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Prognostic significance of amino-acid transporter expression (LAT1, ASCT2, and xCT) in surgically resected tongue cancer

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Background: Amino-acid transporters are necessary for the tumour cell growth and survival, and have a crucial role in the development and invasiveness of cancer cells. But, it remains unclear about the prognostic significance of L-type amino-acid transporter 1 (LAT1), system ASC amino-acid transporter-2 (ASCT2), and xCT expression in patients with tongue cancer. We conducted the clinicopathological study to investigate the protein expression of these amino-acid transporters in tongue cancer.

Methods: Eighty-five patients with surgically resected tongue cancer were evaluated. Tumour sections were stained by immuno-histochemistry for LAT1, ASCT2, xCT, 4F2hc/CD98hc (4F2hc), Ki-67, and microvessel density (MVD) determined by CD34, and p53.

Results: L-type amino-acid transporter 1 and 4F2hc were highly expressed in 61% (52 out of 85) and 45% (38 out of 47), respectively. ASC amino-acid transporter-2 and xCT were positively expressed in 59% (50 out of 85) and 21% (18 out of 85), respectively. The expression of both LAT1 and ASCT2 was significantly associated with disease staging, lymph-node metastasis, lymphatic permeation, 4F2hc expression and cell proliferation (Ki-67). xCT expression indicated a significant association with advanced stage and tumour factor. By univariate analysis, disease staging, lymphatic permeation, vascular invasion, LAT1, ASCT2, 4F2hc, and Ki-67 had a significant relationship with overall survival. Multivariate analysis confirmed that LAT1 was an independent prognostic factor for predicting poor prognosis.

Conclusions: L-type amino-acid transporter 1 and ASCT2 can serve as a significant prognostic factor for predicting worse outcome after surgical treatment and may have an important role in the development and aggressiveness of tongue cancer.

Cancer of the tongue is a malignant tumour treated by surgery, and comprises ∼30% of all malignant tumours of the oral-pharyngeal lesion (Regezi *et al*, 2008). Disease staging remains the most important tool for the clinician in predicting disease outcome in patients with oral tongue squamous cell carcinoma (SQC) (Bello *et al*, 2010). However, it is not the only prognostic factor for predicting poor prognosis. Clarifying poor prognostic factors at the time of diagnosis is essential to enable identification of patients

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whose tumours have high risk if treatment failure. Nowadays, there has been no established biomarker that correlates with the prognosis and therapeutic response in patient with tongue cancer.

Amino-acid transporters are necessary for the tumour cell growth and proliferation, and the overexpression of amino-acid transporters has been described to have a crucial role in the survival and metastasis of cancer cells (Christensen, 1990; Kanai et al, 1998; Yanagida et al, 2001; Fuchs and Bode, 2006; Baek et al, 2012). Recent studies have described that L-type amino-acid transporter 1 (LAT1), system ASC amino-acid transporter-2 (ASCT2), and xCT were significantly linked to the carcinogenesis and tumour pathogenesis (Yanagida et al, 2001; Whitte et al, 2002; Li et al, 2003; Kaira et al, 2008, 2011, 2012; Baek et al, 2012). L-type amino-acid transporter 1 is one of the L-type amino-acid transporters, and transports large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine and requires covalent association with the heavy chain of 4F2 cell-surface antigen (4F2hc/CD98hc) for its functional expression in plasma membrane (Kanai et al, 1998; Yanagida et al, 2001). It has been described to be highly expressed in proliferating tissues, many tumour cell lines and primary human neoplasms (Yanagida et al, 2001; Kaira et al, 2008, 2012). Expression of LAT1 has a close relationship with 4F2hc, cell proliferation, angiogenesis and cell-cycle regulator (Kaira et al, 2011). Recently, the expression of LAT1 has been described to be a significant factor indicating a poor outcome in human neoplasms (Kaira et al, 2008, 2012). However, it remains unclear about the pathological significance of LAT1 expression in patients with tongue cancer. ASC amino-acid transporter-2 is a Na^+-dependent transporter responsible for transport of neutral amino acids including glutamine, leucine, and isoleucine, and is a major glutamine transporter in human hepatoma cells. Recently, ASCT2 has been described to be highly expressed in human neoplasms such as hepatocellular carcinoma, colorectal, and prostate cancer (Whitte et al, 2002; Li et al, 2003; Fuch et al, 2007). Researchers have documented that the protein level of ASCT2 was related to poor outcome and malignant aggressiveness in patients with colorectal and prostate cancer (Whitte et al, 2002; Li et al, 2003). System x_c^- is a cystine-glutamate exchanger transporter, composed of a light chain (xCT) and heavy chain (4F2hc) (Baek et al, 2012; Takeuchi et al, 2013). The expression of xCT has been reported to have a crucial role in the tumour progression and growth of cancer and glutathione-based drug resistance (Huang et al, 2005). Recent research also demonstrated that the overexpression of xCT could be a pathological marker for predicting poor outcome in patients with glioblastoma (Takeuchi et al, 2013). The study using human tissue specimens showed that xCT is highly expressed in patients with lung cancer and breast cancer (Baek et al, 2012).

But, it remains unclear whether the amino-acid transporters such as LAT1, ASCT2, and xCT have a significant relationship with the tumour progression and development of patients with tongue cancer. Therefore, we conducted the clinicopathological study evaluating the prognostic significance of these three amino-acid transporters (LAT1, ASCT2, and xCT) in patients with tongue cancer. In this study, therefore, the expression level of LAT1, ASCT2, and xCT proteins was assessed in the resected tissue specimen and correlated with outcome of patients. In addition, the expression of Ki-67 labelling index, cell-cycle regulator (p53) and angiogenic markers such as microvessel density (MVD) determined by CD34 were also examined by immunohistochemistry.

MATERIALS AND METHODS

Patients. Between November 2000 and January 2012, we analysed 94 consecutive patients with tongue cancer who underwent surgical resection at Gunma University Hospital. Nine patients who had a histological type other than SQC were excluded. Therefore, 85 patients were analysed in this study. The study was approved by the institutional review board.

The age of the patients ranged from 33 to 92 years, and the median age was 69 years. None of the patients had received neo-adjuvant chemotherapy. All surgical specimens were reviewed and classified according to the WHO classification by an experienced pathologist who was unaware of clinical or imaging findings. Pathological tumour-node-metastasis stages were established using the International System for Staging adopted by the American Joint Committee on Cancer and the Union Internationale Centre le Cancer. Histologically, all patients had histology of SQC, and 38, 12, 15, and 20 of the total patients had stage I, II, III, and IV tumours, respectively. Postoperative adjuvant chemotherapy with S-1 (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan), oral administration of tegafur (a fluorouracil derivative drug) and docetaxel were administered to 9, 12, and 3 patients, respectively. The day of surgery was considered as the starting day for measuring postoperative survival. The follow-up duration ranged from 61 to 3452 days (median, 1033 days).

Immunohistochemical staining. The LAT1 expression was determined by immunohistochemical staining with a murine anti-human LAT1 monoclonal antibody 4A2 (provided by Dr H Endou (J-Pharma, Tokyo, Japan), $2\,mg\,ml^{-1}$, dilution; 1:3200) (Sakata et al, 2009). The production and characterisation of the LAT1 antibody has previously been described (Kaira et al, 2008, 2012). The anti-CD98 antibody is an affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:100 dilution) raised against a peptide mapping at the carboxy terminus of CD98 of human origin. CD98 clearly detects 4F2hc and this antigen is SLC3A2/4F2hc/CD98hc, therefore, we used this antibody. The detailed protocol for immunostaining was also published elsewhere (Kaira et al, 2008, 2012). The LAT1 and 4F2hc expression scores were assessed by the extent of staining as follows: 1, ≤10% of tumour area stained; 2, 11–25% stained; 3, 26–50% stained; and 4, ≥51% stained. The tumours in which
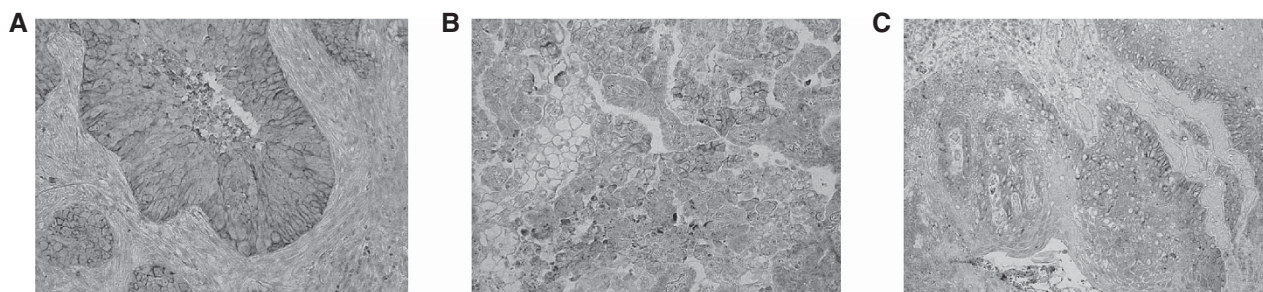

Figure 1. Representative immunohistochemical staining of patient with tongue squamous cell carcinoma. Immunostaining of LAT1 (**A**), ASCT2 (**B**), and xCT (**C**) displays a membranous immunostaining pattern.

stained tumour cells were scored as 3 or 4 were defined as high expression.

Anti-ASCT2 and anti-xCT antibodies are affinity-purified rabbit polyclonal antibody (1:300 dilution) and affinity-purified murine polyclonal antibody (Santa Cruz Biotechnology, Inc., 1:200 dilution), respectively. An oligopeptide (RDSKGLAAAEPTAN) corresponding to amino-acid residues 7–20 of a rabbit polyclonal antibody against ASCT2 was synthesised (see Supplementary Figure S1). The N-terminal cysteine residue was used for conjugation with keyhole limpet haemocyanin. An anti-ASCT2 rabbit polyclonal antibody was produced as described elsewhere (Altman *et al*, 1984). The antiserum was affinity purified as described (Chairoungdua *et al*, 2001). The specificity of the antibody was confirmed as described in Supplementary Procedure. Briefly, HEK 293T cells were transfected with a plasmid encode ASCT2 or the parent plasmid. Crude membrane fractions were isolated, and then subjected to SDS–PAGE and western blotting as described in Khunweeraphong *et al* (2012). Immunohistochemical staining was performed on paraffin sections using a polymer peroxidase method (Histofine Simple Stain MAX PO (MULTI or

Table 1. Patient's demographics according to LAT1, ASCT2, and xCT expression												
		LAT1			ASCT2			xCT			
Variable	Total (*n* = 85)	High (*n* = 52)	Low (*n* = 33)	*P*-value	Positive (*n* = 50)	Negative (*n* = 35)	*P*-value	Positive (*n* = 18)	Negative (*n* = 67)	*P*-value	
Age											
≤65 years/>65 years	36/49	19/33	17/16	0.185	18/32	18/17	0.184	7/11	29/38	0.793	
Sex											
Male/Female	56/29	34/18	22/11	>0.999	31/19	25/10	0.486	12/6	44/23	>0.999	
Differentiation											
WD or MD/PD	73/11	43/9	30/3	0.353	42/10	31/4	0.753	14/4	59/8	0.271	
Stage											
I or II/III or IV	50/35	26/26	24/9	**0.044**	26/24	30/5	**0.001**	7/11	49/18	**0.011**	
Primary tumour status											
T1-2/T3-4	72/13	42/10	30/3	0.236	40/10	34/1	**0.023**	11/7	63/4	**0.001**	
Lymph-node status											
N0/N1-2	31/54	27/25	4/29	**<0.001**	28/22	8/27	**0.004**	10/8	20/47	0.054	
Lymphatic permeation											
Positive/Negative	37/48	30/22	7/26	**<0.001**	27/33	11/24	**0.047**	11/7	27/40	0.181	
Vascular invasion											
Positive/Negative	26/59	22/30	4/29	**0.003**	20/30	7/28	0.061	6/12	21/46	>0.999	
Resected status											
Positive/Negative	15/70	11/41	4/29	0.383	10/40	5/30	0.573	4/14	11/56	0.727	
Adjuvant CTx											
Yes/No	24/61	19/33	5/28	**0.047**	17/33	7/28	0.221	7/11	17/50	0.376	
4F2hc											
High/Low	38/47	33/19	5/28	**<0.001**	27/23	11/24	**0.048**	12/6	26/41	0.059	
Ki-67											
High/Low	43/42	33/19	10/23	**0.003**	29/21	11/24	**0.026**	11/7	29/38	0.195	
CD34											
High/Low	44/41	33/19	11/22	**0.008**	26/24	16/19	0.661	10/8	32/35	0.604	
p53											
Positive/Negative	37/48	25/27	12/21	0.370	21/29	17/18	0.658	8/10	30/37	>0.999	

Abbreviations: ASCT2 = ASC amino-acid transporter 2; CTx = chemotherapy; LAT1 = L-type amino-acid transporter 1; MD = moderate differentiated; PD = poorly differentiated; WD = well differentiated.
The bold entries show a statistically significant difference.

G) kit; Nichirei Corporation, Tokyo, Japan). Briefly, deparaffinised and rehydrated sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. To expose antigens, sections were autoclaved in EDTA (pH 8.0) for 5 min and cooled for 30 min. After rinsing in phosphate-buffered saline (PBS), sections were incubated with affinity-purified anti-ASCT2 antibody (1:300) overnight. Thereafter, they were incubated with the Histofine Simple Stain MAX PO (MULTI or G) kit (Nichirei Corporation). The peroxidase reaction was performed using 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M tris–HCl buffer, pH 7.4. Negative control tissue sections were prepared by omitting the primary antibody. The expression of ASCT2 and xCT was considered as positive only if distinct membrane staining was present. The ASCT2 expression scores were assessed by the extent of staining as follows: 1, ⩽10% of tumour area stained; 2, 11–25% stained; 3, 26–50% stained; and 4, ≧51% stained. The tumours in which stained tumour cells were scored as > 2 were defined as positive expression.

For CD34, Ki-67, and p53, immunohistochemical staining was performed according to the procedures described in previous reports (Kaira *et al*, 2008, 2012). The following antibodies were used: murine monoclonal antibodies against CD34 (Nichirei, 1:800 dilution), Ki-67 (Dako, Glostrup, Denmark; 1:40 dilution), and p53 (D07; Dako, 1:50 dilution). The number of CD34-positive vessels was counted in four selected hot spots in a ×400 field (0.26 mm² field area). The MVD was defined as the mean count of microvessels per 0.26 mm² field area. The median numbers of CD34-positive vessels were evaluated, and the tumours in which stained tumour cells made up more than each median value were defined as high expression. For p53, microscopic examination for the nuclear reaction product was performed and scored. Based on a previous report (Kaira *et al*, 2008, 2012), p53 expression in >10% of tumour cells was defined as positive expression. 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. Approximately 1000 nuclei were counted on each slide. Proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labelling index) in the sample. The median value of the Ki-67 labelling index was evaluated, and the tumour cells with greater than the median value were defined as high expression. The sections were assessed using a light microscopy in a blinded manner 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 analysed using the non-parametric Spearman's rank test. Follow-up for these 85 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 analysed by the log-rank test. Overall survival (OS) was determined as the time from tumour resection to death from any cause. Progression-free survival (PFS) was defined as the time between tumour resection and the first disease progression or death. 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 analysis of the biomarkers was performed on the 85 primary lesions with tongue cancer. Figure 1 shows the representative immuno-histochemical staining of LAT1, ASCT2, and xCT expression. The

immunostaining of LAT1, ASCT2, and xCT was detected in carcinoma cells in tumour tissues and localised predominantly on their plasma membrane. All positive cells revealed strong membranous immunostaining. In total patients, a high LAT1 and 4F2hc expression was recognised in 61% (52 out of 85) and 45% (38 out of 47), respectively (*P* = 0.045). A positive ASCT2 and xCT expression was recognised in 59% (50 out of 85) and 21% (18 out of 85), respectively.

The median number of CD34 was 14 (range, 2–29), and the value of 14 was chosen as a cutoff point. The median value of the Ki-67 labelling index was 21% (range, 5–72), and the value of 21% was chosen as a cutoff point. The high expression of CD34 and Ki-67 was recognised in 52% (44 out of 85) and 51% (43 out of 85), respectively. Positive expression of p53 was recognised in 44% (37 out of 85).

Patient's demographics according to LAT1, ASCT2, and xCT expression status is listed in Table 1. High LAT1 expression was significantly associated with disease staging, lymph-node status, lymphatic permeation, vascular invasion, 4F2hc, Ki-67, and CD34. Positive ASCT2 expression yielded a significant relationship with disease staging, primary tumour status, lymph-node status, lymphatic permeation, 4F2hc, and Ki-67. Positive expression of xCT was significantly associated with disease staging and T factor.

Correlation between LAT1 expression and different variables. Using Spearman's rank correlation, LAT1 had a statistically significant correlation with ASCT2 ($r = 0.608$, $P < 0.001$), xCT ($r = 0.409$, $P < 0.001$), 4F2hc ($r = 0.496$, $P < 0.001$), Ki-67($r = 0.473$, $P < 0.001$), and CD34 ($r = 0.468$, $P < 0.001$). ASC amino-acid transporter-2 yielded a significant correlation with 4F2hc ($r = 0.221$, $P = 0.041$) and Ki-67 ($r = 0.417$, $P < 0.001$); and a significant correlation was observed between xCT and 4F2hc ($r = 0.251$, $P = 0.019$) (Table 2).

Different variables survival analysis. The 5-year survival rate and median survival time for all patients were 31% and 18 months, respectively. Tables 3 and 4 show the univariate and multivariate analysis in OS and PFS. By univariate analysis, disease staging, lymphatic permeation, vascular invasion, LAT1, ASCT2, 4F2hc, and Ki-67 had a significant relationship with OS, and tumour differentiation, lymphatic permeation, LAT1, ASCT2, 4F2hc, and

Table 2. Correlation between LAT1, ASCT2, and xCT, and other biomarkers			
Biomarkers	**Spearman γ**	**95% CI**	***P*-value**
LAT1			
ASCT2	0.608	0.449–0.730	**<0.001**
xCT	0.409	0.209–0.576	**<0.001**
4F2hc	0.496	0.338–0.627	**<0.001**
CD34	0.468	0.298–0.609	**<0.001**
Ki-67	0.473	0.311–0.608	**<0.001**
ASCT2			
4F2hc	0.221	0.026–0.421	**0.041**
CD34	0.127	−0.094–0.337	0.244
Ki-67	0.417	0.217–0.583	**<0.001**
xCT			
4F2hc	0.251	0.035–0.447	**0.019**
CD34	0.029	−0.191–0.247	0.786
Ki-67	0.177	−0.043–0.382	0.105

Abbreviations: ASCT2 = ASC amino-acid transporter 2; 95% CI = 95% confidence interval; LAT1 = L-type amino-acid transporter 1.
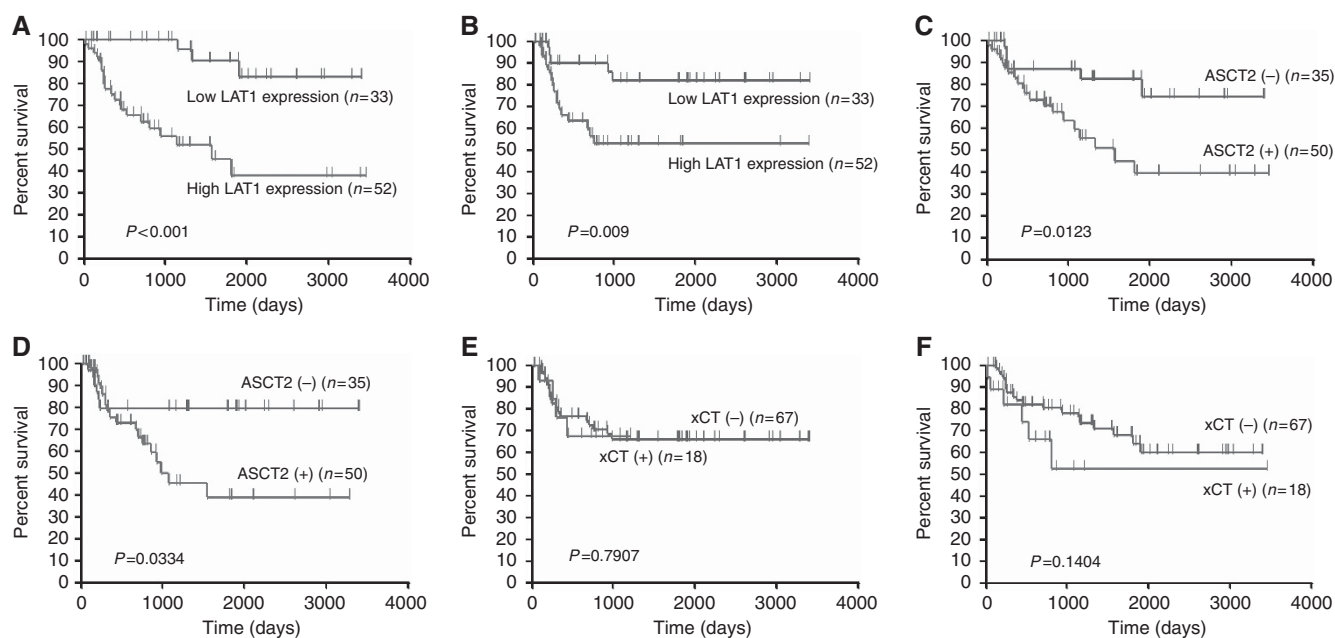The bold entries show a statistically significant difference.

Table 3. Univariate analysis in overall survival and progression-free survival				
	Overall survival		Progression-free survival	
Variables	5-year survival rate (%)	*P*-value	5-year survival rate (%)	*P*-value
Age				
≤65 years/>65 years	64.8/56.3	0.383	61.8/68.3	0.504
Sex				
Male/Female	58.9/71.2	0.847	62.3/72.4	0.585
Differentiation				
WD or MD/PD	64.6/43.6	0.090	40.0/37.8	**0.037**
Stage				
I or II/III or IV	73.4/34.2	**0.003**	72.1/46.9	0.103
Primary tumour status				
T1-2/T3-4	65.6/23.1	0.112	66.9/36.4	0.785
Lymphatic permeation				
Positive/Negative	39.6/74.3	**0.007**	43.7/77.7	**0.004**
Vascular invasion				
Positive/Negative	32.3/72.7	**0.001**	50.5/70.6	0.140
Resected status				
Positive/Negative	54.5/63.0	0.806	59.3/66.8	0.715
Adjuvant chemotherapy				
Yes/No	40.7/71.1	0.217	42.3/73.3	0.058
LAT1				
High/Low	37.8/90.1	<**0.001**	53.0/81.8	**0.009**
ASCT2				
Positive/Negative	39.4/82.3	**0.012**	39.2/79.5	**0.033**
xCT				
Positive/Negative	52.5/73.5	0.140	67.5/65.9	0.791
4F2hc				
High/Low	41.9/75.8	**0.012**	47.9/79.0	**0.023**
Ki-67				
High/Low	46.8/73.3	**0.036**	48.1/81.2	**0.001**
CD34				
High/Low	48.9/75.3	0.182	68.7/72.1	0.617
p53				
Positive/Negative	49.8/71.6	0.111	60.2/70.4	0.542

Abbreviations: ASCT2 = ASC amino-acid transporter 2; LAT1 = L-type amino-acid transporter1; MD = moderate differentiated; PD = poorly differentiated; WD = well differentiated. The bold entries show a statistically significant difference.

Ki-67 were significantly associated with PFS. Figure 2 shows the Kaplan–Meier survival curve in patients with tongue cancer according to the expression status of LAT1, ASCT2, and xCT. Multivariate analysis confirmed that LAT1 was an independent prognostic factor for predicting poor PFS and OS.

DISCUSSION

This is a first study to evaluate the clinical significance of LAT1, ASCT2, and xCT expression in patients with tongue cancer. Of

Table 4. Multivariate analysis in overall survival and progression-free survival				
	Overall survival		Progression-free survival	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age				
⩽65 years/>65 years	0.796 (0.328–1.878)	0.603	0.801 (0.509–1.241)	0.321
Sex				
Male/Female	0.981 (0.336–2.244)	0.701	1.047 (0.352–2.798)	0.928
Stage				
I or II/III or IV	2.204 (0.898–5.487)	0.084	1.365 (0.509–3.653)	0.532
LAT1				
High/Low	6.167 (2.044–32.44)	**0.001**	4.100 (1.263–15.35)	**0.018**
ASCT2				
Positive/Negative	1.289 (0.404–3.594)	0.649	1.367 (0.407–4.299)	0.603

Abbreviations: ASCT2 = ASC amino-acid transporter 2; 95% CI = 95% confidence interval; HR = hazard ratio; LAT1 = L-type amino-acid transporter1. The bold entries show a statistically significant difference.

Figure 2. Kaplan–Meier analysis of overall survival (OS) and progression-free survival (PFS) according to LAT1, ASCT2, and xCT expression. A statistically significant difference in OS and PFS was observed between the patients with high LAT1 and those with low LAT1 tumour expression (OS, $P<0.001$(**A**); PFS, $P=0.009$ (**B**)), and between the patients with positive and negative ASCT2 tumour expression (OS, $P=0.012$ (**C**); PFS, $P=0.033$ (**D**)). No statistically significant difference in OS (**E**) and PFS (**F**) was observed between the patients with positive xCT and those with negative xCT tumour expression.

these three amino-acid transporters, the expression of LAT1 was confirmed to be a significant independent factor for predicting poor prognosis in tongue cancer by multivariate analysis. However, we also found that the positive expression of ASCT2 yielded a significant relationship with worse prognosis after surgical treatment, but not xCT. Our study showed that the expression of LAT1 and ASCT2 was significantly associated with disease staging, lymph-node metastasis, tumour invasiveness, 4F2hc expression, and cell proliferation (Ki-67). 4F2hc has an important role in the functional expression of LAT1, ASCT2, and xCT; therefore, 4F2hc significantly correlated with the expression of these amino-acid

transporters. In our study, the percentage of patients who received adjuvant chemotherapy was significantly higher in patients with high LAT1 expression than in those with low LAT1, but adjuvant chemotherapy did not affect the outcome after surgical treatment. Our study indicated that the prognosis of patients with high LAT1 expression was worse despite of adjuvant chemotherapy. Therefore, our results suggest that the prognostic significance of LAT1 and ASCT2 expression was not affected by adjuvant chemotherapy. The prognostic significance of LAT1 expression has been extensively evaluated in various types of human neoplasms; however, little is known about the clinicopathological significance

of ASCT2 and xCT (Fuchs and Bode, 2006). To our knowledge, there has been no report comparing the clinical significance of three amino-acid transporters of LAT1, ASCT2, and xCT in human tissue specimen. Our data suggest that the expression level of these amino-acid transporters has a crucial role in the development and carcinogenesis of tumour cells, and it may be an important clinical marker of therapy for patients with tongue cancer.

L-type amino-acid transporter 1 is highly expressed in cancer cells in general; however, LAT1 expression in SQC was markedly higher than that of adenocarcinoma (AC) (Kaira et al, 2008, 2012; Sakata et al, 2009; Ichinoe et al, 2011; Furuya et al, 2012; Nobusawa et al, 2013). For example, the percentage of high LAT1 expression in patients with AC showed 29% in pulmonary AC (Kaira et al, 2008), 22% in prostate cancer (Sakata et al, 2009), 43% in breast cancer (Furuya et al, 2012), 43% in gastric cancer (Ichinoe et al, 2011), and 53% in pancreatic AC (Kaira et al, 2012), whereas patients with SQC revealed 91% in pulmonary SQC (Kaira et al, 2008), 56% in oral SQC (Nobusawa et al, 2013), and 61% in tongue cancer (the present study). But, it remains unknown why the extent of LAT1 expression in cancer cells is different between SQC and AC.

As described by previous studies (Kaira et al, 2008, 2012; Sakata et al, 2009; Ichinoe et al, 2011; Furuya et al, 2012; Nobusawa et al, 2013), the results of our study indicated that LAT1 expression has a close relationship with advanced stage, lymph-node metastasis, cell proliferation, angiogenesis, and survival in tongue cancer. A recent report documented that LAT1 provides the essential amino acids that signal to enhance growth of cancer cells via the mammalian target of rapamycin (mTOR)-stimulated translation (Fuchs and Bode, 2006). Likewise, mTOR is also regulated by amino-acid transporter gene expression and trafficking to the plasma membrane in response to growth signals. In in vitro study, the inhibition of LAT1 has been described to reduce the level of phosphorylation of mTOR, p70 ribosomal S6 kinase (p70SK1), and 4E-binding protein-1 (4E-BP1), suggesting that the activation of mTOR signalling pathway is associated with LAT1 expression (Liu et al, 2004; Yamauchi et al, 2009; Imai et al, 2010). Moreover, several authors have described that the inhibition of LAT1 leads to apoptosis by inducing intracellular depletion of amino acids required for the growth of cancers, and induces cell-cycle arrest at G1 phase (Liu et al, 2004; Kim et al, 2010), These investigation have suggest that the inhibition of LAT1 could be an effective therapeutic target for human neoplasms.

ASC amino-acid transporter-2 is highly expressed in various types of cancer cells that require glutamine for their growth and survival (Fuchs and Bode, 2006). An experimental study has described that the inhibition of ASCT2 reduces the availability of glutamine and other amino acids transported by ASCT2, and this could inhibit the survival of cancer cells depending on increased glutamine metabolism (Oppedisano et al, 2012). Therefore, ASCT2 could be the potential target for anticancer therapeutics as well. Clinically, only two reports showed aggressiveness biological behaviour of ASCT2 expression using tumour tissue specimen (Whitte et al, 2002; Li et al, 2003). Although LAT1 enhances tumour cell growth via the mTOR signalling pathway, whereas ASCT2 keeps the cytoplasmic amino-acid pool necessary to drive LAT1 function and fuels energy via delivery of glutamine (Xu and Hemler, 2005; Fuchs and Bode, 2006; Nicklin et al, 2009). Therefore, the expression levels of ASCT2 and LAT1 are coordinately rose in human cancer and these two obligate amino-acid exchangers are closely related to the cellular growth and survival linked to the mTOR signalling pathway (Xu and Hemler, 2005; Nicklin et al, 2009). Nicklin et al (2009) has described that L-glutamine transporter regulates mTOR and autophagy to coordinate cell growth and proliferation. Our study indicated that ASCT2 expression was significantly associated with tumour aggressiveness, cell proliferation, advanced staging,

metastasis, and worse prognosis in patients with tongue cancer. But, the positive expression of ASCT2 was not confirmed to be an independent prognostic factor for poor outcome in tongue cancer by multivariate analysis.

In the present study, the expression of xCT indicated any significant association with advanced stage and primary tumour factor. However, we could not find a significant relationship between xCT expression with tumour cell proliferation, angiogenesis, or survival in tongue cancer. It is expected to examine whether the expression of xCT protein has any relationship with the clinical significance of tumour cells with chemotherapy or radiotherapy resistance. Further study is warranted to investigate the xCT expression using tumour specimens resistant to anticancer therapy.

Limitations of the current study must be addressed. One limitation is that the sample size in this study was small, which may bias our results. Another limitation is that it remains unclear about the optimal cutoff points for the expression level of LAT1, ASCT2, and xCT. Previous studies revealed that there was a strong correlation between the two transporters (LAT1 and ASCT2) and their obligate chaperone (Xu and Hemler, 2005; Fuchs and Bode, 2006; Nicklin et al, 2009). However, the present study shows the relatively low percentage correlation between LAT1 and ASCT2 expression. These disparities may be due to the qualities of these antibodies and the evaluating system to measure their expression level. This is one of the limitations of immunohistochemical studies. Further study is warranted to evaluate a large number of patients with tongue cancer and to investigate the appropriate cutoff level of these amino-acid transporters.

In conclusion, the high expression of LAT1 can serve as an independent prognostic factor for predicting worse outcome after surgical treatment and may have an important role in the metastasis, cell proliferation, angiogenesis, and invasiveness of tongue cancer. The expression of ASCT2 is also closely associated with tumour growth and proliferation, and could be an indicator for predicting poor prognosis as well. The inhibition of LAT1 and ASCT2 may, therefore, be a potential target for anticancer therapy in tongue cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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