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...
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 treat-
ment of various cancers, including lung cancer. Cis-
platin is a highly reactive compound that binds to
DNA, inhibits DNA synthesis and RNA transcrip-
tion, affects the cell cycle, and ultimately induces
apoptosis (32). Chemotherapy using a combination
of cisplatin and vinorelbine improves survival in pa-
tients with NSCLC; however, resistance to cisplatin
occurs often and causes treatment failure (5, 29).
The mechanisms underlying the development of cis-
platin resistance include alterations in the cellular
accumulation of the drug, intracellular drug detoxifi-
cation, DNA damage repair, and apoptotic signaling
pathways (7). Although the biological causes of re-
sistance to cisplatin have not been completely eluci-
dated, the identification of predictive markers of
therapeutic efficacy is an urgent issue.

In this study, we explored novel predictive bio-
markers 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 evalu-
ated its utility as a predictive biomarker of the effect
of platinum-based adjuvant chemotherapy in patients
with lung adenocarcinoma. Furthermore, we con-
formed 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 me-
dium (FUJIFILM Wako Pure Chemical, Osaka, Ja-
pan) supplemented with 10% fetal bovine serum (MP
Biomedicals, Inc., Santa Ana, CA, USA), 100 U/mL of
tenicillin, and 100 μg/mL of streptomycin (Ther-
mo Fisher Scientific, Waltham, MA, USA) at 37°C
in 5% CO₂ and 95% humidified air. Subconfluent
cells were harvested and washed twice with phos-
phate-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. Pa-
tients who had received preoperative chemotherapy
and/or radiotherapy were excluded. Each histologi-
cal 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 patho-
logical parameters were extracted from the medical
records: age at surgery, gender, smoking habits,
pathological TNM (p-TNM) stage, tumor differenti-
ation, 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 de-
defined as the time from surgery to death from the dis-
ease 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 ap-
proved ethical guidelines and agreed to participate
in this study. They could refuse entry and discon-
tinue participation at any time. Written informed con-
sent 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-chol-
amidopropyl] dimethylammonium] propanesulfonate,
10 mM Tris (2-carboxyethyl) phosphine hydrochlo-
ride, 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 centri-
fuged at 20,000 × g for 5 min at 4°C. The supernatant
was alkylated with 1/20 volumes of 400 mM 4-vin-
ylpyridine for 1 h by mixing, followed by the addi-
tion 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 5% 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-D 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 pre-treated 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 NH₄HCO₃, 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 NH₄HCO₃. 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 pre-spotted 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 SI0366364) 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 × 10⁴ 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 × 10³ 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 × 10³ 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 ≥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).

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Table 1  Patient characteristics

| Clinicopathological characteristics | Patients, n (%) |
|-------------------------------------|-----------------|
| Age, years                          | (n = 64)        |
| <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
### Table 2: Antigenic proteins identified in the sera from patients with resected lung adenocarcinoma

| Gene symbol | Protein name | Protein accession | Function | Molecular weight (Da) | Disease-free survival of <1 year | Disease-free survival of ≥5 years |
|-------------|--------------|-------------------|----------|-----------------------|-------------------------------|---------------------------------|
| DDX5        | Heat shock protein 70 kDa, subunit 1 | P13056 | 59,277 | Catalyzes the production of spermidine from putrescine and decarboxylated S-adenosylmethionine | 38,609 | 49,950 |
| NONO        | Non-POU domain-containing octamer-binding protein | Q13478 | 74,322 | Promotes cell cycle control and signal transduction | 64,615 | 65,926 |
| ACTN1       | Alpha-actinin-1 | P12814 | 103,058 | Anchor actin to a variety of intracellular structures | 92,139 | 94,323 |
| KRT18       | Keratin, type I cytoskeletal 18 | P05783 | 48,058 | Involved in the uptake of thrombin-antithrombin complexes by hepatic cells | 49,950 | 51,615 |
| CAPZA1      | F-actin-capping protein subunit alpha-1 | P52907 | 42,231 | Formation of epithelial cell junctions | 40,428 | 42,659 |
| TUBA1C      | Tubulin alpha-1G chain | O00299 | 101,052 | Catalyzes the decarboxylation of isocitrate (ICT) into alpha-ketoglutarate | 72,857 | 74,139 |

**Notes:**
- **Function:**
  - Catalyzes the production of spermidine from putrescine and decarboxylated S-adenosylmethionine
  - Promotes cell cycle control and signal transduction
  - Anchor actin to a variety of intracellular structures
  - Involved in the uptake of thrombin-antithrombin complexes by hepatic cells
  - Formation of epithelial cell junctions
  - Catalyzes the decarboxylation of isocitrate (ICT) into alpha-ketoglutarate

**References:**
- Kuchitsu Y et al. 2023. J Mol Biol. 100, 563-585.
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...
cells compared with the negative-control-siRNA-treated cells \((P < 0.05, \text{Fig. 4A})\). 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 \((P < 0.05 \text{ for all, Fig. 4C})\). 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 \((P < 0.05 \text{ for all})\) and 20 μM cisplatin \((P < 0.05 \text{ for all})\) (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 (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-treated cells \((P < 0.05, \text{Fig. 4A})\). 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 \((P < 0.05 \text{ for all, Fig. 4C})\). 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 \((P < 0.05 \text{ for all})\) and 20 μM cisplatin \((P < 0.05 \text{ for all})\) (Fig. 4D).

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**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
TRAP1 in lung adenocarcinoma

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 IIA 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
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-

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**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). *$P < 0.05$. 

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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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