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Expression of L-type amino acid transporter 1 (LAT1) as a prognostic and therapeutic indicator in multiple myeloma

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Key words

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L-type amino-acid transporter 1 (LAT1) plays a key role in cell growth and survival. To determine the prognostic significance of LAT1 in multiple myeloma (MM), we investigated the expression of LAT1 and its functional subunit, 4Fc heavy chain (CD98), on myeloma cells by immunohistochemistry in 100 newly diagnosed MM patients. High expression (moderate or strong staining intensity) of LAT1 and CD98 was detected in 56% and 45% of patients, respectively. The LAT1 expression score was positively correlated with Ki-67 index ($r = 0.631$, $P < 0.001$), and there was a statistically significant difference in Durie–Salmon stage between patients with high and low LAT1 expression ($P = 0.03$). In 43 patients treated with melphalan and prednisolone, the overall response rate was significantly higher in the high LAT1 expression group (60.0%) than in the low LAT1 expression group (17.6%) ($P = 0.03$). Multivariate analysis confirmed that high expression of LAT1 was a significant prognostic factor for predicting poor overall survival independently from the International Staging System (both $P = 0.01$). Here, we show that the overexpression of LAT1 is significantly associated with high proliferation and poor prognosis in newly diagnosed MM patients. Thus, LAT1 may be a promising pathological marker for identifying high-risk MM.

Multiple myeloma (MM) is a neoplastic disorder characterized by the clonal proliferation of plasma cells that produce monoclonal immunoglobulin. The clinical course of patients with MM is very heterogeneous, with patient survival ranging from a few months to more than 10 years.

To predict the prognosis of MM patients, the evaluation of cellular proliferative activity is regarded with great importance.[1–3] Proliferation status of myeloma cells has been traditionally estimated by the plasma cell labeling index,[4,5] nuclear proliferation antigen Ki-67 index,[6,7] or metaphase cytogenetics.[3] More recently, gene expression profiling has served as a powerful tool for determining clonal aggressiveness in patients with MM.[8] However, these methods are technically difficult for routine clinical use.

Amino acid transporters are essential for the survival and proliferation of normal and transformed cells.[9,10] Among the various types of amino acid transporters, L-type amino acid transporter 1 (LAT1), an isoform of the L-type amino acid transporters, requires a covalent association with the heavy chain of the 4F2 cell-surface antigen (CD98) for its functional expression and preferably transports large neutral amino acids, such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine.[11,12] LAT1 is highly expressed in human cancer cells of the lung,[13] brain,[14] prostate,[15] stomach,[16] and pancreas,[17] and its expression level is closely related to tumor proliferation, angiogenesis, and poor prognosis.

Although myeloma cells naturally produce large amounts of monoclonal immunoglobulin, the exact role of amino acid transporters in myeloma cells remains unknown. The aim of our study is to investigate the expression levels of LAT1 and CD98 on myeloma cells by immunohistochemistry and their correlation with clinicopathological characteristics in patients with newly diagnosed MM.

Materials and Methods

Patients and materials. Between January 2002 and December 2011, 109 patients were diagnosed with MM at the National

Hospital Organization Nishigunma Hospital on the basis of the criteria of the International Myeloma Working Group.[18] Bone marrow aspiration was carried out at the time of diagnosis in all patients, but clot specimens of nine patients were inadequate for histological evaluation. Therefore, a total of 100 patients were analyzed in this study. The study protocol was approved by the institutional ethics committee.

The age of the patients ranged from 35 to 87 years (mean age, 66 years). Clinical staging was carried out according to the Durie–Salmon staging system (DSS)[19] and the International Staging System (ISS).[20] Analyses of cytogenetic abnormalities by conventional G-banding karyotyping as well as routine laboratory tests were carried out at the time of diagnosis. As the initial therapy, 92 patients received conventional chemotherapy, including melphalan and prednisolone (MP), vincristine, doxorubicin, and dexamethasone, and ranimustine, vincristine, melphalan, and dexamethasone. Sequentially, 24 eligible patients underwent single or double high-dose melphalan with autologous stem cell transplantation support. Novel antimyeloma agents, such as bortezomib, lenalidomide, and thalidomide, were administered to 54 patients mostly as salvage treatment. Eight patients did not receive chemotherapy because of their poor general condition. The median follow-up duration was 30.2 months (range, 0.6–147.6 months).

Immunohistochemical staining. Immunohistochemistry was carried out with formalin-fixed, paraffin-embedded sections of aspirated bone marrow clots. LAT1 expression was determined by immunohistochemical staining with a LAT1 antibody (2 mg/mL, anti-human monoclonal mouse antibody, 4A2, provided by Dr. H. Endou [J-Pharma, Tokyo, Japan]; dilution, 1:3200). The production and characterization of the LAT1 antibody has previously been described.[15] The CD98 antibody is an affinity purified rabbit polyclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against a peptide mapping at the carboxy terminus of CD98 of human origin. The detailed protocol for immunostaining has been published elsewhere.[21]

Aggregates of bone marrow plasma cells that were CD138 positive (the protocol for immunostaining is described below) were evaluated for LAT1 and CD98 expression in sequential slides. First, each slide was scanned at ×100 magnification to determine at least three "hot spots", defined as areas with aggregates of plasma cells. The slides were then examined at ×600 magnification the immunostaining intensity of LAT1 and CD98. Cells were considered positive for LAT1 and CD98 only if distinct membrane staining was present. Without prior knowledge of the clinical data, two of the authors independently graded the staining intensity as follows: grade 0, no staining or less than 10% of tumor cells; grade 1+, ≥10% tumor cells with weak staining intensity; grade 2+, ≥10% tumor cells with moderate staining intensity; and grade 3+, ≥10% tumor cells with strong staining intensity. When the scores differed between observers, they were resolved by discussion at a dual-view microscope. Scores of 0 or 1+ were regarded as immunohistochemically low expression and 2+ or 3+ as immunohistochemically high expression.

For determination of the proliferative fraction of myeloma cells, Ki-67 expression in bone marrow plasma cells (i.e., Ki-67 index) was determined. Double-immunostaining for CD138 and Ki-67 was carried out to avoid the inclusion of high proliferative non-myeloma cells, such as erythroid precursors.[22] Briefly, deparaffinized and rehydrated tissue sections were heated at 100°C in antigen retrieval solution (Heat Processor Solution pH9; Nichirei Bioscience, Tokyo, Japan) for 20 min. After cooling to 60°C, the slides were blocked with 3% H_2O_2 in distilled water for 5 min to block endogenous peroxidase activity. They were then incubated with a primary antibody cocktail containing a 1:3 ratio of anti-CD138 antibody (B-A38; Nichirei Bioscience) and anti-Ki-67 antibody (SP6; Nichirei Bioscience) at room temperature for 30 min. After washing with PBS with Tween 20, the slides were incubated with the secondary antibody cocktail containing anti-mouse–HRP and anti-rabbit–alkaline phosphatase (MACH2 double stain polymer detection kit; Biocare Medical, Concord, CA, USA) at room temperature for 30 min and then washed in PBS with Tween 20. The HRP reaction color was developed by incubating the slides with the DAB substrate (MAX PO (MULTI); Nichirei Bioscience) for 10 min. The slides were washed in water, incubated with Fast Red II (Nichirei Bioscience) for 10 min to develop the reaction color from the ALP, and then washed in water again. The slides were rinsed with water, counterstained with hematoxylin, dehydrated, and mounted with Malinol (Muto Pure Chemicals, Tokyo, Japan) permanent mounting media. Ki-67 positive nuclei were manually counted in at least 500 bone marrow plasma cells (CD138 positive), and Ki-67 index were defined as percentages.

Statistical analysis. Probability values of less than 0.05 indicated a statistically significant difference. Fisher's exact test was used to examine the association between two categorical

Table 1. Demographics of 100 newly diagnosed multiple myeloma patients according to L-type amino-acid transporter 1 (LAT1) and CD98 expression status

Variables	LAT1			CD98		
	Low (n = 41)	High (n = 59)	*P*-value	Low (n = 55)	High (n = 45)	*P*-value
Age, years						
≤65	16	27	0.54	23	20	0.84
>65	25	32		32	25	
Sex						
Male	24	33	0.84	33	24	0.55
Female	17	26		22	21	
ISS						
1, 2	28	31	0.58	33	26	0.84
3	13	28		22	19	
Durie–Salmon stage						
I, II	14	9	*0.03*	18	5	*0.02*
III	27	50		37	40	
LDH (IU/L)						
≤220	36	45	0.11	42	39	0.19
>220	4	24		13	5	
CRP (mg/dL)						
≤0.3	25	28	0.22	28	25	0.69
>0.3	16	31		27	20	
Cytogenetic abnormality						
Positive	3	8	0.52	5	6	0.54
Negative	38	51		50	39	
Extra-medullary plasmacytoma						
Positive	4	4	0.71	3	5	0.46
Negative	37	55		52	40	
BMPC						
<60%	20	21	0.22	25	16	0.41
≥60%	21	38		30	29	

BMPC, bone marrow plasma cells; CRP, C-reactive protein; ISS, International scoring system; LDH, lactate dehydorogenase. Italic values indicate statistically significant difference.

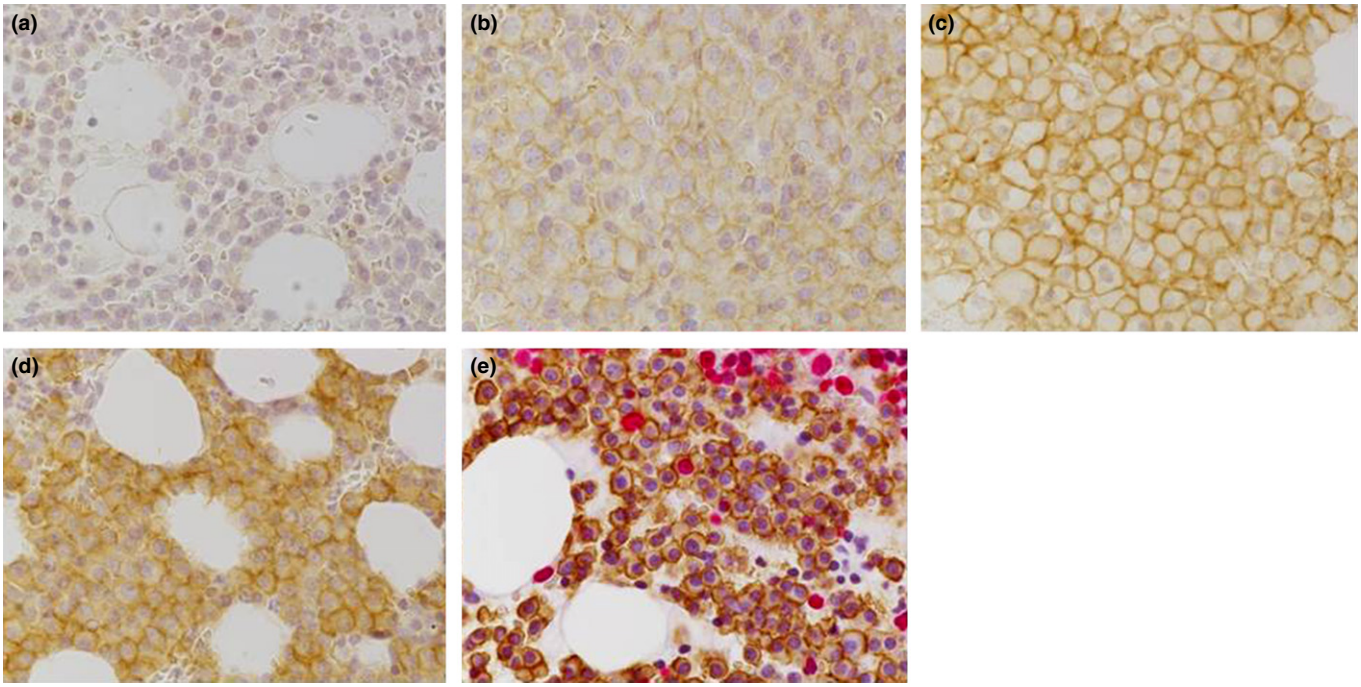

Fig. 1. Immunohistochemical staining of bone marrow tissues in newly diagnosed multiple myeloma patients. Representative images of scores 1+ (weak staining) (a), 2+ (moderate staining) (b), and 3+ (strong staining) for L-type amino acid transporter 1. (d) Representative image of score 3+ (strong) CD98 immunostaining. (e) Double-staining for CD138 (membranous, brown) and Ki-67 (nuclear, red).

variables. The correlation between different variables was analyzed by using the non-parametric Spearman's rank test. Both progression-free survival and overall survival were assessed by the Kaplan–Meier method and compared by the log–rank test. Multivariate analyses were carried out by using Cox's proportional hazards model to identify independent prognostic factors. The statistical analysis was carried out by using the R 1.6 package for Windows (http://cran.ism.ac.jp/).

Results

Immunohistochemical analysis. Patients' demographic information according to the expression status of LAT1 and CD98 and the correlation to parameters are shown in Table 1. Both LAT1 and CD98 expression were significantly associated with the DSS stage ($P = 0.03$ and $P = 0.02$, respectively) but not with the ISS stage. Representative pictures of the immunohistochemical staining of LAT1 according to the expression status, CD98, and double-immunohistochemical staining of CD138 and Ki-67 are shown in Figure 1. LAT1 and CD98 were highly (score ≥2) expressed on myeloma cells in 59% and 45% of patients, respectively. Both LAT1 and CD98 were highly expressed in 38% of patients. The Ki-67 index ranged from 0.2 to 26.0, and the median value was 3.6%.

Correlation of LAT1 and CD98 scores with other variables. Analysis with Spearman's rank correlation showed that the LAT1 score was significantly correlated with Ki-67 index ($r = 0.631$, $P < 0.001$), CD98 score ($r = 0.598$, $P < 0.001$), and the ISS stage ($r = 0.256$, $P = 0.01$) (Table 2). Figure 2 shows that the LAT1 score is closely correlated with Ki-67 index.

Survival analysis. For all patients, the 3-year survival rate and median survival time were 66.0% and 63.6 months, respectively. The Kaplan–Meier survival curves according to the expression status of LAT1 or CD98 are shown in Figure 3. Table 3 summarizes the results of analyses for prognostic factors. Univariate analysis indicated that DSS stage, ISS stage, cytogenetic abnormality, LAT1 expression, and CD98 expression were significant prognostic markers for progression-free survival, whereas age, DSS stage, ISS stage, and LAT1 expression were significant variables for overall survival. Based on the results of univariate analysis, we screened prognostic variables with a cut-off of $P < 0.1$. Multivariate analysis confirmed that high expression of LAT1 was an independent prognostic factor for poor progression-free survival ($P = 0.03$), and ISS stage 3 and high expression of LAT1 were each independent variables for predicting poor overall survival (both $P = 0.01$).

Treatment response to MP. In a subgroup of 45 patients who were initially treated with the MP regimen, response data after three cycles were available for 43 patients. Overall response rate (partial response or better) was significantly higher in the high LAT1 expression group (60.0%) than in the low LAT1 expression group (17.6%) ($P = 0.03$). Conversely, no significant differences were observed between the high CD98 expression group (50.0%) and the low CD98 expression group (36.0%) ($P = 0.53$).

Discussion

Cellular proliferation is one of the most powerful intrinsic prognostic factors in MM. Thus, evaluating proliferative

Table 2. Correlation between L-type amino-acid transporter 1 expression and other parameters in 100 newly diagnosed multiple myeloma patients

Parameters	Spearman γ	*P*-value
CD98 score	0.598	<0.001
Ki-67 index	0.641	<0.001
ISS	0.256	0.010

ISS, International Staging System.

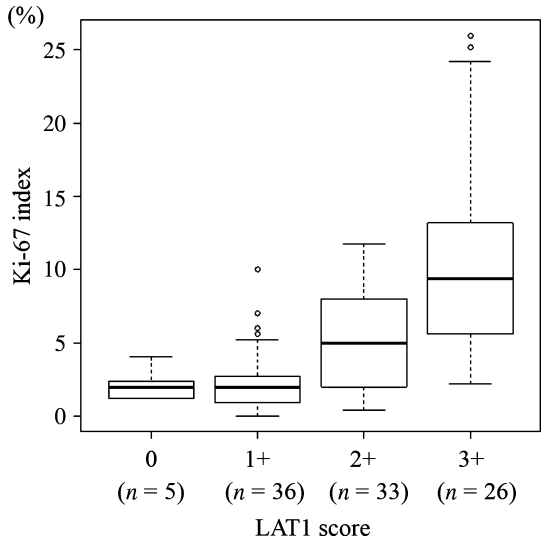

Fig. 2. Comparison of Ki-67 index in 100 newly diagnosed multiple myeloma patients with L-type amino acid transporter 1 (LAT1) immunohistochemical staining scores of 0–3+ in bone marrow tissue. The boundaries of the box show 25th and 75th percentiles, and the line within the box is the median. Whiskers show 10th and 90th percentiles, and the circles indicate outliers.

activity of myeloma cells is important for predicting the prognosis and determining personalized treatment strategies. Although several techniques have been proposed to assess the proliferative status of myeloma cells, these methods are not widely available due to their technical difficulty and high cost. In the present study, we found that the expression level of LAT1 was significantly correlated with the proliferative activity of myeloma cells. Furthermore, multivariate analysis indicated that MM patients with high expression of LAT1 have a shorter survival time independently from ISS stage. Although both ISS and DSS stages are the most commonly used prognostic indicators in patients with newly diagnosed MM, neither system was predictive of tumor aggressiveness, facilitating the need for other potent prognostic markers incorporating the intrinsic variability of myeloma cells.

In several solid tumors, high expression of amino acid transporters is associated with tumor aggressiveness and poor prognosis.[13–16,23,24] However, to our knowledge, this is the first report indicating a relationship between the expression level of amino acid transporter, cellular proliferation, and prognosis in patients with MM. As LAT1 expression was easily assayed by immunohistochemistry on formalin-fixed, paraffin-embedded sections, immunostaining of LAT1 would be a useful pathological marker for identifying high-risk MM.

Another interesting finding in the current study is that the LAT1 expression level appears to correlate with response to MP treatment. Melphalan has been widely used for over 40 years and remains one of the key drugs for MM[25–27]; however, this alkylating agent is toxic to normal hematopoietic stem cells and has carcinogenic potential.[28–30] One possible explanation for our finding is that overexpression of LAT1 could contribute to a high intracellular concentration of melphalan, resulting in enhanced cytocidal activity at the target cells. As melphalan is a

Table 3. Univariate and multivariate analysis of progression-free survival and overall survival in 100 newly diagnosed multiple myeloma patients

	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	3-year PFS (%)	*P*-value	HR (95% CI)	*P*-value	3-year OS (%)	*P*-value	HR (95% CI)	*P*-value
Age, years								
≤65	37.7	0.378			78.7	*0.010*	1.86 (0.91–3.79)	0.09
>65	33.2				56.3			
Sex								
Male	36.5	0.990			71.4	0.440		
Female	33.4				59.0			
Durie–Salmon stage								
I, II	56.5	*0.010*	1.46 (0.76–2.77)	0.25	88.7	*0.020*	1.61 (0.65–3.99)	0.31
III	28.5				59.1			
ISS								
1, 2	44.6	*0.004*	1.50 (0.91–2.48)	0.11	81.6	*<0.001*	2.51 (1.24–5.06)	*0.01*
3	21.2				44.3			
Cytogenetic abnormality								
Positive	10.9	*0.040*	1.77 (0.88–3.58)	0.11	43.6	*0.090*	1.75 (0.74–4.16)	0.21
Negative	38.2				68.7			
Extra-medullary plasmacytoma								
Positive	23.4	0.990			40.0	0.230		
Negative	35.9				68.0			
Expression of LAT1								
High	20.4	*<0.001*	1.88 (1.05–3.37)		55.8	*0.002*	2.47 (1.18–5.15)	*0.01*
Low	54.7				79.5			
Expression of CD98								
High	23.6	*0.020*	1.24 (0.72–2.12)	0.44	56.9	0.320		
Low	42.3				72.0			

CI, confidence interval; HR, hazard ratio; ISS, International Staging System; LAT1, L-type amino acid transporter 1; OS, overall survival; PFS, progression-free survival. Italic values indicate statistically significant difference.

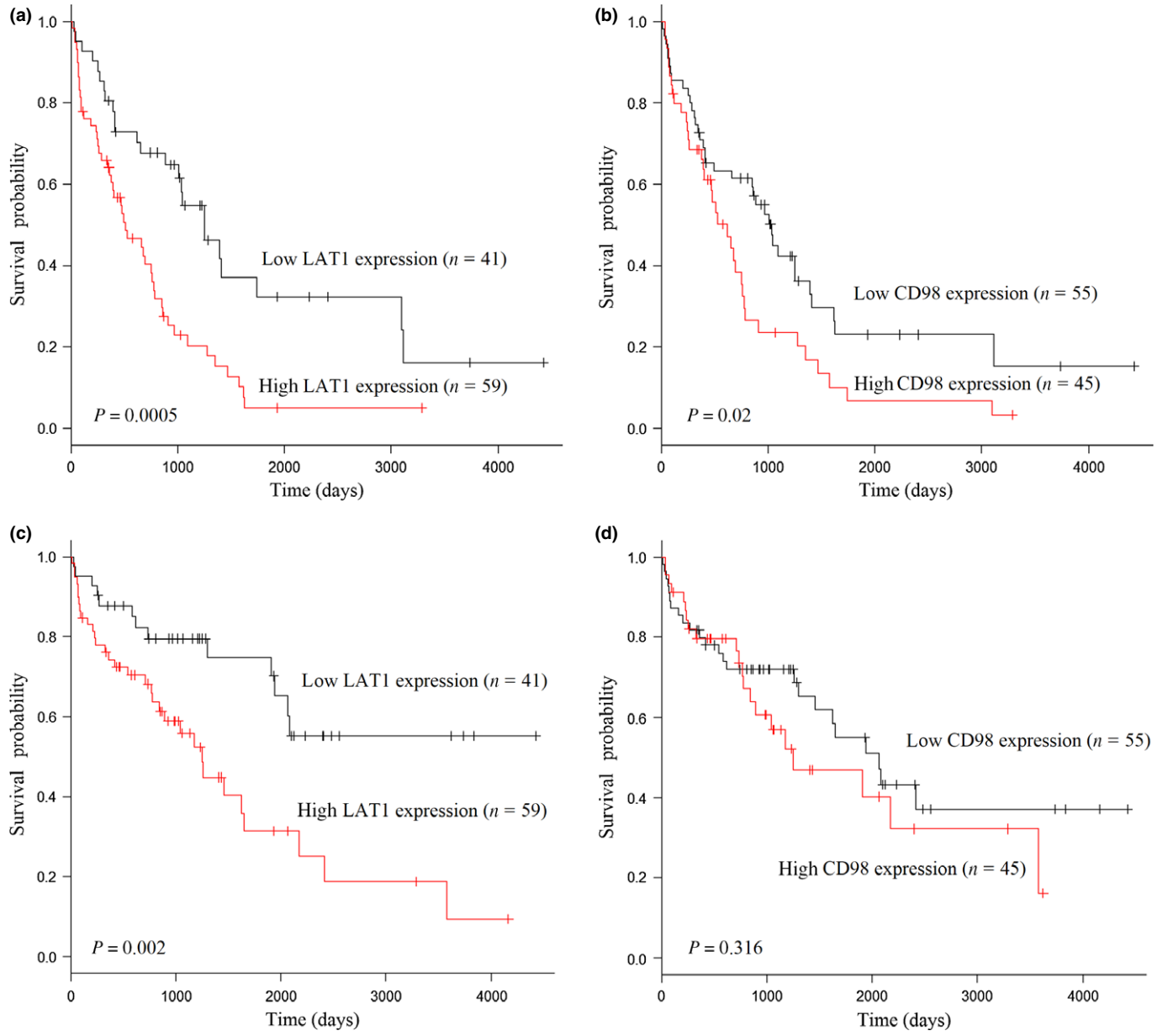

Fig. 3. Kaplan–Meier analysis of survival according to L-type amino acid transporter 1 (LAT1) and CD98 expression in 100 newly diagnosed multiple myeloma patients. Statistically significant differences in progression-free survival were observed between high and low LAT1 expression ($P = 0.0005$) (a) as well as between high and low CD98 expression ($P = 0.02$) (b). Conversely, a significant difference in overall survival was observed between high and low LAT1 expression ($P = 0.002$) (c) but not between high and low CD98 expression ($P = 0.316$) (d).

phenylalanine derivative of mechlorethamine, it could be taken up through LAT1 as well as other neutral amino acids.[31] Lin *et al.*[32] established by *in vitro* studies that Barrett's adenocarcinoma cell lines expressing LAT1 were sensitive to melphalan. Harada *et al.*[33] also reported that downregulation of CD98, a subunit of LAT1, could reduce melphalan uptake by the myeloma cells. Another possible explanation is that the cytocidal activity of melphalan could be dependent on the proliferative status of myeloma cells. The antineoplastic actions of alkylating agents are generally thought to be cell-cycle independent, whereas it has been reported that exponentially dividing cells are more sensitive to the cytotoxicity of melphalan than cells in a stationary phase.[34] Additionally, we found that patients with high LAT1 expression had worse outcome despite their better response to MP treatment. This discrepancy may be explained by the biological features of myeloma cells, in which DNA damaging agents would exert selective pressure and induce the expansion of resistant clones.[35] The Arkansas group using thalidomide and autologous stem cell transplantation has shown that higher complete remission rates are associated with prolonged event-free survival but not final overall survival.[36] Recent reports have also shown that achievement of complete response was not critical for the outcomes of patients with a low-risk gene expression profiling signature[37] or with a preceding course of smoldering disease.[38,39] Our current finding is consistent with these observations and implies that standard-dose melphalan provides a relatively small cytoreductive benefit for MM patients with low LAT1 expression.

Amino acid transporters transport amino acid radiotracers for PET imaging.[40,41] In patients with MM, PET using 2-[^{18}F]-fluoro-2-deoxy-D-glucose, a radio-labeled glucose analogue, has emerged as an imaging technique for initial staging and evaluation of treatment response,[42,43] but the clinical utility of amino acid PET tracers has been uncertain in MM. Recently, we have reported that amino acid PET using L-[3-^{18}F]-α-methyltyrosine (^{18}F-FAMT) could depict active MM lesions.[44] Different from other amino acid PET tracers such as ^{11}C-methionine, ^{18}F-FAMT is selectively transported by LAT1 and thus shows highly tumor-specific accumulation.[41] Indeed, in oral and lung malignancies, the uptake of ^{18}F-FAMT is significantly correlated with LAT1 expression level and tumor progression.[45,46] In the current study, we have also shown a significant correlation of LAT1 expression level and cellular proliferation determined by Ki-67 index in myeloma cells. These results suggest that ^{18}F-FAMT PET could be a promising imaging procedure to evaluate the proliferative activity of MM.

Mammalian target of rapamycin (mTOR) is a possible target for MM therapy. A clinical trial of combination therapy including mTOR inhibitor has been shown to be effective in MM patients.[47] LAT1 expression is correlated with the mTOR pathway, and the inhibition of LAT1 reduces phosphorylation of mTOR and tumor proliferation. Therefore, LAT1 inhibitor could be an alternative therapy to melphalan or mTOR inhibitor, and examination of LAT1 expression or PET imaging with ^{18}F-FAMT might be a predictor of therapeutic effectiveness.

Our study is limited by a relatively small number of patients and its retrospective nature in a single-center referral population. An additional study with a large number of uniformly treated patients is needed to clarify whether the LAT1 expression level could be predictive of the prognosis of patients. Response to the melphalan-based regimens and the outcome in relation to LAT1 expression should also be verified.

In conclusion, our study shows that the expression of LAT1 on myeloma cells is significantly associated with high proliferative activity as well as poor prognosis in patients with newly diagnosed MM. LAT1 expression at the time of MM diagnosis could be predictive of a subgroup of patients with an aggressive clinical course who would benefit from melphalan therapy.

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Disclosure Statement

The authors have no conflict of interest.

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