Identification of Epstein-Barr Virus-Induced Gene 3 as a Novel Serum and Tissue Biomarker and a Therapeutic Target for Lung Cancer

Ryohei Nishino,1,4 Atsushi Takano,1,2,3 Hideto Oshita,1 Nobuhisa Ishikawa,1 Hirohiko Akiyama,5 Hiroyuki Ito,6 Haruhiko Nakayama,6 Yohei Miyagi,7 Eiju Tsuchiya,7 Nobuoki Kohno,4 Yusuke Nakamura,1 and Yataro Daigo,1,2,3*

1Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
2Department of Medical Oncology and 3Cancer Center, Shiga University of Medical Science, Otsu 520-2192, Japan
4Department of Molecular and Internal Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan
5Department of Thoracic Surgery, Saitama Cancer Center, Saitama 362-0806, Japan
6Division of Thoracic Surgery, and 7Molecular Pathology and Genetics Division, Kanagawa Cancer Center, Yokohama 241-0815, Japan
*Request for reprints: ydaigo@ims.u-tokyo.ac.jp

Running title: EBI3, a Novel Biomarker for Lung Cancer

Key words: oncogenes, cancer antigen, biomarker, therapeutic target, lung cancer
Statement of Clinical Relevance

Since there is a significant association between EBI3 expression and poor prognosis of patients with lung cancer, EBI3 positivity in resected specimens could be an index providing information useful to physicians in administration of adjuvant therapy and intensive follow-up of cancer patients who are more likely to suffer a relapse. Since serum levels of EBI3 are specifically high in operable lung cancer patients, serum EBI3 can be used for detecting cancer at an early stage and for monitoring the disease. EBI3 can be classified as a typical oncoantigen and may play important roles in cancer proliferation; therefore, selective inhibition of EBI3 function could be a promising therapeutic strategy with powerful biological activity against lung cancer with a minimal risk of adverse events.
Abstract

Purpose: This study aims to identify novel biomarkers and therapeutic targets for lung cancer.

Experimental Design: We performed gene expression profile analysis of 120 lung cancers in order to screen for genes encoding transmembrane/secretory molecules that are commonly transactivated in lung cancers. Epstein-Barr virus-induced gene 3 (EBI3), which encodes a secretory glycoprotein, was selected as a good candidate. Immunohistochemical staining using tissue microarray consisting of 414 non-small-cell lung cancers was applied to examine the expression level and prognostic value of EBI3. Serum EBI3 levels in 400 individuals for training assays (274 lung cancers and 126 healthy volunteers) and those in 173 individuals for validation analysis (132 lung cancers and 41 healthy volunteers) were measured by ELISA. The role of EBI3 in cancer cell growth was examined by small interfering RNA (siRNA) and cell growth assays using cells stably expressing exogenous EBI3.

Results: Immunohistochemical staining of EBI3 using tissue microarrays revealed that a high level of EBI3 expression was associated with a poor prognosis of lung cancer \( (P = 0.0014) \), and multivariate analysis confirmed it to be an independent prognostic factor \( (P = 0.0439) \). Serum levels of EBI3 in the training set were found to be significantly higher in lung cancer patients than in healthy volunteers; this result was also observed in the validation set. Furthermore, reduction in EBI3 expression by siRNA suppressed cancer cell proliferation, while induction of exogenous EBI3 conferred growth-promoting activity.

Conclusions: EBI3 is a potential serum and tissue biomarker as well as therapeutic target for lung cancer.
Introduction

Lung cancer is the leading cause of cancer deaths in the world (1). Although the overall 5-year survival rate of patients with non-small-cell lung cancer (NSCLC) is only 10%–15%, that of patients with stage IA NSCLC exceeds 80% (2). Several tumor markers such as carcinoembryonic antigen (CEA), serum cytokeratin 19 fragment (CYFRA21-1), and progastrin-releasing peptide (Pro-GRP) are clinically available for lung cancer diagnosis. However, these markers are not entirely useful for screening early stage cancers because of their relatively low sensitivity and specificity (3, 4). Therefore, the development of novel diagnostic tools is necessary.

Since cell surface proteins are considered more accessible to immune mechanisms and drug delivery systems, identification of cancer-specific cell surface and secretory proteins may be an effective approach to develop novel diagnostic biomarkers such as serum biomarkers and antibody-based therapies (5). We have screened genes encoding molecules that are upregulated in lung cancer tissues but not expressed in normal human tissues. For these experiments, we performed cDNA microarray analysis of 27,648 genes or expressed sequence tags (ESTs) in combination with purification of tumor cells by laser microdissection. In addition, we compared the microarray data with the expression profiles of 31 normal tissues (27 adult and 4 fetal organs) (6-10). To verify the biological and clinicopathological significance of the respective gene products, we performed tumor tissue microarray analysis of clinical lung cancer materials as well as loss of function assays using small interfering RNA (siRNA) (11-29). We also used ELISA to determine whether the genes exhibiting a tumor-specific transmembrane/secretory protein are potential diagnostic serum biomarkers (30-35). We also screened for epitope peptides
recognized by human histocompatibility leukocyte-A0201/A2402–restricted cytotoxic T lymphocyte from the list of selected cancer-testis antigens (36-41). This systematic approach revealed that the Epstein-Barr virus-induced gene 3 (EBI3) was frequently transactivated in primary lung cancer.

_EBI3_ encodes a 34-kDa secretory glycoprotein with a signal peptide and 2 fibronectin type III domains (42). The fibronectin type III repeat region is an approximately 100-amino-acid domain that contains binding sites for DNA, heparin, fibrin, and cell surface proteins (43). EBI3 had possible roles in maintenance of pregnancy or immunotolerance of the human maternal body toward the fetus (44). A few studies indicated the expression of _EBI3_ in Hodgkin’s lymphoma and adult T-cell lymphoma/leukemia (45, 46). However, the significance of EBI3 activation in human carcinogenesis and its potential as a therapeutic target as well as a serological/prognostic biomarker were not clarified. We here describe a crucial role of EBI3 activation in human lung cancer development and progression as well as its potential as a novel biomarker and/or molecular therapeutic target for lung cancer.
Materials and Methods

Cell lines and tissue samples. The human lung cancer cell lines used in this study included 8 adenocarcinoma (ADC; A427, A549, LC319, PC-3, PC-9, PC-14, NCI-H1373, and NCI-H1781), one bronchioalveolar carcinoma (BAC; NCI-H358), 6 squamous cell carcinoma (SCC; NCI-H226, NCI-H520, NCI-H2170, NCI-H1703, EBC-1, and RERF-LC-AI), one large cell carcinoma (LCC; LX1), and six small cell lung cancers (SCLC; DMS114, DMS273, SBC-3, SBC-5, NCI-H196, and H446). A human bronchial epithelial cell line (BEAS-2B) was used as a control (Supplementary Table S1). 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 with informed consent as described elsewhere (6, 10). All tumors were staged on the basis of the postsurgical pathologic TNM stage classification of the International Union Against Cancer (47). In addition, a total of 414 NSCLC tissues and adjacent normal lung tissues had been obtained with clinicopathologic data from patients who underwent surgery at Saitama Cancer Center (Saitama, Japan). Summary of patient background was described in Supplementary Table S2. This study and the use of all clinical materials mentioned were approved by institutional ethical committees.

Serum samples. Serum samples were obtained in 2000-2008 with informed consent from 636 individuals comprising 463 training set and 173 validation set for serum EBI3 detection by ELISA. The training set includes serum samples from 274 patients with lung cancers, 63 patients with chronic obstructive lung disease (COPD), and 126 healthy volunteers. These serum samples from NSCLC patients were
obtained at Kanagawa Cancer Center Hospital (Yokohama, Japan; n = 121) and Hiroshima University (Hiroshima, Japan; n=78), and those from SCLC patients were enrolled as a part of the Japanese Project for Personalized Medicine (BioBank Japan; n = 75). Serum samples from COPD were obtained from BioBank Japan, and those from healthy volunteers were obtained from Hiroshima University. The independent validation set for confirming the reproducibility of serum EBI3 as a cancer detection biomarker included serum samples from 101 NSCLC and 31 SCLC patients from BioBank Japan and those from 41 healthy volunteers from Hiroshima University. Summary of patient background is described on Supplementary Table S3. Serum was obtained at the time of diagnosis and stored at -150°C. The use of all clinical materials mentioned was approved by institutional ethical committees.

**Semiquantitative RT-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 primers specific to EBI3 or β-actin (ACTB) as an internal control: EBI3, 5'-TGTTCTCCATGGCTCCCTAC-3' and 5'-AGCTCCCTGACGCTTGTAAC-3'; ACTB, 5'-GAGGTGATAGCATTGCTTTCG-3' and 5'-CAAGTCAGTGTACAGGTAAGC-3'. PCRs were optimized for the number of cycles to ensure product intensity to be within the linear phase of amplification.

**Northern blot analysis.** Human multiple tissue blots covering 16 tissues (BD
Biosciences, Clontech) were hybridized with an α-32P-dCTP-labeled, 404-bp PCR product of EBI3, prepared as a probe using primers 5′-TGTTCTCCATGGCTCCCTAC-3′ and 5′-CTACTTGCCCAGGCTCATTG-3′. Prehybridization, hybridization, and washing were done following the manufacturer’s recommendations. The blots were autoradiographed with intensifying screens at -80°C for 7 days.

**Immunocytochemical analysis.** Immunocytochemical analyses were performed as previously described using a commercially available goat polyclonal anti-human EBI3 antibody (Santa Cruz Biotechnology; Catalog No. sc-26797) (14). On immunocytochemical analyses, we confirmed that the antibody was specific for EBI3 protein, using NSCLC cell lines that endogenously expressed EBI3 as well as cell lines derived from NSCLC or bronchial epithelia that did not express it.

**Tissue microarray and Immunohistochemistry.** Tumor tissue microarrays were constructed with 414 formalin-fixed primary lung cancers as described elsewhere (48). The tissue area for sampling was selected by visual alignment with the corresponding HE-stained section on a slide. Three, four, or five tissue cores (diameter 0.6 mm; height 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.

To investigate the EBI3 protein expression in clinical samples that had been
embedded in paraffin blocks, we stained sections using ENVISION+ kit/horseradish peroxidase (Dako Cytomation) for immunostaining according to the manufacturer’s instructions (12). In brief, antigens were retrieved by heating the sections in Target Retrieval Solution, Citrate pH 6 (Dako Cytomation). A goat polyclonal anti-human EBI3 antibody (Santa Cruz Biotechnology; Catalog No. sc-26797) was added to each slide after blocking of endogenous peroxidase and proteins. The sections were incubated with HRP-labeled anti-goat IgG as the secondary antibody. Substrate-chromogen was added, and the specimens were counterstained with hematoxylin. On immunohistochemical analyses, we confirmed that the antibody was specific for EBI3 protein by antigen blocking assays using EBI3 antigen peptides (Catalog No. sc-26797P; Santa Cruz Biotechnology) that were used for immunization of goats to produce polyclonal anti-human EBI3 antibodies (details were shown in Supplementary Fig. S1 legend).

Three independent investigators semiquantitatively assessed EBI3 positivity without prior knowledge of clinicopathologic data. The intensity of EBI3 staining was evaluated using the following criteria: strong positive (scored as 2+), brown staining in > 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 if two or more investigators independently defined them as such.

**ELISA.** We constructed a sandwich-type ELISA system as described previously (32). In brief, a 96-well microplate (439454; NALGE NUNC International) was coated with a goat polyclonal anti-human EBI3 antibody (Santa Cruz Biotechnology; Catalog No.
(sc-26797) overnight at 4°C. Wells were blocked with PBS (pH 7.4) containing 5% BSA and 0.05% Tween 20 for 2 h and then 3-fold diluted sera were added and incubated for 2 h. A biotinylated polyclonal antibody specific for EBI3 using Biotin Labeling Kit-NH2 (Dojindo Laboratories) was added as a detection antibody and incubated for 2 h, followed by reaction with avidin-conjugated peroxidase (P347; Dako Cytomation) using a Substrate Reagent (R&D Systems). The reaction was stopped by adding 50 µL of 2N sulfuric acid. Color intensity was determined by a photometer at a wavelength of 450 nm, with a reference wavelength of 570 nm. Standard curve of color intensity was made by using immunogen EBI3 peptide for anti-EBI3 antibody production (Catalog No.sc-26797; Santa Cruz Biotechnology). The color intensity gained by applying 20 nM of the EBI3 peptide was tentatively defined as 1 U/mL. Levels of three conventional tumor markers (CEA, CYFRA21-1, and Pro-GRP) in serum were measured by ELISA with a commercially available enzyme test kit (CEA: Hope Laboratories; CYFRA21-1: DRG Instruments GmbH; Pro-GRP: TFB Inc.) according to the supplier’s recommendations.

**RNA interference assay.** Small interfering RNA (siRNA) duplexes (Dharmacon, Inc.; 600 pM) were transfected into lung cancer cell lines A549 and LC319, using 30 µL of Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. The transfected cells were cultured for 7 days. Cell numbers and viability were evaluated by Giemsa staining and triplicate MTT assays (cell counting kit-8 solution; Dojindo Laboratories). To confirm suppression of EBI3 expression, semiquantitative RT-PCR was carried out with synthesized primers specific to EBI3 described above. The
target sequences of the synthetic oligonucleotides for RNAi were as follows: control 1 (On-Target plus; Dharmacon, Inc.; pool of 5'-UGGUUUACAUGUCGACUAA-3'; 5'-UGGUUUACAUGUUUUCUGA-3'; 5'-UGGUUUACAUGUUUUCCUA-3'; 5'-UGGUUUACAUGUGUGUGA-3'); control 2 (Luciferase/LUC: Photinus pyralis luciferase gene), 5'-CGUACGCGGAAUACUUCGA-3'; siRNAs against EBI3-1 (si-EBI3-#1), 5'-CAAUGAGCCUGGGCAAGUA-3'; si-EBI3-#2, 5'-UCACCGAUGUCCAGCUGUU-3'.

**Cell growth assay.** To establish COS-7 cells stably expressing EBI3, plasmids expressing either EBI3 (pcDNA3.1-EBI3-myc/His) or mock plasmids (pcDNA3.1-myc/His) were transfected into COS-7 cells that did not express endogenous EBI3, using FuGENE 6 Transfection Reagent (Roche Diagnostics) according to the manufacturer’s protocol. Transfected cells were cultured in DMEM containing 10% FBS and geneticin (0.4 mg/ml) for 14 days; then 50 individual colonies were trypsinized and screened for stable transfectants by a limiting-dilution assay. Expression of EBI3 protein was determined in each clone by Western blotting and immunostaining. COS-7 transfectants that could stably express EBI3 were seeded onto six-well plates (1 X 10⁴ cells/well), and maintained in medium containing 10% FCS and 0.4 mg/ml geneticin. After 120 hours cell viability was evaluated by MTT assay. Colonies were also stained at the same time. All experiments were done in triplicate.

**Statistical analysis.** Statistical analyses were performed using the StatView statistical program (SAS). Survival curves were calculated from the date of surgery.
to the time of death due to NSCLC or to the last follow-up observation. Kaplan–Meier curves were calculated for each relevant variable and for EBI3 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. The sample size for comparing 2 survival curves was confirmed using a statistical power level of 90% and a 2-sided level of 5% on the basis of observed probability. The risk factors associated with the prognosis of patients with NSCLC were evaluated using Cox's proportional hazard regression model with a step-down procedure. Only variables that were statistically significant in univariate analysis were evaluated by multivariate analysis. The criterion for removing a variable was the likelihood ratio statistic, which was based on the maximum partial likelihood estimate (default $P$ value of 0.05 for removal from the model).

Differences in the serum EBI3 levels among tumor groups, healthy volunteers, and patients with chronic obstructive pulmonary disease (COPD) were analyzed by Mann–Whitney $U$ tests. Serum EBI3 levels before and after surgery were analyzed by the paired $t$-test. The correlations between serum biomarkers were calculated by Spearman’s rank correlation. Serum levels of EBI3, CEA, CYFRA21-1, and Pro-GRP were analyzed by drawing receiver operating characteristic (ROC) curves for their capability of distinguishing between patients with lung cancers and healthy volunteers. Statistical comparison between the area under the ROC curve (AUC) of EBI3 and that of other conventional serum markers was performed by MedCalc software (MedCalc Software, Mariakerke, Belgium), which calculates the difference in AUC and standard errors (SEs), followed by statistical calculation of the $P$ value based on the method proposed by Hanley and Mc Nail (49).
Results

**EBI3 expression in lung cancers and normal tissues.** To screen novel molecule targets that can be used for detection of cancer at an early stage and for development of novel treatment strategies, we first performed genome-wide gene expression profile analysis of 120 lung carcinomas using a cDNA microarray (6-10). Among 27,648 screened genes or ESTs, we identified elevated expression (5-fold or higher) of *EBI3* transcripts in the great majority of lung cancer tissues examined. We subsequently confirmed its overexpression by semiquantitative RT-PCR experiments in 11 of 15 lung cancer tissues and in 14 of 22 lung cancer cell lines (Fig. 1A). We subsequently performed immunocytochemical analysis to examine the subcellular localization of endogenous EBI3 protein in 4 lung cancer cell lines as well as BEAS-2B cells. We found that EBI3 was detected in the cytoplasm of EBI3-positive lung cancer cell lines with granular appearance, while it was not detected in cells in which the *EBI3* transcript was not detected (Fig. 1B). Since EBI3 is a secretory protein, we applied ELISA to evaluate the EBI3 protein levels in the culture media of the same set of cell lines. The amount of detectable EBI3 in culture media was concordant to the expression levels of EBI3 detected by semiquantitative RT-PCR and immunocytochemistry, suggesting that anti-EBI3 antibody is capable of specifically recognizing the secretory EBI3 protein (Fig. 1C).

Northern blot analysis using an *EBI3* cDNA fragment as a probe identified a 1.3-kb transcript that was highly expressed only in placenta (Fig. 2A). We examined the expression of the EBI3 protein in 5 normal tissues (liver, heart, kidney, lung, and placenta) and lung cancer tissues using anti-EBI3 antibodies. EBI3-positive staining was mainly observed in the cytoplasm of placenta and lung cancer cells; however, it
was not detectable in 4 normal tissues (Fig. 2B). We confirmed that the positive
signal by anti-EBI3 antibody obtained in lung cancer tissues was markedly diminished
by preincubation of the antibody with an EBI3 antigen peptide used for immunization
of a goat, which independently indicated its specificity to the EBI3 protein
(Supplementary Fig. S1). We also evaluated EBI3 staining in NSCLC and adjacent
normal lung tissues, confirming the EBI3 protein to be positively stained in the majority
of NSCLC tissues but not in their corresponding normal lungs (Fig. 2C).

**Association of EBI3 expression with poor prognosis of patients with NSCLC.**
To investigate the biological and clinicopathological significance of EBI3 in pulmonary
carcinogenesis, we performed immunohistochemical staining on tissue microarray
containing primary lung tumor tissue sections obtained from 414 patients with NSCLC,
who had undergone surgery. We classified a pattern of EBI3 expression on the
tissue array ranging from absent (scored as 0) to weak/strong positive (scored as 1+
to 2+; Fig. 2D). The number of NSCLC tissues scored as 0, 1, and 2 was 55 (13.3%),
156 (37.7%), and 203 (49.0%), respectively. As shown in Table 1A, male gender (P
= 0.0001 by Fisher's exact test), no smoking history (P = 0.0051), larger tumor size
(pT2–4; P = 0.0012), and non-adenocarcinoma (ADC) histology (P = 0.0003) were
significantly associated with strong EBI3 positivity. As shown in Fig. 2E, the survival
periods of patients with NSCLC showing absent/weak positive EBI3 staining was
significantly longer than those that of patients with strong positive EBI3 staining (P
= 0.0014, log-rank test). To determine the prognostic significance of the clinical
characteristics and EBI3 expression in patients with NSCLC, we performed Cox's
proportional hazard regression analysis on the parameters listed in Table 1B.
Multivariate analysis determined that EBI3 positivity ($P = 0.0439$) as well as age, pathological tumor stage, and pathological node stage were independently useful as prognostic factors for surgically treated patients with NSCLC.

**Serum levels of EBI3 in patients with lung cancer.** Since the *in vitro* findings by ELISA evaluating the EBI3 protein levels in the culture media of lung cancer cells had suggested application of EBI3 as a serum biomarker, we investigated whether the EBI3 protein is detectable in the sera of patients with lung cancer. ELISA experiments detected the EBI3 protein in serological samples obtained from 274 patients with lung cancer, 63 patients with COPD, and 126 healthy volunteers. The mean serum level of EBI3 in patients with lung cancer, patients with COPD, and healthy volunteers was $6.1 \pm 5.2$ U/mL (mean $\pm$ 1 SD), $1.9 \pm 2.6$ U/mL, and $1.5 \pm 1.6$ U/mL, respectively. The serum levels of EBI3 were significantly higher in patients with lung cancer than in those with COPD or in healthy donors ($P < 0.0001$ and $P < 0.0001$, respectively, Mann–Whitney $U$ test), whereas the difference between healthy individuals and patients with COPD was not significant ($P = 0.246$). When classified according to histological type, the serum levels of EBI3 were $6.0 \pm 5.0$ U/mL in patients with lung ADC, $6.1 \pm 4.4$ U/mL in patients with lung SCC, and $6.3 \pm 6.2$ U/mL in patients with SCLC (Fig. 3A); the differences between the 3 histological types were not significant (ADC vs. SCC, $P = 0.4857$; ADC vs. SCLC, $P = 0.8309$; SCC vs. SCLC, $P = 0.4707$). Using ROC curves drawn with the data of the 274 patients with lung cancer and the 126 healthy volunteers, the cutoff level in this assay was set to provide optimal diagnostic accuracy and likelihood ratios (minimal false-negative and false-positive results) for EBI3, i.e., 5.5 U/mL with a sensitivity of 46.7% (128 of 274)
and specificity of 97.6% (123 of 126). According to tumor histology, the proportion of serum EBI3-positive cases was 47.1% for ADC [95% confidence interval (CI), 39.2%–54.9%; 73 of 155], 54.5% for SCC (95% CI, 39.8%–69.2%; 24 of 44), and 41.3% for SCLC (95% CI, 30.2%–52.5%; 31 of 75). The proportion of serum EBI3-positive cases was 3.2% (2 of 63) for COPD. Validation analysis using another independent set of serum samples obtained from patients with lung cancer and healthy volunteers also confirmed significant elevation of EBI3 in the sera of patients with lung cancer (Fig. 3B).

We also compared the serum EBI3 values with the expression levels of EBI3 in primary tumors in the same set of 16 NSCLC cases whose serum had been collected before surgery (8 patients with EBI3-positive tumors and 8 with EBI3-negative tumors). The levels of serum EBI3 showed good correlation with the expression levels of EBI3 in primary tumors (Fig. 3C). We then performed ELISA using 20 pairs (before surgery and 2 months after surgery) of serum samples from patients with lung cancer to monitor the levels of serum EBI3 in these patients. The concentration of serum EBI3 decreased significantly after surgical resection of the primary tumors (Fig. 3D). The results independently support the high specificity and potentiality of serum EBI3 as a biomarker for detecting cancer at an early stage and for monitoring the disease.

**Combination assay using EBI3 and CEA/CYFRA/Pro-GRP as tumor markers.** To evaluate the clinical usefulness of serum EBI3 levels as a tumor detection biomarker, we also measured the serum levels of 3 conventional tumor markers (CEA for ADC, CYFRA21-1 for SCC, and Pro-GRP for SCLC patients) in the same set of
serum samples from patients with cancer and control individuals by ELISA. AUC of EBI3 was significantly larger than that of CEA in lung ADC (difference between areas, 0.131; SE, 0.0401; 95% CI, 0.0521–0.0210; \( P = 0.0011 \)); it was also larger than that of CYFRA21-1 in lung SCC (difference between areas, 0.151; SE, 0.0572; 95% CI, 0.0388–0.0263; \( P = 0.0083 \)), although it was not as good as that of Pro-GRP in SCLC (difference between areas, 0.164; SE, 0.0359; 95% CI, 0.0935–0.0234; \( P < 0.0001 \), Fig. 3E). The correlation coefficient between EBI3 and the 3 conventional tumor markers (CEA, CYFRA21-1, and Pro-GRP) was not significant, indicating that measuring both EBI3 and the conventional tumor markers in serum can improve the overall sensitivity of lung cancer detection. Indeed, a combination assay using EBI3 and CEA in serum could improve the overall sensitivity of ADC detection and diagnosis until 65.8% (102 of 155). The sensitivity of CEA alone was 34.2% (53 of 155) and that of EBI3 was 47.1% (73 of 155). The false-positive rate of the combined assay for the 2 tumor markers among healthy volunteers (control group) was 4.8% (6 of 126). The false-positive rate for either CEA or EBI3 individually were 2.4% (3 of 126). The combination assay using EBI3 and CYFRA21-1 in serum could improve the overall sensitivity of SCC detection until 65.9%. For diagnosing SCC, the sensitivity of CYFRA21-1 alone was 38.6% (17 of 44) and that of EBI3 was 54.5% (24 of 44). The false-positive rate by combination of the 2 tumor markers among healthy volunteers (control group) was 4.8% (6 of 126). The combination assay using EBI3 and Pro-GRP could improve the overall sensitivity of SCLC detection until 72.0% (54 of 75). For diagnosing SCLC, the sensitivity of ProGRP alone was 60.0% (45 of 75) and that of EBI3 was 41.3% (31 of 75). The false-positive rate by combination of the 2 tumor markers among healthy volunteers was 4.0% (5 of 126).
Inhibition of growth of lung cancer cells by siRNA against EBI3. To assess whether EBI3 plays a role in growth or survival of lung cancer cells, we evaluated the knockdown effect of endogenous EBI3 expression by siRNA together with 2 different control siRNA (siRNA for CNT and LUC). Treatment of 2 different NSCLC cells, A549 or LC319, with the effective siRNA reduced the expression of EBI3 (Fig. 4A) and resulted in significant inhibition of cell viability and colony numbers as measured by MTT and colony formation assays (Figs. 4B and 4C). The results suggest that upregulation of EBI3 contribute to the growth or survival of cancer cells.

Growth-promoting effect of EBI3. To disclose the potential role of EBI3 overexpression in tumorigenesis, we transfected either EBI3-expressing plasmids or mock plasmids into COS-7 cells, which normally express very low endogenous EBI3, and established cells that stably expressed EBI3. Two established COS-7 cell lines expressing exogenous EBI3 (COS-7-EBI3-#A and COS-7-EBI3-#B; Fig. 5A) exhibited significant rapid cell growth compared with control mock cells (COS-7-MOCK-M1 and COS-7-MOCK-M2) (Fig. 5B). Furthermore, there was a tendency of COS7-EBI3 cells to form larger colonies than the control cells (Fig. 5C). These data strongly suggest a potential oncogenic effect of EBI3.

Discussion
We performed gene expression profile analysis using cDNA microarrays for screening genes encoding transmembrane/secretory proteins that are upregulated in the majority of lung cancers (5). With subsequent systemic screening systems utilizing
tumor tissue microarray, RNA interference, high-throughput ELISA, and bioinformatics, we demonstrated that EBI3 is a useful serum and cancer tissue biomarker. EBI3 is also a potential target for the development of novel drugs for treating lung cancer.

**EBI3** encodes a secretory glycoprotein; it dimerizes with p28 or interleukin (IL)-12 p35-related subunits to form IL-27 or IL-35 cytokines, respectively (42, 43). IL-35 is a new member of the IL family and has an immune-suppressive function of regulatory T cells in mouse inflammatory tissues; however, the precise function is not well understood in the human immune system (50).

In this study, we demonstrated that EBI3 was expressed in placenta and was also highly expressed in the majority of surgically resected samples from patients with NSCLC. Strong EBI3 expression in NSCLC tissues is associated with poor prognosis. To the best of our knowledge, this is the first study to report that EBI3 expression has a strong prognostic value with regard to human NSCLC. We also found that inhibition of endogenous EBI3 expression by siRNA resulted in marked reduction in lung cancer cell viability. Concordantly, mammalian cells expressing exogenous EBI3 exhibited a significant increase in cellular proliferation. Since EBI3 is a secretory protein, presence of an autocrine/paracrine loop could be an explanation for the underlying mechanism of the growth-promoting effect of EBI3. Although the detailed function of EBI3 in lung carcinogenesis, including identification of its cell surface receptor, should be elucidated in future studies, our results implied that EBI3 may be associated with cancer growth and a highly malignant phenotype of lung tumors. Since EBI3 is considered to be a cancer antigen, it might be a good target for the development of cancer immunotherapy such as cancer vaccines as well as therapeutic strategies selectively targeting EBI3 activity, which could be essential
for cancer cell growth.

We constructed an ELISA system to measure serum levels of EBI3 and showed that serum EBI3 was significantly higher in patients with lung cancer than in patients with COPD and in healthy volunteers. Furthermore, we verified our results in an independent set of serum samples. The positivity of serum EBI3 seems to be associated with the presence of lung tumors because the concentration of serum EBI3 was significantly decreased after surgical resection of primary tumors and the serum EBI3 levels showed good correlation with its expression levels in primary tumor tissue in these patients. Importantly, AUC for serum EBI3 levels was significantly larger than that for CEA and CYFRA21-1, suggesting that EBI3 is a better diagnostic marker for lung cancer. When we classified all lung cancer serum samples used in this study (training and validation sets) into clinical stages of lung cancer, the sensitivity of serum EBI3 for cancer detection was 44.4% for stage I–II lung ADC, 50.0% for stage I–II lung SCC, and 35.2% for SCLC with LD stage (Supplementary Fig. S2, Supplementary Table S4). These results indicate that the sensitivity of serum EBI3 as a single tumor marker for early stage lung cancer is likely to be better than that of CEA and CYFRA21-1, although further validation in larger scale samples with various clinical stages is necessary. Interestingly, the correlation coefficient between serum EBI3 and conventional markers was not significant, and combination assays using both EBI3 and CEA/CYFRA21-1/Pro-GRP improved diagnostic accuracy with a minimal false-positive rate. Our data presented here shows a potential usefulness of EBI3 as a serological biomarker for lung cancers. It should also be noted that we observed EBI3 activation in other types of cancers, such as pancreatic cancer (data not shown), thus suggesting its diagnostic and therapeutic
application for other tumor types.

In conclusion, we have identified EBI3 as a potential serum and tissue biomarker for diagnosis of lung cancer. This molecule is a possible candidate for the development of new anti-cancer drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank BioBank Japan for providing the serum samples.

Grant Support: This work was supported in part by Grant-in-Aid for Scientific Research (B) and Grant-in-Aid for Scientific Research on Innovative Areas from The Japan Society for the Promotion of Science. Y.D. is a member of Shiga Cancer Treatment Project supported by Shiga Prefecture (Japan).
References


49. Hanley JA, McNeil BJ. A method of comparing the areas under receiver

Figure legend

Figure 1. Expression and subcellular localization of EBI3 in lung cancers.

A, Expression of EBI3 in 15 representative pairs of clinical lung cancer tissues (T) and surrounding normal lung (N) tissue samples (top panels) and in 22 lung cancer cell lines (bottom panels) detected by semiquantitative RT-PCR analysis.  
B, Subcellular localization of endogenous EBI3 protein in lung cancer cell lines.  EBI3 was stained in the cytoplasm of the cell with granular appearance in A549, LC319 and PC14 cell lines, whereas no staining was observed in NCI-H2170 and bronchial epithelia derived BEAS-2B cell lines.  
C, Detection of secretory EBI3 protein by ELISA into culture medium from four lung cancer cell lines and BEAS-2B cells.

Figure 2. Expression of EBI3 in normal tissues and lung tumors, and association of strong EBI3 expression with poor prognosis for NSCLC patients.

A, Northern blot analysis of the EBI3 transcript in 16 normal human tissues.  
B, Immunohistochemical analysis of EBI3 protein in representative normal tissues: adult liver, kidney, heart, lung, and placenta as well as three histological types of lung cancers (ADC, adenocarcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer).  
C, Immunohistochemical analysis of EBI3 protein in paired lung tumor tissues and adjacent normal lung tissues (T, lung tumor; N, normal lung).  
D, Examples are shown for strong, weak, and absent EBI3 expression in lung squamous cell carcinoma tissues and a normal lung tissue on tissue microarray.  
E, Kaplan-Meier analysis of survival of patients with NSCLC (P = 0.0014 by log-rank test) according to expression of EBI3 protein.  

Vertical bars on survival curves
indicate censored cases.

**Figure 3.** Serologic concentration of EBI3 protein determined by ELISA in patients with lung cancer or chronic obstructive lung disease (COPD) and healthy volunteers. **A,** EBI3 levels in serum from patients with lung cancer (ADC, SCC, and SCLC) or COPD and healthy volunteers. *Black line* indicates cut off values determined by receiver operating characteristic (ROC) curve. **B,** Validation analysis of serum levels of EBI3 in lung cancer patients and healthy volunteers. *Black line* indicates cut off values. **C,** Serum EBI3 levels (U/mL) and EBI3 staining in tumor tissues in 16 NSCLC patients. **D,** Serum levels of EBI3 before and after curative resection of primary tumors in NSCLC patients (n = 20). *Black lines* indicate cut off values. **E,** Receiver operating characteristic (ROC) curve analysis of serum EBI3 (blue lines) and other conventional tumor markers (CEA as red line, CYFRA21-1 as green line, and ProGRP as yellow line) as serum markers for each histological types of lung cancer. *X axis* indicates 1-specificity; *Y axis,* sensitivity; *AUC,* area under curves; 95%CI, 95% confidential interval.

**Figure 4.** Inhibition of growth of lung cancer cells by siRNAs against EBI3. **A,** Gene knockdown effect on EBI3 expression in A549 cells and LC319 cells by siRNAs against EBI3 (si-EBI3-#1 and #2) and control siRNAs (si-CNT/On-target, si-LUC/Luciferase), analyzed by semiquantitative RT-PCR. **B, C,** MTT assays (**B**) and colony formation assays (**C**) of A549 and LC319 cells transfected with si-EBI3s or control siRNAs. *Columns,* relative absorbance of triplicate assays; *bars,* SD.
**Figure 5.** Enhanced growth of mammalian cells stably overexpressing exogenous EBI3.

*A, B, C*, *in vitro* assays showing the rapid growth of COS-7 cells stably overexpressing EBI3 (COS-7-EBI3-#A and -#B). Two independent transfectants expressing high levels of EBI3 (COS-7-EBI3-#A and -#B, *A*) and controls (COS-7-M1 and -M2) were each cultured in triplicate; after 120 hours the cell viability was evaluated by the MTT assays (*B*) and colony formation assays (*C*).
Table 1A. Association between EBI3-positivity in NSCLC tissues and patients’ characteristics (n = 414)

<table>
<thead>
<tr>
<th></th>
<th>Total n = 414</th>
<th>strong expression n = 203</th>
<th>weak expression n = 156</th>
<th>absent expression n = 55</th>
<th>P-value strong vs weak/absent expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>129</td>
<td>45</td>
<td>55</td>
<td>29</td>
<td>0.0001 +</td>
</tr>
<tr>
<td>Female</td>
<td>285</td>
<td>158</td>
<td>101</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>197</td>
<td>96</td>
<td>82</td>
<td>19</td>
<td>0.9219</td>
</tr>
<tr>
<td>≥65</td>
<td>217</td>
<td>107</td>
<td>74</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>265</td>
<td>112</td>
<td>107</td>
<td>46</td>
<td>0.0003 +</td>
</tr>
<tr>
<td>Non-ADC</td>
<td>149</td>
<td>91</td>
<td>49</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>pT factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>138</td>
<td>52</td>
<td>57</td>
<td>29</td>
<td>0.0012 +</td>
</tr>
<tr>
<td>T2+T3+T4</td>
<td>276</td>
<td>151</td>
<td>99</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>pN factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>264</td>
<td>120</td>
<td>105</td>
<td>39</td>
<td>0.0655</td>
</tr>
<tr>
<td>N1+N2</td>
<td>150</td>
<td>83</td>
<td>51</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>123</td>
<td>47</td>
<td>54</td>
<td>22</td>
<td>0.0051 +</td>
</tr>
<tr>
<td>Smoker</td>
<td>291</td>
<td>156</td>
<td>102</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

ADC, adenocarcinoma

*P < 0.05 (Fisher’s exact test)
Table 1B. Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazards ratio</th>
<th>95% CI</th>
<th>Unfavorable/Favorable</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBI3</td>
<td>1.611</td>
<td>1.198-2.165</td>
<td>Strong(+) / Weak(+) or (-)</td>
<td>0.0016*</td>
</tr>
<tr>
<td>Gender</td>
<td>1.585</td>
<td>1.131-2.221</td>
<td>Male / Female</td>
<td>0.0074*</td>
</tr>
<tr>
<td>Age ( years )</td>
<td>1.477</td>
<td>1.097-1.988</td>
<td>65 ≥ / &lt;65</td>
<td>0.0102*</td>
</tr>
<tr>
<td>Histological type</td>
<td>1.402</td>
<td>1.045-1.883</td>
<td>Non-ADC / ADC¹</td>
<td>0.0244*</td>
</tr>
<tr>
<td>pT factor</td>
<td>2.667</td>
<td>1.836-3.875</td>
<td>T₂+T₃+T₄ / T₁</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>pN factor</td>
<td>2.351</td>
<td>1.756-3.149</td>
<td>N₁+N₂ / N₀</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Smoking history</td>
<td>1.174</td>
<td>0.847-1.626</td>
<td>Smoker / Never smoker</td>
<td>0.3345</td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBI3</td>
<td>1.366</td>
<td>1.009-1.851</td>
<td>Strong(+) / Weak(+) or (-)</td>
<td>0.0439*</td>
</tr>
<tr>
<td>Gender</td>
<td>1.278</td>
<td>0.883-1.851</td>
<td>Male / Female</td>
<td>0.1936</td>
</tr>
<tr>
<td>Age ( years )</td>
<td>1.659</td>
<td>1.224-2.247</td>
<td>65 ≥ / &lt;65</td>
<td>0.0011*</td>
</tr>
<tr>
<td>Histological type</td>
<td>0.950</td>
<td>0.688-1.312</td>
<td>Non-ADC / ADC¹</td>
<td>0.7559</td>
</tr>
<tr>
<td>pT factor</td>
<td>2.125</td>
<td>1.445-3.127</td>
<td>T₂+T₃+T₄ / T₁</td>
<td>0.0001*</td>
</tr>
<tr>
<td>pN factor</td>
<td>2.256</td>
<td>1.666-3.055</td>
<td>N₁+N₂ / N₀</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

¹ADC, adenocarcinoma

*P < 0.05
Fig. 1

A

Clinical lung cancers

<table>
<thead>
<tr>
<th>ADC</th>
<th>SCC</th>
<th>SCLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA1</td>
<td>LCA2</td>
<td>LCA3</td>
</tr>
<tr>
<td>N</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>LCA4</td>
<td>LCA5</td>
<td>LCA6</td>
</tr>
<tr>
<td>N</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>LCA7</td>
<td>LCA8</td>
<td>LCA9</td>
</tr>
<tr>
<td>N</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>LCA10</td>
<td>LCA11</td>
<td>LCA12</td>
</tr>
<tr>
<td>N</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>LCA13</td>
<td>LCA14</td>
<td>LCA15</td>
</tr>
<tr>
<td>N</td>
<td>T</td>
<td>N</td>
</tr>
</tbody>
</table>

Lung cancer cell lines

<table>
<thead>
<tr>
<th>ADC</th>
<th>BAC</th>
<th>SCC</th>
<th>LCC</th>
<th>SCLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H1373</td>
<td>NCI-H358</td>
<td>NCI-H2170</td>
<td>RERF-LCA1</td>
<td>DMS114</td>
</tr>
<tr>
<td>NCI-H1781</td>
<td>NCI-H226</td>
<td>NCI-H1703</td>
<td>EBC-1</td>
<td>DMS273</td>
</tr>
<tr>
<td>NCI-H520</td>
<td>NCI-H2170</td>
<td></td>
<td></td>
<td>SBC-3</td>
</tr>
<tr>
<td>NCI-H446</td>
<td></td>
<td></td>
<td></td>
<td>SBC-5</td>
</tr>
<tr>
<td>NCI-H1796</td>
<td></td>
<td></td>
<td></td>
<td>BEAS-2B</td>
</tr>
</tbody>
</table>

EBI3
ACTB

Research. on September 22, 2017. © 2011 American Association for Cancer Research. Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited. Author Manuscript Published OnlineFirst on August 17, 2011; DOI: 10.1158/1078-0432.CCR-11-0060
Fig. 1

EBI3

<table>
<thead>
<tr>
<th>A549</th>
<th>LC319</th>
<th>PC14</th>
<th>NCI-H2170</th>
<th>BEAS-2B</th>
</tr>
</thead>
</table>

EBI3(+)

EBI3(-)

Airway epithelia

DAPI
Fig. 1

Levels of EBI3 in culture media (fold change compared by A549)

<table>
<thead>
<tr>
<th>Airway epithelia</th>
<th>EBI3(+)</th>
<th>EBI3(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC319</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H2170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEAS-2B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2

Normal tissues

Heart
Brain
Placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small intestine
Colon
Leukocyte

(kb)

0.24
1.35
2.4
4.4
7.5
9.5

4.4
2.4
1.35
0.24
0.24
Fig. 2

B

Liver  Kidney  Heart  Lung

Placenta  ADC  SCC  SCLC

Lung cancer
Fig. 2

<table>
<thead>
<tr>
<th>ADC-1</th>
<th>ADC-2</th>
<th>ADC-3</th>
<th>ADC-4</th>
<th>ADC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>SCC-1</td>
<td>SCC-2</td>
<td>SCC-3</td>
<td>SCC-4</td>
<td>SCC-5</td>
</tr>
<tr>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Fig. 2

Lung Cancer Mortality of patients

Absent or weak expression (n=211)

Strong expression (n=203)

Survival Rate (% of patients)

Postoperative Days

P = 0.0014

Number of patients at risk

Strong EBI3 expression

Absent or weak EBI3 expression

203

211

191

169

157

134

106

84

65

40

20

10

6

2

3000

2500

2000

1500

1000

500

0

100

80

60

40

20

0
**Fig. 3**

Levels of serum EBI3 (U/mL)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mean ± SD</th>
<th>No. of Cases</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>1.5 ± 1.6</td>
<td>126</td>
<td>0.246</td>
</tr>
<tr>
<td>COPD</td>
<td>1.9 ± 2.6</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>6.0 ± 5.0</td>
<td>155</td>
<td>0.486</td>
</tr>
<tr>
<td>SCC</td>
<td>6.1 ± 4.4</td>
<td>44</td>
<td>0.471</td>
</tr>
<tr>
<td>SCLC</td>
<td>6.3 ± 6.2</td>
<td>75</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.0001

Cut off 5.5 U/mL

Note: The figure shows the levels of serum EBI3 for different groups, with healthy volunteers having the lowest levels and SCC and SCLC having the highest levels.
**Fig. 3**

B

**Levels of serum EBI3 (U/mL)**

- Cut off 5.5 U/mL

<table>
<thead>
<tr>
<th>No. of Cases examined</th>
<th>Healthy volunteers</th>
<th>ADC</th>
<th>SCC</th>
<th>SCLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>1.3±1.2</td>
<td>6.0±5.8</td>
<td>6.4±4.8</td>
<td>5.0±4.5</td>
</tr>
</tbody>
</table>

*P<0.0001
<table>
<thead>
<tr>
<th>Serum EBI3-positive cases</th>
<th>Serum EBI3-negative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum EBI3 9.0U/mL, ADC, stage IA</td>
<td>Serum EBI3 11.7U/mL, ADC, stage IB</td>
</tr>
<tr>
<td>Serum EBI3 5.9U/mL, SCC, stage IB</td>
<td>Serum EBI3 &lt; cut off, ADC, stage IA</td>
</tr>
<tr>
<td>Serum EBI3 7.7U/mL, ADC, stage IIA</td>
<td>Serum EBI3 9.5U/mL, ADC, stage IB</td>
</tr>
<tr>
<td>Serum EBI3 &lt; cut off, ADC, stage IIA</td>
<td>Serum EBI3 &lt; cut off, ADC, stage IB</td>
</tr>
<tr>
<td>Serum EBI3 6.0U/mL, ADC, stage IIIA</td>
<td>Serum EBI3 &lt; cut off, ADC, stage IIB</td>
</tr>
<tr>
<td>Serum EBI3 8.5U/mL, ADC, stage IIB</td>
<td>Serum EBI3 &lt; cut off, ADC, stage IIB</td>
</tr>
<tr>
<td>Serum EBI3 13.6U/mL, SCC, stage IIIA</td>
<td>Serum EBI3 &lt; cut off, SCC, stage IIIB</td>
</tr>
<tr>
<td>Serum EBI3 &lt; cut off, SCC, stage IIIB</td>
<td>Serum EBI3 &lt; cut off, SCC, stage IIIB</td>
</tr>
</tbody>
</table>
Fig. 3

Levels of serum EBI3 (U/mL)

Pre Post

Cut off 5.5U/mL

P<0.0001

Levels of EBI3 (U/mL) show significant increases post-treatment compared to pre-treatment levels.
Fig. 3

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>AUC</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC (n=155)</td>
<td>0.808</td>
<td>0.757-0.852</td>
</tr>
<tr>
<td></td>
<td>0.677</td>
<td>0.619-0.731</td>
</tr>
<tr>
<td>SCC (n=44)</td>
<td>0.858</td>
<td>0.796-0.907</td>
</tr>
<tr>
<td></td>
<td>0.707</td>
<td>0.633-0.774</td>
</tr>
<tr>
<td>SCLC (n=75)</td>
<td>0.804</td>
<td>0.743-0.857</td>
</tr>
<tr>
<td></td>
<td>0.968</td>
<td>0.934-0.988</td>
</tr>
</tbody>
</table>

EBI3 = Ectodomain Binding Ig-like Substance H (EBI3), CEA = Carcinoembryonic Antigen (CEA), CYFRA21-1 = Cytokeratin 19 Fragments, proGRP = Progastrin-Releasing Peptide
Fig. 4

A

<table>
<thead>
<tr>
<th></th>
<th>A549</th>
<th></th>
<th>LC319</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA-</td>
<td></td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>EBI3</td>
<td>#1</td>
<td>#2</td>
<td>CNT</td>
</tr>
<tr>
<td>ACTB</td>
<td></td>
<td></td>
<td>LUC</td>
</tr>
</tbody>
</table>

EBI3

ACTB

B

Relative absorbance (490/630nm)

0.0 0.5 1.0 1.5 2.0 2.5

#1  #2  CNT  LUC

* P<0.001

C

#1  #2  CNT  LUC

#1  #2  CNT  LUC

* P<0.001

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Fig. 5

Panel A: Western blot analysis of EBI3 and ACTB in COS-7 cells transfected with either EBI3(+) or MOCK constructs. M1 and M2 represent different experimental conditions.

Panel B: Bar graph showing relative absorbance at 490/630 nm for samples A, B, M1, and M2. *P<0.001 indicates statistical significance.

Panel C: Photographs of cell cultures under different experimental conditions, depicting cell growth and morphology.
Clinical Cancer Research

Identification of Epstein-Barr Virus-Induced Gene 3 as a Novel Serum and Tissue Biomarker and a Therapeutic Target for Lung Cancer

Ryohei Nishino, Atsushi Takano, Hideto Oshita, et al.

Clin Cancer Res  Published OnlineFirst August 17, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-0060

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/08/17/1078-0432.CCR-11-0060.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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

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

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