Relationship between LAT1 Expression and Response to Platinum-based Chemotherapy in Non-small Cell Lung Cancer Patients with Postoperative Recurrence

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Abstract. *Background: The aim of this study was to investigate whether L-type amino acid transporter 1 (LAT1) expression can predict poor outcome after chemotherapy in patients with non-small cell lung cancer (NSCLC). Materials and Methods: Immunohistochemistry was carried out to examine the expression of LAT1, CD98, vascular endothelial growth factor (VEGF), Ki-67, phosphorylation of Akt (p-Akt), phosphorylation of mammalian target of rapamycin (p-mTOR) and p53 in resected lung tumor specimens obtained from 56 patients treated with platinum-based chemotherapy. Results: Positive LAT1 and CD98 expression was recognized in 45% (25/56) and 34% (19/56), respectively. In NSCLC (N=56), LAT1, CD98, VEGF, Ki-67 and p53 were significant factors for predicting poor outcome, and adenocarcinoma was an independent factor for predicting favorable prognosis. LAT1 expression was closely associated with chemoresistance. In adenocarcinoma (N=37), a statistically significant inverse relationship was observed between the expression of LAT1, VEGF and Ki-67 and epidermal growth factor receptor (EGFR) mutation, and positive expression of LAT1 and VEGF was an independent factor for predicting poor prognosis after chemotherapy. Conclusion: LAT1 expression may be useful for predicting response and outcome after systemic chemotherapy.*

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Key Words: LAT1, CD98, recurrence, advanced NSCLC, *EGFR* mutation, prognosis, chemotherapy. Ki-67, p-Akt, p-mTOR.

L-type amino acid transporter 1 (LAT1) is one of the system L amino acid transporters, transportsing large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine and histidine (1-3). LAT1 requires covalent association with the heavy chain of 4F2hc cell surface antigen (CD98) for its functional expression on the plasma membrane (1). LAT1 provides cancer cells with the essential amino acids not only for protein synthesis, but also for the stimulation of growth of cancer cells via the mammalian target of rapamycin (mTOR) (4-6). Previous studies have shown that LAT1 is highly expressed in various human neoplasms (3, 6, 7). Recently, we had documented that a positive expression of LAT1 was a significant factor in predicting poor prognosis in stage I-III non-small cell lung cancer (NSCLC) (7).

An inhibitor of system L amino acid transporter has been documented to reduce the level of phosphorylation of mTOR and its downstream signaling molecules in NSCLC (6). mTOR is a key intracellular kinase, integrating proliferation and survival pathways, and is associated with the resistance to epidermal growth factor receptor (EGFR) inhibitors.

Recent studies describe that an inhibitor of mTOR causes antitumor activity in EGFR-resistant NSCLC cell lines and xenografts (8, 9). Although LAT1 expression may be related to the resistance to EGFR inhibitors, there is still no data about the relationship between LAT1 expression and EGFR inhibitors in human neoplasms.

NSCLC is the leading cause of cancer death and has a poor prognosis. To improve the outcome of patients, biomarkers that may predict prognosis and the chemotherapeutic response should be established. In advanced disease, performance status has been consistently shown to be the most powerful prognostic tool for survival rates (10). Recently, investigators have documented several promising markers for chemotherapeutic response and overall survival in advanced NSCLC (11).

However, there is no established clinical marker which correlates with the response to the treatment and the prognosis in patients with advanced NSCLC.

Although LAT1 expression is closely associated with poor outcome after surgery in patients with resectable NSCLC (7, 12-15), it is unknown whether the expression of LAT1 has any prognostic significance after chemotherapy in patients with advanced NSCLC. In advanced NSCLC, an adequate specimen is not available for immunohistochemical staining. Immunohistochemistry has been documented as being appropriate for evaluating the expression of LAT1 in tumor specimens (6, 7). As the use of a biopsy specimen biases the immunohistochemical analysis of LAT1 expression, it may be difficult to evaluate the relationship between LAT1 expression and outcome after chemotherapy in patients with unresectable NSCLC. Therefore, we conducted this study to investigate the prognostic significance of LAT1 expression in NSCLC patients with postoperative recurrence who received platinum-based chemotherapy. In addition, CD98, Ki-67, mTOR signaling pathway, vascular endothelial growth factor (VEGF) and p53 were correlated with outcome after chemotherapy, and the relationship between these markers and *EGFR* mutation status was also examined.

Materials and Methods

Patients. Between March 2003 and May 2008, 973 consecutive patients with resectable NSCLC underwent curative resection at Shizuoka Cancer Center. One hundred and forty-six patients had a postoperative recurrence. Among them, 60 patients had received platinumed-based chemotherapy against recurrent disease after curative surgical resection of primary lung tumors, however, specimens of 4 patients were not available. Therefore, a total of 56 patients were enrolled in this immunohistochemical study. The age of the patients ranged from 45 to 77 years, and the mean age was 66 years. None of the patients had received neo-adjuvant or adjuvant chemotherapy. Tumor recurrence was confirmed by bronchoscopy and/or radiological imaging in addition to clinical course. The tumor histology was classified on the basis of the World Health Organization (WHO) criteria. Pathologic tumor-node-metastasis (TNM) stages were established using the International System for Staging Lung Cancer adopted by the American Joint Committee on Cancer and the Union Internationale Centre le Cancer (16). Patient demographics are listed in Table I. The study protocol was approved by the Institutional Review Board.

All patients were treated by platinum doublet chemotherapy as first-line chemotherapy. Twenty (36%) patients received EGFR tyrosine kinase inhibitor (TKI) therapy (gefitinib or erlotinib) as second or third line chemotherapy. We used the Response Evaluation Criteria in Solid Tumors to assess response to chemotherapy (17). Response based on target (and non-target lesions) was defined as follows: complete response (CR), disappearance of all target (non-target) lesions; partial response (PR), ≥30% reduction in size (or disappearance of one or more non-target lesions); stable disease (SD), less than 30% decrease and less than 20% increase in size (or the persistence of one or more non-target lesions); progressive disease (PD), more than 20% increase in size (or the appearance of new non-target lesions and/or progression of existing non-target lesions). The overall response was defined as the best response recorded from the start of treatment until disease progression or recurrence, confirmed by repeated assessments performed no less than 4 weeks after the criteria for response were first met.

Table I. *Patient demographics*.

Characteristic	N=56
Age (years)	
≤65/> 65	26/30
Gender	
Male/female	39/17
Performance status	
0/1	43/13
Smoking history	
Yes/no	43/13
Histology	
AC/non-AC	37/19
Recurrence pattern	
Local relapse	13
Distant metastasis	43
Pathological stage	
IA/IB/IIA/IIB/IIIA/IIIB	10/9/6/8/12/11
Metastatic site	
Lung	21
Bone	10
Brain	9
Liver	4
Lymph node	15
Other	7
Pleural effusion	7
Chemotherapy regimen	
Carboplatin+paclitaxel	29
Cisplatin+gemcitabine	18
Cisplatin+docetaxel	5
Cisplatin+vinorelbine	2
Cisplatin+amrubicin	1
Cisplatin+S-1	1
Treatment response	
Complete response	1
Partial response	17
Stable disease	28
Progressive disease	10

AC: Adenocarcinoma; pathological stage, pathological stage at the time of radical surgery.

Immunohistochemical staining. LAT1 expression was determined by immunohistochemical staining with an affinity-purified rabbit polyclonal anti-human LAT1 antibody (1.2 mg/ml; 1:3200) (18). An oligopeptide corresponding to amino acid residues 497-507 of human LAT1 (CQKLMQVVPQET) was synthesized. The *N*-terminal cysteine residue was introduced for conjugation with keyhole limpet hemocyanine. Antipeptide antibody was produced as described elsewhere (19). For immunohistochemical analysis, antiserum was affinity-purified as described previously (19). CD98 is an affinity purified goat polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Inc. California, U.S.A) raised against a peptide mapping at the carboxy terminus of CD98 of human origin. The

Table II. *Comparison of positive rate according to biomarkers between responder and non-responder.*

Variable	Positive rate (%)	Responder (CR or PR) (N=18)	Non-responder (SD or PD) (N=38)	*p*-Value
LAT1 (positive/negative)	25/31 (45%)	4/14	21/17	0.02
CD98 (positive/negative)	19/37 (34%)	4/14	15/23	0.24
p-mTOR (positive/negative)	31/25 (55%)	10/8	21/17	1.00
p-Akt (positive/negative)	32/24 (57%)	12/6	20/18	0.39
VEGF (positive/negative)	26/30 (46%)	7/11	19/19	0.57
Ki-67 (positive/negative)	28/28 (50%)	8/10	20/18	0.77
p53 (positive/negative)	28/28 (50%)	8/10	20/18	0.77

CR; Complete response; PR; partial response; SD; stable disease; PD; progressive disease; LAT1; L-type amino acid transporter 1; p-mTOR; phosphorylated mammalian target of rapamycin; VEGF; vascular endothelial growth factor.

detailed protocol for immunostaining has been published elsewhere (7, 12-15). LAT1 and CD98 expression was considered positive only if distinct membrane staining was present. Staining intensity was scored as follows: 1, ≤10% of tumor area stained; 2, 11-25% stained; 3, 26-50% stained; and 4, ≥51% stained. The tumors in which stained tumor cells made up more than 10% of the tumor were graded as positive.

Immunohistochemical staining of the other markers was performed according to the procedure described in the previous reports (7, 14, 20). The following antibodies were used: a rabitt polyclonal antibody against phospho-Akt (1:200 dilution; Abcam, Tokyo, Japan); a rabbit monoclonal antibody against phospho-mTOR (80 dilution; Cell Signaling); a murine monoclonal antibody against MIB-1 (1:40 dilution; Dako, Glostrup, Denmark), specific for human nuclear antigen Ki-67; a monoclonal antibody against VEGF (1:300 dilution; Immuno-Biological Laboratories Co.,Ltd., Japan); a mouse monoclonal antibody against p53 (D07, 1:50 dilution; Dako). The detailed protocol for Ki-67 immunostaining was published elsewhere (7). A highly cellular area of the immunostained sections was evaluated. All epithelial cells with nuclear staining of any intensity were defined as being positive. Approximately 1000 nuclei were counted on each slide. Proliferative activity was assessed as the percentage of MIB-1-stained nuclei (Ki-67 labeling index) in the sample. The expression of VEGF was quantitatively assessed according to the percentage of immunoreactive cells in a total of 1000 neoplastic cells. Staining for p-AKT and p-mTOR was considered positive if membranous and/or cytoplasmic staining was present and a semi-quantitative scoring method was used: 1= <10%, 2=10-25%, 3=25-50%, 4=51-75% and 5=>75% of cells positive. The tumors in which stained tumor cells made up more than 25% of the tumor were graded as positive. For p53, microscopic examination for the nuclear reaction product was performed and scored. According to a previous report (20), p53 expression in more than 10% of tumor cells was defined as being high expression. Sections were assessed using light microscopy in a blinded fashion by at least two of the authors.

EGFR mutation analysis. We investigated *EGFR* gene mutations by peptide nucleic acid (PNA)-locked nucleic acid (LNA) polymerase chain reaction (PCR) clamp, the detailed protocol for PNA-LNA PCR clamp method has been published elsewhere (21).

Statistical analysis. Probability values of <0.05 indicated a statistically significant difference. Fisher's exact test was used to examine the association of two categorical variables. Correlation of different variables was analyzed using the nonparametric Spearman's rank test. Follow-up for these 56 patients was conducted through patient medical records. Survival was recorded from the first day of treatment to the date of death or last follow-up, and the survival curves were calculated according to Kaplan-Meier method. Survival difference was analyzed by the log-rank test. Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Immunohistochemical analysis. LAT1, CD98, Ki-67, VEGF, p-Akt, p-mTOR and p53 immunohistochemical staining were evaluated for the surgically resected 56 primary lesions. The positive rate of these biomarkers is listed in Table II. LAT1 and CD98 immunostaining was detected in carcinoma cells in tumor tissues and localized predominantly on their plasma membrane (Figure 1). Cytoplasmic staining was rarely evident. In the present study, no expression of LAT1 and CD98 protein was observed in any normal epithelial cells of the lung, including bronchial epithelial and alveolar cells. Positive LAT1 and CD98 expression was recognized in 45% (25/56) and 34% (19/56) of cases, respectively (p=0.333). The positive rate of LAT1 expression was significantly higher in non-adenocarcinoma (NAC) (68%; 13/19) than in AC (32%; 12/37) (p=0.0022). Rate of positive CD98 expression was also significantly higher in NAC (63%; 12/19) than in AC (19%; 7/37) (p=0.0002). There was significant correlation between LAT1 and CD98 expression.

The median value of the Ki-67 labeling index was 30% (range, 2-80%) and the value of the cut-off point was 30. The staining pattern of VEGF was uniformly localized in the cytoplasm and/or membrane of neoplastic cells. The median rate of VEGF positivity was 40.0% (range, 5-80%) and the value of cut-off point for VEGF was 40. A positive expression of p-Akt, p-mTOR and p53 was recognized in 57%, 55% and 50% (Table II).

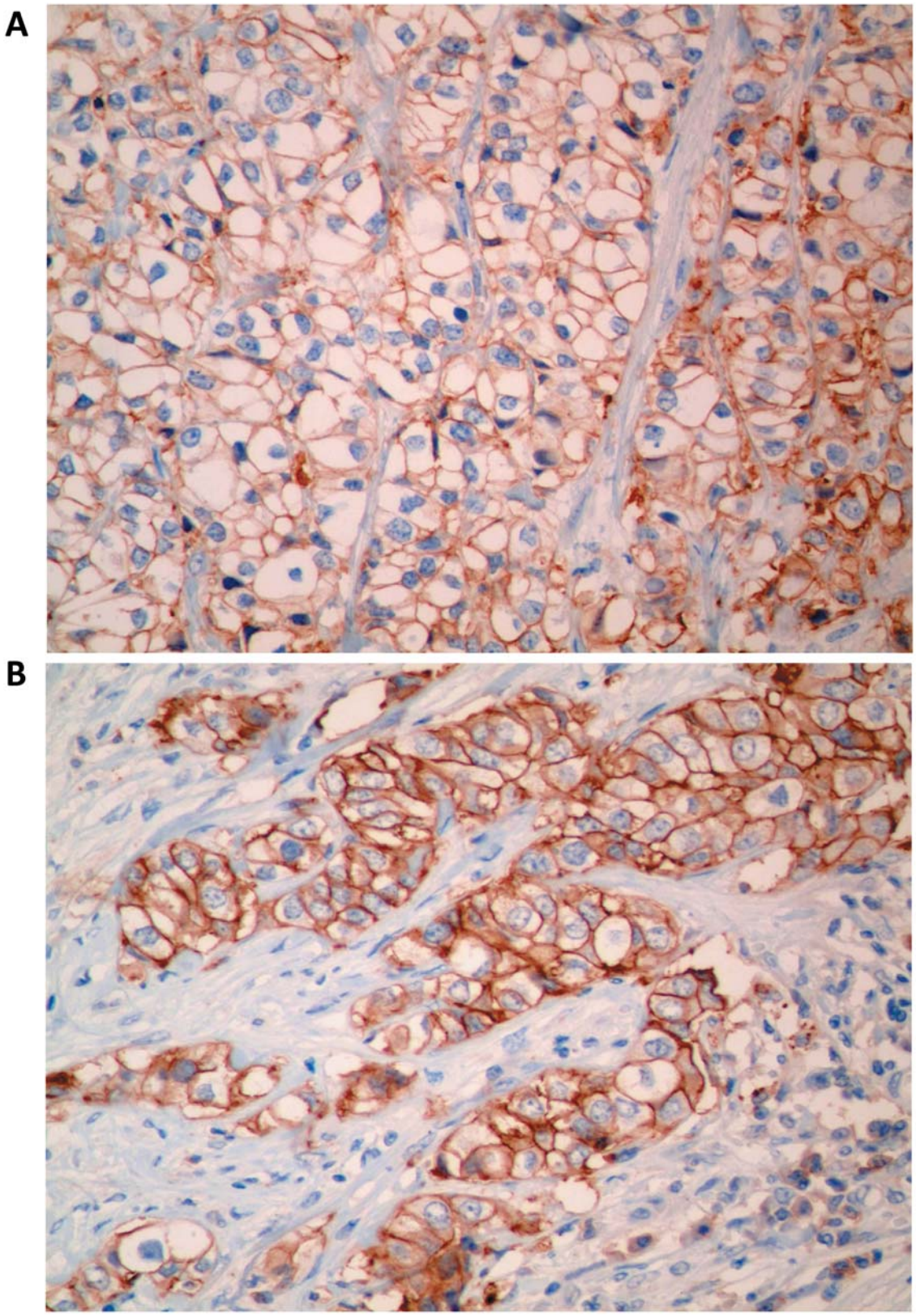

Figure 1. *Immunohistochemical analysis in adenocarcinoma patients without EGFR mutation. The score of LAT1 (A) and CD98 (B) immunostaining shown was grade 4 in both cases, and their immunostaining pattern was membranous (×400).*

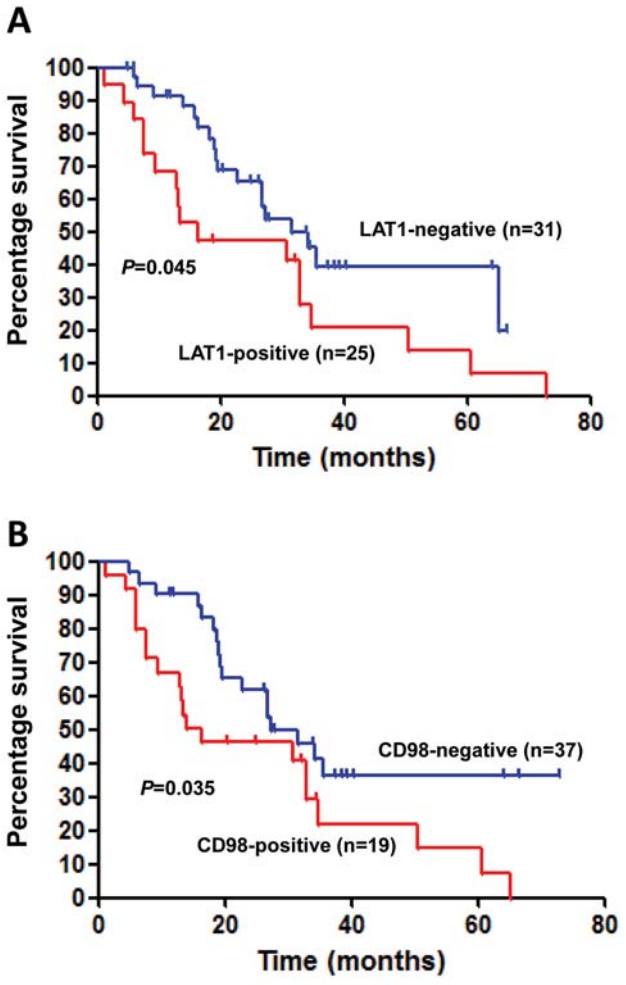

Figure 2. *Kaplan-Meier survival analysis for LAT1 (A) and CD98 (B) expression. Difference in overall survival between subgroups was analyzed using the log-rank test.*

We investigated the relationships between LAT1 expression and the other different variables. A statistically significant correlation was observed between LAT1 expression and smoker status (p=0.041), NAC (p=0.040), and a positive CD98 expression (p<0.001).

Chemotherapy and response. Chemotherapy regimens consisted of carboplatin plus paclitaxel in 29 patients, cisplatin plus gemcitabine in 18 patients, cisplatin plus docetaxel in 5 patients, and other cisplatin-based regimens in 4 patients. The median number of chemotherapy cycles was four (range, one to six). Overall response (CR+PR) was observed in 18 (32%) patients, whereas 28 (50%) patients had a response of SD and 10 (18%) patients had PD. Table II shows the comparison of the positive rate according to these biomarkers between responders (CR or PR) and non-responders (SD or PD). The response rates for patients who were LAT1-positive and -negative were 16% (4 out of 25 patients) and 45% (14 out of 31 patients), respectively. The response rates for patients who were CD98-positive and -negative were 21% (4 out of 19 patients) and 38% (14 of 37 patients), respectively. The positive expression of LAT1 was significantly associated with non-responce to chemotherapy (p=0.02), but there were no correlations between the expression of CD98, p-Akt, p-mTOR, Ki-67, VEGF, and p53 and response to chemotherapy.

Relationship between different variables and overall survival. The median progression-free and overall survival times were 7.5 months and 30.8 months, respectively. Table III shows the survival analysis of all patients (N=56). In the univariate analysis, age, smoking status, histology and the expression of LAT1, CD98, VEGF, Ki-67 and p53 were significantly associated with poor overall survival. Figure 2 shows the Kaplan-Meier survival curves for patients with positive and negative for LAT1 and CD98 expression. Multivariate analysis of these biomarkers demonstrated that the histology of adenocarcinoma was an independent prognostic factor for predicting favorable outcome after chemotherapy.

Biomarkers according to EGFR mutation and survival analysis in adenocarcinoma. Next, we investigated the positive rate of biomarkers according to *EGFR* mutations in 37 patients with adenocarcinoma (Table IV). The expression of LAT1 (p<0.001), VEGF (p=0.048) and Ki-67 (p=0.048) was significantly higher in patients (n=14) without *EGFR* mutation than those (n=23) with *EGFR* mutation.

Twelve (85%) out of 14 patients with *EGFR* mutation received gefitinib, whereas 8 (35%) out of 23 patients without *EGFR* mutation were treated by gefitinib. The median survival time of the 14 patients with *EGFR* mutation was significantly longer than that of 23 patients without *EGFR* mutation (p=0.032). In 37 patients with AC, univariate analysis demonstrated that smoking and positive expression of LAT1, VEGF, and Ki-67 were significantly associated with poor overall survival. Multivariate analysis of these biomarkers demonstrated that positive expression of LAT1 was an independent factor predicting poor outcome after chemotherapy in patients with AC.

Discussion

This was a retrospective study to evaluate the prognostic significance of LAT1 expression in relapsed NSCLC patients who received platinum-based chemotherapy. LAT1, CD98, VEGF, Ki-67 and p53 were significant factors for predicting poor outcome after chemotherapy, but these markers were not confirmed as being independent prognostic factors in the multivariate analysis. In the survival analysis of all patients

Table III. *Survival analysis of all patients (N=56).*

Variable		*p*-Value	
	MST (months)	Univariate analysis	Multivariate analysis
Age (years)			
≤65/>65	18.6/32.2	0.035	0.052
Gender			
Male/female	26.6/30.6	0.228	
PS			
0/1	27.1/25.1	0.136	
Smoking			
Yes/no	19.3/64.7	<0.001	0.128
Histology			
AC/non-AC	35.5/14.0	<0.001	0.005
LAT1			
Positive/negative	16.5/31.5	0.045	0.491
CD98			
Positive/negative	16.5/31.5	0.035	0.070
p-mTOR			
Positive/negative	33.3/22.7	0.084	
p-Akt			
Positive/negative	34.3/19.5	0.135	
VEGF			
Positive/negative	19.1/34.8	0.027	0.233
Ki-67			
Positive/negative	18.4/34.8	<0.001	0.137
p53			
Positive/negative	19.1/34.8	0.035	

MST; Median survival time; AC; adenocarcinoma; PS; performance status; LAT1; L-type amino acid transporter 1; p-mTOR; phosphorylated mammalian target of rapamycin; VEGF; vascular endothelial growth factor.

Table IV. *Biomarkers according to EGFR mutation in adenocarcinoma (N=37).*

Variable	EGFR mutation		*p*-Value
	Positive (N=14)	Negative (N=23)	
LAT1 (positive/negative)	0/14 (0%)	12/11 (52%)	<0.001
CD98 (positive/negative)	2/12 (14%)	5/18 (22%)	0.686
p-mTOR (positive/negative)	12/2 (86%)	15/8 (65%)	0.260
p-Akt (positive/negative)	12/2 (86%)	16/7 (70%)	1.000
VEGF (positive/negative)	3/11 (21%)	13/10 (57%)	0.048
Ki-67 (positive/negative)	3/11 (21%)	13/10 (57%)	0.048
p53 (positive/negative)	6/8 (43%)	12/11 (52%)	0.737

EGFR; Epidermal growth factor receptor; LAT1; L-type amino acid transporter 1; p-mTOR; phosphorylated mammalian target of rapamycin; VEGF; vascular endothelial growth factor.

(N=56), a histology of AC was an independent prognostic factor for favorable prognosis. The positive expression of LAT1 was significantly associated with non-response to chemotherapy. In AC patients (N=37), the expression of LAT1, VEGF and Ki-67 was significantly higher in patients with wild type *EGFR* than those with *EGFR* mutation. The expression of LAT1 was an independent factor predicting poor prognosis after chemotherapy in AC patients with postoperative recurrence.

LAT1 is widely expressed in primary human cancer and several cancer cell lines, where it has been shown to play essential roles in growth and survival (2, 3, 22). LAT1 is overexpressed in malignant tumors and is associated with tumor proliferation, angiogenesis, and poor survival. Recently, we documented that a positive LAT1 expression is closely associated with poor outcome in stage I-III NSCLC after curative surgery (7, 12-15). LAT1 expression was significantly associated with disease stage and lymph node metastasis (7). Our previous studies showed that positive LAT1 expression in AC was the strongest prognostic factor among patients with NSCLC (7, 23, 24). In this study, LAT1 expression was associated with resistance to chemotherapy and was an independent prognostic factor in AC with postoperative recurrence, demonstrating an inverse relationship with *EGFR* mutation. Recent reports have documented that AC patients harboring *EGFR* mutation were sensitive to gefitinib and those without *EGFR* mutation have no response to *EGFR* inhibitors (25, 26). Therefore, disease was refractory to gefitinib in patients with a positive LAT1 expression, and advanced AC patients with LAT1 expression may have a worse prognosis because of non-effective gefitinib treatment. However, it is unclear why LAT1 should be highly expressed in AC patients without *EGFR* mutation. Further study is warranted for examining the biological mechanism to the relationship between LAT1 expression and *EGFR* mutation.

Recently, several researchers have documented biomarkers predicting chemoresistance in advanced NSCLC (11). These include high expression of the DNA repair enzyme excision repair cross-complementation group 1 (ERCC1), which has demonstrated predictive value for resistance to platinum agents; uridine diphosphate-gluronosyltransferase polymorphism has demonstrated predictive value for resistance to irinotecan; and low expression levels of ribonucletide reductase have been demonstrated to have predictive value for response to gemcitabine. Incresed β-III tubulin expression has consistently been associated with a poor outcome in NSCLC patients treated by tubulin-binding agents, including vinorelbine and taxanes. However, little is known about the association between LAT1 expression and these proteins involved in chemoresistance (ERCC1 or β-III tubulin). As our sample size was small, a large-scale study should be performed for evaluating whether the overexpression of LAT1 is truly associated with resistance to platinum-based chemotherapy.

Recent experimental studies suggest that the inhibition of LAT1 activity leads to apoptotic cancer cells death by inducing intracellular depletion in neutral amino acids necessary for cancer cell growth. System L amino acid transporter inhibitor enhances anti-tumor activity in various tumor cell lines including human head and neck squamous cell carcinoma cells (Hep-2), NCI-H1395 lung cancer cells, breast adenocarcinoma cells (MCF-7 and MDA-MB-231) and human ovarian cancer cells (SKOV3,IGROV1, OVCAR3 and A2780) (6, 6, 27, 28). Therefore, inhibition of LAT1 would appear to plays a crucial role in the antitumor activity at the *in vitro* level, and it has been proposed that inhibiting LAT1 function could serve as a potential therapeutic for many types of cancer (29).

Currently, paraffin-embedded specimens obtained by bronchial biopsy are the usual materials available for immunohistochemical analysis in advanced NSCLC. But these tumor samples are sometimes too small for the detection of molecular markers in heterogenous tumor tissue by immunohistochemistry. Therefore, we used tumor samples obtained by the curative resection of primary lung tumors, and analyzed whether LAT1 expression was correlated with the survival of patients who received platinum-based chemotherapy after disease recurrence. The overall survival has documented to be superior in patients with postoperative recurrence than in those with stage IV disease (overall survival, 21.3 *versus* 13.3 months, respectively, $p<0.001$) (30). The survival time of patients in our study also seemed to be longer in comparison with that of patients with stage IV disease. As the NSCLC patients with postoperative recurrence had characteristics different from those with stage IV disease, the results of our study may not be consistent with those of patients with stage IV. Therefore, further investigation is warranted for investigating the relationship between LAT1 expression, chemotherapy response and prognosis using biopsy specimens of patients with advanced NSCLC.

In conclusion, LAT1 expression is an independent prognostic factor for overall survival in relapsed AC patients treated with platinum-based chemotherapy. Our study showed the inverse association between LAT1 expression and *EGFR* mutation. Moreover, LAT1 expression was closely related to chemoresistance in NSCLC patients with postoperative recurrence. LAT1 expression may be useful for predicting response and outcome after systemic chemotherapy. We believe that inhibition of LAT1 may increase the sensitivity toward chemotherapeutic agents and provide new and effective targeted therapy of AC with *EGFR* wild-type in the future.

Conflicts of Interest Statement

None of the Authors have any financial or personal relationships with other people or organizations that could inappropriately influence our work.

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