LAMP1 expression is associated with poor prognosis in breast cancer

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Abstract. Lysosome associated membrane protein-1 (LAMP1) is a heavily glycosylated lysosomal membrane protein, which is able to protect the lysosomal membrane from intracellular proteolysis. LAMP1 has been implicated in cancer development and progression. However, LAMP1 expression in breast cancer (BC) and its relationship with the clinical parameters of BC has not yet been fully investigated. In the present study, *LAMP1* expression in BC was characterized by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) on 20 pairs of fresh-frozen BC and corresponding non-cancerous tissues. In addition, tissue microarray immunohistochemistry (TMA-IHC) was conducted on 143 BC and matched non-cancerous tissue samples. The results of RT-qPCR and TMA-IHC demonstrated that *LAMP1* expression in BC tissues was significantly higher than in corresponding non-cancerous tissues. Furthermore, LAMP1 protein expression levels were significantly associated with histological grade (P=0.047), estrogen receptor expression (P=0.003), progesterone receptor expression (P=0.002), molecular classification (P=0.022), lymph node metastasis (P=0.033) and tumor-node-metastasis (TNM) stage (P=0.012). Multivariate analysis using Cox regression models and Kaplan-Meier survival curves demonstrated that LAMP1 expression (P=0.037), molecular classification (P=0.017) and TNM stage (P=0.003) were independent prognostic factors for overall survival. The above data suggested that LAMP1 expression is associated with malignant attributes of BC and may serve as a novel prognostic factor for patients with BC.

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Introduction

Breast cancer (BC) is one of the most common types of cancer in women in the developed and developing world, and remains a leading cause of mortality globally, with ~1,300,000 new cases and ~450,000 mortalities reported annually (1). BC represented 10% of all newly diagnosed cancers, 30% of female malignancies and up to 15% of cancer-associated mortality in 2013 worldwide (2,3). BC is a heterogeneous disease in relation to molecular changes, clinical-pathologic characteristics, responses to therapy and clinical outcomes. The tumorigenesis of BC is a complicated process characterized by genetic and epigenetic alterations that affect major cellular pathways involved in BC development (4). Although notable advances in early detection and therapeutic strategy for BC have been achieved, substantial challenges still remain to improve the prevention and treatment of BC (5). In China, the number of incidences of mortality in patients with BC has doubled during the last three decades (6). Further exploration of the molecular mechanisms underlying BC tumorigenesis and tumor progression is urgently required, as this may contribute to the identification of novel prognostic biomarkers and therapeutic targets for BC.

Lysosome associated membrane protein-1 (LAMP1), also known as CD107a, is a heavily glycosylated lysosomal membrane protein which is involved in protecting the lysosomal membrane from intracellular proteolysis (7,8). Although LAMP1 is primarily expressed in the endosome-lysosomal membrane of cells, it is also expressed in the plasma membrane (9,10). Furthermore, elevated LAMP1 expression at the cell surface has also been detected during platelet and granulocytic cell activation, as well as in metastatic tumor cells (10-12). Thus, cell-surface expressed LAMP1 may serve as a ligand for selectins and may be modulated by tumor cells (13). In a previous study, Furuta *et al* (14) reported high LAMP1 expression in colorectal neoplasm compared with normal mucosa, indicating LAMP1's potential function in cell adhesion and migration. LAMP1 has also been implicated to facilitate cancer progression and tumor metastasis (13,15). As for the prognostic role of LAMP1, Künzli *et al* (16) reported a

positive association between LAMP1 expression and survival status of patients with pancreatic carcinoma. Nevertheless, the relationship between LAMP1 expression and the clinicopathological significance of BC has not been investigated.

In the present retrospective study, the expression of LAMP1 was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in fresh BC samples and immunohistochemistry (IHC) in BC tissue microarrays (TMA). Furthermore, associations between LAMP1 expression and the clinicopathological attributes of patients with BC, particularly its prognostic significance, were further evaluated.

Materials and methods

Patient sample collection. A total of 20 fresh-frozen BC tissues and corresponding non-cancerous tissues were collected from the Department of Pathology, The Affiliated Hospital of Nantong University (Nantong, China). Simultaneously, another 143 paraffin-embedded BC tissue samples and 143 matched non-cancerous tissue samples were also obtained from the Department of Pathology, The Affiliated Hospital of Nantong University, from January 2002 to May 2010. Diagnosis of BC was validated by two pathologists in the department, according to the latest World Health Organization criteria (17). All patients underwent mastectomy and/or axillary dissection (radical or functional, based on clinical and surgical findings). Lymph node metastasis was confirmed by postoperative histological examination. The original clinical data were obtained from hospital medical records, including patient age, histological grade, hormone receptor (ER/PR status), erb-b2 receptor tyrosine kinase 2 (HER2) expression, tumor size, lymph node metastasis and tumor-node-metastasis (TNM) stage (18). None of the patients received preoperative radiotherapy or chemotherapy prior to surgery.

Ethics statement. The Ethics Committee of Nantong University (Nantong, China) and The Affiliated Hospital of Nantong University (Nantong, China) in the present study approved the study protocol. Written informed consent was acquired from all of the patients who were enrolled in the present study.

RT-qPCR in fresh BC tissues. A total of 20 fresh-frozen BC and corresponding non-cancerous tissues were included in the present study. Total RNA from BC tissues and non-cancerous tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and reverse transcribed to cDNA using a Revert Aid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. The primers for *LAMP1*, designed by Primer Express software (version 2.0; Thermo Fisher Scientific, Inc.) were as follows: Forward, 5'-GTT TCT TCA TTC TTT ACT G-3' and reverse, 5'-TCT CTA CTG TTG TAA TGT-3'. β-actin was included as an internal control, using the following primers: Forward, 5'-TAA TCT TCG CCT TAA TAC TT-3' and reverse, 5'-TAA TCT TCG CCT TAA TAC TT-3'. One-step PCR was performed using the DyNAmo Flash SYBR Green
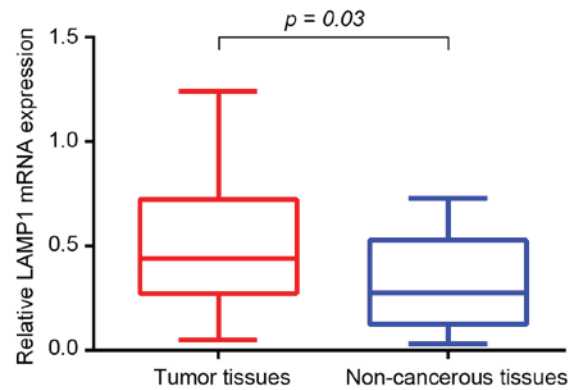

Figure 1. mRNA expression of *LAMP1* in BC and non-cancerous tissues. Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect *LAMP1* mRNA expression in BC compared with non-cancerous tissues using β-actin as the normalization control. *LAMP1* mRNA levels were significantly higher in BC compared with corresponding non-cancerous tissues. LAMP1, lysosome associated membrane protein-1; BC, breast cancer.

qPCR kit (cat. no. F-415XL; Thermo Fisher Scientific, Inc.) on an ABI 7500 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously described (19). Briefly, the PCR conditions were as follows: Initial denaturation at 95˚C for 10 min; denaturation at 95˚C for 15 sec, annealing and extension at 60˚C for 1 min, 40 cycles. All experiments were performed in triplicate. Relative quantification was performed using the $2^{-\Delta\Delta Cq}$ method (20).

IHC using the BC TMA. A total of 143 BC tissue samples and corresponding non-cancerous tissue samples were all fixed in 10% buffered formalin overnight at room temperature and then embedded in paraffin wax. Core tissue biopsies (2 mm in diameter) were taken from individual paraffin embedded sections (5 μm) to make a TMA. IHC analysis was performed as described previously (20-22). Following deparaffinization, endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at room temperature, then washed by PBS (3 times, 5 min each) and incubated with 1% goat normal serum in PBS for 30 min at room temperature. Subsequently, TMA sections were overnight at 4˚C incubated with a primary monoclonal mouse anti-LAMP1 antibody (cat. no. ab25630, 1:200; Abcam, Cambridge, MA, USA) in TBS and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG at room temperature (cat. no. A21010, 1:1,000; Abbkine, Inc, Redlands, CA, USA), followed by washing with TBS. LAMP1 immunostaining was evaluated by two trained pathologists from the Department of Pathology, the Affiliated Hospital of Nantong University under blinded experimental conditions. The percentage of LAMP1 positive cells were scored as follows: 0, 0-19%; 1, 20-39%; 2, 40-59% and 3, 60-100%. LAMP1 staining intensity was also scored as follows: 0, negative; 1, weakly positive; 2, moderately positive and 3, strongly positive. A combined score was generated by taking into consideration the percentage of positive cells and the staining intensity. The cut-off point for a statistically significant LAMP1 expression score in terms of overall survival was set using the X-tile software program (version 3.6.1; Rimm Lab, Yale
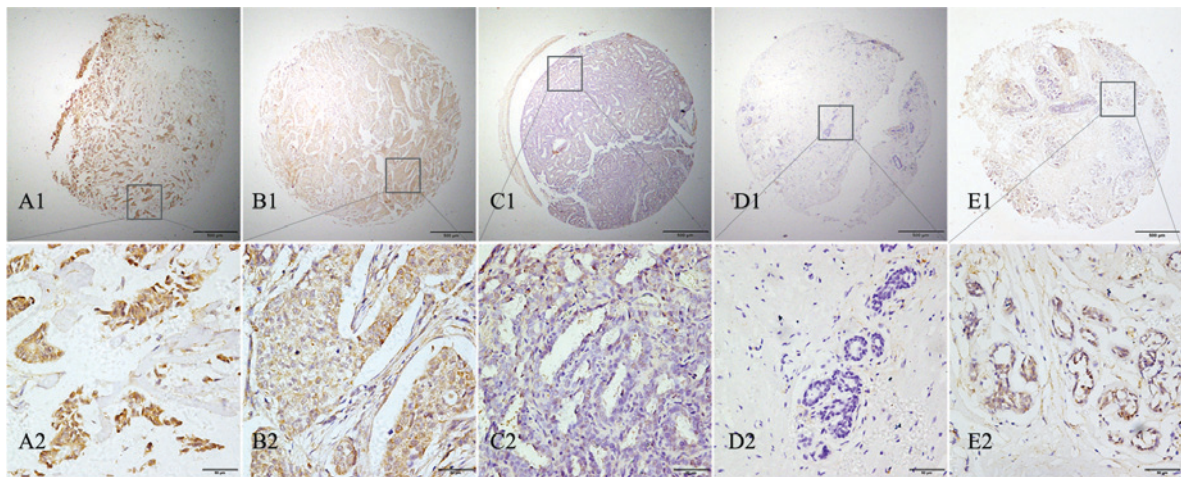
Figure 2. Representative images of LAMP1 protein expression in BC and corresponding non-cancerous tissues evaluated by tissue microarray. (A) High IHC staining of LAMP1 in the cytoplasm of BC cells. (B) High IHC staining of LAMP1 in the cytoplasm of BC cells. (C) Low IHC staining of LAMP1 in the cytoplasm of BC cells. (D) No IHC staining of LAMP1 in non-cancerous breast tissue cells. (E) Low IHC staining of LAMP1 in non-cancerous breast tissue cells. LAMPI, lysosome associated membrane protein-1; BC, breast cancer; IHC, immunohistochemical.

University, New Haven, CT, USA) (http://www.tissuearray.org/rimmlab) (23). The sum of the percentage and intensity scores was used as the final LAMP1 staining score and was defined as follows: <4 suggesting low or no expression and ≥4 indicating high expression.

Statistical analysis. The Wilcoxon signed rank nonparametric test was performed to compare the expression of *LAMP1* mRNA in fresh-frozen BC tissues with corresponding non-cancerous tissues. A χ^2 test was used to evaluate the associations between clinicopathologic variables and LAMP1 protein expression. Univariate and multivariate analyses were conducted using Cox proportional hazard regression models to determine factors that were independently associated with patients' overall survival. Kaplan-Meier survival analysis and log-rank tests were used to calculate survival curves. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed using STATA 16.0 software (StataCorp LP, College Station, TX, USA).

Results

LAMP1 mRNA expression in BC and corresponding non-cancerous tissues by RT-qPCR. The expression of *LAMP1* mRNA was analyzed by RT-qPCR in BC and non-cancerous tissue specimens obtained from 20 patients. *LAMP1* transcript levels were significantly higher in BC tissues compared with corresponding non-cancerous tissues (0.473±0.069 vs. 0.319±0.049, 1.5-fold; Fig. 1).

LAMP1 protein expression is increased in BC tissues compared with corresponding non-cancerous tissues. To investigate LAMP1 protein expression in BC, IHC was conducted on a BC TMA. As presented in Fig. 2, LAMP1 was detected at different intensities and percentages in BC, and was primarily located in the cytoplasm of BC cells. High LAMP1 expression was detected in 64.3% (82/143) of BC samples compared with 42.7% (61/143) of non-cancerous tissue samples (Fig. 2). These results indicated that LAMP1

protein expression was statistically increased in BC tissues compared with corresponding non-cancerous tissues (χ^2=13.5066; P=0.001).

Associations between LAMP1 protein expression and clinical parameters of patients with BC. The relationship between LAMP1 protein expression and important clinical parameters of patients with BC was investigated. As presented in Table I, high LAMP1 protein expression was significantly associated with histological grade (P=0.047), estrogen receptor expression (P=0.003), progesterone receptor expression (P=0.002), molecular classification (P=0.022), lymph node metastasis (P=0.033) and TNM stage (P=0.012). Specifically, the percentage of high LAMP1 expression in positive and negative lymph node metastasis was 65.4% (51/78) and 47.7% (31/65) respectively, and this difference was statistically significant (χ^2=4.537; P=0.033). The data demonstrated that patients with BC with positive lymph node metastasis suffered high incidence of positive LAMP1 expression, which indicates a correlation between high LAMP1 expression and positive lymph node metastasis. In comparison, no significant association was identified between LAMP1 expression and other clinical characteristics, including age, HER2 expression, Ki-67 expression and tumor size.

Survival analysis. In univariate analysis, the overall survival of 143 patients with BC was associated with LAMP1 expression (P=0.002), ER expression (P=0.017), PR expression (P=0.028), molecular classification (P=0.001), histological grade (P=0.008), lymph node metastasis (P=0.002) and TNM stage (P=0.001) (Table II). In multivariate analysis using Cox regression model, only LAMP1 expression (P=0.037), molecular classification (P=0.017) and TNM stage (P=0.003) may serve as independent prognostic factors for overall survival. Kaplan-Meier survival curves also demonstrated that patients with BC with high LAMP1 expression, molecular classification and advanced TNM stage were associated with unfavorable overall survival (Fig. 3).

Table I. Association of LAMP1 expression with clinical parameters in breast cancer.

Groups	No.	LAMP1 High expression, n (%)	Low or no expression, n (%)	χ^2	P-value
Total	143	82	61		
Age (years)					
≤60	101	58 (57.4)	43 (42.6)	0.001	0.975
>60	42	24 (57.1)	18 (42.9)		
Histological grade					
I	47	23 (48.9)	24 (51.1)	6.113	0.047[a]
II	69	38 (55.1)	31 (44.9)		
III	27	21 (77.8)	6 (22.2)		
ER expression					
Positive	109	55 (50.5)	54 (49.5)	8.882	0.003[a]
Negative	34	27 (79.4)	7 (20.6)		
PR expression					
Positive	97	47 (48.5)	50 (51.5)	9.741	0.002[a]
Negative	46	35 (76.1)	11 (23.9)		
HER-2 expression					
Positive	42	29 (69.0)	13 (31.0)	3.331	0.068
Negative	101	53 (52.5)	48 (47.5)		
Ki-67 expression					
High	46	26 (56.5)	20 (43.5)	0.019	0.891
Low	97	56 (57.7)	41 (42.3)		
Molecular classification					
Luminal A	69	34 (49.3)	35 (50.7)	9.638	0.022[a]
Luminal B	40	21 (52.5)	19 (47.5)		
Her-2 overexpression	24	18 (75.0)	6 (25.0)		
Triple negative	10	9 (90.0)	1 (10.0)		
Tumor size (T stage)					
T1	76	37 (48.7)	39 (51.3)	5.599	0.061
T2	54	35 (64.8)	19 (35.2)		
T3+T4	13	10 (76.9)	3 (23.1)		
Lymph node metastasis (N stage)					
N0	65	31 (47.7)	34 (52.3)	4.537	0.033[a]
N1+2+3	78	51 (65.4)	27 (34.6)		
TNM stage					
I	43	17 (39.5)	26 (60.5)	8.909	0.012[a]
II	74	46 (62.2)	28 (37.8)		
III	26	19 (73.1)	7 (26.9)		

[a]$P<0.05$. ER, estrogen receptor; PR, progesterone receptor; HER2, erb-b2 receptor tyrosine kinase 2; TNM, tumor-node metastasis.

Discussion

The lysosomal pathway represents a novel regulator of cell death in cancer through lysosomal membrane permeabilization (24-26). In normal cells, the outcome of cell death depends on the extent of lysosomal damage; limited lysosomal damage leads to cell death by apoptosis while large number of lysosomal breakdown results in cytosolic necrosis (27). The balance between apoptosis and necrosis is crucial for cancer development because cancer cells usually acquire mutations to protect themselves from cell death via the classical apoptotic pathways (28). LAMP1 is among the most abundant lysosomal membrane proteins, and it creates a glycocalyx on the inner side of the lysosomal membrane to protect the membrane from hydrolytic enzymes and degradation (18). High LAMP1 expression has been implicated in cancer

Table II. Univariate and multivariate analysis of prognostic factors in breast cancer for overall survival.

Variables	Yrs	Univariate analysis			Multivariate analysis		
		HR	P-value	95% CI	HR	P-value	95% CI
LAMP1 expression							
Low or no vs. high	5	3.246	0.002[a]	1.551-6.791	2.251	0.037[a]	1.051-4.821
Age (years)							
≤60 vs. >60	5	1.856	0.053	0.993-3.470			
ER expression							
Positive vs. negative	5	0.450	0.017[a]	0.233-0.869			
PR expression							
Positive vs. negative	5	0.489	0.028[a]	0.259-0.925			
Her2 expression							
Positive vs. negative	5	1.859	0.060	0.973-3.551			
Ki-67 expression							
Low vs. high	5	1.076	0.837	0.538-2.149			
Molecular classification							
Luminal A vs. luminal B vs. Her-2 overexpression vs. triple negative	5	1.675	0.001[a]	1.235-2.274	1.483	0.017[a]	1.071-2.053
Histological grade							
I vs. II vs. III	5	1.763	0.008[a]	1.159-2.684	1.499	0.072	0.964-2.333
T stage							
T1 vs. T2 vs. T3+T4	5	1.231	0.368	0.783-1.936			
Lymph node metastasis (N stage)							
N0 vs. N1+2+3	5	1.822	0.002[a]	1.256-2.643			
TNM stage							
I vs. II vs. III	5	2.206	0.001[a]	1.371-3.551	2.195	0.003[a]	1.318-3.655

[a]P<0.05. LAMP1, lysosome associated membrane protein-1; ER, estrogen receptor; PR, progesterone receptor; T, tumor stage: N, lymph node metastasis stage; TNM, tumor-node metastasis; HR, hazard ration; CI, confidence interval.

development and progression, including in astrocytoma, colorectal cancer, pancreatic carcinoma and various other cancer tissues (14,16,29,30). LAMP1 has also been suggested to facilitate cancer metastasis by acting as a ligand for galectin-3, to increase translocation to the cell membrane (31,32). The aforementioned studies all indicate certain malignant characteristics of LAMP1 in human cancers. However, little is known about the function of LAMP1 in BC.

In the present study, RT-qPCR analysis in a small number of BC samples revealed a significantly higher level of *LAMP1* gene expression in BC tissues than in non-cancerous tissues. Subsequently, IHC analysis was conducted in a constructed TMA and the results demonstrated that LAMP1 protein expression in BC was significantly higher than in non-cancerous tissues. These data are consistent with previous studies demonstrating LAMP1 overexpression in various types of cancers (14,16,30). In addition, high LAMP1 expression in BC was associated with certain clinical attributes, including histological grade, ER/PR expression, molecular classification, and TNM stage. Ozaki *et al* (31) stated that LAMP1 expression on the cell surface was associated with the metastatic potential of melanoma cells, and downregulation of LAMP1 significantly

inhibited cancer metastasis. Agarwal *et al* (33) also demonstrated that LAMP1 was well known to act as a carrier of β1,6 branched N-oligosaccharides, which are aberrantly expressed in several human cancers and have malignant potential. The results of the present study are in line with these studies, and further confirm the association between LAMP1 expression and malignant attributes of BC.

To date, studies investigating the prognostic value of LAMP1 are limited; therefore, the association between LAMP1 expression and overall survival was evaluated in patients with BC. Univariate analysis revealed that in addition to cytoplasmic expression of LAMP1, ER and PR expression, molecular classification, histological grade, lymph node metastasis and TNM stage were also associated with survival in patients with BC. Multivariate analysis subsequently demonstrated that high LAMP1 expression, molecular classification and TNM stage were independent predictors of poor prognosis in patients with BC. Kaplan-Meier analysis further verified that patients with BC with high LAMP1 expression suffered a significantly reduced life span. However, the results of the present study contrast with those of a previous study, in which Künzli *et al* (16) reported that patients with pancreatic
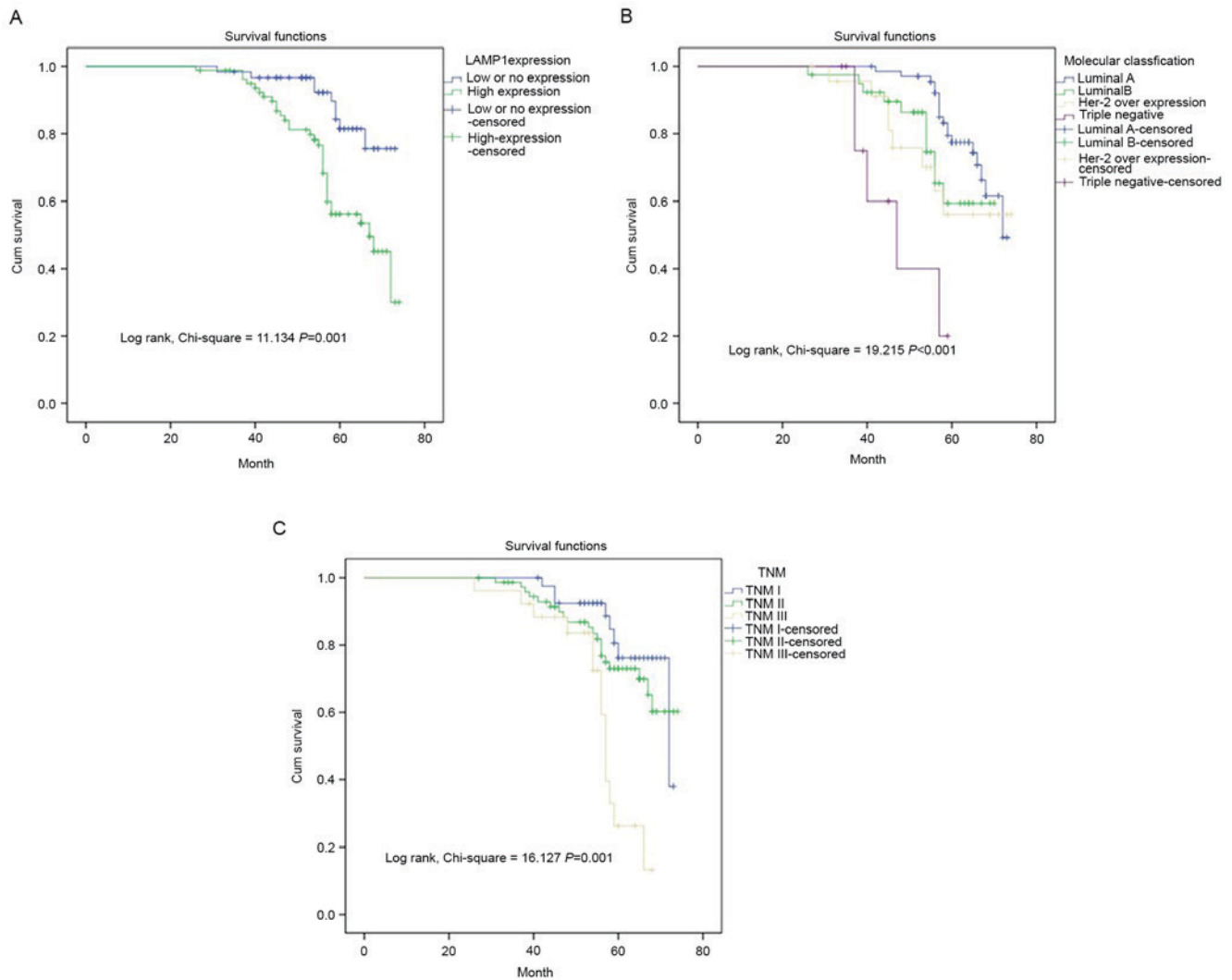
Figure 3. Survival analysis of patients with BC using the Kaplan-Meier method. (A) Overall survival rate in patients with BC with high expression of LAMP1 (green line) was significantly lower than that in patients with BC with low or no LAMP1 expression (blue line). (B) Overall survival rate in patients with BC with TNM stage III (gray line) was significantly lower than that in patients with BC with TNM stage I and stage II (blue and green lines). (C) Overall survival rate in triple negative patients with BC with high HER2 expression (purple line) was significantly lower than that in luminal A (blue line), luminal B (green line), and patients with BC overexpressing HER2 (gray line). BC, breast cancer; LAMP1, lysosome associated membrane protein-1; TNM, tumor-node metastasis; HER2, erb-b2 receptor tyrosine kinase 2.

carcinoma with higher LAMP1 expression had longer survival time. This inconsistency may be due to the differences in the tumor types (pancreatic carcinoma vs. BC), experimental methods (northern blot vs. IHC) or evaluation system (mRNA expression vs. protein expression).

In addition, there are several limitations faced by the present study. For example, the use of archived, convenient BC samples may introduce bias into this retrospective, observational study, hence future studies that include larger sample sizes are necessary to confirm the results of the present study. Secondly, the construction of TMA using small sections of tissue blocks to analyze target protein expression may not be representative of the whole tissue block. Thirdly, the mechanisms underlying of how LAMP1 protein influences the tumor microenvironment in BC remains to be fully elucidated. A series of in vitro and in vivo experiments, including overexpression and knockdown studies, are in progress. We anticipate that our research group will publish related studies to illustrate the mechanisms underlying LAMP1 activity in

BC development. Fourthly, blood samples were not collected but this problem may be addressed when the construction of the Biobank at our hospital is completed.

To the best of our knowledge, the present study was the first to report on the differential expression of LAMP1 in BC, at the gene and protein level. the results indicated that LAMP1 may be a novel prognostic biomarker in patients with BC. Further in vitro mechanistic studies concerning LAMP1 in BC are being conducted by our research group.

Acknowledgements

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