Lineage-Specific Dependency of Lung Adenocarcinomas on the Lung Development Regulator TTF-1

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Abstract
Emerging evidence, although currently very sparse, suggests the presence of “lineage-specific dependency” in the survival mechanisms of certain cancers. TTF-1 has a decisive role as a master regulatory transcription factor in lung development and in the maintenance of the functions of terminal respiratory unit (TRU) cells. We show that a subset of lung adenocarcinoma cell lines expressing TTF-1, which presumably represent those derived from the TRU lineage, exhibit marked dependence on the persistent expression of TTF-1. The inhibition of TTF-1 by RNA interference (RNAi) significantly and specifically induced growth inhibition and apoptosis in these adenocarcinoma cell lines. Furthermore, a fraction of TTF-1–expressing tumors and cell lines displayed an increase in the gene dosage of TTF-1 in the analysis of 214 patients with non–small-cell lung cancer, including 174 adenocarcinomas, showing a tendency of higher frequency of increased gene copies at metastatic sites than at primary sites (P = 0.07, by two-sided Fisher’s exact test). These findings strongly suggest that in addition to the development and maintenance of TRU lineages in normal lung, sustained TTF-1 expression may be crucial for the survival of a subset of adenocarcinomas that express TTF-1, providing credence for the lineage-specific dependency model. [Cancer Res 2007;67(13):6007–11]

Introduction
Accumulating evidence strongly supports the presence of oncogene addiction in certain cancers (1), with lung adenocarcinomas carrying epidermal growth factor receptor (EGFR) mutations among the best examples (2). However, emerging evidence, although currently very sparse, suggests that “lineage-specific dependency” on survival mechanisms that are programmed for the developmental roles in normal progenitor cells of particular lineages may exist in cancer cells (3, 4). The basic helix-loop-helix (bHLH) transcription factor MITF in melanoma was proposed as an archetypal prototype (5–8), whereas we have suggested adding another bHLH protein, achaete-scute homologue 1 (ASH1)/achaete-scutecomplex-like 1 to (5–8), whereas we have suggested adding another bHLH protein, achaete-scute homologue 1 (ASH1)/achaete-scutecomplex-like 1 to (5–8), whereas we have suggested adding another bHLH protein, achaete-scute homologue 1 (ASH1) and TTF-1 deficiency in mice results in lung aplasia (12). These genes are presumed to arise from the presumed progenitor TRU cells. Nkx2.1 or thyroid-specific enhancer-binding protein, is a homeo-domain-containing transcription factor expressed in a very restricted set of cells in the human body, including the TRU cells in the lung, such as alveolar pneumocytes (11). TTF-1 plays essential crucial roles in the development of the peripheral lung, and TTF-1 deficiency in mice results in lung aplasia (12). These data prompted us to investigate the potential involvement of TTF-1 in the pathogenesis of adenocarcinomas, a major fraction of which are presumed to arise from the presumed progenitor TRU cells.

Materials and Methods
TTF-1–RNAi treatment. A TTF-1–RNAi plasmid expressing double-stranded short hairpin RNA (shRNA) was constructed by inserting the siTTF-1 oligonucleotide and a scrambled oligonucleotide containing the loop sequence indicated in lowercase letters (5′-ACCAGCAACACATTGAAATTCAAGGTTTCTCAGGTGA-3′ and 5′-CGTAAACGGAAGCGCAATGCTCA-3′, respectively) into the human H1-promoter-driven expression vectors, pH1-RNApuro and pH1-RNAneo (13). The processing and expression of the small interfering RNA (siRNA) molecule were confirmed by Northern blot analysis using the corresponding oligonucleotide probes. For the TTF-1 expression vector, full-length human TTF-1 cDNA was amplified and inserted into pcDNA3 (Invitrogen) and sequenced thoroughly. RNAi-resistant mutant TTF-1 was constructed by PCR amplification using Pfu Turbo DNA Polymerase (Stratagene) and a primer containing silent mutations: 5′-TATCAAGTACAGTGCACCGCATCGTAAT-3′ (mutated residues are underlined). The derivation and culture conditions of cell lines were as described previously (9, 13). Conditions of transfections and subsequent selection processes are summarized as Supplementary data.

Western blot analysis and flow-cytometric analysis. Western blot analysis was done using the following antibodies: anti-TTF-1 (DAKO), anti-cleaved caspase-3, and anti-cleaved caspase-7 (Cell Signaling Technology). Details of flow-cytometric analysis using cells transfected with the TTF-1–RNAi plasmid are provided as Supplementary data.

Southern blotting, TaqMan gene dosage, real-time reverse transcription-PCR and PCR–single-strand conformational polymorphism analyses. Southern blot analysis was done using a TTF-1 cDNA probe,
essentially as described previously (13). C3β, a T-cell receptor β-chain cDNA fragment, was used as the loading control. TaqMan-based gene dosage analysis was also done to measure the gene copies of TTF-1 using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions.

The sequences of primers and probes used for gene copy analysis are given in Supplementary Table S1. Fold increase in copy number was calculated as the ratio of the TTF-1 signal to the albumin gene signal normalized to that of a DNA mixture from five normal lungs, which was taken to be one. TTF-1 gene expression was analyzed using TaqMan gene expression assay (Assay ID: Hs00968399_m1, Applied Biosystems), according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) amplifications of TTF-1, SP-B, and 18S RNA were done using the primer sets in Supplementary Table S2. The entire coding region of the TTF-1 gene was examined by PCR-single-strand conformational polymorphism (SSCP) analysis using genomic DNAs and the nine sets of primers shown in Supplementary Table S3.

Approval for this study was obtained from the institutional review boards of both Nagoya University and the Aichi Cancer Center to examine a series of 36 adenocarcinomas as well as a series of 214 non–small-cell lung cancers (NSCLC), three small-cell lung cancers (SCLCs), and two carcinoids. The NSCLCs included 174 adenocarcinomas, 8 of which were bronchioalveolar carcinomas, as well as 32 squamous cell carcinomas, 4 large-cell carcinomas, 1 adenosquamous cell carcinoma, and 3 large-cell neuroendocrine carcinomas.

Fluorescence in situ hybridization analysis and immunohistochemistry. Dual-color fluorescence in situ hybridization (FISH) was done using the SK-LC-5 cell line and tumor specimens, which have been shown to contain increased copy numbers of the TTF-1 gene. Two bacterial artificial chromosome DNAs, RP11-465B6, which spans the TTF-1 locus (14q13.2b-14q13.3a), and RP11-368C9 (14q11.2g-14q12a), which corresponds to a locus near the centromere and was used as a reference, were labeled with SpectrumGreen-dUTP and SpectrumOrange-dUTP, respectively, using a nick translation kit (Vysis Inc.) according to the manufacturer's instructions. A monoclonal antibody (clone 8G7G3; DAKO) was used for the immunohistochemical detection of TTF-1 as described previously (11). Molecular localization was visualized with the diaminobenzidine reaction.

Results

Western blot analysis was done to examine TTF-1 expression in a panel of lung adenocarcinoma cell lines (Fig. 1A). NCI-H358 and A427 cells, with readily detectable TTF-1 expression, and NCI-H23, A549, and SK-LC-7 cells, which do not express TTF-1, were selected for further studies. The introduction of human H1-promoter–driven plasmids expressing shRNA directed against TTF-1 mRNA clearly diminished TTF-1 expression in the NCI-H358 and A427 cells (Fig. 1A; data not shown for A427), resulting in a marked reduction in colonies of both NCI-H358 and A427 cells (Fig. 1B and C, data not shown for A427 in Fig. 1B) In contrast, NCI-H23, A549, and SK-LC-7 cells showed no reduction in number of colonies after the same treatment with the TTF-1-shRNA–expressing plasmid (Fig. 1C for HCl-H23 and Supplementary Fig. S1 for A549 and SK-LC-7). Furthermore, the introduction of shRNA-expressing plasmids directed against either scrambled TTF-1 or green fluorescent protein (GFP) sequences caused no reduction in the number of colonies in any of the cell lines tested (Fig. 1B and C as well as Supplementary Fig. S1, data not shown for GFP-shRNA, done with all but A549). Flow-cytometric analysis showed an increase in the sub-G1 population of NCI-H358 cells after the introduction of the TTF-1–RNAi expression construct, suggesting that the induction of apoptosis is part of the mechanism for this reduction (Fig. 1D). The results of Western blot analysis showed increased cleaved caspase-3 and caspase-7 in TTF-1–RNAi–treated NCI-H358 cells, further supporting this notion (Fig. 1D). Taken together, these data strongly suggest that lung adenocarcinoma cell lines expressing TTF-1 are dependent on the sustained expression of the TRU-lineage–specifying transcription factor, TTF-1, for cell survival and proliferation.

To clarify the specificity of the TTF-1–RNAi effects, a TTF-1 expression construct carrying silent mutations at the siRNA-binding site was used. Western blot analysis of 293T cells, which were cotransfected with the TTF-1–RNAi and either mutant or wild-type TTF-1–expressing vectors, confirmed the RNAi-resistant nature of the mutant TTF-1 (Fig. 2A). After cotransfection with the mutant but not wild-type TTF-1, inhibition of cell growth by TTF-1–RNAi treatment was clearly canceled, as shown by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 2B). Flow-cytometric analysis of NCI-H358 cells that were cotransfected with the mutant TTF-1 showed similar abolition of the TTF-1–RNAi–induced induction of apoptosis (Fig. 2C). These findings clearly imply that the TTF-1–RNAi–induced effects were specifically elicited by the inhibition of the expression of TTF-1 itself.

The identification of dependency on sustained TTF-1 expression in adenocarcinoma cells prompted us to investigate alterations in the TTF-1 gene in 36 adenocarcinoma specimens, leading to the identification of more than 5-fold TTF-1 expression in 17% of the specimens in real-time reverse transcription-PCR (RT-PCR) analysis as well as of the absence of somatic mutations in PCR–SSCP analysis (data not shown). We next examined the dosage of the TTF-1 gene as another possible genetic alteration. TaqMan PCR-based duplex gene dosage analysis of the TTF-1 and albumin genes, which gave results similar to those achieved with Southern blot analysis (Fig. 3A), revealed an increase of about 2-fold in a single specimen (data not shown). Because this increase may have been underestimated due to inevitable contamination with normal stromal cells, we further analyzed DNAs from grossly microdissected tumor specimens of another independent cohort consisting of 214 patients with NSCLC, including 174 adenocarcinomas, as well as three patients with SCLC and two with carcinoids. An increase of more than 2.5-fold in the dosage of the TTF-1 gene was detected in four (2.3%) of these 174 adenocarcinomas, including that of patient 38, which had a 20-fold increase, and in two (66.7%) of the three large-cell neuroendocrine carcinomas, which are a rare type of NSCLC known to express exceptional levels of TTF-1 (ref. 14; Fig. 3B). We noted that the frequency of increased gene copies at metastatic sites tended to be higher than that at primary sites (13% versus 2%, respectively, for increases of >2.5-fold; P < 0.07 by two-sided Fisher’s exact test). Dual-color FISH analysis confirmed marked amplification of TTF-1 in the distant metastasis of patient 38 (Fig. 3C). All the samples with increased TTF-1 gene copies were shown by immunohistochemical analysis to express the gene product (data shown for patient 38 in Fig. 3B). Similarly, patient 56 also showed a significantly increased number of TTF-1 gene copies, especially in a metastatic lymph node (Supplementary Fig. S2).

To investigate the potential effects of TTF-1 expression on cell growth in lung epithelial cells, we introduced TTF-1 into an immortalized human peripheral lung epithelial cell line, HPL1D, which does not express TTF-1 (15). Interestingly, both colony formation and MTT assays showed significant growth inhibition in HPL1D cells when exogenously introduced TTF-1 was overexpressed (Fig. 4A and B), whereas RT-PCR analysis showed that the overexpression of TTF-1 induced the expression of surfactant protein B, a differentiation marker for TRU cells (Fig. 4C). These findings suggest that the overexpression of TTF-1 may have inhibitory effects on the growth of normal lung epithelial cells in vitro.

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It is well recognized that various cancer phenotypes are closely associated with specific cell lineages, suggesting that cellular mechanisms that normally govern lineage development also affect carcinogenic processes (16). The lineage-specific dependency concept emphasizes the lineage-specific dependence of cancer cells for their survival and/or proliferation on cellular lineage–governing master regulatory genes and the importance of these genes in shaping the range of cancer-specific genetic and epigenetic alterations in a lineage-specified manner (3). The MITF gene in melanoma was previously presented as an archetypal prototype (5–8). We have suggested adding ASH1 in small-cell lung cancers, to the nearly empty list of prototypic lineage-dependency genes for which experimental evidence already exists (4, 9).

**Figure 1.** Dependency on TTF-1 in adenocarcinomas of the lung. A, Western blot analysis of TTF-1 in a panel of cell lines (top) as well as in NCI-H358 cells after the introduction of a TTF-1–RNAi (siTTF-1)– or its scrambled RNAi (siScr)–expressing constructs (bottom). B, representative phase-contrast micrographs of NCI-H358 cells after puromycin selection, showing marked growth inhibition induced by TTF-1–RNAi. C, representative photographs of Giemsa-stained dishes of TTF-1–RNAi transfectants after neomycin selection, as well as quantitative results that showed markedly reduced numbers of NCI-H358 and A427 cell colonies, but not of NCI-H23 cell colonies. D, detection of induction of apoptosis by flow-cytometric analysis (left) as well as by Western blot analysis using caspase-3 and caspase-7 antibodies (right).

**Discussion**

It is well recognized that various cancer phenotypes are closely associated with specific cell lineages, suggesting that cellular mechanisms that normally govern lineage development also affect carcinogenic processes (16). The lineage-specific dependency concept emphasizes the lineage-specific dependence of cancer cells for their survival and/or proliferation on cellular lineage–governing master regulatory genes and the importance of these genes in shaping the range of cancer-specific genetic and epigenetic alterations in a lineage-specified manner (3). The MITF gene in melanoma was previously presented as an archetypal prototype (5–8). We have suggested adding ASH1 in small-cell lung cancers, to the nearly empty list of prototypic lineage-dependency genes for which experimental evidence already exists (4, 9). Previously, we reported significant distinctions between TRU- and non-TRU–type adenocarcinomas in terms of clinicopathologic features, as well as genetic and epigenetic...
changes (10, 11). Our present findings seem consistent with and support the lineage-specific dependency concept. TTF-1 has already been shown to play a decisive role in the development and maintenance of the normal functions of the lung (12, 17). We have shown here that the sustained expression of TTF-1 is required to support the survival of adenocarcinomas that retain TTF-1 expression, which are presumably derived from the TRU lineage expressing TTF-1 (10). We also observed an increase in the gene dosage of TTF-1, albeit at relatively modest levels, in a small subset of adenocarcinomas both in vitro and in vivo. We noted that increased dosage of the TTF-1 gene tended to occur at a higher frequency in the metastatic sites of lung adenocarcinomas. This observation seems to be consistent with previous reports for certain oncogenes as well as for MITF in metastatic melanomas (18), although additional studies are necessary to elucidate its clinical and functional significance. Considering these data together, there seems to be sufficient evidence to suggest that TTF-1 constitutes one of a currently very sparse list of prototypic lineage-dependency genes.

It is noteworthy that the introduction of TTF-1 paradoxically resulted in significant growth inhibition of an immortalized human peripheral lung epithelial cell line that lacks TTF-1 expression. In this regard, MITF has been suggested to regulate cell-cycle exit and the maintenance of the postmitotic state in melanocytes by transactivating the expression of cyclin-dependent kinase inhibitors (7, 8). This, in turn, provides a probable

Figure 2. Clarification of TTF-1–RNAi effects. A, Western blot analysis confirming the RNAi-resistant nature of mutant TTF-1 carrying silent mutations at the binding site for siRNA. 293T cells were cotransfected with TTF-1–RNAi and either wt- or mut-TTF-1 vectors. B, results of MTT assay of NCI-H358 cells showing the cancellation of the inhibitory effects of the TTF-1–RNAi expression vector by cotransfection with the RNAi-resistant mut-TTF-1, but not with wild-type TTF-1. Transfected cells were selected with puromycin and analyzed on day 7. C, flow-cytometric analysis showing markedly reduced occurrence of TTF-1–RNAi–induced apoptosis in NCI-H358 cells after cotransfection with RNAi-resistant mut-TTF-1.

Figure 3. Gene dosage analysis of TTF-1 in lung cancer cell lines and in lung cancer tissues. A, Southern blot analysis of TTF-1 in a panel of adenocarcinoma cell lines, showing an amplification of about 4-fold in SK-LC-5 cells. Bottom, results of a TaqMan-based gene dosage assay of TTF-1, which confirms the amplification of TTF-1 in SK-LC-5 cells. B, TaqMan-based gene dosage assay for TTF-1 in 214 surgically resected primary NSCLCs, three small-cell lung cancers, and two carcinoids, showing increased gene dosage in a fraction of adenocarcinomas and large-cell neuroendocrine carcinomas. Open and solid circles, surgically resected specimens taken from primary and metastatic sites, respectively. Numbers in parentheses, number of specimens examined. Pt. 38 and Pt. 56, patients 38 and 56, respectively, for which FISH analyses of both primary and metastatic sites were conducted [shown in (C) and Supplementary Fig. S2, respectively]. C, FISH analysis confirming marked amplification of the TTF-1 gene at chromosome 14q13 (green) especially in the recurrent distant metastasis in patient 38. Red, hybridization with a reference probe for a near-centromeric region of 14q.
shown that TRU-type adenocarcinomas carry EGR mutations significantly more frequently than do non–TRU-type adenocarcinomas and have poorer prognoses in the presence of EGR mutations (10, 20), suggesting a potential functional connection between TTF-1 and mutant EGR-mediated signals. We have also observed that TRU-type and non–TRU-type adenocarcinomas have clearly inverse status with regard to various other lung-cancer-related genes, including p53 and KRAS mutations, and in the altered expression of RB, cyclin D1, and cyclooxygenase-2 (10, 11). Future studies are therefore warranted to investigate whether the observed significantly biased occurrence of cancer-specific changes, including EGR mutations, reflect a functional interplay between these genes and the lineage-specific survival gene, TTF-1, in a subset of adenocarcinomas.

In summary, we have shown that sustained TTF-1 expression plays a crucial role in the survival of a subset of adenocarcinomas, adding this master regulator of lung development to the list of lineage-specific survival genes in cancers. Although such examples, supported by solid evidence, are currently negligible, we anticipate that future studies will prove this emerging paradigm of lineage-specific dependency to be applicable to a wide range of human cancers. TTF-1 itself may not be a suitable molecular target because of its important role in maintaining normal lung functions, such as surfactant protein expression. However, it will certainly be interesting to identify the downstream molecule(s) responsible for the survival of adenocarcinoma cells that express TTF-1 because this might ultimately lead to the development of novel therapies for patients who currently have a very dismal prognosis.

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References
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