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FULL PAPER

Expression of granzyme B, heat shock protein 27, and tumor-associated macrophage as predictors of anthracycline-based neoadjuvant chemotherapy response through a subtype approach to local advanced breast cancer

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Systemic therapy as a part of the management of locally advanced breast cancer (LABC) remains a problem because the response varies. Clinical, pathological, or biomolecular markers used as predictor factors for response to chemotherapy at present were not yet established or constantly practiced. The aim of this study was to investigate the relationship among granzyme B expression, tumor-associated macrophage (TAM), heat shock protein 27 (Hsp27), and subtypes of breast cancer patients with LABC as a predictor of response to anthracycline-based neoadjuvant chemotherapy. It was an observational-analytic study. Sixty patients with LABC were recruited for this study. All received 3-4 series of neoadjuvant chemotherapy using anthracycline at a 3-week interval. Overexpression of Granzyme B, Hsp27, and TAM were found at 29, 31, and 27, respectively. Of the twenty-nine patients with overexpression of Granzyme B, only 14 respond to anthracycline-based neoadjuvant chemotherapy. Of the 31 patients with overexpression of Hsp 27, only 12 respond to anthracycline-based neoadjuvant chemotherapy. There are 29 patients with overexpression of Granzyme B, but only 14 respond to anthracycline-based neoadjuvant chemotherapy. There are 27 patients with overexpression of TAM, but only 13 respond to anthracycline-based neoadjuvant chemotherapy. Overexpression of Granzyme B, low expression of TAM, low expression of Hsp27, and different subtypes of breast cancer have not been shown as predictors of response to anthracycline-based neoadjuvant chemotherapy for locally advanced breast cancer. Granzyme B, Hsp27, and TAM expressions were not affected by the breast cancer subtype.

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KEYWORDS

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Introduction

Breast cancer ranks second as the leading cause of cancer death in women. Patients generally come at an advanced stage (stages III–IV) due to a lack of screening in developing countries, where the problems raised, such as operability and response to chemotherapy, cannot be predicted because of the

heterogeneity of tumor cells and are probably already distant metastases [1].

Protocol treatment of advanced breast cancer begins with systemic therapy (neoadjuvant or induction) in the form of chemotherapy or hormonal therapy, sometimes combined with radiation therapy. First-line chemotherapy is anthracycline-based (epirubicin, adryamicin, or doxorubicin) in combination with fluorouracil-epirubicin-cyclophosphamide (FEC) or fluorouracil-adiamicin-cyclophosphamide (FAC), given 3 or 4 times, and then assessed for response before the next treatment modality. Neoadjuvant chemotherapy results showed 7% complete response (CR), 45% partial response (PR), thus providing the opportunity for surgery, either breast-conserving treatment (BCT) or Modified Radical Mastectomy (MRM), 42% had stable disease (SD) or no change (NC), and 7% had progressive disease (PD) [2].

Responses to treatment are not the same because of the heterogeneity of the tumor. Factors that play a role in the response to systemic therapy are patient factors, treatment factors, and disease factors. Patient factors include age, BRCA1 and BRCA2 gene mutations, and the patient's immune system. Disease factors are tumor size (T), lymph node metastasis (N), hormone receptors (estrogen receptor/ER and progestron receptor/PR), overexpression of the gene Human Epidermal Growth Factor Receptor (HER2/neu), multi-drug resistance (MDR) gene, and others [3].

Other factors that contribute to the response to chemotherapy are the expression of Granzyme B, Heat Shock Protein (Hsp) 27, and Tumor-Associated Macrophage (TAM), in which HSP 27 expression is correlated with a poor prognosis or short disease-free interval (DFI), while TAM expression means the ability of cancer invasion and metastasis. Heat shock protein will increase due to stress (heat, inflammation, and drugs) as chaperones that improve cellular homeostasis. Heat shock protein 27 also plays an important role in the apoptotic process, in which inhibition of apoptosis is associated with high HSP 27 expression. Cancer is also an immunogenic disease, so there's a possibility of cancer cell eradication through a specific immune system. Cytotoxic T Lymphocyte (CTL) or CD8+ T cells and natural killer cells (NK) are the major effector cells in immune surveillance mechanisms for eliminating cancer cells. The activity of CTL and NK cells can be determined by the expression of granzyme B and perforin in cancer cells. Granzyme B has the most potent apoptotic ability [4,6].

The St. Gallen International Expert Consensus 2011 proposed a new classification of breast cancer that aims to explain the relationship between breast cancer subtypes and genomic characteristics. Breast cancer is divided into 5 subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), HER2/neu, and Ki-67. This grouping is due to the heterogeneity of breast cancer, both in morphology and biology. There are five subtypes: Luminal A, Luminal B and HER2-negative, Luminal B and HER2-positive, a triple-negative or basal-like subtype, and a HER2-positive type. This grouping relates to the selection and response to therapy [7].

The aim of this study is to investigate the relationship among Granzyme B expression, TAM, Hsp27, and subtypes of breast cancer patients with LABC as a predictor of response to anthracycline-based neoadjuvant chemotherapy.

Experimental

Design study

A cross sectional study was performed from July 2011 until December 2012 at Dr. Soetomo General Hospital. The study protocol was approved by the Medical Ethics Committee of Dr. Soetomo General Hospital (No: 01 / Panke.KKE / I / 2014).

Inclusion and exclusion criteria

The inclusion criteria in this study are: (i) All patients with stage III mammary carcinoma based on Tumour-Node-Metastasis (TNM) system from the American Joint Committee on Cancer (AJCC), seventh edition; (ii) not multiple breast cancers; (iii) the maximum age 65 years; (iv) do not have an immune disease or take immunosuppressant drugs; (v) karnofsky scale >70; (vi) subjects who are willing to take part in the research series.

Meanwhile, the exclusion criteria for this study are: (i) There are other comorbidities that are contraindications for administering anti-cancer chemotherapy, for example, congestive heart defects, a history of acute heart attack (acute myocardial infarction), blood disorders such as thrombopathia, chronic liver disease, and impaired kidney function, and chronic, performance status (Karnoffsky Score) < 70%; (ii) previous history of breast cancer; (iii) the patient has undergone surgery, received treatment with other chemotherapy, or received hormonal therapy previously; and (iv) neurological or psychological disorders that can affect understanding of informed consent. Drop-out criteria are patients who cannot complete 3 or 4 series of neoadjuvant chemotherapy, either because of serious side effects or of their own accord.

Sample size

The sample size was determined according to calculations based on proportions for the cohort research formula as follows:The total sample in our study was 60.

$$P = \frac{\psi}{(1+\psi)}$$

α = 95 %, so the value of $Z_{0.025}$ = 1.96
β = 10 % resulting in 1.285
Odds Ratio Value (ψ) = 4.33

Therapeutic response

The therapeutic response according to the World Health Organization (WHO) criteria is referred to as complete clinical / pathological response: if the tumor disappears either clinically or pathologically for at least 4 weeks; partial clinical response: the tumor has decreased by more than 50% of its original size and no new lesions have been found for at least 4 weeks; no change: if the tumor decrease less than 50% of its original size or enlarges <25% during treatment; progressive disease: if the tumor enlarges >25% of its original size or new lesions are discovered.

Chemotherapy regiment

Anthracycline-based neoadjuvant chemotherapy is a combination regimen of Fluorouracil, Adriamycin/Doxorubicin,

Cyclophosphamide (FAC) or FEC (Fluorouracil, Epirubicin, Cyclophosphamide), with doses F: 500 mg/m^2, A/E: 50 mg/m^2, and C: 500 mg/m^2, given before surgery three times. Administration interval: every 3 weeks.

Granzyme B

Granzyme B was examined by immunohistochemistry staining using the anti-granzyme B antibody (ab4059) abcam® reagent. The interpretations are overexpression and no overexpression. The cut-off value is based on the median value, 9.1 (overexpressed >9.1 and not overexpressed <9.1).

Heat shock protein 27 (Hsp 27)

Hsp 27 was examined using immunohistochemistry staining using the commercial avidin-biotin-peroxidase method (Vector Laboratories, Burlingame, CA). The interpretations are overexpression and no overexpression. The cut-off value for determining overexpression is based on the

median value, 9.1 (overexpressed >9.1 and not overexpressed <9.1).

Tumour-associated macrophage (TAM)

TAM was examined using the immunohistochemistry stain technique using anti-CD68 antibodies. The cut-off value for determining overexpression is based on the median value, namely 7.7. It is said to be overexpressed if > 7.7 and no overexpressed if < 7.7.

Statistical analysis

Statistical tests were performed using the SPSS 18.0 software for Windows. Statistical analysis was carried out to see the relationship and magnitude of significance of each variable, namely Granzyme B, Hsp 27, TAM, ER, PR, and HER2/neu, with the response to the administration of neoadjuvant combination chemotherapy, carried out using correlation and crosstab analysis, the Chi-square test (X^2), and Cramer.

Results and discussion

Subject characteristics

The subjects for this study were 60, with the largest age group being between 41 and 50 years (50%). Regarding the characteristics of menstrual status, 36 (60%) subjects were still menstruating, while 24 (40%) subjects were menopausal. Stage IIIA in the subjects of this study was 26 (43.33%), while stage IIIB was 34 (56.67%) (Table 1).

Table 1 Subject characteristics

Characteristic	Frequency	%
Age		
21-30 years	1	1.67
31-40 years	6	10
41-50 years	30	50
51-60 years	19	31.67
>60 years	4	6.67
Menstrual status		
Menstrual	36	60
Menopause	24	40
Staging		
IIIA	26	43.33
IIIB	34	56.67
Subtype cancer		
Luminal A (LA)	16	26.67
Luminal B (LB)	4	6.67
Triple Negative (TN)	21	35
HER2	19	31.67

Immunohistochemistry (IHC) staining

IHC examination of Granzyme B, TAM, and Hsp 27 showed that overexpression of Granzyme B was found in 31 (51.7%), overexpression of TAM was found in 29 (48.3%), and overexpression of Hsp 27 in 31 (51.7%) (Figures 1, 2, and 3).

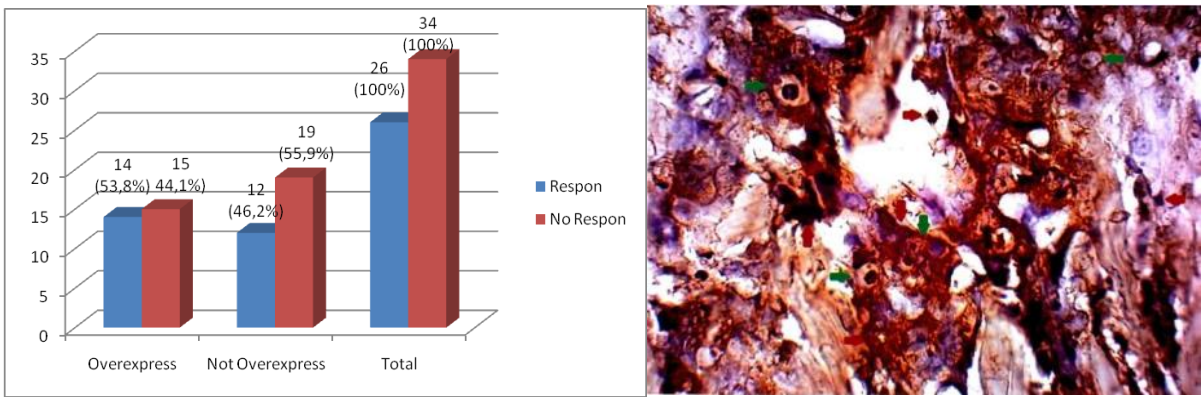

FIGURE 1 The result of IHC examination of Granzyme B. Mammary cancer cells and Granzyme B with 400x magnification. Red arrows show granzyme in breast cancer, and green arrows show apoptotic cells

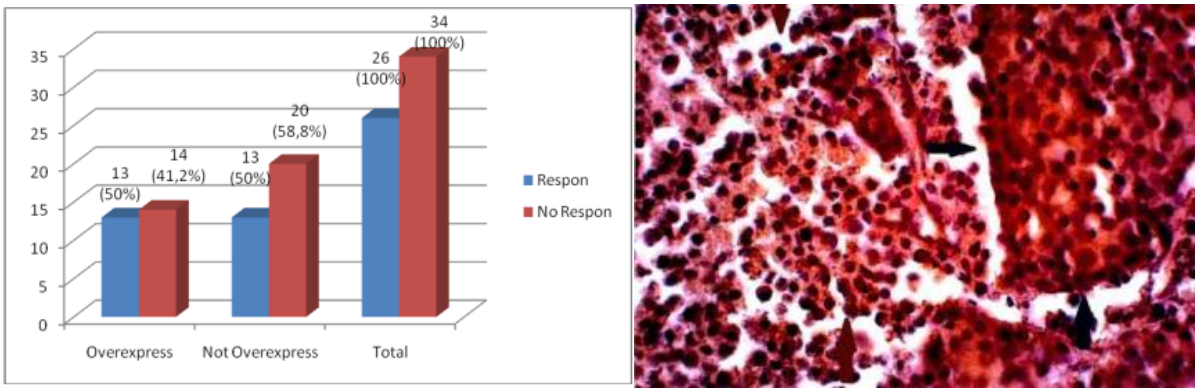

FIGURE 2 The result of IHC examination of TAM. Mammary cancer cells and TAM with 400x magnification. Positive TAM show red arrow; Breast cancer show blue arrow

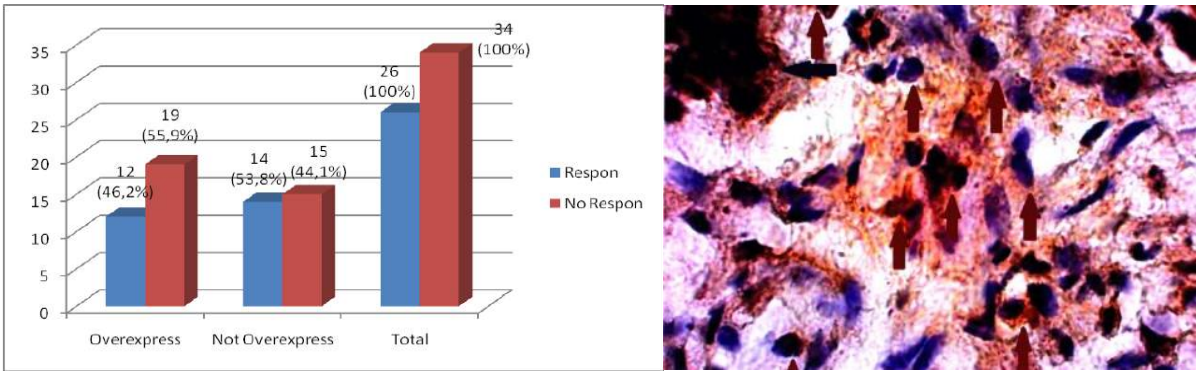

FIGURE 3 The result of IHC examination of Hsp 27. Mammary cancer cells and HSP 27 with1000x magnification. Breast Ca shows a blue arrow; HSP 27 positive shows a red arrow.

Chemotherapy response

Response to neoadjuvant combination chemotherapy was found in 26 patients (43.3%); 8 (13.3%) subjects from the Luminal A subtype, 2 (3.3%) subjects from the Luminal B subtype, 8 (13.3%) subjects from the Triple Negative subtype, and 8 (13.3%) subjects from the HER2/neu subtype. No response was found in 34 (56.7%) subjects; 8 (13.3%) sufferers with the Luminal A subtype, 2 (3.3%) subjects with the Luminal B subtype, and 13 (21.7%) subjects with the Luminal B subtype. Triple Negative and 11 (18.3%) patients with the HER2/neu subtype (Figure 4).

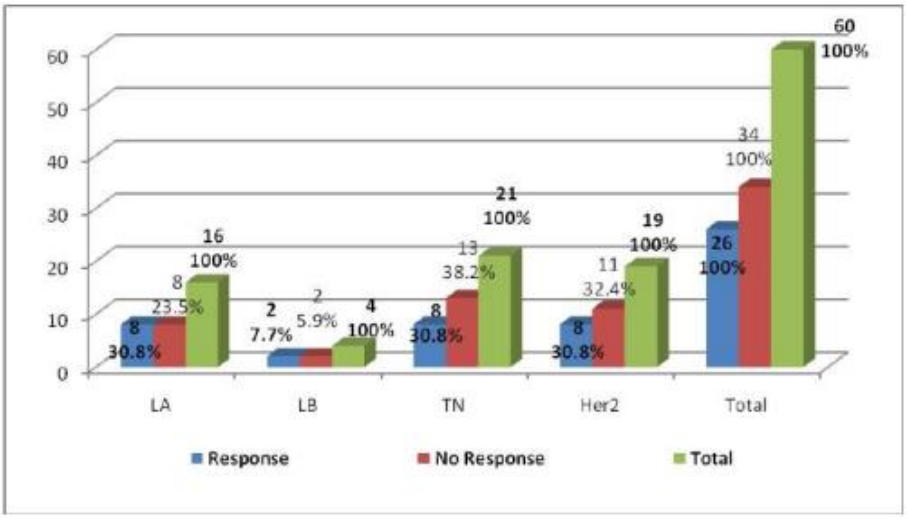

FIGURE 4 Response to chemotherapy in Luminal A, Luminal B, Triple Negative, and HER2/neu subtypes

Relationship between breast cancer subtype and granzyme B, TAM, and HSP 27 overexpression

The results of the relationship between breast cancer subtypes and Granzyme B overexpression show that in the HER2 subtype, 42.1% show overexpression, while in the LA and LB subtypes, 43.8% and 75% show overexpression. Meanwhile, 40% show overexpression in the TN subtype. These results indicate that the LB subtype has the highest Granzyme B overexpression (Figure 5).

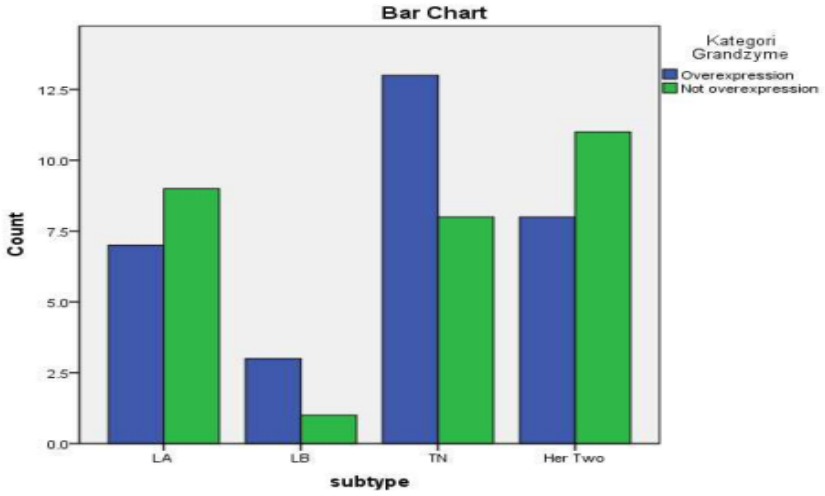

FIGURE 5 Relationship between Granzyme B expression and cancer subtype

Statistical tests showed that Granzyme B overexpression in the four subtypes was not significantly different (p = 0.444).

The results of the relationship between breast cancer subtypes and TAM overexpression show that in the HER2 subtype, 55% show overexpression, while in the LA and LB subtypes, 50% show overexpression, and in the TN subtype, 40% show overexpression. These results indicate that the HER-2 subtype has the highest TAM overexpression. Statistical tests showed that TAM overexpression in the four subtypes was not significantly different (p = 0.872) (Figure 6).

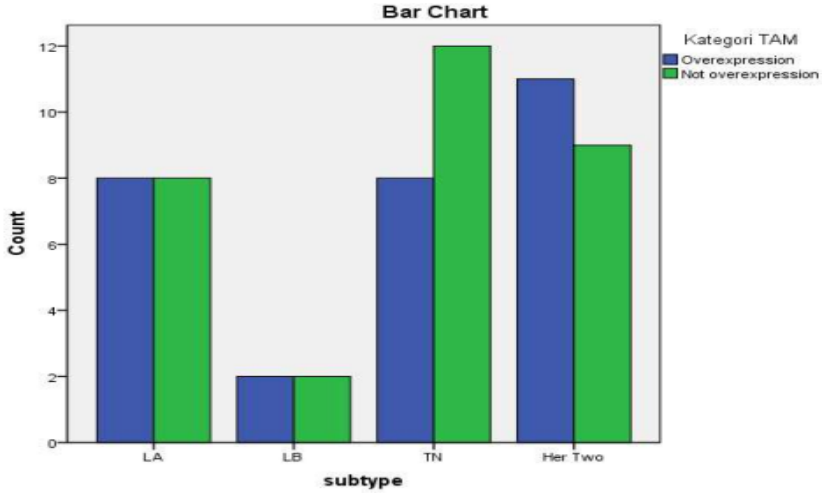

FIGURE 6 Relationship between TAM expression and cancer subtype

The results of the HSP 27 overexpression in the HER2, LA, LB, and TN subtypes show 68.4%, 37.5%, 50%, and 47.6%, respectively. It indicates that the HER2 subtype has the highest Granzyme B overexpression. Statistical tests showed that HSP 27 overexpression in the four subtypes was not significantly different (p = 0.310) (Figure 7).

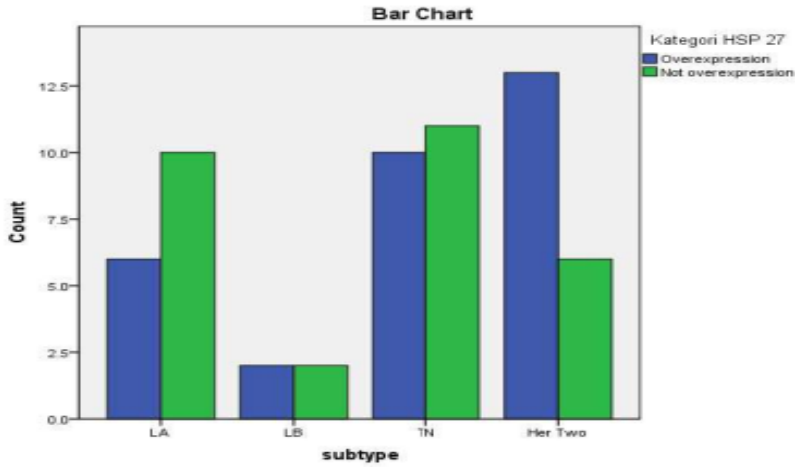

FIGURE 7 Relationship between TAM expression and cancer subtype

Discussion

Breast cancer is the most frequently diagnosed cancer in women worldwide with 2.26 million new cases in 2020. According to the WHO, malignant neoplasms are the greatest worldwide burden for women, estimated at 107.8 million Disability-Adjusted

Life Years (DALYs), of which 19.6 million DALYs are due to breast cancer[8].

Currently, about 80% of patients with breast cancer are individuals aged >50 while at the same time more than 40% are those more than 65 years old. [9,10].

The risk of developing breast cancer increases as follows—the 1.5% risk at age 40, 3% at age 50, and more than 4% at age 70[8].

Interestingly, a relationship between a particular molecular subtype of cancer and a patient's age was observed –aggressive resistant triple-negative breast cancer subtype is most commonly diagnosed in groups under 40 age, while in patients >70, it is luminal A subtype [9].

The results of this study support the results of our study, which showed that the highest frequency was found in the age range of 41-50 years. Although they tend to be younger than previous studies, it is because all subtypes of breast cancer in our research subjects were included.

The response to a combination of neoadjuvant chemotherapy or anthracycline-based preoperative chemotherapy in stage III mammary carcinoma is reported to range between 60 and 90%, both in the primary tumor and in regional lymph node metastases. Clinically complete responses are found in 10-20% and can be even higher if a combination of chemotherapy is added with the Taxane group [11].

In our study, a response to anthracycline-based neoadjuvant combination chemotherapy was found in 43.3% of subjects. Several factors that influence the response to chemotherapy are the degree of differentiation (poorly differentiated), negative hormonal receptors, aneuploid tumors, and a high proliferation fraction. Patients with HER2/neu overexpression have relative resistance to the combination of CMF chemotherapy (Cyclophosphamide, Methotrexate, and 5-Fluorouracyl) and hormonal therapy with tamoxifen. Overexpression of p53 indicates a poor prognosis and is relatively resistant to chemotherapy, while overexpression of bcl2 is a good predictor of prognosis but is resistant to chemotherapy [12].

Research conducted by Mohamed Baker regarding the expression of granzyme B in breast cancer found that granzyme B was found in 62.5% of patients with ductal infiltrating breast cancer with an *in situ* component, 80% of patients with *in situ* breast cancer, and 100% of patients with breast cancer of the in situ type medullary [13].

In this study, granzyme B overexpression was found in 29 patients, of whom 14 responded to anthracycline-based neoadjuvant chemotherapy and 12 did not overexpress but responded to anthracycline-based neoadjuvant chemotherapy.

Granzyme B has the strongest apoptotic ability of all granzymes with caspase-like abilities, namely cleave substrates at aspartic acid residues. Granzyme B can cleave and activate several procaspases, especially caspase 10, directly and directly reduce caspase substrates such as *inhibitor caspase activated DNase* (ICAD) inhibitors which cause DNA fragmentation in target cells [14].

Estrogen can induce granzyme B inhibitor, proteinase inhibitor 9 (PI-9), causing NK cell apoptosis so that immune surveillance fails to damage new cells undergoing transformation. It can explain the mechanism of tumor incidence caused by estrogen [15,16].

These results of this study indicate that the LB subtype has the highest overexpression of Granzyme B. The probability of someone with Granzyme B overexpression responding to anthracycline-based neoadjuvant chemotherapy is 48.33%. With a probability of <50%, the administration of neoadjuvant chemotherapy does not use granzyme B overexpression parameters but uses standard prognostic and predictive factors, namely estrogen receptor, progestrone receptor,

HER2/neu gene expression, and Ki67 expression.

Heat Shock Protein 27 (Hsp27) functions as a chaperone in thermotolerance in vivo and inhibits apoptosis, regulates cell development and differentiation, maintains cell resilience under stress conditions, and plays a role in signal transduction. Hsp27 will experience overexpression during the differentiation and development stages of cells, neurodegenerative disorders, and various types of cancer, including breast cancer, and has been shown to inhibit various stages in the apoptotic pathway, inhibition of the intrinsic pathway mediated by mitochondria, and inhibition of the extrinsic pathway mediated by Fas [17].

The results of this study indicate that the HER2 subtype has the highest overexpression of Hsp 27. Meanwhile, the probability of Hsp27 overexpression responding to anthracycline-based neoadjuvant chemotherapy is 31%. With a probability of <50%, the administration of neoadjuvant chemotherapy does not use Hsp27 overexpression parameters but uses standard prognostic and predictive factors, namely the expression of estrogen receptors, progesterone receptors, HER2/neu, and Ki67 genes. Hsp27 expression has a significant correlation with the ER status of breast cancer patients because Hsp27 expression is regulated by estrogen. Research conducted by Oesterreich (1996) showed that Hsp 27 levels had a positive correlation with ER, PR, and aneuploidy status but not with tumor size or S phase. Hsp-27 expression plays a role in resistance to chemotherapy. Hsp-27 has cytoprotective and anti-apoptotic abilities. Overexpression of Hsp-27 is found in malignancies and various tumor cell lines, which is associated with chemotherapy drug resistance, altered cell growth, increased tumorigenicity, and metastasis [18].

Tumor associated macrophage (TAM) has a pleiotropic function that plays a role in cancer regression or progression, known as the macrophage plasticity reflex. These different effects of TAMs are regulated by the host immune system. Cancer regression by TAMs is mediated by non-specific anti-tumor cytotoxic mechanisms or induction of specific lytic cells. On the other hand, tumor progression by TAM is caused by the release of various cytokines and prostanoids, including prostaglandin E2, which causes an immunosuppressive effect on cell-mediated immunity mechanisms and produces immunosuppressive cytokines such as TGF-β and IL-10 [16,17].

The results of this study indicate that the HER2 subtype has the highest overexpression of Hsp 27. Meanwhile, the probability of Hsp27 overexpression responding to anthracycline-based neoadjuvant chemotherapy is 31%. With a probability of <50%, the administration of neoadjuvant chemotherapy does not use Hsp27 overexpression parameters but uses standard prognostic and predictive factors, namely the expression of estrogen receptors, progesterone receptors, HER2/neu, and Ki67 genes. Hsp27 expression has a significant correlation with the ER status of breast cancer patients because Hsp27 expression is regulated by estrogen. Research conducted by Oesterreich (1996) showed that Hsp 27 levels had a positive correlation with ER, PR, and aneuploidy status but not with tumor size or S phase [4]. Hsp-27 expression plays a role in resistance to chemotherapy. Hsp-27 has cytoprotective and anti-apoptotic abilities. Overexpression of Hsp-27 is found in malignancies and various tumor cell lines, which is associated with chemotherapy drug resistance, altered cell growth, increased tumorigenicity, and metastasis [6,18].

Conclusion

It has not been demonstrated that high levels of granzyme B, low levels of heat shock protein 27, or low levels of tumor-associated macrophages can predict response to

anthracycline-based neoadjuvant chemotherapy. Furthermore, the outcomes of anthracycline-based neoadjuvant chemotherapy for locally advanced breast cancer patients did not exhibit subtype-dependent changes in the expression of tumor-associated macrophage, heat shock protein 27, or granzyme B.

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None.

Authors' Contributions

The study's design and methodology were developed by Desak GA Suprabawati, who also carried out formal research, analysis and wrote the initial draught of the paper, edited the manuscript, curated the data, supplied resources, and oversaw project administration, in addition to validating and supervising the study.

Conflict of Interest

The authors have no conflict of interest to declare.

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