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Original Article

Expression of autophagy related proteins in invasive lobular carcinoma: comparison to invasive ductal carcinoma

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Abstract: The aim of this study is to compare the expression of autophagy related proteins in invasive lobular carcinoma (ILC) with that of autophagy related proteins in invasive ductal carcinoma (IDC), and to determinate its implication. Tissue microarray containing 114 ILC and 692 IDC was constructed, and immunohistochemistry was performed for autophagy related protein (beclin-1, LC3A, LC3B, p62) and Ki-67. No significant difference in expression of autophagy-related proteins between pleomorphic type (n = 12) and classic type (n = 102) of ILC was observed, whereas ILC and IDC showed distinguished features that tumoral beclin-1, stromal LC3A, tumoral LC3B, tumoral p62 were highly expressed in IDC and tumoral BNIP3 was highly expressed in ILC ($P < 0.001$). Beclin-1 expression was correlated with ER negativity ($P = 0.016$) and TNBC type ($P = 0.024$). BNIP3 expression was correlated with ER positivity ($p = 0.040$). Using multivariate Cox analysis, shorter overall survival was associated with tumoral beclin-1 positivity (hazard ratio: 21.19, 95% CI: 1.098-409.1, $P = 0.043$). In conclusion, ILC and IDC showed different expression pattern of autophagy-related proteins in tumor and stroma that demonstrated by higher expression of tumoral beclin-1, stromal LC3A, tumoral LC3B, tumoral p62 in IDC, and higher expression of tumoral BNIP3 in ILC.

Keywords: Autophagy, breast cancer, lobular cancer

Introduction

Breast cancer is one of the most common cancers in women, accounts for about 23% of female carcinoma [1]. Invasive carcinoma of the breast is categorized into the several histologic subtypes, largely into invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) [1]. ILC comprises about 5-15% of invasive carcinoma [2, 3], appears to increase more than IDC in recent decade, along with hormone replacement therapy and alcohol consumption [4, 5]. ILC is distinguished from IDC by clinical and histological features. ILC usually presents as multiple lesions, and tends to involve bilateral breast [6, 7]. Histologically, ILC is characterized by non-cohesive cancer cells lacking e-cadherin [8]. Metastasis sites of ILC include bone, gastrointestinal tract, uterus, meninges, and ovary, which are unusual pattern of involvement in case of IDC. Diffuse serosal involvement is often found in ILC [7, 9, 10].

Cancer cells survive under condition of oxygen and glucose deprivation with angiogenesis and/or aerobic glycolysis. If this regulatory compensation is insufficient to meet the high metabolic demand of highly aggressive malignant tumor, alternative metabolic pathway triggers autophagy, in which cells cannibalize and recycle their cytoplasmic components for energy production [11]. Although autophagy is believed to be important in tumor metabolism, expression of autophagy related protein in ILC is yet uncertain because most of prior studies have analyzed of IDC [12, 13]. Since ILC is distinctive from IDC in clinical, histological, and molecular aspects, we assumed that status of autophagy related proteins are different from IDC. The aims of this study were to compare the expression of autophagy related proteins in ILC to IDC, and to determinate its implication.

Material and methods

Patient selection and clinicopathologic evaluation

Between January 2000 and December 2012, formalin-fixed paraffin-embedded (FFPE) tissue

Table 1. Clones, dilutions, and sources of antibodies used

Antibody	Clone	Dilution	Source
Autophagy related			
Beclin-1	Polyclonal	1:100	Abcam, Cambridge, UK
LC3A	EP1528Y	1:100	Abcam, Cambridge, UK
LC3B	Polyclonal	1:100	Abcam, Cambridge, UK
p62	SQSTM1	1:100	Abcam, Cambridge, UK
BNIP3	Ana40	1:100	Abcam, Cambridge, UK
Molecular subtype-related			
ER	SP1	1:100	Thermo Scientific, CA, USA
PR	PgR	1:50	DAKO, Denmark
HER-2	Polyclonal	1:1500	DAKO, Denmark
Ki-67	MIB-1	1:150	DAKO, Denmark

Table 2. Clinicopathologic characteristics of patients with invasive lobular carcinoma

Parameter	Total N = 114(%)	Classic type N = 102 (%)	Pleomorphic type N = 12 (%)	*P*-value
Age (years)				0.033
< 50	63 (55.3)	60 (58.8)	3 (25.0)	
≥ 50	51 (44.7)	42 (41.2)	9 (75.0)	
Nuclear grade				< 0.001
1/2	102 (89.5)	102 (100.0)	0 (0.0)	
3	12 (10.5)	0 (0.0)	12 (100.0)	
Histologic grade				< 0.001
I/II	109 (95.6)	102 (100.0)	7 (58.3)	
III	5 (4.4)	0 (0.0)	5 (41.7)	
T stage				0.026
T1	67 (58.8)	64 (62.7)	3 (25.0)	
T2/T3	47 (41.2)	38 (37.3)	9 (75.0)	
Lymph node metastasis				0.749
Absent	80 (70.2)	72 (70.6)	8 (66.7)	
Present	34 (29.8)	30 (29.4)	4 (33.3)	
ER				0.158
Negative	7 (6.1)	5 (4.9)	2 (16.7)	
Positive	107 (93.9)	97 (95.1)	10 (83.3)	
PR				0.005
Negative	19 (16.7)	13 (12.7)	6 (50.0)	
Positive	95 (83.3)	89 (87.3)	6 (50.0)	
HER-2				0.002
Negative	107 (93.9)	99 (97.1)	8 (66.7)	
Positive	7 (6.1)	3 (2.9)	4 (33.3)	
Ki-67 LI				< 0.001
≤ 14	92 (80.7)	88 (86.3)	4 (33.3)	
> 14	22 (19.3)	14 (13.7)	8 (66.7)	
Molecular type				< 0.001
Luminal A	86 (75.4)	83 (81.4)	3 (25.0)	
Luminal B	22 (19.3)	15 (14.7)	7 (58.3)	
HER-2	1 (0.9)	0 (0.0)	1 (8.3)	
TNBC	5 (4.4)	4 (3.9)	1 (8.3)	

samples were collected at Severance Hospital, from patients that had resection of breast due to ILC. Tissue samples diagnosed of IDC, no specific type in 2006 were prepared for control group. The study was approved by the Institutional Review Board of Severance Hospital. Patients who had received preoperative chemotherapy were excluded. Histologic examination was performed by hematoxylin and eosin (H&E) staining. All the slides were reviewed retrospectively by breast pathologists (Koo JS). The histological grade was assessed using the Nottingham grading system [14]. Tumor staging was based on the 7th American Joint Committee on Cancer (AJCC) criteria. Disease-free survival (DFS) was calculated from the date of the first curative surgery to the date of the first loco-regional or systemic relapse, or death without any type of relapse. Overall survival (OS) was estimated from the date of the first curative operation to the date of the last follow-up or death from any cause. Clinicopathologic parameters evaluated in each breast cancer included patient age at initial diagnosis, lymph node metastasis, tumor recurrence, distant metastasis, and patient's survival.

Tissue microarray

Through retrospective review of the H&E-stained slides, the most appropriate formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were obtained. The most representative tumor areas were marked on FFPE

Table 3. Expression of autophagy-related proteins in ILC according to cytologic type

Parameter	Total N = 114 (%)	Classic type N = 102 (%)	Pleomorphic type N = 12 (%)	*P*-value
Beclin-1 (T)				0.508
Negative	83 (72.8)	73 (71.6)	10 (83.3)	
Positive	31 (27.2)	29 (28.4)	2 (16.7)	
LC3A (T)				1.000
Negative	112 (98.2)	100 (98.0)	12 (100.0)	
Positive	2 (1.8)	2 (2.0)	0 (0.0)	
LC3B (T)				0.675
Negative	98 (86.0)	88 (86.3)	10 (83.3)	
Positive	16 (14.0)	14 (13.7)	2 (16.7)	
LC3B (S)				0.594
Negative	105 (92.1)	93 (91.2)	12 (100.0)	
Positive	9 (7.9)	9 (8.8)	0 (0.0)	
p62 (T)				0.220
Negative	68 (59.6)	63 (61.8)	5 (41.7)	
Positive	46 (40.4)	39 (38.2)	7 (58.3)	
BNIP3 (T)				0.210
Negative	38 (33.3)	32 (31.4)	6 (50.0)	
Positive	76 (66.7)	70 (68.6)	6 (50.0)	
BNIP3 (S)				1.000
Negative	113 (99.1)	101 (99.0)	12 (100.0)	
Positive	1 (0.9)	1 (1.0)	0 (0.0)	

T: tumor; S: tumor stroma.

Table 4. Expression of autophagy-related proteins in IDC and ILC

Parameter	Total N = 806 (%)	IDC N = 692 (%)	ILC N = 114 (%)	*P*-value
Beclin-1 (T)				< 0.001
Negative	463 (57.4)	380 (54.9)	83 (72.8)	
Positive	343 (42.6)	312 (45.1)	31 (27.2)	
LC3A (T)				0.153
Negative	767 (95.2)	655 (94.7)	112 (98.2)	
Positive	39 (4.8)	37 (5.3)	2 (1.8)	
LC3A (S)				< 0.001
Negative	588 (73.0)	474 (68.5)	114 (100.0)	
Positive	218 (27.0)	218 (31.5)	0 (0.0)	
LC3B (T)				< 0.001
Negative	546 (67.7)	448 (64.7)	98 (86.0)	
Positive	260 (32.3)	244 (35.3)	16 (14.0)	
LC3B (S)				< 0.001
Negative	556 (69.0)	451 (65.2)	105 (92.1)	
Positive	250 (31.0)	241 (34.8)	9 (7.9)	
p62 (T)				< 0.001
Negative	325 (40.3)	257 (37.1)	68 (59.6)	
Positive	481 (59.7)	435 (62.9)	46 (40.4)	
BNIP3 (T)				< 0.001
Negative	506 (62.8)	468 (67.6)	38 (33.3)	
Positive	300 (37.2)	224 (32.4)	76 (66.7)	
BNIP3 (S)				0.109
Negative	775 (96.2)	662 (95.7)	113 (99.1)	
Positive	31 (3.8)	30 (4.3)	1 (0.9)	

T: tumor; S: tumor stroma.

blocks and then 3 mm tissue cores were extracted by punch machine from the selected areas. Extracted tumor spots were inserted in 6 x 5 recipient blocks. Every 2 tissue cores were extracted from each case for TMA construction.

Immunohistochemistry

The antibodies used for immunohistochemistry in this study are shown in **Table 1**. Three micro meter paraffin sections were deparaffinized and rehydrated by xylene and alcohol solution. Immunohistochemistry was performed using the Ventana Discovery XT automated stainer (Ventana Medical System, Tucson, AZ, USA). Antigen retrieval was performed using CC1 buffer (Cell Conditioning 1; citrate buffer pH 6.0, Ventana Medical System). Appropriate positive and negative controls for immunohistochemistry were included.

Interpretation of immunohistochemical results

A cut-off value of 1% or more positively stained nuclei was used to define ER and AR positivity [15]. HER-2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using the following categories: 0 = no immunostaining; 1+ = weak incomplete membranous staining, less than 10% of tumor cells; 2+ = complete membranous staining, either uniform or weak in at least 10% of tumor cells; and 3+ = uniform intense membranous staining in at least 30% of tumor cells [16]. HER-2 immunostaining was considered positive when strong (3+) membranous staining was observed whereas cases with 0 to 1+ were regarded as negative. The cases showing 2+ HER-2 expression were evaluated for HER-2

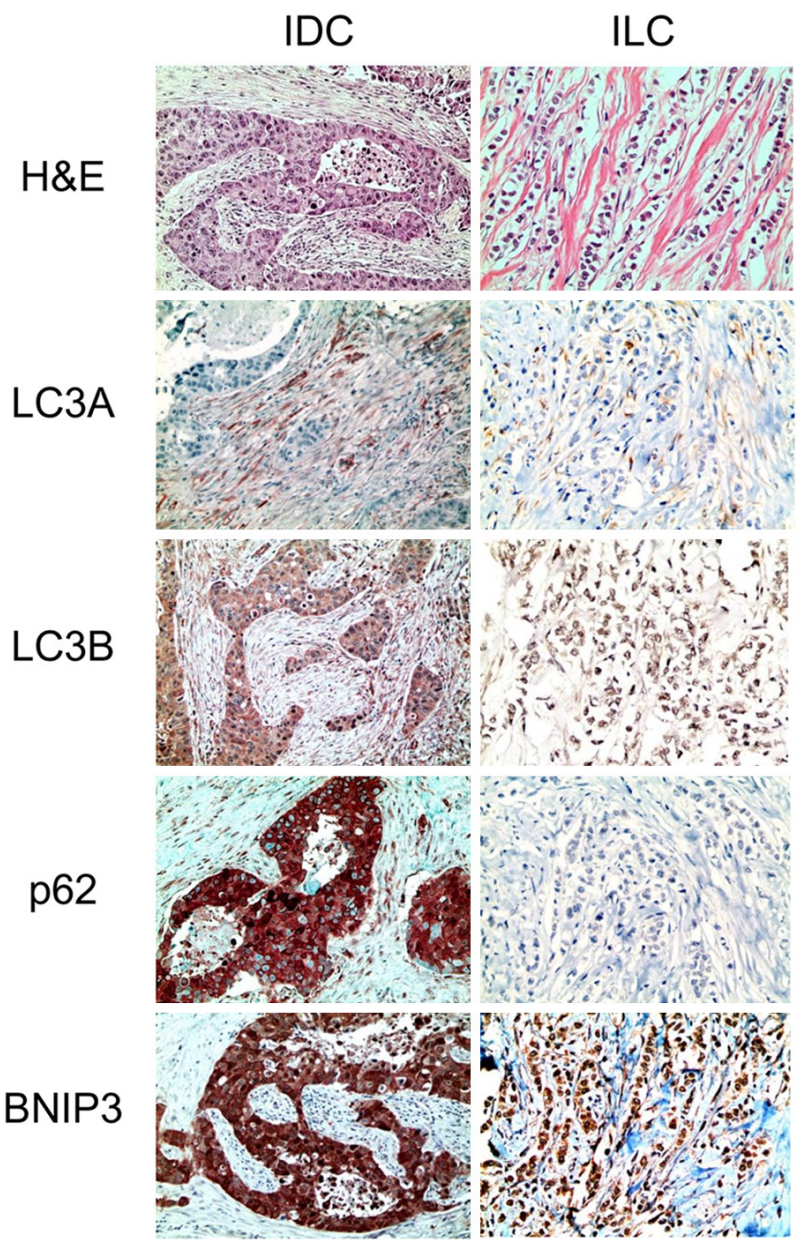

Figure 1. Comparison of expression of autophagy related proteins between invasive ductal carcinoma and invasive lobular carcinoma.

amplification by fluorescent *in situ* hybridization (FISH).

Interpretation of immunohistochemical staining for autophagy and redox-related proteins was determined by multiplying the proportion of stained cell (0% = 0, 1-29% = 1, 30-100% = 2) with the immunostaining intensity (negative = 0, weak = 1, moderate = 2, strong = 3). The final scores of 0-1, 2-4, and 5-6 were interpreted as negative, low positive, and high positive, respectively [17]. Ki-67 labeling indices (LI) were scored by counting the number of positively stained nuclei and expressed as a percentage of total tumor cells.

Tumor phenotype classification

In this study, we classified breast cancer phenotypes according to the immunohistochemistry results for ER, PR, HER-2, and Ki-67 LI. FISH results for HER-2 were as follows [18]: *luminal A type*: ER and/or PR positive, HER-2 negative, and Ki-67 LI < 14%; *luminal B type*: (HER-2 negative) ER and/or PR positive, HER-2 negative, and Ki-67 LI ≥ 14% and (HER-2 positive) ER and/or PR positive and HER-2 overexpressed and/or amplified; *HER-2 type*: ER and PR negative and HER-2 overexpressed and/or amplified; *TNBC type*: ER, PR, and HER-2 negative.

Statistical analysis

Data were statistically processed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL). Student's *t* test and Fisher's exact test were used for continuous and categorical variables, respectively. Statistical significance was assumed when $P < 0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor metastasis and time to survival. Multivariate regression analysis was performed using Cox proportional hazards model.

Results

Basal characteristics of ILC

Clinicopathologic characteristics of ILC are summarized in **Table 2**. Among 114 ILCs, 102 were classic type, and 12 were pleomorphic

Table 5. Expression of autophagy-related proteins in ILC and IDC

Parameters	Total N = 566 (%)	IDC, luminal A and B type N = 452 (%)	ILC N = 114 (%)	*P*-value
Beclin-1 (T)				0.005
Negative	346 (61.1)	263 (58.2)	83 (72.8)	
Positive	220 (38.9)	189 (41.8)	31 (27.2)	
LC3A (T)				0.348
Negative	560 (98.9)	448 (99.1)	112 (98.2)	
Positive	6 (1.1)	4 (0.9)	2 (1.8)	
LC3A (S)				< 0.001
Negative	397 (70.1)	283 (62.6)	114 (100.0)	
Positive	169 (29.9)	169 (37.4)	0 (0.0)	
LC3B (T)				< 0.001
Negative	402 (71.0)	304 (67.3)	98 (86.0)	
Positive	164 (29.0)	148 (32.7)	16 (14.0)	
LC3B (S)				< 0.001
Negative	381 (67.3)	276 (61.1)	105 (92.1)	
Positive	185 (32.7)	176 (38.9)	9 (7.9)	
p62 (T)				< 0.001
Negative	246 (43.5)	178 (39.4)	68 (59.6)	
Positive	320 (56.5)	274 (60.6)	46 (40.4)	
BNIP3 (T)				< 0.001
Negative	347 (61.3)	309 (68.4)	38 (33.3)	
Positive	219 (38.7)	143 (31.6)	76 (66.7)	
BNIP3 (S)				0.143
Negative	548 (96.8)	435 (96.2)	113 (99.1)	
Positive	18 (3.2)	17 (3.8)	1 (0.9)	

T: tumor; S: tumor stroma.

type. Pleomorphic type demonstrates older age ($P = 0.033$), higher nuclear grade ($P < 0.001$), higher histologic grade ($P < 0.001$), higher T stage ($P = 0.026$), PR negativity ($P = 0.005$), HER-2 positivity ($P = 0.002$), higher Ki-67 LI ($P < 0.001$), non-luminal A subtype ($P < 0.001$) than classic type.

Expression of autophagy-related proteins in ILC according to the cytologic type

There was no difference in expression of autophagy related proteins in two subtypes of ILC. Beclin-1, LC3A, and p62 were absent in stromal cells in all cases (**Table 3**).

Comparison of the expression of autophagy-related proteins between IDC and ILC

ILC showed lower expression of tumoral beclin-1, stromal LC3A, tumoral LC3B, and tumoral p62, and higher expression of tumoral BNIP3 than IDC ($P < 0.001$, **Table 4** and **Figure 1**). Because molecular subtypes of ILC are largely composed of luminal type, IDC of luminal types were selected for further comparison. Different expression of autophagy related proteins was identified as above between ILC and IDC of luminal types (**Table 5**).

Correlation between autophagy-related proteins and clinicopathologic factors in ILC

In ILC, beclin-1 expression was correlated with ER negativity ($P = 0.016$), tumoral BNIP3 expression was correlated with ER positivity ($P = 0.040$). Expression of beclin-1 was variably expressed according to molecular subtypes, and was highly expressed in TNBC ($P = 0.024$, **Figure 2**).

Impact of expression status for autophagy-related proteins on prognosis in ILC

Expression of autophagy related proteins in relation to prognosis in ILC were calculated using univariate analysis (**Table 6**). Although no significant autophagy related proteins were found associated with shorter DFS and shorter OS, tendency of shorter OS was revealed with tumoral beclin-1 positivity ($P = 0.062$, **Figure 3**). In multivariate Cox analysis to determine the independent predictors, higher histologic grade (I/II versus III, hazard ratio: 57.45, 95% CI: 5.162-639.5, $P = 0.001$) was significantly associated with shorter DFS, and pleomorphic type (hazard ratio: 38.96, 95% CI: 2.612-581.1, $P = 0.008$) and tumoral beclin-1 positivity (hazard ratio: 21.19, 95% CI: 1.098-409.1, $P = 0.043$) were significantly associated with shorter OS.

Discussion

We examined expression of autophagy related proteins in ILC, and compared it to those in IDC statistically. There was no difference in between the classic type and pleomorphic type of ILC. Pleomorphic type has been reported as to show

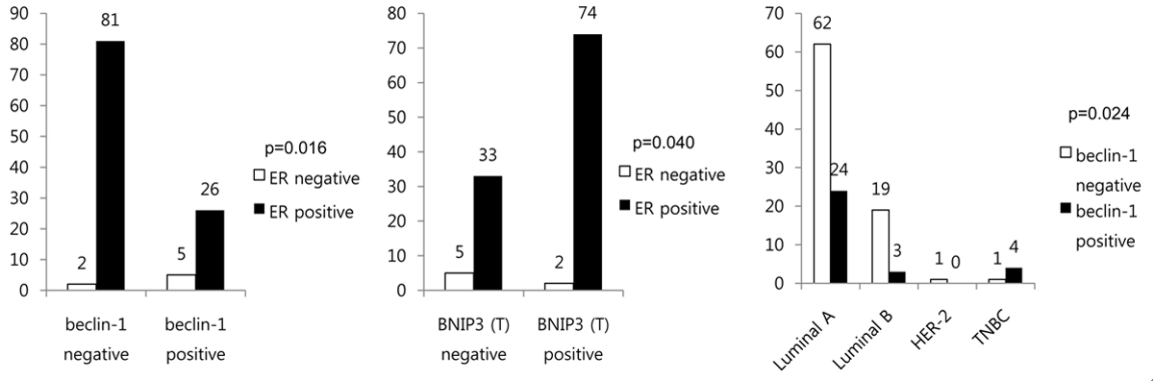

Figure 2. Correlation between the expression of autophagy-related proteins and clinicopathologic factors in invasive lobular carcinoma.

Table 6. Univariate analysis using the log-rank test to determine the impact of autophagy-related protein expression in patients with invasive lobular carcinoma on disease-free survival and overall survival times

Parameter	Disease-free survival		Overall survival	
	95% CI	*P*-value	95% CI	*P*-value
Beclin-1 (T)		n/a		0.062
Negative	n/a		165 (161-170)	
Positive	n/a		84 (77-91)	
LC3A (T)		n/a		n/a
Negative	n/a		n/a	
Positive	n/a		n/a	
LC3A (S)		n/a		n/a
Negative	n/a		n/a	
Positive	n/a		n/a	
LC3B (T)		n/a		0.499
Negative	n/a		163 (157-169)	
Positive	n/a		85 (78-93)	
LC3B (S)		n/a		n/a
Negative	n/a		n/a	
Positive	n/a		n/a	
p62 (T)		0.853		0.954
Negative	163 (156-169)		162 (155-169)	
Positive	93 (89-98)		93 (89-97)	
BNIP3 (T)		0.246		0.281
Negative	159 (147-171)		159 (147-171)	
Positive	88 (86-91)		88 (86-91)	
BNIP3 (S)		n/a		n/a
Negative	n/a		n/a	
Positive	n/a		n/a	

T: tumor; S: tumor stroma.

higher histologic grade, ER negativity, PR negativity, HER-2 positivity, poor prognosis than classic type [19, 20]. It has also been suspected that autophagy-related proteins were usually associated with high grade tumor and poor prognosis [21-25]. In IDC, expression of LC3A and LC3B were also correlated with histologic grade [12, 13]. However, in present study no significant difference was identified between classic type and pleomorphic type. It might be due to the limitation of sample numbers of pleomorphic type, which was far less than numbers of classic type.

Autophagy-related proteins were differently expressed in ILD and IDC. ILC showed lower expression of tumoral beclin-1, stromal LC3A, tumoral LC3B, and tumoral p62, and higher expression of tumoral BNIP3 than IDC ($P < 0.001$).

Although limited number of studies have been carried out about the difference in between ILC and IDC, autophagy-related proteins except BNIP3 were highly expressed in IDC rather than ILC. In general, higher proliferative activity of tumor cells induced increase of autophagy activity to meet the increased energy demand. IDC has been known to have higher Ki-67 LI than ILC [26], which was also confirmed in the present study. However, ILC demonstrated higher BNIP3 expression than IDC. BNIP3 is one of pro-apoptotic Bcl-2 members, induced by HIF-1α which is triggered

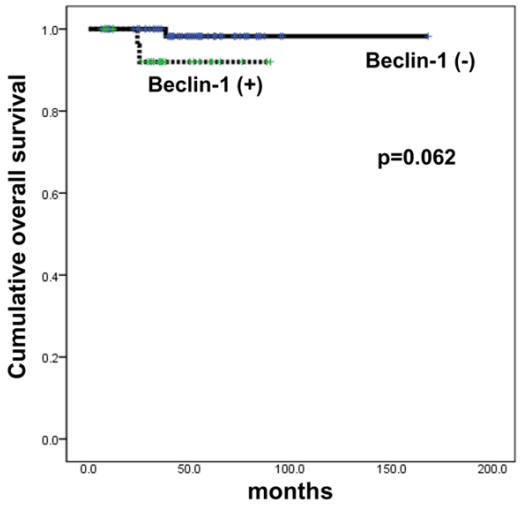

Figure 3. Overall survival according to the status of beclin-1 expression in invasive lobular carcinoma.

in tumor microenvironment like hypoxia, leads to cell death by mitophagy [27]. In previous study, BNIP3 was expressed in normal breast tissues, lost in a significant portion of invasive cancers, which was correlated with poor prognostic features like positive lymph node status and higher mitotic activity index [28]. Higher expression of BNIP3 in ILC than IDC in present study is compatible with prior studies, as Ki-67 LI is lower in ILC, which is also correlated with the loss of BNIP3 in tumor with higher Ki-67 LI.

In the current study, divergent expression of stromal autophagy-related protein between IDC and ILC were observed. LC3A was expressed higher in IDC than ILC. Autophagy-related proteins in tumor stroma has been also been reported in the previous studies [12]. In the breast cancer, autophagy activity in the tumor stroma is explained by reverse Warburg effect, which postulates the metabolic interaction between tumor cell and stromal cell in the breast cancer. Glycolysis, mitochondrial dysfunction, increased autophagy occur in stromal cells by reactive oxygen species released from tumor cells. Tumor cells generate ATP through oxidative phosphorylation, by modifying the lactate produced from glycolysis of stromal cells [29-32]. Stromal cell with increased autophagy activity is defined as cancer-associated fibroblast (CAF), which is characterized by loss of caveolin-1 [31]. Contrary to the stroma in IDC which demonstrates variable histologic features such as desmoplasia and fibrosis, no distinctive alteration is seen in the stroma of ILC. Only subset of breast cancer shows reverse Warburg effect as mentioned in prior study that 15% of tumor stroma in breast cancer showed loss of caveolin-1 [33]. However, this phenomenon in ILC is yet elucidated. In present study, autophagy-related proteins were differently expressed in stroma of IDC and ILC. Further analysis of this different signature of stroma in ILC is required whether CAF and metabolic interaction between tumor cell and stroma are associated in ILC.

We found beclin-1 positivity as independent poor prognostic factor, which was concordant with prior findings of correlation between beclin-1 expression and poor prognosis in ovary cancer [34], stomach cancer [35], larynx cancer [36], and uterine endometrial cancer [37]. However, it is controversial because other studies have demonstrated decreased of low expression of beclin-1 was associated with poor prognosis in cancer [38-40]. It is suspected that this controversy could be originated from dual role of autophagy that regulates both tumor survival and tumor suppression. Further validation in ILC is needed.

In present study, autophagy-related proteins were found in both tumor cell and stroma of ILC. Based on this result, inhibitor of autophagy could allow the target treatment in ILC. To date, as autophagy inhibitor has been discovered as suppressor of diverse tumors [41-44], further exploring of ILC is also required.

In conclusion, the present study analyzed expression pattern of autophagy-related proteins between IDC and ILC that demonstrated by higher expression of tumoral beclin-1, stromal LC3A, tumoral LC3B, tumoral p62 in IDC, and higher expression of tumoral BNIP3 in ILC.

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Disclosure of conflict of interest

None.

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