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Glutamate-cysteine ligase catalytic subunit is associated with cisplatin resistance in lung adenocarcinoma

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Abstract

Background: Cisplatin is a key drug for treating lung adenocarcinoma, and its sensitivity to cisplatin is directly related to prognosis. We aimed to reveal the roles of genes related to glutathione synthesis (glutamate-cysteine ligase catalytic subunit, GCLC) and cystine uptake (cystine/glutamate transporter, xCT and CD44v8-10) in cisplatin resistance and prognosis in lung adenocarcinoma.

Methods: We established cell lines stably expressing GCLC, xCT, standard isoform of CD44, and CD44v8-10, and investigated their sensitivities to cisplatin. We also measured mRNA expression levels of these genes in the tumor tissues from 92 lung adenocarcinoma patients. Patients were divided into high-expression (upper quartile, $N = 23$) and low-expression groups (remaining patients, $N = 69$). Recurrence-free survival, overall survival ($N = 92$), and post-recurrence survival ($N = 22$) were selected as endpoints.

Results: Compared with the control green fluorescent protein-expressing cell line (inhibitory concentration 50:6.9 μM), all the stable cell lines were more resistant to cisplatin (12.9 μM, $P = 0.025$; 13.9 μM, $P = 0.028$; 26.7 μM, $P = 0.001$; 17.7 μM, $P = 0.008$, respectively). In contrast, there was no significant difference in recurrence-free or overall survival between the high- and low-expression groups for any of the genes. However, high expression of GCLC was a risk factor for poorer post-recurrence survival (hazard ratio, 6.26; 95% confidence interval, 1.37–28.7; $P = 0.018$).

Conclusion: High expression levels of genes related to glutathione synthesis and cystine uptake promote cisplatin resistance in lung adenocarcinoma cell lines. High expression of GCLC in tumor tissue may be a potential predictor of treatment failure.

Key words: glutamate-cysteine ligase catalytic subunit, CD44, lung adenocarcinoma, drug resistance, prognosis

Introduction

Lung cancer is a leading cause of cancer-related death worldwide (1), and adenocarcinoma is the most common histological subtype of lung cancer in Japan (2). Platinum doublet therapy has generally been used as conventional chemotherapy for lung cancer patients (3). However, cancer cells often acquire gradual resistance to these anti-cancer agents, including the key lung cancer chemotherapy agent cisplatin, by decreasing their intracellular concentrations (4).

Glutathione conjugates with various electrophilic toxic substances such as cisplatin, thus enhancing their efflux and detoxifying

them (5, 6). Glutathione can also scavenge free radicals, thus aiding the survival of cancer cells exposed to environmental stresses such as reactive oxygen species (7). Glutamate cysteine ligase (GCL) is the first and rate-limiting enzyme in the biosynthesis of glutathione from cysteine, glutamate and glycine. GCL is composed of a large catalytic subunit (GCLC) and a small modified subunit (5, 8). GCLC was recently reported to be involved in tamoxifen resistance in breast cancer (9) and cisplatin resistance in head and neck squamous cell carcinoma (10). Cysteine is mainly supplied from outside of the cell as a form of cystine via a cystine-glutamate transporter, xc(-), which was previously reported to be related to cancer chemoresistance (11, 12).

Cancer stem cells have been reported to play an important role in the efficacy of cancer therapies (13–15). In particular, one of the splicing variant of CD44, CD44v8-10, which is a known cancer stem cell marker, interacts with and stabilizes the xCT subunit of the xc(-), and thereby promotes glutathione synthesis (16). CD44v8-10 has been reported to serve as a marker of poor prognosis in a type of ovarian cancer (17) and in early gastric cancer (18). Inhibition of xCT in CD44v8-10-positive cells is therefore attracting attention as a potential new therapeutic target for improving sensitivity to chemotherapy (19–21).

The impacts of the expression levels of genes related to redox regulation on lung cancer prognosis have not been well studied. In this study, we investigated the prognostic values of tumor expression levels of GCLC, xCT and CD44v8-10 in lung adenocarcinoma.

Patients, materials and methods

Cell cultures

Lenti-X 293t cells (Takara Bio USA, Inc., CA, USA) were cultured under 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum. The human lung cancer cell line NIH-H358 (ATCC) and its transgenic cell lines created as below were cultured under 5% CO_2 at 37°C in RPMI-1640 (Wako Pure Chemical Industries, Ltd) supplemented with 10% fetal bovine serum.

Preparation of lentiviral vectors for exogenous overexpression

The lung adenocarcinoma cell line H358 showed relatively low expression levels of GCLC, xCT and CD44. We therefore created new stable cell lines by infecting them with lentiviral expression vectors for each gene, using the cDNA expression plasmid (CSII-CMV-MCS-IRES2-Bsd) and packaging plasmids (pCMV-VSVG-RSV-Rev and pCAG-HIVgp) (kindly provided by RIKEN BioResource Center) (22). The complete coding DNA sites of human GCLC (NCBI Reference Sequence: NM_001498.3), xCT (NM_014331.3), CD44s (NM_001001391.1), and CD44v8-10 (NM_001001390.1) were targeted. These cDNAs were cloned individually into the multiple cloning sites of the cDNA expression plasmid. Green fluorescent protein (GFP) was also cloned as a control. The resulting cell lines were named as H358-GCLC, H358-xCT, H358-CD44s, H358-CD44v and H358-GFP, respectively. Each cDNA expression plasmid and packaging plasmids were cotransfected into Lenti-X 293t cells using the lipofection agent HilyMax (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The following day, the medium was removed and replaced with new complete culture medium with 10 μM forskolin. Each aliquot of Lenti-X 293t cells was incubated for 48 h at 37°C, and the vector-containing medium was then collected and filtered through a 0.45-μm Millipore filter.

Lentiviral infection

NIH-H358 cells were cultured to 25% confluence, the medium was then removed, and equivalent amounts of vector-containing medium and RPMI-1640 were added. Cells stably expressing each gene were obtained by selection with 10 μg/ml blasticidin S hydrochloride (Wako Pure Chemical Industries, Ltd) for 1week.

Cisplatin treatment and cell viability assay

Each cell line (1×10^4 cells in 100 μl of medium) was seeded in a well of a 96-well plate, and each seeding was performed in triplicate. The medium was changed the following day to medium containing with various concentrations of cisplatin (0–50 μM) for 48 h. Cell viability was then assessed using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. The absorbance at 450 nm was measured using a multilabel counter ARVO MX (Perkin Elmer, Inc., MA, USA). The inhibitory concentration $(IC)_{50}$ was calculated from the rate of cell survival after normalization by the probit transformation.

Patients' selection

Among patients with pathological Stage IB–IIIA lung adenocarcinoma who underwent curative resection at the University of Tokyo Hospital (Tokyo, Japan) between March 2007 and June 2013, 92 patients had tumors large enough to allow the collection of adequate tumor specimens. The following clinical and pathological data were collected retrospectively: age, sex, smoking status, tumor size, pathological TNM classification, pathological stage, lymph node invasion and vascular invasion. Recurrence-free survival (RFS) was defined as the period from the date of lung resection until the date of radiologic evidence of disease recurrence. Overall survival (OS) was defined as the period from the date of lung resection until the date of death or last recall. Post-recurrence survival was defined as the period from the date of recurrence until the date of death or last recall. This study was approved by the Institutional Review Board at the University of Tokyo Hospital, and written informed consent was obtained from each patient.

Measurements of glutathione synthesis-related gene expression

Total RNA was extracted from cultured cells or patient tumor specimens and reverse transcribed into cDNA, as described previously (23). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the following primers: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-CAC CAC CAA CTG CTT AGC AC-3′ and reverse 5′-TGG CAG GTT TTC TAG ACG G-3′; GCLC, forward 5′-ACG GAG GAA CAA TGT CCG AG-3′ and reverse 5′-TAC TGA AGC GAG GGT GCT TG-3′; xCT, forward 5'-CAG GAG AAA GTG CAG CTG AA-3′ and reverse 5′-CTC CAA TGA TGG TGC CAA TG-3′; all CD44, forward 5′-TCG CTA CAG CAT CTC TCG GA-3′ and reverse 5′-TGC TGC ACA GAT GGA GTT GG-3′; and CD44v8-10, forward 5′-GAC AGA ATC CCT GCT ACC AAT A-3′ and reverse 5′-ATG TGT CTT GGT CTC CTG ATA A-3′. The validated primers targeting GAPDH were designed using Primer Analysis Software (OLIGO; Molecular Biology Insights, Inc., CO, USA). The other primers were

optimally designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, last accessed 30 January 2018). The primer set for all CD44 was designed to amplify all splicing variant of CD44 (from exons 2 to 3), whereas that for CD44v8-10 was designed to be specific for CD44v8-10 (from variant exons 8 to 10). qRT-PCR analysis was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA) with Thunderbird SYBR qPCR Mix (TOYOBO CO., LTD, Osaka, Japan). The amplification protocol comprised an initial incubation at 95°C for 1 min and 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 45 s, followed by dissociation-curve analysis to confirm specificity. Relative expression of each gene was calculated after normalization to GAPDH using the -ΔΔCt method.

Statistical analysis

Cell line data were represented as the mean ± standard error based on at least three independent experiments, each conducted in triplicate. IC_{50} values of cell lines were compared using Student's *t*-test.

RFS, OS and post-recurrence survival were analyzed by the Kaplan–Meier method, and differences in survival were analyzed by the log-rank test. Differences were considered significant when the *P* value was <0.05. Statistical analysis was performed using SPSS version 22 (SPSS, Inc., Chicago, IL, USA).

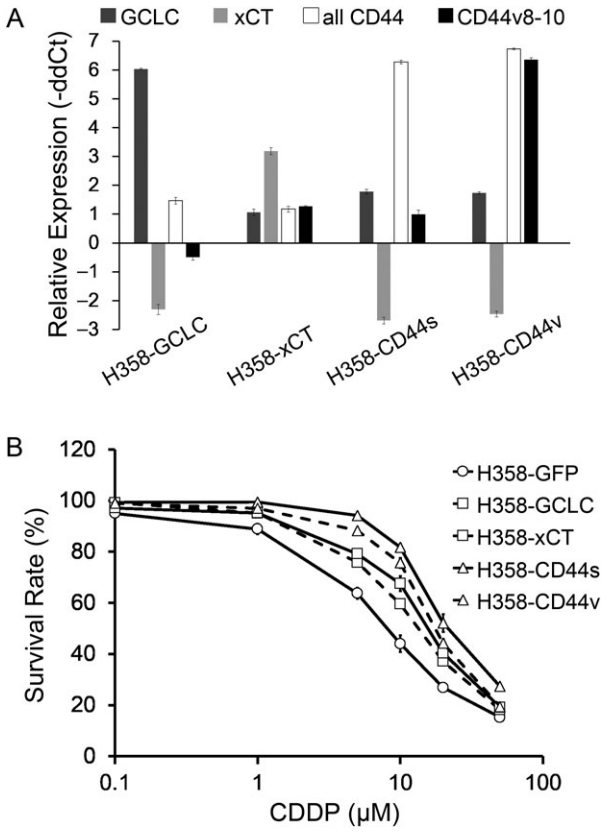

Figure 1. mRNA expression levels of GCLC, xCT, all CD44 and CD44v8-10, and survival rate of cisplatin-exposed stable H358 cell lines. (A) mRNA expression levels in the stable cell lines relative to H358-GFP analyzed by qRT-PCR with GAPDH expression as an internal calibrator. Whiskers indicate the standard error from triplicate experiments. (B) Survival rates of stable cell lines exposed to cisplatin at various concentrations. Whiskers show standard error calculated from the results of more than three independent experiments.

Results

The newly created stable cell lines, H358-GCLC, H358-xCT, H358-CD44s and H358-CD44v, showed increased expression levels of the transfected genes compared with H358-GFP (Fig. 1A). Survival curves of the cell lines exposed to various concentrations of cisplatin are shown in Fig. 1B. The IC_{50} value of each cell line was significantly increased compared with that for H358-GFP (Table 1).

The clinical characteristics of the 92 patients with lung adenocarcinoma are shown in Table 2. The gene expression levels of GCLC, xCT, all CD44 and CD44v8-10 in tumor tissue were investigated by qRT-PCR and compared with normal lung tissues (Fig. S1). The expression levels of these genes in tumors were relatively low, with higher expression levels of GCLC, xCT, all CD44 and CD44v8-10 compared with normal tissues only found in 30, 47, 8 and 28 patients, respectively. To allow us to perform statistical analysis with the same numbers of cases for all genes, we set the top 25%

Table 1. The IC_{50} values of cisplatin in the five stable cell lines

	IC_{50} (μM)	*P* value (vs. H358-GFP)
H358-GFP	6.9 ± 0.7	
H358-GCLC	12.9 ± 1.6	0.025
H358-xCT	13.9 ± 1.1	0.028
H358-CD44s	26.7 ± 0.7	0.001
H358-CD44v	17.7 ± 0.4	0.008

Data presented as mean ± standard error, taken from at least three independent experiments.

Table 2. Clinical and pathological features of lung adenocarcinoma patients

Variable	*N* = 92 (%)
Age (years)	
≤65	30 (32.6)
>65	62 (67.4)
Sex	
Male	54 (58.7)
Female	38 (41.3)
Smoking status	
Never	36 (39.1)
Current/former	54 (58.7)
pT	
1	8 (8.7)
2	71 (77.2)
3	12 (13.0)
4	1 (1.1)
Mean tumor size (mm) ± SD	37.4 ± 16.8
pN	
0	64 (69.6)
1	11 (12.0)
2	17 (18.5)
pStage	
IB	51 (55.4)
II	21 (22.8)
III	20 (21.7)
Vascular invasion	
Positive	39 (42.4)
Lymphatic invasion	
Positive	60 (65.2)

SD, standard deviation.

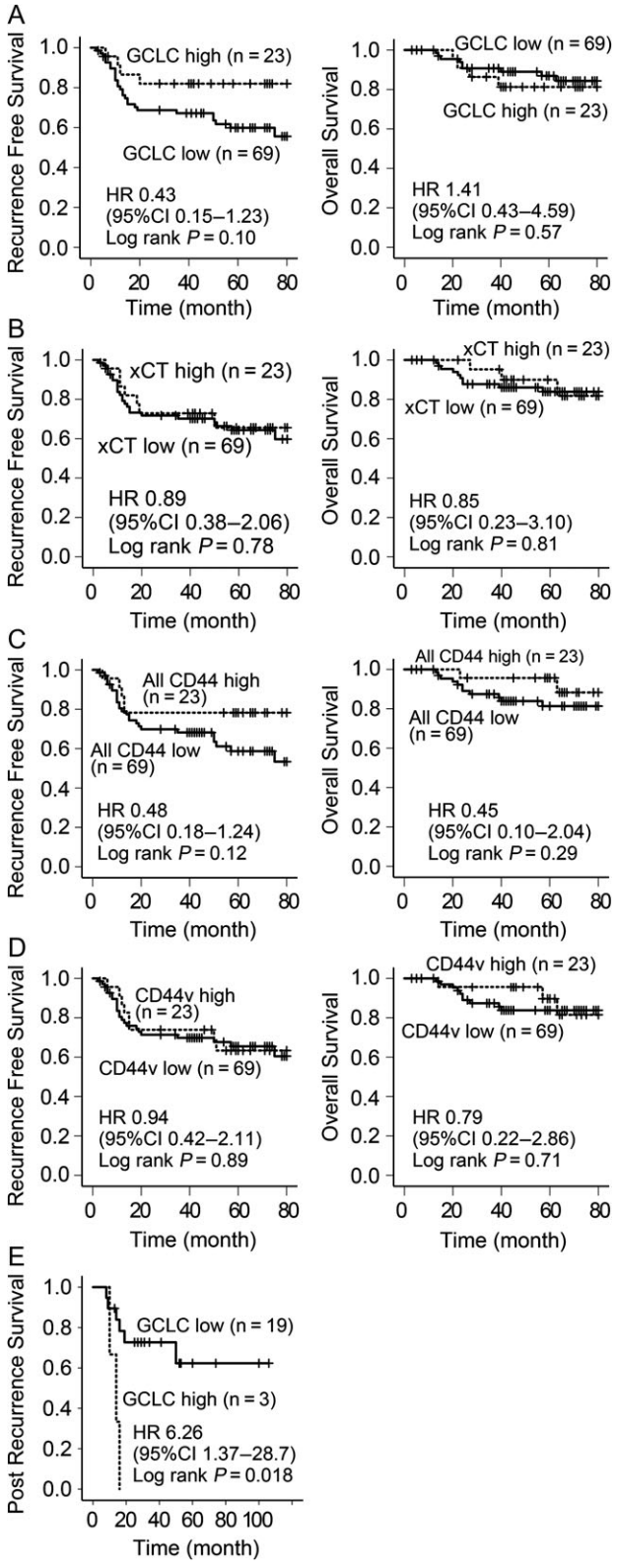

Figure 2. Survival curves of surgically treated lung adenocarcinoma patients. (A) RFS and OS according to GCLC mRNA expression level; (B) according to xCT; (C) according to all CD44; (D) according to CD44v8-10 (A–D: $N = 92$) and (E) post-recurrence survival compared with GCLC expression level ($N = 22$).

(23 patients) as the high-expression group and the remaining patients ($N = 69$) as the low-expression group, such that the expression levels were not lower than in normal lung, except for all CD44. RFS and OS curves are shown in Fig. 2A–D. There were no significant differences in survival between the high- and low-expression groups for any of the genes. High expression of GCLC tended to be associated with a better prognosis in terms of RFS (hazard ratio (HR) 0.43, 95% confidence interval (CI) 0.15–1.23, log rank $P = 0.10$), and a poorer OS (HR 1.41, 95% CI 0.43–4.59, log rank $P = 0.57$), but the differences were not significant. Twenty-two patients received radical treatment for postoperative recurrence (including stereotactic radiation therapy for metastasis, chemotherapy with cisplatin/carboplatin and/or paclitaxel, docetaxel, pemetrexed or epidermal growth factor receptor tyrosine kinase inhibitors). Among these, high GCLC expression was associated with a significantly poorer prognosis in terms of post-recurrence survival (HR 6.26, 95%CI 1.37–28.7, log rank $P = 0.006$) (Fig. 2E), but expression levels of the other genes were not (Fig. S2).

Discussion

The results of the present study showed that lung cancer cells acquired resistance to cisplatin under stable expression of GCLC, xCT, CD44s and CD44v8-10. Interestingly, xCT expression levels tended to decrease when other genes were overexpressed. The transcription factor Nrf2 has previously been reported to regulate the expression of antioxidant proteins, including GCLC and xCT (24), and Nrf2 is activated by oxidative stress (25). We speculated that the intracellular oxidative stress status may be changed by other genes, followed by inactivation of Nrf2. In addition, both standard and variant forms of CD44 contributed to cisplatin resistance. Previous studies using gastric and colon cancer cell lines showed that CD44v8-10 increased cystine uptake by stabilizing xCT on the cell membrane (16). Another study using ovarian and breast cancer cell lines, however, reported that CD44s increased P-glycoprotein-mediated multi-drug resistance (26). Further studies are needed to determine the mechanism responsible for promoting cisplatin resistance in lung cancer.

According to the present analysis, mRNA expression levels of none of the tested genes were significant prognostic factors. However, high expression of GCLC significantly shortened post-recurrence survival in patients after surgery, suggesting that it could be a potential marker for predicting treatment failure. These results appear to contradict the results obtained from the experimental cell lines. One possible reason for this apparent discrepancy is that the mRNA expression levels may not reflect the biological availability of the xCT and CD44 gene products, because they are expressed and function on the cell surface and are stabilized by each other. Higher levels of transmembrane CD44v8-10 protein expression were previously shown to correlate with good prognosis, but conversely, detection of the proteolytically cleaved and soluble extracellular domain of CD44v8-10 in patient ascites samples was correlated with a poorer prognosis (17). Furthermore, our cohort was limited to surgically treated cases, and the recurrence rate and mortality were therefore relatively low, and further studies in more cases may be needed to detect a significant difference in survival rate. In particular, only 22 of 27 recurrent patients had received curative chemotherapy or radiotherapy, and only one received

cisplatin for recurrence. Carboplatin was used as a cisplatin analog in five cases, and 17 patients used platinum agents, including for postoperative adjuvant therapy. Third, GCLC, xCT and CD44 are also expressed in normal lung tissue. Compared with the total mRNA expression levels in background lung tissues from three patients who underwent lung resection for benign lung nodules, most of our tumor cohort showed low expression levels of these genes (Fig. S1). It is also possible that the RNA was contaminated by RNA from cells other than tumor cells.

High mRNA expression of GCLC in cancer tissue is a potential predictor of cisplatin resistance in lung adenocarcinoma patients.

Supplementary data

Supplementary data are available at *Japanese Journal of Clinical Oncology* online.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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