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 (16×16 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-

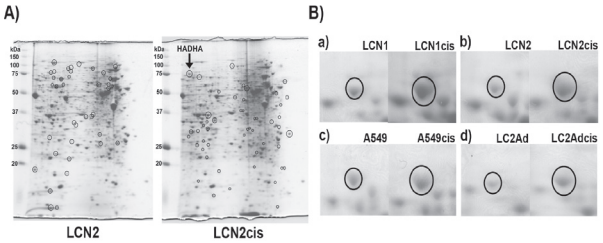

Figure 1. Comparison of Cellular Proteins of Cisplatin-Resistant Cells and Parent Cells on Agarose 2DE. Similar experiments were repeated 3 times, and representative results are shown. A, 2DE maps of cellular proteins of LCN2 and cisplatin-resistant LCN2cis. LCN2cis were cultured with 3,200 ng/ml of 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

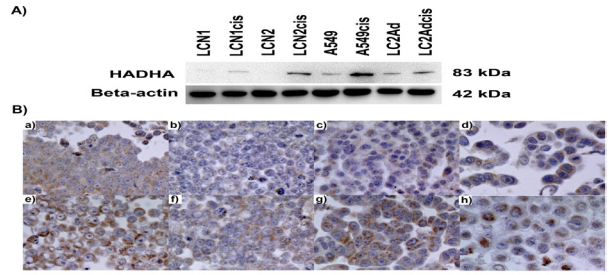

Figure 2. Expression of HADHA in Cisplatin-resistant Cells and their Parent Cells. A, WB of HADHA in the cell lines. Each sample (10 μg) was separated by 10% SDS-PAGE and detected with anti-HADHA polyclonal antibody. The membrane was reprobed with anti-beta actin monoclonal antibody. All cisplatin-resistant 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.

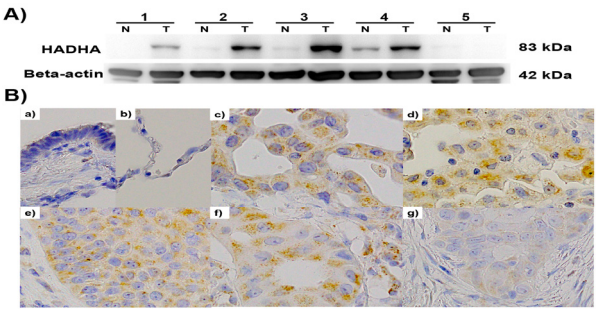

Figure 3. HADHA Expression in Lung Cancer Tissues. A, WB of HADHA in non-neoplastic peripheral lung or lung cancer tissues. HADHA was expressed at levels 1.5–3 times higher in lung cancer cells compared with non-neoplastic peripheral lung tissues. 1, 2, 3, 4, and 5 are the ID of lung cancer patients. 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

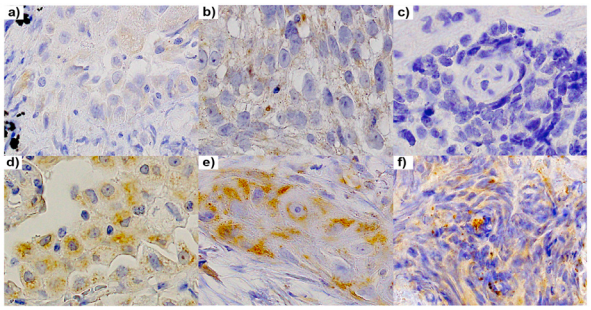

Figure 4. Representative IHC of HADHA in Biopsy Samples of Lung Cancer. a), b), and c) are sections from patients responding to platinum-based chemotherapy, and the staining is evaluated as HADHA-negative (–). d), e), and f) are sections from patients not responding to platinum-based chemotherapy, and 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 2×2 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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