# Characterization of SEZ6L2 cell-surface protein as a novel prognostic marker for lung cancer

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**To identify molecules that might serve as biomarkers or targets for development of novel molecular therapies, we have been screening genes encoding transmembrane/secretory proteins that are up-regulated in lung cancers, using cDNA microarrays coupled with purification of tumor cells by laser microdissection. A gene encoding seizure-related 6 homolog (mouse)-like 2 (SEZ6L2) protein, was chosen as a candidate for such molecule. Semi-quantitative RT-PCR and western-blot analyses documented increased expression of *SEZ6L2* in the majority of primary lung cancers and lung-cancer cell lines examined. SEZ6L2 protein was proven to be present on the surface of lung-cancer cells by flow cytometrical analysis using anti-SEZ6L2 antibody. Immunohistochemical staining for tumor tissue microarray consisting of 440 archived lung-cancer specimens detected positive SEZ6L2 staining in 327 (78%) of 420 non-small cell lung cancers (NSCLCs) and 13 (65%) of 20 small-cell lung cancers (SCLCs) examined. Moreover, NSCLC patients whose tumors revealed a higher level of SEZ6L2 expression suffered shorter tumor-specific survival compared to those with no SEZ6L2 expression. These results indicate that SEZ6L2 should be a useful prognostic marker of lung cancers. (*Cancer Sci* 2006; 97: 737–745)**

Lung cancer is the leading cause of cancer deaths worldwide, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of those cases.[1] Regardless to histological subtypes, the 5-year survival rate of lung-cancer patients is around 10–15%[1,2] and even that of patients diagnosed at stage IA is less than 80%.[2,3] Within the last decade several newly developed cytotoxic agents such as paclitaxel, docetaxel, gemcitabine, and vinorelbine have begun to offer multiple choices for treatment to patients with advanced lung cancer, but each of these regimens confers only a modest survival benefit compared with cisplatin-based therapies.[4,5] Hence, novel therapeutic strategies such as molecular-targeted drugs, siRNAs and immunotherapies (antibodies and cancer vaccines) are eagerly expected. Although the precise pathways involved in lung tumorigenesis still remain unclear,[6] some evidences indicate that tumor cells express cell-surface markers unique to each histological type at particular stages of differentiation. Since cell-surface proteins or secretory autocrine-growth factors are considered to be more accessible to immune mechanisms and drug-delivery systems, identification of cancer-specific cell-surface and/or secretory proteins is likely to be an effective approach to develop novel diagnostic markers and therapeutic strategies.

We have been screening genes encoding molecules that are up-regulated in lung cancers, using cDNA microarrays and tumor cells purified by laser-capture microdissection.[7–15] To verify the biological and clinicopathological significance of the respective gene-products, we have been performing tissue microarray analysis of clinical lung-cancer materials.[11–15] This systematic approach combined with the search of cell-surface and/or secretory proteins by bioinformatics tools identified that SEZ6L2, seizure related 6 homolog (mouse)-like 2 (alias PSK-1), was frequently transactivated in a large population of lung cancers.

Application of multiple strategies for the identification of genes that encode secreted and transmembrane molecules, termed the Secreted Protein Discovery Initiative (SPDI), indicated SEZ6L2 to be one of novel transmembrane proteins.[16] *SEZ6L2* was also identified as a highly homologous gene to mouse *SEZ6* that had been first identified in the course of differential screening of mRNA from cortical neurons treated with pentylentetrazole (PTZ), a drug known to induce epileptic seizures.[17] The *SEZ6L2* encodes a 92.5-kDa protein with a N-terminal signal peptide, five SUSHI domains (SCR repeat), three CUB (initials of the first three identified proteins containing such domains: *c*omplement factor C1r/C1s, embryonic sea urchin protein *u*EGF, and *b*one morphogenetic protein 1) domains, and a C-terminal transmembrane domain. Although there is little information about the function of proteins including SUSHI and CUB domains, they have been postulated that they would be mainly involved in developmental process, cell–cell interaction, and cell adhesion. A previous study using cDNA microarray combined with bioinformatics analysis demonstrated that *SEZ6L2* is one of the 703 genes that are highly expressed in human hepatocellular carcinoma, although its physiological significance in carcinogenesis or its clinicopathological importance has not been clarified.[18]

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In this study, we reveal over-expression of SEZ6L2 protein in a considerable portion of lung cancers, and suggest that SEZ6L2 could be a novel prognostic marker and also a potential target for development of therapeutic antibodies for treatment of the majority of lung tumor.

## Materials and Methods

### Cell lines and clinical tissue samples

The 23 human lung-cancer cell lines used in this study included nine adenocarcinomas (ADCs; A427, A549, LC319, NCI-H1373, PC-3, PC-9, PC-14, NCI-H1666, and NCI-H1781), nine squamous-cell carcinomas (SCCs; EBC-1, LU61, NCI-H520, NCI-H1703, NCI-H2170, RERF-LC-AI, SK-MES-1, NCI-H226, and NCI-H647), one large-cell carcinoma (LCC; LX1), and four small-cell lung cancers (SCLCs; DMS114, DMS273, SBC-3, and SBC-5). A human bronchial epithelial cell line, BEAS2B (American Type Culture Collection; ATCC) was also included in the panel of the cells used in this study. All cells were grown in monolayers in appropriate media supplemented with 10% fetal calf serum (FCS) and were maintained at 37°C in an atmosphere of humidified air with 5% $CO_2$. Surgically resected primary NSCLC samples had been obtained earlier with informed consent.[7] A total of 420 formalin-fixed samples of primary NSCLCs (stage I-IIIA) including 263 ADCs, 116 SCCs, 28 LCCs, 13 adenosquamous carcinomas (ASCs) and adjacent normal lung tissues, had been obtained earlier along with clinicopathological data from patients undergoing surgery at Saitama Cancer Center (Saitama, Japan). ADCs were also classified into two groups: 129 mixed subtypes with bronchioloalveolar-cell carcinoma (BAC) components and 134 unmixed subtypes without BAC (non-BAC). SCLCs from postmortem materials (20 individuals) obtained from Hiroshima University (Hiroshima, Japan), were used in this study. NSCLC specimen and five tissues (heart, liver, lung, kidney, and pancreas) from postmortem materials (2 individuals with ADC) were also obtained from Hiroshima University. This study and the use of all clinical materials obtained with written informed consent were approved by the Institutional Research Ethics Committees. The histological classification of the tumor specimens was performed by the WHO criteria.[19] The postsurgical pathologic tumor-node-metastasis stage was determined according to the guidelines of the American Joint Committee on Cancer.[20]

### Semi-quantitative RT-PCR analysis

Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc. Gaithersburg, MD, USA) according to the manufacturer's protocol. Extracted RNAs and normal human-tissue polyA RNAs were treated with DNase I (Roche Diagnostics, Basel, Switzerland) and then reversely transcribed using oligo $(dT)_{12–18}$ primer and Super-Script II reverse transcriptase (Life Technologies, Inc.). Semi-quantitative RT-PCR experiments were carried out with synthesized *SEZ6L2* gene-specific primers (5′-GGGAGTAT-GAAGTTTCCATCTG-3′ and 5′-GGATGCTGGTTTATTTA-CTGTAGG-3′), or with beta-actin (*ACTB*)-specific primers (5′-ATCAAGATCATTGCTCCTCCT-3′ and 5′-CTGCGCAA-GTTAGGTTTTGT-3′) as an internal control. All PCR reactions involved initial denaturation at 94°C for 2 min followed by 22 (for *ACTB*) or 30 cycles (for *SEZ6L2*) of 94°C for 30 s, 54–60°C for 30 s, and 72°C for 60 s on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

### Northern-blot analysis

Human multiple-tissue blot (16 normal tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte; BD Biosciences Clontech, Palo Alto, CA, USA) was hybridized with a $^{32}$P-labeled PCR product of *SEZ6L2*. The cDNA probes of *SEZ6L2* were prepared by RT-PCR using primers, 5′-GCTATGAGGG-CTTTGAGCTTATC-3′ and 5′-AGAAGCAAAGGTGGAGA-GACTGT-3′. Pre-hybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at –80°C for one week.

### Preparation of anti-SEZ6L2 polyclonal antibody

Rabbit antibodies specific for extracellular portion of SEZ6L2 were raised by immunizing rabbits with 6-histidine fused human SEZ6L2 protein (codons 737–787; accession No. NM_012410), and purified with standard protocols using affinity columns (Affi-gel 10; Bio-Rad Laboratories, Hercules, CA, USA) conjugated with the 6-histidine fused protein. On Western blots we confirmed that the antibody was specific to SEZ6L2, using lysates from NSCLC tissues and cell lines as well as normal lung tissues.

### Western-blot analysis

Cells and tissues were lyzed in lysis buffer; 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 0.5% deoxycholate-Na, 0.1% SDS, plus protease inhibitor (Protease Inhibitor Cocktail Set III; Calbiochem Darmstadt, Germany). We used an ECL western-blotting analysis system (GE Healthcare Bio-sciences, Piscataway, NJ), as previously described.[12,13] SDS-PAGE was performed in 7.5% polyacrylamide gels. PAGE-separated proteins were electroblotted onto nitrocellulose membranes (GE Healthcare Bio-sciences) and incubated with a rabbit polyclonal antihuman SEZ6L2 antibody. A goat antirabbit IgG-HRP antibody (GE Healthcare Bio-sciences) was served as the secondary antibodies for these experiments.

### Flow-cytometric analysis

Lung-cancer cells ($1 \times 10^6$ cells) were incubated with a rabbit polyclonal antihuman SEZ6L2 antibody for detecting the extracellular domain of the protein (0.34 mg/mL) or control rabbit IgG (0.34 mg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 1 h. The cells were washed in PBS and then incubated with AlexaFluor 488-conjugated antirabbit IgG (Molecular Probes, Eugene, OR, USA) at 4°C for 30 min. The cells were washed in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson Labware, Bedford, MA, USA) and analyzed by ModFit software (Verity Software House, Inc. Topsham, ME, USA).

### Immunohistochemistry and tissue microarray

Tumor-tissue microarrays were constructed using 440 formalin-fixed primary lung cancers (420 NSCLCs and 20 SCLCs), according to the method published previously.[21–23] The tissue


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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, Sun Prairie, WI, USA). 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 presence of SEZ6L2 protein in clinical samples that had been embedded in paraffin blocks, we stained the sections as previously described.[11–15] Briefly, 16.25 μg/mL of a rabbit polyclonal antihuman SEZ6L2 antibody was added after blocking of endogenous peroxidase and proteins. The sections were incubated with HRP-labeled antirabbit IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin.

Three independent investigators assessed SEZ6L2 positivity semiquantitatively without prior knowledge of clinicopathological data. The intensity of SEZ6L2 staining was evaluated using following criteria: strong positive (2+), dark brown staining in more than 50% of tumor cells completely obscuring membrane and cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell membrane and cytoplasm; absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted only as strongly positive if reviewers independently defined them as such.

### Statistical analysis

Statistical analyses were performed using the StatView statistical program (SaS, Cary, NC, USA). We used contingency tables to analyze the relationship between SEZ6L2 expression and clinicopathological variables in NSCLC patients. Tumor-specific survival curves were calculated from the date of surgery to the time of death related to NSCLC, or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for SEZ6L2 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were performed with the Cox proportional-hazard regression model to determine associations between clinicopathological variables and cancer-related mortality. First, we analyzed associations between death and possible prognostic factors including age, gender, histological type, pT-classification, and pN-classification, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong SEZ6L2 expression into the model, along with any and all variables that satisfied an entry level of a $P$-value less than 0.05. As the model continued to add factors, independent factors did not exceed an exit level of $P < 0.05$.

### RNA interference assay

Using the vector-based RNA interference (RNAi) system, psiH1BX3.0, which we had established earlier to direct the synthesis of siRNAs in mammalian cells,[10,12,13,15] we transfected 10 μg of siRNA-expression vector with 30 μL of Lipofectamine 2000 (Invitrogen) into two NSCLC cell lines (A549, LC319) that endogenously over-expressed *SEZ6L2*. The transfected cells were cultured for five days in the presence of appropriate concentrations of geneticin (G418). Cell numbers and viability were measured by Giemsa staining and MTT assay in triplicate. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control-1 (EGFP: enhanced green fluorescent protein (GFP) gene, a mutant of *Aequorea victoria* GFP), 5′-GAAGCAGCACGACTTCTTC-3′; control-2 (LUC, luciferase gene from *Photinus pyralis*), 5′-CGTACGCGGAATACTTCGA-3′; control-3 (Scramble: Chloroplast *Euglena gracilis* gene coding for the 5S and 16S rRNA), 5′-GCGCGCTTTGTAGGATTCG-3′; siRNA-SEZ6L2-1 (si-1), 5′-CCAACCGGCTGCTTCTGCA-3′; siRNA-SEZ6L2-2 (si-2), 5′-CTGGAAGTGACCCAGACCA-3′; siRNA-SEZ6L2-3 (si-3), 5′-GCTTCAGGGAAAGTCCCTT-3′. To validate our RNAi system, individual control siRNAs were tested by semiquantitative RT-PCR to confirm the decrease in expression of the corresponding target genes that had been transiently transfected to COS-7 cells. Down-regulation of *SEZ6L2* expression by functional siRNA, but not by controls, was also confirmed in the cell lines used for this assay.

## Results

### SEZ6L2 expression in lung tumors, cell lines, and normal tissues

To search for novel target molecules for development of therapeutic agents and/or diagnostic markers for NSCLC, we first screened genes that showed more than a 3-fold higher level of expression in cancer cells than in normal cells, in half or more of the 37 NSCLCs analyzed by cDNA microarray.[7] Among 23 040 genes screened, we identified the *SEZ6L2* transcript as a good candidate (3-fold or higher expression in 81% of the NSCLC cases), and confirmed its transactivation by semiquantitative RT-PCR experiments in 12 of 15 additional lung-cancer tissues and in 19 of 23 lung-cancer cell lines (NSCLC and SCLC samples), while its expression in normal lung tissue cells or a human bronchial epithelial cell line, BEAS2B, was hardly detectable (Fig. 1a,b).

We subsequently generated rabbit polyclonal antibody specific to human SEZ6L2 and confirmed by western-blot analysis an expression of SEZ6L2 protein in 4 cancer cell lines of lung, in which the *SEZ6L2* transcript had been detected at a high level (Fig. 1c). We found no band in two cell lines, which expressed no *SEZ6L2* transcript. As SEZ6L2 was suggested to be a type I membrane protein, we attempted to validate SEZ6L2 expression on the surfaces of lung-cancer cells using flow-cytometry with anti-SEZ6L2 polyclonal antibody. This analysis indicated that the antibody bound to A549 and EBC-1 cells, in which *SEZ6L2* transcript had been detected at a high level, but not to NCI-H647 cells, which had not expressed *SEZ6L2* (Fig. 1d). We also examined expression of SEZ6L2 protein in NSCLC tissues using the same antibody. Western-blot analysis revealed the increased SEZ6L2 protein expression in tumor tissues in representative pairs of ADC samples analyzed (Fig. 2a). Immunohistochemical analysis of tumor tissues detected positive staining for SEZ6L2 specifically in cancer cells in 7 of the 10 NSCLC cases examined, but the staining was hardly detectable in surrounding normal lung epithelial cells (Fig. 2b–d). Interestingly, the invasive border of the tumor adjacent to the non-cancerous cells showed the tendency of strong staining. SEZ6L2 localized at the plasma membrane as well as in the cytoplasm of tumor cells (Fig. 2e–g).

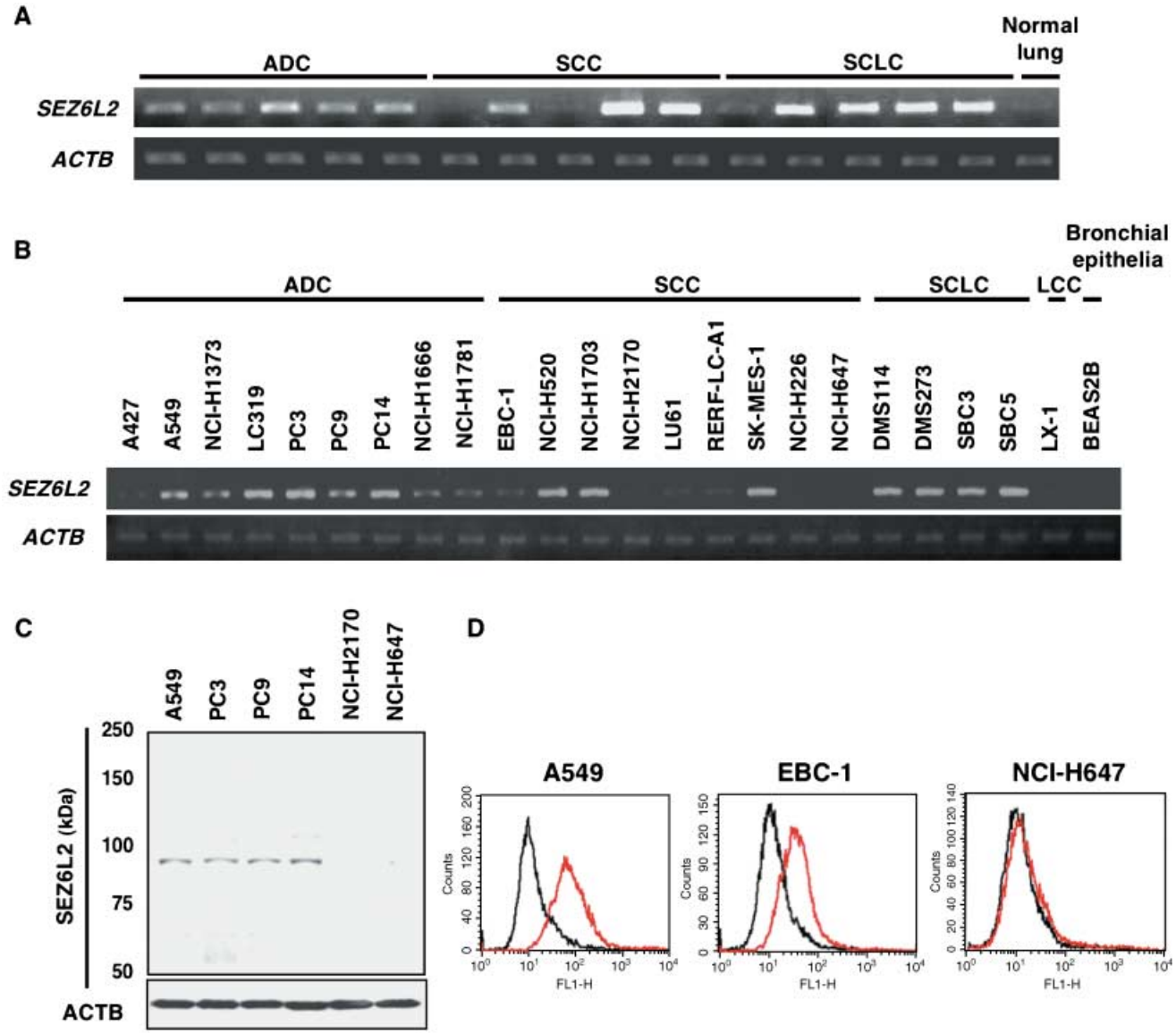


**Fig. 1.** Validation of SEZ6L2 expression and localization in lung cancers. (a) Expression of *SEZ6L2* in 15 clinical lung-cancer samples, examined by semiquantitative RT-PCR. (b) Expression of *SEZ6L2* in 23 lung-cancer cell lines, examined by semiquantitative RT-PCR. (c) Expression of SEZ6L2 protein in 6 lung-cancer cell lines, examined by western-blot analysis. (d) Expression of SEZ6L2 protein on cell surfaces in lung-cancer lines A549, EBC-1, and NCI-H647, evaluated by flow-cytometric analysis. Signal intensity values (*Y*-axis) of cells treated with antihuman SEZ6L2 polyclonal antibody (red) or cells treated with rabbit IgG (control; black) were shown.

Northern-blot analysis using human *SEZ6L2* cDNA as a probe detected a 3.2-kb transcript of weak signal only in brain, pancreas, prostate, and testis among the 16 normal human tissues (data not shown). We also examined expression of SEZ6L2 protein with anti-SEZ6L2 antibody on five normal tissues (heart, liver, lung, kidney, and pancreas), and found that it was hardly detectable in these tissues (Fig. 3a–e) while positive SEZ6L2 staining appeared in lung tumor tissues (Fig. 3f).

### Association of SEZ6L2 expression with poor prognosis of NSCLC patients

To verify the biological and clinicopathological significance of SEZ6L2, we additionally examined the expression of SEZ6L2 protein by means of tissue microarrays containing lung-cancer tissues from 440 patients. We classified a pattern of SEZ6L2 expression on the tissue array ranging from absent (scored as 0) to weak/strong positive (scored as 1+ ~ 2+) (Fig. 4a–d). Of the 420 NSCLC cases examined, SEZ6L2 was strongly stained in 31 (7.4%; score 2+), weakly stained in 296 (70.5%; score 1+), and not stained in 93 cases (22.1%; score 0) (details are shown in Table 1). Weak positive staining (score 1+) was observed in 65% (13 of 20) of SCLC cases examined. As shown in Table 1, gender (higher in female; $P = 0.007$ by Fisher's exact test) and hisotological type (higher in ADC; $P < 0.001$ by Fisher's exact test) were significantly associated with the SEZ6L2 positivity (score 1+ ~ 2+). The median survival time of NSCLC patients was significantly related to the expression levels of SEZ6L2 (3172 days in score 0


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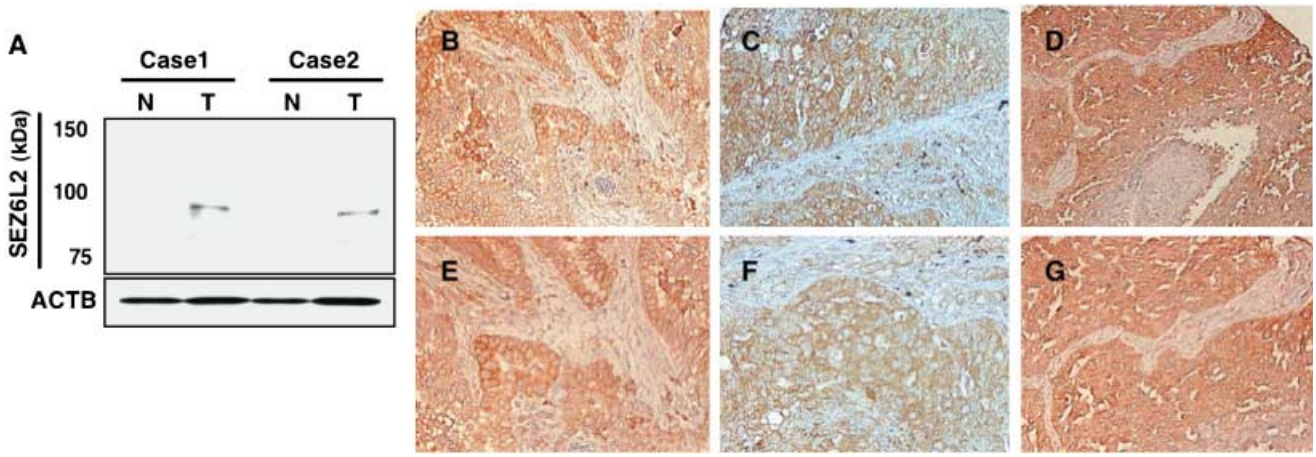


**Fig. 2.** Expression and localization of SEZ6L2 in clinical lung cancer tissues. (a) Western-blot analysis of SEZ6L2 protein in two representative pairs of lung adenocarcinoma samples. (b–g) Representative images of immunohistochemical analysis of SEZ6L2 protein in lung adenocarcinoma tissues. Magnification, ×100 (b, c, d) and ×200 (e, f, g).

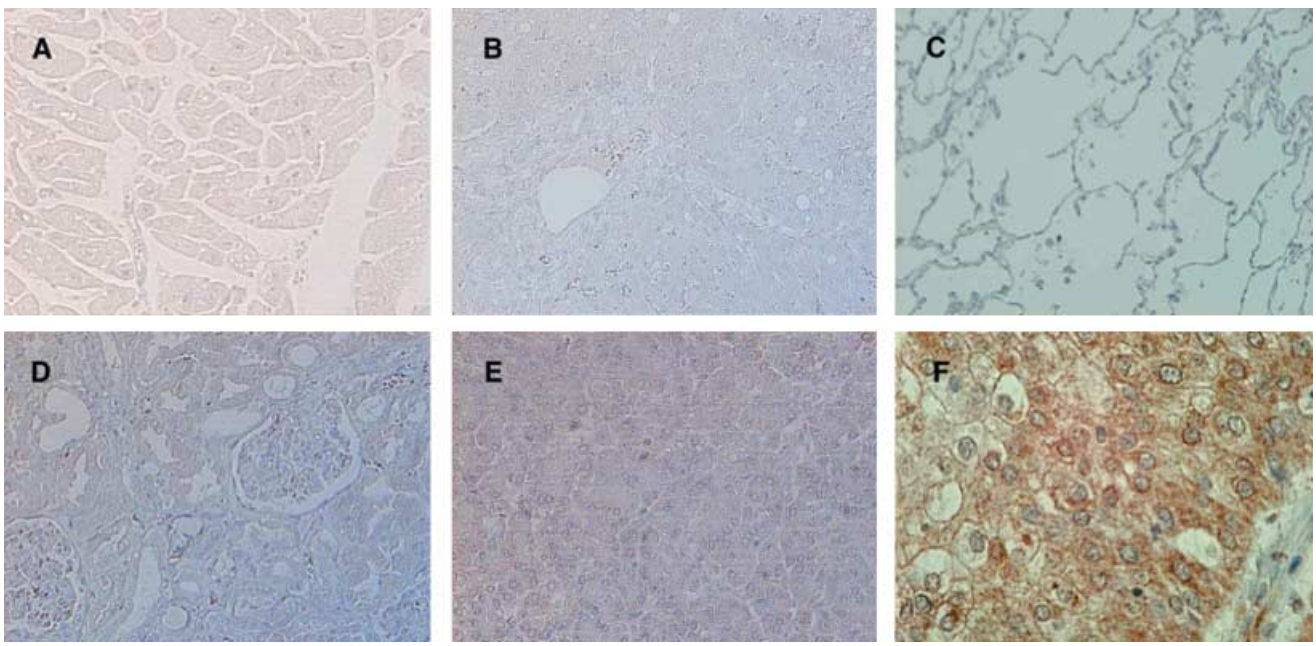


**Fig. 3.** Expression of SEZ6L2 protein in normal organ tissues.(a–f) Immunohistochemical evaluation of SEZ6L2 protein in representative normal tissues; adult heart (a), liver (b), lung (c), kidney (d), and pancreas (e), as well as lung adenocarcinoma tissues (f). Magnification, ×200.

cases, 2346 days in 1+, and 1134 days in 2+; $P = 0.0209$ by log-rank test; Fig. 4e). By univariate analysis, pT stage (T3, T4 *versus* T1, T2), pN stage (N1, N2 *versus* N0), age ($\geq 65$ *versus* $< 65$), gender (Male *versus* Female), histological classification (ADC *versus* other histological types), and strong SEZ6L2 positivity (score 2+ *versus* 0, 1+) were all significantly related to poor tumor-specific survival among NSCLC patients ($P = < 0.0001, < 0.0001, 0.0038, 0.0027, 0.0102$, and $0.0138$, respectively; Table 2). In multivariate analysis of the prognostic factors, pT stage, pN stage, age, and strong SEZ6L2 expression were indicated to be an independent prognostic factor ($P = 0.0001, < 0.0001, < 0.0001, 0.0144$, respectively; Table 2).

## Inhibition of endogenous *SEZ6L2* expression by siRNA in NSCLCs

To assess whether up-regulation of SEZ6L2 plays a role in growth or survival of lung cancer cells, we constructed three independent plasmids that were designed to express siRNA against *SEZ6L2* (si-1, si-2, and si-3), along with three different control plasmids (siRNAs for EGFP, LUC and Scramble). The treatment of NSCLC cells with the three effective and specific siRNAs could reduce expression of *SEZ6L2*, but did not suppress cell growth significantly (data not shown), suggesting that up-regulation of SEZ6L2 is not directly related to growth or survival of cancer cells.

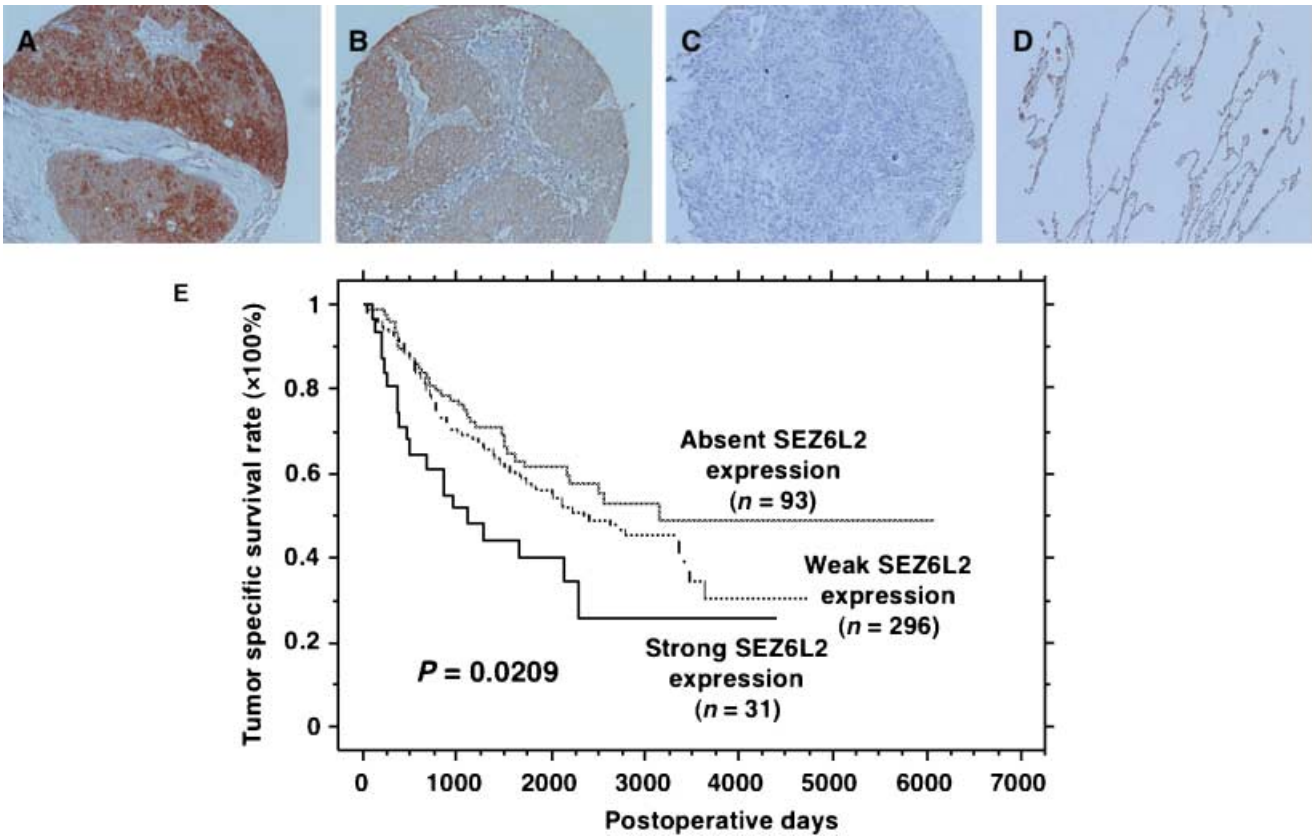


**Fig. 4.** Association of increased SEZ6L2 expression with poorer clinical outcomes among NSCLC patients. (a–d) Immunohistochemical evaluation of SEZ6L2 protein expression on tissue microarrays. Examples are shown for strong (a), weak (b), or absent (c) SEZ6L2 expression in lung SCCs, and for no expression in normal lung (d). Magnification, ×100. (e) Kaplan-Meier analysis of tumor-specific survival in 420 patients with NSCLCs according to the level of SEZ6L2 expression ($P = 0.0209$; log-rank test).

**Table 1. Associabon between SEZ6L2-positivity in NSCLC tissues and patients' characteristics ($n = 420$)**

| | Total $n = 420$ | SEZ6L2 strong positive $n = 31$ | SEZ6L2 weak positive $n = 296$ | SEZ6L2 absent $n = 93$ | $P$ value strong/ weak vs absent |
|---|---|---|---|---|---|
| Gender | | | | | |
| Male | 290 | 20 | 195 | 75 | |
| Female | 130 | 11 | 101 | 18 | 0.007† |
| Age (years) | | | | | |
| < 65 | 207 | 15 | 147 | 45 | |
| ≥ 65 | 213 | 16 | 149 | 48 | NS |
| Histological type | | | | | |
| ADC | 263 | 24 | 198 | 41 | |
| SCC | 116 | 3 | 77 | 36 | < 0.001*† |
| Others | 41 | 4 | 21 | 16 | |
| pT factor | | | | | |
| T1+T2 | 301 | 22 | 212 | 67 | |
| T3+T4 | 119 | 9 | 84 | 26 | NS |
| pN factor | | | | | |
| N0 | 259 | 14 | 180 | 65 | |
| N1+N2 | 161 | 17 | 116 | 28 | NS |
| Smoking history | | | | | |
| Never smoker | 129 | 10 | 96 | 23 | |
| Smoker | 291 | 21 | 200 | 70 | NS |

ADC, adenocarcinoma; SCC, squamous cell carcinoma.
Others, large cell carcinoma plus adenosquamous cell carcinoma.
*ADC versus other histology.
†$P < 0.05$ (Fisher's exact test).
NS, no significance.


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

| Variables | Hazards ratio | 95% CI | Unfavorable/Favorable | *P*-value |
|---|---|---|---|---|
| Univariate analysis | | | | |
| SEZ6L2 | 1.789 | 1.126–2.841 | Strong(+)/ Weak(+) or (–) | 0.0138* |
| Age (years) | 1.520 | 1.145–2.018 | 65 ≥ / < 65 | 0.0038* |
| Gender | 1.640 | 1.187–2.265 | Male / Female | 0.0027* |
| Histological type | 1.444 | 1.091–1.912 | others/ADC† | 0.0102* |
| pT factor | 1.889 | 1.411–2.528 | T3+T4/T1+T2 | < 0.0001* |
| pN factor | 2.930 | 2.197–3.908 | N1+N2/N0 | < 0.0001* |
| Multivariate analysis | | | | |
| SEZ6L2 | 1.814 | 1.126–2.922 | Strong(+)/ Weak(+) or (–) | 0.0144* |
| Age (years) | 1.930 | 1.442–2.581 | 65 ≥ / < 65 | < 0.0001* |
| Gender | 1.413 | 0.989–2.019 | Male / Female | 0.0572 |
| Histological type | 1.184 | 0.861–1.628 | others ADC† | 0.2982 |
| pT factor | 1.787 | 1.329–2.404 | T3+T4/T1+T2 | 0.0001* |
| pN factor | 2.356 | 1.761–3.153 | N1+N2/N0 | < 0.0001* |

†ADC, adenocarcinorna.
*$P < 0.05$.

## Discussion

Molecular-targeted therapies are expected to be highly specific to malignant cells, with minimal adverse reactions due to their well-defined mechanisms of action. Equally desirable prospects are minimally invasive, and highly sensitive and specific new diagnostic methods that would adapt readily to clinical settings. From this point of view, tumor-specific transmembrane/secretory proteins should have significant advantages because they are located either at the cell surface or within the extracellular space; if they are present in serum, it makes them easily accessible as molecular markers. Some tumor-specific markers already available, such as CYFRA or Pro-GRP, are transmembrane/secretory proteins.[24,25] In addition, an example of rituximab (Rituxan), a chimeric monoclonal antibody against CD20-positive lymphomas, provides proof of the concept that targeting specific cell-surface molecules can result in significant clinical benefits.[26] During the course of our attempt to identify novel cancer-specific cell-surface or secretory proteins, we have been exploiting the power of genome-wide expression analysis to select genes that are over-expressed on the surface of cancer cells.[11,14] Functional and immunohistochemical analysis of candidate molecules on tissue microarray has revealed that SEZ6L2 is likely to be a potential target for development of novel tools for diagnosis and treatment of lung cancer.

SEZ6L2 was indicated to be a type I transmembrane protein with extracellular SUSHI and CUB domains by the bioinformatics tools.[16] SUSHI domains are known as complement control protein (CCP) modules, which exist in a wide variety of complements and adhesion proteins,[27] and are involved in protein-protein or protein–ligand interactions.[28] On the other hand, CUB domains are structurally related to immunoglobulins and play important roles in cell adhesion.[29] Some members of the proteins with these domains that include a number of serine protein kinases, complement components, cubulin, spermadhesin, bone morphogenetic protein 1, and others involved in cell adhesion or interaction with extracellular matrix components, were shown to have key functions in embryonic development.[30–34] In this study, we demonstrated that SEZ6L2 protein was expressed in a great majority of surgically resected NSCLC specimens, whereas it was scarcely expressed in normal tissues. Furthermore, the higher SEZ6L2 expression level was associated with shorter cancer-specific survival periods. This is, to our best knowledge, the first study to show prognostic value of SEZ6L2 expression in human cancers. Our observations implied that over-expressed SEZ6L2 may be associated with further malignant progression or unique phenotype in a subset of NSCLCs. However, reduction of SEZ6L2 expression by siRNA against *SEZ6L2*, did not suppress cell growth significantly, suggesting that up-regulation of SEZ6L2 is unlikely to be essential to growth or survival of cancer cells.

Recently, CUB domain-containing protein 1 (CDCP1), a novel member of CUB family proteins, was described as a marker for metastatic tissues over-expressed in colorectal cancer as well as in breast and lung carcinomas.[35] In mice models, expression levels of CDCP1 protein correlated with the metastatic ability of human epidermoid carcinoma cell line, HEp3 variant.[36] The CDCP1 molecule also contains intracellular binding sites for SH2 and SH3 domains and was phosphorylated at tyrosine residues by an Src kinase family member.[36] Since SEZ6L2 has a short C-terminal cytoplasmic domain with the sequence of Asn-Pro-X-Tyr, this motif is a potential target for tyrosine phosphorylation by Src family proteins.[37] A combination of our results and possible biological functions of the proteins with similar domains, suggest that activation of SEZ6L2 in cancer cells may modulate the cell adhesion, or the interaction and communication of cancer cells with the extracellular matrix or ligands, and may also function in some signal transduction, which possibly result in the promotion of tumor cell motility or invasion, and their subsequent highly malignant phenotype. In fact, immunohistochemical study demonstrated that SEZ6L2 localized at the plasma membrane as well as in the cytoplasm of tumor cells. Furthermore, SEZ6L2 protein expression was likely to increase at the invasive border of the tumor adjacent to the non-cancerous cells, thus partly supporting our hypothesis (Fig. 2b–g). Further elucidation of the mechanism implied by these observations should reveal important new information

about cell-cell communication, differentiation, and cancer progression.

Tumor tissue microarray is a powerful method to validate clinicopathological significance of candidate molecular markers using a large number of clinical samples, however, considerable heterogeneity exists within lung cancer with respect to morphology and the expression of biomarkers. We took 3–5 tissue-cores from each donor tumor block, all of which were carefully reviewed by the pathologists to cover most of histological subtypes within each tumor, which enabled us to correlate more detailed tumor subtypes with SEZ6L2 expression. Lung ADC has been classified in five histological subtypes; BAC, acinar, papillary, solid with mucin, and ADC with mixed subtypes. BAC is the subtype showing no invasive features, and good prognosis can be expected for patients with non-invasive BAC, although most lung ADC have mixed subtypes, which have invasive components.[38–40] We divided the 263 ADC cases into two subgroups with or without BAC component. Of the 129 cases with BAC subtype, 16 (12.4%) revealed strong positive staining of SEZ6L2 and 92 (71.3%) showed weakly positive staining. Of the 134 cases with non-BAC subtype, 8 (6.0%) were judged to be strong positive and 101 (75%) were to be weak positive, suggesting that SEZ6L2 staining was not associated with these BAC histology.

In summary, we have shown that over-expressed SEZ6L2 is likely to be an essential contributor to malignant features of NSCLCs. The data reported here imply the possibility of SEZ6L2 as a potential prognostic marker for lung cancers. Moreover, this molecule is a possible target for development of therapeutic approaches such as molecular-targeted antibodies to any types of cancers over-expressing this molecule on the cell surface.

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## References

1 Jemal A, Tiwari RC, Murray T *et al*. Cancer statistics, 2004. *CA Cancer J Clin* 2004; **54**: 8–29.
2 Naruke T, Goya T, Tsuchiya R, Suemasu K. Prognosis and survival in resected lung carcinoma based on the new international staging system. *J Thorac Cardiovasc Surg* 1998; **96**: 440–7.
3 Chang MY, Sugarbaker DJ. Surgery for early stage non-small cell lung cancer. *Semin Surg Oncol* 2003; **21**: 74–84.
4 Schiller JH, Harrington D, Belani CP *et al*. Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002; **346**: 92–8.
5 Smit EF, van Meerbeeck JP, Lianes P *et al*. European Organization for Research and Treatment of Cancer Lung Cancer Group. Three-arm randomized study of two cisplatin-based regimens and paclitaxel plus gemcitabine in advanced non-small-cell lung cancer: a phase III trial of the European Organization for Research and Treatment of Cancer Lung Cancer Group – EORTC 08975. *J Clin Oncol* 2003; **21**: 3909–17.
6 Huber RM, Stratakis DF. Molecular oncology – perspectives in lung cancer. *Lung Cancer* 2004; **45**: S209–13.
7 Kikuchi T, Daigo Y, Katagiri T *et al*. Expression profiles of non-small cell lung cancers on cDNA microarrays: Identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. *Oncogene* 2003; **22**: 2192–205.
8 Kakiuchi S, Daigo Y, Tsunoda T *et al*. Genome-wide analysis of organ-preferential metastasis of human small cell lung cancer in mice. *Mol Cancer Res* 2003; **1**: 485–99.
9 Kakiuchi S, Daigo Y, Ishikawa N *et al*. Prediction of sensitivity of advanced non-small cell lung cancers to gefitinib (Iressa, ZD1839). *Hum Mol Genet* 2004; **13**: 3029–43.
10 Suzuki C, Daigo Y, Kikuchi T, Katagiri T, Nakamura Y. Identification of COX17 as a therapeutic target for non-small cell lung cancer. *Cancer Res* 2003; **63**: 7038–41.
11 Ishikawa N, Daigo Y, Yasui W *et al*. ADAM8 as a novel serological and histochemical marker for lung cancer. *Clin Cancer Res* 2004; **10**: 8363–70.
12 Kato T, Daigo Y, Hayama S *et al*. A novel human tRNA-dihydrouridine synthase involved in pulmonary carcinogenesis. *Cancer Res* 2005; **65**: 5638–46.
13 Furukawa C, Daigo Y, Ishikawa N *et al*. PKP3 oncogene as prognostic marker and therapeutic target for lung cancer. *Cancer Res* 2005; **65**: 7102–10.
14 Ishikawa N, Daigo Y, Takano A *et al*. Increases of amphiregulin and transforming growth factor-alpha in serum as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancers. *Cancer Res* 2005; **65**: 9176–84.
15 Suzuki C, Daigo Y, Ishikawa N *et al*. ANLN plays a critical role in human lung carcinogenesis through activation of RHOA and by involvement in PI3K/AKT pathway. *Cancer Res* 2005; **65**: 11314–25.
16 Clark HF, Gurney AL, Abaya E *et al*. The secreted protein discovery initiative (SPDI), a large-scale effort to identify novel human secreted and transmembrane proteins: a bioinformatics assessment. *Genome Res* 2003; **13**: 2265–70.
17 Shimizu-Nishikawa K, Kajiwara K, Kimura M, Katsuki M, Sugaya E. Cloning and expression of SEZ-6, a brain-specific and seizure-related cDNA. *Brain Res Mol Brain Res* 1995; **28**: 201–10.
18 Patil MA, Chua MS, Pan KH *et al*. An integrated data analysis approach to characterized genes highly expressed in hepatocellular carcinoma. *Oncogene* 2005; **24**: 3737–47.
19 Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E. *Histological Typing of Lung and Pleural Tumors: World Health Organization International Histological Classification of Tumors*, 3rd edn. Berlin: Springer 1999.
20 Sobin LH, Wittekind CH, eds. *UICC TNM Classification of Malignant Tumors*, 5th edn. New York: John Wiley 1997.
21 Callagy G, Cattaneo E, Daigo Y *et al*. Molecular classification of breast carcinomas using tissue microarrays. *Diagn Mol Pathol* 2003; **12**: 27–34.
22 Callagy G, Pharoah P, Chin SF *et al*. Identification and validation of prognostic markers in breast cancer with the complementary use of array-CGH and tissue microarrays. *J Pathol* 2005; **205**: 388–96.
23 Chin SF, Daigo Y, Huang HE *et al*. A simple and reliable pretreatment protocol facilitates fluorescent in situ hybridisation on tissue microarrays of paraffin wax embedded tumour samples. *Mol Pathol* 2003; **56**: 275–9.
24 Pujol JL, Grenier J, Daures JP, Daver A, Pujol H, Michel FB. Serum fragment of cytokeratin subunit 19 measured by CYFRA 21–1 immunoradiometric assay as a marker of lung cancer. *Cancer Res* 1993; **53**: 61–6.
25 Miyake Y, Kodama T, Yamaguchi K. Pro-gastrin-releasing peptide (31–98) is a specific tumor marker in patients with small cell lung carcinoma. *Cancer Res* 1994; **54**: 2136–40.
26 Hennessy BT, Hanrahan EO, Daly PA. Non-Hodgkin lymphoma: an update. *Lancet Oncol* 2004; **5**: 341–53.
27 Norman DG, Barlow PN, Baron M, Day AJ, Sim RB, Campbell ID. Three-dimensional structure of a complement control protein module in solution. *J Mol Biol* 1991; **219**: 717–25.
28 Kirkitadze MD, Barlow PN. Structure and flexibility of the multiple domain proteins that regulate complement activation. *Immunol Rev* 2001; **180**: 146–61.
29 Duke-Cohan JS, Gu J, McLaughlin DF, Xu Y, Freeman GJ, Schlossman SF. Attractin (DPPT-L), a member of the CUB family of cell adhesion and guidance proteins, is secreted by activated human T lymphocytes and modulates immune cell interactions. *Proc Natl Acad Sci U S A* 1998; **95**: 11336–41.
30 Nakamura F, Tanaka M, Takahashi T, Kalb RG, Strittmatter SM. Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. *Neuron* 1998; **21**: 1093–100.
31 Li SW, Sieron AL, Fertala A, Hojima Y, Arnold WV, Prockop DJ. The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1. *Proc Natl Acad Sci U S A* 1996; **93**: 5127–30.


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32 Stohr H, Berger C, Frohlich S, Weber BH. A novel gene encoding a putative transmembrane protein with two extracellular CUB domains and a low-density lipoprotein class A module: isolation of alternatively spliced isoforms in retina and brain. *Gene* 2002; **286**: 223–31.
33 Feinberg H, Uitdehaag JC, Davies JM *et al*. Crystal structure of the CUB1-EGF-CUB2 region of mannose-binding protein associated serine protease-2. *EMBO J* 2003; **22**: 2348–59.
34 Hartigan N, Garrigue-Antar L, Kadler KE. Bone morphogenetic protein-1 (BMP-1). Identification of the minimal domain structure procollagen C-proteinase activity. *J Biol Chem* 2003; **278**: 18045–9.
35 Scherl-Mostageer M, Sommergruber W, Abseher R, Hauptmann R, Ambros P, Schweifer N. Identification of a novel gene, CDCP1, overexpressed in human colorectal cancer. *Oncogene* 2001; **20**: 4402–8.
36 Hooper JD, Zijlstra A, Aimes RT *et al*. Subtractive immunization using highly metastatic human tumor cells identifies SIMA135/CDCP1, a 135 kDa cell surface phosphorylated glycoprotein antigen. *Oncogene* 2003; **22**: 1783–94.
37 Sudol M. From Src Homology domains to other signaling modules: proposal of the 'protein recognition code'. *Oncogene* 1998; **17**: 1469–74.
38 Noguchi M, Morikawa A, Kawasaki M *et al*. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995; **75**: 2844–52.
39 Sakurai H, Maeshima A, Watanabe S *et al*. Grade of stromal invasion in small adenocarcinoma of the lung: histolopathological minimal invasion and prognosis. *Am J Surg Pathol* 2004; **28**: 198–206.
40 Awaya H, Takeshima Y, Yamasaki M, Inai K. Expression of MUC1, MUC2, MUC5AC, and MUC6 in atypical adenomatous hyperplasia, bronchioloalveolar carcinoma, adenocarcinoma with mixed subtypes, and mucinous bronchioloalveolar carcinoma of the lung. *Am J Clin Pathol* 2004; **121**: 644–53.