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TRAP1 is a predictive biomarker of platinum-based adjuvant chemotherapy benefits in patients with resected lung adenocarcinoma

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ABSTRACT

Platinum-based adjuvant chemotherapy after complete resection has become a standard treatment for patients with stage II to IIIA non-small cell lung cancer; however, not all patients exhibit survival benefits. Therefore, the development of predictive biomarkers for selecting a subgroup of patients who may show improved survival after these treatments is important. Among the 42 proteins identified here using a proteomics analysis that were recognized by autoantibodies in pretreated sera from patients with lung adenocarcinoma who received platinum-based adjuvant chemotherapy, the tumor necrosis factor-receptor-associated protein 1 (TRAP1) was detected in patients with a short disease-free survival. TRAP1 expression was immunohistochemically analyzed in 64 patients with completely resected stage II and IIIA lung adenocarcinoma treated with platinum-based adjuvant chemotherapy. TRAP1 expression was significantly associated with higher p-TNM stage ($P = 0.005$) and lymph node metastasis ($P = 0.017$). Moreover, TRAP1 expression was significantly correlated with a shorter disease-free survival ($P = 0.028$). Furthermore, TRAP1-siRNA-treated LC-2/ad cells derived from lung adenocarcinoma exhibited significantly reduced proliferation and increased sensitivity to cisplatin. These results suggest that TRAP1 expression is a valuable biomarker for predicting the poor survival of platinum-based adjuvant chemotherapy in patients with resected lung adenocarcinoma.

Lung cancer is the leading cause of cancer-related morbidity and mortality worldwide (33). It is the highest number of cancer deaths and poor prognosis in Japan, and lung adenocarcinoma is the most common histological type in non-small cell lung cancer (NSCLC). Several clinical trials have demonstrated that cisplatin-based adjuvant chemotherapy improves the prognosis of NSCLC patients compared with surgery alone (3, 9, 30, 36). At present, adjuvant platinum-based chemotherapy after complete surgical resection is the standard therapy for patients with stage II to IIIA NSCLC, to reduce the risk of recurrence (27). However, not all patients show a survival benefit, and some patients suffer from side effects. Therefore, the development of predictive biomarkers for selecting a subgroup of patients who may show improved survival after this treatment modality is an important issue.

Autoantibodies are generated in response to antigens and are present in the sera of patients with various autoimmune diseases (11). They are also

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frequently found in the sera of patients with various tumors, even at early stages (10, 14, 18, 37). Hanash has suggested that the detection of autoantibodies to identify novel cancer biomarkers is an attractive strategy, because the immune system accompanies biological amplifications (13). Thus, a search for useful biomarkers based on autoantibodies has been proposed.

Cis-diaminodichloroplatinum (II) (cisplatin) is an anticancer agent that is used widely for the treatment of various cancers, including lung cancer. Cisplatin is a highly reactive compound that binds to DNA, inhibits DNA synthesis and RNA transcription, affects the cell cycle, and ultimately induces apoptosis (32). Chemotherapy using a combination of cisplatin and vinorelbine improves survival in patients with NSCLC; however, resistance to cisplatin occurs often and causes treatment failure (5, 29). The mechanisms underlying the development of cisplatin resistance include alterations in the cellular accumulation of the drug, intracellular drug detoxification, DNA damage repair, and apoptotic signaling pathways (7). Although the biological causes of resistance to cisplatin have not been completely elucidated, the identification of predictive markers of therapeutic efficacy is an urgent issue.

In this study, we explored novel predictive biomarkers of the effect of platinum-based adjuvant chemotherapy via two-dimensional immunoblotting using mixed pretreated sera of patients with lung adenocarcinoma who had received platinum-based adjuvant chemotherapy as primary antibodies. We identified tumor necrosis factor-receptor-associated protein 1 (TRAP1) in this screening and evaluated its utility as a predictive biomarker of the effect of platinum-based adjuvant chemotherapy in patients with lung adenocarcinoma. Furthermore, we confirmed the effect of TRAP1 on the proliferation and cisplatin sensitivity of lung adenocarcinoma cells.

MATERIALS AND METHODS

Cell lines. A549 and LC-2/ad cells derived from lung adenocarcinoma were purchased from the American Type Culture Collection (Manassas, VA, USA) and RIKEN BioResources Bank Center (Tokyo, Japan), respectively. Cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (MP Biomedicals, Inc., Santa Ana, CA, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO_2 and 95% humidified air. Subconfluent cells were harvested and washed twice with phosphate-buffered saline without divalent ions, then stored at −80°C until use in proteomics analysis.

Patients. A total of 64 consecutive patients with lung adenocarcinoma at pathological stage II to IIIA who underwent surgery and received platinum-based adjuvant chemotherapy between January 2003 and December 2012 at the Kitasato University Hospital were included in this retrospective cohort study. Patients who had received preoperative chemotherapy and/or radiotherapy were excluded. Each histological diagnosis was based on the criteria of the 2015 World Health Organization Classification of Lung and Pleural Tumors (35). The pathological stage was determined according to the 7th edition of the TNM classification (12). The following clinical and pathological parameters were extracted from the medical records: age at surgery, gender, smoking habits, pathological TNM (p-TNM) stage, tumor differentiation, vascular invasion, lymphatic invasion, pleural invasion, viability status, disease-free survival, and overall survival. Disease-free survival was defined as the period from the surgery to first recurrence or death from the disease, and overall survival was defined as the time from surgery to death from the disease or the end of the follow-up.

The study was approved by the Ethics Committee of Kitasato University School of Medicine (B07-06) and was performed according to the Declaration of Helsinki protocol. All patients were informed about the aim of the study and gave consent to donate samples. All patients were approached based on approved ethical guidelines and agreed to participate in this study. They could refuse entry and discontinue participation at any time. Written informed consent was obtained from all patients.

Agarose two-dimensional gel electrophoresis. A549 and LC-2/ad cells were solubilized in lysis buffer containing (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonium] propanesulfonate, 10 mM Tris (2-carboxyethyl) phosphine hydrochloride, 2.5% pH 3–10 pharmalyte (GE Healthcare, Chicago, IL, USA), and complete mini EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany) by use of an ultrasonic homogenizer (VP-050; TAITEC, Saitama, Japan). Cells were centrifuged at 20,000 × *g* for 5 min at 4°C. The supernatant was alkylated with 1/20 volumes of 400 mM 4-vinylpyridine for 1 h by mixing, followed by the addition of the same volume of 400 mM dithiothreitol, to quench the reaction. After centrifugation at 20,000 × *g*

for 30 min at 4°C, the proteins were purified using a 2-D Clean-Up Kit (GE Healthcare) according to the manufacturer's instructions. Finally, the concentration of proteins was quantified using the Bio-Rad Protein Assay solution (BIO-RAD Laboratories, Hercules, CA, USA). Two-dimensional gel electrophoresis with agarose (agarose 2-DE) was performed according to our previous study (25). The first-dimensional agarose isoelectric focusing (IEF) gel (80 mm in length and 2.5 mm in inner diameter) was prepared using a single pH 3–10 pharmalyte. Seventy-five microgram of protein extracted from the two cell lines were equally mixed, applied to the cathodic end of the agarose IEF gel, and loaded using increasing voltage (20 min at 100 V, 15 min at 300 V, 15 min at 500 V, 1 h at 700 V, and 1 h at 900 V) at 4°C. After fixation in 10% trichloroacetic acid and 5% sulfosalicylic acid for 3 min at room temperature (RT) with gentle shaking, the gels were rinsed in distilled water 3 times for 15 min each at RT. The agarose gels were immersed in sodium dodecyl sulfate (SDS) treatment solutions [0.06 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue] for 15 min at RT with mild shaking. The agarose gels were then placed at the top of the second-dimensional 10% polyacrylamide gel and loaded with a constant current (20 mA per gel). Two pieces of gel were prepared: one was transferred to a polyvinylidene difluoride (PVDF) membrane (Merck-Millipore Corp., Bedford, MA, USA), for immunoblotting, while the other was visualized using Coomassie brilliant blue (CBB) staining solution (APRO SCIENCE, Tokushima, Japan).

2-DE immunoblotting. The proteins that were separated on the 2-DE gel were transferred to a PVDF membrane overnight at RT with constant voltage (10 V). After blocking with 0.5% casein for 1 h at RT, the membranes were reacted with mixed pretreated sera (1 : 100 dilution) of three patients with lung adenocarcinoma containing 0.025% casein/Tris-buffered saline (TBS) overnight at 4°C. The sera of patients who received adjuvant chemotherapy and exhibited a disease-free survival of <1 year or ≥5 years were used here. The membranes were washed three times with TBS containing 0.1% tween 20 (TBS-T) and then reacted with a horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG polyclonal antibody (1 : 1000 dilution; Dako, Glostrup, Denmark) containing 0.025% casein/TBS for 45 min at RT. After washing three times with TBS-T, the immunoreactive spots on the membranes were visualized by incubation with the Stable DAB solution (Invitrogen, Carlsbad, CA, USA) for 15 min at RT.

Identification of the proteins that were recognized by the autoantibodies. The methods used to identify the antigenic proteins that were recognized by the autoantibodies were described previously (23). The protein spots that matched the immunoreactive spots were excised manually from CBB-stained 2-DE gels, destained with 50% acetonitrile/50 mM NH_4HCO_3, dehydrated with 100% acetonitrile, and dried under vacuum conditions. Gel pieces were rehydrated in digestion solution containing 10 ng/μL trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega, Madison, WI, USA), and incubated for 24 h at 37°C with a minimum volume of 25 mM NH_4HCO_3. Subsequently, the digested tryptic peptide solutions were collected, the gels were washed once in 5% trifluoroacetic acid/50% acetonitrile, and the solution was collected in the same tube. Finally, solutions containing digested tryptic peptides were spotted onto a prespotted AnchorChip 96 Set for Proteomics (Bruker Daltonics, Bremen, Germany), according to the manufacturer's recommendations. The samples were subjected to peptide mass fingerprint and MS/MS analyses for protein identification using an Autoflex III mass spectrometer (Bruker Daltonics). The combined spectral data were connected with MASCOT (http://www.matrixscience.com/) for a database search, and the identification of corresponding proteins was performed using the IPI human database version 3.84 (90,166 sequences and 36,304,241 residues, http://www.ebi.ac.uk/IPI/IPIhuman.).

Immunohistochemical staining. Formalin-fixed (10%) and paraffin-embedded tissues were processed into 3-μm-thick sections. Immunohistochemical staining was performed using a Leica Bond-Max automated system and the BOND polymer Refine Detection Kit (Leica Biosystems, Bannockburn, IL), based on the previous report of Nomura *et al.* (26). Tissues were first deparaffinized with AutoDewaxer and antigens were retrieved with BOND Epitope Retrieval Solution 2 at 100°C for 20 min. After washing and peroxidase blocking for 10 min, tissues were reacted with mouse anti-TRAP1 monoclonal antibody (1 : 800 dilution; Merck-Millipore Corp.) in the Bond Primary Antibody Diluent (Leica Biosystems) for 15 min, Post Primary solution for 8 min, Polymer solution for 8 min, and DAB-Chromogen for 10 min. Finally, tissues were counterstained with hematoxylin for 5 min.

Evaluation of immunohistochemical staining. The TRAP1 staining exhibited a granular pattern in the cytoplasm of tumor cells. TRAP1 staining was calculated and multiplied by the staining score, to obtain a semi-quantitative H-score based on the intensity and percentage of stained tumor cells over an average of three areas (17). The staining intensity was defined as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. In cases in which 100% of the tumor cells are positive for TRAP1 and the overall staining intensity is 3, the H-score is 300.

Transfection of siRNA. For siRNA transfection, two sequences of the TRAP1 siRNA (FlexiTube GeneSolution siRNA SI00115171 and SI03066364) and negative-control siRNA (AllStars Negative Control siRNA; Qiagen, Venlo, Netherlands) were used at a final concentration of 20 nM. A total of 2.5×10^4 LC-2/ad cells were cultured in a 24-well plate (Sumitomo Bakelite, Tokyo, Japan). After 3.5 h of culture, cells were transfected with the HiPerFect Transfection Reagent (QIAGEN). Cells were used in subsequent assays after 48 h of culture.

RNA isolation and quantitative real-time RT-PCR (qRT-PCR). Total RNA was extracted from siRNA-treated LC-2/ad cells using an miRNeasy Mini Kit (Qiagen) and reverse transcribed to cDNA using the PrimeScript™ RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturer's instructions. The specific primers used to detect TRAP1 were as follows: forward, 5'-CTG TAC AGC CGC AAA GTC CTC A-3'; and reverse, 5'-GGA ATG TCC TCA TGT CCA CCA-3' (TaKaRa Bio Inc.). qRT-PCR was performed using a LightCycler 96 machine (Roche Diagnostics, Mannheim, Germany) and TB Green Premix Ex Taq™ II (TaKaRa). The data were analyzed using the LightCycler Software ver1.1 (Roche Diagnostics). The level of expression of the TRAP1 mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase.

One-dimensional (1-D) immunoblotting. Proteins were extracted from LC-2/ad cells using the M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples (1.5 μg) were separated by SDS-PAGE using e-PAGEL 5–20% (ATTO, Tokyo, Japan) and transferred onto PVDF membranes. After blocking with 0.5% casein for 1 h at RT, membranes were reacted with an anti-TRAP1 antibody (1 : 1000 dilution) containing 0.5% casein for 2 h at RT. Membranes were subsequently reacted with a 1 : 1000-diluted HRP-conjugated rabbit anti-mouse IgG polyclonal antibody (Dako) containing 0.5% casein for 30 min at RT. Finally, the immunoreactive bands on the membranes were detected using the Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore Corp.) and images were captured using the ATTO Cool Saver System (ATTO).

MTS assay for cell proliferation; drug-sensitivity assay. An MTS assay was performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) to evaluate the proliferation and cisplatin sensitivity of cells. Transfected cells were seeded at 1×10^3 cells/well in 96-well plates and cultured for 0, 24, 48, and 72 h. Subsequently, 20 μL of MTS reagent was added to each well and the cells were incubated for an additional 2 h at 37°C. The reaction product was measured at 492 and 630 nm as a reference wavelength using a ChroMate 4300 microplate reader (Awareness Technology Inc., Palm City, FL, USA). To evaluate the sensitivity to cisplatin, cells (2.5×10^3 cells/well) were treated with cisplatin at a concentration of 1.25, 2.5, 5, 10, and 20 μM for 48 h. MTS assays were then performed as described above. Each experiment was performed in triplicate.

Statistical analysis. The relationships between TRAP1 expression and clinicopathological parameters were analyzed using the Mann-Whitney *U*-test. The patients were divided into two groups according to the median TRAP1 H-score (median score, 239). The disease-free survival and overall survival of the patients were estimated using the Kaplan-Meier method, and the log-rank test was used to evaluate the significance of the differences in the recurrence or survival rate between the TRAP1-high (H-score $\geq$239) and TRAP1-low (H-score <239) groups. A multivariate analysis was performed using the Cox proportional hazards regression model to investigate the relationships between TRAP1 expression and other clinicopathological parameters, and to estimate the independent predictive value for the effect of treatment. The *P*-value of the MTS assay was estimated using Student's *t*-test. All statistical analyses were performed using StatFlex version 6.0 (Artech Co., Ltd., Osaka, Japan). All reported *P*-values were two-sided. Significance was set at $P < 0.05$.

RESULTS

Patient characteristics

The clinicopathological characteristics of the patients with lung adenocarcinoma are summarized in Table 1. A total of 34 men and 30 women were included in the study, with ages ranging from 41 to 75 years (median, 65 years); among these patients 35 (54.7%) individuals were smokers. Fourteen patients (21.9%) had stage II and 50 patients (78.1%) had stage IIIA disease. The overall follow-up duration ranged from 5.8 to 149.7 months (median, 49.7 months). At the end of the follow-up, 27 patients were alive, 29 patients had died of lung cancer, three patients died from other causes, and five patients were lost to follow-up.

2-DE Immunoblotting analysis

The whole proteins extracted from A549 and LC-2/ad cells were separated by 2-DE, transferred to the PVDF membranes, and reacted with mixed sera of three patients with adenocarcinoma with a disease-free survival of <1 year or ≥5 years, as primary antibodies (Fig. 1). Seventy and 33 immunoreactive spots were detected by the pretreated sera from patients with a disease-free survival <1 year (Fig. 1A) and ≥5 years (Fig. 1B), respectively. In total, 35 proteins (including TRAP1) were identified in the former and seven proteins were identified in the latter group, respectively (Table 2).

Table 1 *Patient characteristics*

Clinicopathological characteristics	Patients, n (%) ($n = 64$)
Age, years	
<60	28 (43.7)
≥60	36 (56.3)
Gender	
Male	34 (53.1)
Female	30 (46.9)
Smoking habit	
Never smoker	29 (45.3)
Smoker	35 (54.7)
Tumor size	
≤3 cm	23 (35.9)
>3 cm	41 (64.1)
Tumor differentiation	
Well	8 (12.5)
Moderately/Poorly	56 (87.5)
p-TNM stage	
Stage II	14 (21.9)
Stage IIIA	50 (78.1)
Chemotherapy regimen	
Cisplatin-based	44 (68.8)
Carboplatin-based	20 (31.2)
Vital status	
Alive	27 (42.2)
Lung cancer-related death	29 (45.3)
Death by other causes	3 (4.7)
Unknown	5 (7.8)

p-TNM, pathological TNM

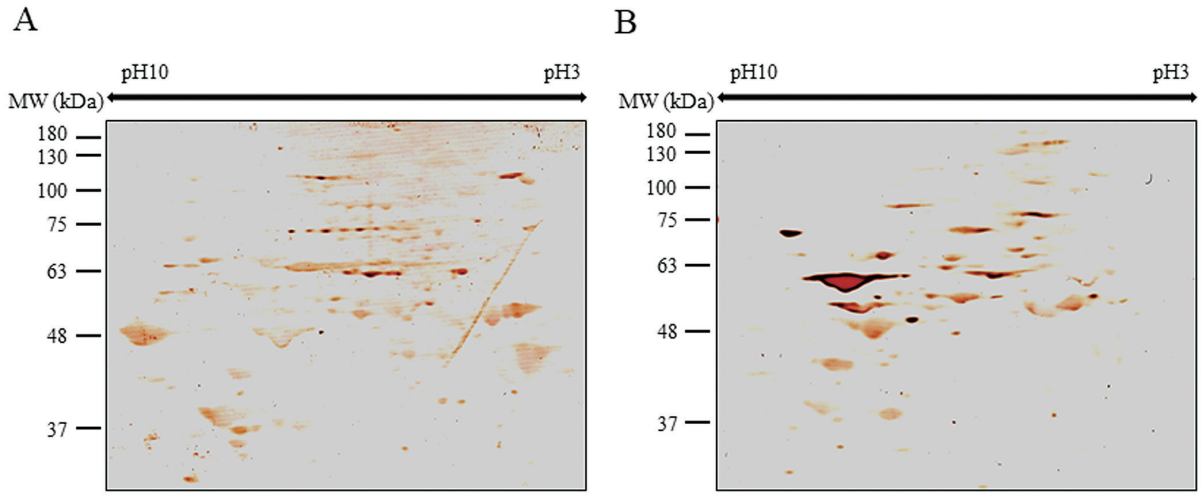

Fig. 1 2-DE immunoblotting detection of proteins that were recognized by autoantibodies in pre-treated sera from patients with resected lung adenocarcinoma who received platinum-based adjuvant chemotherapy. The proteins extracted from A549 and LC-2/ad lung adenocarcinoma cells were separated by 2-DE. Immunoblot analysis was performed using mixed pre-treated sera from patients with lung adenocarcinoma with a disease-free survival of <1 year (A) and ≥5 years (B) as primary antibodies. The immunoreactive spots were visualized using DAB solution.

Table 2 *Antigenic proteins identified in the sera from patients with resected lung adenocarcinoma*

Gene symbol	Protein name	Protein accession	Molecular weight (Da)	Function
Disease-free survival of <1 year				
DDX5	Probable ATP-dependent RNA helicase DDX5	P17844	69,148	pre-mRNA splicing
NONO	Non-POU domain-containing octamer-binding protein	Q15233	54,232	DNA- and RNA binding protein, involved in several nuclear processes
EEF1A1	Elongation factor 1-alpha 1	P68104	50,141	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis
GOT2	Aspartate aminotransferase, mitochondrial	P00505	47,518	Catalyzes the irreversible transamination of the L-tryptophan metabolite L-kynurenine to form kynurenic acid
LDHA	L-lactate dehydrogenase A chain	P00338	36,689	Glycolysis
HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37,430	Associates with nascent pre-mRNAs, packaging them into hnRNP particles.
AKR1C3	Aldo-keto reductase family 1 member C3	P42330	36,853	Catalyzes the conversion of aldehydes and ketones to alcohols
AKR1B10	Aldo-keto reductase family 1 member B10	O60218	36,020	Catalyzes the NADPH-dependent reduction of a wide variety of carbonyl-containing compounds to their corresponding alcohols
PGK1	Phosphoglycerate kinase 1	P0055	44,615	Glycolytic enzyme
ALDOA	Fructose-bisphosphate aldolase A	P04075	39,420	Glycolysis and gluconeogenesis
TKT	Transketolase	P29401	67,878	Catalyzes the transfer of a two-carbon ketol group from a ketose donor to an aldose acceptor, via a covalent intermediate with the cofactor thiamine pyrophosphate
LMNA	Prelamin-A/C	P02545	74,139	Accelerate smooth muscle cell senescence
TRAP1	Heat shock protein 75 kDa, mitochondrial	Q12931	80,110	Chaperone that expresses an ATPase activity
EEF2	Elongation factor 2	P13639	95,338	Catalyzes the GTP-dependent ribosomal translocation step during translation elongation
HSPA4	Heat shock 70 kDa protein 4	P34932	94,331	Structurally related to aconitase
ACTN1	Alpha-actinin-1	P12814	103,058	Anchor actin to a variety of intracellular structures
VCP	Transitional endoplasmic reticulum ATPase	P55072	89,322	Involved in the formation of the transitional endoplasmic reticulum
HSPA1B	Heat shock 70 kDa protein 1B	P0DMV9	70,052	Implicated in a wide variety of cellular processes
CCT5	T-complex protein 1 subunit epsilon	P48643	59,671	Assists the folding of proteins upon ATP hydrolysis
PPP1R16B	Protein phosphatase 1 regulatory inhibitor subunit 16B	Q96T49	63,551	Acts as a positive regulator of pulmonary endothelial cell (EC) barrier function
TUBB4B	Tubulin beta-4B chain	P68371	49,831	Tubulin is the major constituent of microtubules
TUBA1C	Tubulin alpha-1C chain	Q9BQE3	49,895	Tubulin is the major constituent of microtubules
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	P31930	52,646	A component of the ubiquinol-cytochrome c reductase complex
KRT19	Keratin, type I cytoskeletal 19	P08727	44,106	Involved in the organization of myofibers
KRT18	Keratin, type I cytoskeletal 18	P05783	48,058	Involved in the uptake of thrombin-antithrombin complexes by hepatic cells
CAPZA1	F-actin-capping protein subunit alpha-1	P52907	32,923	Formation of epithelial cell junctions
SRM	Spermidine synthase	P19623	33,825	Catalyzes the production of spermidine from putrescine and decarboxylated S-adenosylmethionine
CLIC1	Chloride intracellular channel protein 1	O00299	26,923	Insert into membranes and form chloride ion channels
PAFAH1B3	Platelet-activating factor acetylhydrolase IB subunit gamma	Q15102	25,734	Inactivates paf by removing the acetyl group at the sn-2 position
ESD	S-formylglutathione hydrolase	P10768	31,463	involved in the detoxification of formaldehyde
IDH3A	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	P50213	39,592	Catalyzes the decarboxylation of isocitrate (ICT) into alpha-ketoglutarate
DCPS	m7GpppX diphosphatase	Q96C86	38,609	Catalyzes the cleavage of a residual cap structure following the degradation of mRNAs by the 3'->5' exosome-mediated mRNA decay pathway
NANS	Sialic acid synthase	Q9NR45	40,308	Produces N-acetylneuraminic acid (Neu5Ac) and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid
AHCY	Adenosylhomocysteinase	P23526	47,716	Competitive inhibitor of S-adenosyl-L-methionine-dependent methyl transferase reactions
TUFM	Elongation factor Tu, mitochondrial	P49411	49,542	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis
Disease-free survival of ≥5 years				
AKR1C2	Aldo-keto reductase family 1 member C2	P52895	36,735	Concert with the 5-alpha/5-beta-steroid reductases to convert steroid hormones into the 3-alpha/5-alpha and 3-alpha/5-beta-tetrahydrosteroids
CS	Citrate synthase, mitochondrial	O75390	51,712	Catalyzing the first step of citric acid cycle
EIF4A3	Eukaryotic initiation factor 4A-III	P38919	46,871	ATP-dependent RNA helicase
HSP90AB1	Heat shock protein HSP 90-beta	P08238	83,264	Promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction
HSP90AA1	Heat shock protein HSP 90-alpha	P07900	84,660	Promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction
NDUFS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	P28331	79,468	Transfer of electrons from NADH to the respiratory chain
CCDC110	Coiled-coil domain-containing protein 110	Q8TBZ0	96,726	Protein binding

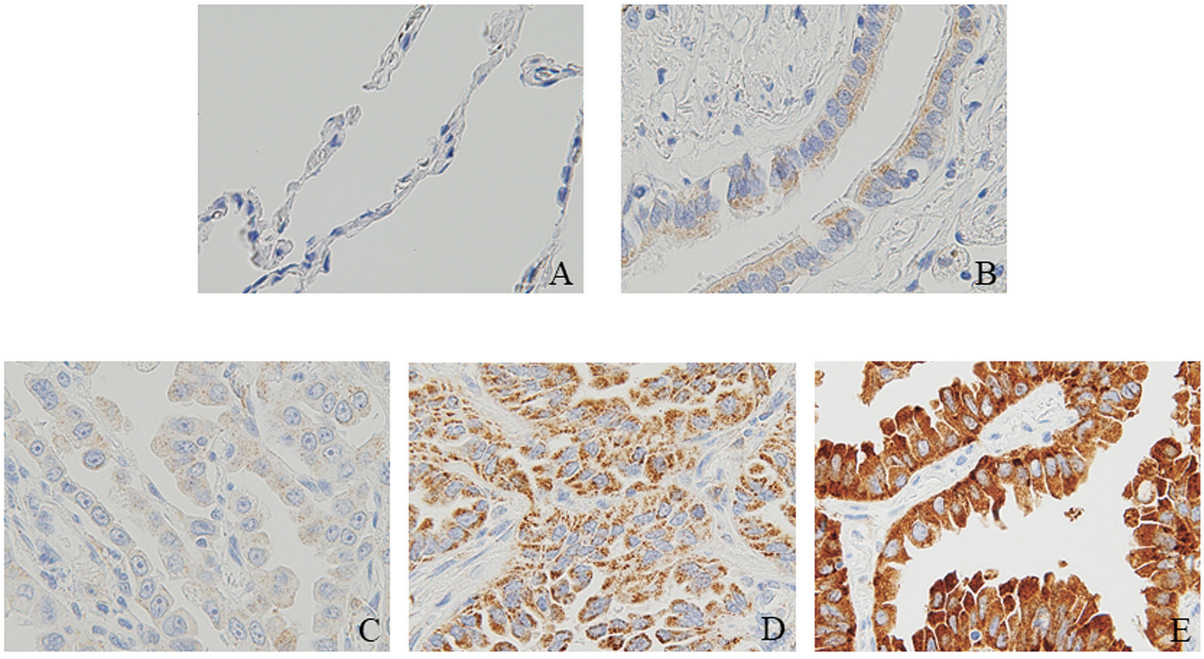

Fig. 2 TRAP1 expression in normal lung tissues and lung adenocarcinomas. No obvious staining was observed in alveolar epithelial cells (A). Weak granular expression of TRAP1 was observed in the cytoplasm of bronchial epithelial cells (B). In lung adenocarcinomas, granular cytoplasmic expression of TRAP1 was observed in tumor cells at various intensities, depending on the case (C, D, E). The H-scores of TRAP1 expression in tumor cells of C, D, and E were 100, 200, and 300, respectively. Original magnification, 400×.

Relationship between TRAP1 expression and clinicopathological characteristics

In normal lung tissues, TRAP1 expression was not found in alveolar epithelial cells (Fig. 2A), whereas weak TRAP1 granular expression was observed in bronchial epithelial cells (Fig. 2B). In patients with lung adenocarcinoma, granular cytoplasmic expression of TRAP1 with varying intensity was observed in tumor cells. The representative images for TRAP1 with H-scores as 100, 200, and 300 are presented in Fig. 2C, D, and E, respectively. The relationships between TRAP1 expression and clinicopathological characteristics in adenocarcinomas are summarized in Table 3. TRAP1 expression was significantly associated with higher p-TNM stage ($P = 0.005$) and lymph node metastasis ($P = 0.017$). There was no significant correlation between TRAP1 expression and age, gender, smoking habit, tumor size, tumor differentiation, vascular invasion, lymphatic invasion and pleural invasion.

Based on histological subtypes, TRAP1-high expression was detected in 2 out of 7 acinar (28.6%), 1 out of 2 lepidic (50%), 4 out of 6 micropapillary (66.7%), 21 out of 38 papillary (55.3%), 4 out of 9 solid (44.4%), and none of the 2 invasive mucinous (0%) adenocarcinomas.

Kaplan-Meier estimate of disease-free survival and overall survival in patients with adenocarcinoma with high and low TRAP1 expression

All 64 patients with lung adenocarcinoma were included in the survival analysis. The overall follow-up duration ranged from 5.8 to 149.7 months (median, 49.7 months). The 5-year cumulative disease-free survival probability was 18.8% for the TRAP1-high expression group and 43.8% for the TRAP1-low expression group; this difference was significant ($P = 0.028$, Fig. 3A). However, TRAP1 expression was not associated with overall survival ($P = 0.696$, Fig. 3B).

Relationship between TRAP1 expression and recurrence, as assessed using univariate- and multivariate analyses

The Cox-proportional hazards model was applied to estimate the relationship between TRAP1 expression and recurrence (Table 4). In univariate analysis, the hazards ratio (HR) of the TRAP1-high expression group compared with the TRAP1-low group was 1.95 (95% confidence interval (CI), 1.07–3.56; $P = 0.030$), which indicated that the TRAP1-high status increased the hazard of recurrence. In multivariate analysis, p-TNM stage was independently associated

Table 3 *Relationship between TRAP1 expression and clinicopathological characteristics in lung adenocarcinoma*

Clinicopathological parameters	total	TRAP1 expression	
		Median H-score	*P*-value
Age, years			
<60	28	238	0.968
≥60	36	244	
Gender			
Male	34	231	0.201
Female	30	252	
Smoking habit			
Never smoker	29	247	0.438
Smoker	35	239	
Tumor size			
≤3 cm	23	240	0.506
>3 cm	41	230	
Tumor differentiation			
Well	8	232	0.536
Moderately/Poorly	56	243	
p-TNM stage			
Stage II	14	206	0.005
Stage IIIA	50	252	
Nodal status			
N0	5	171	0.017
N1/N2/N3	59	245	
Vascular invasion			
No	4	203	0.608
Yes	50	251	
Lymphatic invasion			
No	3	171	0.482
Yes	55	245	
Pleural invasion			
No	25	237	0.655
Yes	39	240	

p-TNM, pathological TNM

with a shorter disease-free survival (HR, 4.01; 95% CI, 1.41–11.4; $P = 0.009$), whereas TRAP1 expression was not significantly correlated with disease-free survival (HR, 1.56; 95% CI, 0.85–2.88; $P = 0.151$). Thus, TRAP1 expression was not an independent predictor of disease-free survival.

Transfection of TRAP1 siRNA, and cell proliferation and drug sensitivity assays

To investigate the roles of TRAP1 in lung-adenocarcinoma-derived LC-2/ad cells, we knocked down TRAP1 mRNA expression using two TRAP1 siRNAs (TRAP1_siRNA#1 and TRAP1_siRNA#2) and performed cell proliferation and cisplatin sensitivity assays. Transfection of LC-2/ad cells with TRAP1_siRNA#1 or siRNA#2 significantly suppressed TRAP1 mRNA expression in TRAP1-knockdown cells compared with the negative-control-siRNA-treared cells ($P < 0.05$, Fig. 4A). Moreover, we found that TRAP1 protein expression was completely inhibited (Fig. 4B). The proliferation capability of TRAP1-knockdown cells and negative-control-siRNA-transfected cells were examined using the MTS assay. At 24, 48, and 72 h after transfection, the proliferation capability of TRAP1-knockdown cells was significantly reduced compared with the negative-control-siRNA-treated cells ($P < 0.05$ for all, Fig. 4C). Furthermore, the sensitivity of TRAP1-knockdown cells to cisplatin was also examined using the MTS assay after 48 h of treatment with this drug at a concentration of 0, 1.25, 2.5, 5, 10, and 20 μM. TRAP1-knockdown cells exhibited a significantly reduced cell viability compared with the negative-control-siRNA-treated cells in the presence of 10 μM cisplatin ($P < 0.05$ for all) and 20 μM cisplatin ($P < 0.05$ for all) (Fig. 4D).

DISCUSSION

In the present study, to identify novel useful predictive biomarkers of the efficacy of platinum-based adjuvant chemotherapy, we performed 2-DE immunoblotting to compare antigenic proteins that were recognized by autoantibodies in pre-treated sera from patients with lung adenocarcinoma with complete resection who had received platinum-based adjuvant chemotherapy and exhibited a disease-free survival of <1 year or ≥5 years. Among the proteins that were recognized specifically in each of the two groups, we focused on TRAP1, which was only detected in the sera of the patients with a shorter disease-free survival.

TRAP1 is a member of the HSP90 family that is localized primarily in mitochondria, but can be found widely in various subcellular compartments (2, 6). TRAP1 maintains a compartmentalized protein folding environment and performs organellar quality control, especially in tumor cells (15). TRAP1 expression is usually upregulated in tumor cells, while the protein is expressed at very low levels or is undetectable in their normal counterparts (2). Recently, several studies demonstrated that TRAP1 levels are increased in tumor cells, including glioblastoma (34), colon (8, 28), breast (21, 39), prostate (19), and lung cancer cells (1). In contrast, other studies reported the downregulation of TRAP1 in bladder and renal cancers (38) and that a lower TRAP1 expression level is correlated with poor prognosis in patients with ovarian cancer (4). Therefore, TRAP1 was considered to play a potential dual

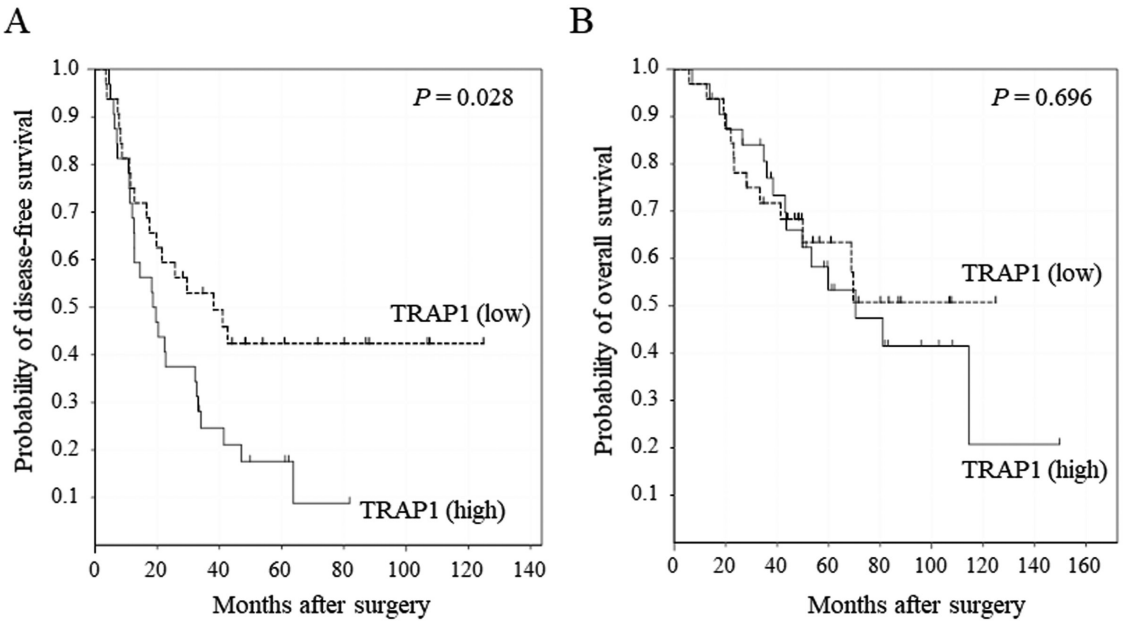

Fig. 3 Cumulative survival of patients with lung adenocarcinoma according to TRAP1 expression, as estimated using the Kaplan-Meier method. Panels A and B report the disease-free survival and overall survival, respectively. High-TRAP1 expression was significantly associated with a shorter disease-free survival in patients with resected lung adenocarcinoma who received platinum-based adjuvant chemotherapy ($P = 0.028$), whereas there was no correlation between TRAP1 expression and overall survival ($P = 0.696$).

Table 4 *Univariate and multivariate analyses of the effect of TRAP1 expression on recurrence*

Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P*-value	HR	95%CI	*P*-value
TRAP1 expression						
High (≥239) vs. Low (<239)	1.95	1.07–3.56	0.030	1.56	0.85–2.88	0.151
Age, years						
≥60 vs. <60	1.33	0.73–2.43	0.344	n/d	n/d	n/d
Gender						
Female vs. Male	1.07	0.59–1.92	0.827	n/d	n/d	n/d
Smoking habit						
Never smoker vs. Smoker	1.2	0.66–2.17	0.558	n/d	n/d	n/d
p-TNM stage						
stage IIIA vs. stage II	4.53	1.62–12.67	0.004	4.01	1.41–11.4	0.009
Tumor differentiation						
Moderately/Poorly vs. Well	0.64	0.28–1.44	0.277	n/d	n/d	n/d
Tumor size						
>3 cm vs. ≤3 cm	1.63	0.87–3.07	0.131	n/d	n/d	n/d
Vascular invasion						
Yes vs. No	2.24	0.54–9.32	0.268	n/d	n/d	n/d
Nodal status						
N1/N2/N3 vs. N0	1.47	0.45–4.74	0.521	n/d	n/d	n/d
Lymphatic invasion						
Yes vs. No	1.64	0.39–6.81	0.500	n/d	n/d	n/d
Pleural invasion						
Yes vs. No	1.52	0.83–2.81	0.178	n/d	n/d	n/d
Intrapulmonary metastasis						
Yes vs. No	2.17	0.85–5.54	0.104	n/d	n/d	n/d

CI, confidence interval; HR, hazard ratio; n/d, not done; p-TNM, pathological TNM

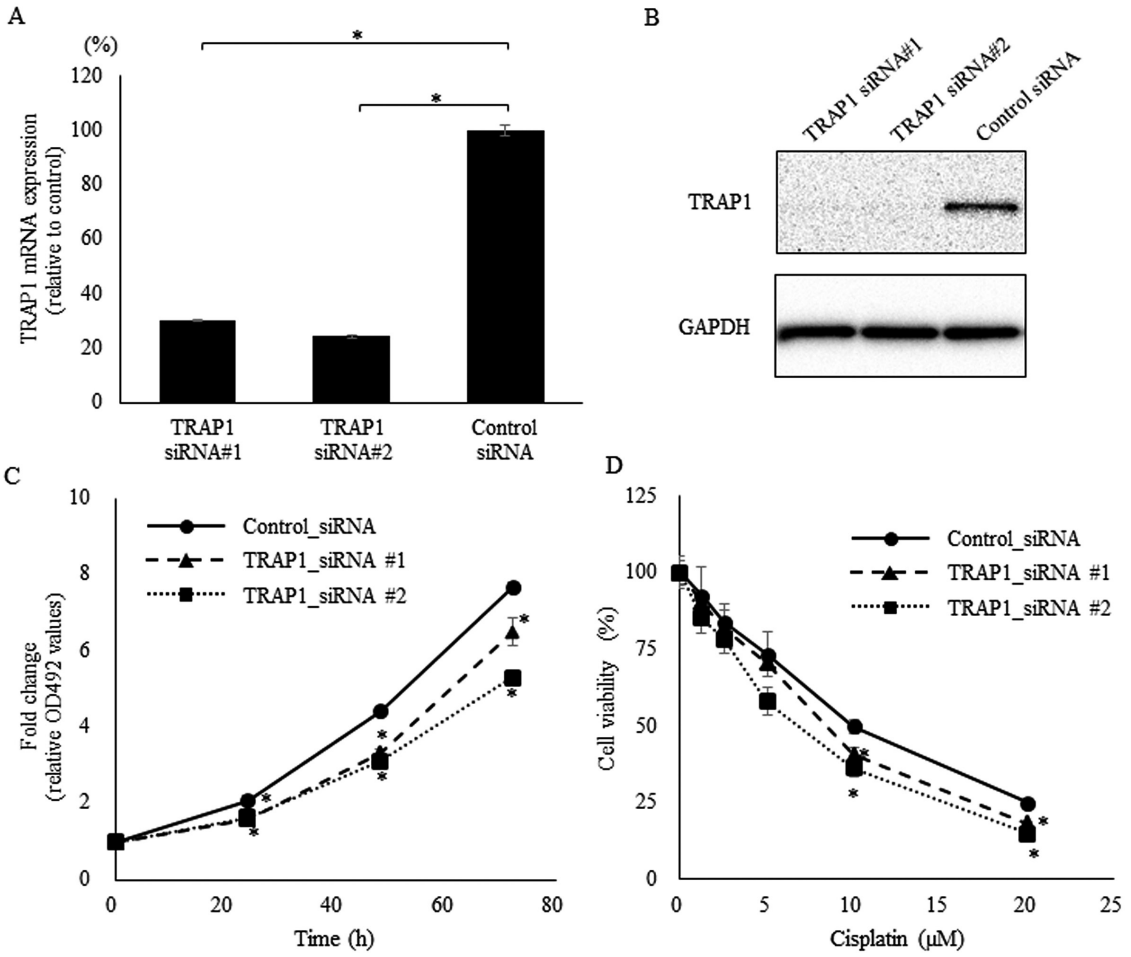

Fig. 4 Transfection of a TRAP1 siRNA inhibited cell proliferation and increased sensitivity to cisplatin. The successful knockdown of TRAP1 expression by two independent TRAP1-siRNA sequences in lung-adenocarcinoma-derived LC-2/ad cells was demonstrated by quantitative real-time RT-PCR ($P < 0.05$, A) and western blot analysis (B). The proliferation capability of TRAP1-knockdown cells was significantly suppressed compared with the negative-control-siRNA-treated cells, as assessed by MTS assay ($P < 0.05$, C). Treatment with 10 and 20 μM cisplatin for 48 h significantly reduced the viability of TRAP1-knockdown cells compared with negative-control-siRNA-treated cells, as assessed by MTS assay ($P < 0.05$ for all, D). *$P < 0.05$.

role as an oncogene or onco-suppressor depending on cancer type (22). Regarding lung cancer, Agorreta *et al.* reported that the upregulation of TRAP1 is associated with an increased risk of disease recurrence in patients with NSCLC (1). However, to our knowledge, no study has focused on the expression levels of TRAP1 in patients with lung adenocarcinoma who received platinum-based adjuvant chemotherapy.

In this study, to evaluate the utility of TRAP1 as a predictive biomarker of the efficacy of platinum-based adjuvant chemotherapy, immunohistochemistry was performed on lung tissues obtained from patients with lung adenocarcinoma who received platinum-based adjuvant chemotherapy. TRAP1 exhibited granular cytoplasmic expression in tumor cells. We confirmed that TRAP1 expression was correlated with a higher p-TNM stage and lymph node metastasis. Furthermore, TRAP1 expression was associated with a shorter disease-free survival, whereas it was not an independent predictor of recurrence in patients with lung adenocarcinoma who received platinum-based adjuvant chemotherapy. This can possibly be explained by the small size of our sample and the design of the experiment (retrospective study at a single institute). According to these results, the expression of TRAP1 may affect recurrence and chemoresistance in patients with lung adenocarcinoma. Therefore, TRAP1 may be a valuable predictive biomarker of platinum-based ad-

juvant chemotherapy and of a selected subgroup of patients who may show improved survival after this treatment modality.

A recent study indicated that TRAP1 plays an important role in cell growth and protection from apoptosis (1). In addition, the authors demonstrated that suppression of TRAP1 reduces cell proliferation and leads to apoptosis (1). Similarly, Liu *et al.* reported that TRAP1 controls genes that are related to the cell cycle and metastasis, and upregulates genes that are involved in promoting cell proliferation and tumor growth through the TNF pathway; in contrast, its downregulation is likely to lead to decreased proliferation with an increase in metastatic potential (20). In tumor cells, adaptive responses to stress, such as enhanced DNA repair and defense against oxidative stress, may lead to escape from apoptosis and may participate in the acquisition of drug resistance (8). Recently, Montesan *et al.* reported that overexpression of TRAP1 was associated with a phenotype of resistance to cisplatin-induced DNA damage and apoptosis (24), probably via the interaction between TRAP1 and cyclophilin D, inhibition of reactive oxygen species activity and production, and modulation of the mitochondrial permeability transition pores (2, 16). In fact, overexpression of TRAP1 increased resistance to 5-fluorouracil, oxaliplatin, and irinotecan in colorectal carcinoma cells (8). Based on these observations, we investigated the role of TRAP1 in the proliferation and drug sensitivity of lung adenocarcinoma cells. Transfection with TRAP1 siRNAs inhibited the proliferation capability of LC-2/ad cells. Mitochondria are a major source of ATP and are involved in cell proliferation in tumor cells (31). A precious study demonstrated that the suppression of TRAP1 expression leads to a decrease in ATP production (1). Therefore, we presumed that the inhibition of TRAP1 expression led to a decrease in ATP levels and consequently reduced cell proliferation. Moreover, the viability of TRAP1-knockdown LC-2/ad cells in the presence of cisplatin was significantly decreased compared with the negative-control-siRNA-treated cells. These data suggest that TRAP1 regulates the proliferation and sensitivity to cisplatin of lung adenocarcinoma cells. However, further studies are necessary to investigate the biological mechanisms and related signaling pathways of TRAP1.

In conclusion, we demonstrated that TRAP1 expression was significantly associated with a higher p-TNM stage, lymph node metastasis, and a shorter disease-free survival in patients with lung adenocarcinoma who received platinum-based adjuvant chemotherapy. Furthermore, knockdown of TRAP1 reduced the proliferation of, and raised the sensitivity to cisplatin in, lung adenocarcinoma cells. The expression of TRAP1 may be a useful predictive marker of the efficacy of platinum-based adjuvant chemotherapy for lung adenocarcinoma and of a selected subgroup of patients who may show improved survival after this treatment modality.

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