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RESEARCH

Clinicopathological Significance of L-type Amino Acid Transporter 1 (LAT1) Expression in Patients with Adenoid Cystic Carcinoma

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Abstract The expression of L-type amino acid transporter 1 (LAT1) is correlated with tumor cell growth and survival. However, the clinicopathological significance of LAT1 expression in adenoid cystic carcinoma (ACC) remains to be elucidated. The aim of this study is to investigate the clinicopathological significance of LAT1 expression in ACC. A total of 30 patients with ACC were retrospectively reviewed. Tumor sections were stained by immunohistochemistry for LAT1, p53, and CD98, and cell proliferation and microvessel density (MVD) were determined by Ki-67 and CD34, respectively. High LAT1 and CD98 expression were observed in 27 % (8/30) and 23 % (7/30) of samples, respectively ($p>0.999$). The high expression of LAT1 was significantly correlated with cell proliferation (Ki-67) and the cell cycle regulator p53. By univariate analysis, solid histological pattern, maxillary tumor site, LAT1, CD98, Ki-67 and p53 were significantly associated with poor prognosis. Multivariate analysis demonstrated that the high expression of LAT1 was an independent prognostic factor for predicting poor prognosis after surgical resection. LAT1 is a promising clinical marker to predict the outcome after surgery in patients with ACC.

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Introduction

Adenoid cystic carcinoma (ACC) is a rare epithelial tumor of the major and minor salivary glands and comprises approximately 1 % of all malignant tumors of oral and maxillofacial origin [1]. Histologically, ACC is a biphasic tumor comprised of ductal and myoepithelial components. The three major growth patterns are tubular, cribriform, and solid. One of the important prognostic factors is the histological grade, which is determined by the solid tumor component percentage [2]. To improve patient outcomes, it is important to identify clinical markers that may predict prognosis and response to specific therapies. However, there are no established biomarkers that correlate with the outcome and therapeutic response in patients with ACC.

Amino acid transporters play an essential role in the growth and proliferation of both normal and transformed cells [3, 4]. L-type amino acid transporter 1 (LAT1) is one of the L-type amino acid transporters and is responsible for the transport of large neutral amino acids, such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine and histidine. LAT1 requires the covalent association of the heavy chain of 4F2 cell surface antigen (CD98) for its functional expression within the plasma membrane [5, 6]. LAT1 has been described as highly expressed in proliferating tissues, tumor cell lines and primary human tumors [6–14]. LAT1 expression is closely related to CD98 expression, cell proliferation, angiogenesis and cell cycle regulators [14, 15]. Recently, the expression of LAT1 was described as a significant factor predicting poor outcomes in various human cancers [8–15]. However, the pathological significance of LAT1 expression in ACC patients remains to be elucidated.

Based on this research, we evaluated the clinicopathological significance of LAT1 protein expression in resected tissue specimens of ACC patients. In addition, we determined the correlation between LAT1 expression and CD98, Ki-67 labeling index, the p53 cell cycle regulator and angiogenic markers such as microvessel density (MVD), as determined by CD34.

Material and Methods

Patients

We analyzed 32 consecutive patients with pathologically confirmed ACC who underwent surgical resection at the Gunma University Hospital between 1988 and 2006. The specimens from 2 patients were not available. Therefore, a total of 30 patients were analyzed in the study. The authors' approach to the evaluation and resection of these tumors has been described previously [16]. All surgical specimens were reviewed and classified according to the WHO classification by an experienced pathologist who was unaware of the clinical or imaging findings. Pathologic tumor-node-metastasis (TNM) stages were established according to the Classification of Malignant Tumors by the International Union against Cancer (UICC) by measuring distant metastasis. A staging system proposed by Arriaga et al. was used to stage external auditory meatus carcinomas [17]. The study protocol was approved by the institutional review board.

Immunohistochemical Staining

The whole sections of formalin-fixed paraffin embedded tissue were used. LAT1 expression was determined by immunohistochemical staining with an LAT1 antibody (2 mg/mL, anti-human monoclonal mouse antibody, 4A2, provided by Dr. H. Endou [J-Pharma, Tokyo, Japan], 1:3200 dilution). The production and characterization of the LAT1 antibody has been described previously [11]. CD98 was detected using an affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., 1:100 dilution) raised against a peptide mapping to the carboxy terminus of human CD98. The detailed protocol for immunostaining has been published elsewhere [8]. The LAT1 and CD98 expression scores were assessed by the extent of staining 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 were scored as 3 or 4 were defined as high-expression tumors.

For CD34, Ki-67 and p53, immunohistochemical staining was performed according to the procedures described in a previous report [14]. Mouse monoclonal antibodies against CD34 (Nichirei, Tokyo, Japan, 1:800 dilution), Ki-67 (Dako, Glostrup, Denmark, 1:40 dilution) and p53 (D07; Dako, 1:50 dilution) were used. The number of CD34-positive vessels was counted in four selected hotspots in a 400z field (0.26 mm^2 field area). MVD was defined as the mean microvessel count per 0.26 mm^2 field area. The median numbers of CD34-positive vessels were evaluated, and the tumors in which stained tumor cells exceeded the median value were defined as high-expression tumors. For p53, microscopic examination of the nuclear reaction product was performed and scored. Based on a previous report [14], positive p53 expression was defined as expression in more than 10 % of the tumor cells. For Ki-67, a highly cellular area of the immunostained sections was evaluated. All epithelial cells with nuclear staining of any intensity were defined as high-expression cells. Approximately 1,000 nuclei were counted on each slide. Proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index) in the sample. The median value of the Ki-67 labeling index was evaluated, and

the tumors exceeding the median value were defined as high-expression tumors. The sections were assessed using light microscopy 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. The correlation between different variables was analyzed using the nonparametric Spearman's rank test. The follow-up for these 30 patients was conducted using the patient medical records. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. Overall survival (OS) was determined as the time from tumor resection to death from any cause. Progression-free survival (PFS) was defined as the time between tumor resection and the first disease progression or death. Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analyses was performed using JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Patients' Demographics

The median age of the ACC patients was 56 years, ranging from 24 to 80 years. No patients had received chemotherapeutic agents or radiotherapy before surgery. The primary sites were as follows: 11 major salivary glands (8 parotid, 1 submandibular and 2 sublingual), 17 minor salivary glands (7 maxillary, 5 oral cavity, 2 nasal cavity and 3 nasopharynx), and 2 others (2 external auditory meatus). The histological patterns consisted of 7 solid, 13 cribriform and 10 tubular tumors. The day of surgery was considered the starting day for measuring postoperative survival. The median follow-up duration for all patients was 69 months (range, 7 to 312 months).

Immunohistochemical Analysis

Immunohistochemical analyses of the biomarkers were performed on the 30 primary lesions with ACC. Figure 1 presents representative immunohistochemical staining images of LAT1 and CD98 expression. LAT1 immunostaining was detected in carcinoma cells in tumor tissues and was localized predominantly to the plasma membrane. All positive cells exhibited strong membranous LAT1 immunostaining. Cytoplasmic staining was rarely evident. The patient demographics according to LAT1 expression status

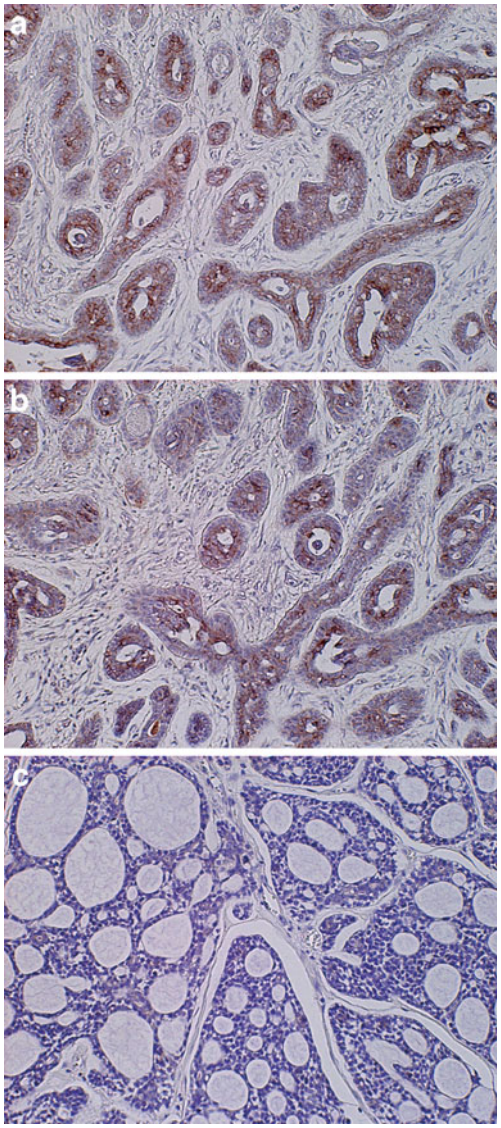

Fig. 1 Immunohistochemical staining of tissue from a 32-year-old man with an adenoid cystic carcinoma. Immunostaining of LAT1 (**a**) and CD98 (**b**) demonstrates a membranous immunostaining pattern. Figure 1c demonstrates negative staining for LAT1 in a patient with adenoid cystic carcinoma

is presented in Table 1. Overall, high LAT1 and CD98 expression were observed in 27 % (8/30) and 23 % (7/30) of patient samples, respectively ($p>0.999$). Of the 8 patients with high LAT1 expression, 7 (87.5 %) exhibited high CD98 expression, and all of the patients with high CD98 expression exhibited high LAT1 expression.

The median number of CD34-positive vessels was 7 (range, 0–15), and the value of 7 was chosen as a cutoff point. The median value of the Ki-67 labeling index was 3 % (range, 0–20), which was chosen as the cutoff point. High CD34 and Ki-67 expression was recognized in 33 % (10/30) and 47 % (14/30) of patient samples, respectively. Positive p53 expression was recognized in 50 % (15/30) of

Table 1 Patient's demographics

Variable	Total	LAT1 expression		
	(n=30)	High (n=8)	Low (n=22)	p-value
Age				
≤60 year	18	4	14	0.677
>60 year	12	4	8	
Sex				
Male	21	4	17	0.195
Female	9	4	5	
Histology				
S	7	3	4	0.344
C or T	23	5	18	
Tumor site				
Maxillary	7	3	4	0.344
Other	23	5	18	
Stage				
I or II	17	4	13	0.697
III or IV	13	4	9	
CD98				
High	7	7	0	<0.001
Low	23	1	22	
Ki-67				
High	14	7	7	0.012
Low	16	1	15	
CD34				
High	10	5	5	0.025
Low	20	2	18	
p53				
Positive	15	7	8	0.035
Negative	15	1	14	

LAT1 L-type amino acid transporter 1; *S* solid; *C* cribriform; *T* tubular

samples. High LAT1 expression was significantly associated with CD98, Ki-67, MVD and p53.

Correlation Between LAT1 Expression and Different Variables

Using Spearman's rank correlation, the LAT1 scoring exhibited a statistically significant correlation with CD98 (r=0.876, p<0.001) and Ki-67 (r=0.394, p=0.019) but not CD34 (r=0.069, p=0.693).

Univariate and Multivariate Survival Analyses

Overall, the 5-year survival OS and PFS rates were 65 % and 55 %, respectively. Figure 2 presents the Kaplan-Meier survival curve in patients with high and low LAT1 and CD98 expression. Univariate and multivariate analyses of OS and PFS are presented in Table 2. The univariate analysis for OS demonstrated that the solid histological pattern, maxillary tumor site and high LAT1, CD98, Ki-67 and p53 expression were significantly associated with poor prognosis. The statistically significant prognostic factors for PFS included the maxillary tumor site and high LAT1, CD98 and Ki-67 expression. According to the results of univariate log-rank test, we screened prognostic factors with cutoff value of p<0.025 because of the small sample size. Multivariate analysis confirmed that a high LAT1 expression was an independent prognostic factor for predicting poor OS, and LAT1 expression exhibited a borderline significance for poor PFS.

Next, we analyzed the different variables according to maxillary tumor site and solid histological pattern. Of the 7 patients with a maxillary tumor site, all (100 %) patients exhibited advanced disease stages (3 stage III and 4 stage IV), 4 (57 %) patients exhibited a solid tumor pattern, 3 (43 %) patients exhibited high LAT1 expression, 2 (29 %) patients exhibited high CD98 expression, 5 (71 %) patients possessed a high Ki-67 score, no (0 %) patients exhibited high MVD, and 4 (57 %) patients were positive for p53. Only the proportion of patients with tumors of an advanced disease stage (III or IV) was significantly higher in patients with a maxillary tumor site than those with a non-maxillary site (p=0.006). By contrast, 5 (71 %) of the 7 patients with a solid tumor pattern exhibited advanced disease stages (all stage IV), 3 (43 %) patients exhibited high LAT1 expression, 2 (29 %) patients exhibited high CD98 expression, 6 (86 %) patients possessed a high Ki-67 index, 2 (29 %) patients exhibited high MVD, and 5 (71 %) patients expressed positive p53. The patients with solid tumor pattern exhibited a significantly higher cell proliferation (Ki-67 index) compared to those with non-solid tumor patterns (p=0.031).

Discussion

To our knowledge, this is the first report demonstrating that both LAT1 and CD98 are expressed in human ACC, indicating that the co-expression of LAT1 and CD98 plays an important role in large neutral amino acids permeating tumor cells of ACC. We observed a significant association between LAT1 and CD98 expression (r=0.876, p<0.001), and the expression levels of LAT1 and CD98 were almost parallel. The expression of LAT1 exhibited a significant relationship with cell proliferation (Ki-67) and cell cycle regulation (p53). Univariate analyses demonstrated that the solid histological pattern, maxillary tumor site, and LAT1, CD98, Ki-67 and p53 expression were significantly associated with poor prognosis. By multivariate analyses, the expression of LAT1 was an independent prognostic factor for predicting poor outcomes after surgical resection. In the present research, no statistically significant correlation was observed between LAT1 expression and disease staging in ACC patients, which is inconsistent with the previous

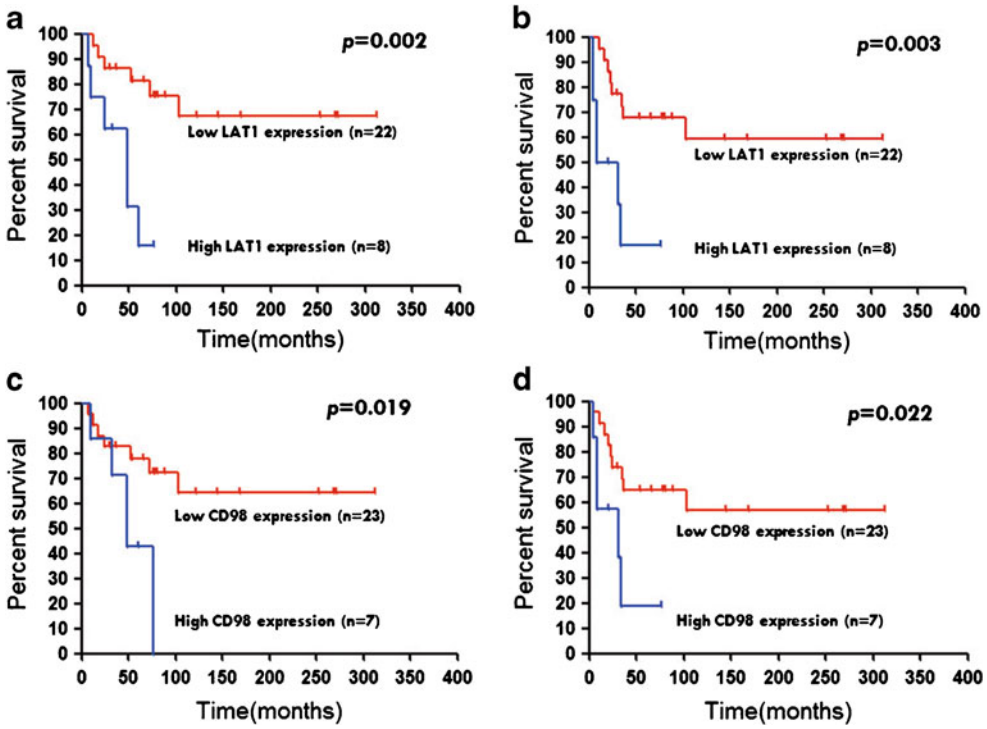

Fig. 2 Kaplan-Meier analysis of overall survival (OS) and progression-free survival (PFS) according to LAT1 and CD98 expression. A statistically significant difference in OS and PFS was observed between patients with high and low LAT1 expression [OS, $p=0.002$ (**a**); PFS, $p=0.003$ (**b**)] and between the patients with high and low CD98 expression [OS, $p=0.019$ (**c**); PFS, $p=0.022$ (**d**)]

studies on various carcinomas of other origin [8, 9, 13–15]. However, our results indicate that LAT1/CD98 may play a crucial role in the malignant potential such as cell proliferation and pathogenesis of ACC.

High LAT1 expression was observed in 27 % (8/30) of ACC patients in the present study. Previous studies disclosed that LAT1 expression was elevated in 51 % (163/321) of lung cancer patients [8], 53 % (51/97) of pancreatic cancer patients [15], 22 % (25/114) of prostate cancer patients [11], 43 % (56/129) of breast cancer patients [13] and 41 % of gastric cancer patients (36/87) [12]. The rate of high LAT1 expression in ACC is similar to that of prostate cancer and seems to be lower than in aggressive tumors such as lung cancer, breast cancer and pancreatic cancer. Previous reports demonstrated that LAT1 and CD98 are co-expressed at high rates in various human cancers [8, 13, 15], and the results of our study also indicate that LAT1 co-expression with CD98 was observed in 87.5 % of patients with high LAT1 expression. These results suggest that LAT1 requires CD98 for its functional expression in patients ACC tumor cells. We analyzed the association of the Ki-67 labeling index with LAT1 expression in ACC and observed a significant correlation between LAT1 expression and the tumor proliferative index, consistent with previous descriptions [8, 13, 15]. However, ACC is typically presented as a slow-growing, firm, unilobular mass in the gland, and our study documented that the median value of Ki-67 labeling index (3 %) was markedly low in ACC as compared to aggressive malignancies such as lung cancer (32 %), pancreatic cancer (27 %) and breast cancer (40 %). In our series, all patients with maxillary site tumors presented at an advanced stage, and a solid histological pattern was significantly correlated with tumor cell proliferation. However, our research indicated that the maxillary tumor site and solid histological pattern did not strongly correlate with high LAT1 expression in ACC patients. Previous studies reported that positive Ki-67 expression in tumor cells exhibited a strong positive correlation with the solid pattern in ACC but not with disease stage [18], which is consistent with our data. In previous research, angiogenesis has been described as significantly correlated with tumor stage, aggressiveness and decreased survival in ACC patients [19]. In our study, angiogenesis was closely associated with the LAT1 expression. Our results suggest that the level of LAT1 expression protein plays a crucial role in tumor proliferation and angiogenesis within ACC cells, independent of maxillary tumor location and solid pattern histology.

Several researchers have reported that poor prognosis in ACC exhibits a significant relationship with advanced stage, solid histological pattern and distant metastasis [20–22]. Although the expression of p53 and Ki-67 has been reported to correlate with a poor outcome, these biomarkers have not provided sufficient evidence for predictive prognostic factors [18, 20, 23]. The tumors arising from the maxillary antrum are often clinically silent and may present at advanced stages disease with extensive invasion. Therefore, such tumors have been thought to be associated with poor outcome. However, there are no biomarkers for predicting poor outcome in tumors arising from the maxillary antrum. Because many reports have documented that the histological subtype influences the outcome of the disease, the histological subtypes of low malignancy (cribriform and tubular) have a better prognosis than

Table 2 Univariate and Multivariate analysis in overall survival and progression-free survival

Variables	Overall survival			Progression-free survival		
	5-year survival rate (%)	*p*-value (Univariate)	Hazard ratio 95 % CI *p*-value (Multivariate)	5-year survival rate (%)	*p*-value (Univariate)	Hazard ratio 95 % CI *p*-value (Multivariate)
Age						
≤60 year	71.3	0.975		46.1	0.556	
>60 year	64.8			66.7		
Sex						
Male	77.5	0.085		62.6	0.219	
Female	55.5			33.3		
Histology						
S	21.4	0.005	0.136	28.5	0.074	
C or T	76.5			63.4		
Tumor site						
Maxillary	42.8	0.016	0.411	19.1	0.025	0.116
Other	72.1			64.5		
Stage						
I or II	80.7	0.118		69.3	0.082	
III or IV	43.9			35.9		
LAT1						
High	15.6	0.002	0.019	16.7	0.003	0.054
Low	81.3			67.6		
CD98						
High	17.8	0.019	0.076	19.1	0.022	0.242
Low	77.8			64.7		
Ki-67						
High	44.6	0.026		31.7	0.026	
Low	80.7			74.5		
CD34						
High	45.7	0.391		50.0	0.745	
Low	74.3			57.8		
p53						
Positive	38.1	0.026		42.2	0.176	
Negative	86.7			66.7		

95 % CI 95 % confidence interval; *S* solid; *C* cribriform; *T* tubular; *LAT1* L-type amino acid transporter1

those of a high degree of malignancy (solid) [2]. In the present study, LAT1 expression, CD98 expression, solid histological pattern, maxillary tumor location, Ki-67 index and p53 expression were significantly correlated with worse survival, and the high expression of LAT1 nearly overlapped that of CD98. Previous reports have documented that the cooperative expression of LAT1 with CD98 is a better prognostic marker for predicting poor outcome than LAT1 expression alone [9, 24]. In ACC patients, LAT1 co-expression with CD98 may also be closely associated with decreased survival. ACC has the tendency to recur locally and to develop distant metastasis. Thus, ACC exhibits a problematic pathology, and there is a lack of reliable information regarding the molecular markers for predicting the malignant potential of ACC. It is our hope that discoveries can be made that will greatly influence the treatment and prognosis of ACC patients. Current reviews have documented many biomarkers that have been studies as potential prognostic indicators of ACC, but additional prognostic information can be obtained only by using Ki-67 and p53 staining [25]. Given the results of our study, LAT1 appears to be a promising indicator as an independent marker of poor prognosis when compared with Ki-67 and p53. Further study warrants a larger, multicenter cohort trial to confirm the results of our study.

To date, chemotherapy use for ACC is not promising, and surgical treatment with wide margins has been considered the treatment of choice. Recent reviews discussed systemic chemotherapy for the treatment of metastatic or locally recurrent advanced ACC [26]. Major objective responses are infrequent in advanced ACC, and the optimal regimen is not established yet. Therefore, prospective clinical trials of chemotherapy should be conducted to explore standard chemotherapy to

achieve a survival benefit in patients with recurrent or locally advanced ACC. Recently, several experimental studies have reported that the majority of amino acids into human oral cancer cells are mediated by LAT1 and its associating protein CD98 [27] and that the inhibition of LAT1 expression leads to the inhibition of cell growth in human oral cancer [28]. The results of these studies suggest that blocking the activity of LAT1 could markedly suppress the growth of oral cancer cells and induce tumor apoptosis [27, 28]. On the other hand, the potential of targeted therapy for LAT1 have been investigated in tumor cell lines using 2-aminobicyclo-(2,2,1)-heptane-2-carboxtylic acid (BCH), an inhibitor of L-amino acid transporters, and the inhibition of LAT1 function has been described to play a crucial antitumor role in tumor cell lines [29, 30]. In ACC patients, further study is warranted to investigate the potential new targeted therapies using LAT1 inhibitors.

Because of the rarity of ACC, our survival analysis is limited by a small sample size. However, our series had a long-term follow-up of at least 5 years, and there was a sufficient follow-up duration to evaluate the prognostic value of slow-growing ACCs.

In conclusion, high LAT1 expression can serve as an independent prognostic factor to predict poor outcomes after surgical resection and may be an important indicator of therapy for patients with ACC. We believe that blocking LAT1 inhibits the growth of ACC and that LAT1 may become an attractive molecular target for advanced ACC.

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Statement of Conflicts of Interest 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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