L-Type Amino Acid Transporter 1 (LAT1) Expression in Malignant Pleural Mesothelioma

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[4], MASAHIRO ENDO[5], HARUHIKO KONDO[3], TAKASHI NAKAJIMA[6] and NOBUYUKI YAMAMOTO[1]

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

Abstract. *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 expressed in malignant pleural mesothelioma (MPM). Twenty-one patients were included in this study. Tumor sections were stained by immunohistochemistry for LAT1, glucose transporter 1 (GLUT1), GLUT3, hypoxia inducible factor-1α (HIF-1α), hexokinase I, vascular endothelial growth factor (VEGF), microvessel density (MVD) by determination of CD34, epidermal growth factor receptor (EGFR), phosphatase and tensin analog (PTEN), p-AKT, p-manmalian target of rapamycin (mTOR), p-S6K, p53 and BCL-2. LAT1 was overexpressed in approximately 50% of the patients with MPM. LAT1 expression was closely correlated with CD98, hypoxic markers, the mTOR pathway, Ki-67 and p53. The overexpression of LAT1 was closely associated with poor outcome in patients with MPM. LAT1 is closely associated with tumor development and progression in patients with MPM.*

Malignant pleural mesothelioma (MPM) is an aggressive tumor with a poor prognosis and an increasing incidence in many countries. To improve the prognosis of patients, molecular markers that may predict the outcome and therapeutic response should be established. Disease staging and performance status have been consistently shown to be the most powerful prognostic indicators of survival rates of MPM (1). However, no biomarker which correlates with the response to treatment and the prognosis in patients with MPM has been established.

Amino acids are essential not only for protein synthesis but also as a carbon and nitrogen source in the synthesis of purine and pyrimidine nucleotides, amino sugars and glutathione. L-Type amino acid transporter 1 (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 the proper recruitment of the light chain to the plasma membrane (4). LAT1 is known to be highly expressed in many tumor cell lines and primary human tumors (2, 5-8), where it has been shown to play essential roles in growth and survival. The stimulation of growth of cancer cells occurs *via* mammalian target of rapamycin (mTOR) (6, 7). Recent studies have shown that the expression of LAT1/4F2hc correlates 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 the pathogenesis and development of MPM in patients.

Glucose transporter (GLUT) is thought to be a possible intrinsic marker of hypoxia and the expression of GLUT has been found to be regulated by hypoxia in a hypoxia-inducible factor-1 (HIF-1)-dependent mannet (12, 13). One of the factors responsible for the up-regulation of GLUT 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 anaerobic metabolic mechanisms (14). GLUT1 and GLUT3 are expressed at high levels in a variety of carcinomas (15). mTOR is a

Correspondence to: Kyoichi Kaira, MD, Division of Thoracic Oncology, Shizuoka Cancer Center, 1007 Shimonagakubo Nagaizumi-cho, Sunto-gun, Shizuoka, 411-8777, Japan. Tel: +81 559895222, Fax: +81 559895634, e-mail: kkaira1970@yahoo.co.jp

Key Words: LAT1, CD98, mesothelioma, hypoxia, prognosis, mTOR.

downstream component of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway involved in the regulation of cell proliferation, angiogenesis and metabolism. Epidermal growth factor receptor (EGFR) is an upstream component of the PI3K/AKT/mTOR signaling pathway in human neoplasms and is overexpressed in many carcinomas. Recent experimental studies have documented that LAT1, like GLUT, is subject to regulation in hypoxia, and the overexpression of 4F2hc increased the level of GLUT1 protein with increased glucose uptake *in vitro* (16, 17). These results suggest that hypoxia contributes to the regulation of not only glucose but also amino acid metabolism. However, there is still no data on the relationship between LAT1, and GLUT1, hypoxia and mTOR in tumor cells.

In a recent review, molecular biology including GLUT1, HIF-1α, VEGF, EGFR, p53 and BCL-2, has been described to play an important role in the pathogenesis of MPM (1). Although many studies have clearly indicated that LAT1 is associated with cancerous or proliferative cells, the clinical significance of LAT1 expression remains unknown in MPM. Therefore, an immunohistochemical study was conducted to examine how LAT1 is expressed in patients with MPM. In addition, the correlation of LAT1 expression with hypoxic markers (GLUT1, GLUT3, hexokinase I, HIF-1α, VEGF and microvessel density [MVD] determined by CD34), the mTOR pathway (EGFR, phosphatase and tensin analog [PTEN], phospho(p)-AKT, p-mTOR and p-S6K), cell cycle control (p53) and apoptosis (BCL-2) in patients with MPM, was also investigated.

Materials and Methods

Patients. Between August 2003 and May 2009, 25 consecutive patients with MPM were included in this study. Out of these patients, four were excluded from further studies because a tissue specimen was not available. Thus, a total of 21 patients were analyzed in the study. The study protocol was approved by the Institutional Review Board.

The median age of the patients was 66 years (range, 19-79 years). Eighteen patients were men and 3 were women. Eleven out of the 21 patients had undergone surgical resection, 6 surgical biopsy, and the remaining 4 patients only percutaneous needle-core biopsy. Disease stage was classified according to the TNM staging system proposed by the International Mesothelioma Interest Group (IMIG) (18). Sixteen patients had tumor histology of epithelial type, two biphasic types, one sarcomatous type, and two unspecific types. Out of the total patients, 8, 1, 5 and 7 had stage I, II, III and IV tumors, respectively. As the initial treatment, 11 patients underwent surgery, 5 systemic chemotherapy, 2 thoracic radiotherapy and 3 patients had best supportive care alone. Including neoadjuvant therapy and relapse after surgery, 17 out of 21 patients had systemic chemotherapy. The clinical course was assessed by analyzing outpatient medical records and by making telephone inquiries. The day of definite diagnosis of MPM was considered the starting day for counting overall survival. The follow-up duration ranged from 6 to 76 months (median, 18 months).

Immunohistochemical staining. Immunohistochemical staining was performed according to the procedures described in previous reports (9, 11, 19). The following antibodies were used: rabbit polyclonal antibodies against GLUT1 (AB15309, 1:200 dilution; Abcam, Tokyo, Japan) and GLUT3 (AB15311, 1:100 dilution; Abcam); a rabbit monoclonal antibody against hexokinase I (AB55144, 1:200 dilution; Abcam); a mouse monoclonal antibody against HIF-1α (NB100-123; 1:50 dilution; Novus Biologicals, Inc., Littleton, Canada); a monoclonal antibody against VEGF (1:300 dilution; Immuno-Biological Laboratories Co.Ltd., Fujioka, Japan); mouse monoclonal antibodies against CD34 (1:800 dilution; Nichirei, Tokyo, Japan), Ki-67 (1:40 dilution; Dako, Glostrup, Denmark) and EGFR (1:100 dilution; Novocastra Laboratories Ltd., Newcastle, UK); rabbit monoclonal antibody against PTEN (1:50 dilution; Cell Signaling); rabbit polyclonal antibody against p-Akt (1:200 dilution; Abcam); a rabbit monoclonal antibodies against p-mTOR (1:80) and p-S6K (1:100 dilution; Cell Signaling both); and mouse monoclonal antibodies against p53 (D07, 1:50 dilution) and BCL-2 (1:100 dilution; both Dako). LAT1 expression was determined by immunohistochemical staining with an affinity-purified rabbit polyclonal anti-human LAT1 antibody (1.2 mg/ml; 1:3200) (20). An oligopeptide corresponding to amino acid residues 497-507 of human LAT1 (CQKLMQVVPQET) was synthesized. The *N*-terminal cysteine residue was introduced for conjugation with keyhole limpet hemocyanine. Antipeptide antibody was produced as described elsewhere (21). For immunohistochemical analysis, the antiserum was affinity-purified as described previously (21). For 4F2hc (CD98), an affinity purified goat polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Inc. USA) raised against a peptide mapping at the carboxy terminus of CD98 of human origin was used.

The detailed protocol for immunostaining assessment was as published elsewhere (2). The expression of GLUT1, GLUT3 and EGFR was considered positive if distinct membrane staining was present. Five fields (×400) were analyzed to determine the frequency of HIF-1α-stained nuclei and hexokinase I-stained cytoplasm. 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 was found. For GLUT1, GLUT3, EGFR, HIF-1α, hexokinase I, 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 positive cells. The tumors scoring 3 or above were graded as positive. LAT1 and CD98 expression was considered positive only if distinct membraneous 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 a total of 1000 neoplastic cells. The number of CD34-positive vessels was counted in four selected hot spots in a ×400 field (0.26 mm^2 field area). MVD was defined as the mean count of microvessels per 0.26 mm^2 field area. The median rate of VEGF positivity and the median numbers of CD34-positive vessels were evaluated, and the tumors in which stained tumor 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. According to a previous report (19), p53 expression in more than 10% of tumor cells was defined as positive expression. The expression of BCL-2 was considered to be positive if there was staining of the epithelial

component of the tumor. 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 having high expression. Approximately 1000 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 tumor cells with more than the median value were defined as having high expression. The sections were assessed using a light microscope 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. Follow-up for these 21 patients was conducted through 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. 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. Each protein revealed a unique profile pattern of expression. The immunohistochemical staining of LAT1, CD98, GLUT1, GLUT3, hexokinase I, HIF-1α, VEGF, CD34, Ki-67, EGFR, PTEN, p-AKT, p-mTOR, p-S6K, p53 and BCL-2 was evaluated for the 21 tumor lesions. Figure 1 shows representative immunohistochemical staining of LAT1 and CD98. LAT1 and CD98 immunostaining was detected in carcinoma cells in tumor tissues and localized predominantly on their plasma membrane. A positive LAT1 and CD98 expression was recognized in 47.6% (10/21) and 61.9% (13/21), respectively (p=0.535). GLUT1 and GLUT3 were detected in the tumor cells and localized predominantly on their plasma membrane. Positivity rate of GLUT1 and GLUT3 expression was recognized in 66.7% and 90.5% of the tumor samples, respectively. Positive expression of HIF-1α was predominantly expressed in the cytoplasm with some nuclear staining, and was recognized in 90.5% of the tumor samples. Positive expression of hexokinase I was found in the cytoplasm and/or membrane of the neoplastic cells, and was recognized in 76.2% of the tumor samples. The median value of the Ki-67 labeling index was 28% (range, 5-65%). The median rate of VEGF positivity was 70.0% (range, 25-88%), and the median numbers of CD34-positive vessels was 29 (12–58). Positive expression of EGFR, PTEN, p-AKT, p-mTOR and p-S6K was 42.8%, 47.6%, 61.9%, 42.8% and 76.2%, respectively. Positive expression of p53 and BCL-2 was recognized in 66.7% and 47.6% of the tumor samples, respectively.

LAT1 expression and other variables. The distribution of the other variables according to LAT1 expression is listed in Table I. A statistically significant difference between in the expression of CD98, VEGF, p-Akt, p-mTOR, p-S67 and p53 LAT1- positive and -negative tumors was found. Figure 2 shows the comparison between LAT1 positive and negative expression according to the positive rate for the different biomarkers.

Table I. *Distribution of the variables according to LAT1 expression*.

Variable		Total (n=21)	LAT1+ (n=10)	LAT1– (n=11)	*p*-Value
Age	≤65/>65	9/12	4/6	5/6	1.000
Gender	Male/female	18/3	7/3	11/0	0.090
Stage	I+II/III+IV	9/12	3/7	6/5	0.386
Histology	Epi/non-epi	15/6	8/2	7/4	0.635
CD98	Positive/negative	13/8	10/0	3/8	0.001
Glut1	Positive/negative	14/7	8/2	6/5	0.361
Glut3	Positive/negative	18/3	10/0	8/3	0.214
HIF-1α	Positive/negative	19/2	10/0	9/2	0.476
Hexokinase I	Positive/negative	16/5	9/1	7/4	0.310
VEGF	High/low	11/10	8/2	3/8	0.029
CD34	High/low	10/11	7/3	3/8	0.086
Ki-67	High/low	11/10	7/3	4/7	0.198
EGFR	Positive/negative	9/12	6/4	3/8	0.198
PTEN	Positive/negative	10/11	6/4	4/7	0.394
p-AKT	Positive/negative	13/8	9/1	4/7	0.023
p-mTOR	Positive/negative	9/12	7/3	2/9	0.029
p-S6K	Positive/negative	16/5	10/0	6/5	0.035
p53	Positive/negative	13/8	9/1	4/7	0.023
BCL-2	Positive/negative	10/11	5/5	5/6	1.000

LAT1: L-Type amino acid transporter 1; Epi: epithelial type; non-epi: non-epithelial type; 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; p-mTOR: phosphorylated mammalian target of rapamycin; p-AKT: phosphorylated AKT; p-S6K: phosphorylated S6K; CD34: indicator of microvessel density; Ki-67: indicator of cell proliferation; p53: cell cycle regulator; BCL-2: indicator of apoptosis.

Correlation between LAT1 expression and different biomarkers. The correlation between LAT1 and the different biomarkers using the scoring, positivity rate of the number of vessels was analyzed using Spearman's rank correlation and a significant correlation was found between LAT1 and CD98, GLUT3, HIF-1α, VEGF, CD34, Ki-67, p-AKT, p-mTOR and p-S6K expression (Table II).

Relationship between different variables and overall survival. The median survival time (MST) was 17.6 months for all the patients. Table III shows the survival analysis in relation to the different variables of all the patients (N=21). In the univariate analysis, disease stage, LAT1 and CD34 were significantly associated with poor overall survival. Figure 3 shows the Kaplan-Meier survival curves of the patients with positive or negative LAT1 and CD98 expression. According

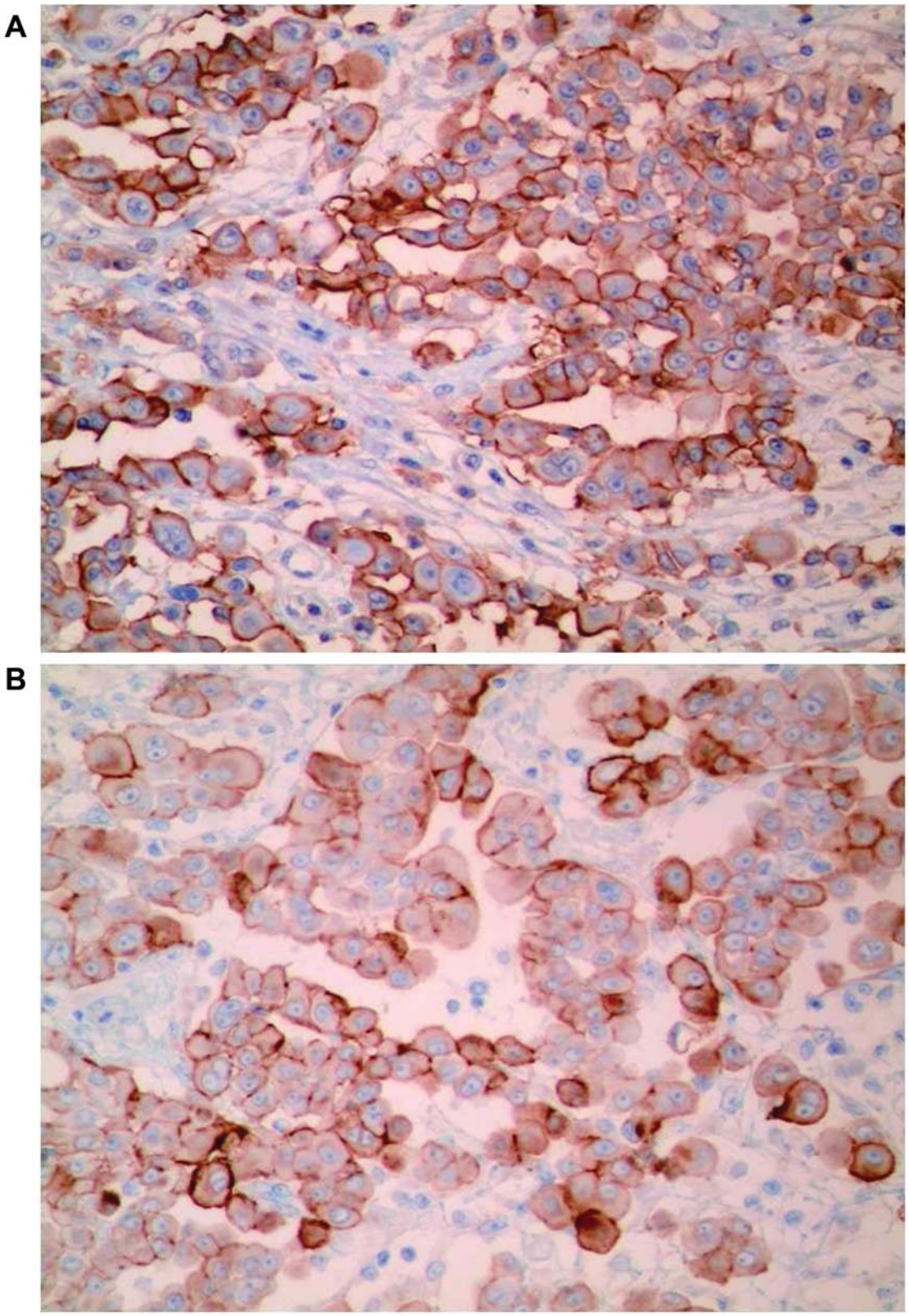
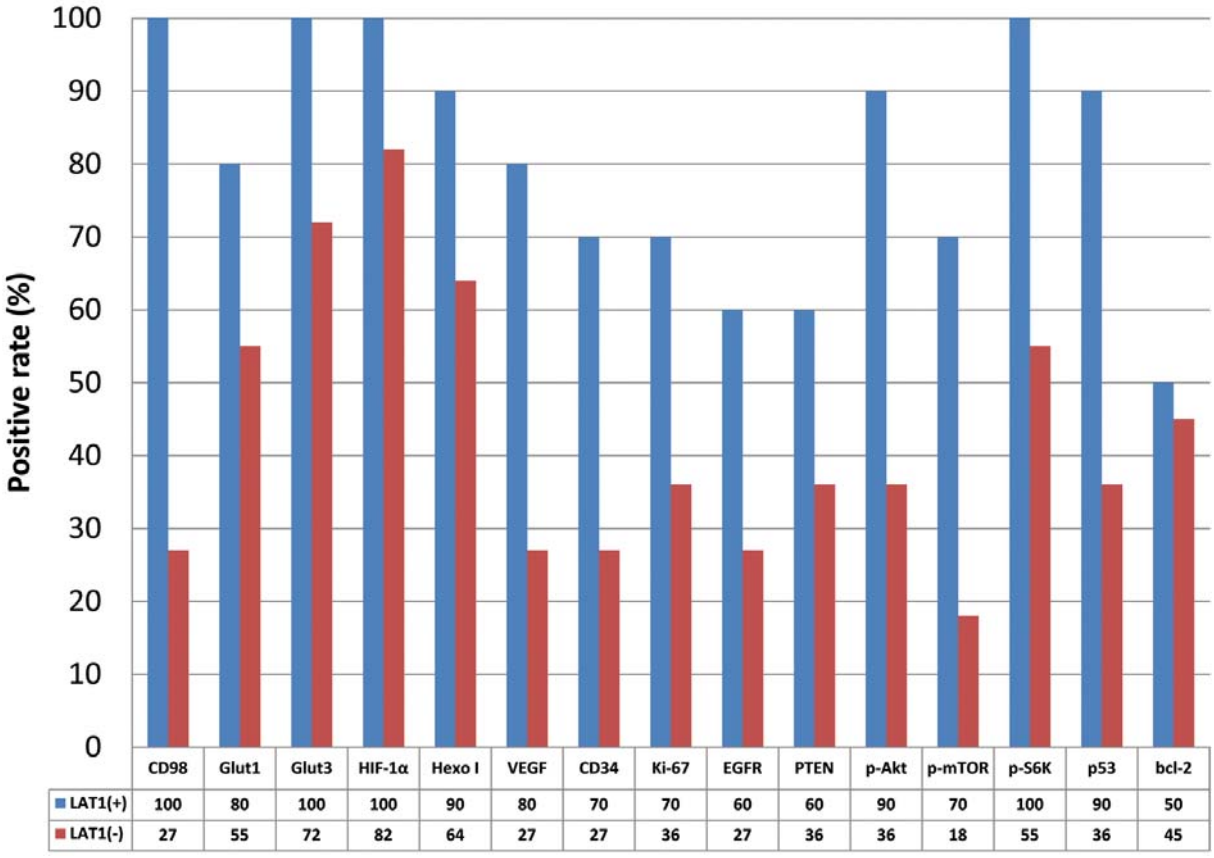


Figure 1. *Representative immunohistochemical staining. LAT1 (A) and CD98 (B) immunostaining of grade 4 and score 4, with membranous immunostaining pattern respectively.*



	CD98	Glut1	Glut3	HIF-1α	Hexo I	VEGF	CD34	Ki-67	EGFR	PTEN	p-Akt	p-mTOR	p-S6K	p53	bcl-2
LAT1(+)	100	80	100	100	90	80	70	70	60	60	90	70	100	90	50
LAT1(-)	27	55	72	82	64	27	27	36	27	36	36	18	55	36	45

Figure 2. *Comparison of the positive rate for different biomarkers according to LAT1 expression. A statistically significant difference in the expression of CD98, VEGF, p-AKT, p-mTOR, p-S6K and p53 was observed between patients with LAT1 positive and negative tumors, but there was no significant difference in the other biomarkers.*

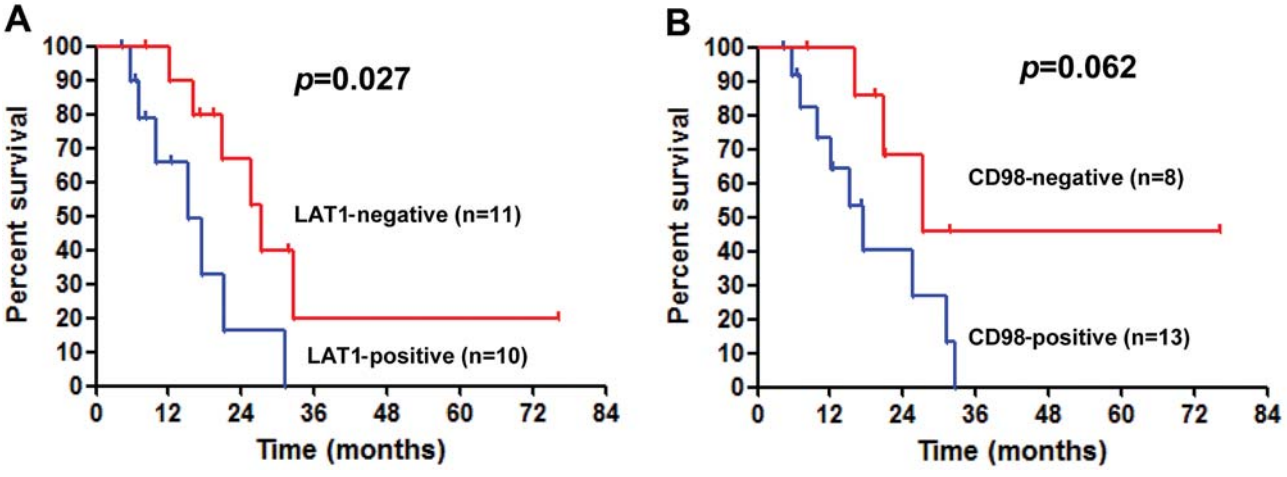


Figure 3. *Kaplan-Meier survival analysis in relation to LAT1 (A) and CD98 expression (B). Difference in overall survival between subgroups was analyzed using log-rank test.*

Table II. *Correlation between LAT1 and other biomarkers.*

Biomarker	Spearman γ (95% CI)	*p*-Value
CD98	0.517 (0.067 to 0.792)	0.023
GLUT1	0.359 (–0.127 to 0.707)	0.130
GLUT3	0.536 (0.094 to 0.801)	0.018
HIF-1α	0.523 (0.076 to 0.795)	0.021
Hexokinase I	0.388 (–0.094 to 0.722)	0.100
VEGF	0.676 (0.307 to 0.868)	0.001
CD34	0.539 (0.098 to 0.803)	0.017
Ki-67	0.531 (0.087 to 0.799)	0.019
EGFR	0.392 (–0.089 to 0.725)	0.096
PTEN	0.234 (–0.260 to 0.631)	0.334
p-AKT	0.556 (0.122 to 0.811)	0.013
p-mTOR	0.549 (0.112 to 0.808)	0.014
p-S6K	0.571 (0.067 to 0.792)	0.023

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; p-mTOR: phosphorylated mammalian target of rapamycin; p-AKT: phosphorylated AKT; p-S6K: phosphorylated S6K; CD34: indicator of microvessel density; Ki-67: indicator of cell proliferation; 95 CI: 95% confidence interval.

Table III. *Univariate and multivariate analyses of overall survival.*

Variable		Univariate analysis		Multivariate analysis
		MST (months)	*p*-Value	*p*-Value
Age, years	≤65/>65	13.8/23.2	0.191	
Gender	Male/female	20.9/NR	0.319	
Stage	I+II/III+IV	24.0/16.3	0.043	0.303
Histology	Epi/non-epi	20.9/23.3	0.777	
LAT1	Positive/negative	15.3/27.2	0.027	0.924
CD98	Positive/negative	17.4/27.2	0.062	0.515
GLUT1	Positive/negative	20.2/NR	0.105	
GLUT3	Positive/negative	20.1/21.3	0.761	
HIF-1α	Positive/negative	20.2/NR	0.275	
Hexokinase I	Positive/negative	20.2/NR	0.275	
VEGF	High/low	20.2/19.0	0.170	
CD34	High/low	13.6/23.2	0.044	0.408
Ki-67	High/low	16.3/23.2	0.643	
EGFR	Positive/negative	20.2/19.1	0.809	
PTEN	Positive/negative	28.3/17.3	0.118	
p-AKT	Positive/negative	19.1/22.4	0.744	
p-mTOR	Positive/negative	19.1/20.1	0.618	
p-S6K	Positive/negative	20.9/14.1	0.994	
p53	Positive/negative	19.2/24.5	0.526	
BCL-2	Positive/negative	23.2/17.3	0.252	

LAT1: L-Type amino acid transporter 1; epi: epithelial type; non-epi: non-epithelial type; 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; p-mTOR: phosphorylated mammalian target of rapamycin; p-AKT: phosphorylated Akt; p-S6K: phosphorylated S6K; CD34: microvessel density; Ki-67: indicator of cell proliferation; p53: cell cycle regulator; BCL-2: indicator of apoptosis; MST: median survival time; NR: not reached.

to the results of univariate log-rank test, disease stage, LAT1 and CD34 were significant prognostic factors; multivariate analysis demonstrated that none was an independent prognostic factor for predicting poor outcome.

Discussion

This is the first study to evaluate the LAT1 expression in patients with MPM, and positive LAT1 expression was found in 47.6% of the patients' samples with no significant difference between epithelial and non-epithelial types. LAT1 expression in MPM seemed to correspond to that in NSCLC (51%) (2). However, the present study was a preliminary investigation, with a small sample size, therefore a large-scale study is warranted to confirm the results.

The LAT1 expression was closely correlated with 4F2hc (CD98), glucose metabolism (GLUT3), hypoxia (HIF-1α), angiogenesis (VEGF and CD34), cell proliferation (Ki-67), the AKT/mTOR signal pathway (p-AKT, p-mTOR and p-S6K) and the cell cycle regulator (p53).

An experimental study demonstrated that LAT1 expression was closely related to the growth of liver metastases in a rat model (22), while the expression of LAT1 was significantly higher in the metastatic sites of human neoplasms than in the primary sites (8). Moreover, LAT1 expression was significantly associated with lymph node metastasis, cell proliferation and angiogenesis in NSCLC (2, 11). In patients with MPM, LAT1 expression also appears to play a crucial role in the development of cell proliferation and angiogenesis, and has a significant correlation with CD98. In particular, CD98, VEGF, the AKT/mTOR pathway and p53 seemed to be closely related to the overexpression of LAT1 in patients with MPM. Amino acid nutrition in mammalian cells is coupled to cell signaling *via* mTOR (6, 7) and coordinates the signal with cell growth and cell cycle progression (6). *In vitro* studies, the inhibition of LAT1 has been documented to reduce the level of phosphorylation of mTOR and p70S6K, indicating the close relationship between LAT1 and the mTOR pathway (6, 7). Our results also suggest that the expression of LAT1 plays an essential role in the activation of the AKT/mTOR pathway. If tumor cells have excess amounts of amino acids and LAT1 is overexpressed in the tumor cells, the kinase activity of mTOR may be stimulated, initiating a signaling cascade and regulating protein synthesis and cell proliferation. Since mTOR is a upstream of HIF-1α, VEGF, GLUT1 and GLUT3, the activation of mTOR may stimulate the expression of these hypoxic markers. Therefore, the

overexpression of LAT1 may be closely associated with not only CD98 but also the hypoxic condition, the mTOR pathway, cell proliferation and the cell cycle.

LAT1 has been described as a promising pathological factor for predicting prognosis in lung cancer and brain tumors (2, 20). The present preliminary data also indicated that the expression of LAT1 is useful for predicting poor outcome in patients with MPM. Biomarkers such as GLUT1, HIF-1α, VEGF, EGFR and p53 have also been suggested as prognostic factors to predict poor outcome in MPM, although no prognostic biomarker has yet been fimly established (1). As LAT1 expression was closely related to the regulation of these hypoxia and proliferative markers, we believe that LAT1 may have an important role in the pathogenesis and tumor progression of MPM in patients.

In conclusion, LAT1 is overexpressed in approximately 50% of patients with MPM. LAT1 expression is closely correlated with CD98, hypoxia markers, the mTOR pathway, Ki-67 and p53. Although this was a preliminary study, LAT1 was useful for predicting poor prognosis, and may be an important clinical marker for therapy for MPM. The inhibition of LAT1 function may also be cessation of tumor growth and provide a new and effective therapeutic target in MPM in the future

Conflicts of Interest Statement

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

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 staff of the Pathology Department in Shizuoka Cancer Center for their technical assistance in the immunohistochemical analyses.

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Received June 5, 2011
Revised November 8, 2011
Accepted November 9, 2011