CAXII Is a Sero-Diagnostic Marker for Lung Cancer

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Abstract

To develop sero-diagnostic markers for lung cancer, we generated monoclonal antibodies using pulmonary adenocarcinoma (AD)-derived A549 cells as antigens by employing the random immunization method. Hybridoma supernatants were immunohistochemically screened for antibodies with AMeX-fixed and paraffin-embedded A549 cell preparations. Positive clones were monocloned twice through limiting dilutions. From the obtained monoclonal antibodies, we selected an antibody designated as KU-Lu-5 which showed intense membrane staining of A549 cells. Based on immunoprecipitation and MADLI TOF/TOF-MS analysis, this antibody was recognized as carbonic anhydrase XII (CAXII). To evaluate the utility of this antibody as a sero-diagnostic marker for lung cancer, we performed dot blot analysis with a training set consisting of sera from 70 lung cancer patients and 30 healthy controls. The CAXII expression levels were significantly higher in lung cancer patients than in healthy controls in the training set ($P<0.0001$), and the area under the curve of ROC was 0.794, with 70.0% specificity and 82.9% sensitivity. In lung cancers, expression levels of CAXII were significantly higher in patients with squamous cell carcinoma (SCC) than with AD ($P=0.035$). Furthermore, CAXII was significantly higher in well- and moderately differentiated SCCs than in poorly differentiated ones ($P=0.027$). To further confirm the utility of serum CAXII levels as a sero-diagnostic marker, an additional set consisting of sera from 26 lung cancer patients and 30 healthy controls was also investigated by dot blot analysis as a validation study. Serum CAXII levels were also significantly higher in lung cancer patients than in healthy controls in the validation set ($P=0.030$). Thus, the serum CAXII levels should be applicable markers discriminating lung cancer patients from healthy controls. To our knowledge, this is the first report providing evidence that CAXII may be a novel sero-diagnostic marker for lung cancer.

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Introduction

Lung cancer is the leading cause of cancer death, comprising 13% (1.6 million) of the total cancer cases and 18% (1.4 million) of the cancer deaths in the world in 2008 [1,2].

Tumor markers have been detected in sera, urine, and tissues from patients with malignant tumors, and can be used for an exact diagnosis, discrimination of benign or malignant tumors, follow-up after therapies, and prediction of the patient's outcome. At present, some sero-diagnostic markers are used for lung cancer, such as carcinoembryonic antigen (CEA) and sialyl Lewis X antigen (SLX) for adenocarcinoma (AD), and cytokeratin 19 fragment (CYFRA) and squamous cell carcinoma antigen (SCCa) for squamous cell carcinoma (SCC) [3]. The positive rates of CEA, SLX, CYFRA, and SCCa are reportedly 57, 40~50, 50~60, and 60~80%, respectively. However, it has been reported that these markers do not show sufficient tumor or organ specificities; for example, SLX can show false-positive results in the presence of pulmonary tuberculosis and pulmonary fibrosis, and CYFRA can elevate with interstitial pneumonia and renal failure.

Antibodies are usually developed using purified proteins or synthetic peptides. We have exhaustively generated monoclonal antibodies (MoAbs) against various tumor-associated proteins using the pulmonary AD-derived A549 cell as an antigen with the random immunization method [4], and over 1,000 MoAbs have been obtained [5]. This method is expected to generate antibodies against proteins with tumor-specific post-translational modifications, which are difficult to obtain by conventional immunization methods.

Carbonic anhydrase XII is a transmembrane zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide to form bicarbonate ($H_2O+CO_2 \Leftrightarrow H^+ + HCO_3^-$), and is a member of the alpha carbonic anhydrase (CA) family. CAXII has been proposed to be involved in the acidification of the extracellular microenvironment, which is suitable for rapid tumor growth. CAXII overexpression was initially detected in renal cell carcinoma, and subsequent studies confirmed its expression in

various human cancers, such as diffuse astrocytoma, breast, pancreatic, and ovarian carcinoma, as well as in non-small cell lung cancer (NSCLC) [6–11]. Its expression was influenced both by factors related to differentiation and hypoxia in breast cancer *in vivo*, and was associated with a more favorable prognosis in invasive breast carcinoma patients [12]. Higher CAXII expression was also correlated with a better overall and disease-specific survival in patients with resectable NSCLC [13]. However, no study has clarified CAXII in sera and its clinical utility as a sero-diagnostic marker for patients with malignant tumors.

In this study, the specificity of the obtained anti-CAXII antibody was confirmed by immunohistochemistry (IHC) and immunoblotting with lung cancer cell lines and lung cancer tissues. To further confirm its utility as a sero-diagnostic marker, CAXII levels in sera from patients with lung cancer were studied by dot blot analysis.

Table 1. Clinicopathological characteristics of the patients.

Characteristics		Training set (N = 70)	Validation set (N = 26)
Age	<70	40 (57.1%)	19 (73.1%)
	≥70	30 (42.9%)	7 (26.9%)
Gender	Male	52 (74.3%)	16 (61.5%)
	Female	18 (25.7%)	10 (38.5%)
Stage	I	19 (27.2%)	17 (65.4%)
	II	11 (15.7%)	2 (7.7%)
	III	26 (37.1%)	4 (15.4%)
	IV	14 (20.0%)	3 (11.5%)
Tumor differentiation	Well	7 (13.2%)	11 (52.4%)
(NSCLC)	Moderate	10 (18.9%)	5 (23.8%)
	Poor	18 (34.0%)	4 (19.0%)
	Unknown	18 (34.0%)	1 (4.8%)
Tumor size	<3 cm	24 (34.3%)	15 (57.7%)
	≥3 cm	45 (64.3%)	6 (23.1)
	Unknown	1 (1.4%)	5 (19.2)
Nodal status	N0	23 (32.9%)	18 (69.3%)
	N1	12 (17.1%)	1 (3.8%)
	N2	23 (32.9%)	5 (19.2%)
	N3	12 (17.1%)	2 (7.7%)
Distant metastasis	M0	56 (80.0%)	23 (88.5%)
	M1	14 (20.0%)	3 (11.5%)
Histological type	AD[a]	29 (41.4%)	20 (77.0%)
	SCC[b]	21 (30.0%)	0 (0.0%)
	SCLC[c]	17 (24.3%)	5 (19.2%)
	LCNEC[d]	3 (4.3%)	1 (3.8%)

[a]Adenocarcinoma.
[b]Squamous cell carcinoma.
[c]Small cell lung carcinoma.
[d]Large cell neuroendocrine carcinoma.
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Materials and Methods

1. Cell lines

The A549 and LC-2/ad cells derived from lung AD were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and RIKEN BioResource Center (Ibaraki, Japan), respectively. The RERF-LC-AI cells derived from lung SCC was purchased from the RIKEN BioResource Center. The N231 cells derived from SCLC were purchased from the American Type Culture Collection (Rockville, MD, USA). LCN1, a large cell neuroendocrine carcinoma (LCNEC) line, was established in our laboratory [14]. These cells were grown in RPMI-1640 medium (SIGMA, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS; Biowest, Miami, FL, USA), 100 units/ml of penicillin, and 100 µg/ml of streptomycin (GIBCO, Auckland, New Zealand). After harvesting and washing twice with phosphate-buffered saline without divalent ions (PBS-), sub-confluent cells were stored at −80°C for proteomics analysis or fixed in 10% formalin and embedded in paraffin for immunohistochemistry. A549 cells were also AMeX-fixed [15] for immunohistochemical screening. The SP2/O-Ag14 cells derived from a mouse myeloma were purchased from the RIKEN BioResource Center, and were grown in RPMI-1640 medium supplemented with 1× 8-azaguanine (50× Hybri-Max, SIGMA), 10% FBS, penicillin, and streptomycin.

2. 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. All patients and healthy controls were approached based on approved ethical guidelines, and those who agreed to participate in this study were required to sign consent forms. Patients could refuse entry and discontinue participation at any time. All participants provided written consent.

2.1. Sera. Sera from 70 patients with lung cancer (AD: 29, SCC: 21, SCLC: 17, and LCNEC: 3) and 30 healthy controls were used in the training set. In addition, a validation set consisting of sera from 26 patients with lung cancer (AD: 20, SCLC: 5, and LCNEC: 1) and 30 healthy controls was also studied. The clinicopathological characteristics of the patients data are summarized in Table 1.

Patient sera were collected at Kitasato University Hospital, and healthy control sera were provided by Kyowa Medex Co., Ltd. (Tokyo, Japan) and kept at −80°C until use.

3. Generation of monoclonal antibodies

A549 cell lysate was prepared with PBS(-) using an ultra-sonic homogenizer (UH-50; SMT Company, Tokyo, Japan). Five-week-old female BALB/c mice were immunized intra-peritoneally with 50 mg wet- weight of A549 cell lysate in 500 µl of PBS(-) 3 times with a two-week interval. The antibody titer was tested by IHC using 100-times diluted sera from the immunized mice as the first antibody on AMeX-fixed A549 cells. Three days prior to cell fusion, the animal with the highest titer was intra-peritoneally boosted by the same amount of A549 lysate. Hybridoma preparation and IHC screening with AMeX-fixed A549 cells were previously described [4,5].

4. Proteomics analysis

4.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were extracted from each of A549, LC-2/ad, RERF-LC-AI, N231, and LCN1 cells with detergent lysis buffer [16] using an ultra-sonic homogenizer. Ten µg each of extracted proteins were boiled and separated by SDS-PAGE with 10% polyacrylamide gel at a constant current of 20 mA. After SDS-PAGE, proteins in gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Billerica, MA, USA) for immunoblotting.

4.2. Immunoblotting. Blotting membranes were blocked with 0.5% casein from bovine milk (Sigma, St. Louis, MO, USA) for 30 min at RT. The membranes were then reacted with non-

diluted hybridoma supernatant for 1 hr at RT, followed by incubation with 1,000-times diluted horseradish peroxidase-conjugated rabbit anti-mouse IgG polyclonal antibody (Dako, Glostrup, Denmark) with 0.025% Casein for 45 min at RT. Finally, signals were developed using Immobilon Western HRP reagent (Millipore Corp.).

4.3. Determination of antibody isotype. To determine the isotype of the established KU-Lu-5 antibody, we used the IsoStrip^{TM} Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

4.4. Immunoprecipitation. The immunoprecipitation method used in this study was previously described [17]. In brief, A549 cells were washed with PBS (-) and treated with radio-immunoprecipitation assay (RIPA) buffer containing Complete-mini EDTA-free (Roche Diagnostics) on ice for 30 min. After centrifugation at 15,000 rpm for 30 min at 4°C, the supernatant was collected and precleared with protein G sepharose (50% slurry) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) at 4°C overnight. To conjugate the primary antibody, 250 µL of primary antibody (KU-Lu-5 hybridoma supernatant) and 20 µL of protein G sepharose beads suspended in RIPA buffer were incubated with mixing at 4°C overnight. After centrifugation, the antibody-sepharose conjugate and 500 µg of total cellular protein from the precleared supernatant were incubated with mixing at 4°C for 4 hrs. The immunoprecipitates were collected by centrifugation at 15,000 rpm for 5 min at 4°C. After washing four times with RIPA buffer, the supernatant was carefully removed and the pellets were resuspended in 15 µL of 1×Laemmili's buffer. Then, 15 µL of samples were boiled and separated by SDS-PAGE with 10% polyacrylamide gel. After SDS-PAGE, gels were Zn-stained with the Negative Gel Stain MS kit (Wako Pure Chemical, Tokyo, Japan) according to the manufacturer's instructions.

4.5. Identification of antigen protein. *4.5.1. In-gel digestion.* The protein spot was excised from the SDS-PAGE gel and minced to 1 mm^3, destained with destaining solution (Wako Pure Chemical), dehydrated with 100% (v/v) ACN, and dried under vacuum conditions. Tryptic digestion was performed with a minimal volume of digestion solution which contained 20 ng/µl of trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI, USA) and 25 mM NH_4HCO_3 for 24 hrs at 37°C. After incubation, digested protein fragments eluted in solution were collected, and gels were washed once in 5% (v/v) trifuloroacetic acid /50% (v/v) ACN and collected in the same tube.

4.5.2. Protein identification. The collected peptide fragments were analyzed using autoflex III matrix-associated laser desorption/ ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS; Bruker Daltonik, Bremen, Germany). A disposable plate, spotted α-cyano-4-hydroxycinnamic acid matrix for samples, and PAC Peptide Calibstandard for calibration (Prespotted AnchorChip 96 set for Proteomics, Bruker Daltonik) were used. Peptide mass fingerprints (PMF) were measured, and then a few peaks obtained from PMF were further measured for their tandem mass spectra as parent masses. MASCOT (http://www.matrixscience.com) using the IPI Human database (93,289 sequences; 36,994,704 residues), released on 3 May, 2011 (http://www.matrixscience.com), was used to determine proteins from PMF and tandem mass data.

5. Immunoblot analysis with recombinant CAXII protein

Recombinant CAXII protein and Venus protein as a negative control with GST-tag were prepared using a wheat germ cell-free system [18]. Fourteen µg each of recombinant CAXII and Venus proteins were boiled and separated with SDS-PAGE, followed by immunoblotting with KU-Lu-5 antibody, as mentioned in 2.4.1.

6. Immunohistochemical staining

Three-µm-thick sections, made from 10% formalin-fixed and paraffin-embedded lung cancer cell lines and 37 surgically resected lung cancers (AD: 28, SCC: 9) were deparaffinized in xylene, rehydrated in a descending ethanol series, and then treated with 3% hydrogen peroxide for 20 min. After the antigen was retrieved by autoclaving in 0.01 mol/L citrate buffer (pH 6.0) with 0.1% Tween 20 at 121°C for 10 min, the sections were reacted with non-diluted KU-Lu-5 hybridoma supernatant for 16–18 hrs at room temperature (RT). After rinsing in TBS three times for 5 min each, the sections were reacted with ChemMate Envision reagent (Dako) for 30 min at RT. Finally, the sections were visualized with Stable DAB solution (Invitrogen Corp.) and counterstained with Mayer's hematoxylin.

7. Dot blot analysis

7.1. Sample preparation. *7.1.1. Removal of albumin and IgG from serum samples.* The removal of albumin and IgG from sera was performed using a ProteoExtract Albumin/IgG Removal kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. A 60-µL sample of each sera was diluted with 540 µL of binding buffer, and allowed to pass the column by gravity flow. The flow-through fraction was collected in a collection tube. To wash the column, binding buffer was allowed to pass the column by gravity flow. The flow-through fraction was collected in the same collection tube.

7.1.2. Desalting and concentration by ultrafiltration. The albumin- and IgG-depleted samples were buffer-exchanged and concentrated using 10-kDa molecular-weight cut-off ultra-filtration VIVASPIN 2 (Sartorius, Gottingen, Germany). The samples were centrifuged at 6,000×g at 4°C until less than 100 µL, and then the buffer was exchanged for PBS (-) with concentration at 6,000×g at 4°C until concentrated to less than 50 µL. The concentrated samples were adjusted to a final volume of 60 µL with PBS (-).

7.2. Dot blot analysis. One µl each of albumin- and IgG-depleted samples diluted to 1:20 with PBS(-) and mouse IgG (purified in our laboratory) for a positive control were spotted on a PVDF membrane (Millipore Corp.) using the automatic dot blot system with a 256-solid pin configuration (Kakengeneqs Inc., Chiba, Japan). Two sheets of membrane were prepared for one set of experiment. Spotted membranes were washed in TBS for 10 min, and blocked with 0.5% casein (Sigma) for 1 hr at RT. One membrane was then reacted with non-diluted KU-Lu-5 hybridoma supernatant, and the other membrane was reacted with antibody diluting solution [20-times diluted 0.5% casein with 0.1% Tween 20 added TBS (TBS-T)] for 30 min at RT. After rinsing in TBS-T 3 times for 5 min each, membranes were incubated with 1,000-times diluted horseradish peroxidase-conjugated rabbit anti-mouse IgG polyclonal antibody (Dako) for 30 min at RT. Finally, signals were developed with Immobilon Western reagent (Millipore Corp.). The data were analyzed using DotBlotChipSystem Ver. 4.0 (Dynacom Co., Ltd., Chiba, Japan). Each normalized signal was presented as the ratio of the positive intensity versus the negative background intensity.

8. Statistical analysis

Serum CAXII levels in patients with lung cancer and healthy controls were statistically analyzed using the Mann-Whitney *U*-test. Sensitivity, specificity, and predictive values were calculated with the SPBS software package (Ver. 9.42 for Windows) for each variable at a corresponding cut-off. Discriminant function analysis

was performed to classify patients in the "lung cancer" vs. "healthy control" group, according to the status of the biomarkers, using the SPBS software package. The area under the curve (AUC) and best cut-off point were calculated employing receiver operating characteristic (ROC) analysis. Results were considered significant when $P<0.05$.

Results

1. Confirmation of antibody titer in mouse sera

The antibody titer was tested by IHC with 1,000-times diluted sera of immunized mice as the first antibody on AMeX-fixed A549 cells. As a result, the sera from immunized mice contained antibodies that reacted with various components of A549 cells.

Using AMeX-fixed A549 cell preparations for the immunohistochemical screening of hybridomas, we finally established 188 MoAbs in total and a further study was performed with the KU-Lu-5 clone, which showed intense staining in A549 cells (Fig. 1 A).

2. Identification of antigen protein

In order to identify the antigen protein recognized by the KU-Lu-5 antibody, we performed IP with lysate from A549 cells. The results of IP are shown in Fig. 1 B, C. The antigenic protein was observed at roughly 40 kDa. To determine the antigenic protein recognized by KU-Lu-5 antibody, we excised and collected the spot from the Zn-stained gel, and proceeded with in-gel digestion. After analysis employing a MALDI-TOF/TOF MS and a MASCOT search, the protein was determined as isoform 2 of carbonic anhydrase XII (CAXII, accession: IPI00221392), which is composed of 343 amino acids with a predicted M.W. of 38,384 Da. The result was confirmed by immunoblot analysis with recombinant CAXII protein using KU-Lu-5 hybridoma supernatant as the first antibody (Fig. 1 D). The immunoglobulin isotype of KU-Lu-5 antibody was determined as IgG_1, κ.

3. Immunoblot analysis

Expression of CAXII was detected only in A549 cells as a roughly 40-kDa protein, and no clear band was detected in other cells used in this study (Fig. 2 A).

4. Immunohistochemical staining for CAXII

Immunohistochemically, membranous expression of CAXII was observed only in A549 cells (Fig. 2 B). Membranous staining was detected in 2 of the 28 ADs (7.1%) and in 2 of the 9 SCCs (22.2%) (Fig. 2 C, D).

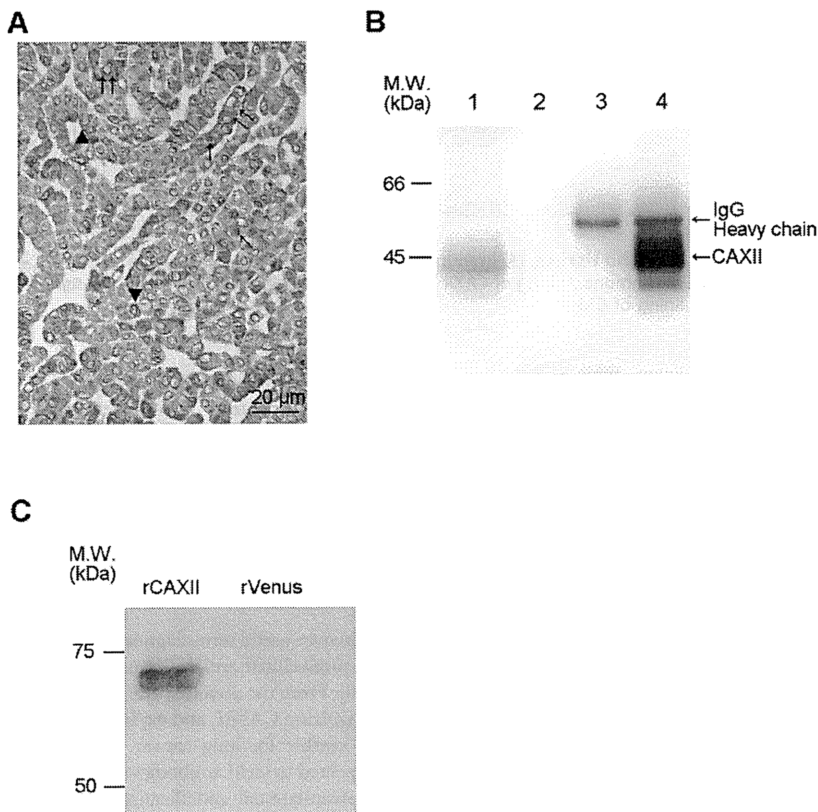

Figure 1. Production of anti-CAXII monoclonal antibody and its antigen identification. (A) The antibody titer was tested immunohistochemically using 1,000-times diluted sera of immunized mice as the first antibody on AMeX-fixed A549 cells, which were used as an immunogen. The sera of immunized mice contained antibodies that reacted with various cell components, such as the nucleus (↑), plasma membrane (▲), and cytoplasm (↑ ↑). (B) Immunoprecipitation with KU-Lu-5 antibody. Immunoblot analysis using KU-Lu-5 hybridoma supernatant as the first antibody [lane 1: A549 lysate, lane 2: A549 lysate combined with protein G, lane 3: KU-Lu-5 antibody combined with protein G, lane 4: A549 lysate combined with KU-Lu-5 antibody]. Lanes 2 to 3 are negative controls, and immunoprecipitated product with KU-Lu-5 antibody was detected in lane 4 (↑). (C) Confirmation of identified antigen protein. KU-Lu-5 antibody reacted with recombinant CAXII protein (64 kDa), but not with recombinant Venus protein.
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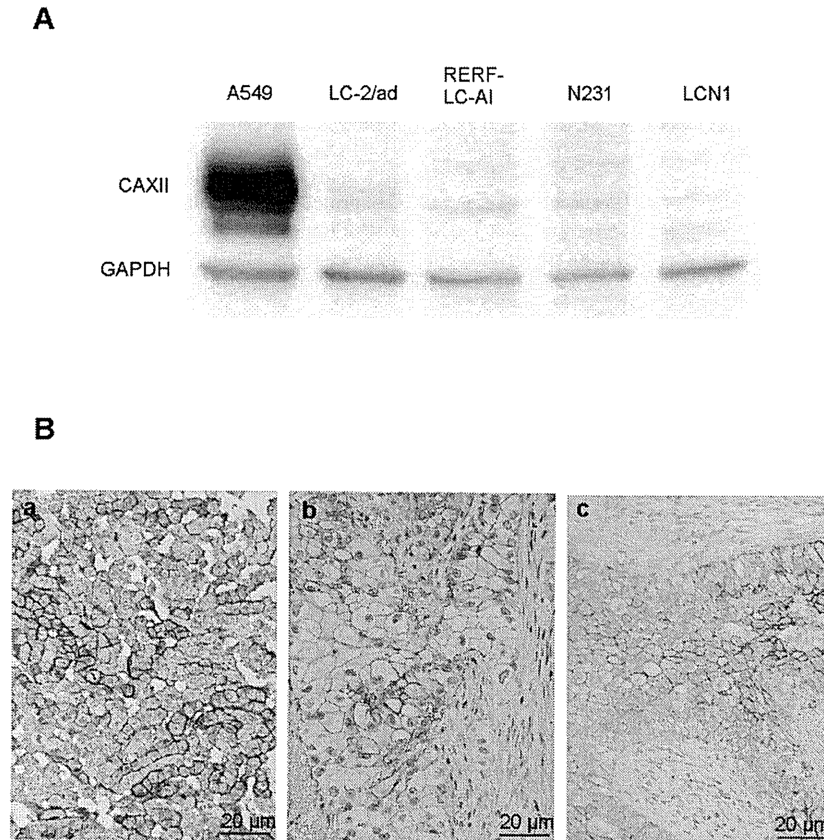

Figure 2. Expression of CAXII antibody in lung cancer cell lines and tissues. (A) Immunoblot analysis of CAXII in lung cancer cell lines. CAXII was detected as an approximately 40-kDa protein with A549 cells. (B) Immunostaining of CAXII in A549 cells (a), adenocarcinoma (b), and squamous cell carcinoma (c) of the lung, and each showed membranous staining of CAXII.
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5. Serum CAXII in patients with lung cancer

The serum CAXII levels were significantly higher in lung cancer patients than in healthy controls in the training set ($P<0.0001$). Relative values of serum CAXII levels ranged from 0.101 to 4.01 (median: 1.520) in lung cancer patients, but 0.006 to 1.679 (median: 0.290) in healthy controls (Fig. 3 A). In lung cancer, CAXII serum levels of SCC patients were significantly higher than those of AD patients ($P=0.03$) (Fig. 3 A). The area under the ROC curve (AUC) between lung cancers and healthy controls was 0.794 (Fig. 3 B). When an optimal cut-off value of 0.387 for CAXII was applied, the diagnostic sensitivity and specificity for lung cancer were 82.9 and 70.0, respectively, and the negative and positive predictive values were 0.617 and 0.863, respectively. Furthermore, within SCCs, serum CAXII levels were significantly higher in patients with well- and moderately differentiated tumors than those with poorly differentiated ones ($P=0.027$) (Fig. 4 A), and tended to be higher in patients with a tumor size of less than 3 cm rather than more than 3 cm ($P=0.0538$). However, there was no difference in the smoking history of patients (Fig. 4 B). CAXII levels in stage I, II, and III ADs were 1.501, 0.704, and 1.001, respectively, and CAXII levels in stage I, II, and III SCCs were 1.764, 2.093, and 1.854, respectively. These data were summarized in Table 2. No relations between the CAXII serum levels and tumor stage or presence of metastasis were identified for either ADs or SCCs. To further confirm the utility of serum CAXII levels as a sero-diagnostic marker, 56 additional samples of sera were analyzed by dot blot analysis as a validation study. The serum CAXII levels were also significantly higher in lung cancer patients than in healthy controls in the validation set ($P=0.030$). Relative values of serum CAXII levels ranged from 0.000 to 8.023 (median: 3.921) in lung cancer patients, but 0.000 to 8.331 (median: 2.806) in healthy controls (Fig. 5). When an optimal cut-off value of 3.086 for applied, the diagnostic sensitivity and specificity for lung cancer were 65.4 and 70.0, respectively.

Discussion

In this study, aiming to discover useful sero-diagnostic markers for lung cancer, we generated monoclonal antibodies using lung AD-derived A549 cells as antigens. From the obtained 188 antibodies, we focused on an antibody recognizing CAXII, and explored its clinical utility as a sero-diagnostic marker for lung cancer. This random immunization method is expected to yield antibodies against tumor-specific proteins with post-translational modifications, which are difficult to obtain by conventional immunization methods. Actually, several authors have reported that monoclonal antibodies generated by this method are useful as diagnostic and prognostic markers for cancers [5,17,19]. Battke *et al.* [20] established a 6A10 antibody recognizing CAXII using a similar immunization methodology. However, the obtained antibodies were limited to those only reacting with cell surface antigens because of using flow cytometry for the screening of hybridomas. In the present study, the hybridomas were immunohistochemically screened which facilitated the obtaining of

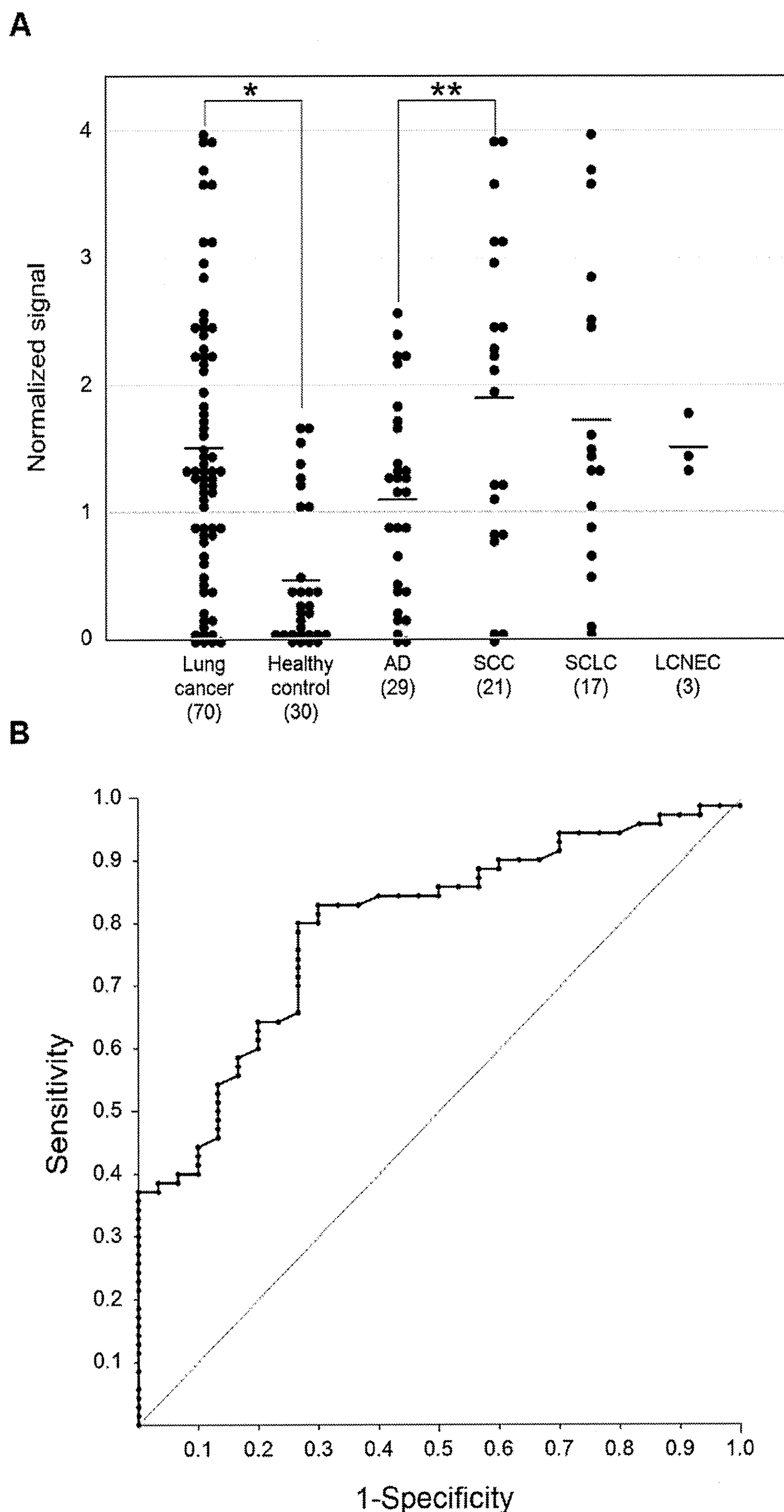

Figure 3. Serum CAXII levels in patients with lung cancer and healthy controls in the training set. Serum CAXII levels in patients with lung cancer and healthy controls. (A) The median CAXII level in the sera from healthy controls was 0.29, and that in sera from lung cancer patients was 1.52. Serum CAXII levels were significantly higher in lung cancer patients (*P<0.001). Furthermore, serum CAXII levels were higher in SCCs than ADs (**P = 0.0381). (B) Receiver-operating characteristic curve analysis of CAXII as a serum marker for lung cancer. The corresponding areas under the curves were 0.794 for CAXII. With a 70.0% specificity, the sensitivity of CAXII for lung cancer was 82.9%, at a cut-off value corresponding to 0.387.
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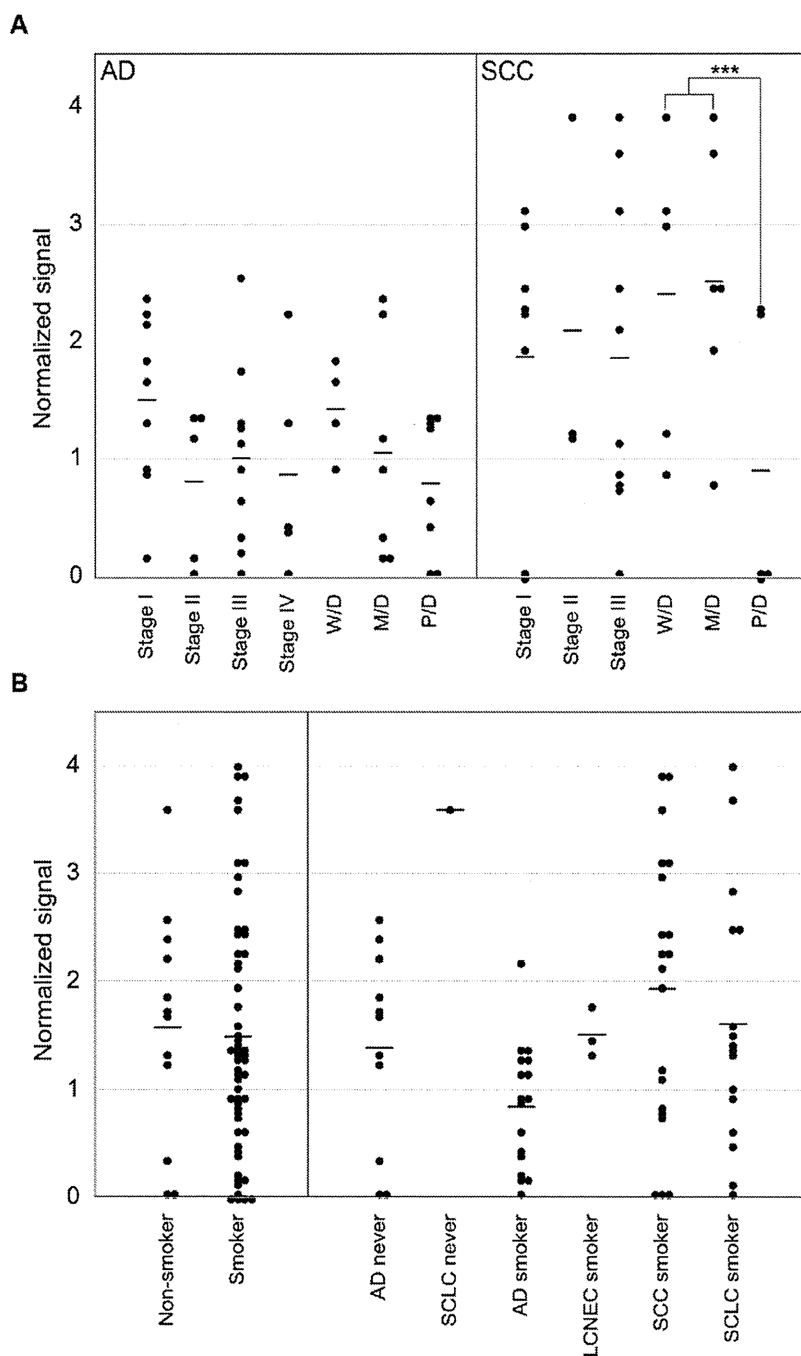

Figure 4. Correlation between serum CAXII levels and patients' clinicopathological characteristics. (A) CAXII levels in sera from patients with ADs and SCCs with a focus on the stage and differentiation. In SCCs, CAXII levels were significantly higher in well- and moderately differentiated tumors than in poorly differentiated ones (***P = 0.0272). In ADs, no significant difference based on the differentiation extent was detected. (B) Smoking history in lung cancer patients. The median CAXII level in the sera from non-smokers was 1.56, and that in smokers was 1.54, showing no significant difference.
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antibodies reacting with tumor-associated proteins localized in several intra-cellular compartments. The CAs constitute a family of ubiquitous enzymes with important roles in many physiological and pathological processes which reversely catalyse the conversion of CO_2+H_2O to HCO_3^- and H^+, contributing to regulation of the intracellular pH [6–11,19]. Several clinical studies have shown a clear relationship between high CAXII expression levels in tumor cells and a favorable prognosis.

Table 2. Serum CAXII levels in ADs and SCCs.

		Average value (AD)	Average value (SCC)
Stage	I	1.501	1.764
	II	0.704	2.093
	III	1.001	1.854
	IV	0.654	0.000
Tumor differentiation	Well	1.424	2.403
	Moderate	1.046	2.511
	Poor	0.727	0.742

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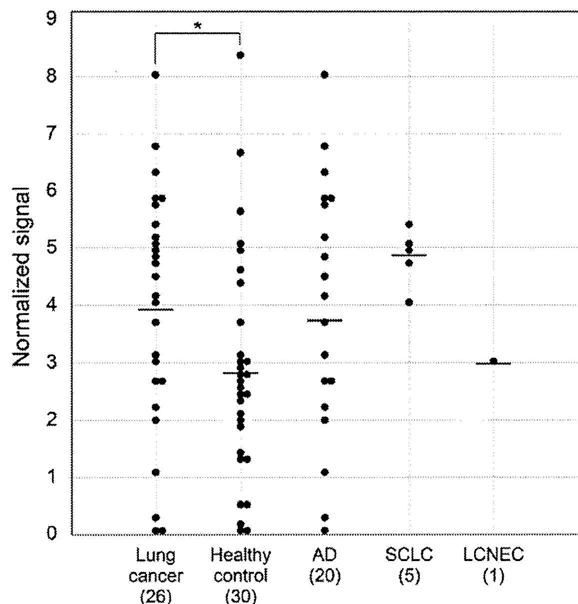

Figure 5. Serum CAXII levels in patients with lung cancer and healthy controls in the validation set. To confirm the utility of serum CAXII levels as a sero-diagnostic marker, 56 additional sera were analyzed by dot blot analysis as a validation study. The serum CAXII levels were also significantly higher in lung cancer patients than in healthy controls (P = 0.030). Relative values of serum CAXII levels ranged from 0.000 to 8.023 (median: 3.921) in lung cancer patients, but 0.000 to 8.331 (median: 2.806) in healthy controls.
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Watson *et al.* [12] reported that CAXII was expressed in 75% of invasive breast carcinoma cases, and was significantly associated with a lower histological grade (P = 0.001), positive estrogen receptor status (P<0.01), and negative epidermal growth factor receptor overexpression (P<0.001). Using univariate analysis, CAXII-positive tumors were associated with a lower relapse rate (P = 0.04) and a better OS (P = 0.01). On the other hand, although 98% of astrocytomas were immunohistochemically positive for CAXII, higher CAXII expression levels were correlated with a higher histological grade and a poorer patient outcome either by univariate (P = 0.010) or multivariate (P = 0.039) survival analysis [9].

An immunohistochemical study of the expression of CAXII in lung cancer was reported by Ilie *et al.* [13]. CAXII overexpression was observed in 105/555 cases, and was significantly associated with a better differentiation (P = 0.015) and SCC histological type (P<0.001). Furthermore, high CAXII expression was also significantly correlated with better overall and disease-specific survival. From our results, CAXII levels were higher in sera from SCC patients than ADs (Fig. 3 A). Also, they correlated more favorably with differentiation (Fig. 4 A). Integrating these results, CAXII may not only be a candidate tissue marker, but also a sero-diagnostic marker for lung cancer.

Although the association of CAXII expression and clinicopathologic factors and patient outcome in different tumors has been reported, to our knowledge, no study concerning the serum CAXII protein levels or its autoantibody levels in patients with tumors has been reported.

To confirm the possibility of CAXII as a sero-diagnostic marker, we measured its serum levels in patients with lung cancer and healthy controls. We demonstrated that the CAXII protein flowed out into the sera and its 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 = 0.030). It is possible that the gap in the P-value between the training set and validation set is caused by the fact that serum levels of CAXII of SCC patients were generally higher than those of patients with other histologies, and the validation set included no SCC case. Taken together, the serum CAXII levels should be applicable markers discriminating lung cancer patients from healthy controls. Currently, CT scan or chest X-ray is the main method of lung cancer screening [21]. Mazzone et al. suggested that blood and breath tests should also be included for lung cancer screening, because they are both easy to perform and free of risks related to test administration [21,22]. In this study, we analyzed CAXII levels in sera from lung cancer patients and healthy controls using monoclonal antibody, and our results suggested that the serum CAXII level was a useful sero-diagnostic marker for lung cancer.

Author Contributions

Conceived and designed the experiments: MK TM Y. Sato. Performed the experiments: MK KY RN TM. Analyzed the data: MK TM SR. Contributed reagents/materials/analysis tools: SR YK NG SXJ MS AI Y. Satoh NM. Wrote the paper: MK TM RN Y. Sato.

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RESEARCH COMMUNICATION

HADHA is a Potential Predictor of Response to Platinum-based Chemotherapy for Lung Cancer

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Abstract

To identify a cisplatin resistance predictor to reduce or prevent unnecessary side effects, we firstly established four cisplatin-resistant sub-lines and compared their protein profiles with cisplatin-sensitive parent lung cancer cell lines using two-dimensional gel electrophoresis. Between the cisplatin-resistant and -sensitive cells, a total of 359 protein spots were differently expressed (>1.5 fold), and 217 proteins (83.0%) were identified. We focused on a mitochondrial protein, hydroxyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase alpha subunit (HADHA), which was increased in all cisplatin-resistant cells. Furthermore, pre-treated biopsy specimens taken from patients who showed resistance to platinum-based treatment showed a significantly higher positive rate for HADHA in all cases (p=0.00367), including non-small cell lung carcinomas (p=0.002), small-cell lung carcinomas (p=0.038), and adenocarcinomas (p=0.008). These results suggest that the expression of HADHA may be a useful marker to predict resistance to platinum-based chemotherapy in patients with lung cancer.

Keywords: Cisplatin - HADHA - lung cancer - two-dimensional gel electrophoresis

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Introduction

Lung cancer is the leading cause of cancer-related death in the world, and the five-year overall survival rate is still below 16% (Jemal et al., 2009). Chemotherapy and radiotherapy are playing an important role in the management of lung cancer. A reason for the poor survival rate is that lung cancer tends to acquire resistance to anti-cancer drugs, and clinical drug resistance to platinum-based chemotherapy is considered to be a major impediment to the treatment of lung cancer.

Cis-diamino-dichloroplatinum (II) (cisplatin) is a common therapeutic agent used for chemotherapy in various cancers including lung cancer. Cisplatin is a cytotoxic compound, which inhibits transcription and DNA replication and induces apoptosis (Gonzalez et al., 2001). For lung cancer patients, cisplatin was found to be more effective than radiotherapy, and the combination of cisplatin and vinorelbinn improved survival (Pepe et al., 2007). Furthermore, cisplatin improved the survival rate in patients older than 65 years with acceptable toxicity (Pepe et al., 2007). The overcoming of cisplatin resistance may save more patients. Although it was reported that cisplatin resistance rose due to a decrease of blood flow in the tumor and increased DNA repair (Stewart, 2007), the mechanisms underlying cisplatin resistance have not yet been clarified, and an effective cisplatin resistance prediction marker has not been identified. Thus, the identification of markers predicting cisplatin resistance would improve patients' quality of life by avoiding unnecessary side effects.

Some studies have demonstrated predictive markers of resistance to chemotherapy employing proteomics methods (Urbani et al., 2005; Aggarwal et al., 2009; Cicchillitti et al., 2009; Michele et al., 2009; ChengJ et al., 2010; Lee et al., 2010). In most reports, the cells that survived after culturing with chemotherapeutic treatment for 24–72 h were used as the drug-resistant cell lines. However, these cells were not exactly resistant to the drug because the short-term cultured cells were not stable and most were eliminated on long-term culture with the drug. In this report, we established four lung cancer sub-lines: A549cis, LC2Adcis, LCN1cis, and LCN2cis, which were resistant and stably grew in medium supplemented with cisplatin at a concentration of 3,200 ng/ml for over 12 months. The protein expression of

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these four cisplatin resistant sub-lines was compared with that of their parent cell lines: LCN1, LCN2, A549, and LC2Ad, by two-dimensional gel electrophoresis (2DE). We identified 217 different proteins that were differently expressed more than 1.5-fold. We found a marked increase in the expression of hydroxyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase alpha subunit (HADHA) in the cisplatin-resistant sub-lines. HADHA also increased in lung cancers of cisplatin-resistant patients. It has been reported that HADHA is a factor that inhibits the effect of nonsteroidal anti-inflammatory drugs against colorectal cancer growth (Baldwin G S et al., 1998). HADHA is also expressed at a higher level in non-metastatic breast than in metastatic cancer (Xu X et al., 2010), and a decrease of HADHA was observed in hepatocellular cancer compared to non-neoplastic controls in hepatitis B virus-associated hepatocellular cancer patients (Kim S Y et al., 2009). In this study, we provide evidence that HADHA may be a useful marker of the response of lung cancer to cisplatin.

Materials and Methods

Cell lines

The cell lines were pulmonary large cell neuroendocrine carcinomas (LCNEC) (LCN1 and LCN2) and adenocarcinomas (AD) (A549 and LC2Ad). LCN1 and LCN2 were established in our laboratory (Jiang SX and et al., 2004). A549 was purchased from the American Type Culture Collection (Rockville, MD, USA). LC2/Ad was purchased from the RIKEN BioResource Center (Ibaraki, Japan). All cell lines were grown in RPMI1640 supplemented with 10% fetal bovine serum (Biowest, Miami, FL, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Harvested cells were washed with phosphate-buffered saline and stored at –80°C.

Cisplatin-resistant sub-lines

Cisplatin-resistant sub-lines (LCN1cis, LCN2cis, A549cis, and LC2Adcis) were established by culturing the above four cell lines for 6 months with cisplatin (Randa inj., Nippon Kayaku Co., Ltd., Tokyo, Japan), starting from a concentration of 25 to 3,200 ng/ml. All cisplatin-resistant sub-lines were stably grown at a concentration of 3,200 ng/ml cisplatin for over 12 months in our laboratory.

Tissues

Biopsy samples from 46 patients with lung cancer at Kitasato University Hospital were used in this study. They were divided into 31 ADs, 5 squamous cell carcinomas (SCCs), and 10 small cell lung carcinomas (SCLCs). All 46 patients were treated with platinum-based chemotherapy after the biopsy samples were taken. The total number of patients undergoing cisplatin-based chemotherapy was 30 and, of these 30 patients, 22 patients were treated with gemcitabine, 7 patients were treated with irinotecan, and 1 patient was treated with etoposide. The remaining 16 patients were treated with carboplatin-based chemotherapy: 14 patients were treated with paclitaxel, and 2 patients were treated with etoposide. The responses to chemotherapy were assessed by RECIST (version 1.1): 16 patients were assessed as showing a partial response (PR), 16 patients were assessed as showing stable disease (SD), and 14 patients were assessed as showing progressive disease (PD). There were no patient with a complete response (CR). Three ADs and two SCCs that were surgically resected were also used.

This study was approved by the Ethics Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave consent to donate their samples.

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. All patients and all healthy controls were approached using approved ethical guidelines and those who agreed to participate in this study, were required to sign consent forms. Patients could refuse entry and discontinue participation at any time. All participants provided written consent.

Agarose two-dimensional gel electrophoresis (2DE)

Cell lines were solubilized by an ultra-sonic homogenizer (UT-50; SMT Company, Tokyo, Japan) in 7 M urea containing 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylam monio]propanesulfonic acid, 10 mM tris (2-carboxyethyl)phosphine hydrochloride, and 2.5% pharmalyte, pH 3-10 (GE Healthcare, Piscataway, NJ, USA), and they were centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was alkylated with 1/20 volumes of 400 mM 4-vinylpyridine for 1 h, and then excess 4-vinylpyridine was quenched with the same volume of 400 mM dithiothreitol. After being centrifuged at 15,000 rpm for 30 min at 4°C, interfering components were removed with a 2-D Clean-up kit (GE Healthcare) according to the manufacturer's instructions. After being centrifuged at 50,000 rpm for 30 min at 4°C, protein in each sample was quantified employing Bio-Rad Protein Assay solution (Bio-Rad Laboratories, Hercules CA, USA). The agarose 2DE method (Oh-Ishi M et al., 2000; Nagashio R et al., 2010) was used with some modifications for this study. The first-dimensional agarose isoelectric focusing gel (105 mm in length and 2.5 mm in inner diameter) was made with pharmalyte, pH 3-10 (GE Healthcare). The second-dimension separation was achieved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with 12% polyacrylamide gel (16x16 cm, N-1111, NIHON EIDO Corp., Tokyo, Japan). The extracted protein (280 μg) was applied at the cathodic end of an agarose IEF gel, and loaded at 4°C in stepwise voltages (100 V for 9.3 h, 300 V for 15 min, 500 V for 15 min, 700 V for 1 h, 900 V for 4 h, and 150 V for 4 h). After fixation of the gel in 10% trichloroacetic acid and 5% sulfosalicylic acid, it was washed in distilled water. Each agarose gel was placed on the top of a second dimensional SDS-PAGE gel, and loaded with a constant current at 25 mA. After 2DE, the proteins were visualized by CBB PhastGel Blue R (Amersham Pharmacia Biotech, Uppsala, Sweden)

staining. Each agarose 2DE was performed in triplicate. The stained gels were scanned using a high-resolution scanner (GT-9800; Epson, Tokyo, Japan). Stained spots were merged and analyzed using the Prodigy SameSpots software (Nonlinear Dynamics, Newcastle, UK). Spots with 1.5-fold differences between cisplatin-sensitive and -resistant cells were selected as differentially expressed spots.

In-gel digestion

The spot was excised from the 2DE gel, destained with 50 mM ammonium hydrogen carbonate containing 50% acetonitrile, dehydrated with 100% acetonitrile, and dried under vacuum conditions. Tryptic digestion was performed in 25 mM ammonium hydrogen carbonate with 20 ng/μl trypsin for 24 h at 37°C (Trypsin Gold, Mass Spectrometry Grade; Promega, Madison, WI, USA). After digestion, digested peptides were collected from the solution. The gel was washed again with 50% acetonitrile plus 5% trifluoroacetic acid, and the extract was added to the same tube.

Protein identification

Tryptic peptides were spotted on a Prespotted AnchorChip 96 set for proteomics (Bruker Daltonik GmbH, Bremen, Germany) according to the manufacturer's recommendations. MS spectra were analyzed in an Autoflex III TOF/TOF (Bruker Daltonik GmbH) in reflector mode by summarizing 1,000 signal spectra (5×200) with a 50-Hz laser in the mass range from 580 to 4,000 Da applying the following instrument settings: ion source 1, 19.00 kV; ion source 2, 16.60 kV; lens, 8.55 kV; reflector 1, 21.00 kV; reflector 2, 9.70 kV; reflector detector, 1,400 V; suppression up to 500 Da by deflection. Then, MS/MS spectra of tryptic peptides were further measured in MS/MS mode using the following instrument settings: ion source 1, 6.00 kV; ion source 2, 5.30 kV; lens, 3.00 kV; reflector 1, 27.00 kV; reflector 2, 11.65 kV; lift 1, 19.00 kV; lift 2, 4.20 kV; reflector detector, 1,400 V. Fragment ion spectra from MS and MS/MS were submitted to MASCOT (http://www.matrixscience.com/) for a database search. The corresponding proteins were identified from the following database: IPI human 20081114 (74,049 sequences, 31,194,560 residues; http://www.ebi.ac.uk/IPI/IPIhuman.html/).

Western blotting (WB)

The cells were lysed by an ultra-sonic homogenizer on ice in 62.5 mM Tris-buffer (pH 6.8) containing 2% SDS, 0.001% bromophenol blue, 5% 2-mercaptoethanol, 10% glycerol, and 1 mM phenylmethyl-sulfonyl fluoride. The extracted proteins (10 μg) were boiled and separated by 1-dimensional 10% SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After being blocked with 0.5% casein for 40 min at room temperature (RT), the membrane was reacted for 2 h at RT with 800-times diluted rabbit anti-human HADHA polyclonal antibody (Sigma-Aldrich, Steinheim, Germany). After washing 3 times for 5 min each with 10 mM Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated with 1,000-times-diluted horseradish peroxidase conjugated goat anti-rabbit Ig polyclonal antibody for 30 min at RT (Dako, Glostrup, Denmark). The bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Immunohistochemical staining (IHC)

Three-μm-thick sections of formalin-fixed and paraffin-embedded lung cancer tissues or cell preparations were deparaffinized in xylene and rehydrated in a descending ethanol series, and then treated with 3% hydrogen peroxide for 20 min. The antigen was retrieved by autoclaving in 10 mM citrate buffer (pH 6.0) with 0.1% Tween-20 for 10 min at 121°C. After blocking with 0.5% casein for 10 min, the sections were reacted with 200-times-diluted rabbit anti-human HADHA polyclonal antibody (Sigma-Aldrich) for 18 h at RT. After washing in 10 mM Tris-buffered saline, the sections were reacted with ChamMate ENVISION reagent (Dako) for 30 min at RT. Finally, the sections were visualized using Stable DAB solution (Invitrogen, Carlsbad, CA, USA) and counterstained with Mayer's hematoxylin.

Evaluation of IHC

The HADHA staining intensity was scored as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), and 3+ (strongly positive). At least moderately positive (2+) tumor cells were considered as HADHA-positive. The 2 x 2 chi square test was used for the statistical evaluation of IHC data. $P < 0.05$ was considered to show a significant difference.

Results

Comparison of the protein expression profile between cisplatin-resistant sub-lines and their parent lines

To investigate the cisplatin resistance marker, we established four lung cancer sub-lines (A549cis, LC2Adcis, LCN1cis, and LCN2cis) that were resistant to 3,200 ng/ml of cisplatin. These cell lines grew with a doubling time similar to their parent lines (data not shown). Figure 1A is a 2DE protein map of LCN2 and LCN2cis. More than 1,500 protein spots were separated on 2DE, and the circled spots were defined as up-regulated (>1.5-fold ratio of means) in comparison with spots of the counterpart cell lines. The results are summarized in Table 1. A total of 359 differentially expressed spots were revealed from the four groups of paired cell lines. Of the 359 spots, 298 (83%) proteins were identified, and tryptic digestion and mass spectrum analysis revealed 217 different proteins.

Table 1. Summary of Agarose 2DE Analysis

Number of differentially expressed spots compared with parent lines		
Cisplatin-resistant cells	Up-regulated	Down-regulated
LCN1cis	54	33
LCN2cis	55	39
A549cis	69	60
LC2Adcis	28	21
Total	206	153

A total of 359 spots were analyzed by TOF-MS, and 298 (83%) spots were identified. By tryptic digestion and mass spectrum analysis, 217 different proteins were revealed

Table 2. Proteins Showing Similar Changes in More Than Two Paired Cell Lines

Accession number	Gene symbol	Protein name	Localization	Function	Expression in cisplatin-resistant cells (fold)
IPI00029733	AKR1C1	Aldo-keto reductase family member C1	Cytoplasm	Enzyme(Reductase)	LC2Adcis 2.7 LCN2cis 3.2
IPI00005668	AKR1C2	Aldo-keto reductase family 1 member C2	Cytoplasm	Enzyme (Dehydrogenase)	A549cis 2.5 LC2Adcis 2.8
IPI00218918	ANXA1	Annexin A1	Plasma membrane	Calcium-binding protein	A549cis 2.7 LCN2cis 1.6
IPI00295386	CBR1	Carbonyl reductase (NADPH) 1	Cytoplasm	Enzyme: Oxidoreductase	A549cis 1.5 LCN1cis 1.8 LCN2cis 2.3
IPI00027626	CCT6A	T-complex protein 1 subunit zeta	Cytoplasm	Chaperone	A549 0.63 LCN2 0.63
IPI00908424	CDC2	Cell division cycle 2 isoform 3	Cytoplasm	Serine/ threonine kinase	LC2Adcis 0.63 LCN1cis 0.59
IPI00015947	DNAJB1 (HSP40)	DnaJ homolog subfamily B member 1	Cytoplasm	Heat shock protein	A549cis 1.5 LCN2cis 1.5
IPI00843975	EZR	Ezrin	Cytoplasm	Anchor protein	A549cis 0.59 LCN2cis 0.59
IPI00219757	GSTP1	Glutathione S-transferase P	Cytoplasm	Enzyme: Glutathione transferase	LC2Adcis 1.5 LCN2cis 1.5
IPI00031522	HADHA	Hydroxyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase alpha subunit	Mitochondrion	Enzyme: Dehydrogenase	LCN1cis 1.5 LCN2cis 2.0 A549cis 1.5 LC2Adcis 2.5
IPI00396378	HNRNPA2B1	Isoform B1 of heterogeneous nuclear ribonucleoproteins A2/B1	Nucleus	Ribonucleoprotein	LC2Adcis 0.67 LCN2cis 0.67
IPI00216592	HNRNPC	Isoform C1 of heterogeneous nuclear ribonucleoproteins C1/C2	Nucleus	RNA-binding protein	LC2Adcis 0.63 LCN1cis 0.53
IPI00220327	KRT1	Keratin, type 2 cytoskeletal 1	Plasma membrane	Structural protein	A549cis 0.53 LCN1cis 0.63
IPI00444262	NCL	Nucleolin	Nucleolus	RNA-binding protein	A549cis 5.3 LC2Adcis 1.5 LCN1cis 4.8 LCN2cis 2.4
IPI00025252	PDIA3	Protein disulfide-isomerase A3	Endoplasmic reticulum	Enzyme: Isomerase	LCN1cis 0.67 LCN2cis 0.67
IPI00639981	PFKP	Phosphofructokinase, platelet	Cytoplasm	Enzyme: Phosphotransferase	A549cis 0.40 LCN1cis 0.63
IPI00000874	PRDX1	Peroxiredoxin-1	Cytoplasm	Enzyme: Peroxidase	A549cis 2.4 LC2Adcis 1.6 LCN1cis 1.5
IPI00010201	PSMD8	proteasome 26S non-ATPase subunit 8	Cytoplasm	Ubiquitin proteosome system protein	A549cis 2.4 LCN1cis 1.8
IPI00000494	RPL5	60S ribosomal protein L5	Ribosome	Ribosomal subunit	LCN1cis 0.63 LCN2cis 0.35
IPI00008530	RPLP0	60S acidic ribosomal protein P0	Ribosome	Ribosomal subunit	A549cis 2.6 LCN1cis 2.3
IPI00427330	SBDS	Ribosome maturation protein SBDS	Nucleolus	Unclassified	A549cis 1.5 LCN2cis 1.7
IPI00893645	SEPT2	Putative uncharcterized protein SEPT2	Cytoplasm	GTPase	A549cis 0.38 LCN1cis 0.59
IPI00140420	SND1	Staphylococcal nuclease domain-containing protein 1	Nucleus	Transcription regulatory protein	A549cis 0.38 LCN2cis 0.59
IPI00031420	UGDH	UDP-glucose 6-dehydrogenase	Unknown	Enzyme: Dehydrogenase	A549cis 1.5 LC2Adcis 1.9
IPI00418471	VIM	Vimentin	Intermediate filament	Cytoskeletal protein	A549cis 2.2 LC2Adcis 2.0 LCN2cis 2.5

About 50% of the identified proteins were cytoplasmic proteins, and 41% of the proteins function in metabolism. From the 217 identified proteins, we picked up 25 proteins that showed similar up- or down-regulation in more than two paired cell lines (Table 2). From the 25 proteins, we focused on HADHA, a mitochondrial protein, because it was over-expressed in all cisplatin-resistant sub-lines and mitochondria play an important role in cisplatin sensitivity. Compared with their parent lines, the fold up-regulation of HADHA in cisplatin-resistant cells was: LCN1cis, 1.5-

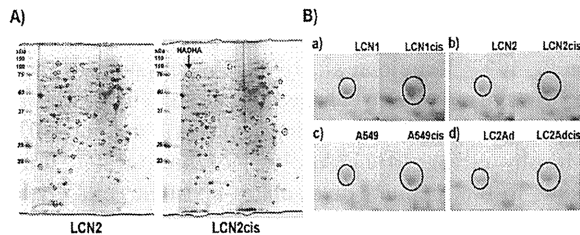

Figure 1. Comparison of Cellular Proteins of Cisplatin-Resistant Cells and Parent Cells on Agarose 2DE. Similar experiments were repeated 3 times, and representative results are shown. A, 2DE maps of cellular proteins of LCN2 and cisplatin-resistant LCN2cis. LCN2cis were cultured with 3,200 ng/ml of cisplatin. Each sample (280 μg) was resolved with agarose 2DE and stained with coomassie blue. Circled spots are increased proteins (>1.5 fold) either in cisplatin-resistant cells or in parent lines. HADHA increased about 2.0-fold in LCN2cis cells compared with LCN2. B, HADHA spots in cisplatin-resistant cells and parent lines on agarose 2DE. HADHA spots of cisplatin-resistant cells and those of parent lines—LCN1 (a), LCN2 (b), A549 (c), and LC2Ad (d)—are shown as circles

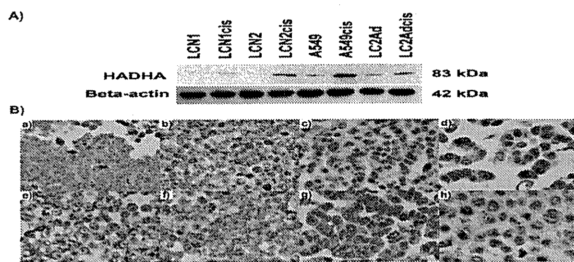

Figure 2. Expression of HADHA in Cisplatin-resistant Cells and their Parent Cells. A, WB of HADHA in the cell lines. Each sample (10 μg) was separated by 10% SDS-PAGE and detected with anti-HADHA polyclonal antibody. The membrane was reprobed with anti-beta actin monoclonal antibody. All cisplatin-resistant cells expressed higher levels of HADHA than their parent cells. B, IHC of HADHA in the cell lines. More positive tumor cells and intenser staining were observed in the resistant cells than parent cells

fold; LCN2cis, 2.0-fold; A549cis, 1.8-fold; LC2Adcis, 2.5-fold (Figure 1B).

IHC and WB analyses of HADHA expression between 8 cell lines and lung cancer tissues

Next, we confirmed the up-regulated HADHA expression in the cisplatin-resistant sub-lines by WB (Figure 2A), and all cisplatin-resistant cell lines expressed significantly higher HADHA than their parent lines. By IHC, more positive cells and more intensive staining were observed in cisplatin-resistant sub-lines than in the parent lines (Figure 2B). These results suggest that the rate of HADHA-expressing cells increased in cell lines that acquired cisplatin resistance.

HADHA expression was also examined in lung cancer tissues (Figure 3). HADHA expression in lung cancer tissues was 1.5–3 times higher than in corresponding non-neoplastic peripheral lung tissues by WB (Figure 3A), and the intensity of HADHA staining was stronger in lung cancer tissues than in normal bronchial epithelium based on IHC samples (Figure 3B). These results suggest that lung cancer cells tend to express higher HADHA than non-neoplastic peripheral lung tissues.

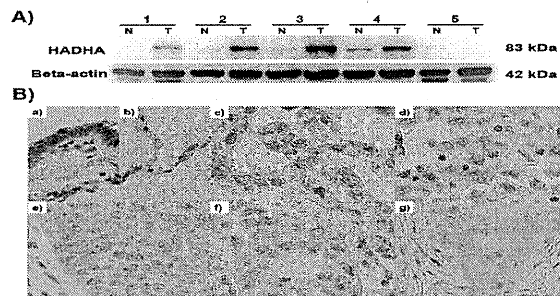

Figure 3. HADHA Expression in Lung Cancer Tissues. A, WB of HADHA in non-neoplastic peripheral lung or lung cancer tissues. HADHA was expressed at levels 1.5–3 times higher in lung cancer cells compared with non-neoplastic peripheral lung tissues. 1, 2, 3, 4, and 5 are the ID of lung cancer patients. N, non-neoplastic peripheral lung tissues; T, tumor; 1, 2, and 4 are adenocarcinomas (AD); 3 and 5 are squamous cell carcinomas (SCC). B, IHC of HADHA in non-neoplastic peripheral lung tissues or lung cancer tissues. The positive rate of HADHA-expressing cells was higher in lung cancer cells than in non-neoplastic peripheral lung tissues. a) normal bronchial epithelium of ID 1, b) normal alveolar epithelium of ID 4, c) AD of ID 1, d) AD of ID 2, e) SCC of ID 3, f) AD of ID 4, g) SCC of ID 5

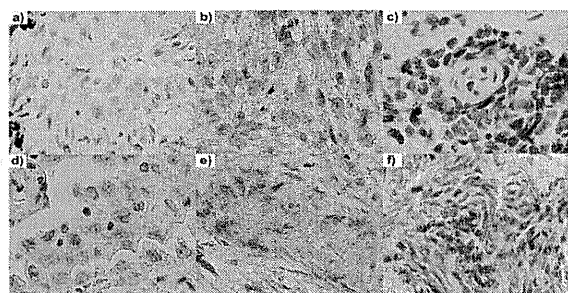

Figure 4. Representative IHC of HADHA in Biopsy Samples of Lung Cancer. a), b), and c) are sections from patients responding to platinum-based chemotherapy, and the staining is evaluated as HADHA-negative (–). d), e), and f) are sections from patients not responding to platinum-based chemotherapy, and the staining is evaluated as HADHA-positive (+)

The relationship between HADHA expression and clinical data among lung cancer biopsy samples

To clarify the relation between HADHA expression and resistance to platinum-based chemotherapy, we compared the IHC results of 46 biopsy samples taken before clinical treatment with their clinical data. The patients with weak or negative HADHA-expressing tumors (Figure 4a, b, c) were responders to platinum-based chemotherapy, while patients with HADHA-positive tumors (Figure 4d, e, f) were non-responders to chemotherapy. The results are summarized in Table 3. HADHA expression was significantly associated with the response to platinum-based chemotherapy. In all lung cancers, the cancer tissues of chemotherapy non-responding patients expressed HADHA (p=0.000367). Similar results were also observed in patients with NSCLC (p=0.002121), SCLC (p=0.038433), or AD (p=0.005772). There was no difference in SCC samples (p=0.136037), possibly due to the small number of cases. In addition, although five HADHA-positive patients responded to the chemotherapy, one developed brain metastases and two

Table 3. Expression of HADHA in Biopsy of Lung Cancer

Response	HADHA-positive	HADHA-negative	p-value*
Total			
Responder (CR + PR)	31% (5/16)	69% (11/16)	p=0.000367
Non-responder (SD + PD)	90% (27/30)	10% (3/30)	
AD			
Responder (CR + PR)	56% (5/9)	44% (4/9)	p=0.005772
Non-responder (SD + PD)	100% (22/22)	0% (0/22)	
SCC			
Responder (CR + PR)	0% (0/2)	100% (2/2)	p=0.136037
Non-responder (SD + PD)	67% (2/3)	33% (1/3)	
SCLC			
Responder (CR + PR)	0% (0/5)	100% (5/5)	p=0.038433
Non-responder (SD + PD)	60% (3/6)	40% (2/5)	

*Results of 2x2 chi square test; NSCLC, non-small cell lung carcinoma; AD, adenocarcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma. CR, complete response; PR, partial response; PD, progressive disease; SD, stable disease. Responder (CR + PR), chemotherapy sensitive; Non-responder (SD + PD), chemotherapy-resistant

showed relapsed lung cancer within two to three months. Our results suggest that HADHA expression in lung cancer has an impact on chemotherapy sensitivity and the prognosis.

Discussion

To identify new markers that predict the sensitivity to platinum-based chemotherapy, we compared the protein profiles of parent-sensitive lung cancer cell lines and their cisplatin-resistant sub-lines using a proteomics method, agarose 2DE. From 217 differently expressed proteins, we found that HADHA, a mitochondrial protein, increased significantly in cisplatin-resistant cells. Mitochondria and their components are related to the effect of and resistance to platinum-based chemotherapy. The mitochondrial DNA (mtDNA) mutations derived from patients with mitochondrial encephalopathy suppressed apoptosis induced by cisplatin (Shidara et al., 2005). It was also shown that DNA-damaging agents might cause mtDNA mutation, and leukemia cells with more mutant mtDNA were chemoresistant and survived after chemotherapy (Carew et al., 2003). Furthermore, mtDNA mutations could confer chemoresistance to human pancreatic cancer cell lines (Mizutani et al., 2009), and mitochondrial poisons were a useful therapeutic strategy for cisplatin-resistant cancer (Andrews and Albright, 1992). The cell lines with a low density of mitochondria were more sensitive to cisplatin than the parent lines (Qian W et al., 2005). Thus, HADHA, a mitochondrial protein, may also be a factor associated with resistance to the cisplatin chemotherapy.

HADHA is a part of a complex enzyme called mitochondrial trifunctional protein. Mitochondrial trifunctional protein binds to the mitochondrial inner membrane and plays a significant role in the last three steps of the beta-oxidation cycle of long-chain acyl-CoAs (Uchida et al., 1992; Kamijo et al., 1994). HADHA exhibits two enzyme activities, long-chain 2-enoyl-coenzyme A hydratase and long-chain 3-OH-acyl-CoA dehydrogenase, and both of them are required in the beta-oxidation of polyunsaturated fatty acids (Uchida et al., 1992; Kamijo et al., 1994). Polyunsaturated fatty acids were reported to enhance the cytotoxicity of several antineoplastic agents including cisplatin (Conklin, 2002; Murphy et al., 2011). Adding polyunsaturated fatty acids to the medium of cisplatin-resistant cells could enhance cisplatin sensitivity and increase the amount of total platinum binding to DNA, with a consequent increase in the formation of platinum-DNA adducts (Timmer-Bossacha et al., 1989). These reports indicate that the loss of polyunsaturated fatty acids might be one of the reasons for cisplatin resistance. In addition to HADHA, we also found increases of two enzymes in cisplatin-resistant cells: 2,4 dienoyl-CoA reductase (up-regulated 1.5-fold in LCN1cis) and enoyl-CoA isomerase (up-regulated 1.9-fold in LCN2cis), and both were involved in the beta-oxidation of polyunsaturated fatty acids. Up-regulation of these enzymes might confer cisplatin resistance to cells by enhancing beta-oxidation and decreasing polyunsaturated fatty acids. It was also reported that nonsteroidal anti-inflammatory drugs inhibited the intrinsic enzyme activities of HADHA, and this inhibition caused a reduction of long-chain fatty acid oxidation, resulting in the inhibition of cell proliferation in human colorectal cancer cell lines (Baldwin et al., 1998). Thus, HADHA may be required for cell proliferation in cancer. In this study, HADHA was highly expressed in cisplatin-resistant lung cancer patients, indicating a role in cisplatin resistance. The mechanism of cisplatin resistance conferred by HADHA needs further study.

This is the first report providing evidence that HADHA might be a useful marker to predict the response to platinum-based chemotherapy of lung cancer. The detection of high-level HADHA expression might prevent or reduce the side effects of chemotherapy and improve the quality of life of patients.

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