Correlation Expression of Autophagy Marker LC3B with Histopathological Grading and Molecular Subtypes in Invasive Breast Carcinoma of No Special Type

Rahayu Asih Putri[a], Causa Trisna Mariedina[b], Betty[c]
Department of Anatomical Pathology, Faculty of Medicine,
Universitas Sumatera Utara, Medan, Indonesia
raputri24@gmail.com

ABSTRACT
Background: Breast cancer is one of the cancers with the highest incidence in women. Invasive breast carcinoma is found in most breast cancers and infiltrates the tissues. LC3B is a marker of autophagy and is an important protein involved in the formation of autophagosomes. The LC3B protein acts as a tumor suppressor. Therefore, lack of LC3 expression has been reported to be associated with survival and high mortality rates in TNBC.
Objective: To analyze correlation expression of autophagy marker LC3B with histopathological grading and molecular subtypes in invasive breast carcinoma of no special type (IBC-NST).
Methods: This study was an analytic study with a cross sectional approach on 40 samples paraffin block with histopathological diagnosed as IBC-NST. Slides were made with routine staining of hematoxyllin eosin and immunohistochemistry LC3B. LC3B expressed on cytoplasm of tumor cells. Scores for LC3B based on multiplication proportion and intensity of staining. Correlation expression of LC3B with histopathological grading and molecular subtypes in IBC-NST was statistically tested.
Results: Most patients with IBC-NST occur age 40-49 years, with average age 50,1 years, youngest age 27 years and oldest age 73 years. Most tumor size according to T2 criteria. Most molecular subtypes were luminal. Most histopathological grading was grade 3. Immunohistochemical expression of LC3B in IBC-NST was found to be highest with strong expression.
Conclusions: The study showed a significant correlation between immunohistochemical expression of LC3B with histopathological grading (p-value 0.0001). There was a significant correlation between immunohistochemical expression of LC3B with molecular subtype (p-value <0.05). LC3B has a role as a tumor suppressor, by inducing autophagy is an effective therapeutic strategy in IBC-NST especially TNBC.

Keywords: Breast cancer, IBC-NST, LC3B, histopathological grading, molecular subtype

1. Introduction

Invasive breast cancer (IBC) refers to a heterogeneous group of malignant epithelial neoplasms occurring in the mammary glands.[1] Invasive breast cancer of no special type (IBC-NST), previously known as invasive ductal carcinoma, is the most common subgroup (40-80%).[2,3] This type refers to a large heterogeneous group of IBCs that cannot be classified morphologically into any special type of histology. IBC-NST has similar or slightly worse prognostic and treatment characteristics, with a 10-year growth rate of 65-78% versus 80% of all breast cancers.[4]

Breast cancer is the most frequently diagnosed cancer in women worldwide with 2.26 million new cases in 2020 and is the leading cause of cancer death in women worldwide. In the United States, breast cancer alone is estimated to account for 29% of all new cancers in women. Breast cancer accounts for 11.6% of cancers in both women and men, making it the second most common cancer overall. The incidence rate of IBC has increased in most low- and middle-income countries in recent decades.[2,4]

Histopathological grading has become a simple and inexpensive method for assessing tumor behavior. The prognosis of breast cancer is determined by the grading and stage of breast cancer. The

widely used system is based on the Nottingham Grading System (NGS) by assessing tubular/glandular formation, nuclear pleomorphism, and a number of mitoses. The World Health Organization (WHO) divides IBC grading into grade 1 (well-differentiated), grade 2 (moderately differentiated), and grade 3 (poorly differentiated).[1]

Breast cancer is heterogeneous at the molecular level, with different gene expression patterns leading to differences in behavior and prognosis. Over the past few years, there have been many attempts to characterize and classify breast carcinomas at the molecular level in order to adapt treatment effectively. Molecular classification of carcinomas is largely based on immunohistochemical assessment of biomarkers (ER, PR HER2, and Ki-67). Based on the gene expression profile, breast cancer is divided into 4 subtypes, namely luminal A, luminal B, HER2-enriched, and triple negative breast cancer (TNBC).[1]

The autophagic catabolic process was first defined by Christian De Duve with the name meaning "self-eating". Autophagy overcomes the pathophysiological process to maintain homeostasis, cellular biological function, metabolism, and cell survival, which is the main process of degradation and intracellular recycling.[5] Autophagy is a physiological cellular process that is crucial for development and can occurs in response to nutrient deprivation or metabolic disorders. Interestingly, autophagy plays a dual role in cancer cells, in some situations it is cytoprotective (prevents tumor development by preventing reactive oxygen species/ROS and damaged mitochondria), whereas in others it is cytotoxic (contributes to living cancer of tumor cells by supporting the recycling of nutrients and maintain the anti-apoptotic pathway).[5,6,7,8,9] Autophagy can be helpful in promoting or inhibiting breast cancer. On the one hand, through the function of protein and organelle quality control, autophagy can maintain gene stability, prevent chronic tissue damage, cell injury, and inflammation, and inhibit the accumulation of p62 oncogenic protein aggregates, thereby preventing tumor initiation, proliferation, invasion, and metastasis. Therefore, here autophagy acts as a tumor inhibition (tumor suppressor) mechanism, especially in the early stages of tumorigenesis. On the other hand, immediately before the tumor develops into a late stage, autophagy can work as a mechanism of cell protection, cell survival, and cell defense, maintain functional mitochondria, reduce DNA damage, and increase cancer cell survival and resistance to stress (such as malnutrition, hypoxia, metabolic stress, DNA damage, and chemotherapy), and maintain tumor metabolism, growth, and survival. It is very important to define role of autophagy which is expected to be useful for effective treatment strategies in breast cancer cells.[10,11,12,13]

Microtubule-associated light chain 3B (LC3B) is one of the autophagy markers that is often used to determine the presence of autophagy activity. This is because the LC3B protein is a very important protein involved in the formation of autophagosomes (maturation phase), so the detection of LC3B has been considered as one way to measure autophagy activity.[7] Lack of LC3 expression has been reported to be associated with survival. Low LC3 expression in TNBC patients is associated with high mortality rates. LC3 suppresses TNBC in adult tumor cells and cancer stem cells. Suggested that tumor development is strongly associated with defective autophagic processes.[14] Whether its role is positive or negative has not been fully elucidated. Therefore, researchers are interested in examining whether there is a correlation between immunohistochemical expression of LC3B with histopathological grading and molecular subtypes in IBC-NST.

2. Material and methods

This research is an analytic study with a cross sectional approach and was conducted at the Department of Anatomic Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan and the Anatomic Pathology Unit, H. Adam Malik Hospital, Medan. The research was conducted from January 2022 to May 2022 after obtaining approval from the Health Research Ethics Committee, Faculty of Medicine, Universitas Sumatera Utara.

Sample of this study was a paraffin block from patients who had been diagnosed histopathologically as IBC-NST that met inclusion and exclusion criteria that met the inclusion and exclusion criteria. Samples were taken using consecutive sampling technique. Inclusion criteria included adequate clinical

data (include age, tumor size and molecular subtype) and representative preparations of paraffin slides and blocks, derived from postoperative mastectomy or lumpectomy tissue diagnosed histopathologically as IBC-NST. The exclusion criteria for this study were unrepresentative slides for processing and re-evaluation and paraffin slides or blocks derived from minimal tissue biopsy results

Histopathological grading was performed using Nottingham grading. Tumor grade is determined based on 3 parameters Tubular/glandular formation, nuclear pleomorphism and number of mitoses. (1) Tubular or glandular formations were given if tubular formation is found in > 75% of all tumors scored 1. If tubular formation is found in 10-75% of all tumors scored 2. If tubular formation is only <10% of all tumors scored 3 (2) Nuclear pleomorphism is given a score of 1 if cells are small, regular and uniform, a score of 2 if nucleus is enlarged and nucleus is moderately varied, a score of 3 if size and shape of nucleus are highly variable; (3) Number of mitoses is based on sum of all mitotic figures in 10 large visual fields (400x). Microscope used in this study has a field diameter of 0.5 mm, so mitotic assessment with a field area of 0.196 mm2 is a score of 1 for number of mitoses 7/10 LPB, a score of 2 for number of mitoses 8-14/10 LPB, and a score of 3 for mitotic count 15/10 LPB. Scores from each category will be summed and interpreted as follows: 1 = Grade 1, if total score is 3-5; 2 = Grade 2, if total score is 6 or 7; 3 = Grade 3, if total score is 8 or 9.[1]

Molecular subtypes were assessed using the results of the ER, PR, HER2 and Ki67 immunohistochemical examinations. Obtained from secondary data obtained from medical records. Based on this immunohistochemical profile IBC is divided into several molecular subtypes and categorized as follows 1 = Luminal (luminal A, luminal B HER2 negative and luminal B HER2 positive); 2 = HER2 positive (non-luminal); 3 = TNBC.[1]

LC3B is a member of the Atg8 protein family that includes the LC3 and GABARAP subfamilies. The LC3B was stained in the cytoplasm, using primary antibody LC3B, rabbit, polyclonal with 1:200 dilution (Cat. No. GTX127375; GeneTex, Inc).[15,16] The expression of LC3B was evaluated using a proportion score and an intensity score. The proportion score is scored as follows: 0% number of positive stained cell scored 0, <10% scored 1, 10%-50% scored 2, and >50% scored 3. The intensity of staining was as follow: 0 = no intensity, 1 = weak intensity, 2 = moderate intensity, 3 = strong intensity. Then, the staining index was calculated by multiplying the proportion of positive cells by the staining intensity score. The total score obtained ranges from 0-9: Total score 0-4 = Weak expression, total score 5-9 = Strong expression.[14,17] Assessment of correlation immunohistochemical expression of LC3B and histopathological grading on IBC-NST using Somers'd test. Assessment of correlation immunohistochemical expression of LC3B with molecular subtypes in IBC-NST using eta test.

3. Results

The number of samples used in this study were 40 samples from parraffin block/slide histopathology diagnosed with IBC-NST at the Anatomic Pathology Unit of H. Adam Malik Hospital Medan and the Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara. All samples have met the inclusion criteria

Table 1 shows the frequency distribution of research subjects based on age. The highest age group was 40-49 years old with 13 people (32,5%) followed by subjects 50-59 years old with 12 people (30%). The least age group is <30 years old as many as 2 people (5%). The average age of patients with IBC-NST in this study was 50,1 years with an age range of 27-73 years.

Table 1. Frequency Distribution of Research Subjects Based on Age

Age (years old)	Frequency	%
<30	2	5,0
30 - 39	4	10,0
40 - 49	13	32,5
50 - 59	12	30,0
>59	9	22,5
Total	**40**	**100**

Table 2 shows the frequency distribution of research subjects based on tumor size of IBC-NST. The most tumor size was T2 with 21 samples (52,5%). The second highest tumor size is T3 with 10 samples (25%).

Table 2. Frequency Distribution of Research Subjects Based on Tumor Size

Tumor size (cm)	Frequency	%
≤ 2 cm (T1)	9	22,5
2 - 5 cm (T2)	21	52,5
> 5 cm (T3)	10	25,0
Tumor any size with direct extension to chest wall and/or skin (skin ulceration or nodule) (T4)	0	0
Total	**40**	**100**

Table 3 shows the frequency distribution of research subjects based on molecular subtype of IBC-NST. The most molecular subtype was luminal with 21 samples (52,5%). The second highest molecular subtype is HER2 positive (non-luminal) with 11 samples (27,5%).

Table 3. Frequency Distribution of Research Subjects Based on Molecular Subtype

Molecular subtype	Frequency	%
Luminal	21	52,5
HER2 positif (Non luminal)	11	27,5
TNBC	8	20,0
Luminal	21	52,5
Total	**40**	**100**

Table 4 shows the frequency distribution of research subjects based on histopathological grading of IBC-NST. The most histopathological grade was grade 3 with 14 samples (35%).

Table 4. Frequency Distribution of Research Subjects Based on Histopathological Grading of IBC-NST

Grade	Frequency	%
1	13	32,5
2	13	32,5
3	14	35,0
Total	**50**	**100**

Table 5 shows the frequency distribution of research subjects based on immunohistochemical expression of LC3B in IBC-NST. Strong expression were found in 24 samples (60%) while weak expressions were found in 16 samples (40%).

Table 5. LC3B immunohistochemical expression frequency distribution on IBC-NST

LC3B expression	Frequency	%
Weak	16	40,0
Strong	24	60,0
Total	**50**	**100**

Table 6 shows the results of the analysis of the correlation between the immunohistochemical expression of LC3B and the histopathological grading of IBC-NST. There is a significant correlation between immunohistochemical expression of LC3B and histopathological grading (p-value= 0,0001), where the stronger immunohistochemical expression of LC3B, the lower grade (grade 1 and grade 2). On the other hand, the weaker immunohistochemical expression of LC3B, the higher histopathological grade (grade 3)

Table 6. Correlation expression of LC3B with histopathological grading in IBC-NST

LC3B expression	Grade			p-value*
	1	2	3	
Weak	2 (5,0)	3 (7,5)	11 (27,5)	0,0001
Strong	11 (27,5)	10 (25)	3 (7,5)	

**Somers'd*

Table 7 shows the results of the analysis of the correlation between the immunohistochemical expression of LC3B and the moleculer subtype of IBC-NST. There is a significant correlation between immunohistochemical expression of LC3B and molecular subtype (p-value <0,05), where the stronger immunohistochemical expression of LC3B, higher probability of occurrence in luminal molecular subtype. On the other hand, the weaker immunohistochemical expression of LC3B, higher probability that it will occur in molecular subtype of TNBC.

Table 7. Correlation expression of LC3B with moleculer subtype in IBC-NST

LC3B expression	Moleculer subtype			p-value*
	Luminal	HER2 (non-luminal)	TNBC	
Weak	2 (5,0)	3 (7,5)	11 (27,5)	<0,05
Strong	11 (27,5)	10 (25)	3 (7,5)	

*Eta test

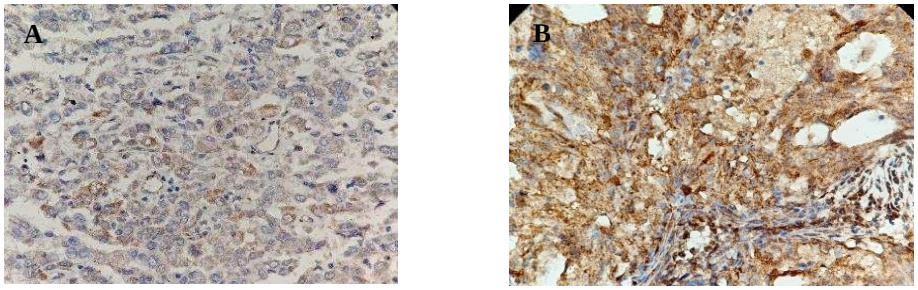

Fig. 1. LC3B immunohistochemical expression. (a) weak expression (x400); (b) strong expression (x400).

4. Discussion

The number of samples diagnosed as IBC-NST in this study were 40 samples, of which 32.5% were aged 40-49 years, with a mean age of 50,1 years, where youngest age was 27 years and oldest age was 73 years. The results of this study are not much different from previous studies. Laurinavicius et al. in 2016 stated that IBC occurs in women aged ≤55 years.[18] Kang et al. in 2020 reported that the age of most breast cancer patients was 40-49 years.[19] Kumarguru et al. in 2020 found that the average age of IBC patients was 53.14 years, with an age range of 24-77 years.[20] Epidemiological studies show that many risk factors are involved in the development of breast cancer in women, one of which is age. Increasing age is one of the risk factors for breast cancer, presumably due to the influence of long-term hormonal exposure, especially the hormone estrogen. In addition, the effects due to the accumulation of chemical substances in foods that are carcinogenic and contain high fat that are consumed from a young age often appear after a person enters old age where the decline in the body's immune system has begun to weaken, resulting in an increase in carcinogenesis over time.[21,22,23] The discovery of young patients in this study, namely 27 years, proves that breast cancer can occur at a young age. In Asian countries there is a shift in age to be younger in breast cancer patients. This is probably due to lifestyle changes such as a diet that is low in fiber, high in fat, especially trans fats combined with a lack of physical exercise and specific risk factors in each individual such as premature age of menarche and a tendency to genetically BRCA1 or BRCA2 mutations. hereditary (familial breast cancer) which in this case is different for each individual and environment.[23]

In this study, the most tumor size was found on the T2 criteria (2-5cm) as much as 52.5%, in accordance with the research conducted by Xu et al. in 2020 stated that the largest tumor size was found at T2 of 67.5%.[24] Guay et al. in 2022 found that the largest size was 2.2 cm (T2).[25] However, this is not in line with the research conducted by Widiana, et al. in 2020 reported that the largest tumor size in breast cancer cases was in accordance with the T4 criteria as much as 47%.[26] The staging of breast cancer is determined by the characteristics of the cancer, such as the size of the tumor and the type of hormone receptors. Cancer staging helps to determine the prognosis and outcome of breast cancer patients and can be used to determine the best treatment options for patients. TNM staging system by AJCC with one of the assessments depending on the size of the tumor.[27] The difference in the results of this study may lie in the difference in the level of knowledge and awareness of the health of each patient. Including early screening for breast cancer, which is different for each patient.

The most molecular subtypes of IBC-NST were luminal, as many as 52.5%, consisting of luminal A, luminal B-HER2 negative, and luminal B-HER2 positive. The results of this study are not much different from previous studies. Laurinavicius et al. in 2016 stated that the most common molecular subtype in IBC found was hormone receptor (Luminal) at 68%.[18] Kang et al. in 2020 reported that the most molecular subtypes in breast cancer were hormone receptor positive and HER2 negative as much as 65.9%.[19] Guay et al. in 2022 found that the most molecular subtypes in IBC were ER positive, PR positive, and HER2 negative as much as 37%.[25] Si et al. in 2015 reported that Luminal B HER2 was

negative as much as 40.7%.[28] Breast cancer is heterogeneous at the molecular level, with different patterns of gene expression leading to differences in behavior and prognosis. Over the past few years, there have been many attempts to characterize and classify breast carcinomas at the molecular level in order to adapt treatment effectively. Molecular classification of breast carcinoma is still largely based on immunohistochemical assessment of biomarkers (ER, PR, HER2, and Ki-67). Based on the gene expression profile, breast cancer is divided into 4 subtypes, namely luminal A, luminal B, HER2-enriched, and triple negative breast cancer (TNBC).[1] The luminal subtype has characteristics that affect hormone receptors on immunohistochemical examination. This subtype is a major predictive factor for hormone therapy. Luminal A is said to have a better prognosis than the other subtypes. In contrast to luminal A, luminal B tends to have a poorer prognosis, although treatment with hormone therapy is the same. The cell proliferation index was higher in luminal B than in luminal A. Patients with HER2-positive subtypes and TNBC had a worse prognosis than those with luminal subtypes. In this subtype, combination therapy between chemotherapy and targeted therapy is used. Luminal is a subtype that is more often found in the elderly, while positive subtypes such as TNBC and HER2 are more common in young people.[26,29] Based on the statements previously described, the results of this study found similar things where the luminal subtype was most commonly found.

The most histopathological grade of IBC-NST was grade 3, which was 35%, in accordance with previous studies conducted by Laurinavicius et al. in 2016 stated that the most IBC cases were found in grade 3 as much as 60%.[18] Bolhasani et al. in 2020 reported that the most cases of IBC were grade 3 as many as 35.48%.[30] In contrast to the results of the study reported by Dooijeweert et al. in 2019 that the most cases of IBC were grade 2 (47.6%).[31] Histopathological grade is one of the important and independent prognostic factors in breast cancer and is associated with survival and cancer-free disease in breast cancer.[31,32] Other studies even demonstrated that histopathological grade can predict tumor behavior more accurately than other prognostic factors, such as tumor size. Therefore, histopathological grade is an important clinical contributor and is widely used as a guide in the management of breast cancer therapy.[32] This grading system can be used to determine the choice of chemotherapy. Grades 2 and 3 are eligible for adjuvant chemotherapy, whereas grade 1 is not recommended.[31]

Assessment of LC3B immunohistochemical expression in IBC-NST was found to be the most with strong expression, as much as 60%. The results of this study are not much different from the research conducted by Wang et al. in 2018 reported that the strong immunohistochemical expression of LC3B in gastric cancer was 64.5%.[33] Choi et al. in 2012 reported that the positive immunohistochemical expression of LC3B in breast cancer was 51.3%.[34] However, this was not in line with the results of the study by Cha et al. in 2014 who found that the most negative LC3B immunohistochemical expression was found in IBC as much as 67.7%.[35] Strong LC3B immunohistochemical expression was associated with more aggressive behavior. Several studies have revealed that strong LC3B expression can predict poor outcome in breast cancer patients with molecular subtypes of TNBC. These findings suggest that autophagy plays a role in various types of cancer.[36]

Based on the results of the analysis, that there was a significant relationship between the immunohistochemical expression of LC3B and histopathological grading (p-value = 0.0001), where the stronger the immunohistochemical expression of LC3B, the lower the grade (grade 1 and grade 2). On the other hand, the weaker the immunohistochemical expression of LC3B, the higher the histopathological grade (grade 3). The results of this study are supported by Wu et al. in 2015 reported that there was a significant relationship between LC3B immunohistochemical expression and histopathological grade in colorectal cancer (p value = 0.021).[37] In contrast to the results reported by Zhao et al. in 2013 reported that there was no significant relationship between LC3B and tumor grade (p value = 0.290).[36] Abdelbary et al. in 2017 reported that there was no significant relationship between LC3B immunohistochemical expression and histopathological grade (p value = 0.55).[38] Based on the results in this study and previous studies which showed that there was a relationship between LC3B expression and histopathological grading, conclusions can be drawn. it is necessary to assess the histopathological grading accurately so that it can predict the possible expression of LC3B. Where, the lower the histopathological grade, the stronger the immunohistochemical expression of LC3B.

Autophagy can play a role in cancer cell death and tumor cell suppression. Induction of cell death due to autophagy has been proposed as a mechanism of cell death because autophagosome accumulation and autolysosomes have been found in the cytoplasm of dying cells, in the absence of activation of the apoptotic process. Cell death due to autophagy can occur under conditions of prolonged stress, which can lead to excessive protein and organelle turnover that exceeds the capacity of a cell.[7]

Based on the results of the analysis, that there was a significant relationship between the immunohistochemical expression of LC3B and the molecular subtype (p-value <0.05), where the stronger the immunohistochemical expression of LC3B, the higher the probability that it would occur in the luminal molecular subtype. On the other hand, the weaker the immunohistochemical expression of LC3B, the higher the probability that it will occur in the molecular subtype of TNBC. Based on research that has been done by. Ladoire et al. in 2015 found that there was a significant relationship between the immunohistochemical expression of LC3B with molecular subtypes (p value = 0.016), where the most was the strong expression of the molecular subtype in luminal A as much as 67.7%.[39] Choi et al. in 2013 reported that there was a significant relationship between the immunohistochemical expression of LC3B with molecular subtypes where the immunohistochemical expression of LC3B was weakly positive for the molecular subtype in TNBC (p value <0.001).[34] However, this was not in line with the results of research conducted by Chen et al. in 2013 found that there was no significant relationship between LC3B immunohistochemical expression and positive estrogen receptor at the time of diagnosis (p value = 0.066) and after surgery (p value = 0.532).[40] Chang et al. in 2016 in his study reported the negative expression of LC3 in TNBC and suggested that LC3 deficiency can control TNBC in adult tumor cells and cancer stem cells. These results also show that LC3 suppresses TNBC in adult tumor cells and cancer stem cells. In conclusion, his research shows that cancer stem cells are associated with the development of autophagy in TNBC. During the progression and progression of TNBC cancer, autophagy of cancer stem cells/progenitor cells is low. Therefore, the rational conclusion is that inducing autophagy may be an effective therapeutic strategy in TNBC.[41] Shen et al. in his study also found a weak expression of LC3 in ovarian cancer. In ovarian cancer, autophagy capacity is decreased due to low levels of LC3 expression. Therefore, autophagy inducers can be used for the treatment of ovarian cancer.[42]

5. Conclusion

Based on the data and analysis that has been carried out, in this study it was concluded that there is a significant correlation between immunohistochemical expression of LC3B and histopathological grading (p-value= 0,0001). And There is a significant correlation between immunohistochemical expression of LC3B and molecular subtype (p-value <0,05)

Competing interests

Author has no financial interests relevant to product or company described in this article.

Acknowledgment

We would like to thank all staff of the Department of Anatomical Pathology, Universitas Sumatera Utara, Private hospitals and private clinics in Medan, Indonesia for all their assistance and cooperation.

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