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Clinicopathological Significance of LAT1 and ASCT2 in Patients With Surgically Resected Esophageal Squamous Cell Carcinoma

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Background: Amino acid transporters are highly expressed in various human cancers. L-type amino acid transporter 1 (LAT1) and system alanine-serine-cysteine amino acid transporter-2 (ASCT2) play a crucial role in tumor progression and survival. However, the clinicopathological significance of these transporters in patients with esophageal squamous cell carcinoma (ESCC) remains unclear.
Methods: One hundred and fifty-seven patients with surgically resected ESCC were evaluated. Immunohistochemical analysis was performed for LAT1, ASCT2, CD98, Ki-67, and micro-vessel density (MVD), as determined by CD34 expression.
Results: LAT1 and ASCT2 were positively expressed in 59% (93/157) and 48% (76/157) of tumors respectively. LAT1 and ASCT2 expression significantly correlated with T factor, N factor, lymphatic permeation, vascular invasion, and CD98 expression. The 5-year survival rates of LAT1-high and -low and ASCT2-high and -low expressing patients were 62.0% and 69.6% ($P < 0.05$) and 59.6% and 70.1% ($P = 0.068$), respectively. The combined positive expression of LAT1 and ASCT2 was a significant prognostic factor in univariate analysis.
Conclusion: High expression of LAT1 and ASCT2 correlates with metastasis and invasion. Accordingly, these proteins could serve as prognostic biomarkers and therapeutic targets for treating patients with surgically resectable ESCC.
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KEY WORDS: esophageal cancer; LAT1; ASCT2; immunohistochemistry; prognostic factor

INTRODUCTION

Malignant tumor cells require more nutrients than non-malignant cells because of their rapid growth. Amino acids are essential nutrients for cells, not only because they are a nitrogen source for the synthesis proteins, nucleotides, glutathione, and amino sugars, but also because they activate mammalian target of rapamycin (mTOR) via a nutrient signaling pathway that regulates cell survival [1–3]. It has been reported that some types of amino acid transporters are up-regulated in tumor cells, but not in normal tissues [1,4].

L-type amino acid transporter 1 (LAT1) is an isoform of amino acid transporter system L. It transports large neutral (branched chain) or aromatic amino acids, such as leucine, valine, phenylalanine, tryptophan, methionine, and histidine, which are essential for fundamental cellular activities including growth, proliferation, and maintenance [5]. Leucine, in particular, is considered as the most effective amino acid that stimulates protein synthesis via nutrient signaling pathways [6]. For functional expression on the plasma membrane, LAT1 must be covalently associated with the heavy chain of the 4F2 cell surface antigen (CD98) [7].

System alanine-serine-cysteine amino acid transporter-2 (ASCT2) is a sodium (Na+)-dependent transporter responsible for transporting neutral amino acids, such as glutamine, leucine, isoleucine, alanine, serine, and cysteine [8]. The ASCT2 glutamine transport system supports amino acid exchange by LAT1 [9]. Therefore, it is critical for cell growth and survival.

These amino acid transporters have the potential to be used as either therapeutic targets or biomarkers for malignant tumors in patients [6,10–13]. In this study, we investigated the clinicopathological importance of LAT1 and ASCT2 expression in esophageal squamous cell carcinoma (ESCC) patients.

MATERIALS AND METHODS

Patients

We analyzed 157 ESCC patients who underwent curative resection without neo-adjuvant chemotherapy or chemo-radiation therapy at Gunma University Hospital between January 2000 and December 2010. Patient characteristics are listed in Table I. The patient cohort was comprised of 140 men and 17 women, with age ranging from 41 to 83 years (median, 64 years). All patients were identified as squamous cell carcinoma (SCC) by histopathological examination. Among the 157 patients, 3, 39, 31, 58, and 26 were diagnosed with tumors at p-Stage 0, 1, 2, 3, and 4, respectively. The tumor specimens were histologically characterized in accordance to the criteria of World Health Organization (WHO) Classification of Tumors of the Digestive System [14]. Pathological findings are described according to the guidelines of the Union for International Cancer Control TNM

Kyoichi Kaira and Tatsuya Miyazaki equally contributed to this study.

Conflicts of interest: We (all authors) have no financial or personal relationships with other people or organizations that could inappropriately influence our work.

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TABLE I. Patient's Characteristics

Age	Median (range)	64 (41–83)
Gender	Male	140
	Female	17
Histopathological type	SCC	157
	Others	0
Differentiation	Well	29
	Mod	86
	Por	42
Depth of tumor (pT)[a]	0	2
	1	61
	2	18
	3	70
	4	6
Lymph node metastasis (pN)[a]	0	58
	1	42
	2	38
	3	19
Distant metastasis (pM)[a]	0	130
	1	27
Lymphatic permeation[a]	No	23
	Yes	134
Vascular invasion[a]	No	36
	Yes	121
pStage[a]	0	3
	I	39
	II	31
	III	58
	IV	26

[a]UICC 7th edition.
SCC, squamous cell carcinoma; pStage, pathological Stage; Well, well differentiated; Mod, moderately differentiated; Por, poorly differentiated.

(tumor-node-metastasis) Classification of Malignant Tumors, 7th edition [15]. The post-operative clinical course was assessed by analyzing out-patient medical records. The date of surgery was considered as Day 1 for the calculation of post-operative survival. The follow-up duration ranged from 22 days to 78 months, with the median being 30 months. Tumor recurrence was confirmed by clinical course and gastrointestinal fiber optic and/or radiological imaging.

Immunohistochemical Staining

LAT1 expression was determined by immunohistochemical staining using the murine anti-human LAT1 monoclonal antibody 4A2 (provided by Dr. H. Endou, J-Pharma, Tokyo, Japan; 2 mg/ml; 1:3200 dilution). An affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX; 1:100 dilution) raised against the C-terminus of human CD98 was used to detect CD98. An affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.; 1:300 dilution) was used to detect ASCT2. Immunostaining was performed as described in a previously published protocol [16]. Normal tonsil tissue was used as a positive control. For negative control, the tissue samples were not incubated with the primary antibody.

LAT1, CD98, and ASCT2 expression levels were considered positive when distinct membrane staining was present regardless of cytoplasmic staining. This allowed us to select the functional populations of these amino acid transporters. Staining intensity was scored as follows: 0, no staining; 1, 1–10% of tumor area stained; 2, 11–25% of tumor area stained; 3, 26–50% of tumor area stained; and 4, $\geq$51% of tumor area stained. Scores of 0, 1, and 2 were defined as low expression, whereas 3 and 4 were defined as high expression. Tumor staining intensity scores over 2 were graded as positive.

Similar to LAT1, immunohistochemical staining of CD34 and Ki-67 was performed using Immunosaver (Nishin EM, Tokyo, Japan) for optimal antigen retrieval. The antibodies used were as follows: a mouse monoclonal antibody against CD34 (Nichirei, Tokyo, Japan; 1:200 dilution) and a mouse monoclonal antibody against MIB-1 specific for human nuclear antigen Ki-67 (Dako, Glostrup, Denmark; 1:100 dilution). Four highly cellular areas of the immunostained sections were selected to determine the Ki-67 labeling index. Tumor cells with nuclear staining of any intensity were defined as positive. Over 1,000 nuclei were counted on each slide. The Ki-67 labeling index was calculated as the ratio of positive tumor cells to all measured cells. To estimate micro-vessel density (MVD), the four areas with the highest neo-vascularization were identified as hot spots in a 400× magnification field of view (0.26 mm^2) for each slide. Any brown-stained endothelial cells or cell clusters that were clearly separate from adjacent micro-vessels, tumor cells, or other connective tissue elements were considered as single micro-vessels. Micro-vessels in sclerotic areas within the tumor and adjacent areas of unaffected esophageal tissue were not counted. The number of CD34-positive vessels was counted and the mean count of positive cells per 400× field of view was calculated as the MVD.

Statistical Analysis

The χ^2-test and Fisher's exact test were used to examine the association between two categorical variables. The results of immunohistochemical staining were expressed as mean $\pm$ the standard deviation. The correlation between LAT1, CD98, ASCT2, CD34, and Ki-67 were analyzed using the non-parametric Spearman's rank correlation. *P*-values less than 0.05 were considered indicative of statistical significance. Statistical analyses were performed using StatMate V version 5.01.

RESULTS

Immunohistochemical Analysis

The 157 primary ESCC lesions were analyzed by immunohistochemistry. Figure 1 shows representative images of LAT1, ASCT2, CD98, CD34, and Ki-67 expression. LAT1, ASCT2, and CD98 immunostaining was detected in carcinoma cells, but not in vascular endothelial or interstitial tumor cells. LAT1 and CD98 expression was localized predominantly to the plasma membrane. However, ASCT2 expression was observed in the cytoplasm and plasma membrane. We defined "positive staining" as distinct membrane staining of LAT1, CD98, and ASCT2, regardless of cytoplasmic staining. In the 157 tumor samples analyzed, high LAT1, CD98, and ASCT2 expression was identified in 59.2% (93/157), 58.6% (92/157), and 48.4% (76/157), respectively. On a scale of 1–4, the average LAT1, CD98, and ASCT2 expression scores were 2.6 ± 1.00, 2.6 ± 1.04, and 2.4 ± 0.97, respectively. Among the 93 patients with high LAT1 expression (score of 3 or 4), 75 (80.6%) displayed high expression (score of 3 or 4) and 18 displayed low expression (score of 0, 1, or 2) of CD98. Among the 76 patients with high ASCT2 expression (score of 3 or 4), 52 (68.4%) displayed high expression (score of 3 or 4) and 24 displayed low expression (score of 0, 1, or 2) of CD98.

The median value of the Ki-67 labeling index was 27% (range, 1–74%), which was chosen as the cut-off point. The median MVD value was 13 (range, 0–80), which was chosen as the cut-off point.

Demographics of Patients According to LAT1 and ASCT2 Expression

The demographic distribution of the other variables according to LAT1 and ASCT2 expression is listed in Table II. A statistically significant difference in patient age ($P < 0.05$), tumor depth ($P < 0.05$), lymph node metastasis ($P < 0.05$), lymphatic permeation ($P < 0.05$), venous invasion ($P = 0.001$), and CD98 expression ($P < 0.001$) was

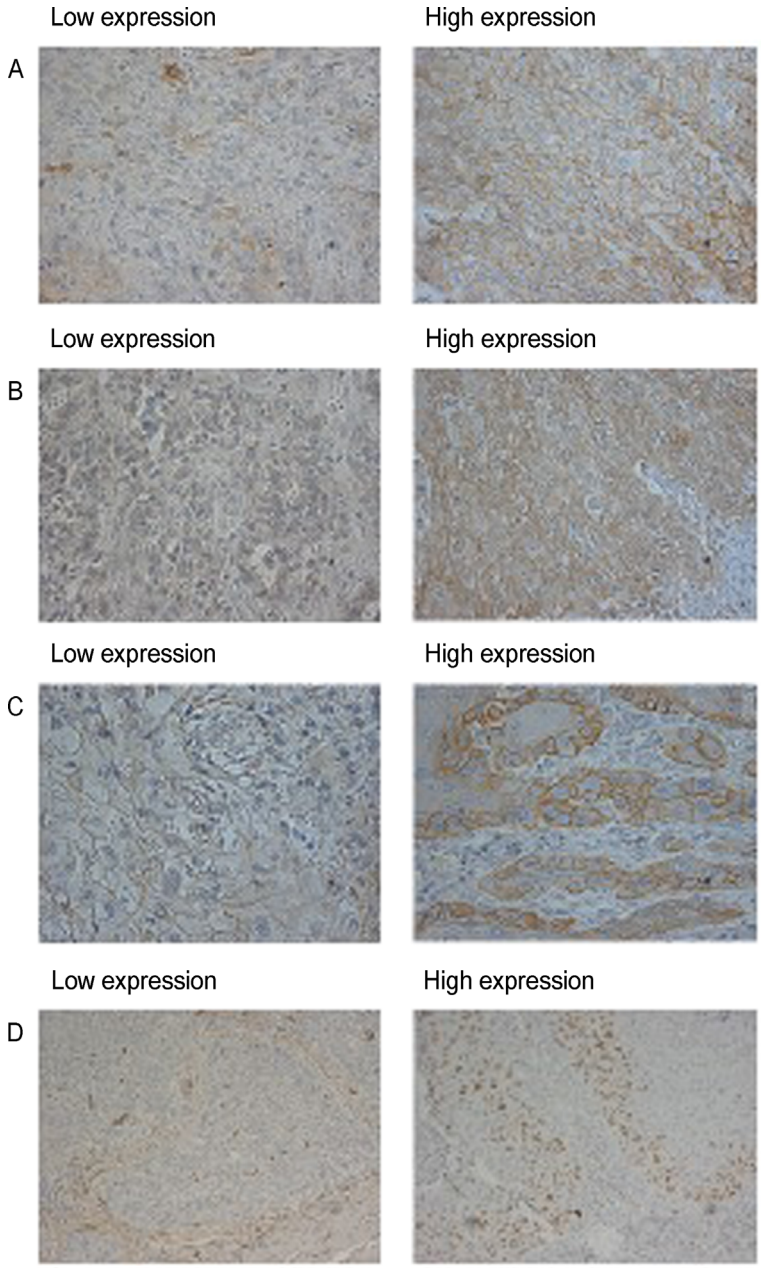

Fig. 1. Immunohistochemical staining of the resected tumor in a 400× magnification; the score of LAT1 (**A**), ASCT2 (**B**), and CD98 (**C**) immunostaining was evaluated with five phases, and defined low (score 0–2) and high (score 3, 4). Either of their immunostaining demonstrates a membranous pattern. Every figure shows examples of low expression and high expression. (**D**) shows examples of CD 34 and Ki-67 expression. Four highly cellular areas of the immunostained sections were selected and evaluated to assess the Ki-67 labelling index. All tumor cells with nuclear staining of any intensity were defined as positive. Ki-67-labelling index was calculated as the ratio of positive tumor cells to all measured cells. To estimate micro-vessel density (MVD), the four highest neovascularization areas were identified as hot spots. Any brown stained endothelial cell or cell cluster that was clearly separate from adjacent micro-vessels, tumour cells, and other connective tissue elements was considered as a single micro-vessel. The number of CD34-positive vessels was counted and the mean count of positive cells per 400× magnification field of view was calculated as MVD. IHC, immunohistochemistry; ESCC, esophageal squamous cell carcinoma; LAT1, L-type amino-acid transporter 1; ASCT2, ASC amino acid transporter-2; MVD, micro-vessel density.

observed between samples with high and low LAT1 expression. There was also a statistically significant difference in tumor depth ($P < 0.05$), lymph node metastasis ($P < 0.05$), lymphatic permeation ($P < 0.05$), venous invasion ($P < 0.05$), and CD98 expression ($P < 0.05$) between samples with high and low ASCT2 expression.

When LAT1 and ASCT2 expression scores were added, the high expression group was defined as having a total combined score of greater than 4 and the low expression group was defined as having a total combined score of less than 4. A statistically significant difference in p-Stage ($P < 0.05$), tumor depth ($P < 0.001$), lymph node metastasis

TABLE II. Patient's Demographics According to LAT1 and ASCT2 Expression

		LAT1			ASCT2			LAT1 + ASCT2		
Variables	Total (n = 157)	Low (n = 64)	High (n = 93)	*P*-value	Low (n = 81)	High (n = 76)	*P*-value	Negative (n = 105)	Positive (n = 52)	*P*-value
Age										
≤65	86	26	60	0.004	43	43	0.748	53	33	0.130
>65	71	38	33		38	33		52	19	
Gender										
M	140	59	81	0.434	69	71	0.125	93	47	>0.999
F	17	5	12		12	5		12	5	
pT										
T1	63	33	30	0.020	42	21	0.002	53	10	<0.001
T2-4	94	31	63		39	55		52	42	
pN										
No	58	31	27	0.018	37	21	0.021	55	13	0.006
Yes	99	33	66		44	55		60	39	
pM										
No	131	57	74	0.131	69	62	0.668	92	39	0.066
Yes	26	7	19		12	14		13	13	
pStage										
0 + I + II	73	36	37	0.051	45	28	0.025	55	18	0.042
III + IV	84	28	56		36	48		50	34	
ly.										
No	23	16	7	0.005	17	6	0.024	21	2	0.007
Yes	134	48	86		64	70		84	50	
v.										
No	36	23	13	0.001	25	11	0.022	30	6	0.016
Yes	121	41	80		56	65		75	46	
Differ.										
WD + MD	115	48	67	0.717	64	51	0.106	78	37	0.704
PD	42	16	26		17	25		27	15	
CD98										
Low	65	47	18	<0.001	41	24	0.023	53	10	<0.001
High	92	17	75		40	52		52	42	
Ki-67										
Low	82	33	49	>0.999	46	36	0.265	57	25	0.500
High	75	31	44		35	40		48	27	
CD34										
Low	80	37	43	0.193	42	38	0.874	57	23	0.308
High	77	27	50		39	38		48	29	

LAT1 and ASCT2 expression showed significant association with tumor depth (pT), lymph node metastasis (pN), lymphatic permeation, vascular invasion and CD98 expression. LAT1 also showed significant association with patient's age. The combination of LAT1 and ASCT2 expression (Positive; double positive, Negative; other combinations) enforced this tendency.

LAT1, L-type amino-acid transporter 1; ASCT2, ASC amino acid transporter-2; M, male; F, female; pT, pathologically tumor invasion depth; pN, pathologically lymph node metastasis; pM, distant metastasis; pStage, pathological stage; ly., lymphatic permeation; v., venous invasion; Differ., Differ tumor differenciation; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; Ki-67, Ki-67 index.

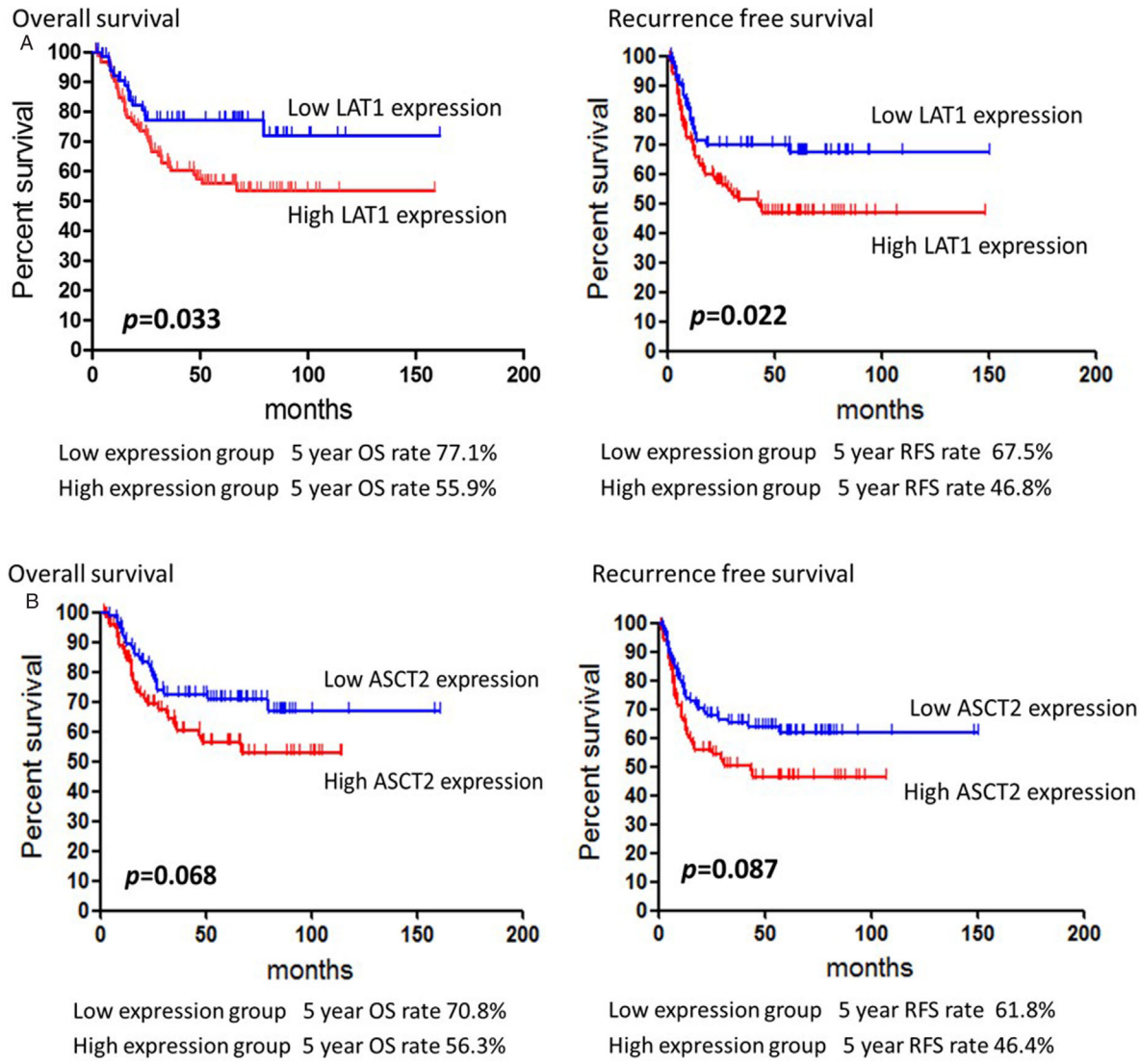

Fig. 2. Kaplan–Meier analysis of overall survival (OS) and recurrence-free survival (RFS) according to LAT1 (**A**) and ASCT2 (**B**) expression. A statistically significant difference in OS and RFS was observed between patients with high and low LAT1 expression [OS, $P = 0.033$; PFS, $P = 0.022$ (**A**)] but not between the patients with high and low ASCT2 expression [OS, $P = 0.068$; RFS, $P = 0.087$ (**B**)]. LAT1, L-type amino-acid transporter 1; ASCT2, ASC amino acid transporter-2; OS, overall survival; RFS, recurrence free survival.

($P < 0.05$), lymphatic permeation ($P < 0.05$), venous invasion ($P < 0.05$), and CD98 expression ($P < 0.001$) was observed in samples with a high combined LAT1 and ASCT2 expression versus a low combined expression.

Postoperative Survival Analysis

The 5-year overall survival (OS) rate was 65.2%, and the 5-year recurrence-free survival (RFS) rate was 59.1% in our study cohort. The high LAT1 expression group had a worse prognosis than the low expression group (Fig. 2A). Similarly, high ASCT2 expression correlated with a worse prognosis than low ASCT2 expression (Fig. 2B). Worse prognosis after surgery was significantly associated with the pathological disease stage, lymphatic permeation, vascular invasion, LAT1 expression, and co-expression of LAT1 and ASCT2 as determined by univariate analysis. LAT1 was a statistically independent factor of poor prognosis, whereas ASCT2 was not. Comparing the group with double-positive expression of LAT1 and ASCT2 with the other groups, this group was associated with worse OS and RFS rates (Fig. 3). The OS and RFS rates in the double-positive group were 60.0% and 48.6%, respectively.

Multivariate analysis was performed for all patients (Table III). To ensure that this population was consistent with previous studies, LAT1 and ASCT2 single and co-expressing tumor samples were analyzed. Disease stage and lymphatic permeation were independent prognostic factors for predicting poor RFS. Disease stage was also an independent prognostic factor for OS. LAT1 and ASCT2 co-expression was not a significantly independent prognostic factor, although this group had the worst prognosis. These results suggest that co-operation of LAT1 and ASCT2 might lead to increased cancer spread, invasion, and worse prognosis in the early stages of cancer growth.

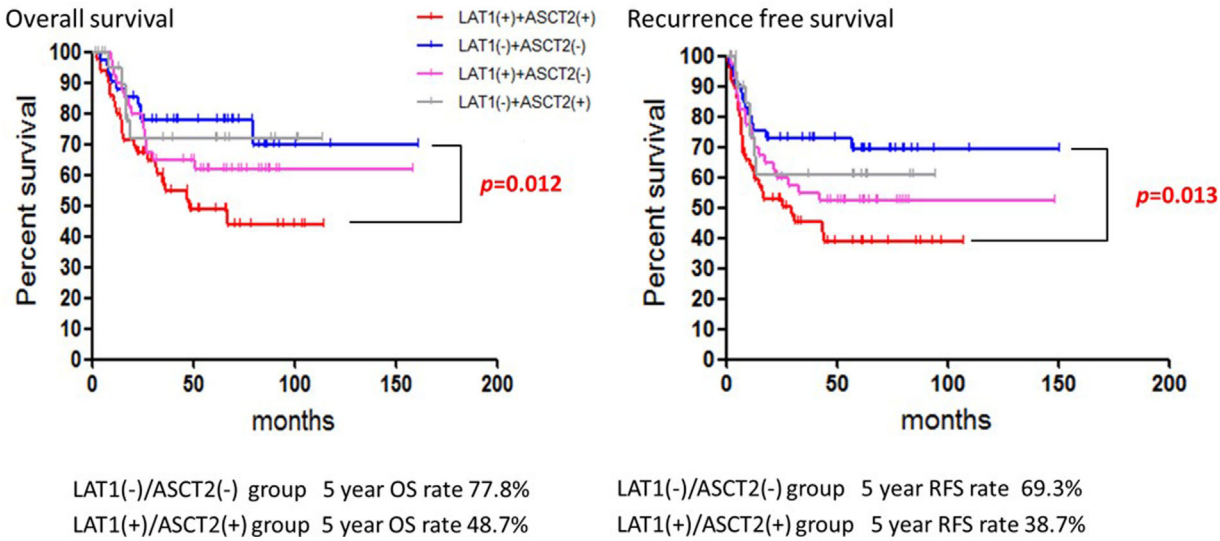

Fig. 3. Kaplan–Meier analysis of overall survival (OS) and recurrence-free survival (RFS) according to the combination LAT1 and ASCT2 expression. A statistically significant difference in OS and RFS was observed between patients with double positive expression and double negative expression [OS, $P = 0.012$; PFS, $P = 0.013$]. LAT1, L-type amino-acid transporter 1; ASCT2, ASC amino acid transporter-2; OS, overall survival; RFS, recurrence free survival.

DISCUSSION

To our knowledge, this is the first clinicopathological study evaluating the prognostic significance of LAT1 and ASCT2 expression in patients with surgically resected ESCC. In this study, we demonstrated the clinicopathological significance of amino acid transporters LAT1 and ASCT2 as potential prognostic markers and therapeutic targets.

According to the experimental data available to date, it is clear that both LAT1 and ASCT2 are expressed in primary human cancers and several cancer cell lines. In addition, they play essential roles in cancer cell growth and survival. Fuchs et al. [1] investigated the number of expressed sequence tags for LAT1 and ASCT2 detected by cDNA Northern blot in the National Cancer Institute's Cancer Genome Anatomy Project (CGAP) database. This study demonstrates that LAT1 and ASCT2 are both up-regulated in cancerous tissues from a variety of organs including the brain, colon, eye, head, and neck, kidney, liver, lung, lymph nodes, mammary glands, muscle, ovary, pancreas, placenta, skin, and stomach, compared with normal tissue from these organs.

Kobayashi et al. [17] reported that LAT1 was only expressed in the basal layer of the esophageal wall in non-cancerous esophageal mucosa. However, in squamous cell carcinoma tissues, LAT1 was expressed throughout the tumor, as determined by immunohistochemical staining. They also demonstrated that LAT1 expression was higher in cancer tissues than that in the non-cancerous tissues, and that the expression

TABLE III. Univariate and Multivariate Analysis of RFS and OS

Variables		Recurrence free survival			Overall survival		
		Univariate		Multivariate	Univariate		Multivariate
		5-yr rate (%)	*P*-value	*P*-value	5-yr rate (%)	*P*-value	*P*-value
Age	≦65/>65	48/64	0.119		61/68	0.361	
Gender	M/F	53/64	0.479		62/76	0.377	
p-Stage	I + II/III + IV	73/38	<0.001	0.005	85/47	<0.001	<0.001
ly.	yes/no	47/95	<0.001	0.04	58/94	0.002	0.246
v.	yes/no	44/88	<0.001	0.141	55/94	<0.001	0.062
Differ.	WD+MD/PD	57/45	0.079		67/54	0.199	
LAT1	High/Low	47/68	0.022		56/77	0.033	
ASCT2	High/Low	46/62	0.087		56/71	0.068	
LAT1+ASCT2	Positive/Negative	39/69	0.013	0.143	49/78	0.012	0.097
CD98	High/Low	49/62	0.071		57/73	0.057	
Ki-67	High/Low	50/58	0.711		64/63	0.932	
CD34	High/Low	48/59	0.116		59/68	0.32	

Pathological stage, lymphatic invasion, vascular invasion, high LAT1 expression, and the combination of both LAT1 and ASCT2 positive expression were statistically risk factor in recurrence free survival (RFS) and overall survival (OS) in univariate analysis. Among these factors, pathological stage was the only statistically independent poor prognostic factor in RFS ($P = 0.005$) and OS ($P < 0.001$).

Univariate, univariate analysis; Multivariate, multivariate analysis; 5-yr SR, 5 year survival rate; LAT1, L-type amino-acid transporter 1; ASCT2, ASC amino acid transporter-2; M, male; F, female; ly., lymphatic permeation; v., venous invasion; Differ., Differ tumor differenciation; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; LAT1, L-type amino-acid transporter 1; ASCT2, ASC amino acid transporter-2; Ki-67, Ki-67 index.

increased as the depth of tumor invasion, tumor size, and degree of differentiation increased. Accordingly, this study shows that both LAT1 and ASCT2 were mainly expressed in tumor tissues. This was especially evident when we defined positive expression as protein expression on the cell membrane. Although we observed no obvious heterogeneity in LAT1 or ASCT2 expression, in some cases these amino acid transporters were predominantly expressed on the borders of tumor nests. However, the scoring system applied is not very strict and may tolerate slight heterogeneity to some extent. Therefore, we believe that these results may not have significantly influenced the scoring system used or the outcome of the study.

We previously reported a link between LAT1, ASCT2, and CD98 expression and pancreatic cancer development [18]. Our present study shows a significant association between the expression of LAT1 and ASCT2 and that of CD98 ($P < 0.001$ and $P = 0.023$, respectively). We also reported a relationship between LAT1 and ASCT2 expression and clinicopathological factors such as disease stage, lymph node metastasis, and vascular invasion in several types of cancer such as biliary tract cancer [12], pancreatic cancer [18], tongue cancer [19], thymic carcinoma [20], malignant pleural mesothelioma [21], rectal cancer [22], adenoid cystic carcinoma [23], and lung cancer [24]. In accordance with these studies, our present study shows that LAT1 and ASCT2 are highly expressed in patients with ESCC; the percentage of tumors expressing these markers was 59% and 48%, respectively. There was a significant association between LAT1 and ASCT2 expression and the depth of tumor invasion, lymph node metastasis, pathological disease stage, lymphatic invasion, and vascular invasion. However, no significant association between LAT1 and ASCT2 expression and the degree of differentiation of the tumor was observed in this study.

Expression of both LAT1 and ASCT2 has been reported to be closely associated with poor prognosis in some cancers [18,25–27]. In our study, high LAT1 and ASCT2 expression were both related to poor prognosis. Furthermore, the double positive group had a poorer prognosis than that of any other LAT1 and ASCT2 combination groups. This group also had a poorer prognosis than the high LAT1 or ASCT2 expression groups. The high expression levels of LAT1 and ASCT2 significantly correlated with lymph node metastasis, lymphatic permeation, vascular invasion, and CD98 expression. In contrast to the previous studies, there was no significant correlation between these amino acid transporters and cell proliferation or angiogenic markers (Ki-67 and CD34, respectively). These results indicate that the amino acid transporters LAT1, ASCT2, and CD98 play important roles in tumorigenesis and expansion, and that LAT1 and ASCT2 complement each other and co-operate to contribute to the malignant characteristics of cancer cells. Although over-expression of LAT1 and ASCT2 was not identified as an independent prognostic indicator for patients with esophageal cancer, we believe that these amino acid transporters play a crucial role in the pathogenesis and tumor progression of esophageal cancer. In addition, they could be promising biomarkers for predicting negative outcome after surgery.

Human papillomavirus (HPV) is now recognized as a carcinogenic factor in ESCC [28]. Previous studies have reported that in patients with HPV-positive oropharyngeal squamous cell carcinoma, high CD98 expression was associated with a poorer prognosis than patients with low CD98 expression [29]. CD98 is a promising cancer stem cell enrichment marker in head and neck squamous cell carcinoma [30], and cancer stem cells are implicated in resistance to anti-cancer therapy. Although CD98 and HPV can contribute to poor convalescence, an association between HPV and LAT1 or ASCT2 has not been reported. Therefore, we did not investigate if any of the tumors in our study were HPV-positive.

LAT1 and ASCT2 are considered to be closely related to cellular growth and survival signaling via the mTOR pathway [31]. Fuchs et al. hypothesized that mTOR regulates amino acid transporter gene expression and transportation to the plasma membrane in response to growth signals in cancer cells [1]. Moreover, LAT1 provides the essential amino acids needed to enhance cancer cell growth via mTOR-stimulated translation. In addition, ASCT2 maintains the cytoplasmic amino acid pool necessary to drive LAT1 function, suppress apoptosis, and fuel the energy economy via net delivery of glutamine.

Increased activity of the mTOR signaling pathway is an adverse prognostic factor in human cancers such as cervical, hepatic, gastric, and biliary tract cancers. However, increased activity of this pathway is associated with better prognosis in ovarian cancer and lung adenocarcinoma. As for ESCC, phosphorylated mTOR expression was reported to be independently associated with poor prognosis, which supports the argument for mTOR as a therapeutic target [32].

Several experimental studies have demonstrated that a reduction in mTOR phosphorylation through the inhibition of amino acid transporters can lead to cell cycle arrest at the G1 phase, and can also have anti-tumor effects in the C6 glioma-bearing rat model [25,33,34] and in non-small cell lung cancer [33,35,36]. Recently, the importance of ASCT2-mediated glutamine uptake and the mTOR pathway in cancer was highlighted. Previous studies have demonstrated a close relationship between ASCT2-mediated glutamine transport and the mTOR signaling pathway in human cancer cells such as lung cancer, melanoma, and leukemia cells. These studies found that inhibition of ASCT2 caused G1 cell cycle arrest by suppressing the mTOR pathway via ASCT2-mediated glutamine uptake, resulting in the suppression of tumor cell growth and proliferation [10,37,38]. Oppedisano et al. [39] reported that inhibition of ASCT2 reduced the availability of glutamine and other amino acids transported by ASCT2, possibly impairing the survival of cancer cells that depend on increased glutamine metabolism. These data suggest that: (i) glutamine is essential for the activation of the mTOR signaling pathway, in which glutamine uptake is mediated by amino acid transporters; and (ii) LAT1 and ASCT2 inhibitors could potentially be used as effective molecular targets for the treatment of human neoplasms. The expression levels of LAT1 and ASCT2 show a parallel increase in various human cancers, and these two obligate amino acid exchangers are closely correlated with cellular growth and survival signaling linked to the mTOR pathway.

Squamous cell carcinomas account for about 90% of all esophageal cancer cases in Japan. Various therapeutic methods that combine surgery, chemotherapy, and radiation therapy have been developed, but esophageal cancer has a poor prognosis and is the 6th leading cause of cancer death worldwide. It has a high relapse rate after surgery. Indeed, although adjuvant or neo-adjuvant chemotherapy contributes to improved patient survival, these therapeutic methods cause serious side effects in the majority of patients, and only some patients are able to profit from these treatments. To improve convalescence following esophageal cancer, the immediate discovery of new therapeutic tools is required [40]. Therefore, the identification of biomarkers that can be used to determine whether post-operative adjuvant therapy is necessary is an urgent matter. Proteomics studies aimed at identifying biomarkers of esophageal cancer have been performed worldwide. One report described newly found biomarkers associated with the early diagnosis, therapeutic effect, prognosis, and molecular mechanisms of carcinogenesis and progression [41]. Among these proteins, transglutaminase 3 [40], calreticulin [42], 78-kDa glucose-regulated protein [42], apolipoprotein A-1 [43], serum amyloid A [43], and transthyretin [43] were reported to be prognostic biomarkers. A correlation between alpha-actinin 4 expression and lymph node metastasis was also shown [44,45]. Squamous cell carcinoma antigen 1 [44] and annexin A2 [46,47] correlated with lymph node metastasis and histological tumor differentiation.

To date, we reported that high LAT1 and ASCT2 expression levels correlate with disease progression, angiogenesis, and metastasis. As such, they could be significant prognostic markers in human tumor tissues [18,24,48,49]. Similarly, the results of our present study indicate that LAT1 and ASCT2 expression is significantly correlated with

tumor invasion, lymph node metastasis, micro-vessel invasion, and poor prognosis. These proteins have the potential to be prognostic biomarkers as well as therapeutic targets that act through the mTOR signaling pathway.

The limitations of this study must be mentioned. There was a major male bias in tumor sampling. This is because of: (i) the gender gap in the outbreak frequency of ESCC in Japan; and (ii) because this study was carried out on consecutive cases. The sample size of this study was not large enough to perform sub-group analysis. Amino acid transporters are considered to play an important role in the early phases of cancer development; therefore, sub-group analysis according to disease stage would have been ideal. Neither LAT1 nor ASCT2 expression completely correlated with CD98 expression. This is because CD98 serves as a chaperone for other SLC7 family transporters, and ASCT2 does not directly bind to CD98. In this study, the expression of LAT1 correlated with CD98. In our study, we did not take into account any additional therapy received after surgery. Therefore, the outcome of the study may have been affected by the adjuvant therapy used. Furthermore, the prognostic analysis might be incorrect since a few of patients in our study cohort died during the course of the study. In the future, LAT1, ASCT2, and CD98 expression analysis should be investigated for sub-groups that received adjuvant chemotherapy. Further investigation is warranted for a more accurate analysis.

In conclusion, the high expression levels of LAT1 and ASCT2 means that they could potentially be used as powerful prognostic markers or therapeutic targets for patients with ESCC. A treatment strategy that inhibits both LAT1 and ASCT2 function may prove to be an effective therapy for ESCC.

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