Am J Transl Res 2011;3(5):468-478
www.ajtr.org /ISSN:1943-8141/AJTR1109002

Original Article

LAT1 expression is closely associated with hypoxic markers and mTOR in resected non-small cell lung cancer

Kyoichi Kaira[1], Noboru Oriuchi[2], Toshiaki Takahashi[1], Kazuo Nakagawa[3], Yasuhisa Ohde[3], Takehiro Okumura[3], Haruyasu Murakami[1], Takehito Shukuya[1], Hirotsugu Kenmotsu[1], Tateaki Naito[1], Yoshikatsu Kanai[6], Masahiro Endo[4], Haruhiko Kondo[3], Takashi Nakajima[5], and Nobuyuki Yamamoto[1]

[1]Division of Thoracic Oncology, [3]Division of Thoracic Surgery, [4]Division of Diagnostic Radiology, and [5]Division of Pathology, Shizuoka Cancer Center, 1007 Shimonagakubo Nagaizumi-cho, Sunto-gun, Shizuoka, 411-8777, Japan; [2]Department of Diagnostic Radiology and Nuclear medicine, Gunma University Graduate School of Medicine, Showa-machi, Maebashi, Gunma 371-8511, Japan; [6]Division of Bio-system Pharmacology, Graduate School of Medicine, Osaka University, Osaka, Japan

Received September 17, 2011; accepted October 3, 2011; Epub October 7, 2011; Published October 31, 2011

Abstract: Aim: L-type amino acid transporter 1 (LAT1) is known to be highly expressed in various human neoplasms. However, little is known about how LAT1 is associated with glucose metabolism, hypoxia and mammalian target of rapamycin (mTOR) signaling pathway in non-small cell lung cancer (NSCLC). The aim of this study is to evaluate the relationship between LAT1 expression, and hypoxic marker and mTOR pathway in resected NSCLC. Methods: One hundred and sixty patients were included in this study. Tumors sections were stained by immunohistochemistry for LAT1, glucose transporter 1 (Glut1), hypoxia inducible factor-1α (HIF-1α), hexokinase I, vascular endothelial growth factor (VEGF), microvessel density (MVD) by determinate by CD34, epidermal growth factor receptor (EGFR), Phosphatase and tensin analog (PTEN), phosph-Akt, phosph-mTOR and phosph-S6K. Results: A positive LAT1 and CD98 expression were recognized in 36.8% (59/160) and 33.7% (54/160), respectively (p=0.640). LAT1 expression was significantly associated with CD98, hypoxic markers (Glut1, HIF-1α, hexokinase I, VEGF and CD34) and mTOR pathway (EGFR, a loss of PTEN, p-mTOR and p-S6K), especially in lung adenocarcinoma (AC). The expression profile of these biomarkers was significantly higher in non-AC than in AC, but almost these biomarkers were equally expressed between AC (n=16) and non-AC (n=43) patients with a positive LAT1 expression. Overexpression of LAT1 was closely associated with poor outcome in patient with AC. Conclusion: LAT1 expression is closely correlated with hypoxic markers and mTOR pathway in patients with resected NSCLC.

Keywords: LAT1, hypoxia, mTOR, glucose transporter, NSCLC

Introduction

Tumor cells have an increased demand for nutrients such as glucose, amino acids, fatty acids, vitamins and micronutrients. This demand is increased by availability of nutrients through vascular formation and enhanced by cellular entry of nutrients through upregulation of specific transporters. Cancer cells enhance glucose uptake via induction of glucose transporter 1 (Glut1) and Glut3, and meet their amino acid demands by inducting L-type amino acid transporter 1 (LAT1) / 4F2hc (CD98) [1, 2]. As tumor cells selectively regulate these nutrient transporter to support their progression and metastases, these nutrient transporters have potential as therapeutic targets for cancer treatment.

Amino acids are essential not only for protein synthesis but also as carbon and nitrogen source in the synthesis of purine and pyrimidine nucleotides, amino sugars, and glutathione. LAT1 is one of the transporters that is responsible for system L amino acid transporter activity [2, 3]. A light chain (LAT1) constitutes the actual transporter, and a heavy chain (4F2hc, also known as CD98) serves as a chaperone for proper recruitment of the light chain to the plasma membrane [4]. LAT1 is known to be highly expressed in various human neoplasms [2, 5-6]. Previous studies have documented that LAT1 plays an essential role not only for protein synthesis but also the stimulation of growth of cancer cells *via* mammalian target of rapamycin (mTOR) [6, 7]. Recently, we described that the expression of LAT1/4F2hc correlate with cell proliferation and angiogenesis, and LAT1/4F2hc could be a significant prognostic

factor for predicting poor outcome in non-small cell lung cancer (NSCLC) [2, 8-11]. However, little is known about how LAT1 is associated with glucose metabolism and mTOR signaling pathway in human neoplasms.

Glut 1(and also Glut3) is thought to be a possible intrinsic marker of hypoxia, and the expression of Glut 1 has been found to be regulated by hypoxia in a hypoxia-inducible factor-1 (HIF-1)-dependent way [12, 13]. One of the factors responsible for the upregulation of Glut1 in tumor cells is HIF-1α, which is considered to support tumor growth by the induction of angiogenesis via the expression of the vascular endothelial growth factor (VEGF) and also by high and anaerobic metabolic mechanisms [14]. In addition, mTOR is a downstream component of the PI3K/Akt pathway involved in the regulation of cell proliferation, angiogenesis, and metabolism. Epidermal growth factor receptor (EGFR) is an upstream component of the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR signaling pathway in human neoplasms and is overexpressed in many cancers. One in vitro study demonstrated that LAT1, like the Glut1 glucose transporter, is subject to regulation in hypoxia, although hypoxia causes divergent changes in the levels of the mRNA for LAT1 and Glut1 [15]. Ohno et al described that overexpression of 4F2hc increased the amount of Glut1 protein with increased glucose uptake in vitro, whereas, siRNA-mediated 4F2hc gene suppression markedly reduced Glut1 protein [16]. Their results suggest that 4F2hc stabilizes Glut1 protein and contributes to the regulation of not only amino acid but also glucose metabolism. In vivo levels, however, there is still no data about the relationship between LAT1, and Glut1, hypoxia and mTOR in tumor cells. Based on these backgrounds, we conducted an immunohistochemical study to examine how LAT1 expression is correlated with hypoxic markers [Glut1, Glut3, Hexokinase I, HIF-1α, VEGF and microvessel density (MVD) determinate by CD34] and EGFR pathway [EGFR, Phosphatase and tensin analog (PTEN), phospho-Akt, phospho-mTOR and phospho-S6K] in patients with resected NSCLC.

Materials and methods

Patients

Between September 2002 and October 2004, 168 consecutive patients with resectable NSCLC underwent curative resection at Shizuoka Cancer Center. Of these patients, 8 patients were excluded for further studies because the tissue specimens were not available. Thus, a total of 160 patients (97 men, 63 women) were eligible in the study. The study protocol was approved by the institutional review board.

The age of the patients ranged from 39 to 89 years, and the mean age was 67 years. None of the patients had received neo-adjuvant or adjuvant chemotherapy. The tumor stage was determined by diagnostic imaging including computed tomography and 2-[^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography (^{18}F-FDG PET). All surgical specimens were reviewed and classified according to the WHO classification by an experienced lung pathologist who was unaware of clinical or imaging findings. 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 [17]. Histologically, 106 patients had adenocarcinoma (AC), 50 had squamous cell carcinoma (SQC), and 4 had large cell carcinoma (LCC). Of the total patients, 106, 25 and 29 had stage I, II and III tumors, respectively. As postoperative adjuvant therapies, platinum-based chemotherapy, radiation, and oral administration of tegafur (a fluorouracil derivative drug) were administered to 2, 1 and 6 patients, respectively. Intraoperative therapy was not performed on any patient. The day of surgery was considered the starting day for counting postoperative survival. The follow-up duration ranged from 6 to 97 months (median, 73 months).

Immunohistochemical staining

Immunohistochemical staining was performed according to the procedure described in the previous reports [1, 2, 11]. The following antibodies were used: a rabbit polyclonal antibody against GLUT1 (AB15309, Abcam, Tokyo, Japan, 1:200 dilution); a rabbit polyclonal antibody against GLUT3 (AB15311, Abcam, Tokyo, Japan, 1:100 dilution); a rabbit monoclonal antibody against Hexokinase I (AB55144, Abcam, Tokyo, Japan, 1:200 dilution); a mouse monoclonal antibody against HIF-1α (NB100-123, Novus Biologicals, Inc., Littleton, 1:50 dilution); a monoclonal antibody against VEGF (Immuno-Biological Laboratories Co.,Ltd., Japan, 1:300

dilution); a mouse monoclonal antibody against CD34 (Nichirei, Tokyo, Japan,1:800 dilution); a mouse monoclonal antibody against EGFR (Novovastra laboratories Ltd., Newcastle, UK, 1:100 dilution); a rabbit monoclonal antibody against PTEN (Cell signaling, 50 dilution); a rabitt polyclonal antibody against phosph-Akt (Abcam, Tokyo, Japan, 1:200 dilution); a rabbit monoclonal antibody against phosph-mTOR (Cell signaling, 80 dilution); a rabbit monoclonal antibody against phosph-S6K (Cell signaling, 100 dilution). LAT1 expression was determined by immunohistochemical staining with an affinity-purified rabbit polyclonal anti-human LAT1 antibody (1.2 mg/mL; 1:3200) [18, 19]. 4F2hc (CD98) is an affinity purified goat polyclonal antibody (Santa Cruz Biotechnology, Inc. 1:200 dilution) raised against a peptide mapping at the carboxy terminus of CD98 of human origin.

The expression of Glut1, Glut3 and EGFR was considered positive if distinct membrane staining was present. Five fields (X400) were analyzed to determine the frequency of the HIF-1α stained nuclei and hexokinase I stained cytoplasm. For Glut1, Glut3, EGFR, HIF-1α and hexokinase I, 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. The detailed protocol for immunostaining was published elsewhere [1, 2]. 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.

The expression of VEGF was quantitatively assessed according to the percentage of immunoreactive cells in the total of 1000 neoplastic cells. The number of CD34-positive vessels was counted in four selected hot spots in a X 400 field (0.26 mm^2 field area). MVD was defined as the mean count of microvessels per 0.26 mm^2 field area. p-Akt, p-mTOR and p-S6K were considered positive if membranous and/or cytoplasmic staining was present, and PTEN was positive if nuclear staining. For p-Akt, p-mTOR, p-S6K and PTEN, 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. Sections were assessed using a light microscopic in a blinded fashion by at least two of the authors.

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. The Kaplan-Meier method was used to estimate survival as a function of time, and survival difference were 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

The immunohistochemical staining of these biomarkers was evaluated for the 160 primary lesions (**Figure 1**). A positive LAT1 and CD98 expression were recognized in 36.8% (59/160) and 33.7% (54/160), respectively (p=0.640). Rate of positive LAT1 expression was significantly higher in non-adenocarcinoma (AC) (15.1%; 16/106) than in AC (79.6%; 43/54) (p<0.001). Positive rate of CD98 expression was also significantly higher in non-AC (79.6%; 43/54) than in AC (19.8%; 21/106) (p<0.001). A positive rate of Glut1 and Glut3 expression was recognized in 55.6% (89/160) and 6.8% (11/160), respectively. A positive expression of HIF-1α was predominantly recognized in the cytoplasm with some nuclear staining, and was recognized in 76.2% (122/160). A positive expression of hexokinase I was recognized in the cytoplasm and/or membrane of neoplastic, and was recognized in 53.7% (86/160). The median rate of VEGF positivity was 33% (range, 3–86%), and the value of 33% was chosen as a cutoff point. Positive expression was recognized in 50.0% (80/160). The median numbers of CD34 was 25 (3–53), and the value of 25% was chosen as a cutoff point. Positive expression was recognized in 49.3% (79/126). A positive expression of EGFR, PTEN, p-AKT, p-mTOR and p-

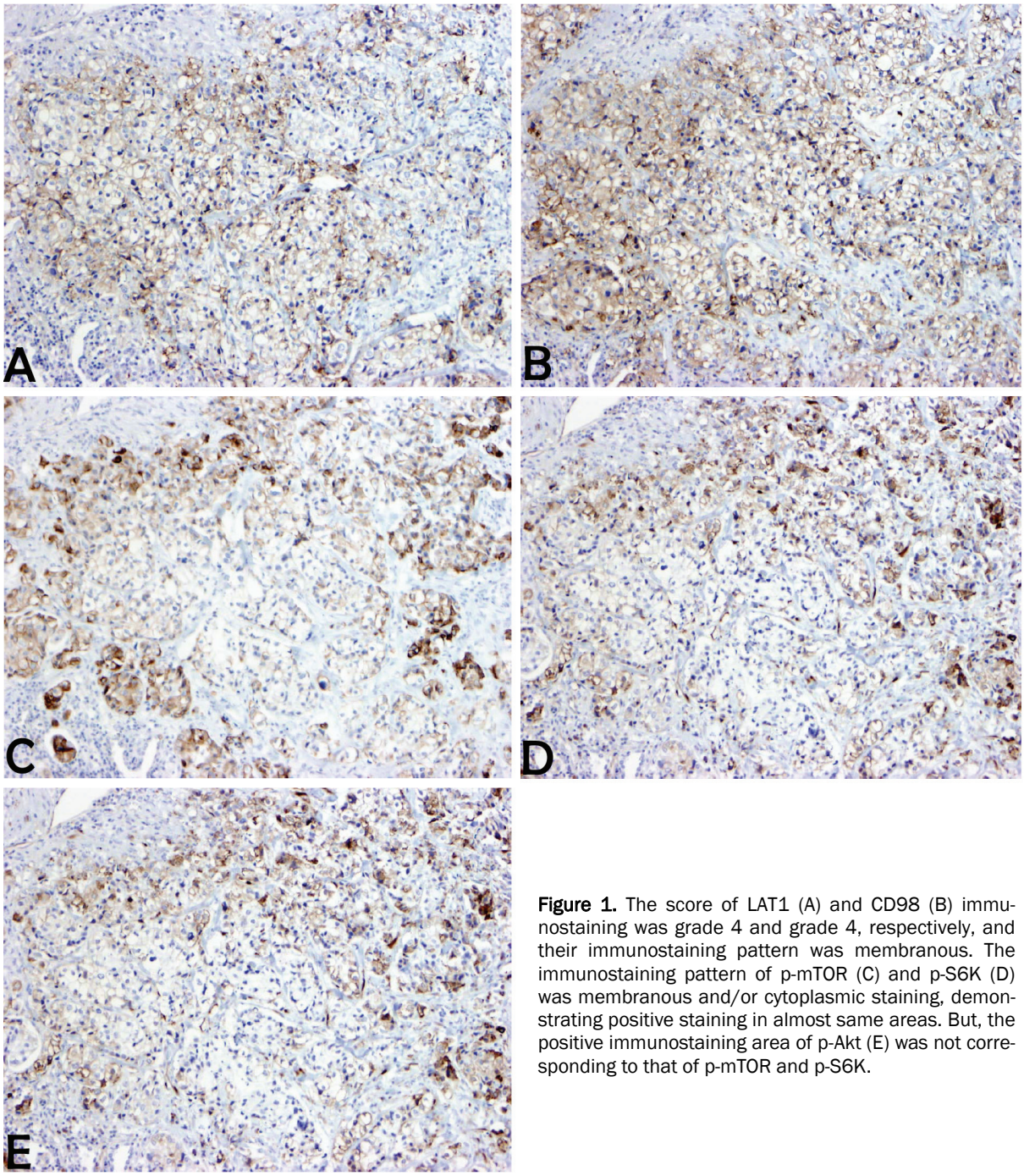

Figure 1. The score of LAT1 (A) and CD98 (B) immunostaining was grade 4 and grade 4, respectively, and their immunostaining pattern was membranous. The immunostaining pattern of p-mTOR (C) and p-S6K (D) was membranous and/or cytoplasmic staining, demonstrating positive staining in almost same areas. But, the positive immunostaining area of p-Akt (E) was not corresponding to that of p-mTOR and p-S6K.

S6K 3 was 55.0% (88/160), 21.8% (35/160), 48.1% (77/160), 60.0% (96/160) and 65.0% (104/160), respectively.

Demographics of patients according to LAT1 expression

Different variables according to LAT1 expression are listed in **Table 1**. A statistically significant difference between LAT1 positive and negative expression was observed in the gender, histology, pleural involvement, vascular invasion, lymphatic permeation, CD98, Glut1, HIF-1α, hexokinase I, VEGF, CD34, EGFR, PTEN, p-mTOR and p-S6K. Table A1 shows the different variables according to LAT1 expression in AC and

Table 1. Different variables according to LAT1 expression

Different variables		Total (n=160)	LAT1 (+) (n=59)	LAT1(-) (n=101)	*p*-value
Age	≤65 / >65	71 / 89	24 / 35	47 / 54	0.512
Gender	Male / Female	97 / 63	45 / 14	52 / 49	**0.002**
Stage	I+II / III	131 / 29	48 / 11	83 / 18	1.000
Histology	AC / Non-AC	106 / 54	16 / 43	90 / 11	**<0.001**
LN meta	Yes / No	41 / 119	16 / 43	25 / 76	0.851
PI.	Yes / No	40 / 120	22 / 37	18 / 83	**0.008**
Vas.	Yes / No	63 / 97	31 / 28	32 / 69	**0.011**
Ly.	Yes / No	61 / 99	33 / 26	28 / 73	**0.007**
CD98	Positive / Negative	54 / 96	53 / 6	11 / 90	**<0.001**
Glut1	Positive / Negative	89 / 71	53 / 6	36 / 65	**<0.001**
Glut3	Positive / Negative	11 / 149	6 / 53	5 / 96	0.215
HIF-1α	Positive / Negative	122 / 38	58 / 1	64 / 37	**<0.001**
Hexokinase I	Positive / Negative	107 / 53	53 / 6	54 / 47	**<0.001**
VEGF	Positive / Negative	80 / 80	46 / 13	34 / 67	**<0.001**
CD34	Positive / Negative	79 / 81	44 / 15	35 / 66	**<0.001**
EGFR	Positive / Negative	88 / 72	44 / 15	44 / 57	**<0.001**
PTEN	Positive / Negative	35 / 125	3 / 56	32 / 69	**<0.001**
p-Akt	Positive / Negative	77 / 83	24 / 35	53 / 48	0.189
p-mTOR	Positive / Negative	96 / 64	51 / 8	45 / 56	**<0.001**
p-S6K	Positive / Negative	104 / 56	45 / 14	59 / 42	**0.025**

Abbreviation: AC, adenocarcinoma; LAT1, L-type amino acid transporter 1; LN meta; lymph node metastases; PI., pleural involvement; Vas., vascular invasion; Ly., lymphatic permeation; Glut, glucose transporter; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin.

non-AC. In AC patients, the frequency of pleural involvement, lymphatic permeation, CD98, Glut1, HIF-1α, hexokinase I, VEGF, CD34, PTEN, p-mTOR and p-S6K was significantly higher in patients with a positive LAT1 expression than those with a negative LAT1 expression. In non-AC patients, CD98, Glut1 and p-mTOR yielded a statistically significant difference between LAT1

positive and negative expression.

Table A2 shows comparison of different biomarkers between AC and non-AC patients according to LAT1 expression. The expression of all molecular biomarkers except for Glut3 and p-S6K was significantly higher in patients with non-AC than those with AC. In 59 patients with a positive LAT1 expression, only Glut1 expression was significantly higher in non-AC than in AC, whereas, p-Akt expression was higher in AC as compared with non-AC. In 101 patients with a negative LAT1 expression, the frequency of Glu1, HIF-1α, VEGF, CD34 and EGFR was significantly higher in non-AC than in AC.

Correlation between LAT1 expression and different biomarkers

Using Spearman's rank correlation, a significant correlation was found between LAT1, and CD98, Glut1, HIF-1α, hexokinase I, VEGF, CD34, EGFR, a loss of PTEN, p-mTOR and p-S6K expression (**Table 2**). In AC patients, CD98, Glut1, HIF-1α, hexokinase I, VEGF, CD34, a loss of PTEN, p-mTOR and p-S6K expression yielded a statistically significant correlation. In non-AC patients, CD98, Glut1, a loss of PTEN, p-mTOR and p-S6K expression yielded a statistically significant correlation.

Next, we also evaluated the correlation between Glut1 expression and different biomarkers (Table A3). For all patients, a significant correlation was found between Glut1, and CD98, LAT1, HIF-1α, hexokinase I, VEGF, CD34, EGFR, a loss of PTEN, p-mTOR and p-S6K expression.

Relationship between different variables and overall survival

The 5-year survival rate was 77.5% for all patients. **Table 3** shows the survival analysis in all patients. In the univariate analysis, gender, disease stage, histology and the expression of LAT1, CD98, Glut1, HIF-1α, hexokinase I, CD34, EGFR and a loss of PTEN were significantly associated with poor overall survival. **Figure 2** shows the Kaplan-Meier survival curves in patients with positive and negative for LAT1 and CD98 expression. In AC patients, gender, disease stage, and the expression of LAT1, CD98, Glut1, HIF-1α, hexokinase I, CD34, EGFR and a loss of PTEN were significantly associated with poor overall survival. In non-AC patients, disease stage only yielded a statistically significant difference in overall survival. According to the results of univariate log-rank test, we screened prognostic factors with cut-off of $p<0.05$; gender, disease stage, histology, LAT1, CD98, Glut1, HIF-1α, hexokinase I, CD34, EGFR and a loss of PTEN. Multivariate analysis demonstrated that disease stage was an independent prognostic factor for predicting poor outcome.

Discussion

This is a clinicopathological study to investigate whether LAT1 expression is correlated with hypoxic markers and EGFR/Akt/mTOR pathway in NSCLC. LAT1 expression was significantly associated with CD98, hypoxic markers and mTOR pathway. Although the expression profile of these biomarkers was significantly higher in non-AC than in AC, almost these biomarkers were equally expressed between AC and non-AC patients with a positive LAT1 expression. In AC patients with LAT1 expression, CD98 and hypoxic markers are markedly overexpressed with the activation of EGFR pathway. However, only three biomarkers of Glut1, CD98 and p-mTOR were overexpressed in non-AC patients with LAT1 expression. Moreover, our survival analysis indicated that LAT1 expression was closely associated with poor outcome in patient with AC. Although it remains unknown whether LAT1 actually regulate the overexpression of CD98 and hypoxic markers, and the activation of mTOR pathway or not, our results suggest that these molecular markers are closely related to the overexpression of LAT1 as a prognostic factor for predicting poor outcome in NSCLC, especially lung adenocarcinoma.

Amino acid nutrition in mammalian cells is couple to cell signaling via mTOR and coordinates the signal with cell growth [20]. If tumor cells have excess amounts of amino acids, the kinase activity of mTOR is stimulated, initiating a signaling cascade and regulating protein synthesis and cell proliferation. One of the proteins whose synthesis is regulated by the mTOR pathway is HIF-1α, which is closely linked to tumor growth. In various cancer cell lines, inhibition of LAT1 has been documented to reduce the level of phosphorylation of mTOR and p70S6K, and these reports indicate the close relationship between LAT1 and mTOR pathway in the level of in vitro [6, 7, 20]. Our report is the first report to evaluate whether LAT1 is closely associated

Table 2. Correlation between LAT1 and other biomarkers

Biomarkers	Total (n=160)		AC (n=106)		Non-AC (n=54)	
	Spearman γ (95% CI)	*p*-value	Spearman γ (95% CI)	*p*-value	Spearman γ (95% CI)	*p*-value
CD98	0.842 (0.789 – 0.883)	**<0.001**	0.650 (0.520 – 0.751)	**<0.001**	0.765 (0.622 – 0.858)	**<0.001**
Glut1	0.563 (0.441 – 0.663)	**<0.001**	0.296 (0.105 – 0.466)	**0.002**	0.337 (0.071 – 0.558)	**0.018**
Glut3	0.068 (-0.092 – 0.225)	0.388	0.005 (-0.191 – 0.202)	0.953	0.213 (-0.062 – 0.459)	0.117
HIF-1α	0.381 (0.236 – 0.510)	**<0.001**	0.232 (0.036 – 0.410)	**0.017**	0.149 (-0.128 – 0.406)	0.275
Hexokinase I	0.383 (0.238 – 0.512)	**<0.001**	0.237 (0.041 – 0.414)	**0.014**	0.097 (-0.180 – 0.360)	0.480
VEGF	0.452 (0.316 – 0.571)	**<0.001**	0.294 (0.102 – 0.464)	**0.002**	-0.010 (-0.282 – 0.263)	0.942
CD34	0.448 (0.310 – 0.567)	**<0.001**	0.254 (0.060 – 0.430)	**0.008**	0.168 (-0.109 – 0.421)	0.219
EGFR	0.356 (0.209 – 0.488)	**<0.001**	0.086 (-0.112 – 0.278)	0.380	0.066 (-0.209 – 0.333)	0.627
PTEN	-0.362 (-0.493 – -0.214)	**<0.001**	-0.249 (-0.425 – -0.054)	**0.010**	-0.274 (-0.509 – -0.001)	**0.042**
p-Akt	-0.148 (-0.300 – 0.001)	0.061	0.189 (-0.008 – 0.372)	0.053	-0.038 (-0.308 – 0.236)	0.778
p-mTOR	0.453 (0.316 – 0.571)	**<0.001**	0.409 (0.231 – 0.561)	**<0.001**	0.423 (0.170 – 0.624)	**0.001**
p-S6K	0.202 (0.044 – 0.350)	**0.010**	0.264 (0.071 – 0.439)	**0.006**	0.404 (0.148 – 0.610)	**0.002**

Abbreviation: AC, adenocarcinoma; LAT1, L-type amino acid transporter 1; Glut, glucose transporter; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin; 95CI, 95% confidence interval.

with mTOR pathway in the level of human neoplasms. Our results suggest that the phosphorylation of mTOR has a regulatory role for the overexpression of LAT1 regardless of the histological type of NSCLC. In the level of cell lines, LAT1 expression was also correlated with the phosphorylation of mTOR and p70S6K in both squamous cell carcinoma and adenocarcinoma cell lines [6, 7, 20]. Among various oncogenic signaling pathway, PI3K/Akt is the most important as the upstream effectors of mTOR. However, LAT1 expression was not correlated

Table 3. Univariate analysis in overall survival

Different variables		Total (n=160)		AC (n=106)		Non-AC (n=54)	
		5-yr rate (%)	*p*-value	5-yr rate (%)	*p*-value	5-yr rate (%)	*p*-value
Age	≤65 / >65	81.6 / 73.0	0.145	82.3 / 81.8	0.980	58.8 / 80.0	0.093
Gender	M / F	72.1 / 85.7	**0.043**	75.9 / 88.4	**0.031**	67.4 / 63.4	0.844
Stage	I+II / III	83.9 / 48.2	**<0.001**	89.4 / 52.3	**<0.001**	73.9 / 37.5	**0.003**
Histology	AC / Non-AC	82.0 / 68.5	**0.015**	NA	NA	NA	NA
LAT1	P / N	71.1 / 81.1	**0.034**	68.7 / 84.4	**0.009**	72.0 / 63.6	0.612
CD98	P / N	70.3 / 82.2	**0.029**	71.4 / 84.7	**0.035**	69.7 / 63.6	0.640
Glut1	P / N	67.4 / 88.6	**<0.001**	66.6 / 91.0	**<0.001**	68.0 / 75.0	0.783
Glut3	P / N	81.8 / 76.3	0.756	85.7 / 81.8	0.994	75.0 / 65.3	0.532
HIF-1α	P / N	72.9 / 89.4	**0.016**	77.4 / 91.6	**0.034**	67.3 / 68.5	0.989
Hexokinase I	P / N	71.0 / 90.5	**0.005**	74.5 / 91.4	**0.004**	66.6 / 66.7	0.993
VEGF	P / N	73.7 / 81.2	0.143	75.6 / 85.5	0.092	72.0 / 68.5	0.088
CD34	P / N	68.3 / 86.4	**0.001**	71.0 / 88.2	**0.016**	63.4 / 76.9	0.148
EGFR	P / N	68.1 / 88.9	**<0.001**	72.7 / 88.7	**0.001**	63.6 / 80.0	0.146
PTEN	P / N	88.5 / 73.5	**0.009**	90.3 / 78.6	**0.021**	75.0 / 64.0	0.531
p-Akt	P / N	74.0 / 80.7	0.407	75.8 / 90.9	**0.011**	66.6 / 69.2	0.675
p-mTOR	P / N	76.0 / 79.6	0.472	80.3 / 84.0	0.765	70.0 / 64.2	0.791
p-S6K	P / N	75.9 / 80.3	0.332	78.2 / 89.1	0.097	71.4 / 63.1	0.790

Abbreviation: Abbreviation: AC, adenocarcinoma; LAT1, L-type amino acid transporter 1; Glut, glucose transporter; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin; 95CI, 95% confidence interval; P/N, Positive / Negative; NA, not applicable; 5-yr rate, 5-year survival rate.

with the phosphorylation of Akt. Considering the results of our study and previous in vitro data, LAT1 seems to regulate multiple cellular activities including tumor cell proliferation and metastases through mTOR pathway. Recently, the inhibition of mTOR signaling pathway is believed to be a promising therapeutic option in various neoplasms. Therefore, LAT1 may have potential as drug targets for cancer therapy.

Malignant cells demonstrated increased glucose uptake in vitro and in vivo [21], and this

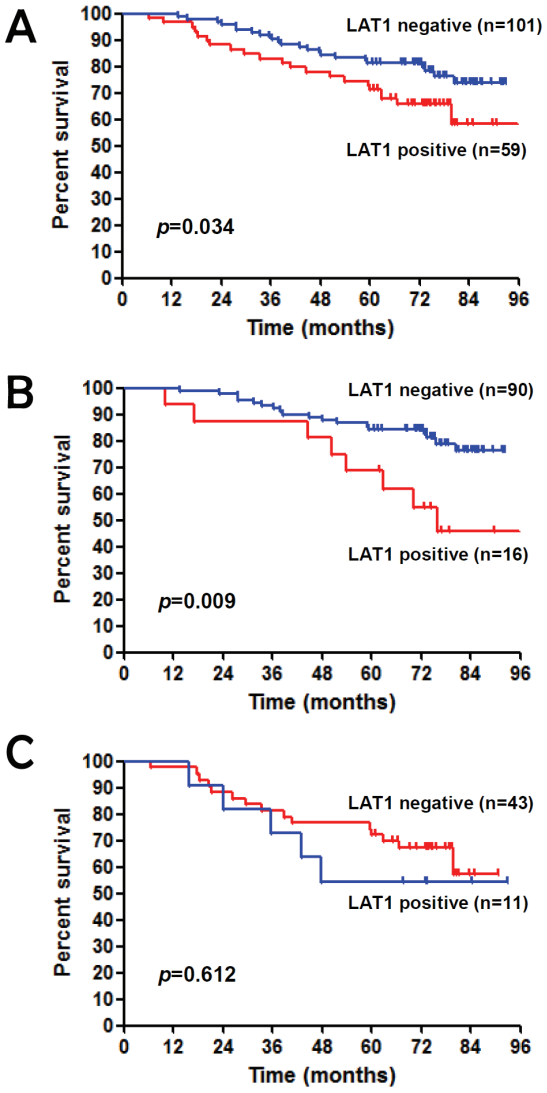

Figure 2. Kaplan-Meier survival analysis for LAT1 expression in all patients (A), adenocarcinoma patients (B) and non-adenocarcinoma (C). Difference in overall survival between subgroups was analyzed using log-rank test.

process is thought to be mediated by glucose transporters. Our results indicated that LAT1 was closely correlated with Glut1 expression but not Glut3. HIF-1α is known to stimulate the transcription of glycolytic enzymes and angiogenesis, and is a downstream component of mTOR pathway. In the present study, Glut1 expression was significantly correlated with CD98, HIF-1α and the phosphorylation of mTOR. Although in vitro data also indicated that CD98 stabilizes Glut1 and increase glucose uptake, our results suggest that CD98 plays a crucial role on the regulation of not only amino acid metabolism by LAT1 but also glucose metabolism by Glut1 in patients with NSCLC. In vitro study demonstrated that hypoxia regulate the activity of LAT1, thus HIF-1α may be necessary for the expression of LAT1-mediated amino acid transport. Amino acid and glucose metabolism in tumor cells are regulated by HIF-1 or CD98, and HIF-1α is upregulated by hypoxia and induces Glut1 and LAT1 expression. Therefore, LAT1 may have a positive correlation with Glut1 expression. Since it remains unclear whether HIF-1α is closely associated with the regulation of LAT1 expression in cancer cells, further study is warranted.

We have previously described the difference of LAT1 expression profile between AC and non-AC in human neoplasms [2, 8]. LAT1 expression was significantly higher in SQC or LCC than in AC. Our results indicated that the expression of hypoxic markers and the activation of mTOR pathway are markedly increasing in AC with a positive LAT1 expression as compared to non-AC. In addition, LAT1 expression has a prognostic significance in patients with AC, which is supported by previous study [9]. The role as a prognostic factor of LAT1 may be associated with the difference of hypoxic markers or mTOR pathway between AC and non-AC.

Previous in vitro studies suggest that the inhibition of LAT1 has potential as new option for cancer therapy. However, further investigation is warranted for confirming the therapeutic potential by in vivo data. In our study, LAT1 expression is correlated with hypoxia and Glut1 expression, ^{18}F-FDG PET has been investigated for monitoring tumor response to treatment [22, 23]. The amount of ^{18}F-FDG uptake in human neoplasm is determined by the presence of glucose metabolism, hypoxia and angiogenesis [1]. Thus, ^{18}F-FDG PET may be useful for monitoring the response of NSCLC treated by LAT1-targeted therapy.

In conclusion, LAT1 expression is closely correlated with hypoxic markers and mTOR pathway. These molecular markers are markedly increasing in AC with a positive LAT1 expression, and this may be closely related to the overexpression of LAT1 as a prognostic factor for predicting poor outcome in AC. It is necessary to investigate whether these hypoxic markers is associ-

ated with the regulation of LAT1 expression in human neoplasms. If the relationship between these hypoxic markers and LAT1 expression is clearly elucidated, the radiological modalities such as ^{18}F-FDG PET will be useful for the therapeutic monitoring of LAT1 inhibitor in future.

Acknowledgements

This work was supported in part by Grant 21790793 (K. K) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and National Hospital Organization Policy Based Medical Services. We thank all staffs of pathology department in Shizuoka Cancer Center for their technical assistance of immunohistochemical analysis.

Address correspondence to: Dr. Kyoichi Kaira, Division of Thoracic Oncology, Shizuoka Cancer Center, 1007 Shimonagakubo Nagaizumi-cho Sunto-gun, Shizuoka, 411-8777, Japan Tel:+81 55 989 5222; Fax:+81 55 989 5634; E-mail: kkaira1970@yahoo.co.jp

References

[1] Kaira K, Endo M, Abe M, Nakagawa K, Ohde Y, Okumura T, Takahashi T, Murakami H, Tsuya A, Nakamura Y, Naito T, Hayashi I, Serizawa M, Koh Y, Hanaoka H, Tominaga H, Oriuchi N, Kondo H, Nakajima T and Yamamoto N. Biologic correlation of 2-[^{18}F]-fluoro-2-deoxy-D-glucose uptake on positron emission tomography in thymic epithelial tumors. J Clin Oncol 2010; 28: 3746-3753.

[2] Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Tanaka S, Ishizuka T, Kanai Y, Endou H, Nakajima T and Mori M. Prognostic significance of L-type amino acid transporter 1 expression in resectable stage I-III nonsmall cell lung cancer. Br J Cancer 2008; 98: 742-748.

[3] Kanai Y, Segawa H, Miyamoto K, Uchino H, Takeda E and Endou H. Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). J Biol Chem 1998; 273: 23629-23632.

[4] Oxender DL, Christensen HN. Evidence for two types of mediation of neutral amino acid transport in Ehrlich cells. Nature 1963; 197: 765-767.

[5] Fuchs BC, Bode BP. Amino acid transporters ASCT2 and LAT1 in cancer: Partners in crime? Seminars in Cancer Biology 2006; 15: 254-266.

[6] Yamauchi K, Sakurai H, Kimura T, Wiriyasermkul P, Nagamori S, Kanai Y and Kohno N. System L amino acid transporter inhibitor enhances anti-tumor activity of cisplatin in a head and neck squamous cell carcinoma cell line. Cancer Letters 2009; 276: 95-101.

[7] Imai H, Kaira K, Oriuchi N, Shimizu K, Tominaga H, Yanagitani N, Sunaga N, Ishizuka T, Nagamori S, Promchan K, Nakajima T, Yamamoto N, Mori M and Kanai Y. Inhibition of L-type amino acid transporter 1 has antitumor activity in non-small cell lung cancer. Anticancer Res 2010; 30: 4819-4828.

[8] Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Tanaka S, Ishizuka T, Kanai Y, Endou H, Nakajima T and Mori M. L-type amino acid transporter 1 and CD98 expression in primary and metastatic sites of human neoplasms. Cancer Sci 2008; 99: 2380-2386.

[9] Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Ishizuka T, Kanai Y, Nakajima T and Mori M. Prognostic significance of L-type amino acid transporter 1 (LAT1) and 4F2 heavy chain (CD98) expression in stage I pulmonary adenocarcinoma. Lung Cancer 2009; 66: 120-126.

[10] Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Kawashima O, Kamide Y, Ishizuka T, Kanai Y, Nakajima T and Mori M. CD98 expression is associated with poor prognosis in resected non-small cell lung cancer with lymph node metastasis. Ann Surg Oncol 2009; 16: 3473-3481.

[11] Kaira K, Oriuchi N, Shimizu K, Ishikita T, Higuchi T, Imai H, Yanagitani N, Sunaga N, Hisada T, Ishizuka T, Kanai Y, Endou H, Nakajima T, Endo K and Mori M. Correlation of angiogenesis with ^{18}F-FMT and ^{18}F-FDG uptake in non-small cell lung cancer. Cancer Sci 2009; 100: 753-758.

[12] Vleugel MM, Greijer AE, Shvarts A, van der Groep P, van Berkel M, Aarbodem Y, van Tinteren H, Harris AL, van Diest PJ and van der Wall E. Differential prognostic impact of hypoxia induced and diffuse HIF-1 alpha expression in invasive breast cancer. J Clin Pathol 2005; 58: 172-177.

[13] Elson DA, Ryan HE, Snow JW, Johnson R and Arbeit JM. Coordinate up-regulation of hypoxia inducible factor (HIF)-1a and HIF-1 target genes during multi-stage epidermal carcinogenesis and wound healing 1. Cancer Res 2000; 60: 6189-6195.

[14] Ryan HE, Polni M, McNulty W, Elson D, Gassmann M, Arbeit JM and Johnson RS. Hypoxia-inducible factor-1α is a positive factor in solid tumor growth. Cancer Res 2000; 60: 4010-4015.

[15] Soado RJ, Li JY, Tsukamoto H and Pardridge WM. Hypoxia induces de-stabilization of the LAT1 large neural amino acid transporter mRNA in brain capillary endothelial cells. J Neurochem 2003; 85: 1037-1042.

[16] Ohno H, Nakatsu Y, Sakoda H, Kushiyama A, Ono H, Fujishiro M, Otani Y, Okubo H, Yoneda M, Fukushima T, Tsuchiya Y, Kamata H, Nishimura F, Kurihara H, Katagiri H, Oka Y and Asano T. 4F2hc stabilizes GLUT1 protein and increases glucose transport activity. Am J Physiol Cell Physiol 2011; 286: C1047-1054.

[17] Mountain CF. Revision in the International System for Staging Lung Cancer. Chest 1997; 11: 1710-1717.

[18] Nawashiro H, Otani N, Shinomiya N, Fukui S, Ooigawa H, Shima K, Matsuo H, Kanai Y and Endou H. L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors. Int J Cancer 2006; 119: 484-492.

[19] Chairoungdua A, Segawa H, Kim JY, Miyamoto K, Haga H, Fukui Y, Mizoguchi K, Ito H, Takeda E, Endou H and Kanai Y. Identification of an amino acid transporter associated with the cystinuria-related type II membrane glycoprotein. J Biol Chem 1999; 274: 28845-28848.

[20] Fan X, Ross DD, Arakawa H, Ganapathy V, Tamai I and Nakanishi T. Impact of system L amino acid transporter 1 (LAT1) on proliferation of human ovarian cancer cells: A possible target for combination therapy with proliferative aminopeptidase inhibitors. Biochem Pharmacol 2010; 80: 811-8.

[21] Younes M, Brown RW, Stephenson M, Gondo M and Cagle PT. Over expression of Glut1 and Glut3 in stage I nonsmall cell lung carcinoma is associated with poor survival. Cancer 1997; 80: 1046-1051.

[22] Pöttgen C, Levegrün S, Theegarten D, Marnitz S, Grehl S, Pink R, Eberhardt W, Stamatis G, Gauler T, Antoch G, Bockisch A and Stuschke M. Value of ^{18}F-fluoro-2-deoxy-D-glucose-positron emission tomography/computed tomography in non-small-cell lung cancer for prediction of pathologic response and times to relapse after neoadjuvant chemoradiotherapy. Clin Cancer Res 2006; 12: 97-106.

[23] Sunaga N, Oriuchi N, Kaira K, Yanagitani N, Tomizawa Y, Hisada T, Ishizuka T, Endo K and Mori M. Usefulness of FDG-PET for early prediction of the response to gefitinib in non-small cell lung cancer. Lung Cancer 2008; 59: 203-210.

Table A1.

Different variables according to LAT1 expression in AC and non-AC

Different variables		Adenocarcinoma				Non-adenocarcinoma			
		Total (n=106)	LAT1 (+) (n=16)	LAT1 (-) (n=90)	*p*-value	Total (n=54)	LAT1 (+) (n=43)	LAT1 (-) (n=11)	*p*-value
Age	≤65 / >65	51 / 55	10 / 6	41 / 49	0.279	20 / 34	14 / 29	6 / 5	0.293
Gender	M / F	54 / 52	11 / 5	43 / 47	0.175	43 / 11	34 / 9	9 / 2	1.000
Stage	I+II / III	86 / 20	12 / 4	74 / 16	0.497	46 / 8	36 / 7	10 / 1	1.000
LN meta	Yes / No	23 / 83	3 / 13	20 / 70	1.000	20 / 34	14 / 29	6 / 5	0.293
Pl.	Yes / No	27 / 79	11 / 5	16 / 74	**<0.001**	12 / 42	11 / 32	1 / 10	0.421
Vas.	Yes / No	34 / 72	8 / 8	26 / 64	0.143	29 / 25	23 / 20	6 / 5	1.000
Ly.	Yes / No	34 / 72	10 / 6	24 / 66	**0.007**	27 / 27	23 / 20	4 / 7	0.500
CD98	P / N	21 / 85	12 / 4	9 / 81	**<0.001**	43 / 11	41 / 2	2 / 9	**<0.001**
Glut1	P / N	39 / 67	11 / 5	28 / 62	**0.009**	50 / 4	42 / 1	8 / 3	**0.023**
Glut3	P / N	7 / 99	2 / 14	5 / 85	0.284	4 / 50	4 / 39	0 / 11	0.570
HIF-1α	P / N	69 / 37	16 / 0	53 / 37	**0.001**	52 / 2	42 / 1	10 / 1	0.369
Hexo I	P / N	60 / 46	14 / 2	46 / 44	**0.006**	48 / 6	39 / 4	9 / 2	0.590
VEGF	P / N	36 / 70	11 / 5	25 / 65	**0.003**	43 / 11	35 / 8	8 / 3	0.676
CD34	P / N	38 / 68	10 / 6	28 / 62	**0.023**	41 / 13	34 / 9	7 / 4	0.429
EGFR	P / N	44 / 62	9 / 7	35 / 55	0.271	44 / 10	35 / 8	9 / 2	1.000
PTEN	P / N	31 / 75	1 / 15	30 / 60	**0.035**	4 / 50	2 / 41	2 / 9	0.180
p-Akt	P / N	61 / 45	13 / 3	49 / 41	0.055	15 / 39	11 / 32	4 / 7	0.475
p-mTOR	P / N	56 / 50	15 / 1	41 / 49	**<0.001**	40 / 14	36 / 7	4 / 7	**0.003**
p-S6K	P / N	69 / 37	15 / 1	54 / 36	**0.009**	35 / 19	30 / 13	5 / 6	0.166

Abbreviation: AC, adenocarcinoma; LAT1, L-type amino acid transporter 1; LN meta; lymph node metastases; Pl., pleural involvement; Vas., vascular invasion; Ly., lymphatic permeation; Glut, glucose transporter; HIF-1α, hypoxia inducible factor-1 alpha; Hexo I, Hexokinase I; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin; M / F, Male / Female; P/N, Positive / Negative.

Table A2.

Comparison of biomarkers between adenocarcinoma and non-adenocarcinoma according to LAT1 expression

Different variables		Total patients (n=160)			LAT1 expression (+) (n=59)			LAT1 expression (-) (n=101)		
		AC (n=106)	**Non-AC** (n=54)	*p*-value	**AC** (n=16)	**Non-AC** (n=43)	*p*-value	**AC** (n=90)	**Non-AC** (n=11)	*p*-value
CD98	Positive / Negative	21 / 85	43 / 11	**<0.001**	12 / 4	41 / 2	0.537	9 / 81	2 / 9	0.342
Glut1	Positive / Negative	39 / 67	50 / 4	**<0.001**	11 / 5	42 / 1	**0.004**	28 / 62	8 / 3	**0.015**
Glut3	Positive / Negative	7 / 99	4 / 50	1.000	2 / 14	4 / 39	0.658	5 / 85	0 / 11	1.000
HIF-1α	Positive / Negative	69 / 37	52 / 2	**<0.001**	16 / 0	42 / 1	1.000	53 / 37	10 / 1	**0.048**
Hexokinase I	Positive / Negative	60 / 46	48 / 7	**<0.001**	14 / 2	39 / 4	0.658	46 / 44	9 / 3	0.137
VEGF	Positive / Negative	36 / 70	43 / 11	**<0.001**	11 / 5	35 / 8	0.486	25 / 65	8 / 3	**0.004**
CD34	Positive / Negative	38 / 68	41 / 13	**<0.001**	10 / 6	34 / 9	0.312	28 / 62	7 / 4	**0.045**
EGFR	Positive / Negative	44 / 62	44 / 10	**<0.001**	9 / 7	35 / 8	0.089	35 / 55	9 / 2	**0.009**
PTEN	Positive / Negative	31 / 75	4 / 50	**0.001**	1 / 15	2 / 41	1.000	30 / 60	2 / 9	0.494
p-Akt	Positive / Negative	63 / 43	15 / 39	**<0.001**	14 / 2	11 / 32	**<0.001**	49 / 41	4/ 7	0.342
p-mTOR	Positive / Negative	56 / 50	40 / 14	**0.010**	15 / 1	36 / 7	0.426	41 / 49	4 / 7	0.750
p-S6K	Positive / Negative	58 / 38	35 / 19	0.726	15 / 1	30 / 13	0.084	43 / 37	5 / 6	0.740

Abbreviation: AC, adenocarcinoma; LAT1, L-type amino acid transporter 1; Glut, glucose transporter; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin.

Table A3.

Correlation between Glut1 and other biomarkers

Biomarkers	Total (n=160)		AC (n=106)		Non-AC (n=54)	
	Spearman γ (95% CI)	*p*-value	Spearman γ (95% CI)	*p*-value	Spearman γ (95% CI)	*p*-value
CD98	0.552 (0.430 – 0.654)	**<0.001**	0.367 (0.183 – 0.526)	**<0.001**	0.301 (0.031 – 0.530)	**0.025**
LAT1	0.563 (0.441 – 0.663)	**<0.001**	0.296 (0.105 – 0.466)	**0.002**	0.337 (0.071 – 0.558)	**0.018**
Glut3	0.132 (-0.027 – 0.286)	0.094	0.219 (0.023 – 0.399)	**0.024**	-0.116 (-0.377 – 0.161)	0.398
HIF-1α	0.654 (0.552 – 0.737)	**<0.001**	0.615 (0.475 – 0.724)	**<0.001**	0.436 (0.185 – 0.633)	**<0.001**
Hexokinase I	0.678 (0.581 – 0.756)	**<0.001**	0.706 (0.591 – 0.793)	**<0.001**	0.219 (-0.056 – 0.464)	0.107
VEGF	0.725 (0.639 – 0.793)	**<0.001**	0.739 (0.635 – 0.817)	**<0.001**	0.289 (-0.017 – 0.520)	**0.032**
CD34	0.680 (0.584 – 0.757)	**<0.001**	0.698 (0.581 – 0.787)	**<0.001**	0.263 (-0.014 – 0.500)	0.052
EGFR	0.478 (0.345 – 0.592)	**<0.001**	0.356 (0.171 – 0.517)	**<0.001**	0.296 (0.025 – 0.526)	**0.027**
PTEN	-0.417 (-0.541 – -0.276)	**<0.001**	-0.350 (-0.512 – 0.164)	**<0.001**	-0.133 (-0.394 – 0.144)	0.329
p-Akt	0.094 (-0.064 – 0.250)	0.235	0.442 (0.268 – 0.588)	**<0.001**	0.082 (-0.194 – 0.347)	0.547
p-mTOR	0.356 (0.209 – 0.488)	**<0.001**	0.368 (0.184 – 0.527)	**<0.001**	0.106 (-0.171 – 0.368)	0.439
p-S6K	0.172 (0.013 – 0.323)	**0.028**	0.257 (0.063 – 0.432)	**0.008**	0.161 (-0.116 – 0.416)	0.237

Abbreviation: AC, adenocarcinoma; LAT1, L-type amino acid transporter 1; Glut, glucose transporter; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin; 95CI, 95% confidence interval.