Characterization of a Cleavage Stimulation Factor, 3’ pre-RNA, Subunit 2, 64 kDa (CSTF2) as a Therapeutic Target for Lung Cancer

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Abstract

Purpose: This study aims to discover novel biomarkers and therapeutic targets for lung cancers.

Experimental Design: We screened for genes showing elevated expression in the majority of lung cancers by genome-wide gene expression profile analysis of 120 lung cancers obtained by cDNA microarray representing 27,648 genes or expressed sequence tags. In this process, we detected a gene encoding cleavage stimulation factor, 3’ pre-RNA, subunit 2, 64 kDa (CSTF2) as a candidate. Immunohistochemical staining using tissue microarray consisting of 327 lung cancers was applied to examine the expression of CSTF2 protein and its prognostic value. A role of CSTF2 in cancer cell growth was examined by siRNA experiments.

Results: Northern blot and immunohistochemical analyses detected the expression of CSTF2 only in testis among 16 normal tissues. Immunohistochemical analysis using tissue microarray showed an association of strong CSTF2 expression with poor prognosis of patients with non–small cell lung cancer (P = 0.0079), and multivariate analysis showed that CSTF2 positivity is an independent prognostic factor. In addition, suppression of CSTF2 expression by siRNAs suppressed lung cancer cell growth, whereas exogenous expression of CSTF2 promoted growth and invasion of mammalian cells.

Conclusions: CSTF2 is likely to play an important role in lung carcinogenesis and be a prognostic biomarker in the clinic. Clin Cancer Res; 17(18); 5889–900. ©2011 AACR.

Introduction

Primary lung cancer is the leading cause of cancer deaths in the world, and nonsmall cell lung cancer (NSCLC) accounts for approximately 80% of them (1). Detailed molecular mechanism of lung carcinogenesis remains unclear, although various genetic alterations in lung cancer were reported (2). The patient with an advanced lung cancer often suffers from the fatal disease progression in spite of the improvement in surgical treatment and chemoradiotherapy (1). Therefore, it is extremely important to understand the biology of lung cancer and to develop more effective treatments to improve the prognosis of patients.

Within the past 2 decades, some newly developed cytotoxic agents such as paclitaxel, docetaxel, gemcitabine, and vinorelbine have appeared to offer multiple choices for treatment of patients with advanced NSCLC; however, these regimens could show only a modest survival benefit compared with conventional platinum-based regimens (3). To date, a lot of molecular targeting agents have been used in the clinical practice of lung cancer treatment, including tyrosine kinase inhibitors of epidermal growth factor or VEGF as well as monoclonal antibodies against them (4, 5). However, only a small proportion of patients can choose these treatment regimens due to the problem of toxicity, and the ratio of patients who show good response is limited even if all kinds of treatments are applied.

Systematic analysis of expression levels of thousands of genes by a cDNA microarray technology is an effective approach to identify diagnostic and therapeutic target molecules involved in carcinogenic pathways (2). To identify potential molecular targets for diagnosis and/or treatment of lung cancers, we previously analyzed genome-wide gene expression profiles of 120 lung cancer tissue samples by means of a cDNA microarray consisting of 27,648 genes or expressed sequence tags (EST) and tumor-cell populations purified by laser microdissection (6–10). To verify the biological and clinicopathologic significance of the respective gene products, we have established a screening system by combination of the tumor tissue microarray analysis of clinical lung cancer materials and RNA interference.
Translational Relevance

Because there is a significant correlation of cleavage stimulation factor, 3’ pre-RNA, subunit 2, 64 kDa (CSTF2) expression with poor prognosis for patients with lung cancers, CSTF2 positivity in resected specimens could be an index that provides useful information to physicians in applying adjuvant therapy and intensive follow-up to the cancer patients who are likely to relapse. Because CSTF2 is likely to play important roles in cell proliferation and invasion, further functional analysis should contribute to the development of a new therapeutic strategy targeting this pathway.

Primary lung tumors and adjacent normal lung tissue samples used for immunostaining on tissue microarrays had been obtained from 327 patients (196 adenocarcinomas, 98 squamous cell carcinomas, 23 large-cell carcinomas, and 10 adenosquamous carcinomas; 99 female and 228 male patients; median age of 64.7 years with a range of 29–85 years) undergoing surgery at Saitama Cancer Center. Those patients that received resection of their primary cancers did not receive any preoperative treatment, and among them only patients with positive lymph node metastasis were treated with platinum-based adjuvant chemotherapies after their surgery. This study and the use of all clinical materials mentioned were approved by individual institutional ethics committees.

Materials and Methods

Cell lines and tissue samples

Fifteen human lung cancer cell lines used in this study included 5 adenocarcinomas (NCI-H1781, NCI-H1373, LC319, A549, and PC-14), 5 squamous cell carcinomas (SK-MES-1, NCI-H520, NCI-H1703, NCI-H2170, and LUC1), 1 large-cell carcinoma (LX1), and 4 small-cell lung cancers (SBC-3, SBC-5, DMS114, and DMS273; details are shown in Supplementary Table S1). All cells were grown in monolayer in appropriate media supplemented with 10% fetal calf serum (FCS) and were maintained at 37°C in humidified air with 5% CO2. Human small airway epithelial cells were grown in optimized medium (Cambrex Bioscience, Inc.). Primary NSCLC tissue samples as well as their corresponding normal tissues adjacent to resection margins had been obtained earlier with informed consent from patients having no anticancer treatment before tumor resection (6, 10). All tumors were staged on the basis of the pathologic tumor-node-metastasis classification of the International Union Against Cancer (45). Formalin-fixed primary lung tumors and adjacent normal lung tissue samples used for immunostaining on tissue microarrays had been obtained from 327 patients (196 adenocarcinomas, 98 squamous cell carcinomas, 23 large-cell carcinomas, and 10 adenosquamous carcinomas; 99 female and 228 male patients; median age of 64.7 years with a range of 29–85 years) undergoing surgery at Saitama Cancer Center. Those patients that received resection of their primary cancers did not receive any preoperative treatment, and among them only patients with positive lymph node metastasis were treated with platinum-based adjuvant chemotherapies after their surgery. This study and the use of all clinical materials mentioned were approved by individual institutional ethics committees.

Northern blot analysis

Human multiple tissue blots covering 16 tissues (BD Bioscience) were hybridized with a 32P-labeled 520-bp PCR product of CSTF2 that was prepared as a probe, using primers 5’-GCTAGTCAGGGACACAGGAAT-3’ and 5’-TCGTTAATTGGACTG-3’. Elevated CSTF2 expression was reported in male germ cells of mice and rats (44). In spite of the evidence of CSTF2 function as a member of CSTF in vitro, the significance of CSTF2 activation in carcinogenesis and its potential as a biomarker and a therapeutic target were not described.

We here report that overexpression of CSTF2 is critical for lung cancer growth and is a prognostic factor for NSCLC patients.

Western blotting

Tumor cells were lysed in lysis buffer: 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% sodium deoxycholate, and Protease Inhibitor Cocktail Set III (Calbiochem). The protein content of each lysate was determined by a Bio-Rad protein assay kit with bovine serum albumin as a standard. Ten micrograms of each lysate was resolved on 7.5% to 12% denaturing polyacrylamide gels (with 3% polyacrylamide stacking gel) and transferred electrophoretically onto a nitrocellulose membrane (GE Healthcare Biosciences). After blocking with 5% nonfat dry milk in Tris-buffered saline and Tween 20 (TBST), the membrane was incubated for 1 hour at room temperature with a rabbit polyclonal anti-CSTF2 antibody (ATLAS, Inc.; catalog no. HPA000427) that was proved to be specific to human CSTF2, by Western blot

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analysis using lysates of lung cancer cell lines. Immunoreactive proteins were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Bio-sciences) for 1 hour at room temperature. After washing with TBST, we used an enhanced chemiluminescence Western blotting analysis system (GE Healthcare Bio-sciences), as previously described (12).

Immunofluorescence analysis
SBC-5, A549, and LC319 cells were seeded on cover-slips. The cultured cells washed twice with cold PBS(−) were fixed in 4% paraformaldehyde solution for 60 minutes at 4°C and rendered permeable by treatment for 5 minutes with PBS(−) containing 0.1% Triton X-100. Cells were covered with CAS Block (Zymed) for 10 minutes to block nonspecific binding before the primary antibody reaction. Then the cells were incubated with antibody to human CSTF2 (ATLAS, Inc.; catalog no. HPA000427) for 1 hour at room temperature, followed by incubation with Alexa488-conjugated goat anti-rabbit antibodies (Molecular Probes; 1:1,000 dilution) for 1 hour. Images were captured on a confocal microscope (TCS SP2-AOBS; Leica Microsystems).

Immunohistochemistry and tissue microarray
To investigate the clinicopathologic significance of the CSTF2 protein in clinical lung cancer samples that had been formalin fixed and embedded in paraffin blocks, we stained the sections by Envisionþ Kit/HRP (DakoCytomation) in the following manner. For antigen retrieval, slides were immersed in Target Retrieval Solution (pH 9; DakoCytomation) and boiled at 108°C for 15 minutes in an autoclave. A rabbit polyclonal anti-human CSTF2 antibody (ATLAS, Inc.; catalog no. HPA000427) was added to each slide after blocking of endogenous peroxidase and proteins, and the sections were incubated with HRP-labeled anti-rabbit IgG [Histofine Simple Stain MAX PO (G); Nichirei] as the secondary antibody. Substrate-chromogen was added, and the specimens were counterstained with hematoxylin.

Tumor tissue microarrays were constructed with 327 formalin-fixed primary NSCLCs which had been obtained at Saitama Cancer Center (Saitama, Japan) with an identical protocol to collect, fix, and preserve the tissues after resection (5). Considering the histologic heterogeneity of individual tumors, tissue area for sampling was selected on the basis of visual alignment with the corresponding hematoxylin and eosin–stained section on a slide. Three, 4, or 5 tissue cores (diameter, 0.6 mm; depth, 3–4 mm) taken from a donor tumor block were placed into a recipient paraffin block, using a tissue microarrayer (Beecher Instruments). A core of normal tissue was punched from each case, and 5-μm sections of the resulting microarray block were used for immunohistochemical analysis. Three independent investigators semiquantitatively assessed CSTF2 positivity without prior knowledge of clinicopathologic data. Because the intensity of staining within each tumor tissue core was mostly homogeneous, the intensity of CSTF2 staining was semiquantitatively evaluated by the following criteria: strong positive (scored as 2+), dark brown staining in more than 50% of tumor cells completely obscuring nucleus; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell nucleus; absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted as strongly positive if 2 or more investigators independently defined them as such.

Statistical analysis
Statistical analyses were carried out with the StatView statistical program (SaS). Strong CSTF2 immunoreactivity was assessed for association with clinicopathologic variables such as gender, age, histologic type, smoking, and pathologic tumor-node-metastasis stage by using the Fisher’s exact test. Survival curves were calculated from the date of surgery to the time of death related to NSCLC or to the last follow-up observation. Kaplan–Meier curves were calculated for each relevant variable and for CSTF2 expression; differences in survival times among patient subgroups were analyzed by the log-rank test. Univariate and multivariate analyses were done with the Cox proportional hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality. First, we analyzed associations between death and possible prognostic factors including gender, age, histology, smoking, pT classification, and pN classification, taking into consideration 1 factor at a time. Second, multivariate analysis was applied on backward (stepwise) procedures that always forced strong CSTF2 expression into the model, along with any and all variables that satisfied an entry level of $P < 0.05$. As the model continued to add factors, independent factors did not exceed an exit level of $P < 0.05$.

RNA interference assay
To evaluate the biological functions of CSTF2 in lung cancer cells, we used siRNA duplexes against CSTF2 gene. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: si-CSTF2-#1, 5′-GGCUUUAGUCCCGGGCAGA-3′; si-CSTF2-#2, 5′-CUAAGUGGCGGAAUUJAUC-3′; control 1 (EGFP: enhanced green fluorescence protein [GFP] gene, a mutant of Aequorea victoria GFP], 5′-GAAGCAGACCGACUUCJUC-3′; control 2 (LUC: luciferase gene from Photinus pyralis), 5′-CGUACGGGAUACUUCUCU-3′. Lung cancer cell lines, A549 and LC319, were plated onto 10-cm dishes (8.0 × 10⁵ per dish) and transfected with either of the siRNA oligonucleotides (100 nmol/L), using 30 μL of Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions. After 7 days of incubation, cell viability was assessed by MIT assay.

Flow cytometry
Cells transfected with siRNA oligonucleotides were plated at densities of 8 × 10⁵ per 100-mm dish. Cells were collected in PBS and fixed in 70% cold ethanol for 30 minutes. After treatment with 100 μg/mL RNase
(Sigma–Aldrich), the cells were stained with 50 μg/mL propidium iodide (Sigma Aldrich) in PBS. Flow cytometry was done on a Cell Lab Quanta SC (Beckman Coulter) and analyzed by CXP Analysis software ver.2.2 (Beckman Coulter). The cells selected from at least 20,000 ungated cells were analyzed for DNA content.

**Cell growth assay**

COS-7 and HEK293T cells that hardly expressed endogenous CSTF2 were plated at densities of 5.0 × 10^5 cells per 100-mm dish, transfected with plasmids designed to express CSTF2 (pcDNA3.1/myc-His-CSTF2) or mock plasmids (pcDNA3.1/myc-His). COS-7 and HEK293T cells were cultured for 7 days in medium containing 0.4 and 0.9 mg/mL of geneticin (Invitrogen), respectively. Cell viability was assessed by MTT assay. Colony numbers were also counted with ImageJ software after the cells had been fixed and stained by Giemsa solution.

**Matrigel invasion assay**

COS-7 and HEK293T cells, transfected with plasmids designed to express CSTF2 (pcDNA3.1/myc-His-CSTF2) or mock plasmids (pcDNA3.1/myc-His), were grown near confluence in appropriate media containing 10% FCS. The cells were harvested by trypsinization, washed in the media without addition of serum or protease inhibitor, and suspended in the media at a concentration of 5.0 × 10^5/mL. Before preparing the cell suspension, the dried layer of Matrigel matrix (Becton Dickinson Labware) was rehydrated with the media for 2 hours at room temperature. The media (0.75 mL) containing 10% FCS was added to each lower chamber in 24-well Matrigel invasion chambers, and 0.5 mL (2.5 × 10^6 cells) of cell suspension was added to each insert of the upper chamber. The plates of inserts were incubated for 24 hours at 37°C. Then the chambers were processed, and cells invading through the Matrigel were fixed and stained by Giemsa as directed by the supplier (Becton Dickinson Labware).

**Results**

**Expression of CSTF2 in lung cancers and normal tissues**

To screen novel target molecules for development of therapeutic agents and/or biomarkers for lung cancer, we first carried out genome-wide gene expression profile analysis of 120 lung cancers by using a cDNA microarray consisting of 27,648 genes or ESTs (6–10). We identified the CSTF2 transcript to be overexpressed (3-fold or higher) in the majority of lung cancer samples examined, whereas CSTF2 expression was scarcely detectable in any of 29 normal tissues except testis. We confirmed CSTF2 overexpression by semiquantitative RT-PCR experiments in 12 of 15 lung cancer tissues and in all of 15 lung cancer cell lines examined (Fig. 1A). In addition, we detected expression of CSTF2 proteins in 9 lung cancer cell lines by Western blot analysis, using anti-CSTF2 antibody (Fig. 1B). To determine the subcellular localization of endogenous CSTF2 in lung cancer cells, we carried out immunofluorescence analysis with anti-CSTF2 antibody and found its staining in the nucleus of SBC-5, A549, and LC319 cells (Fig. 1C).

Northern blot analysis with a CSTF2 cDNA as a probe identified a 2.6-kb transcript only in testis among 16 normal human tissues examined (Fig. 2A). In addition, we examined expression of CSTF2 protein in 5 normal tissues (heart, lung, liver, kidney, and testis) as well as lung cancers, using anti-CSTF2 antibody. Positive staining of CSTF2 was observed in the nucleus of testicular cells and lung cancer cells, but not in other normal tissues (Fig. 2B).

**Association of CSTF2 overexpression with poor prognosis for NSCLC patients**

To verify the biological and clinicopathologic significance of CSTF2 in pulmonary carcinogenesis, we carried out immunohistochemical staining on tissue microarrays containing primary NSCLC tissues from 327 patients who underwent curative surgical resection at Saitama Cancer Center. CSTF2-positive staining with the anti-CSTF2 polyclonal antibody was observed in the nucleus of lung cancer cells, but staining was negative in any of their adjacent normal lung cells or stromal cells surrounding tumor cells (Fig. 2C). We classified CSTF2 expression levels on the tissue array ranging from absent (scored as 0) to weak/strong positive (scored as 1+ or 2+; Fig. 2D). Of the 327 NSCLCs, CSTF2 was strongly stained in 77 cases (24%, score 2+), weakly stained in 165 cases (50%, score 1+), and not stained in 85 cases (26%; score 0; details are shown in Table 1). We then examined a correlation of CSTF2 expression levels (strong positive vs. weak positive/absent) with various clinicopathologic variables and found that strong CSTF2 expression was associated with poor prognosis for patients with NSCLC after the resection of primary tumors (P = 0.0079, log-rank test; Fig. 2E; Supplementary Table S2), but not associated with any other clinicopathologic variables. In addition, we used univariate analysis to evaluate associations between patient prognosis and several clinicopathologic factors including gender (male vs. female), age (≥65 vs. <65 years), histology (nonadenocarcinoma vs. adenocarcinoma), smoking history (smoker vs. nonsmoker), pT stage (tumor size; T2–T3 vs. T1), pN stage (lymph node metastasis; N1–N2 vs. N0), and CSTF2 expression (score 2+ vs. 0, 1+). All of these parameters except smoking history were significantly associated with poor prognosis (Table 2). Multivariate analysis by the Cox proportional hazard model indicated that pT stage, pN stage, age, and strong CSTF2 positivity were independent prognostic factors for NSCLC (Table 2).

**Inhibition of growth of lung cancer cells by siRNA for CSTF2**

To assess whether upregulation of CSTF2 plays a role in growth or survival of lung cancer cells, we transfected synthetic oligonucleotide siRNAs against CSTF2...
(si-CSTF2-#1 and si-CSTF2-#2) or control siRNAs (si-EGFP and si-LUC) into A549 and LC319 cells in which CSTF2 was endogenously overexpressed. The mRNA levels of CSTF2 in cells transfected with si-CSTF2-#1 and si-CSTF2-#2 were significantly decreased in comparison with those transfected with either control siRNAs (Fig. 3A). Cell viability and colony numbers investigated by MTT assays were reduced significantly in the cells transfected with si-CSTF2-#1 and si-CSTF2-#2 (Fig. 3B). To clarify the mechanism of tumor suppression by siRNAs against CSTF2, we carried out flow cytometric analysis of the tumor cells transfected with these siRNAs and found a significant increase in the cells of sub-G₁ fraction 72 hours after the treatment (Fig. 3C and D).

**Enhancement of mammalian cell proliferation and invasion by CSTF2**

To examine a potential role of CSTF2 in tumorigenesis, we constructed plasmids designed to express CSTF2 (pcDNA3.1/myc-His-CSTF2) and transfected them into mammalian COS-7 and HEK293T cells. Exogenous CSTF2 expression in the transfected cells was confirmed by Western blot analysis (Fig. 4A). We carried out MTT and colony formation assays and found that growth of the
COS-7 and HEK293T cells transfected with CSTF2 was significantly enhanced compared with those transfected with the mock vector (Fig. 4B; Supplementary Fig. S1).

Because strong CSTF2 expression was associated with poor prognosis of NSCLC patients, we next examined by Matrigel assay the effect of CSTF2 expression on cellular invasion. We transfected plasmids designed to express CSTF2 into COS-7 and HEK293T cells and found that overexpression of CSTF2 significantly enhanced the invasive activity of cells through Matrigel (Supplementary Fig. S2). The result suggests that CSTF2 could contribute to the highly malignant phenotype of cancer cells.

**Discussion**

To develop molecular-targeting anticancer drugs that are expected to be highly specific to malignant cells, with minimal risk of adverse reactions, we established an effective screening system to identify proteins that were activated specifically in lung cancers. First, we analyzed a genome-wide expression profile of 120 lung cancer samples through the genome-wide cDNA microarray system containing 27,648 genes coupled with cancer cell purification by laser microdissection (6–10). After verification of very low or absent expression of such genes in normal...
organs by cDNA microarray analysis and multiple-tissue Northern blot analysis, we analyzed the protein expression of candidate targets among hundreds of clinical samples on tissue microarrays, investigated loss of function phenotypes using RNA interference systems, and further defined biological functions of the proteins. Through these analyses, we identified candidate genes for the development of novel serum biomarkers (30–35), therapeutic
drugs (11–29), and/or immunotherapy (35–41), which were upregulated in cancer cells but not expressed in normal organs, except testis, placenta, and/or fetus tissues. In this study, we report that CSTF2 encoding a member of cleavage stimulation factors was frequently overexpressed in the great majority of clinical lung cancer samples and cell lines, and that its gene product plays an indispensable role in the growth and invasion of lung cancer cells.

### Table 1. Association between CSTF2 positivity in NSCLC tissues and patients’ characteristics

<table>
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<tr>
<th></th>
<th>Total (N = 327)</th>
<th>CSTF2 strong positive (n = 77)</th>
<th>CSTF2 weak positive (n = 165)</th>
<th>CSTF2 absent (n = 85)</th>
<th>P (strong vs. weak/absent)</th>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>228</td>
<td>55</td>
<td>120</td>
<td>53</td>
<td>0.7775</td>
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<tr>
<td>Female</td>
<td>99</td>
<td>22</td>
<td>45</td>
<td>32</td>
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<tr>
<td>Age, y</td>
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<tr>
<td>&lt;65</td>
<td>150</td>
<td>33</td>
<td>77</td>
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<td>≥65</td>
<td>177</td>
<td>44</td>
<td>88</td>
<td>45</td>
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<td>45</td>
<td>97</td>
<td>54</td>
<td>0.7910</td>
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<tr>
<td>Non-ADC&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>32</td>
<td>68</td>
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<td>234</td>
<td>51</td>
<td>124</td>
<td>59</td>
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<td>Nonsmoker</td>
<td>93</td>
<td>26</td>
<td>41</td>
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<td>pT factor</td>
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<tr>
<td>T1</td>
<td>135</td>
<td>26</td>
<td>68</td>
<td>41</td>
<td>0.1458</td>
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<tr>
<td>T2 + T3</td>
<td>192</td>
<td>51</td>
<td>97</td>
<td>44</td>
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<td>N0</td>
<td>208</td>
<td>45</td>
<td>107</td>
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<td>N1 + N2</td>
<td>119</td>
<td>32</td>
<td>58</td>
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Abbreviation: ADC, adenocarcinoma.
<sup>a</sup>The category “non-ADC” comprises squamous cell carcinoma plus large-cell carcinoma and adenocarcinoma.

### Table 2. Cox proportional hazards model analysis of prognostic factors in patients with NSCLCs

<table>
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<tr>
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<th>HR</th>
<th>95% CI</th>
<th>Unfavorable/favorable</th>
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<tr>
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<td>CSTF2</td>
<td>1.617</td>
<td>1.130–2.314</td>
<td>Strong(+)weak(+) or (-)</td>
<td>0.0085&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Gender</td>
<td>1.512</td>
<td>1.037–2.066</td>
<td>Male/female</td>
<td>0.0317&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Age, y</td>
<td>1.672</td>
<td>1.188–2.353</td>
<td>≥65/≤65</td>
<td>0.0032&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Histology</td>
<td>1.434</td>
<td>1.033–1.989</td>
<td>Non-ADC/ADC</td>
<td>0.0311&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Smoking</td>
<td>1.252</td>
<td>0.858–1.826</td>
<td>Smoker/nonsmoker</td>
<td>0.2437</td>
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<td>pT factor</td>
<td>2.176</td>
<td>1.505–3.146</td>
<td>T2 + T3/T1</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>pN factor</td>
<td>1.965</td>
<td>1.416–2.728</td>
<td>N1 + N2/N0</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Multivariate analysis</td>
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<td>CSTF2</td>
<td>1.545</td>
<td>1.078–2.214</td>
<td>Strong(+)weak(+) or (-)</td>
<td>0.0177&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Gender</td>
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<td>0.850–1.969</td>
<td>Male/female</td>
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<td>Age, y</td>
<td>1.858</td>
<td>1.306–2.643</td>
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<td>Histology</td>
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<td>0.696–1.464</td>
<td>Non-ADC/ADC</td>
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<td>pT factor</td>
<td>1.695</td>
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<td>T2 + T3/T1</td>
<td>0.0085&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>pN factor</td>
<td>1.990</td>
<td>1.413–2.802</td>
<td>N1 + N2/N0</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
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NOTE: The category “non-ADC” comprises squamous cell carcinoma plus large-cell carcinoma and adenocarcinoma.

Abbreviations: ADC, adenocarcinoma; CI, confidence interval.
<sup>a</sup>P < 0.05.
Figure 3. Inhibition of growth of NSCLC cells by siRNAs against CSTF2. A, expression of CSTF2 in response to siRNA treatment for CSTF2 (si-CSTF2-#1 or si-CSTF2-#2) or control siRNAs (si-EGFP or si-LUC) in A549 and LC319 cells, analyzed by semiquantitative RT-PCR (top). B, MTT assays of the tumor cells transfected with si-CSTF2s or control siRNAs. C, flow cytometric analysis of the A549 cells and LC319 cells 72 hours after transfection of the siRNAs for CSTF2 (si-CSTF2-#1) and control siRNAs (si-EGFP). D, left, percentage of cells at each phase; right, averaged percentage of cells at sub-G1 phase of triplicate assays.
CSTF2 encodes a 557–amino acid protein with an N-terminal RNP-type RNA-binding domain, a long Pro- and Gly-rich region, and a pentapeptide repeat region that forms an extended α-helix and comprises one of a multisubunit complex of CstF required for polyadenylation and 3′-end cleavage of mammalian pre-mRNAs (46). CstF cooperates with cleavage-polyadenylation specificity factor to define the site of polyadenylation by recognizing the highly conserved AAUAAA hexanucleotide and more divergent GU-rich situated downstream of the actual cleavage site (47). The polyadenylation and 3′-end cleavage of pre-mRNAs are important modifications for proper transcription, splicing, transport, translation, and stability of mRNAs. This factor for polyadenylation is reported to change the length of 3′-UTR of mRNA and thus the binding sites of microRNAs (miRNA), which tend to bind to these regions (48). Loss of miRNA complementary sites in 3′-UTR of some oncogenes is one of the mechanisms of oncogene activation. In cancer cells, 3′-UTR of some oncogenes is likely to be shortened by alternative cleavage and polyadenylation, resulting in avoidance of silencing by miRNA and activation of oncogenes in cancer cells (49). It is still unknown why CSTF2 was overexpressed in testis and cancer cells; however, CSTF2 may play important roles for activation of some oncogenes by changing the length of 3′-UTR of their mRNAs in cancer cells.

To further elucidate the downstream pathways of CSTF2 in lung cancer cells, we transfected siRNAs against CSTF2 (si-CSTF2 #1) or EGFP (control siRNA) into lung cancer A549 cells that overexpressed CSTF2, and screened by microarray analysis as described previously (16) genes that were downregulated in accordance with the knockdown of CSTF2 expression at 24 and 48 hours after siRNA transfection. The self-organizing map clustering analysis for selecting genes showed the same expression pattern to CSTF2 in a time-dependent manner and subsequently the gene ontology database analysis identified 4 candidate downstream pathways of CSTF2 including genes related to a member of interleukin, TGF-β, chemokine, and heterotrimeric G protein, some of which were reported to have important roles in carcinogenesis (Supplementary Tables S3 and S4). Although further detailed analyses are necessary to determine the direct targets of CSTF2, the information implies the biological importance of CSTF2 in human carcinogenesis.

In this study, we showed that CSTF2 gene was overexpressed in most lung cancers and likely to play an important role in the growth of lung cancers. Moreover, clinicopathologic data through our tissue microarray experiments showed that patients with NSCLC, in which CSTF2 was highly expressed, had shorter survival periods than those with CSTF2 weak positive/negative tumors. This is the first study that proves a potential of tissue
CSTF2 expression as a prognostic biomarker for human cancers.

Somatic epidermal growth factor receptor (EGFR) mutation was reported to be biologically important for the clinical response of NSCLCs to EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib (5, 50). To examine the relevance of CSTF2 expression to this subgroup of NSCLCs with EGFR mutation, we examined the correlation between CSTF2 overexpression and EGFR mutation status in NSCLC tissues using specific antibodies to E746-A750 deletion and those to a point mutation (L858R) in EGFR, and found that EGFR mutation was not associated with CSTF2 strong expression (P = 0.2555, Fisher’s exact test; Supplementary Table S5; Supplementary Fig. S3). The combined results suggest that further functional analysis and screening to selectively inhibit CSTF2 function by small molecule compounds and/or nucleic acid drugs could be a potential therapeutic strategy that is expected to have a powerful biological activity against broad clini-copathologic types of lung cancers.

In summary, CSTF2 might play an important role in the growth and progression of lung cancers. CSTF2 overexpression in resected specimens may be a useful indicator of adjuvant therapy to lung cancer patients who are likely to have poor prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Characterization of a Cleavage Stimulation Factor, 3’ pre-RNA, Subunit 2, 64 kDa (CSTF2) as a Therapeutic Target for Lung Cancer

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