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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 × *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