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

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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-mammalian target of rapamycin (mTOR), p-56K, 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 manner (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
Materials and Methods

Patients. Between August 2003 and May 2009, 25 consecutive patients with MPM were included in this study. 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 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; I: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 antiseraum 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 hexokinasen stained cytoplasm. p-AKT, p-mTOR and p-S6K were considered positive if membranous and/or cytoplasmatic staining was present, and PTEN was positive if nuclear staining was found. For GLUT1, GLUT3, EGFR, HIF-1α, hexokinasen 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 membranous staining was present. Staining intensity was scored as follows: 1, ≤10% of tumor area stained; 2, 11-25% stained; 3, 26-50% stained; 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 mm² field area). MVD was defined as the mean count of microvessels per 0.26 mm² 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.

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.
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.
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 firmly 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.

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