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SHORT COMMUNICATION

Cytoskeleton-Associated Protein 4 Is a Novel Serodiagnostic Marker for Lung Cancer

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Our aim was to develop a serodiagnostic marker for lung cancer. Monoclonal antibodies were generated, and one antibody designated as KU-Lu-1, recognizing cytoskeleton-associated protein 4 (CKAP4), was studied further. To evaluate the utility of KU-Lu-1 antibody as a serodiagnostic marker for lung cancer, reverse-phase protein array analysis was performed with sera of 271 lung cancer patients and 100 healthy controls. CKAP4 was detected in lung cancer cells and tissues, and its secretion into the culture supernatant was also confirmed. The serum CKAP4 levels of lung cancer patients were significantly higher than those of healthy controls ($P < 0.0001$), and the area under the curve of receiver-operating characteristic curve analysis was 0.890, with 81.1% sensitivity and 86.0% specificity. Furthermore, the serum CKAP4 levels were also higher in patients with stage I adenocarcinoma or squamous cell carcinoma than in healthy controls ($P < 0.0001$). Serum CKAP4 levels may differentiate lung cancer patients from healthy controls, and they may be detected early even in stage I non—small cell lung cancer. Serum CKAP4 levels were also significantly higher in lung cancer patients than in healthy controls in the validation set ($P < 0.0001$). The present results provide evidence that CKAP4 may be a novel early serodiagnostic marker for lung cancer. *(Am J Pathol 2018, 188: 1328—1333; https://doi.org/10.1016/j.ajpath.2018.03.007)*

Most lung cancers are initially diagnosed at an advanced stage, and so the disease is associated with a poor prognosis, being the leading cause of cancer-related death worldwide.[1] The identification of patients at a resectable early stage of cancer is thus extremely important. Therefore, the identification of biomarkers to diagnose early-stage lung cancer is anticipated. However, tumor markers for lung cancer, such as carcinoma embryonic antigen, sialyl Lewis X antigen, and cytokeratin 19 fragment 21-1, are not suitable for early tumor detection because of their low specificity and/or sensitivity.

We have exhaustively generated monoclonal antibodies against various tumor-associated proteins using lung cancer cell lines as antigens with the random immunization method.[2] One of the antibodies, KU-Lu-1, reacted with only tumor cells and tumor stromal fibroblasts in lung cancer tissues and not normal lung tissues. By immunoprecipitation and mass spectrometry, it was confirmed that the KU-Lu-1 antibody recognized cytoskeleton-associated protein 4 (CKAP4) (Supplemental Figure S1). The present study demonstrates the utility of the KU-Lu-1 antibody as an early serodiagnostic marker for lung cancer by the reverse-phase protein array (RPPA).

Materials and Methods

Cell Lines and Culture Supernatants

The LCN1 line derived from a pulmonary large-cell neuroendocrine carcinoma was established in our laboratory.[3] N231 derived from a small-cell lung carcinoma was

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purchased from the ATCC (Rockville, MD). A549 derived from lung adenocarcinoma (AC) was purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan), and RERF LC-AI derived from lung squamous cell carcinoma (SCC) was purchased from the RIKEN BioResource Center (Ibaraki, Japan). These cells were grown in RPMI 1640 medium (Merck, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Miami, FL), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific Inc., Waltham, MA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Subconfluent cells were harvested and washed twice with phosphate-buffered saline without bivalent ions, stored at −80°C for proteomic analysis, or fixed in 10% formalin and embedded in paraffin for immunocytochemistry.

For immunoblotting with culture supernatants, the cells were washed three times with phosphate-buffered saline without bivalent ions, and they were additionally incubated with protein-free hybridoma medium (PFHM-II; Thermo Fisher Scientific Inc.) for 24 hours. The culture supernatants were collected and centrifuged twice to remove cells at $100 \times g$ for 10 minutes at room temperature, and then the cell debris was removed by a Minisart syringe filter (Sartorius, Gottingen, Germany). Thirty milliliters of the culture supernatant obtained from each cell line was concentrated to 400 μL by ultrafiltration (Amicon Ultra-15 centrifugal filter units with a 30-kDa molecular weight cutoff membrane; Merck), according to the manufacturer's instructions.

Tissue and Serum Samples

Frozen fresh lung cancer tissues were obtained from the Kitasato University Hospital (Kanagawa, Japan) and kept at −80°C until use for proteomic analysis.

A tissue microarray of 70 continuous cases of paired lung cancers and their normal lung tissues, surgically resected at Kitasato University Hospital, was fixed in 10% formalin and embedded in paraffin. The cases consisted of 42 ACs, 24 SCCs, 2 small-cell lung carcinomas, and 2 large-cell neuroendocrine carcinomas, and they were prepared according to the protocol of Tissue Microprocessor KIN-type 1 (Azumaya, Tokyo, Japan).

Sera from 271 patients with lung cancer and 100 healthy controls were used as the training set. In addition, a validation set consisting of sera from 100 patients with lung cancer and 38 healthy controls was also studied. Patient and control sera were collected at Kitasato University Hospital and kept at −80°C until use.

Ethics Statement

All samples were collected in accordance with the ethical guidelines and written consent mandated, and this study was approved by the Ethics Committee of Kitasato University School of Medicine (B07-06). All patients and healthy controls were approached on the basis of approved ethical guidelines, and all agreed to participate in this study and provided written consent. The patients could refuse entry and discontinue participation at any time.

Immunoblotting

A total of 10 μg of each protein extracted from the cell lines or tissues or 1.5-mL equivalents of culture supernatant with detergent lysis buffer were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking with 0.5% casein in 0.01 mol/L Tris-HCl (pH 7.5) and 150 mmol/L NaCl, the membranes were reacted with the nondiluted hybridoma supernatant of KU-Lu-1 antibody for 2 hours at room temperature. The immunoblotting method used was described in our previous report.[2] Finally, immunoreactive bands on the membranes were detected with Immobilon Western Chemiluminescent HRP Substrate (Merck) and captured with ATTO Cool Saver System (ATTO, Tokyo, Japan).

Immunohistochemical Staining

Sections (3 μm thick) were deparaffinized in xylene, rehydrated in a descending ethanol series, and then treated with 3% hydrogen peroxide for 10 minutes. Antigen was retrieved by autoclaving in 0.01 mol/L citrate buffer (pH 6.0) with 0.1% Tween 20 for 10 minutes at 121°C. After blocking with 2% normal swine serum for 10 minutes, the sections were reacted with nondiluted KU-Lu-1 hybridoma supernatant for 2 hours at room temperature, and then reacted with ChemMate ENVISION (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Finally, the sections were visualized by the stable DAB solution (Thermo Fisher Scientific Inc.) and counterstained with Mayer's hematoxylin.

RPPA Analysis

RPPA analysis was performed in almost the same way except for the use of nondiluted hybridoma supernatant KU-Lu-1 as the first antibody, as described in our previous study.[4] Serum samples were diluted 1:100 with 0.01% Triton X-100/phosphate-buffered saline without bivalent ions and spotted onto a high-density amino-group−induced glass slide for dimethyl sulfoxide (SDM0011; Matsunami Glass Ind, Ltd, Osaka, Japan). Finally, the stained slides were scanned on a microarray scanner (Genepix 4000B; Molecular Devices, Sunnyvale, CA). The fluorescence intensity, defined as the median net value of quadruple samples, was determined using the Genepix pro 6.0 software package (Molecular Devices).

Statistical Analysis

Serum levels of CKAP4 in patients with lung cancer and healthy controls were analyzed using the *U*-test. The area

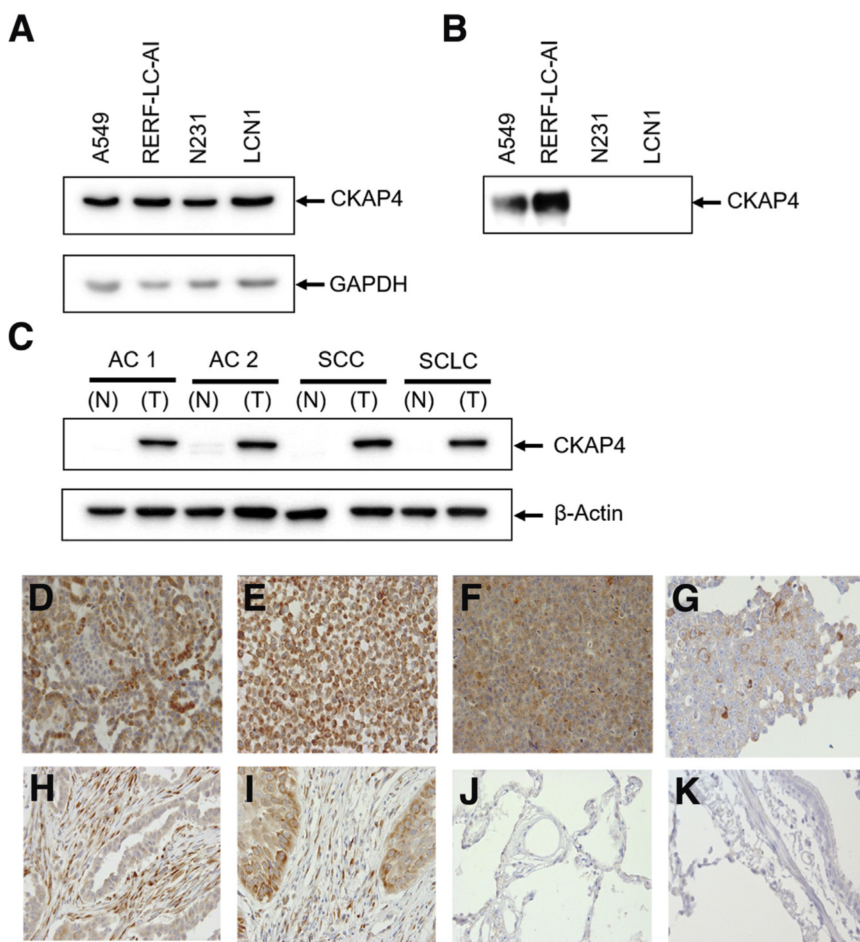

Figure 1 Immunoblot analysis using anti-CKAP4 antibody with whole-cell lysates from A549, RERF-LC-AI, N231, LCN1, and lung tissue samples. **A:** CKAP4 protein is detected at approximately 63 kDa in all lung cancer cells. **B:** CKAP4 protein is also detected at approximately 63 kDa in culture supernatants of A549 and RERF-LC-AI cells. **C:** The CKAP4 protein is expressed only in lung cancer tissues, and not in their normal counterparts. **D–K:** CKAP4 is observed at various intensities in the cytoplasm of lung cancer cells [A549 (**D**), RERF LC-AI (**E**), N231 (**F**), and LCN1 (**G**)] and tissues [adenocarcinoma (AC; **H**) and squamous cell carcinoma (SCC; **I**)], but not in normal lung epithelium [alveolar epithelium (**J**) and bronchial epithelium (**K**)]. Original magnification, ×400 (**D–K**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N, normal lung tissue; SCLC, small-cell lung carcinoma; T, tumor tissue.

under the curve and best cutoff point were calculated using receiver-operating characteristic curve analysis with the StatFlex statistical software package version 5.0 (Artech Co, Ltd, Osaka, Japan). The levels of CKAP4 were divided into a high-expression group (signal intensity $\geq$ median value) and a low-expression group (signal intensity $<$ median value). The relationships between CKAP4 levels and clinicopathological parameters in AC or SCC patients were assessed by the χ^2 test. $P < 0.05$ was considered significant.

Results

Immunoblot Analysis and Immunohistochemical Staining for CKAP4

The CKAP4 protein was expressed in all lung cancer cell lines on immunoblot analysis with the KU-Lu-1 antibody (Figure 1A). Next, the culture supernatants were investigated, and the secretion of CKAP4 in A549 and RERF-LC-AI lines was observed (Figure 1B). The expression of CKAP4 was further examined in lung cancer tissues and their paired normal lung tissues. The expression of CKAP4 was observed only in lung cancer tissues, and not in normal tissues (Figure 1C).

To confirm the immunoblotting data, immunohistochemical staining was subsequently performed using the four cell lines and a tissue microarray with paired cancer and normal lung tissues. The expression of CKAP4 was localized in the cytoplasm of all lung cancer cell lines to various extents (Figure 1, D–G). In lung cancer tissues, the expression of CKAP4 was also observed in the cytoplasm of tumor cells and fibroblasts of the tumor stroma (Figure 1, H and I). However, no obvious staining was observed in normal bronchial epithelial cells, alveolar cells, or stromal cells of normal lung tissues (Figure 1, J and K). These data confirmed the results of immunoblot analysis.

Significantly Elevated Serum CKAP4 Levels in Patients with Lung Cancer

To investigate serum CKAP4 levels in lung cancer patients, RPPA analysis was performed. The serum CKAP4 levels were significantly higher in lung cancer patients than in healthy controls in the training set ($P < 0.0001$) (Figure 2A). Relative values of serum CKAP4 levels ranged from 0.27 to 50.9 (median, 1.72) in lung cancer patients, and from 0.06 to 2.34 (median, 0.77) in healthy controls. On the basis of receiver-operating characteristic curve analysis, an

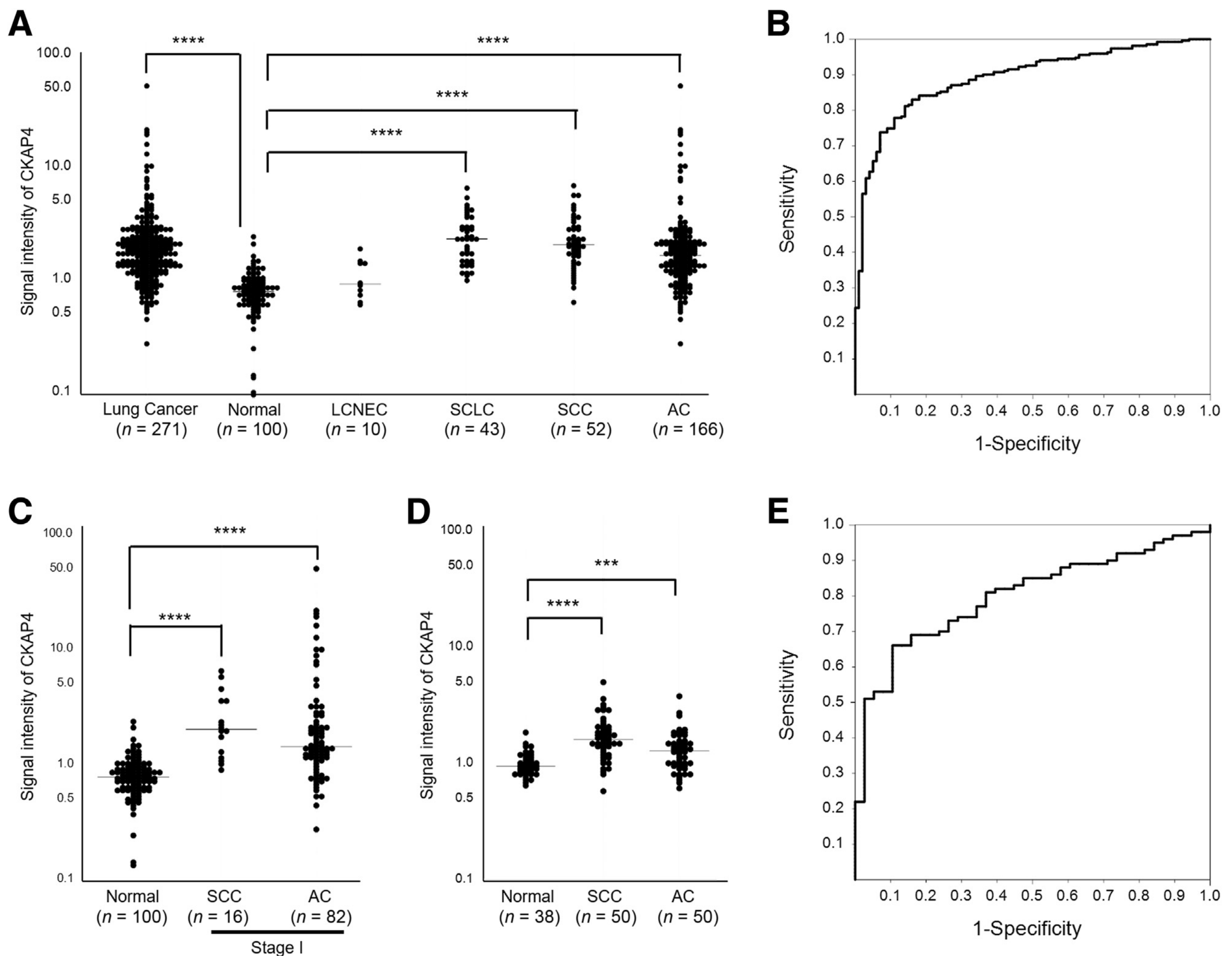

Figure 2 Serum CKAP4 levels in patients with lung cancer and healthy controls. **A:** In the training set, the median CKAP4 levels in sera from lung cancer patients and healthy controls were 1.72 and 0.77, respectively. Serum levels of CKAP4 are significantly higher in lung cancer patients than in healthy controls. Median values indicated in black bars [lung cancer, 1.72; normal, 0.77; large-cell neuroendocrine carcinoma (LCNEC), 0.89; small-cell lung carcinoma (SCLC), 2.25; squamous cell carcinoma (SCC), 2.00; and adenocarcinoma (AC), 1.99]. **B:** Receiver-operating characteristic curve analysis of CKAP4 as a serodiagnostic marker for lung cancer in the training set. The corresponding area under the curve is 0.89 for CKAP4. Using a cutoff value of 1.09, lung cancers were discriminated from healthy controls with 81.1% sensitivity and 86.0% specificity. **C:** In the training set, serum CKAP4 levels are significantly higher in stage I ACs and SCCs than in healthy controls. Median values are indicated in black bars (normal, 0.77; stage I SCC, 2.02; and stage I AC, 1.41). **D:** To confirm the utility of the serum CKAP4 level as a serodiagnostic marker, 138 additional sera were analyzed by reverse-phase protein array analysis as a validation set. The median CKAP4 levels in sera from lung cancer patients and healthy controls are 1.45 and 0.94, respectively. Serum levels of CKAP4 are significantly higher in lung cancer patients than in healthy controls. Median values indicated in black bars (normal, 0.94; SCC, 1.60; and AC, 1.28). **E:** In the validation set, receiver-operating characteristic curve analysis of CKAP4 as a serodiagnostic marker for lung cancer. The corresponding area under the curve is 0.80 for CKAP4. Using a cutoff value of 1.14, lung cancers were differentiated from healthy controls with 69.0% sensitivity and 84.2% specificity. ***$P < 0.001$, ****$P < 0.0001$.

optimal cutoff value of 1.09 for CKAP4 was applied, and the diagnostic sensitivity and specificity for lung cancer patients were 81.1% and 86.0%, respectively. The negative and positive predictive values of lung cancer were 63.0% and 94.0%, respectively. The area under the curve for CKAP4 levels in lung cancer patients was 0.89 compared with healthy controls (Figure 2B). Moreover, the serum CKAP4 levels were also significantly higher in patients, even with stage I AC and SCC, than in healthy controls ($P < 0.0001$) (Figure 2C). On the basis of receiver-operating characteristic curve analysis, an optimal cutoff value of 1.03 for CKAP4 was applied, and the diagnostic sensitivity and specificity for stage I lung cancer patients were 78.6% and 84.0%, respectively. The area under the curve for CKAP4 levels in lung cancer patients, compared with healthy controls, was 0.85 (data not shown). These data suggest that serum CKAP4 is a useful early serodiagnostic marker for lung cancer. The relationships between serum CKAP4 levels and clinicopathological characteristics of the patients are summarized in Supplemental Table S1. When a median value of 1.61 was applied as a cutoff point in AC patients, serum CKAP4 levels were significantly correlated with the age ($P = 0.0050$) and distant metastasis ($P = 0.0052$). Although it was not significant, serum CKAP4 levels also showed an association with the nodal status ($P = 0.0617$). To further confirm the utility of the

serum CKAP4 level as a serodiagnostic marker, 138 additional sera underwent RPPA analysis as a validation study. The serum CKAP4 levels were also significantly higher in lung cancer patients than in healthy controls in the validation set ($P < 0.0001$). Relative values of serum CKAP4 levels ranged from 0.58 to 5.06 (median, 1.45) in lung cancer patients, but values were 0.64 to 1.86 (median, 0.94) in healthy controls (Figure 2D). When an optimal cutoff value of 1.17 was applied, the diagnostic sensitivity and specificity for lung cancer were 69.0% and 84.2%, respectively. The area under the curve for CKAP4 levels in lung cancer patients was 0.80 compared with healthy controls (Figure 2E). There were no significant correlations between serum CKAP4 levels and the sex, stage, tumor differentiation, tumor size, or T-factor. No correlation was detected in SCC patients.

Discussion

In this study, aiming to identify useful serodiagnostic markers for lung cancer, monoclonal antibodies were generated using LCN1 cells derived from a large-cell neuroendocrine carcinoma as an immunogen. From a group of obtained monoclonal antibodies, KU-Lu-1 antibody, which recognized CKAP4, was selected. CKAP4 is a 63-kDa non-glycosylated and reversibly palmitoylated type II transmembrane protein that has been shown to anchor rough endoplasmic reticulum and microtubules in epithelial cells.[5] Although CKAP4 was described as an endoplasmic reticulum—resident protein, it is also now known to be expressed on the surface of vascular smooth muscle cells, where it acts as a receptor for tissue plasminogen activator and on the plasma membrane of type II pneumocytes as a receptor for SP-A.[6,7] Moreover, CKAP4 has been identified as a functional cell surface receptor for frizzled-8 protein-related antiproliferative factor.[8] Antiproliferative factor markedly inhibits normal bladder epithelial cell growth and also inhibits the proliferation of bladder carcinoma cells.[9]

In this study, to confirm the expression of CKAP4, immunohistochemistry was performed with four histologic types of lung cancer cells. The expression of CKAP4 was observed in all cell lines and, therefore, the histologic specificity was considered to be low. Moreover, in lung cancer tissues, CKAP4 was found to be localized in the cytoplasm of tumor cells and tumor stromal fibroblasts in lung cancer tissues, regardless of histologic types, but not in normal alveolar and bronchial epithelium or normal stromal fibroblasts. Recently, Franco et al[10] reported that cancer-associated fibroblasts play an important role in tumor cell growth and invasiveness. Thus, the KU-Lu-1 antibody may be a useful marker of cancer-associated fibroblasts related to a poor prognosis.

Recently, Kimura et al[11] identified CKAP4 as a receptor of Dickkopf1. Expressions of Dickkopf1 and CKAP4 were frequently observed in tumor lesions of human pancreatic and lung cancers, and the simultaneous expression of both proteins in tumor tissues was inversely correlated with the prognosis and relapse-free survival.[11] Because there was a report that CKAP4 was secreted through exosomes of colon and ovarian cancer cells and detected in urine as an exosomal protein,[12−14] CKAP4 was considered to be secreted into the serum in the same way as exosomal CKAP4 protein of urine. However, the mechanism of CKAP4 secretion remains unknown. Moreover, to our knowledge, no study has been conducted on the serum levels of CKAP4 in cancer patients, including those with lung cancer. In this study, it was first confirmed that CKAP4 was secreted into culture supernatants of lung cancer cells. On the basis of this observation, serum CKAP4 levels of lung cancer patients were measured by RPPA analysis. CKAP4 protein levels in patients with lung cancer were significantly higher than in healthy controls in both the training set ($P < 0.0001$) and validation set ($P < 0.0001$) by RPPA analysis. More important, the serum CKAP4 levels in patients, even those with stage I AC and SCC, were significantly higher than in healthy controls. In addition, the serum CKAP4 levels were also significantly correlated with distant metastasis ($P = 0.0052$) of lung AC patients. At present, some serodiagnostic markers are used for lung cancer, such as carcinoma embryonic antigen, cytokeratin 19 fragment, and SCC antigen.[15] Across disease stages I to IV, the sensitivities of serum carcinoma embryonic antigen, cytokeratin 19 fragment, and SCC antigen are reportedly 30% to 52%, 17% to 81%, and 24% to 39%, respectively. In this study, the sensitivity of serum CKAP4 was 81.6% in the training set and 69.0% in the validation set, and these rates are higher than those of serodiagnostic markers. Furthermore, the sensitivity of serum CKAP4 was also higher, even in stage I disease. These data suggest that CKAP4 may change current practices regarding the treatment of lung cancer patients. Furthermore, the diagnostic accuracies may be markedly improved by the combination of CKAP4 and conventional markers. The results suggest that CKAP4 is a novel early serodiagnostic marker for lung cancer.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.ajpath.2018.03.007*.

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