RASEF is a Novel Diagnostic Biomarker and a Therapeutic Target for Lung Cancer

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Abstract

Genome-wide gene expression profiling revealed that the Ras and EF-hand domain containing (RASEF) transcript was significantly transactivated in the majority of lung cancers. Using lung cancer cells, transient expression of RASEF promoted cell growth, whereas RASEF knockdown not only reduced its expression but resulted in growth suppression of the cancer cells. Immunohistochemical staining using tumor tissue microarrays consisting of 341 archived non–small cell lung cancers (NSCLC) revealed the association of strong RASEF positivity with poor prognosis (P = 0.0034 by multivariate analysis). Mechanistically, RASEF interacted with extracellular signal-regulated kinase (ERK) 1/2 and enhanced ERK1/2 signaling. Importantly, inhibiting the interaction between RASEF and ERK1/2 using a cell-permeable peptide that corresponded to the ERK1/2-interacting site of RASEF, suppressed growth of lung cancer cells. This study demonstrates that elevated RASEF promoted cell growth via enhanced ERK signaling and is associated with poor prognosis of NSCLC.

Implications: RASEF may play an important role in lung carcinogenesis and could serve as a viable prognostic biomarker and target for the development of new molecular therapies.

Introduction

Lung cancer is the most common cause of cancer-related death in the world and its incidence has been increasing (1). In spite of the use of advanced surgical treatments combined with radiotherapy and chemotherapy, the overall 5-year survival rate of patients with lung cancer still remains at 20% (2). Development of molecular-targeted drugs such as gefitinib and bevacizumab have improved treatment modalities of lung cancer, but fatal adverse events such as interstitial pneumonitis caused by gefitinib or severe hemorrhage by bevacizumab were reported (3–4). Therefore, further development of new agents targeting cancer-specific molecules with no or minimum risk of adverse effect is urgently awaited. To date, clinical and pathologic staging have been the most reliable information for physicians in the choice of therapy. However, considering that about 30% of patients with stage I non–small cell lung cancer (NSCLC) who had undergone curative surgery suffered recurrent diseases (2), it is important to develop more precise prognostic biomarkers for selecting patients who should be treated with adjuvant therapies and intensively followed after surgical treatment.

Systematic analysis of expression levels of thousands of genes using a cDNA microarray technology is an effective approach for identifying molecules involved in pathways of carcinogenesis or those associated with efficacy/resistance to anticancer therapy; some of such genes or their gene products may be good target molecules for the development of novel therapies and/or cancer biomarkers (5). To identify such molecules, particularly oncoantigens, we had conducted genome-wide expression profile analysis of 120 clinical lung cancer tissue samples, coupled with enrichment of tumor cells by laser microdissection, and then compared the expression profile data with those in 31 normal human tissues (27 adult and 4 fetal organs; refs. 6–10). To verify the clinicopathologic significance of the respective gene products, we have established a screening system by a combination of the tumor-tissue microarray analysis of clinical lung cancer materials and RNA interference technique (11–41). This systematic approach revealed that Ras and EF-hand domain-containing protein (RASEF) is likely to be a novel molecule that was overexpressed commonly in primary lung cancers and was essential for cell growth and/or survival of cancer cells.

RASEF contains a Rab GTPase domain in the C-terminal region and is considered as a member of Rab GTPase protein family. Unlike other Rab proteins, RASEF contains 2 EF-hand domains which are generally known to be important for binding to calcium ions in the N-terminus and a coiled-coil motif in an internal region (42). The functional
relevance of RASEF activation to carcinogenesis as well as its detailed biologic function has not yet been defined. Here we report the first evidence that RASEF plays a significant role in lung cancer cell growth possibly through its interaction with extracellular signal-regulated kinase (ERK) 1/2, and suggest that RASEF could be a promising prognostic biomarker and therapeutic target for lung cancer.

Materials and Methods

Cell lines and tissue samples
Lung cancer cell lines and human bronchial epithelial cells (BEAS-2B) used in this study are listed in Supplementary Table S1. A427, A549, NCI-H1373, NCI-H1781, NCI-H358, NCI-H226, NCI-H520, NCI-H2170, NCI-H1703, DMS114, DMS273, NCI-H196, NCI-H446, and BEAS-2B were from American Type Culture Collection in 2003, 2010, and 2011, and tested and authenticated for Experimental Animals and European Collection of Animal Cell Cultures in 2002, and tested and authenticated for polymorphic STR markers. PC-3, SBC-3, and SBC-5 cells were from Japanese Collection of Research Bioresources (JCRB) in 2001 and 2010, and tested and authenticated by DNA profiling for polymorphic short-tandem repeat (STR) markers. PCR-3, SBC-3, and SBC-5 cells were from RIKEN BioResource Center in 2001 and 2010, and tested and authenticated by DNA profiling for polymorphic STR markers. PC-14, EBC-1, and RERF-LC-A1 cells were from RIKEN BioResource Center in 2001 and 2010, and tested and authenticated by DNA profiling for polymorphic STR markers. LC319 cells were from Aichi Cancer Center in 2003, and tested and authenticated by DNA profiling for single-nucleotide polymorphism, mutation, and deletion analysis. PC-9 and LXI cells were from Tokyo Medical University and Central Institute for Experimental Animals and European Collection of Animal Cell Cultures in 2002, and tested and authenticated by DNA profiling for single-nucleotide polymorphism, mutation, and deletion analysis. All cells were grown in monolayer in appropriate medium supplemented with 10% fetal calf serum (FCS) and maintained at 37 °C in humidified air with 5% CO₂. Primary lung cancer samples had been obtained earlier as previously described (6, 10). All tumors were staged on the basis of the pTNM pathologic classification of the UICC (International Union Against Cancer; ref. 43). A total of 341 formalin-fixed samples of primary NSCLCs [100 female and 241 male patients; median age of 65 with a range of 35–85 years; 93 never smoke cases and 248 ex- or current smokers; 205 adenocarcinomas (ADC), 105 squamous cell carcinomas (SCC), 11 adenosquamous cell carcinoma (ASC), 20 large cell carcinoma (LCC); 141 pT1, 157 pT2, and 43 pT3 cases; 223 pN0, 42 pN1, and 76 pN2 cases; see Supplementary Table S2] had been obtained earlier along with clinicopathologic data from patients undergoing surgery at Saitama Cancer Center (Saitama, Japan). Independent set of 243 formalin-fixed samples of primary NSCLCs for validation study was obtained by Hokkaido University and its affiliated hospitals (Sapporo, Japan). These patients received resection of their primary cancers, and among them only patients with positive lymph node metastasis were treated with cisplatin-based adjuvant chemotherapies after their surgery. This study and the use of all clinical materials mentioned were approved by institutional Ethical Committees.

Semiquantitative reverse transcription-PCR
A total of 3 μg aliquot of mRNA from each sample was reversely transcribed to single-stranded cDNAs using random primer (Roche Diagnostics) and SuperScript II (Invitrogen). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following sets of synthesized gene-specific primers or with β-actin (ACTB)-specific primers as an internal control: RASEF, 5'-GCGTGCAGACCTGTGACC-3' and 5'-GGATTTGTCCTCCGAG-3'; cyclin D1 (CCND1), 5'-CCTCCGGTGTCCACCTTCAAA-3' and 5'-CCAGGTTCCTCCGAGGGAAGTG-3'; cyclin-dependent kinase inhibitor 1A (CDKN1A), 5'-TTAGCAGGGAACAAAGGATT-3' and 5'-ATTAGCTTGCTGAGGAGAG-3'; and ACTB, 5'-GAGGTGATAGCATTGCTTTCG-3' and 5'-CAAGTGACTGAGGCGATCAG-3'. PCRs were optimized for the number of cycles to ensure product intensity to be within the linear phase of amplification.

Western blot analysis
Cell lysates from lung cancer cell line or normal airway epithelial cells were subjected to Western blotting. In brief, cells were incubated in 1 mL lysis buffer (0.5% NP-40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of protease inhibitor (Protease Inhibitor Cocktail Set III; Calbiochem). Western blotting were done using an ECL Western-blotting analysis system (GE Healthcare Bio-Sciences). Northern blot analysis

Immunocytochemical analysis
Cells were plated onto glass coverslips (Becton Dickinson Discovery Labware), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 3 minutes at room temperature. Nonspecific binding was blocked by Casblock (ZYMED) for 10 minutes at room temperature. The cells were then incubated overnight at 4°C with a rabbit polyclonal antibody to RASEF (Catalog No. 11569-1-AP, Proteintech Group, Inc.) diluted in PBS containing 1% bovine serum albumin (BSA). After being washed with PBS, the cells were stained by Alexa 488-conjugated secondary antibody (Invitrogen) for 60 minutes at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Inc.) containing 4',6-diamidino-2-phenylindole (DAPI) and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS; Leica Microsystems).

Northern blot analysis
Human multiple tissue blots covering 16 tissues (BD Biosciences) were hybridized with an [α-32P]-dCTP-labeled,
421-bp PCR product of RASEF that was prepared as a probe using primers 5'-GGCTGACATTTGGACACTG-3' and 5'-CAAGTTCCAGGAGGACTG-3'. Prehybridization, hybridization, and washing were done following the manufacturer’s specifications. The blots were autoradiographed with intensifying screens at –80°C for 10 days.

**Immunohistochemistry and tissue microarray**

To investigate the clinicopathologic significance of RASEF overexpression in lung cancers, we stained tissue sections using ENVISION+ Kit/HRP (DakoCytomation). Anti-RASEF rabbit polyclonal antibody (Catalog No. 11569-1-AP, Proteintech Group, Inc.) was added after blocking of endogenous peroxidase and proteins, and each section was incubated with HRP-labeled anti-rabbit IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin.

Tumor tissue microarrays were constructed with formalin-fixed 341 primary lung cancers, each of which had been obtained with an identical protocol to collect, fix, and preserve the tissues after resection (12–18). The tissue area for sampling was selected on the basis of visual alignment with the corresponding hematoxylin and eosin (H&E)-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 with a tissue microarray (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 RASEF positivity without prior knowledge of clinicopathologic information. The intensity of RASEF staining was evaluated using the following criteria: strong positive (scored as 2+), brown staining in greater than 50% of tumor cells completely obscuring cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell cytoplasm; and absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted as strongly positive only if 2 or more investigators independently defined them as such.

**Statistical analysis**

Statistical analyses were done using the StatView statistical program (SAS). Tumor-specific 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 RASEF expression; differences in survival times among patient subgroups were analyzed using 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 age, gender, smoking status, pathologic tumor classification, and pathologic node classification, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong RASEF expression into the model, along with any and all variables that satisfied an entry level of a P value of < 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 biologic functions of RASEF in lung cancer cells, we used small-interfering RNA (siRNA) duplexes against RASEF. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control 1 (EGFP, enhanced green fluorescent protein [GFP]), a mutant of Aequorea victoria GFP, 5'-GAAGCAG-CACGACUUUCUC-3'; control 2 (LUC, luciferase gene from Photinus pyralis), 5'-CGTACGCGGAATACTTTC-GA-3'; siRNA-RASEF-#1, 5'-GGTATGATCCCTTGATACCAAA-3'; siRNA-RASEF-#2, 5'-CTTCATCCCGTAGATCAGA-3'. siRNAs were transfected into lung cancer cell lines, A549 and NCI-H2170 using 30 μL of Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Cell numbers and viability were measured by Giemsa staining and triplicate MTT assays (cell counting kit-8 solution; Dojindo Laboratories) at 5 days after the transfection. Expression of endogenous RASEF protein was detected by Western blotting.

**Cell growth assays**

Endogenous RASEF-negative BEAS-2B and DMS114 cells transfected either with RASEF expression vector (pCAGGSn3FH-RASEF), which express RASEF with 3 Flag sequences (DYKDHDGDYKDHDIDYKDDDDK) at the NH2-terminal or with mock vector (pCAGGSn3FH) were seeded onto 6-well plates (5 × 104 cells/well), and maintained in medium containing 10% fetal bovine serum (FBS) and gentamicin. After 120 hours, cell proliferation was evaluated by the MTT assay using Cell Counting Kits (Dojindo Laboratories).

**Immunoprecipitation assay**

To examine the interaction between endogenous RASEF and ERK1/2, immunoprecipitation was conducted with a rabbit polyclonal anti-RASEF antibody (Catalog No. 11569-1-AP, Proteintech Group, Inc.) at 4°C for 2 hours after incubation of extracts from NCI-H2170 cells at 4°C for 1 hour with protein G-Agarose beads as described previously (14). The immunoprecipitates were washed 5 times with lysis buffer, and were subjected to Western blotting with a mouse monoclonal anti-ERK1/2 antibody (Catalog. No. 4696; Cell Signaling Technology).

**Identification of ERK1/2-interacting sites on RASEF**

To define the ERK1/2-interacting sites on RASEF protein, we constructed various vectors expressing partial RASEF protein with Flag-tag at its N-terminus, and transfected either of them into RASEF-negative DMS114 cells. Immunoprecipitation using anti-Flag M2 agarose and subsequent immunoblotting with anti-ERK1/2 antibody were conducted as described above.
Synthesized dominant-negative peptide

To further investigate the biologic importance of the interaction between RASEF and ERK1/2 that had been confirmed by above-mentioned assays in lung cancer cell growth, the 3 different 23-amino-acid polypeptides covering the ERK1/2-interacting site on RASEF 520–575 with a membrane-permeable 11 residues of arginine (11R) at its N-terminus (11R-RASEF 520–542, RRRRRRRRRR-GG-SALSQPQTDLVDDNASKFSSQKAY; 11R-RASEF 536–558, RRRRRRRRRR-GGG-FSSQKAYKIVALGDAV-GKSSF; 11R-RASEF 553–575, RRRRRRRRRR-GGG-GKSSFLMRCLKNFRENISATLG) were synthesized as previously described (17, 20, 28, 29). Scramble peptides (SCR) derived from the 11R-RASEF 553 to 575 peptides that showed growth suppressive effect on cancer cells were synthesized as a control (RRRRRRRRRRR-GGG-RSENKMLFRFGEFTLLKGCINA). Peptides were purified by preparative reverse-phase high-performance liquid chromatography with the purity of more than 95%. Two RASEF-positive cell lines A549 and NCI-H2170, and RASEF-negative bronchial epithelial cells BEAS-2B were cultured in the presence of either of these peptides in media at the concentration of 5, 10, or 15 μmol/L for 5 days. The medium was replaced at every 48 hours with the above-mentioned concentrations of each peptide, and the viability of cells was evaluated by the MTT assay.

Results
RASEF expression in lung cancers and normal tissues

To identify novel molecules that can be applicable for the development of novel biomarkers and treatments on the basis of the biologic characteristics of cancer cells, we had conducted genome-wide gene expression profile analysis of 120 lung carcinomas using a cDNA microarray (6–10). Among 27,648 genes or expressed sequence tags screened, we identified elevated expression (5-fold or higher) of RASEF transcript in the great majority of the lung cancer samples examined. We confirmed by semiquantitative RT-PCR experiments RASEF expression in 9 of 12 clinical lung cancers, but its expression was hardly detectable in their corresponding normal lung tissues (Fig. 1A). We also observed overexpression of RASEF in 14 of 22 lung cancer cell lines, but did not detect its expression in BEAS-2B airway epithelial cells (Fig. 1B). To evaluate the expression levels and subcellular localization of RASEF protein in lung cancer cells, we conducted Western blotting and immunofluorescence analyses using a rabbit anti-RASEF polyclonal antibody and RASEF-positive lung cancer A549 and NCI-H2170 cells, and RASEF-negative DMS114 cells as well as BEAS-2B airway epithelial cells. The band was detected by Western blotting at the molecular weight of about 90 kDa in RASEF-positive A549 and NCI-H2170 cells, whereas no signal was detected in RASEF-negative DMS114 and BEAS-2B cells (Fig. 1C). As there are several predicted phosphorylation sites on RASEF, we treated the lysate of NCI-H2170 cells with phosphatase, and observed by immunoblotting the disappearance of the upper weak signals, suggesting the phosphorylation of a part of RASEF protein in cancer cells (Supplementary Fig. S1). By immunofluorescence analysis, we also detected RASEF protein mainly in the cytoplasm of RASEF-positive A549 and NCI-H2170 cells, but not in RASEF-negative DMS114 and BEAS-2B cells (Fig. 1D).

Northern blot analysis using a RASEF cDNA fragment as a probe identified a 5.8-kb transcript only in prostate and testis, but not in any other normal tissues examined (Fig. 2A). By immunohistochemical analysis, we also examined the expression of RASEF protein in 6 normal human tissues (liver, heart, kidney, lung, prostate, and testis) and lung cancer tissues (ADC, SCC, and SCLC). Strong-positive RASEF staining was mainly observed in cytoplasm of lung tumor cells, and weakly in prostate and testicular cells, but its staining was hardly detectable in the remaining 4 normal tissues (Fig. 2B). The comparison of RASEF staining in NSCLC and adjacent normal tissues from 10 patients who underwent surgery revealed that RASEF protein was highly expressed in NSCLC tissues, but not in adjacent normal lung tissues (Fig. 2C).

Association of RASEF expression with poor prognosis for patients with NSCLC

To investigate the biologic and clinicopathologic significance of RASEF in pulmonary carcinogenesis, we carried out immunohistochemical staining on tissue microarray containing 341 NSCLC cases that underwent surgical resection, using a rabbit polyclonal antibody specific to RASEF. We classified a pattern of RASEF expression on the tissue array ranging from absent (scored as 0) to weak/strong positive (scored as 1+ to 2+; representative images of staining were shown in Fig. 2D). Of the 341 NSCLCs, RASEF was strongly stained in 126 (37%) cases (score 2+), weakly stained in 150 (44%) cases (score 1+), and not stained in 65 (19%) cases (score 0; Table 1A). Using the scores of RASEF staining, we examined the association between RASEF positivity and prognosis of patients with NSCLC, and found that the prognosis of NSCLC was likely to be poorer in patients with the higher scores of RASEF positivity than those with the lower scores, although there is no significant difference of survival periods between patients with NSCLC with weak RASEF-positive tumors and those with RASEF-negative (Supplementary Fig. S2) tumors. Therefore, we next examined correlation of RASEF expression (strong positive vs. weak positive/absent) with prognosis of patients as well as various clinicopathologic parameters such as age, gender, smoking status (never smoker vs. current or former smoker), pathologic tumor stage (tumor size; T1 vs. T2 and T3), pathologic node stage (node status; N0 vs. N1 and N2), and histology (ADC vs. other histologic types), and found that strong RASEF positivity was associated with poor prognosis of patients with NSCLC after the resection of primary tumors (P < 0.0001 log-rank test; Fig. 2E). In addition, we found that high levels of RASEF expression were significantly correlated with tumor size (T factor; P = 0.0006; Table 1A). Furthermore, we applied univariate analysis to evaluate associations between patient prognosis and several factors including age, gender, smoking status,
Pathologic tumor stage, pathologic node stage, histology, and RASEF status (score 0, 1+ vs. score 2+). All those variables except smoking status were significantly associated with poor prognosis. Multivariate analysis using a Cox proportional hazard model indicated that RASEF \((P = 0.0034)\) as well as other 3 factors (age, tumor size, and lymph node metastasis) were independent prognostic factors for patients with surgically treated NSCLC (Table 2). To further confirm the independent prognostic value of strong RASEF expression, we conducted subgroup analysis of stage I NSCLCs by log-rank test, and found that strong RASEF positivity was associated with poor prognosis of patients with stage I NSCLC \((P = 0.0004)\). We also confirmed prognostic value of RASEF in another independent set of 243 patients with postoperative NSCLC \((P = 0.0382)\) log-rank test; Supplementary Fig. S3).

**Growth effect of RASEF protein**

To disclose the role of RASEF in the growth or survival of cancer cells, we suppressed endogenous RASEF expression using 2 siRNAs against RASEF (si-RASEF-#1 and -#2), along with 2 control siRNAs (siRNAs for EGFP and LUC).
Transfection of si-RASEFs into lung cancer cells decreased the level of RASEF protein, and resulted in significant reduction of cell viability and colony numbers measured (Fig. 3A–C; statistical analysis of colony formation assay is in Supplementary Fig. S4). These results suggest that RASEF is indispensable for cell growth or survival of lung cancer cells.

We further evaluated the role of RASEF in cell growth by introducing RASEF expression vector or mock plasmid into BEAS-2B bronchial epithelial cells and DMS114 lung cancer cells, which scarcely expressed endogenous RASEF. We observed significantly rapid growth of the cells transfected with RASEF expression vector compared with those with mock plasmid (Fig. 3D and E). These data further imply RASEF to be important for growth of cells.

**Elevation of phosphorylated ERK1/2 by RASEF expression**

Since it has been reported that some Rab proteins were involved in the positive regulation of Mitogen-activated protein kinase (MAPK) cascade that is well-known to be
crucial for cell proliferation (44, 45), we subsequently examined the possibility that RASEF could affect the activity of MAPK cascade in lung cancer cells. We first investigated by Western blot analysis of lung cancer cells the phosphorylation levels of 3 MAPK molecules, c-Raf, MEK1/2, and ERK1/2 according to the levels of RASEF introduction or reduction. Transfection of RASEF expression vector into endogenous RASEF-negative DMS114 cells increased the levels of phospho-ERK1/2 (pERK1/2) compared with that of mock vector, whereas the levels of total ERK1/2 protein were not different between the cells transfected with RASEF expression vector and those with mock vector (Fig. 4A). In addition, transfection of siRNAs for RASEF into endogenous RASEF-positive NCI-H2170 cells suppressed RASEF expression, and resulted in significant decrease of the phosphorylated ERK1/2 (pERK1/2), but not total ERK1/2 (Fig. 4B). The levels of pMEK1/2 and pc-Raf as well as total MEK1/2 and total c-Raf, which are upstream kinases of ERK1/2 were not changed in these 2 assays (Fig. 4A and B), suggesting that RASEF protein expression could selectively enhance ERK1/2 phosphorylation. Moreover, transfection of RASEF expression vector into RASEF-negative DMS114 cells increased the phosphorylation levels of RSK (pRSK (T359/S363)), but did not affect the levels of total RSK which is one of the substrate of ERK1/2 kinase, whereas inhibition of RASEF protein expression by siRNAs for RASEF in RASEF-positive NCI-H2170 cells reduced the levels of pRSK (T359/S363), but not those of total RSK, implying that RASEF protein expression could activate downstream cascade of ERK1/2 such as RSK (Fig. 4A and B).

We also evaluated by semiquantitative RT-PCR the expression levels of CCND1, CCNB1, and CDKN1A which were known to be transcriptionally up- or downregulated by MAPK pathway (46, 47). CCND1 and CCNB1 are reported to be transactivated by the activation of MAPK pathway, whereas CDKN1A is negatively regulated. As expected, overexpression of RASEF protein in RASEF-negative BEAS-2B and DMS114 cells increased the expression of both CCND1 and CCNB1, and reduced the expression of CDKN1A (Fig. 4C). On the other hand, suppression of RASEF protein expression by siRNAs in RASEF-positive A549 and NCI-H2170 cells reduced the expression of both CCND1 and CCNB1, and increased the expression of CDKN1A (Fig. 4D). These results indicate that the presence of RASEF protein might activate ERK1/2 signaling pathway in lung cancer cells.

Since cell-based assays suggested that the presence of total RASEF protein in lung cancer cells could elevate the levels of phospho-ERK1/2, but not those of total ERK1/2 protein, we subsequently evaluated the association between total RASEF protein expression and the levels of phospho-ERK1/2 in 8 lung cancer cell lines and BEAS-2B cells (Supplementary Fig. S5A). The Spearman correlation coefficient indicated that relative ERK1/2 phosphorylation, which was defined as phospho-ERK signal/total ERK protein signal, was significantly correlated with expression levels of total RASEF protein (Supplementary Fig. S5B), suggesting that the presence of total RASEF protein could increase the levels of phospho-ERK1/2.

We further carried out immunohistochemical staining on tissue microarray of 323 NSCLCs using a rabbit anti-
phospho-ERK1/2 (pERK1/2) polyclonal antibody, and compared its expression with total RASEF protein positivity, which were classified as strong positive, weak positive, or absent staining. We confirmed that strong total RASEF protein positivity was significantly correlated with strong phospho-ERK1/2 positivity using chi-square test ($\chi^2 = 16.778$, $P < 0.0001$; representative images were shown in Supplementary Fig. S6).

**Table 1.** Association between RASEF-positivity in NSCLC tissues and patients' characteristics ($n = 341$)

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<th>Weak expression $n = 150$</th>
<th>Absent expression $n = 65$</th>
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Abbreviations: non-ADC, squamous cell carcinoma plus large-cell carcinoma and adenosquamous cell carcinoma.

$^aP < 0.05$ (Fisher exact test).

**Table 2.** Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

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<th>Variables</th>
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<th>$P$-value Strong positive/weak positive or absent</th>
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<td>1.065-2.441</td>
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<td>$0.0239^a$</td>
</tr>
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<td>Smoking status</td>
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<td>0.902</td>
<td>0.855-1.941</td>
<td>Strong positive/weak positive or absent</td>
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<td>1.427</td>
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<td>1.007-2.023</td>
<td>Strong positive/weak positive or absent</td>
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<td>Pt factor</td>
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<td>2.171</td>
<td>1.874-4.255</td>
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<td>PN factor</td>
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<td>1.791-3.596</td>
<td>Strong positive/weak positive or absent</td>
<td>$&lt;0.0001^a$</td>
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Abbreviations: non-ADC, squamous cell carcinoma plus large-cell carcinoma and adenosquamous cell carcinoma.

$^aP < 0.05$. 

Oshita et al.
Identification of ERK1/2-interacting sites on RASEF

To investigate the molecular biologic mechanism of regulation of ERK1/2 phosphorylation by total RASEF protein, we examined the interaction between endogenous RASEF and ERK1/2 by immunoprecipitation assay using extracts from lung cancer NCI-H2170 cells and anti-RASEF antibody, and detected binding of endogenous RASEF to endogenous ERK1/2 (Fig. 5A), suggesting that phosphorylation of ERK1/2 was likely to be increased through its interaction with RASEF. The results support the hypothesis that total RASEF protein expression might play an important role in a subset of clinical lung cancers probably through its interaction with and subsequent enhancement of the phosphorylation levels of ERK1/2.

To narrow down the ERK1/2-interacting sites on RASEF, we first constructed 3 vectors expressing partial RASEF protein with Flag-tag (RASEF1–240, RASEF170–520, and RASEF455–740; Fig. 5B) and transfected either of them into DMS114 cells. Immunoprecipitation assay indicated that the only COOH-terminal portion of RASEF...
(RASEF_{55..740}) including a RAB domain was able to bind to endogenous ERK1/2 (Fig. 5C). To further define the minimal ERK1/2-interacting sites, we constructed 4 additional vectors expressing 55-amino-acid protein derived from a COOH-terminal portion of RASEF (RASEF_{520..575}, RASEF_{575..630}, RASEF_{630..685}, and RASEF_{685..740}; Fig. 5A) and transfected either of them into DMS114 cells. Immunoprecipitation assay with anti-Flag M2 agarose and subsequent Western blotting with anti-ERK1/2 antibody revealed that RASEF_{520..575} was able to bind to ERK1/2, but other peptides were not (Fig. 5D). These experiments indicate that the 55-amino-acid polypeptide in RASEF (codons 520–575) should be responsible for interacting with ERK1/2.

Figure 4. Enhanced phosphorylation of ERK1/2 by RASEF in lung cancer cells. A, expression of MAPK signal molecules and their phosphorylation levels in DMS114 cells transfected with RASEF expression vector or mock plasmid. B, expression of MAPK signal molecules and their phosphorylation levels in NCI-H2170 cells transfected with siRNAs for RASEF (si-RASEF#2) or control siRNAs (si-LUC). C and D, expression levels of downstream target genes of MAPK cascade were regulated by RASEF expression in lung cancer cells. Total RNA from BEAS-2B and DMS114 cells transfected with RASEF expression vector or mock plasmid (C) and A549 and NCI-H2170 cells transfected with siRNAs for RASEF (si-RASEF#2) or control siRNAs (si-LUC; D) were subjected to reverse-transcription reaction, followed by PCR reaction to evaluate the expression levels of CCND1, CCNB1, and CDKN1A transcription. Western blotting with antiphosphorylated ERK1/2 antibody was conducted to confirm the change of ERK1/2 phosphorylation according to RASEF expression.
Growth inhibition of lung cancer cells by dominant-negative peptides inhibiting RASEF–ERK1/2 interaction

To examine whether RASEF–ERK1/2 interaction could be essential for cancer cell growth, we synthesized 3 different kinds of 23-amino acid polypeptides covering the ERK1/2-interacting site on RASEF at codons 520 to 575 with membrane-permeable 11 residues of arginine (11R) at its N-terminus (11R-RASEF520–542, 11R-RASEF536–558, and 11R-RASEF553–575; Fig. 6A). Initially, we evaluated the effect of inhibition of the RASEF–ERK1/2 interaction on lung cancer cell growth, we incubated RASEF-positive A549 and NCI-H2170 cells, and RASEF-negative BEAS-2B cells with each of the 3 peptides at the final concentration of 5, 10, or 15 μmol/L in culture media. The MTT assay revealed that only 11R-RASEF553–575 peptide showed growth suppressive effect on A549 and NCI-H2170 cells on a dose-dependent manner, but not on BEAS-2B cells (Fig. 6B and C). We also confirmed by immunoprecipitation assay using antibodies to RASEF and ERK1/2 that the endogenous RASEF–ERK1/2 binding was inhibited by addition of 11R-RASEF553–575 peptides, but not its control scramble peptides into culture media of lung cancer NCI-H2170 cells (Fig. 6D). The results suggest that RASEF–ERK1/2 interaction plays a critical role in lung cancer cell growth.

Discussion

Recent accumulation of knowledge in cancer genomics introduced new strategies for cancer treatment such as molecular-targeted therapy (5). Molecular-targeted drugs are expected to be highly specific to malignant cells, with minimal adverse effects due to their well-defined mechanisms of action. To find such molecules, we established an effective screening system to identify proteins that were activated specifically in lung cancer cells. The strategy was as follows: (i) identification of upregulated genes in 120 lung cancer samples through the genome-wide gene expression profiles analysis, covering 27,648 genes or ESTs, coupled with laser microdissection; (ii) verification of very low or absent expression of genes in normal organs by cDNA
microarray analysis and multiple-tissue Northern blot analysis; (iii) confirmation of the clinicopathologic significance of their overexpression using tissue microarray consisting of hundreds of NSCLC tissue samples; and (iv) verification of the targeted genes whether they are essential for the survival or growth of lung cancer cells by siRNA. Through this screening system, we have found that RASEF is overexpressed commonly in clinical lung cancer samples and cell lines, and its gene product plays an important role in the growth of lung cancer cells.

RASEF is described as a member of Rab GTPase family which generally plays important roles in vesicle trafficking. To date, a few studies indicated aberrant expression of RASEF in human cancers, but results were contradictory;
RASEF was reported to be downregulated in uveal melanomas, whereas it was overexpressed in esophageal squamous cell carcinomas (48, 49). To examine the mechanism of RASEF activation and overexpression in lung cancer, we checked previous publications and databases for RASEF including the data of CGH and genome sequencing (http://cancer.sanger.ac.uk/cosmic/gene/). However, mis-sense mutation was indicated in 10 of 904 (1.1%) lung cancers, but no amplification or translocation of RASEF gene was reported in lung cancers. Therefore, we speculate that overexpression of RASEF may be mainly caused by epigenetic mechanism. Further analysis of RASEF including screening of activating mutation by functional assays and/or mechanism of epigenetic regulation of RASEF might further clarify the oncogenic function of RASEF.

In this study, we confirmed that inhibition of expression of endogenous RASEF by siRNA resulted in marked reduction of cell viability of lung cancer cells, and that exogenously introduced RASEF promoted cell growth. Moreover, tissue microarray analysis using 2 independent sample sets revealed that strong RASEF expression was a prognostic factor for patients with surgically treated NSCLC. Interestingly, several openly available microarray databases also independently support our data that the higher expression of RASEF is likely to associate with the poorer prognosis of patients with lung cancer (ProgoScan; http://www.prognoscan.org/). The data suggest that RASEF contributes to viability and malignant potential of lung cancer cells and should be a clinically promising prognostic biomarker and novel molecule target for this disease.

Several Rab proteins have been reported to activate the MAPK cascade that is an important intracellular signaling pathway for cell proliferation, cell survival, development, cell cycle, angiogenesis, and cell migration. Rab11 regulates JNK pathway as well as typical MAPK cascade during Drosophila wing development (44), whereas RBE1 (Rab-like protein 1) is thought to promote cell growth through activation of ERK signaling in breast cancer cells (45). We examined whether the presence of total RASEF protein could affect the activity of MAPK signal molecules by transfection of siRNAs for RASEF or RASEF expression vectors into lung cancer cells, and found that the presence of total RASEF protein could elevate the levels of phospho-ERK1/2, but not the amount of total ERK1/2 protein in lung cancer cells probably through its interaction with total ERK1/2.

Somatic EGFR mutation was reported to be biologically important for the clinical response of NSCLCs to EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib (3, 5). To examine the relevance of strong RASEF expression to this type of NSCLCs with EGFR mutation, we examined the association between RASEF overexpression and EGFR mutation status in NSCLC tissues (325 cases available) by using specific antibodies to E746-A750 deletion and those to a point mutation (L858R) in EGFR, and found that there was no exclusive or inclusive relationship between strong RASEF expression and EGFR mutation status, although the frequency of EGFR mutation was higher in NSCLCs with strong RASEF positive compared with those with RASEF weak positive or negative (44% vs. 25%, P = 0.0012, Chi-square test). RASEF may play an essential role in various types of lung cancer including those with other driver mutations.

Since inhibition of interaction between RASEF and ERK1/2 by RASEF-derived peptides with a membrane-permeable 11 residues of arginine (11R) at its N-terminus inhibited the growth of RASEF-positive lung cancer cells, targeting RASEF–ERK1/2 interaction as well as RASEF itself by small molecules and/or nucleic acid drugs is one of the possible therapeutic approaches for cancer. The MAPK cascade is well characterized to play a critical role in human carcinogenesis, and has been the subject of intense research for discovery of novel anticancer drugs. Selumetinib (AZD6244, ARRY-142886), a selective inhibitor of mitogen-activated protein kinase kinase 1/2 (MEK1/2), was reported to be effective for a subset of patients with cancer in clinical trials, but adverse reactions are also observed in a certain portion of the patients (50). Therefore, targeting therapy against cancer-specific coactivator or mediator of MAPK cascade, which is expressed specifically in cancer tissues, but not in normal tissues, should be an alternative approach for cancer treatment with less adverse events. Although the function of RASEF in carcinogenesis remains unclear, therapeutic strategy targeting RASEF and/or RASEF–ERK1/2 interaction is thought to have a great potential.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: Y. Daigo
Acquisition of data (proposed animals, acquired and managed patients, provided facilities, etc.): H. Oshita, R. Nishino, T. Fujimoto, T. Kata, H. Akiyama, Y. Daigo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Oshita, R. Nishino, M. Aragaki, Y. Nakamura, Y. Daigo
Writing, review, and/or revision of the manuscript: H. Oshita, N. Kohno, Y. Nakamura, Y. Daigo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Takano, E. Tsuchiya, N. Kohno
Study supervision: Y. Nakamura, Y. Daigo

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