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BECN1 protein expression is associated with poor survival in triple negative locally advanced breast cancer

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Abstract. – OBJECTIVE: The role that Beclin 1 (BECN1) plays in the development and progression of cancer mediated by autophagy, as well as its differential expression in breast cancer cell lines and mammary tumor tissue according to the molecular subtype, has been demonstrated. The objective of this study was to evaluate the association of BECN1 cytoplasmic expression with clinical and pathologic response, recurrence, disease-free survival (DFS) and overall survival (OS) in patients with locally advanced breast cancer (LABC), according to immunophenotype.

PATIENTS AND METHODS: 64 patients with non-triple negative LABC and 20 patients with triple negative LABC who received preoperative chemotherapy were included in an observational, analytical and retrospective study to evaluate the cytoplasmic expression of BECN1 protein by immunohistochemistry in microarrays of breast cancer tissue obtained before treatment. Association between BECN1 and clinicopathological characteristics, clinical and pathologic response to preoperative chemotherapy and recurrence, were analyzed using Chi-square or Fisher's exact test. Postoperative DFS and OS were assessed by Kaplan-Meir curves, and the difference according to BECN1 expression was evaluated using the log-rank test. The bivariate analysis was performed using the Cox Proportional Hazards Model. A *p*-value of < 0.05 was considered statistically significant.

RESULTS: BECN1 staining revealed positive expression in 62.5% of patients with non-triple negative and 60.0% with triple negative LABC. No association was observed between BECN1 expression and clinical or pathological response or recurrence. An association of the BECN1 expression with lower OS in triple negative breast cancer was found (HR = 5.19; 95% CI 1.12-24.02; *p* = 0.035).

CONCLUSIONS: Results showed an association of the cytoplasmic expression of BECN1 with a lower OS, which could be a poor prognostic biomarker in triple negative LABC.

Key Words:
Breast cancer, BECN1, Biomarker, Overall survival, Disease free survival, Prognosis.

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Introduction

Autophagy is a self-digesting process that removes long-lived proteins and damaged organelles using the lysosomal degradation mechanisms of the cell[1,2]. Normally, constitutive levels of autophagy are needed for maintaining homeostasis; in the context of cancer, up-regulation of this process serves to promote cell survival under conditions of cellular stress[3]. Various studies[1-15] have suggested its important role in the development and progression of cancer and in the response to chemotherapy.

BECN1 is a tumor suppressor gene located on chromosome 17q21 that is monoallelically deleted in approximately 40% of human breast[4,5], ovarian and prostate cancers[6]. It encodes BECN1, an autophagy protein that is essential for autophagosome formation[4,6].

BECN1 prevents breast cancer growth through a mechanism involving E-cadherin, a breast tumor suppressor whose expression is completely lost in most invasive lobular carcinomas[7]. The data suggest that BECN1 promotes the localization of E-cadherin in the plasma membrane, which restricts tumor growth and metastasis[8].

BECN1 messenger ribonucleic acid (mRNA) is differentially expressed according to the molecular subtype of breast cancer. The low expression of *BECN1* mRNA correlates with the overexpression of human epidermal growth factor receptor 2 (HER2/Neu)[6] and is significantly lower in the HER2/Neu enriched and triple negative compared to luminal A/B breast cancer subtypes[5]. Furthermore, low *BECN1* mRNA expression has been associated with decreased survival in breast cancer patients, even when the luminal A subtype and estrogen receptor (ER)-negative subtypes (HER2/Neu enriched and triple negative) are independently analyzed[5,9].

Regarding protein BECN1, a decrease in its expression has been reported in glioblastomas, ovarian and esophageal cancer[10-12]; but an increase in expression in colorectal and gastric carcinomas[1]. In invasive breast cancer samples, positive expression of BECN1 has been reported in 42.4% of cases, with a decrease in the intensity of expression in breast cancer tissue compared to adjacent normal breast tissue[13]. High expression of the BECN1 protein has also been reported in invasive ductal breast carcinoma, but low expression in invasive lobular carcinoma[14]. In addition, it has been reported that patients whose breast cancer tumors express BECN1 before neoadjuvant treatment with exemestane show a lower clinical (25 *vs.* 67%) and pathological response (0 *vs.* 41%) compared to those who do not express BECN1[15].

Because the treatment of locally advanced breast cancer (LABC) requires the evaluation of the response to neoadjuvant chemotherapy and provides the possibility of identifying predictive and prognostic factors with potential use in the clinic[16], the objective of this study was to evaluate the association of cytoplasmic BECN1 expression with clinical and pathologic response, recurrence, disease free survival (DFS) and overall survival (OS) in patients with LABC, according to immunophenotype.

Patients and Methods

Setting and Participants

This observational, analytical, and retrospective study was approved by the National Commission on Scientific Research and the Ethics Committee on Health Research of the Instituto Mexicano del Seguro Social (IMSS), (CONBIOÉTICAA09CEI01520130424 registration number R-2013-785-036 and R-2017-785-059). Data were obtained from clinical records and paraffin-embedded breast cancer tissue samples from 84 women with LABC who received preoperative chemotherapy and were treated at the Unidad Médica de Alta Especialidad, Hospital de Gineco Obstetricia No. 3, "Dr. Víctor Manuel Espinosa de los Reyes Sánchez", Centro Médico Nacional La Raza and at the Unidad Médica de Alta Especialidad, Hospital de Gineco Obstetricia No. 4 "Luis Castelazo Ayala", IMSS from February 1, 2009 to July, 31 2014. All study participants consented to the analysis of tissue samples and clinical data.

A list of women with breast cancer was made and those diagnosed with LABC or those who received preoperative chemotherapy were selected. All the women's clinical records had information on the clinical, pathological and prognostic variables. Women who had a paraffin-embedded tumor tissue sample obtained before the start of treatment were included in the study. Women who used oral contraceptives or hormone replacement therapy three months prior to the tumor biopsy and who were receiving chemotherapy treatment for a second primary tumor or were diagnosed with inflammatory breast cancer were excluded.

Information was obtained on age, family history of cancer, personal pathological history of chronic degenerative diseases (diabetes mellitus and systemic arterial hypertension) and other neoplasms, menarche age, age at first pregnancy to term, number of pregnancies, history of hormone use and menopause state. Information on tumor size (cm) and clinical stage at diagnosis according to the American Joint Committee on Cancer (AJCC), histological type, tumor size in the surgical specimen (cm), metastatic lymph nodes, tumor border, lymphovascular invasion, expression of estrogen receptor alpha (ERα), progesterone receptor (PR) and HER2/Neu according to the recommendations of the American Society of Clinical Oncology (ASCO)[17,18] was also collected. To date, the expression status of ERα, PR and HER2/Neu, but not Ki-67, are used routinely to guide clinical decisions on the use of systemic therapy[19], thus the molecular classification of breast cancer was established as follows: luminal A (ERα positive, PR negative or positive and HER2/Neu negative), luminal B (ERα positive, PR negative or positive and HER2/Neu positive), HER2/Neu enriched (ERα negative, PR negative and HER2/Neu positive) and triple negative (ERα negative, PR negative and HER2/Neu negative)[20]. The type of neoadjuvant chemotherapy treatment, the size of the residual tumor (cm) and the number of residual lymph nodes were recorded.

Tissue Microarray Construction

For each patient, formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from breast cancer tumor samples collected prior to neoadjuvant treatment.

Microtome slices (4 µm) were obtained using the Leica RM2125 Microtome (Leica Biosystems, Wetzlar, Germany) and stained with hematoxylin and eosin (H&E). These sections were reviewed by an experienced pathologist to identify, define and mark representative areas of the tumor. Once the area was marked, it served as a guide for the extraction of the core needle biopsy from the FFPE donor tissue block.

Tissue cylinders with diameters of 1 mm and height of 3 mm were perforated from the targeted tumor regions and transferred to acceptor array blocks in a specific row and column using an Advanced Tissue Arrayer (ATA 100) (CHEMICON International Inc., Temecula, CA, USA).

The location of each sample by row and column was carefully recorded to ensure that the staining data would be properly linked to the clinical data.

Depending on the amount of tumor tissue available, duplicate or triplicate samples from different areas of the tumor were placed on the tissue microarray (TMA). Samples of other tissues were used as positive controls for the BECN1 (testis with high expression; appendix, renal and pulmonary tissues with medium expression; and cerebellum, liver, prostate, and cervix with low expression) and were placed in each TMA. After construction of the acceptor array block, it was covered with Surgipath Paraplast (Leica Biosystems, Wetzlar, Germany) at 60ºC and incubated in an oven at 60ºC for 10 minutes. Subsequently, multiple consecutive 4 µm cuts were made. One of those sections was placed on a microscope slide and stained with H&E for histological verification of the suitability of the arrayed tumor tissues. Consecutive sections were individually transferred to positively charged slides (Biocare Medical, Concord, CA, USA) for immunohistochemistry (IHC) assays[21].

Tissue Microarray Staining

Each section of the TMA was subjected to immunohistochemical staining for the BECN1. Immunodetection was automated using the Ventana BenchMark System *(*Fritz Hoffmann-La Roche, Basilea, Switzerland*)*. Anti-Beclin 1 antibody [EPR1733Y] (cat. no. ab51031; 1:100 dilution) (ABCAM, Cambridge, UK) was used in the staining process with an incubation period of 30 minutes. The negative control was a sample in which the primary antibody was omitted. The UltraView Universal Diaminobenzidine (DAB) detection kit was used (Ventana Medical Systems, Inc. Tucson, Arizona, USA*)*. The slides were dehydrated using ascending alcohol solutions, rinsed in a xylol solution, and mounted using synthetic resin. TMA sections were visualized with a DM750 Leica microscope (Leica Microsystems, Wetzlar, Germany) and digital images were captured using Leica Application Suite (LAS) EZ software (Leica Microsystems, Wetzlar, Germany*)*.

Immunohistochemistry Scoring

TMA sections were independently analyzed by two experienced pathologists and blinded to clinical data (inter-observer agreement greater than 0.90). The TMA slides contained samples from each of the 84 patients included in the study. Each core sample was analyzed to determine the inten-

sity and extent of cytoplasmic staining. A previously validated scoring system was used to combine the staining of intensity (scored 1 to 3) and the extent of stained cells (1 to 100%) in an IHC score. The intensity of staining was rated as 1 = weak, 2 = moderate, and 3 = strong. The extent of staining was converted to a number with 1 = 0 to < 10%, 2 = 10 to < 50%, and 3 = 50 to 100%. The general IHC score was determined based on the two previous variables, positive when both scores were two or more and otherwise negative[22].

Clinical Outcome Assessment

The response to neoadjuvant chemotherapy based on the size of the tumor and axillary lymph nodes was recorded. The clinical response was assessed on two occasions performed less than 4 weeks apart (clinical examination and mammography). The pathological response to neoadjuvant chemotherapy assessed by histopathological analysis was defined as: complete (total resolution of the tumor and axillary lymph nodes); partial (decrease of 50% or more of the perpendicular diameters of measurable lesions, without the appearance of new lesions or progression of any lesion); stabilization (decrease <50% but no increase >25% in the largest perpendicular diameters of measurable lesions; progression (increase in the size of the tumor or axillary lymph nodes of 25% or more, appearance of new lesions or metastatic disease)[23].

The follow-up period was established as the time from diagnosis to the last evaluation (December 2019) for the cases that were censored (patients with study end date and lost to follow-up) or recurrence/death in patients who completed the follow-up. OS was defined as the time from the date of diagnosis to the date of death or to the date of last follow-up if the patients were still alive. DFS was defined as the time from the date of primary surgery to the date of recurrence. The categories analyzed for DFS were disease recurrence at a local, regional, or distant site by clinical or radiological evaluation; and all were considered DFS events.

Statistical Analysis

Based on the study by Ueno et al[15], a sample size calculation was performed to detect a difference between two proportions. The Statulator Sample Size Calculator (beta) (http://statulator.com/SampleSize/ss2P.html) was used. The proportion of clinical and pathological responses reported with and without BECN1 expression was considered, with a power of 0.80 and an α level of 0.05, estimating a total sample size of 23 cases with and 23 cases without expression of BECN1 to demonstrate statistically significant differences. Data were entered and analyzed with the Statistical Package for the Social Sciences (SPSS) software computer package v.25.0 (IBM, Armonk, New York, USA). The normality of quantitative variables was tested using the Shapiro-Wilk test. Due to non-normal distribution of most variables, nonparametric tests were applied to all comparisons. The patients were classified into two groups according to the immunophenotype in triple negative and non-triple negative breast cancer. Quantitative variables were expressed as minimum, median, and maximum, and the differences between groups were examined using the Mann-Whitney U test. The qualitative variables were expressed as frequencies and percentages (%) and the differences between groups were analyzed using the Chi-square or Fisher´s exact test. Postoperative DFS and OS were assessed using Kaplan-Meir curves, and the difference according to BECN1 expression was evaluated using the log-rank test. The bivariate analysis was performed using the Cox Proportional Hazards Model. All tests were 2-tailed and statistical significance was considered for *p*-values less than 0.05.

Results

Patient Characteristics

A total of 84 patients were included in the study. The median age was 54 years (range, 29-88 years). Sixty-four patients with non-triple negative breast cancer tumor and twenty patients with triple negative tumors were analyzed for the expression of the BECN1.

BECN1 cytoplasmic staining revealed positive expression in 40 patients (62.5%) with non-triple negative and in 12 patients with triple negative breast cancer (60.0%) ($p = 0.841$). Figure 1 shows examples of positive and negative expression of BECN1.

The clinicopathological characteristics and the expression of BECN1 according to the immunophenotype of breast cancer are summarized in Table I. Only in non-triple negative breast cancer, a tumor size > 5 cm and lobular histological type were more frequently observed in patients without expression of BECN1. No association was observed between the clinicopathological charac-

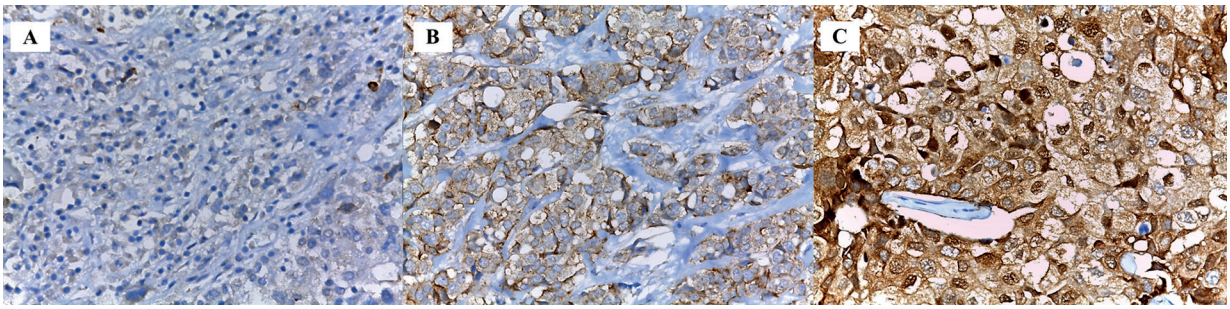

Figure 1. Immunohistochemistry of cytoplasmic BECN1 (400×). In (**A**) weak staining is observed in > 10% of cells, negative result; in (**B**) moderate staining is observed in > 10% of the cells, positive result; in (**C**), intense staining is observed in > 10% of the cells, positive result.

teristics and BECN1 expression in triple negative breast cancer.

Regarding preoperative chemotherapy in patients with non-triple negative and triple negative breast cancer, the sequential regimen with four cycles of 5-Fluorouracil 500 mg/m^2, Epirubicin 75 mg/m^2 and Cyclophosphamide 500 mg/m^2 (FEC) every three weeks, followed by four cycles of Docetaxel 75 mg/m^2, was the most used in 24 (75.0%) and 10 (62.5%) patients respectively. This treatment was followed by the same drugs with variations in the number of cycles (2 to 6 cycles) and in the dose of FEC (500, 75-100 and 500-600 mg/m^2 respectively) in six (18.8%) and four (25%) patients respectively; or with addition of other drugs (Paclitaxel 175 mg/m^2 and/or Carboplatin 350 mg/m^2) in one (3.1%) and two (12.5%) patients respectively, and one (3.1%) patient with non-triple negative breast cancer, who received exclusively Tamoxifen 20 mg/day.

Five patients (15.6%) with non-triple negative breast cancer and one patient (6.3%) with triple negative breast cancer presented clinical complete response (cCR) and pathologic complete response (pCR). Two patients (6.3%) with non-triple negative breast cancer and one patient (6.3%) with triple negative breast cancer presented clinical complete response (cCR) but pathologic partial response (pPR). Three patients (9.4%) with non-triple negative breast cancer presented apparent clinical partial response (cPR), but with pCR. No association was observed between the clinical or pathological response to neoadjuvant chemotherapy and the expression of BECN1 in either group (Table II).

There were 25 patients (39.0%) with local or systemic recurrence in the non-triple negative breast cancer group and six patients (30.0%) in the triple negative group. In neither group was BECN1 expression associated with local or systemic recurrence (Table II).

When exclusively analyzing patients who received standard preoperative chemotherapy, no association of BECN1 expression was observed with clinical (p = 0.678 and p = 0.475) and pathological (p = 0.229 and p = 1.0) response, or recurrence (p = 0.436 and p = 0.325) in non-triple negative and triple negative breast cancer group, respectively.

Mean time to recurrence was 48.7 and 45.7 months in non-triple negative and triple negative breast cancer group respectively. In the group of non-triple negative breast cancer, the bivariate analysis (Table III) and the multivariate analysis showed an association of pregnancies ≥ 1 (HR = 0.16; 95% CI 0.03 - 0.88; p = 0.035) and ductal histological type (HR = 0.11; 95% CI 0.02 - 0.49; p = 0.004) with a longer DFS. Clinical stages IIIB and IIIC at diagnosis (HR = 6.19; 95% CI 1.39 - 27.48; p = 0.017) and the use of preoperative non-standard chemotherapy (HR = 3.49; 95% CI 1.08 - 11.22; p = 0.036) were associated with lower DFS. There were no significant differences in clinical stage (p = 0.476), histological type (p = 0.714) or molecular subtype (p = 0.694) between the patients who received or did not receive the standard regimen of preoperative chemotherapy. In the triple negative breast cancer group, only the tumor size in the surgical specimen > 5 cm was associated with a lower DFS in triple negative breast cancer group. No association of BECN1 expression was observed with DFS in either of two groups (Table III).

During the study period, there were 28 (43.7%) and 12 (60%) deaths in the non-triple negative and triple negative breast cancer group, respectively. The mean time to death was 46.1 and 43.3 months

Table I. Clinicopathological characteristics and expression of BECN1.

	Non-triple negative breast cancer[a]			Triple negative breast cancer[a]		
	BECN1 (-)	BECN1 (+)	p*	BECN1 (-)	BECN1 (+)	p*
Age	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
Years old	53 (29-86)	58 (38-88)	0.255	50 (34-67)	55 (39-69)	0.270
≤ 50 years	11 (45.8)	12 (30.0)	0.282	5 (62.5)	4 (33.3)	0.362
> 50 years	13 (54.2)	28 (70.0)		3 (37.5)	8 (66.7)	
Diabetes mellitus	**n = 22 (35.5)**	**n = 40 (64.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	17 (77.3)	31 (77.5)	1.000	6 (75.0)	12 (100.0)	0.147
Yes	5 (22.7)	9 (22.5)		2 (25.0)	0 (0.0)	
Systemic arterial hypertension	**n = 23 (36.5)**	**n = 40 (63.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	15 (65.2)	21 (52.5)	0.430	6 (75.0)	7 (58.3)	0.642
Yes	8 (34.8)	19 (47.5)		2 (25.0)	5 (41.7)	
Other neoplasms	**n = 23 (36.5)**	**n = 40 (63.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	23 (100)	35 (87.5)	0.149	7 (87.5)	11 (91.7)	1.000
Yes	0 (0.0)	5 (12.5)		1 (12.5)	1 (12.5)	
Family history of cancer	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	18 (75.0)	25 (62.5)	0.412	4 (50.0)	11 (91.7)	0.109
Yes	6 (25.0)	15 (37.5)		4 (50.0)	1 (8.3)	
Menarche age	**n = 20 (40.0)**	**n = 30 (60.0)**		**n = 8 (47.1)**	**n = 9 (52.9)**	
Years	13 (8-16)	13 (10-19)	0.337	12 (12-14)	12 (10-15)	0.888
Age at first pregnancy to term	**n = 16 (32.7)**	**n = 23 (67.3)**		**n = 6 (40.0)**	**n = 9 (60.0)**	
Years	22 (17-33)	20 (15-35)	0.159	24 (19-30)	22 (14-29)	0.328
Pregnancies	**n = 21 (40.4)**	**n = 31 (59.6)**		**n = 8 (42.1)**	**n = 11 (57.9)**	
Number	3 (0-10)	3 (0-11)	0.762	2 (0-5)	3 (1-11)	0.206
Nulligest	1 (4.8)	3 (9.7)	0.639	1 (12.5)	0 (0.0)	0.421
≥ 1	20 (95.2)	28 (90.3)		7 (87.5)	11 (100.0)	
History of hormone use	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	21 (87.5)	39 (97.5)	0.144	8 (100.0)	12 (100.0)	-
Yes	3 (12.5)	1 (2.5)		0 (0.0)	0 (0.0)	
Menopausal state	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	9 (37.5)	8 (20.0)	0.151	4 (50.0)	4 (33.3)	0.648
Yes	15 (62.5)	32 (80.0)		4 (50.0)	8 (66.7)	
Tumor size at diagnosis	**n = 21 (35.0)**	**n = 39 (65.0)**		**n = 7 (36.8)**	**n = 12 (63.2)**	
cm	7.0 (2.5-18.0)	4.5 (1.5-15.0)	0.023	8.0 (2.0-11.0)	7.5 (2.5-17.5)	0.837
≤ 5 cm	7 (33.3)	27 (69.2)	0.013	1 (14.3)	3 (25.0)	1.000
> 5 cm	14 (66.7)	12 (30.8)		6 (85.7)	9 (75.0)	
Clinical stage at diagnosis	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
IIB and IIIA	16 (66.7)	19 (47.5)	0.195	3 (37.5)	5 (41.7)	1.000
IIIB and IIIC	8 (33.3)	21 (52.5)		5 (62.5)	7 (58.3)	
Histological type	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
Ductal	9 (37.5)	29 (72.5)	0.009	6 (75.0)	9 (75.0)	1.000
Lobular or mixed	15 (62.5)	11 (27.5)		2 (25.0)	3 (25.0)	
Tumor size in the surgical specimen	**n = 23 (36.5)**	**n = 40 (63.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
cm	3.0 (0.0-8.0)	2.9 (0.0-15.0)	0.949	2.8 (0.0-8.0)	4.8 (0-14.0)	0.238
≤ 5 cm	19 (82.6)	33 (82.5)	1.000	7 (87.5)	8 (66.7)	0.603
> 5 cm	4 (17.4)	7 (17.5)		1 (12.5)	4 (33.3)	
Metastatic lymph nodes	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
Number	4 (0-13)	4 (0-36)	0.400	0 (0-7)	2 (0-14)	0.181
Tumor border	**n = 23 (37.1)**	**n = 39 (62.9)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
Negative	20 (87.0)	37 (94.9)	0.350	8 (100.0)	12 (100.0)	-
Positive	3 (13.0)	2 (5.1)		0 (0.0)	0 (0.0)	

Continued

Table I *(Continued)*. Clinicopathological characteristics and expression of BECN1.

	Non-triple negative breast cancer[a]			Triple negative breast cancer[a]		
	BECN1 (-)	BECN1 (+)	*p**	BECN1 (-)	BECN1 (+)	*p**
Lymphovascular invasion	**n = 16 (33.3**	**n = 32 (66.4)**		**n = 5 (40.0)**	**n = 9 (60.0)**	
Negative	1 (6.3)	2 (6.3)	1.000	4 (80.0)	3 (33.3)	0.266
Positive	15 (93.8)	30 (93.8)		1 (20.0)	6 (66.7)	
Molecular subtype	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
Luminal A	19 (79.2)	23 (57.5)	0.119	-	-	-
Luminal B	3 (12.5)	5 (12.5)		-	-	
HER2/Neu enriched	2 (8.3)	12 (30.0)		-	-	
Type of preoperative chemotherapy	**n = 15 (46.9)**	**n = 17 (53.1)**		**n = 7 (43.7)**	**n = 9 (56.3)**	
4FEC-4D	11 (73.3)	13 (76.5)	1.000	4 (57.1)	7 (77.8)	0.596
Other	4 (26.7)	4 (23.5)		3 (42.9)	2 (22.2)	

[a]Data shows the median, minimum and maximum or frequency and percentage. *Statistical significance of the chi square/Fisher exact test or Mann-Whitney U test.

in the non-triple negative and triple negative breast cancer group, respectively. In the non-triple negative breast cancer group, the bivariate analysis (Table IV) and the multivariate analysis showed an association of ductal histological type (HR = 0.19; 95% CI 0.07-0.51; $p = 0.001$) with a longer OS, whereas clinical stages IIIB and IIIC at the time of diagnosis (HR = 3.45, 95% CI 1.57-7.56; $p = 0.002$) were associated with lower OS. The triple negative breast cancer group showed an association of BECN1 expression with lower OS (Table IV). There were 2/8 (25%) and 10/12 (83.3%) deaths in patients who had negative or positive expression of BECN1, respectively. The mean time to death was 52.37 and 37.16 months in patients who had negative or positive expression of BECN1, respectively. Kaplan-Meier analysis showed that the positive expression of BECN1 is associated with lower rates of OS (Log Rank test $p = 0.019$) (Figure 2).

Table II. Response to neoadjuvant chemotherapy, recurrence, and expression of BECN1.

	Non-triple negative breast cancer[a]			Triple negative breast cancer[a]		
	BECN1 (-)	BECN1 (+)	*p**	BECN1 (-)	BECN1 (+)	*p**
Clinical response	**n = 15 (46.9)**	**n = 17 (53.1)**		**n = 7 (43.7)**	**n = 9 (56.3)**	
Partial, stabilization or progression	11 (73.3)	14 (82.4)	0.678	7 (100.0)	7 (77.8)	0.475
Complete	4 (26.7)	3 (17.6)		0 (0.0)	2 (22.2)	
Pathological response	**n = 15 (46.9)**	**n = 17 (53.1)**		**n = 7 (43.7)**	**n = 9 (56.3)**	
Partial, stabilization or progression	13 (86.7)	11 (64.7)	0.229	7 (100.0)	8 (88.9)	1.000
Complete	2 (13.3)	6 (35.3)		0 (0.0)	1 (11.1)	
Local recurrence	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	22 (91.7)	35 (87.5)	0.702	7 (87.5)	8 (66.7)	0.603
Yes	2 (8.3)	5 (12.5)		1 (12.5)	4 (33.3)	
Systemic recurrence	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	14 (58.3)	29 (72.5)	0.280	7 (87.5)	8 (66.7)	0.603
Yes	10 (41.7)	11 (27.5)		1 (12.5)	4 (33.3)	
Recurrence (local or systemic)	**n = 24 (37.5)**	**n = 40 (62.5)**		**n = 8 (40.0)**	**n = 12 (60.0)**	
No	13 (54.2)	26 (65.0)	0.436	7 (87.5)	7 (53.8)	0.325
Yes	11 (45.8)	14 (35.0)		1 (12.5)	5 (41.7)	

[a]Data shows frequency and percentage. *Statistical significance of the chi square/Fisher exact test.

Table III. Clinicopathological characteristics and disease-free survival.

	Non-triple negative breast cancer			Triple negative breast cancer		
	HR	95% CI	p*	HR	95% CI	p*
Age > 50 years old	0.80	0.35-1.82	0.601	1.08	0.22-5.43	0.922
Diabetes mellitus	0.76	0.26-2.23	0.614	2.02	0.23-17.45	0.522
Systemic arterial hypertension	0.87	0.38-1.97	0.739	0.28	0.03-2.41	0.247
Other neoplasms	0.84	0.20-3.56	0.808	0.04	0.0-3.94	0.655
Family history of cancer	1.33	0.60-2.93	0.485	0.51	0.59-4.39	0.539
Pregnancies ≥ 1	0.27	0.08-0.80	**0.019**	22.30	0.00-4.37	0.674
History of hormone use	1.84	0.43-7.92	0.412	No patient used hormones		
Postmenopause	1.15	0.48-2.75	0.759	1.81	0.32-10.09	0.498
Tumor size at diagnosis > 5 cm	0.93	0.41-2.10	0.855	1.37	0.16-11.80	0.770
Clinical stage at diagnosis IIIB and IIIC	2.33	1.05-5.17	**0.037**	2.91	0.34-24.9	0.329
Histological type ductal	0.39	0.16-0.91	**0.030**	1.52	0.28-8.33	0.627
Tumor size in the surgical specimen > 5 cm	0.93	0.41-2.10	0.855	6.46	1.24-33.64	**0.027**
Positive tumor border	0.845	0.20-3.59	0.819	No patient had positive tumor border		
Lymphovascular invasion	22.45	0.10-51842.83	0.431	6.97	0.80-60.67	0.079
Luminal B	3.10	1.36-7.06	**0.007**	Does not apply		
Preoperative non-standard chemotherapy	3.58	1.23-10.41	**0.019**	2.82	0.56-14.33	0.210
BECN1 positive	0.96	0.44-2.13	0.933	4.24	0.49-36.49	0.188

*Statistical significance of the Cox regression test.

Discussion

Autophagy may play a role as a continuously occurring tumor suppressive mechanism at basal levels in normal cells to remove old cellular components, toxic materials, damaged organelles and misfolded or damaged proteins, reduce oxidative stress, and protect cells from DNA damage[24,25]. It is a complementary process to the ubiquitin-proteasome system, responsible for the

Table IV. Clinicopathological characteristics and overall survival.

	Non-triple negative breast cancer			Triple negative breast cancer		
	HR	95% CI	p*	HR	95% CI	p*
Age > 50 years old	1.73	0.76-3.94	0.190	3.69	0.99-13.75	0.052
Diabetes mellitus	1.1	0.44-2.73	0.842	0.97	0.12-7.51	0.973
Systemic arterial hypertension	1.43	0.67-3.04	0.355	0.66	0.20-2.19	0.493
Other neoplasms	0.91	0.22-3.85	0.899	1.09	0.14-8.46	0.937
Family history of cancer	0.47	0.19-1.17	0.106	0.20	0.03-1.58	0.128
Pregnancies ≥ 1	0.85	0.20-3.65	0.823	22.80	0.001-4.55	0.536
History of hormone use	1.41	0.33-5.97	0.637	No patient used hormones		
Postmenopause	1.63	0.66-4.03	0.288	3.25	0.87-12.22	0.080
Tumor size at diagnosis > 5 cm	0.84	0.37-1.90	0.681	1.26	0.28-5.81	0.761
Clinical stage at diagnosis IIIB and IIIC	2.90	1.33-6.32	**0.007**	0.83	0.26-2.63	0.757
Histological type ductal	0.22	0.08-0.59	**0.002**	1.07	0.29-3.96	0.920
Tumor size in the surgical specimen > 5 cm	0.84	0.37-1.90	0.681	3.31	0.97-11.36	0.057
Positive tumor border	0.35	0.05-2.61	0.307	No patient had positive tumor border		
Lymphovascular invasion	1.45	0.19-10.75	0.713	2.98	0.80-11.06	0.102
Luminal B	2.21	1.09-4.48	**0.027**	Does not apply		
Preoperative non-standard chemotherapy	2.31	0.76-6.99	0.138	1.32	0.34-5.19	0.684
BECN1 positive	1.45	0.66-3.21	0.358	5.19	1.12-24.02	**0.035**

*Statistical significance of the Cox regression test.

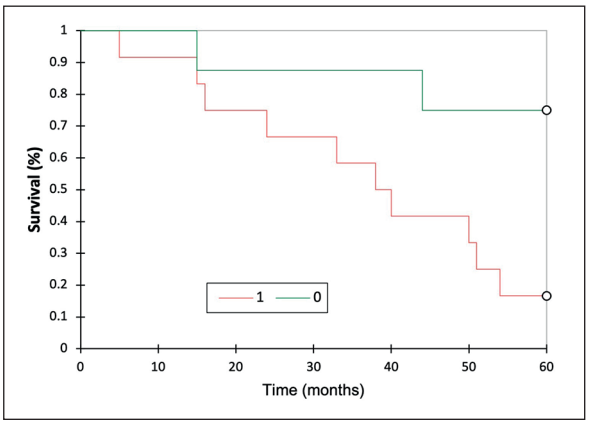

Figure 2. Cumulative overall survival (OS) curves of locally advanced breast cancer (LABC) patients with (1) or without (0) BECN1 expression (number of patients = 20, number or events = 12).

recycling of proteins, capture and degradation of mitochondria, Golgi complex, endoplasmic reticulum, and other cellular components such as proteins and cellular aggregates. Autophagy has been associated with cell death, which is important for cell development, differentiation, aging, and remodeling under certain stress conditions, such as nutrient deprivation, hypoxia, metabolic and therapeutic stress, such as chemotherapy and radiation, among others. However, it is a process that is also used by cancer cells. Both healthy and cancer cells use it to recycle components and save energy[26]. Dysregulation of autophagy alters physiological processes, which is why it has been implicated in tumor progression and survival of cancer cells under stress, contributing to treatment resistance[24,26-28]. Autophagy consists of several phases that are regulated by the expression of at least 15 genes/proteins, including BECN1, which has come to be considered a marker of autophagy[24]. Despite the dual role that has been attributed to BECN1 as a tumor suppressor gene[4] and at the same time as a gene involved in the survival of tumor cells[27,29,30], its potential has been demonstrated in various studies as a prognostic biomarker[13,31,32] and resistance to treatment in breast cancer[15,33]. Therefore, the objective of this study was to evaluate the association of BECN1 expression in breast cancer tissue with the prognosis of patients with LABC according to their immunophenotype.

Hence, our cases were classified according to their immunophenotype as non-triple negative and triple negative. We initially evaluated the prevalence of BECN1 expression. Subsequently, we analyze the association of BECN1 expression with the clinical and pathological characteristics of the patients, including those variables that have been considered as poor prognostic factors. Finally, we evaluated the association of BECN1 expression with response to neoadjuvant chemotherapy, recurrence, DFS and OS.

Given that in advanced stages of breast cancer, the tumor is larger and may be subjected to greater stress in its microenvironment due to deprivation of oxygen and nutrients, it would be expected that the autophagy mechanism would be activated in response to this stress and will achieve cellular homeostasis by reusing components of the cell itself[27,28]. In our study, a positive expression of BECN1 in 62.1% of non-triple negative and 60% of triple negative tumors was observed, which was higher than that reported in other studies. Won et al[13] observed positive expression of BECN1 in 42.4% of 125 breast cancer tissues, while Choi et al[30] reported positive expression in 29.9% of 489 tumor samples analyzed[30]. Both studies reported having studied breast cancer with different tumor sizes and different lymph node involvement, so it can be inferred that samples in different clinical stages were reviewed. This was one of the possible reasons why we observed a higher percentage of samples with positive expression of BECN1 in our study, that only included locally advanced tumors.

The lower expression of BECN1 has been associated with histopathological characteristics of poor prognosis such as a higher clinical stage, a higher histological grade[32] and lobular histological type[14]. A statistically significant association with the clinical stage and histological grade was not found. However, an absence of BECN1 expression in larger tumors and in those with lobular histological type, specifically in the group of patients with non-triple negative breast cancer, was observed.

On the other hand, it has been reported that the expression of BECN1 before neoadjuvant chemotherapy with exemestane is associated with poor clinical and pathological response. An increase in BECN1 expression has even been reported after neoadjuvant treatment with exemestane, suggesting that autophagy is also involved in the cellular response to endocrine treatment[15]. This study only evaluated the expression of BECN1 before neoadjuvant treatment without finding an association with the clinical or pathological response. It was not possible to evaluate changes in the expression of BECN1 before and after neoadjuvant treatment.

Regarding the recurrence of breast cancer, autophagy has been reported to be a critical mechanism for the survival of disseminated tumor cells that remain quiescent, even for decades, when alterations in the tumor microenvironment can trigger signals that lead to their proliferation. Genetic or pharmacological inhibition of autophagy has been shown to decrease survival and metastasis of dormant breast cancer cells in preclinical mouse models and human cell cultures[34]. Therefore, a possible association of BECN1 expression with breast cancer recurrence is expected.

Regardless of the immunophenotype and clinical stage, no association of BECN1 expression in cancer cells with recurrence DFS, or OS has been reported in patients with invasive ductal breast cancer[31,35]. However, Morikawa et al[35] reported that the absence of BECN1 expression in cancer cells in conjunction with the expression of BECN1 in mesenchymal stromal cells was associated with local recurrence, postoperative lymph nodes metastasis, and a shorter DFS. In our study of patients with LABC, regardless of the immunophenotype, a statistically significant difference in the recurrence rate, and DFS as a function of BECN1 expression in cancer cells was not found. As mesenchymal cells of the stroma were not analyzed, our results were similar to those previously reported[31,35]. In accord with studies of patients with breast cancer, the variables that were associated with a reduction in DFS were clinical stage at diagnosis, luminal B histological type and the use of preoperative non-standard chemotherapy[36].

Our data showed that the expression of BECN1 in tumor tissue is associated with a lower OS compared to patients who did not express BECN1. This differs from that reported by Choi et al[31] where deaths in the group of patients with BECN1 expression were not observed, and by Cha et al[14] where statistically significant differences in OS related to the expression of BECN1 were not observed. The difference can be explained by the higher rate of autophagy that has been reported in triple negative breast tumors, since they present a higher mitotic index, higher proliferation rate and central necrosis. Tumor cells are subjected to increased stress due to hypoxia in the tumor microenvironment and require autophagy as an adaptive process to meet their nutrient needs, thus contributing to tumor cell survival and proliferation[31,37]. It is noteworthy that the clinicopathological factors of poor prognosis for DFS and OS, such as clinical stage, histological type, and standard preoperative chemotherapy, usually reported in the literature, were only significant in the group of non-triple negative tumors. Despite having found an association of BECN1 expression with OS in patients with triple negative breast cancer, significant differences in the expression of BECN1 when comparing triple negatives and non-triple negatives were not observed.

The study of the prognosis of patients with breast cancer according to the immunophenotype is of special importance, as it has been reported in cell lines that BECN1 can negatively regulate estrogen signaling and growth response as well as contribute to the development of resistance to antiestrogens[33]. Evidence has shown that binding of progesterone and hydroxyprogesterone to the PR (particularly the beta isoform) induces the expression of BECN1 and activates autophagy, which leads to growth inhibition, reduced survival, and induction of cellular senescence as a protective mechanism against breast cancer cells[38]. The association between BECN1 deletion and HER2/Neu amplification has been reported[6]. HER2/Neu binds to BECN1 in breast cancer cells, and to ensure autophagic response, BECN1 must be released from the complex formed with HER2/Neu[3,39]. The overexpression HER2/Neu does not affect basal autophagy but does reduce stress-induced autophagy in breast cancer cells. HER2/Neu positive breast cancers with or without *BECN1* mRNA expression and HER2/Neu negative tumors (mostly triple negatives) with reduced BECN1 are characterized by a decreased expression of other autophagy genes, an increased glycolysis and increased expression of proliferation genes, compared to HER2/Neu negative tumors with a high expression of BECN1 (mainly positive hormone receptors)[6].

Particularly in triple negative breast cancers, the complete loss of BECN1 inhibits proliferation, colony formation and migratory capacity and invasiveness by reversing the epithelial-mesenchymal transition signals of the MDA-MB-231 cell line[40]. In triple negative breast cancer tumors, increased expression of *BECN1* mRNA[40] and protein in the cytosol and decreased expression of the protein in the nucleus has been observed[31].

The analysis of The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) databases showed a decrease in the expression

of *BECN1* mRNA and BECN1 in HER2/Neu positive and triple-negative tumors, and a lower expression of BECN1 with poor prognosis in patients with breast cancer[5], unlike our study which exclusively included patients with LABC.

Conclusions

Our results showed an association of BECN1 cytoplasmic expression with a lower OS. The integration of the available evidence and our findings suggests that in triple negative LABC, the cytoplasmic expression of BECN1 could constitute a poor prognostic marker. Prospective studies are necessary to evaluate the expression of BECN1 at the cytoplasmic and nuclear level of tumor cells, and mesenchymal stromal cells. It is important to have a larger sample size to allow the association analysis to be carried out with greater control of variables that could confuse or modify the observed effect; such as clinical stage, immunophenotype and the neoadjuvant chemotherapy regimen received.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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