Dikkopf-1 as a Novel Serologic and Prognostic Biomarker for Lung and Esophageal Carcinomas

Takumi Yamabuki,[1,2] Atsushi Takano,[1] Satoshi Hayama,[1] Nobuhisa Ishikawa,[1] Tatsuya Kato,[1] Masaki Miyamoto,[2] Tomoo Ito,[3] Hiroyuki Ito,[5] Yohei Miyagi,[6] Haruhiko Nakayama,[5] Masahiro Fujita,[4] Masao Hosokawa,[4] Eiju Tsuchiya,[6] Nobuoki Kohno,[7] Satoshi Kondo,[2] Yusuke Nakamura,[1] and Yataro Daigo[1]

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; Departments of [2]Surgical Oncology and [3]Surgical Pathology, Hokkaido University Graduate School of Medicine; [4]Keiyukai Sapporo Hospital, Sapporo, Japan; Divisions of [5]Thoracic Surgery and [6]Molecular Pathology and Genetics, Kanagawa Cancer Center, Kanagawa, Japan; and [7]Department of Molecular and Internal Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Abstract

Gene expression profile analysis of lung and esophageal carcinomas revealed that Dikkopf-1 (*DKK1*) was highly transactivated in the great majority of lung cancers and esophageal squamous cell carcinomas (ESCC). Immunohistochemical staining using tumor tissue microarrays consisting of 279 archived non–small cell lung cancers (NSCLC) and 280 ESCC specimens showed that a high level of DKK1 expression was associated with poor prognosis of patients with NSCLC as well as ESCC, and multivariate analysis confirmed its independent prognostic value for NSCLC. In addition, we identified that exogenous expression of DKK1 increased the migratory activity of mammalian cells, suggesting that DKK1 may play a significant role in progression of human cancer. We established an ELISA system to measure serum levels of DKK1 and found that serum DKK1 levels were significantly higher in lung and esophageal cancer patients than in healthy controls. The proportion of the DKK1-positive cases was 126 of 180 (70.0%) NSCLC, 59 of 85 (69.4%) SCLC, and 51 of 81 (63.0%) ESCC patients, whereas only 10 of 207 (4.8%) healthy volunteers were falsely diagnosed as positive. A combined ELISA assays for both DKK1 and carcinoembryonic antigen increased sensitivity and classified 82.2% of the NSCLC patients as positive whereas only 7.7% of healthy volunteers were falsely diagnosed to be positive. The use of both DKK1 and ProGRP increased sensitivity to detect SCLCs up to 89.4%, whereas false-positive rate in healthy donors was only 6.3%. Our data imply that DKK1 should be useful as a novel diagnostic/prognostic biomarker in clinic and probably as a therapeutic target for lung and esophageal cancer. [Cancer Res 2007;67(6):2517–25]

Introduction

Lung cancer is the leading cause of cancer-related death in the world. Despite some advances in early detection and recent improvements in its treatment, the prognosis of the patients with lung cancer still remains poor (1). Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies of the digestive tract, and at the time of diagnosis, most of the patients are at advanced stages (2). In spite of the use of modern surgical techniques combined with various treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate of ESCC still remains at 40% to 60% (3) and that of lung cancer is only 15% (1). Several tumor markers, such as ProGRP, NSE, cytokeratin 19 fragment (CYFRA 21-1), squamous cell carcinoma antigen (SCC), and carcinoembryonic antigen (CEA), are elevated in serum of lung cancer patients (4–6). Similarly, SCC, CEA, and CYFRA 21-1 are elevated in the serum of ESCC patients and are used in clinic for diagnosis as well as in follow-up of the patients (2, 7). The sensitivities of CEA and CYFRA 21-1 were 25% and 57% in lung SCC and 50% and 27% in lung adenocarcinoma, respectively. The sensitivity of CEA was reported to be 30% in ESCC (8). The positive rate of serum SCC in patients with ESCC was reported to be 18% in stage I, 22% in stage II, 34% in stage III, and 37% in stage IV. The incidence of CEA positivity in patients with stage IV ESCC was only 16%. Although CEA was not a prognostic factor, SCC was shown to be an independent prognostic factor from pathologic tumor-node-metastasis (TNM) factors by multivariate analysis (2). These facts indicate that no tumor marker has been sufficiently useful for detection of lung cancer and ESCC at potentially curative stage, and a limited number of practical prognostic biomarker are presently available for selection of treatment modalities for individual patients.

To isolate potential molecular targets for diagnosis, treatment, and/or prevention of lung and esophageal carcinomas, we did a genome-wide analysis of gene expression profiles of cancer cells from 101 lung cancer and 19 ESCC patients by means of a cDNA microarray consisting of 27,648 genes (9–14). To verify the biological and 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 and esophageal cancer materials with RNA interference technique and cell growth/invasion assays (15–23). In this process, we identified Dikkopf-1 (DKK1) as a novel serologic and histochemical biomarker as well as a therapeutic target for lung and esophageal cancers.

DKK1 encodes a secreted protein, which plays a crucial role in head formation in vertebrate development, and is known as a negative regulator of the Wnt signaling pathway in colon cancer cells (24, 25). DKK1 binds to LRP5/6 and Kremen proteins and induces LRP endocytosis, which prevents the formation of

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Requests for reprints: Yataro Daigo, Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-Ward, Tokyo 108-8639, Japan. Phone: 81-3-5449-5457; Fax: 81-3-5449-5406; E-mail: ydaigo@ims.u-tokyo.ac.jp.

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Wnt-Frizzled-LRP5/6 receptor complexes (25). Other studies have shown overexpression of *DKK1* in Wilms' tumor, hepatoblastoma, and hepatocellular carcinoma (HCC), indicating a potential oncogenic function of DKK1 (26, 27). In spite of these studies, there has been no report describing the significance of activation of DKK1 in human cancer progression and its potential as a diagnostic and therapeutic target.

We report here identification of DKK1 as a novel diagnostic and prognostic biomarker and a potential target for therapeutic agents/antibodies and also provide evidence for its possible role in human pulmonary and esophageal carcinogenesis.

Materials and Methods

Cell lines and tissue samples. The 23 human lung cancer cell lines used in this study included nine adenocarcinomas (A427, A549, LC319, PC-3, PC-9, PC-14, NCI-H1373, NCI-H1666, and NCI-H1781), two adenosquamous carcinomas (ASC; NCI-H226 and NCI-H647), seven SCCs (EBC-1, LU61, NCI-H520, NCI-H1703, NCI-H2170, RERF-LC-AI, and SK-MES-1), one large cell carcinoma (LX1), and four small cell lung cancers (SCLC; DMS114, DMS273, SBC-3, and SBC-5). The human esophageal carcinoma cell lines used in this study were as follows: nine SCC cell lines (TE1, TE2, TE3, TE4, TE5, TE6, TE8, TE9, and TE10) and one adenocarcinoma cell line (TE7; ref. 28). All cells were grown in monolayer in appropriate medium supplemented with 10% FCS and maintained at 37°C in humidified air with 5% CO_2. Human small airway epithelial cells used as a normal control were grown in optimized medium (small airway growth medium) from Cambrex Bioscience, Inc. (East Rutherford, NJ). Primary lung cancer and ESCC samples had been obtained earlier with informed consent (9–11). Clinical stage was judged according to the International Union Against Cancer TNM classification (29). Formalin-fixed primary lung tumors and adjacent normal lung tissue samples used for immunostaining on tissue microarrays had been obtained from 279 patients (161 adenocarcinomas, 96 SCCs, 18 large cell carcinomas, and 4 ASCs; 96 female and 183 male patients; median age of 63.3 with a range of 26 to 84 years) undergoing curative surgery at Hokkaido University (Sapporo, Japan). A total of 280 formalin-fixed primary ESCCs (27 female and 253 male patients; median age of 61.5 with a range of 38 to 82 years) and adjacent normal esophageal tissue samples had also been obtained from patients undergoing curative surgery at Keiyukai Sapporo Hospital (Sapporo, Japan). This study and the use of all clinical materials mentioned were approved by individual institutional Ethical Committees.

Serum samples. Serum samples were obtained with written informed consent from 207 healthy control individuals (168 males and 39 females; median age of 50.3 with a range of 31 to 61 years) and from 88 nonneoplastic lung disease patients with chronic obstructive pulmonary disease (COPD) enrolled as a part of the Japanese Project for Personalized Medicine (BioBank Japan) or admitted to Hiroshima University Hospital (78 males and 10 females; median age of 67.6 with a range of 54 to 84 years). All of these patients were current and/or former smokers [the mean (±1 SD) of pack-year index (PYI) was 70.0 ± 42.7; PYI was defined as the number of cigarette packs (20 cigarettes per pack) consumed a day multiplied by years]. Serum samples were also obtained with informed consent from 125 lung cancer patients (78 males and 47 females; median age of 68.0 with a range of 40 to 86 years) admitted to Hiroshima University Hospital as well as Kanagawa Cancer Center Hospital and from 140 patients with lung cancer who were registered in the BioBank Japan (100 males and 40 females; median age of 64.5 with a range of 41 to 89 years). These 265 lung cancer cases included 112 adenocarcinomas, 68 SCCs, and 85 SCLCs. Serum samples were also obtained with informed consent from 81 ESCC patients who were admitted to Keiyukai Sapporo Hospital or who were registered in the BioBank Japan (69 males and 12 females; median age of 62.0 with a range of 37 to 74 years). These serum samples from a total of 346 cancer patients were selected for the study based on the following criteria: (*a*) patients were newly diagnosed and previously untreated and (*b*) their tumors were pathologically diagnosed as lung or esophageal cancers (stages I–IV). Serum was obtained at the time of diagnosis and stored at −150°C.

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, Basel, Switzerland) and SuperScript II (Invitrogen, Carlsbad, CA). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following sets of synthesized primers specific to *DKK1* or with β-actin (*ACTB*)-specific primers as an internal control: *DKK1*, 5′-TAGAGTCTAGAACG-CAAGGATCTC-3′ and 5′-CAAAAACTATCACAGCCTAAAGGG-3′; *ACTB*, 5′-GAGGTGATAGCATTGCTTTCG-3′ and 5′-CAAGTCAGTGTACAGG-TAAGC-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 23 tissues (BD Biosciences, Palo Alto, CA) were hybridized with an [α-^{32}P]dCTP-labeled, 776-bp PCR product of *DKK1* that was prepared as a probe using primers 5′-CATCAGACTGTGCCTCAGGA-3′ and 5′-CAAAAACTATCA-CAGCCTAAAGGG-3′. Prehybridization, hybridization, and washing were done following the manufacturer's specifications. The blots were autoradiographed with intensifying screens at −80°C for 7 days.

Western blotting. Tumor tissues or 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, Protease Inhibitor Cocktail Set III (EMD Biosciences, Inc., San Diego, CA)]. The protein content of each lysate was determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard. Each lysate (10 μg) was resolved on 10% to 12% denaturing polyacrylamide gels (with 3% polyacrylamide stacking gel) and transferred electrophoretically to a nitrocellulose membrane (GE Healthcare Biosciences, Piscataway, NJ). After blocking with 5% nonfat dry milk in TBS-Tween 20 (TBST), the membrane was incubated with primary antibodies for 1 h at room temperature. Immunoreactive proteins were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Biosciences) for 1 h at room temperature. After washing with TBST, the reactants were developed using the enhanced chemiluminescence kit (GE Healthcare Biosciences). A commercially available rabbit polyclonal antibody to human DKK1 (hDKK1; Santa Cruz Biotechnology, Santa Cruz, CA) was hybridized by Western blot analysis using lysates of lung cancer and ESCC tissues and cell lines as well as normal tissues.

Immunocytochemical analysis. Cells were plated on glass coverslips (Becton Dickinson Labware, Franklin Lakes, NJ), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature. Nonspecific binding was blocked by Casblock (ZYMED, San Francisco, CA) for 10 min at room temperature. Cells were then incubated for 60 min at room temperature with primary antibodies diluted in PBS containing 3% BSA. After being washed with PBS, the cells were stained by FITC-conjugated secondary antibody (Santa Cruz Biotechnology) for 60 min at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA) containing 4′,6-diamidino-2-phenylindole and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS; Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry and tissue microarray. To investigate the DKK1 protein in clinical samples that had been embedded in paraffin blocks, we stained the sections in the following manner. Briefly, 3.3 μg/mL of a rabbit polyclonal anti-hDKK1 antibody (Santa Cruz Biotechnology) were 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, Tokyo, Japan] as the secondary antibody. Substrate-chromogen was added, and the specimens were counterstained with hematoxylin.

Tumor tissue microarrays were constructed with formalin-fixed 279 primary lung cancers and 280 primary esophageal cancers as described elsewhere (30–32). The tissue area for sampling was selected based on visual alignment with the corresponding H&E-stained section on a slide. Three, four, or five 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 microarrayer (Beecher Instruments, Sun Prairie, WI). 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 DKK1 positivity without prior knowledge of clinicopathologic data as reported previously (16–21). The intensity of DKK1 staining was evaluated using the following criteria: strong positive (scored as 2+), dark 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 only if reviewers independently defined them as such.

Statistical analysis. Statistical analyses were done using the StatView statistical program (SAS, Cary, NC). Tumor-specific survival curves were calculated from the date of surgery to the time of death related to non-SCLC (NSCLC) or ESCC or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for DKK1 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, 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 DKK1 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 $P < 0.05$.

ELISA. Serum levels of DKK1 were measured by ELISA system, which had been originally constructed. First, a rabbit polyclonal antibody specific to DKK1 was added to a 96-well microplate (Nunc, Roskilde, Denmark) as a capture antibody and incubated for 2 h at room temperature. After washing away any unbound antibody, 5% BSA was added to the wells and incubated for 16 h at 4°C for blocking. After a wash, 3-fold diluted sera were added to the wells and incubated for 2 h at room temperature. After washing away any unbound substances, a biotinylated polyclonal antibody specific for DKK1 using Biotin Labeling Kit-NH_2 (DOJINDO, Kumamoto, Japan) was added to the wells as a detection antibody and incubated for 2 h at room temperature. After a wash to remove any unbound antibody-enzyme reagent, HRP-streptavidin was added to the wells and incubated for 20 min. After a wash, a substrate solution (R&D Systems, Inc., Minneapolis, MN) was added to the wells and allowed to react for 30 min. The reaction was stopped by adding 100 μL of 2 N sulfuric acid. Color intensity was determined by a photometer at a wavelength of 450 nm, with a reference wavelength of 570 nm. Levels of CEA in serum were measured by ELISA with a commercially available enzyme test kit (Hope Laboratories, Belmont, CA) according to the supplier's recommendations. Levels of ProGRP in serum were measured by ELISA with a commercially available enzyme test kit (TFB, Tokyo, Japan) according to the manufacturer's protocol. Differences in the levels of DKK1, CEA, and ProGRP between tumor groups and a healthy control group were analyzed by Mann-Whitney U tests. The levels of DKK1, CEA, and ProGRP were evaluated by receiver operating characteristic (ROC) curve analysis to determine cutoff levels with optimal diagnostic accuracy and likelihood ratios. The correlation coefficients between DKK1

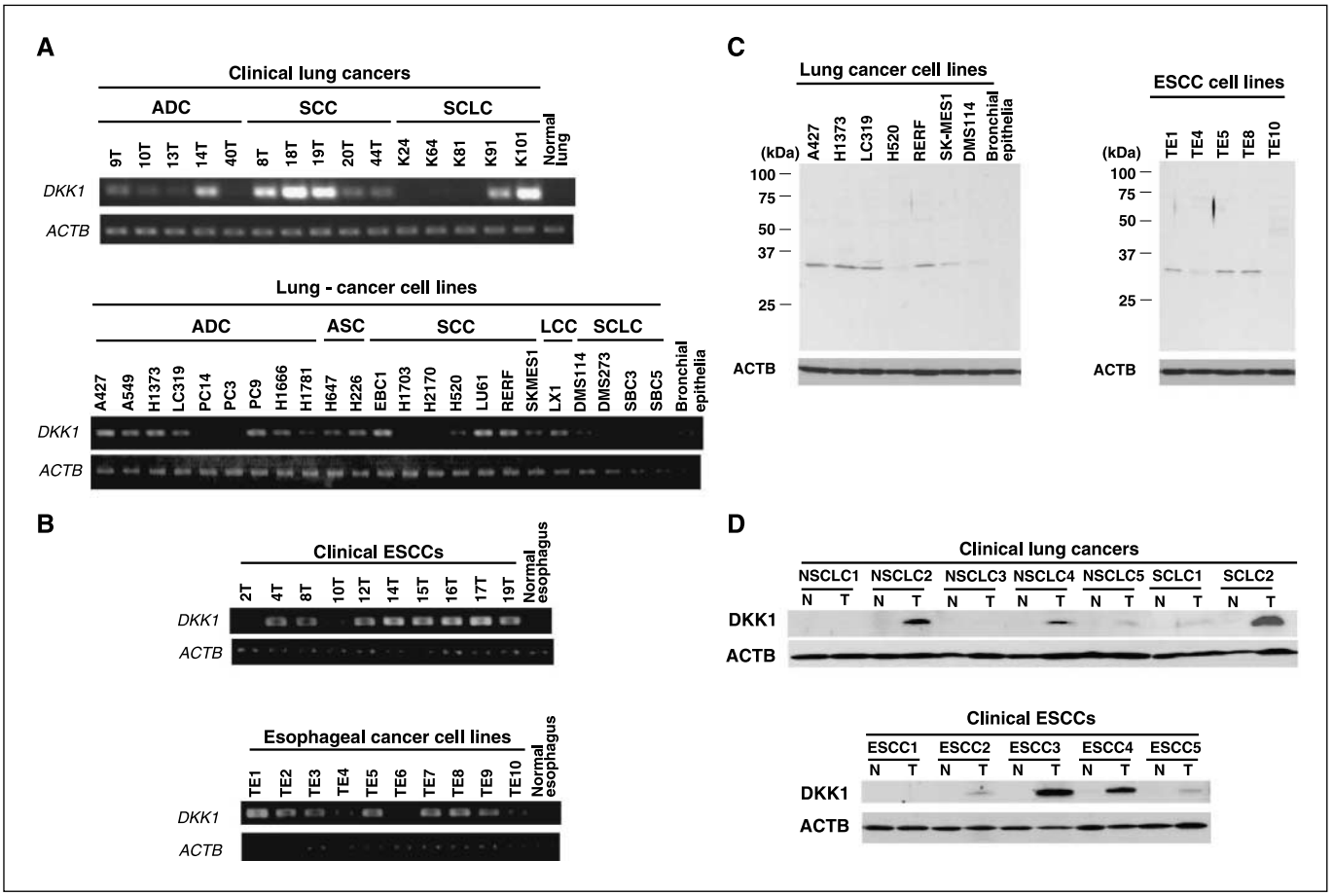

Figure 1. Expression of DKK1 in tumor tissues and cell lines. *A,* expression of *DKK1* in a normal lung tissue and 15 clinical lung cancer samples [lung adenocarcinoma (*ADC*), lung SCC, and SCLC; *top*] and 23 lung cancer cell lines (*bottom*) detected by semiquantitative RT-PCR analysis. *B,* expression of *DKK1* in a normal esophagus and 10 clinical ESCC tissue samples and 10 ESCC cell lines detected by semiquantitative RT-PCR analysis. *C,* expression of DKK1 protein in seven lung cancer cell lines (*left*) and in five ESCC cell lines (*right*) examined by Western blot analysis. *D,* expression of DKK1 protein in representative pairs of five NSCLC and two SCLC samples (*top*) and in those of five ESCCs (*bottom*).

and CEA/ProGRP were calculated with Spearman rank correlation. Significance was defined as $P < 0.05$.

Matrigel invasion assay. NIH3T3 and COS-7 cells transfected either with p3XFLAG-tagged (COOH terminus) plasmids expressing DKK1 or with mock plasmids were grown to near confluence in DMEM containing 10% FCS. The cells were harvested by trypsinization, washed in DMEM without addition of serum or proteinase inhibitor, and suspended in DMEM at concentration of 1×10^5/mL. Before preparing the cell suspension, the dried layer of Matrigel matrix (Becton Dickinson Labware) was rehydrated with DMEM for 2 h at room temperature. DMEM (0.75 mL) containing 10% FCS was added to each lower chamber in 24-well Matrigel invasion chambers, and 0.5 mL (5×10^4 cells) of cell suspension was added to each insert of the upper chamber. The plates of inserts were incubated for 24 h at 37°C. After incubation, the chambers were processed; cells invading through the Matrigel were fixed and stained by Giemsa as directed by the supplier (Becton Dickinson Labware).

Results

DKK1 expression in lung and esophageal cancers and normal tissues. To identify novel molecules that can be applicable to detect presence of cancer at an early stage and to develop novel treatments based on the biological characteristics of cancer cells, we did genome-wide expression profile analysis of lung carcinoma and ESCC using a cDNA microarray (9–14). Among 27,648 genes screened, we identified elevated expression (3-fold or higher) of *DKK1* transcript in cancer cells in the great majority of the lung and esophageal cancer samples examined. We confirmed its overexpression by means of semiquantitative RT-PCR experiments in 11 of 15 lung cancer tissues, in 16 of 23 lung cancer cell lines, in 8 of 10 ESCC tissues, and in 7 of 10 ESCC cell lines (Fig. 1*A* and *B*).

We subsequently confirmed by Western blot analysis overexpression of 35-kDa DKK1 protein in 8 of 12 cancer cell lines using anti-DKK1 antibody in concordance with high levels of *DKK1* transcript (Fig. 1*C, left* and *right*). Western blot analysis also revealed the increased DKK1 protein expression in tumor tissues in representative pairs of lung cancer and ESCC samples analyzed (Fig. 1*D, top* and *bottom*).

Northern blot analysis using a *DKK1* cDNA fragment as a probe identified a transcript of ~1.8 kb that was highly expressed in placenta and at a very low level in prostate, but its transcript was hardly detectable in any other normal tissues (Fig. 2*A*). We did immunofluorescence analysis to examine the subcellular localization of endogenous DKK1 in ESCC cell line TE8 and NSCLC cell line LC319 and found that DKK1 was located at cytoplasm of tumor cells with granular appearance (representative data of TE8 cells are shown in Fig. 2*B*).

Association of DKK1 expression with poor prognosis. To investigate the biological and clinicopathologic significance of DKK1 in pulmonary and esophageal carcinogenesis, we carried out immunohistochemical staining on tissue microarray containing tissue sections from 279 NSCLC and 280 ESCC cases that underwent curative surgical resection. DKK1 staining with polyclonal antibody specific to DKK1 was mainly observed at cytoplasm of tumor cells but was not detected in normal cells (Fig. 3*A* and *B*). We classified a pattern of DKK1 expression on the tissue array ranging from absent (scored as 0) to weak/strong positive (scored as 1+ to 2+). Of the 279 NSCLCs, DKK1 was strongly stained in 125 (44.8%) cases (score 2+), weakly stained in 102 (36.6%) cases (score 1+), and not stained in 52 (18.6%) cases (score 0; details are shown in Supplementary Table S1A). The median survival time of NSCLC patients was significantly shorter in accordance with the higher expression levels of DKK1 ($P = 0.0039$, log-rank test; Fig. 3*C*). We also applied univariate

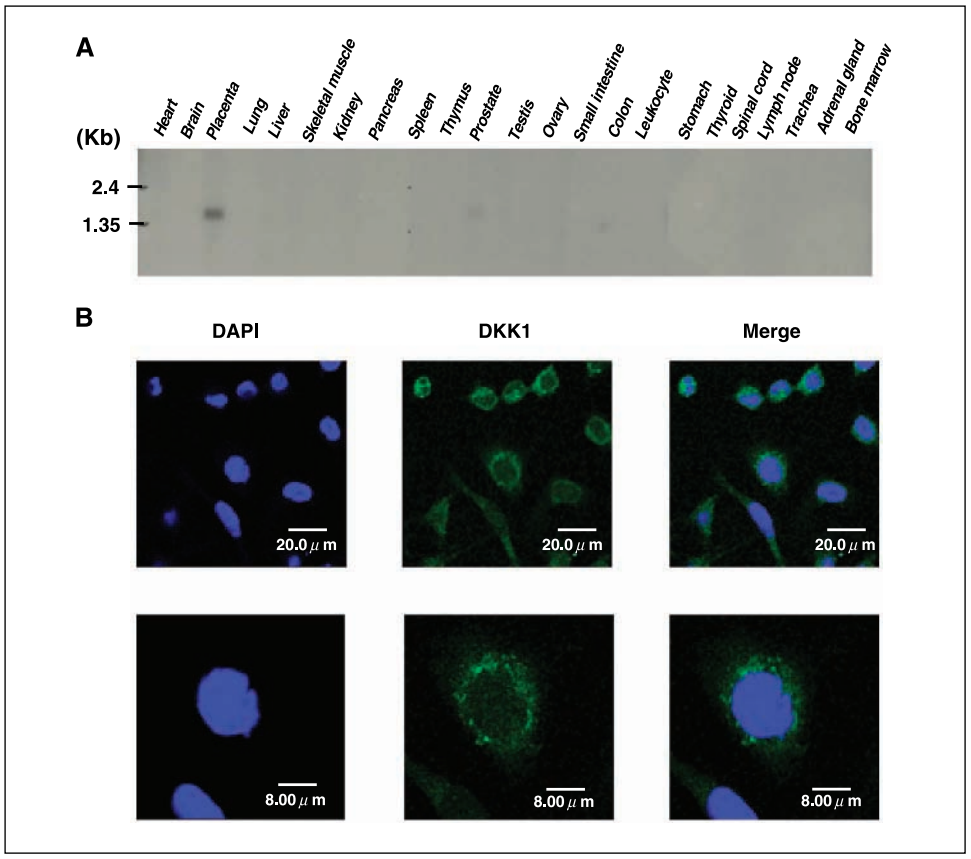

Figure 2. Expression of DKK1 in normal tissues and its subcellular localization. *A,* Northern blot analysis of the *DKK1* transcript in 23 normal adult human tissues. A strong signal was observed in placenta and a very weak signal in prostate. *B,* subcellular localization of endogenous DKK1 protein in TE8 cells. DKK1 was stained at the cytoplasm of the cell with granular appearance. *DAPI,* 4′,6-diamidino-2-phenylindole.

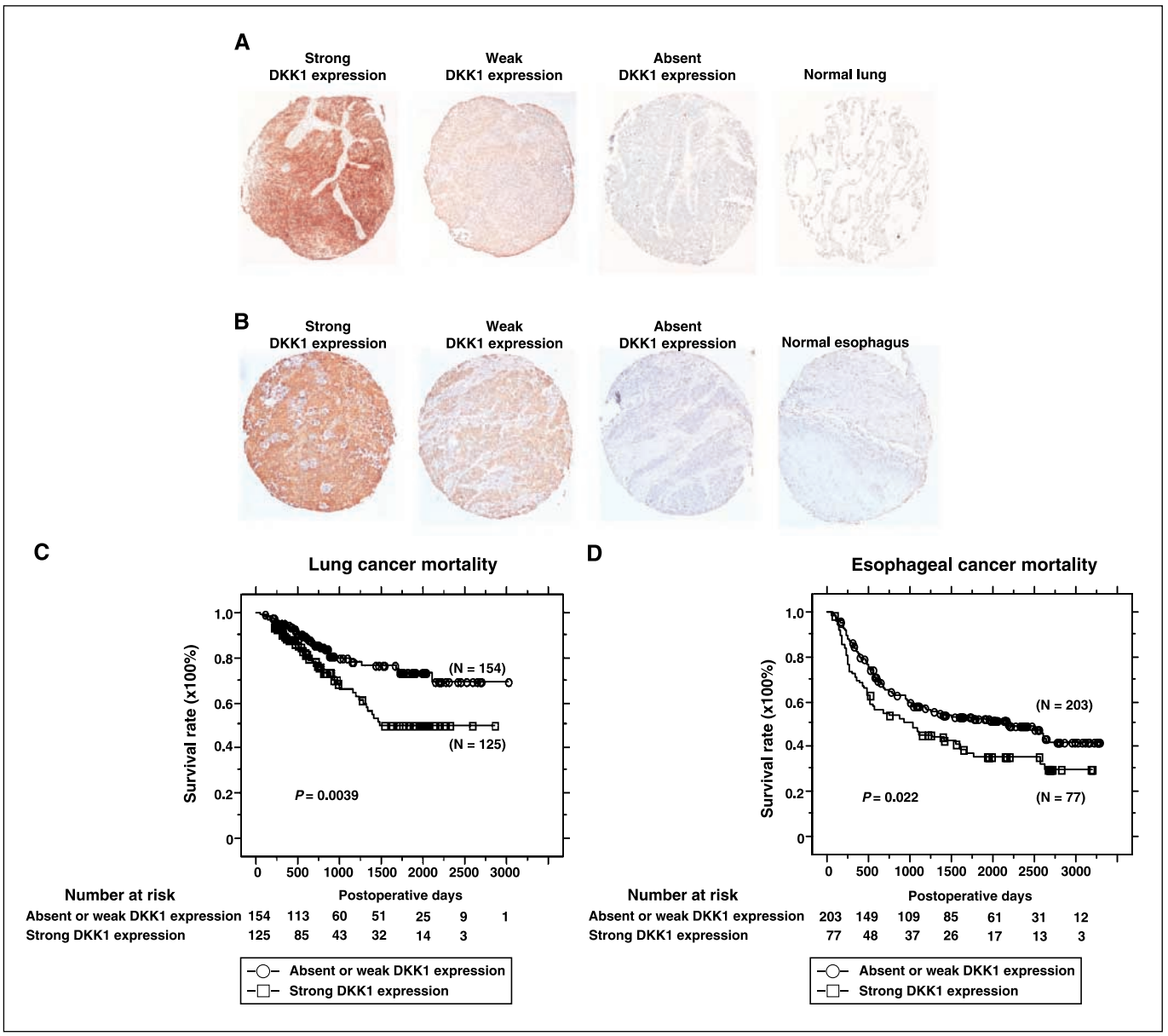

Figure 3. Association of DKK1 overexpression with poor prognosis of NSCLC and ESCC patients. *A* and *B,* examples for strong, weak, and absent DKK1 expression in cancer tissues and a normal tissue. Original magnification, ×100. *A,* lung SCC. *B,* ESCC. *C* and *D,* Kaplan-Meier analysis of survival of patients with NSCLC (P = 0.0039, log-rank test; *C*) and ESCC (P = 0.022, log-rank test; *D*) according to expression of DKK1.

analysis to evaluate associations between patient prognosis and several factors, including age, gender, pathologic tumor stage (tumor size; T_1 + T_2 versus T_3 + T_4), pathologic node stage (node status; N_0 versus N_1 + N_2), and DKK1 status (score 0, 1+ versus score 2+). All those variables were significantly associated with poor prognosis. Multivariate analysis using a Cox proportional hazard model determined that DKK1 (P = 0.0287) as well as other three factors (age, pathologic tumor stage, and pathologic node stage) were independent prognostic factors for surgically treated NSCLC patients (Supplementary Table S1B).

Of the 280 ESCC cases examined, DKK1 was strongly stained in 77 (27.5%) cases (score 2+), weakly stained in 99 (35.4%) cases (score 1+), and not stained in 104 (37.1%) cases (score 0; details are shown in Supplementary Table S2A). The median survival time of ESCC patients was significantly shorter in accordance with the higher expression levels of DKK1 (P = 0.022, log-rank test; Fig. 3*D*). We also applied univariate analysis to evaluate associations between ESCC patient prognosis and several factors, including age, gender, pathologic tumor stage (tumor depth; T_1 + T_2 versus T_3 + T_4), pathologic node stage (node status; N_0 versus N_1), and DKK1 status (score 0, 1+ versus score 2+). All those variables were significantly associated with poor prognosis. In multivariate analysis, DKK1 status did not reach the statistically significant level as independent prognostic factor for surgically treated ESCC patients enrolled in this study (P = 0.1479), whereas pathologic tumor and pathologic node stages as well as gender did so, suggesting the relevance of DKK1 expression to these clinicopathologic factors in esophageal cancer (Supplementary Table S2B).

Serum levels of DKK1 in patients with lung cancer or ESCC. Because *DKK1* encodes a secreted protein, we investigated whether

the DKK1 protein is secreted into sera of patients with lung or esophageal cancer. ELISA experiments detected DKK1 protein in serologic samples from the great majority of the 346 patients with lung or esophageal cancer. The mean (±1 SD) of serum levels of DKK1 in 265 lung cancer patients were 30.7 ± 22.0 units/mL and those in 81 ESCC patients were 38.8 ± 34.6 units/mL. In contrast, the mean (±1 SD) serum levels of DKK1 in 207 healthy individuals were 6.1 ± 5.0 units/mL and those in 88 patients with COPD, who were current and/or former smokers, were 7.2 ± 6.2 units/mL. The levels of serum DKK1 protein were significantly higher in lung or esophageal cancer patients than in healthy donors or in COPD patients ($P < 0.001$, Mann-Whitney U test); the difference between healthy individuals and COPD patients was not significant ($P = 0.427$). According to histologic types of lung cancer, the serum levels of DKK1 were 32.2 ± 21.6 units/mL in 112 adenocarcinoma patients, 25.6 ± 17.6 units/mL in 68 SCC patients, and 33.0 ± 24.8 units/mL in 85 SCLC patients (Fig. 4*A*); the differences among the three histologic types were not significant. High levels of serum DKK1 were detected even in patients with earlier-stage tumors (Fig. 4*B*). Using ROC curves drawn with the data of these 346 cancer

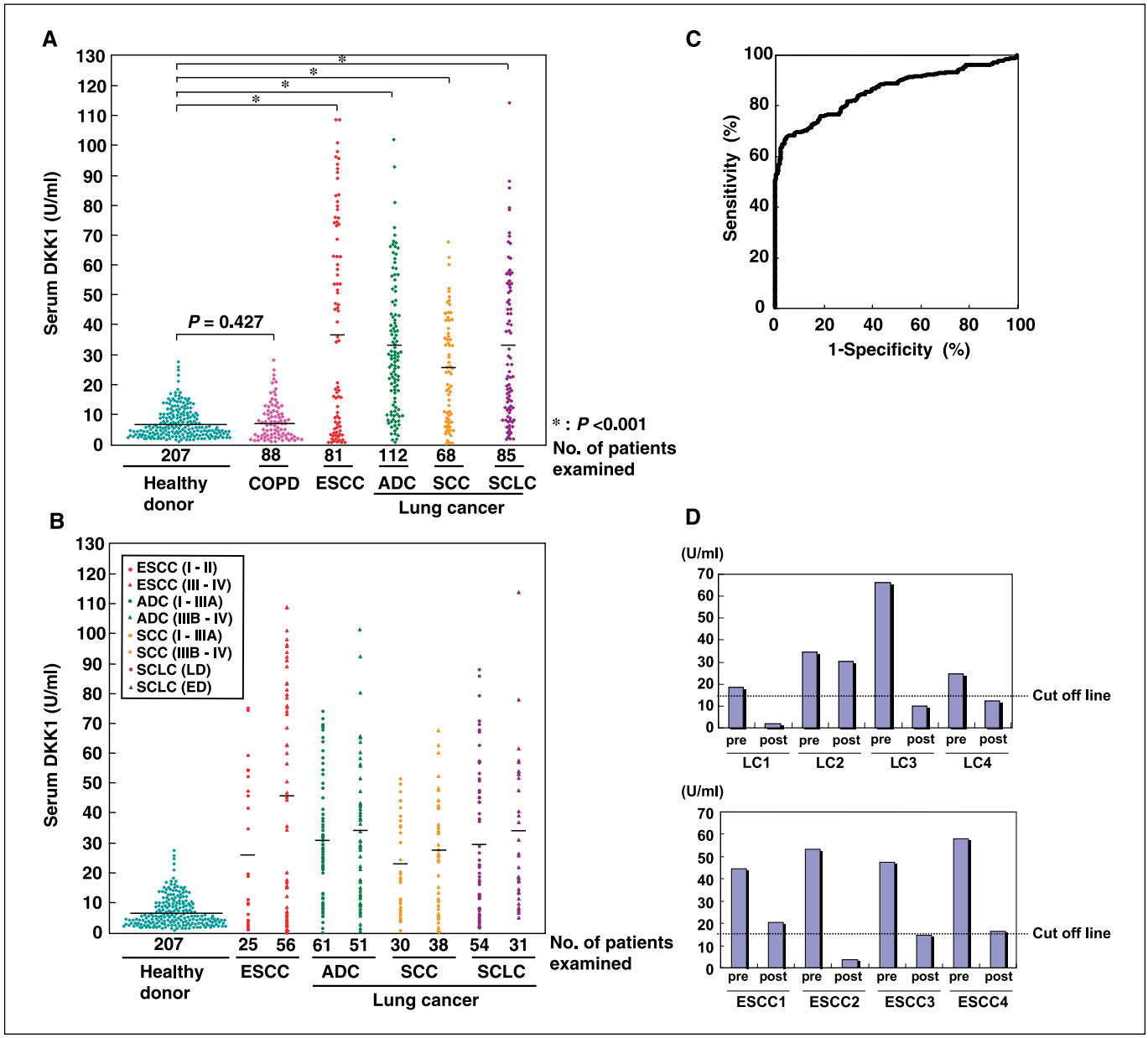

Figure 4. Serologic concentration of DKK1 determined by ELISA in patients with ESCC or lung cancer and in healthy controls or nonneoplastic lung disease patients with COPD. *A,* distribution of DKK1 in sera from patients with ESCC, lung adenocarcinoma, lung SCC, or SCLC. *Black lines,* average serum levels. Differences were significant between ESCC patients and healthy individuals/COPD patients ($P < 0.001$, respectively, Mann-Whitney U test), between adenocarcinoma patients and healthy individuals/COPD patients ($P < 0.001$), between SCC patients and healthy individuals/COPD patients ($P < 0.001$), and between SCLC patients and healthy/COPD individuals ($P < 0.001$). The difference between healthy individuals and COPD patients was not significant ($P = 0.427$). *B,* distribution of DKK1 in sera from patients at various clinical stages of ESCC, lung adenocarcinoma, lung SCC, or SCLC. *LD,* limited disease; *ED,* extensive disease. *C,* ROC curve analysis of DKK1 as serum markers for lung and esophageal cancers. *X axis,* 1-specificity; *Y axis,* sensitivity. *D,* serologic concentration of DKK1 before and after surgery (postoperative days at 2 months) in patients with NSCLC (*top*) and ESCC (*bottom*). The cutoff level was set to provide optimal diagnostic accuracy and likelihood ratios (minimal false-negative and false-positive results) for DKK1 [i.e., 14.7 units/mL with a sensitivity of 68.2% (236 of 346) and a specificity of 95.2% (197 of 207)].

patients and 207 healthy controls (Fig. 4*C*), the cutoff level in this assay was set to provide optimal diagnostic accuracy and likelihood ratios (minimal false-negative and false-positive results) for DKK1 [i.e., 14.7 units/mL with a sensitivity of 68.2% (236 of 346) and a specificity of 95.2% (197 of 207)]. According to tumor histology, the proportions of the serum DKK1-positive cases was 74.1% for adenocarcinoma (83 of 112), 63.2% for SCC (43 of 68), 69.4% for SCLC (59 of 85), and 63.0% for ESCC (51 of 81). The proportions of the serum DKK1-positive cases were 11.4% (10 of 88) for COPD. We then did ELISA experiments using paired preoperative and postoperative (2 months after the surgery) serum samples from lung cancer and ESCC patients to monitor the levels of serum DKK1 in the same patients. The concentration of serum DKK1 was dramatically reduced after surgical resection of primary tumors (Fig. 4*D, top* and *bottom*). The results independently support the high specificity and the great potentiality of serum DKK1 as a biomarker for detection of cancer at an early stage and for monitoring of the relapse of the disease.

To evaluate the clinical usefulness of serum DKK1 level as a tumor detection biomarker, we also measured by ELISA the serum levels of two conventional tumor markers (CEA for NSCLC and ProGRP for SCLC patients) in the same set of serum samples from cancer patients and control individuals. ROC analyses determined the cutoff value of CEA for NSCLC detection to be 2.5 ng/mL [with a sensitivity of 32.2% (58 of 180) and a specificity of 97.1% (201 of 207); Fig. 5*A*]. The correlation coefficient between serum DKK1 and CEA values was not significant (Spearman rank correlation coefficient: $\rho = -0.026$; $P = 0.728$), indicating that measuring both markers in serum can improve overall sensitivity for detection of NSCLC to 82.2% (148 of 180); for diagnosing NSCLC, the sensitivity of CEA alone is 32.2% (58 of 180) and that of DKK1 is 70.0% (126 of 180). False-positive rates for either of the two tumor markers among normal volunteers (control group) were 7.7% (16 of 207), although the false-positive rates for each of CEA and DKK1 in the same control group were 2.9% (6 of 207) and 4.8% (10 of 207), respectively.

ROC analyses for the patients with SCLC determined the cutoff value of ProGRP as 46.0 pg/mL, with a sensitivity of 63.5% (54 of 85) and a specificity of 98.6% (204 of 207; Fig. 5*B*). The correlation coefficient between serum DKK1 and ProGRP values was not significant (Spearman rank correlation coefficient: $\rho = -0.012$; $P = 0.910$), also indicating that measurement of serum levels of both markers can improve overall sensitivity for detection of SCLC to 89.4% (76 of 85); for diagnosing SCLC, the sensitivity of ProGRP alone was 63.5% (54 of 85) and that of DKK1 was 69.4% (59 of 85). False-positive cases for either of the two tumor markers among normal volunteers (control group) were 6.3% (13 of 207), although the false-positive rates for ProGRP and DKK1 in the same control group were 1.5% (3 of 207) and 4.8% (10 of 207), respectively.

Activation of cellular migration by DKK1. As the immunohistochemical analysis on tissue microarray had indicated that lung and esophageal cancer patients with DKK1-positive tumors showed shorter cancer-specific survival period than those with DKK1-negative tumors, we examined a possible role of DKK1 in cellular migration and invasion using Matrigel assays using NIH3T3 and COS-7 cells. As shown in Fig. 6, transfection of *DKK1* cDNA into either cell line significantly enhanced its invasive activity through Matrigel compared with cells transfected with mock vector.

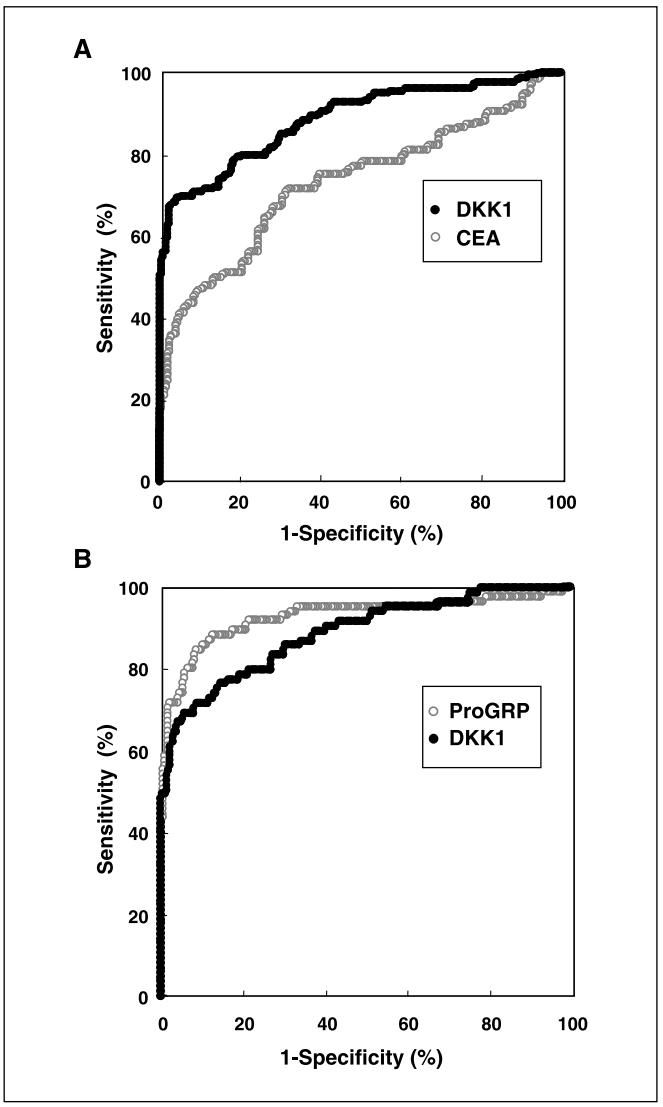

Figure 5. ROC curve analysis of DKK1, CEA, and ProGRP. *A,* ROC curve analysis of DKK1 (*black*) and CEA (*gray*) as serum markers for NSCLC. *X axis,* 1-specificity; *Y axis,* sensitivity. *B,* ROC curve analysis of DKK1 (*black*) and ProGRP (*gray*) as serum markers for SCLC. *X axis,* 1-specificity; *Y axis,* sensitivity.

Discussion

In spite of improvement of modern surgical techniques and adjuvant chemoradiotherapy, lung cancer and ESCC are known to reveal the worst prognosis among malignant tumors. Therefore, it is now urgently required to develop novel diagnostic biomarkers for early detection of cancer and for the better choice of adjuvant treatment modalities to individual patients. We did a genome-wide expression profile analysis of 101 lung cancers and 19 ESCC cells after enrichment of cancer cells by laser microdissection using a cDNA microarray containing 27,648 genes (9–14). Through the analyses, we identified several genes that could be potentially good candidates for development of novel diagnostic markers, therapeutic drugs, and/or immunotherapy (15–23). Among them, the genes encoding putative tumor-specific transmembrane or secretory proteins are considered to have significant advantages because they are present on the cell surface or within the extracellular space, and/or in serum, making them easily accessible as molecular markers and therapeutic targets. In this study, we selected one of such genes, *DKK1*, encoding a secretory protein, and examined the protein expression status by means of tissue microarray and ELISA

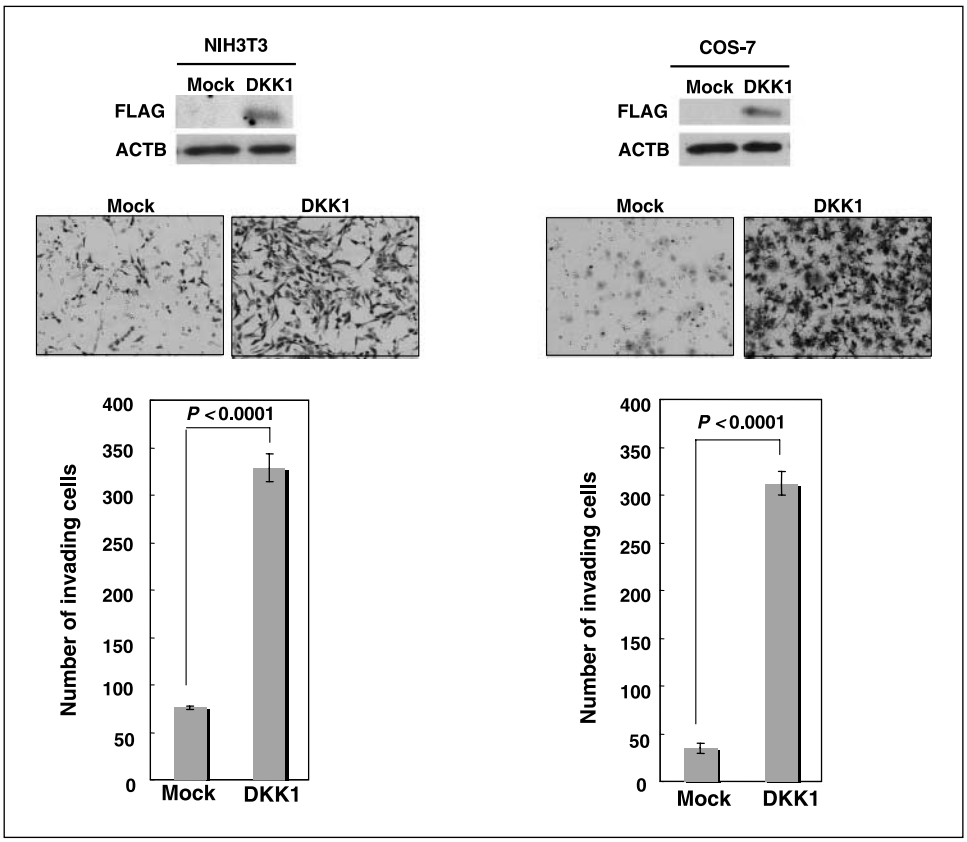

Figure 6. Enhanced invasiveness of mammalian cells transfected with DKK1-expressing plasmids. Assays showing the invasive nature of NIH3T3 (*left*) and COS-7 cells (*right*) in Matrigel matrix after transfection with expression plasmids for hDKK1. *Top,* transient expression of DKK1 in NIH3T3 and COS-7 cells detected by Western blot analysis; *middle* and *bottom,* Giemsa staining (×200 magnification) and the number of cells migrating through the Matrigel-coated filters. Assays were done thrice, each in triplicate wells.

for evaluating it for usefulness as diagnostic and prognostic biomarker(s) for lung cancer and/or ESCC.

DKK1 is a 35-kDa protein that contains a signal peptide sequence and two cysteine-rich domains (33) and is a secreted protein that functions as a negative regulator of the Wnt signaling and plays a crucial role in head formation in vertebrate development (33–37). In addition, DKK1 was reported to be a downstream target of β-catenin/T-cell factor and participates in a negative feedback loop in the Wnt signaling in colon cancer cells (24, 25).

A family of hDKK-related genes was composed of DKK1, DKK2, DKK3, and DKK4 together with a unique DKK3-related protein, termed Soggy (Sgy). hDKK1, hDKK2, hDKK3, and hDKK4 contain two distinct cysteine-rich domains in which the positions of 10 cysteine residues are highly conserved among family members. hDKK1 and hDKK4, but not hDKK2, hDKK3, or Sgy, were shown to suppress the Wnt-induced secondary axis induction in *Xenopus* embryos (38). DKK4 was found to show high specificity for gastric cancer by serial analysis of gene expression and quantitative RT-PCR (39). Other studies have shown overexpression of *DKK1* in Wilms' tumor, hepatoblastoma, and HCC (26, 27), but clinical significance of DKK1 protein as a serologic/histochemical marker in human cancer or as a therapeutic target was not investigated previously. Like the DKK1 protein, Wnt inhibitory factor-1 and Frizzled-related protein (FRP) were known to be secreted molecules, which have been indicated to bind to Wnt proteins and inhibit their activity (40–42). These two proteins were associated with human cancers, including colorectal carcinoma (43). FRP-4 protein showed markedly increased expression levels in colorectal cancers compared with normal mucosa but no significant association with pathologic features or with patient outcome (44). Because various DKK family proteins had been described as being overexpressed in human cancers (39, 44), DKK1 seemed likely to have a potential role in development or progression of certain types of human tumor, although it was indicated as a negative feedback factor for the activated Wnt signaling pathway in colon cancer.

In this study, we showed that induction of exogenous expression of DKK1 enhanced the cellular migration/invasive activity of mammalian cells. Concordantly, tissue microarray analyses revealed correlation of the strong DKK1 staining in primary NSCLC and ESCC tissues with poorer prognosis of the patients. Although the detailed function of DKK1 in lung and esophageal carcinogenesis is unknown, our results implied that DKK1 expression could promote dissemination of tumors by stimulating cell invasion. DKK1 was described as a secreted protein, which could bind to LRP5/6 and Kremen proteins, induce LRP endocytosis, and prevent the formation of the Wnt-Frizzled-LRP5/6 receptor complexes (25). However, when we analyzed mRNA expression of *DKK1* and *LRP5/6* in lung and esophageal cancer cell lines and cancer tissues by semiquantitative RT-PCR, the expression pattern of *LRP5/6* was not concordant with that of *DKK1* (data not shown). Additional studies to identify unknown binding partners and receptors of DKK1 in human lung and esophageal cancers should contribute to a better understanding of the signaling pathway mediated by DKK1 overexpression.

We also found high levels of DKK1 protein in serologic samples from lung cancer and ESCC patients. As a half of the serum samples used for this study were derived from patients with early-stage cancers, DKK1 should be useful for diagnosis of even early-stage

cancers. To examine the feasibility for applying DKK1 as the diagnostic tool, we compared serum levels of DKK1 with those of CEA or ProGRP, two conventional diagnostic markers for NSCLCs and SCLCs, about its sensitivity and specificity for diagnosis. An assay combining both markers (DKK1 + CEA or DKK1 + ProGRP) increased the sensitivity to about 80% to 90% for lung cancer (NSCLC as well as SCLC), significantly higher than that of CEA or ProGRP alone, whereas 6% to 8% of healthy volunteers were falsely diagnosed as positive. Although further validation using a larger set of serum samples covering various clinical stages will be required, our data presented here sufficiently show a potential clinical usefulness of DKK1 itself as a serologic/histochemical marker for lung and esophageal cancers.

In conclusion, we have shown DKK1 as a potential biomarker for diagnosis of lung and esophageal cancers as well as for prediction of the poor prognosis of the patients with these diseases. DKK1 was overexpressed in the great majority of lung and esophageal cancer tissues we examined, and its serum levels were elevated in the sera of a large proportion of the patients. DKK1, combined with other tumor markers, could significantly improve the sensitivity of cancer diagnosis, although it could be used at initial diagnosis to identify patients who might benefit from early systemic treatment. Moreover, this molecule is also a likely candidate for development of therapeutic approaches, such as antibody therapy, to inhibit the cancer progression.

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Takumi Yamabuki, Atsushi Takano, Satoshi Hayama, et al.

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