Immunohistochemical analysis

TTF1 immunohistochemical analysis was published previously (3, 4). Briefly, 5 μmol/L FFPE sections were rehydrated, antigen retrieval was performed using a steamer (pH = 9), intrinsic peroxidase activity was quenched with 3% hydrogen peroxide, and 5% goat serum solution was used to block nonspecific binding before incubating with a primary antibody against TTF1 (dilution 1:100, clone 8G7G3/1; Cell Marque). After three washes, slides were incubated with Dako Envision + Dual Link. After three additional washes, slides were incubated with Dako chromagen substrate and counterstained with hematoxylin. Sections were scored by a thoracic pathologist by multiplying intensity (0–3+) and extent (0–100%) of staining by light microscopy (range, 0–300; ref. 3).

Drug sensitivity of cell lines

Drug resistance data (IC50 value) were downloaded from the GDSC website (21), the CCLE website (22), in addition to IC50 values generated in house measured by MTS assay three or more times in NSCLC (non–small cell lung cancer) cell lines (10). IC50 values were compared between cell lines in the highest and lowest third of NKX2-1 mRNA expression by the t-test.

Results

Clinical characteristics of LUAD cohorts and robust quantification of TTF1 by RPPA

To investigate the molecular profiles of TTF1-negative tumors, TTF1 expression was quantified at the protein and gene-expression levels and correlated with genomic and proteomic profiles from two large independent cohorts of LUAD. These included: 1) a cohort of 93 surgically resected LUADs from patients treated at MDACC (the "PROSPECT" cohort) and 2) 181 LUADs from TCGA.

Clinical features and select mutational profiles of the 93 tumors with RPPA data from the PROSPECT cohort are shown in Table 1. The clinical characteristics of the overall TCGA cohort were recently published (12) and included 181 resected LUAD tumors with RPPA proteomic data (Table 1 shows clinical and mutational characteristics of those tumors with full protein data used in this study). Both cohorts were similar in terms of patients' characteristics, including previous smoking status and mutation status. Both cohorts included a range of stage I–III tumors, although a majority were stage I (52% of both). Former and current smokers made up >80% of patients in both cohorts; KRAS mutations were present in 28% and 26% of patients (PROSPECT and TCGA, respectively); EGFR mutations in 3% and 15% (PROSPECT and TCGA, respectively).

Because TTF1 is typically measured in clinical practice by IHC, we first compared the quantification of TTF1 protein by IHC (the gold standard) with TTF1 protein levels measured by RPPA using the PROSPECT cohort. TTF1 protein expression, as measured by IHC, was quantified by a thoracic pathologist on a scale of 0 to 300 that reflects degree (0–3+) and extent (0%–100% of tumor cells) of staining positive for TTF1 (11). Fourteen tumors had an IHC score equal to zero (“TTF1 negative”) and 79 had positive values ranging from 7 to 300 (median 137). IHC scores were then correlated with TTF1 protein levels measured by RPPA (Fig. 1A) and NKX2-1 (the gene name for TTF1) mRNA levels (Fig. 1B) from the same tumors by Spearman rank correlation.

As shown in Fig. 1A and B, IHC scores were highly correlated with both RPPA protein measurements (Rho = 0.57) as well as mRNA (Rho = 0.61; P < 0.001 for both). Although IHC-negative tumors (score = 0) represented a majority of tumors with the lowest TTF1 levels by RPPA, some IHC-negative tumors had relatively higher levels when measured by RPPA. The range of TTF1 levels observed among IHC-negative tumors is likely due to a combination of the greater sensitivity of the RPPA platform to detect low levels of TTF1 (23), and the presence of TTF1-expressing bronchial epithelial cells (non–cancer cells; ref. 24) that represent a fraction of the total protein lysate used for RPPA. Indeed, the correlation between TTF1/NKX2-1 RNA and RPPA levels was greater than those of IHC with either technology consistent with this contention. Nevertheless, because cases in the PROSPECT and TCGA cohorts were required to have >70% tumor content, the extent to which non-cancer cells contribute to the levels of TTF1/NKX2-1 in the RPPA and gene-expression data is expected to be limited.

The strong correlation between IHC score, TTF1 protein (RPPA) levels, and NKX2-1 mRNA levels (Fig. 1A–C) indicates that the experimental methods subsequently used in this study to define TTF1 status (e.g., RPPA and mRNA levels) were robust and highly correlated with the clinical gold-standard of TTF1 quantification by IHC. By way of cross-validation, mRNA and protein (RPPA) expression values were compared in both PROSPECT and TCGA patients revealing very strong correlations (Fig. 1D Rho = 0.8, P < 0.001 for PROSPECT and Rho = 0.78 P < 0.001 for TCGA; Supplementary Fig. S1).

Low TTF1 expression is associated with worse outcome in LUAD

Measurements of TTF1 by RPPA revealed a bimodal distribution of low and high TTF1–expressing tumors in the PROSPECT and TCGA cohorts (Fig. 2A). Because of the highly quantitative nature of RPPA that allows for protein detection even at extremely low levels, we used this bimodal split to define TTF1-negative
versus TTF1-positive tumors. Using this approach, we classified 25% of tumors as TTF1-negative, similar to the 15% to 20% of LUADs reported in the literature as being TTF1-negative by IHC (25). Tumors classified as TTF1-positive by RPPA had corresponding median IHC scores of 145 (range, 0–300), whereas TTF1-negative tumors had median IHC scores of 20 (range, 0–190).

LUAD patients with TTF1-negative tumors as assessed by IHC have previously been described to have worse clinical outcomes (4). We confirmed this observation in our cohorts and investigated the association of TTF1 levels with other clinical characteristics. TTF1-negative tumors were found across all stages and grades of LUAD, with no significant difference in TTF1 levels between stages in either PROSPECT or TCGA cohorts (Fig. 2B). Although smoking history was also not statistically significantly associated with TTF1 expression ($P = 0.309$ for PROSPECT and $P = 0.242$ for TCGA), TTF1-negative tumors were infrequent among never smokers (2/8 in PROSPECT and 1/27 in TCGA; Fig. 2C).

Consistent with previously published studies (4), clinical outcome for patients with TTF1-negative tumors was worse in both cohorts (Fig. 2D). For example, patients with TTF1-negative, locally advanced (stage III) disease had significantly reduced overall survival (OS) among the TCGA LUAD cohort ($P < 0.001$), with TTF1-negative stage III PROSPECT patients also trending toward shorter survival ($P = 0.07$). Similar trends were observed in stage I and II disease where TTF1-negative tumors were associated with shorter survival; however, these did not reach statistical significance ($P = 0.17$ and 0.24 for TCGA). Consistent with findings from the TCGA (12), which described a low prevalence of TTF1-negative tumors in the terminal respiratory unit (TRU) group of LUAD, only 6% of TTF1-negative tumors in our analysis were classified as TRU.

However, we found no difference in the frequency of TTF1-negative tumors between the proximal proliferative and proximal inflammatory subtypes [47% (8/17) for both].

**Identification of druggable mutations in TTF1-negative LUAD**

Next, we assessed the relationship between TTF1 status and the incidence of established or potentially druggable mutations. For the most common of these, $EGFR$ mutations, prior studies have reported a lower incidence of $EGFR$ mutations among TTF1-negative LUAD (3). In our analysis, comparison of TTF1 expression between $EGFR$ mutant and wild-type tumors in the TCGA.
TTF1-negative and LUAD biopsy samples into new expression by RPPA (A) categorizing reveals bimodal distribution of TTF1 density plot analysis of both cohorts associated with worse clinical outcome. -mutated LUAD (Fig. 3A). Only two EGFR cohort showed high levels of TTF1 protein in the vast majority of EGFR-mutated LUAD (Fig. 3A). Only two EGFR-mutated TCGA samples (one patient had an L861Q alteration and the other both G719A and S768I alterations) were observed among 17 TTF1-negative tumors. However, these mutations are non-classical (26) and their potential association with EGFR TKI response in patients is not fully characterized. Although the G719A and S768I mutations individually are associated with increased EGFR inhibitor sensitivity, there are conflicting reports as to how these mutations interact when co-occurring in the same tumor (27). Similar to the TCGA cohort, in the PROSPECT group, tumors with EGFR mutations (n = 5) had some of the highest levels of TTF1 across the entire group and no EGFR mutations occurred in TTF1-negative cancers (Fig. 3A).

Analysis comparing TTF1 expression between wild-type and mutant groups for other significantly mutated genes in the TCGA cohort (12) identified additional associations between TTF1 status and mutations. Here, we show that BRAF mutations (n = 13) are associated with high TTF1 expression and no BRAF mutations are observed among TTF1-negative tumors (Fig. 3A), in agreement with a previous study that reported BRAF V600E mutations in TTF1-positive LUAD (28). Given the recent granting of breakthrough therapy designation for the BRAF inhibitor dabrafenib in BRAF-mutant NSCLC (29–31), this observation may have clinical relevance for the selection of LUAD patients for BRAF testing.

Other potentially druggable mutations occurring preferentially in TTF1-positive LUAD included HER2 (ERBB2) mutations (n = 21, all TTF1 positive in TCGA) and PIK3CA mutations (all 13 occurring in the TTF1-positive group in the TCGA cohort; 6/7 in PROSPECT). In contrast, there was no significant association observed between STK11 (LKB1), KRAS, ALK (fusion), or TP53 alterations and TTF1 status. Mutations in RIT1 and NF1 were recently identified as novel candidate driver genes in oncogene-negative LUAD by the TCGA. Of these, we found RIT1 mutations to be more frequent in TTF1-negative LUAD 12% (2/17) versus TTF1-positive 2% (3/164), although this was not statistically significant (P = 0.07). As expected, the overall oncogene-positive group of TCGA tumors had a trend toward higher TTF1 protein expression as compared with oncogene-negative tumors (P = 0.07).

We then investigated mutations that were enriched in the TTF1-negative group. This analysis revealed a new association between mutations in kelch-like ECH-associated protein 1 (KEAP1) and TTF1-negative tumors (Fig. 3B and C). Specifically, KEAP1 mutations were observed more frequently in TTF1-negative tumors (53% of TTF1-negative tumors as compared with 16% of TTF1-positive; P = 0.001). KEAP1 is a substrate adapter protein for the E3 ubiquitin ligase complex that targets the transcription factor nuclear factor (erythroid 2)-like 2 (Nrf2) for ubiquitination and degradation by the proteasome. Nrf2 is a master regulator of the antioxidant response, the accumulation of which has been shown to be associated with poor prognosis in a number of cancers, including lung cancer (32, 33). Nrf2 is being explored as a therapeutic target in preclinical studies in pancreatic and breast cancers.
Integrated analysis reveals distinct protein signature in TTF1-negative LUAD

To identify potential therapeutic targets or activated pathways in TTF1-negative tumors, we next performed an integrated analysis comparing protein and mRNA expression in TTF1-negative versus TTF1-positive tumors from PROSPECT and TCGA. In the PROSPECT cohort, 127 total or phosphorylated proteins were measured by RPPA and differences in protein expression between TTF1-positive and -negative tumors were assessed by t-test. This analysis revealed significant, global differences in protein expression in TTF1-negative tumors with 71 of 127 (56%) proteins and/or phospho-proteins significantly different between TTF1-negative and -positive tumors (at a P value of <0.05; Supplementary Table S1). Of these 71 proteins, 14 were expressed at lower levels in TTF1-negative samples, including a number of phosphorylated proteins in the MAPK and PI3K/mTOR pathways—suggesting relatively lower levels of activation of these pathways in TTF1-negative tumors. In contrast, of the 58 proteins that were higher in TTF1-negative LUAD, there was striking enrichment of proteins involved in DNA repair, cell-cycle regulation, and apoptosis (Fig. 4A).

These findings were then compared with differences between TTF1-negative and -positive tumors in the TCGA cohort. Again, we observed a large number of protein markers (55/160) that were different between the TTF1-negative and -positive groups (P < 0.05; Supplementary Table S2). Similar to PROSPECT, proteins expressed at lower levels in TTF1-negative tumors were highly enriched for those in the PI3K/mTOR (e.g., p-mTOR and pAkt) and MAPK (e.g., pMAPK) pathways and their shared downstream target pS6 (Fig. 4A). Conversely, as in the PROSPECT cohort, the 36 targets higher in TTF1-negative LUAD were often involved in DNA repair, cell-cycle regulation, and apoptosis (Fig. 4A).

To identify those proteins with the strongest relationship to TTF1 status, we compared the common protein differences...
between the two cohorts. 25 proteins were commonly associated with TTF1 status (positively or negatively correlated) in both cohorts (Fig. 4B). These included Cyclin B1 ($P = 0.049$ in PROSPECT, $P < 0.001$ in TCGA), thymidylate synthase (TS; $P < 0.001$, $P = 0.005$), Chk1 ($P < 0.001$, $P = 0.017$), Nrf2 ($P = 0.036$, $P = 0.001$; all higher in TTF1-negative tumors), and phosphorylated S6 kinase ($P = 0.002$, $P < 0.001$; lower in TTF1-negative tumors). The higher incidence of mutations in genes such as PIK3CA, EGFR, BRAF, and HER2 in the TTF1-positive tumors may, in part, explain the higher levels of phosphorylated S6 seen in TTF1-positive tumors, because pS6 is a downstream target of pathways activated by these genes (e.g., PI3K and MAPK). Comparing TTF1 and other proteins as continuous variables in PROSPECT also showed significant correlations between TTF1 and 22 of the 25 commonly associated proteins ($FDR/C20 < 0.05$, Supplementary Table S3).

Having observed higher expression of several DNA repair genes in TTF1-negative tumors (e.g., Mre11, Chk1, and PCNA), we applied our previously characterized “DNA repair score” (20) to the protein expression data in PROSPECT and TCGA. As shown in Fig. 4C, the DNA repair score was higher in TTF1-negative samples from the PROSPECT cohort ($P = 0.037$).
TCGA data also trended toward a higher DNA repair score in TTF1-negative tumors \((P = 0.072)\), but the analysis was limited as the TCGA RPPA analysis includes only 9 of the 17 components of the score. As we would predict, based on the higher frequency of \(\text{KEAP1}\) loss in TTF1-negative LUAD (described above in Fig. 3B) and its role as a negative regulator of Nrf2, we also observed higher expression of Nrf2 protein in TTF1-negative tumors (TCGA \(P = 0.001\), PROSPECT \(P = 0.005\); Fig. 3D). Nrf2 and TTF1 proteins also showed a negative correlation when examined as continuous variables (TCGA \(R^2 = 0.27\), \(P < 0.001\)), further confirming the relationship.

TTF1-negative and -positive LUAD have clear differences at the mRNA level (Supplementary Fig. S2). Therefore, as a method of validating the observed differences in total protein levels between the TTF1-positive and -negative tumors, we then compared differences in the corresponding mRNA levels for 18 targets where the antibody used detected total (rather than phosphorylated) protein levels. Five of 15 total proteins identified as more highly expressed in TTF1-negative tumors were also significantly elevated in these tumors at the mRNA level \((P < 0.05)\). These were Cyclin E1, Chk1, 4EBP1, Smad3, and N-Cadherin that were higher at the protein and mRNA levels in both cohorts (Fig. 4D). The only target commonly lower in TTF1-negative tumors across both cohorts and both platforms was TTF1 itself (of three proteins identified).

We have previously observed increased expression of DNA repair proteins in SCLC (small cell lung cancer), which we hypothesize to be driven through increased activity of the transcription factor E2F1 (11). We thus looked for alterations in the TTF1-negative population that could affect E2F1 regulation. The activity of the transcription factor E2F1 can be regulated in a number of ways, including by RB1 (11, 20), \(\text{CDKN2A} (\text{p16}; \text{ref. 34})\), and \(\text{c-Myc} (35)\). Our analysis reveals no difference in RB1 protein, copy number or mutation rate between the TTF1-positive and -negative groups. TTF1-negative LUAD does, however, show higher levels of \(\text{c-Myc}\) expression (Fig. 4B), potentially driving E2F1 activity through miR-17-5p and miR-20a (35). In addition, copy-number analysis in the TCGA cohort reveals \(\text{CDKN2A}\) loss to be strongly associated with reduced TTF1 expression \((P < 0.001)\) with \(\text{CDKN2A}\) copy-number loss observed in 7

*Figure 5.* Therapeutic targets in TTF1-low LUAD cell lines. Comparison of the top and bottom thirds of cell lines by \(\text{NKX2-1}\) expression (A) to IC\(_{50}\) values from drug sensitivity databases identify TTF1-low LUAD cell lines as being more sensitive to agents that impair DNA repair (B and C). Potential mechanisms for the higher expression of thymidylate synthase and DNA repair proteins via E2F1 in TTF1-negative LUAD and elevated PI3K–MAPK signaling in TTF1-positive LUAD (D) combining genetic and protein observations. Proteins/genes in red are higher/more active in TTF1-negative, those in black in TTF-positive LUAD. Copy-number loss of \(\text{SDOX}\) or gain of \(\text{CDKN2A}\) can both lead to increased E2F1.
of 17 TTF1-negative tumors. RB1 and CDKN2A both inhibit E2F1; RB1 and CDKN2A loss, thus, similarly enhance E2F1 activation (20, 34), and may therefore also contribute to the higher DNA repair score seen in TTF1-negative LUAD. These potential mechanisms for driving E2F1 in TTF1-negative LUAD are shown in Fig. 5D. The inverse relationship between TTF1 status and TS (another target of E2F1; ref. 36) is in agreement with previous observations that low TS and high TTF1 expression associate with a better response to pemetrexed in non-squamous NSCLC (37, 38).

Other proteins expressed at higher levels in TTF1-negative cohorts, including c-Myc, 4EBP1, and EGFR, are downstream targets of the transcription factor sex determining region Y-box 2 (SOX2; ref. 39). SOX2 has previously been characterized as both a marker (40) and driver (41, 42) of lung squamous carcinoma where SOX2 amplification is commonly observed and results in increased tumor formation, migration, and proliferation through the activation of hedgehog signaling (23). Copy-number analysis between TTF1-negative and -positive tumors in the TCGA cohort reveals SOX2 copy-number gain in the TTF1-negative population ($P = 0.001$). SOX2 copy-number gain has been associated with increased SOX2 expression (43), suggesting a potential mechanism for the higher expression of targets such as c-Myc (and therefore E2F1, which can be activated by c-Myc; Fig. 5D), 4EBP1, and EGFR in TTF1-negative tumors. An analysis of gene expression across all tumors in the larger PROSPECT dataset (LUAD and lung squamous carcinoma) shows an inverse correlation between SOX2 and TTF1 (Rho = −0.39, $P < 0.001$) further supporting that higher SOX2 may be one mechanism driving the molecular differences observed in TTF1-negative disease. Furthermore, SOX2 is also frequently amplified in SCLC (42), suggesting that TTF1-negative LUAD may be molecularly similar to SCLC. Interestingly, the proteomic differences between TTF1-negative and -positive overlap with those observed between SCLC and NSCLC (11).

**Therapeutic targets in TTF1-low LUAD**

As illustrated above, most of the known targetable populations in LUAD (e.g., EGFR and BRAF mutants, ALK fusions) are found in TTF1-positive adenocarcinomas. Because TTF1-negative patients have worse clinical outcomes and currently lack validated therapeutic targets of the transcription factor sex determining region Y-box 2 (SOX2; ref. 39). SOX2 has previously been characterized as both a marker (40) and driver (41, 42) of lung squamous carcinoma where SOX2 amplification is commonly observed and results in increased tumor formation, migration, and proliferation through the activation of hedgehog signaling (23). Copy-number analysis between TTF1-negative and -positive tumors in the TCGA cohort reveals SOX2 copy-number gain in the TTF1-negative population ($P = 0.001$). SOX2 copy-number gain has been associated with increased SOX2 expression (43), suggesting a potential mechanism for the higher expression of targets such as c-Myc (and therefore E2F1, which can be activated by c-Myc; Fig. 5D), 4EBP1, and EGFR in TTF1-negative tumors. An analysis of gene expression across all tumors in the larger PROSPECT dataset (LUAD and lung squamous carcinoma) shows an inverse correlation between SOX2 and TTF1 (Rho = −0.39, $P < 0.001$) further supporting that higher SOX2 may be one mechanism driving the molecular differences observed in TTF1-negative disease. Furthermore, SOX2 is also frequently amplified in SCLC (42), suggesting that TTF1-negative LUAD may be molecularly similar to SCLC. Interestingly, the proteomic differences between TTF1-negative and -positive overlap with those observed between SCLC and NSCLC (11).

As illustrated above, most of the known targetable populations in LUAD (e.g., EGFR and BRAF mutants, ALK fusions) are found in TTF1-positive adenocarcinomas. Because TTF1-negative patients have worse clinical outcomes and currently lack validated therapeutic targets, there is a critical unmet need to develop more effective therapies for these patients. To identify drugs that may have activity in TTF1-negative LUAD, we compared in *vitro* drug sensitivity (based on IC₅₀ values) between cell lines with the highest versus lowest TTF1 expression (based on NKX2-1 mRNA levels; Fig. 5A). As with PROSPECT and TCGA patient tumors, mRNA and protein were highly correlated for NKX2-1/TTF1 (Rho = 0.61, $P < 0.001$). This analysis assessed 129 targeted drugs and/or chemotherapies using in *vitro* data from our groups and public databases (21, 22).

Using this approach, we identified ten drugs with relatively greater activity in cell lines with low TTF1 expression (Fig. 5B and Supplementary Fig. S3, $P < 0.05$). Consistent with our observations of higher expression of individual DNA repair proteins and a higher DNA repair score in TTF1-negative tumors in patient tumors (above), five of the eight drugs to which TTF1-low cell lines were more sensitive target components of DNA repair as either primary or secondary-target effects (Fig. 5C, Supplementary Table S4). NVP-BEZ235 ($P < 0.001$), for example, although developed as a PI3K inhibitor, is also a potent inhibitor of ATM, and recent reports suggest that its primary antitumor effect is through ATM inhibition (44). Other drugs identified—GD0941, AZD8055, and ABT-888 (veliparib)—inhibit DNA-PK, ATM, and PARP, respectively, all components of DNA repair. When the DNA-repair signature is applied to the cell lines, the cell lines in the bottom third of *NKX2-1* expression have a higher score than those in the top third (Supplementary Fig. S4, $P = 0.063$).

Among the chemotherapies tested, paclitaxel (a standard frontline chemotherapy for NSCLC) showed significantly less activity in cell lines with low TTF1 expression ($P = 0.023$). This decreased sensitivity to a chemotherapy drug commonly used in locally advanced disease (e.g., with chemoradiation), and in the metastatic setting may contribute to the worse outcomes of TTF1-negative patients. A similar trend was observed with a second chemotherapy, pemetrexed ($P = 0.158$) that has previously been reported to be more effective in TTF1-positive and thymidylate synthase low-expressing LUAD (45).

**Discussion**

TTF1 is a clinical marker of LUAD that is typically scored as positive (1–3+) or negative (0) by IHC. In this study, using two independent clinical cohorts, we tested a new approach to measuring TTF1 protein expression using RPPA. Measurements by RPPA were highly correlated with the gold-standard of IHC, as well as with mRNA expression levels. TTF1 levels in patient tumors (based on RPPA and gene expression) were bimodally distributed, with approximately 25% falling into the group with lowest TTF1 expression (subsequently referred to in this analysis as TTF1-negative). Consistent with previous reports, TTF1-negative tumors from the PROSPECT and TCGA cohorts had worse survival outcomes. However, we did not observe significant differences in smoking status, stage, or other clinical characteristics based on TTF1 status.

Gene sequencing performed in PROSPECT and TCGA afforded the opportunity to investigate the relationship between TTF1-status and -specific gene mutations. One of the most striking and novel observations from this analysis was that BRAF mutations were observed only in patients with very high TTF1 levels. BRAF inhibition has recently received breakthrough therapy designation for the treatment of BRAF-mutant NSCLC (29–31). However, the frequency of this alteration in the overall population of LUAD is low (<10%; 12). Therefore, our observation suggests that BRAF testing is likely to be the most clinically relevant and cost effective in TTF1-positive LUADs. Other mutations such as EGFR and PIK3CA also occurred most commonly in tumors with the highest levels of TTF1 protein expression (top third of TTF1 expression). The prevalence of these mutations may explain the higher activation of the mTOR–AKT and MAPK pathways (downstream targets of EGFR, BRAF, and PI3K) in TTF1-positive tumors observed in our proteomic analysis. Copy-number gain at the *NKX2-1* locus is seen in 15% of TTF1-positive patients in the TCGA cohort, explaining the observed TTF1 levels in a subset of TTF1-positive patients. For patients without copy-number alterations, however, increased *NKX2-1* transcription may account for the observed levels of *NKX2-1* mRNA and resulting TTF1 protein. *NKX2-1* is promoted by a number of transcription factors, including specificity protein 1 (Sp1; ref. 46), that is a direct target of ErbB4 and the ERK and Akt pathways—all of which we show are more highly expressed in TTF1-positive LUAD (47, 48).
In contrast, KEAP1 mutations were enriched in TTF1-negative tumors (53% vs. 16%, \( P = 0.001 \)) with resulting overexpression of its protein target Nrf2 (Nrf2 is expression is high in all TTF1-negative LUAD). Drugs with potential activity against Nrf2 have been described, suggesting that KEAP1-Nrf2 alterations in TTF1-negative disease may represent a therapeutic vulnerability for future investigation. For example, alkaloid trigonelline, an inhibitor of Nrf2, renders pancreatic cancer more susceptible to apoptosis (49); ochratoxin inhibits the Nrf2 oxidative stress response pathway (50); and brusatol increases intracellular ROS-sensitizing mammospheres to taxol (51). Thus, an Nrf2 inhibitor or the inhibition of Nrf2 by increasing the expression of caveolin (52) may be of therapeutic benefit in all TTF1-negative LUAD.

Among the other proteins that we found to be highly expressed in TTF1-negative LUAD, several were targets of the SOX2 transcription factor (e.g., cMyc, 4EBP1, and Cyclin D1; refs. 39, 53). In contrast with TTF1, SOX2 is a marker of lung squamous carcinoma and is also frequently amplified in SCLC (42). To further explore a potential role for SOX2 in TTF1-negative adenocarcinomas, we performed a copy-number analysis of SOX2 in the TCGA LUAD cohort. This revealed a subset of TTF1-negative TCGA tumors with SOX2 copy-number gain (n = 3/17). The higher expression of SOX2 targets in TTF1-negative LUAD, combined with more frequent SOX2 amplification events in this group, suggest that the biology of TTF1-negative LUAD may be more similar to squamous lung cancers.

Finally, our results suggest a potential role for DNA repair inhibitors in TTF1-negative LUAD based on 1) proteomic tumor profiles showing higher expression of individual DNA repair proteins and the DNA repair score in TTF1-negative LUAD and 2) greater sensitivity of TTF1-negative LUAD models to DNA repair inhibitors. Previously, our group demonstrated that lung cancer models of SCLC with elevated DNA repair scores are more sensitive to PARP inhibition (20). Consistent with this, we found that TTF1-negative LUAD cell lines had higher DNA repair scores and demonstrated greater sensitivity to the PARP inhibitor veliparib (Fig. 4B). Our previous work in SCLC has shown not only a correlation between DNA repair protein expression and sensitivity to PARP inhibition, but also an inverse correlation to PI3K pathway activity (20)—an observation recapitulated here that may apply more broadly to lung cancers. Association between DNA repair protein expression and susceptibility to DNA repair antagonists is of particular clinical importance, given that the PARP inhibitor veliparib is in clinical trial testing for NSCLC patients (NCT02106546). In addition, several other small-molecule inhibitors with activity against DNA repair targets were also among the top drugs with preferential activity in TTF1-negative cell lines. These included GDC0941, NVP-BEZ235, and AZD8055, which have activity against DNA-PK, ATR, and ATM. In summary, molecular profiling shows TTF1-negative LUAD lack BRAF mutations, but have potential sensitivity to agents targeting the KEAP1/Nrf2 and DNA repair pathways, observations with clinical implications both for which patients should be screened for treatment with BRAF inhibitors and the potential for targeted treatments in this subset of LUAD.

Disclosure of Potential Conflicts of Interest
L.A. Byers is a consultant/advisory board member for AbbVie and BioMint. J.V. Heymach reports receiving commercial research grants from AstraZeneca and is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Exelixis, Genentech, GlaxoSmithKline, Lilly, and Syntha. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: R.J.G. Cardnell, L.A. Byers
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Behrens, Y.H. Fan, X. Tang, J.D. Minna, J. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.J.G. Cardnell, C. Behrens, L. Diao, P. Tong, G.B. Mills, I.V. Heymach, J. Wang, L.A. Byers
Writing, review, and/or revision of the manuscript: R.J.G. Cardnell, J.D. Minna, G.B. Mills, I.I. Wistuba, L.A. Byers
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.B. Mills, J.V. Heymach, L.A. Byers
Study supervision: J. Wang, L.A. Byers
Other (grant support): J.D. Minna

Grant Support
This work was supported by DoD PROSPECT grant W81XWH-07-1-0306; TCGA (S1124-CA143883-05); NIH Cancer Center Support Grant (CCSG) CA016672 (MDACC); NIH SPORE grant P50CA097007 (MDACC), MDACC Physician Scientist Award (to L.A. Byers), and NCI Cancer Clinical Investigator Team Leadership Award (P30 CA016672; to L.A. Byers).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 22, 2014; revised March 18, 2015; accepted April 8, 2015; published OnlineFirst April 15, 2015.

References


19. The results shown here are in whole or part based upon data generated by the TCGA Research Network. Available from: http://cancergenome.nih.gov/.


An Integrated Molecular Analysis of Lung Adenocarcinomas Identifies Potential Therapeutic Targets among TTF1-Negative Tumors, Including DNA Repair Proteins and Nrf2

Robert J.G. Cardnell, Carmen Behrens, Lixia Diao, et al.

Clin Cancer Res  Published OnlineFirst April 15, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-14-3286

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2015/04/16/1078-0432.CCR-14-3286.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.