RESEARCH ARTICLE

Expression of CD8+ and Foxp3+ T Lymphocyte as Predictor for Response to Neoadjuvant Chemotherapy in Stage III Breast Cancer

I Nyoman Wawan Tirtha Yasa[1,*], I Wayan Sudarsa[2], I Wayan Niryana[3], Ni Putu Sriwidyani[4], I Made Jawi[5], I Wayan Putu Sutirta Yasa[6], Ida Bagus Tjakra Wibawa Manuaba[2], Ida Bagus Made Suryawisesa[2]

[1]Doctoral Study Program, Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar 80232, Indonesia
[2]Division Surgical Oncology, Departement of Surgery, Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar 80232, Indonesia
[3]Department of Neurosurgery, Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar 80232, Indonesia
[4]Department ofAnatomical Pathology, Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar 80232, Indonesia
[5]Department of Pharmacology, Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar 80232, Indonesia
[6]Department of Clinical Pathology, Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar 80232, Indonesia

*Corresponding author. Email: nyomanwawan@yahoo.com

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Abstract

BACKGROUND: Neoadjuvant chemotherapy (NAC) in breast cancer is usually utilized to eradicate micro-metastasis, induce apoptosis in tumor cells, and reduce the primary tumor size, enabling surgical intervention. Recent studies have shown that tumor-infiltrating lymphocytes (TILs), especially cytotoxic CD8+ T cells and immunosuppressive Foxp3+ regulatory T cells, influence tumor response to treatment. However, their role as predictive markers for NAC response remains unclear. Therefore, this study was performed to investigate whether high expression of CD8+ and low expression of Foxp3+ T lymphocytes are associated with better response to NAC in stage III breast cancer patients.

METHODS: Total of 60 biopsy samples from stage III breast cancer patients were included, comprising 30 subjects in the response group (+) and 30 subjects in the non-response group (−). The expression levels of CD8+ and Foxp3+ T lymphocytes in tumor tissue were assessed semi-quantitatively by immunohistochemistry (IHC), using a scoring system based on the proportion and intensity of positively stained cells (Black's grading criteria).

RESULTS: Stage III breast cancer with high expression of CD8+ T lymphocytes was significantly associated with a better response to NAC (p=0.004; OR=6.882). Meanwhile, low expression of Foxp3+ T lymphocytes was not significantly associated with chemotherapy response (p=0.067; OR=3.250). A higher tumor grade was also associated with an improved response to treatment. The probability of achieving a positive response to NAC in subjects presenting with high CD8+ expression, low Foxp3+ expression, and high tumor grade was estimated at 96.98%.

CONCLUSION: The combination of high expression of CD8+ T lymphocyte, low expression of Foxp3+ T lymphocyte and high tumor grade might be useful to predict good response to NAC in stage III breast cancer.

KEYWORDS: CD8+ T lymphocyte, Foxp3+ T lymphocyte, neoadjuvant chemotherapy, breast cancer

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Introduction

Breast carcinoma is the most commonly diagnosed cancer in women worldwide and ranks as the second leading cause of cancer-related deaths globally.(1) In Indonesia, breast carcinoma is the second most prevalent malignancy among women after cervical cancer.(2) In Bali, over 70% of breast cancer patients treated at Sanglah General Hospital present with advanced disease (stage III and IV).(3)

Patients with stage III breast cancer, categorized as locally advanced breast cancer (LABC), typically have large tumors and/or extensive regional lymph node involvement without distant metastasis. These patients are commonly treated with combination neoadjuvant chemotherapy (NAC) to reduce tumor size and enable breast-conserving surgery while targeting micrometastases early.(4,5) But response rates to NAC remain variable and difficult to predict. While some patients achieve substantial tumor shrinkage or pathological complete response, others show minimal or no response. This variability underscores the need for predictive biomarkers for chemotherapy responsiveness.

The immune system is increasingly recognized as playing a key role in modulating tumor behavior and treatment outcomes in breast carcinoma.(6,7) Tumor-infiltrating lymphocytes (TILs), particularly cytotoxic $CD8^+$ T cells and regulatory $Foxp3^+$ T cells, have been associated with prognosis and therapeutic responses.(8–11) $CD8^+$ T cells mediate anti-tumor immunity by directly killing tumor cells, and their presence has been correlated with improved outcomes.(12–15) Conversely, $Foxp3^+$ regulatory T cells (Tregs) suppress the immune response, promote immune evasion, and have been linked with poor prognosis when present in high numbers.(16–18)

Recent evidence suggests that chemotherapy can alter the immune microenvironment by modulating the balance of effector and suppressor T cells. Anthracycline-based chemotherapy, for instance, has been shown to reduce $Foxp3^+$ Treg levels and enhance cytotoxic T cell function in some breast cancer subtypes.(19,20) Despite these findings, the predictive role of $CD8^+$ and $Foxp3^+$ expression specifically in stage III breast cancer patients receiving NAC has not been fully elucidated. Most previous studies have either evaluated overall TIL density or did not focus on this specific clinical stage and therapeutic setting.

Therefore, this study was performed to investigate the expression levels of $CD8^+$ and $Foxp3^+$ tumor-infiltrating lymphocytes as risk factors associated with the response to combination NAC in patients with stage III breast carcinoma. The results might help identify potential immunological markers that can predict therapeutic outcomes and support personalized treatment strategies.

Methods

Study Design

This was a nested case-control design study, involving samples of stage III breast carcinoma patients who had received combination NAC Cyclophosphamide, Adriamycin/Epirubicin and 5 Fluorouracil (CAF/CEF), with complete medical records and meeting the specified inclusion and exclusion criteria. This study was conducted in the Surgical Oncology Division and Surgery Laboratory at the Faculty of Medicine, Universitas Udayana/Sanglah Hospital, and the protocol of this study was approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Udayana (Approval No. 482/Skrt/XII/2024).

Sample Recruitment

The study population included all stage III breast carcinoma patients treated at Sanglah Hospital, Denpasar, confirmed by histopathological examination and who had received 3 cycles of CAF/CEF NAC. The inclusion criteria for this study consisted of patients diagnosed with stage III breast carcinoma who had undergone three cycles of combination NAC using either the CAF (Cyclophosphamide, Adriamycin, and 5-Fluorouracil) or CEF (Cyclophosphamide, Epirubicin, and 5-Fluorouracil) regimens, administered at three-week intervals. The selection between CAF and CEF regimens was based on the oncologist's clinical judgment, taking into account the subject's cardiac function, tolerance to anthracyclines, comorbidities, and drug availability at the time of treatment. In cases where there was a delay in chemotherapy administration, the maximum allowable interval between cycles was four weeks. Subjects were excluded from the study if they had previously received any form of treatment, including surgery, chemotherapy, radiotherapy, or hormonal therapy. Additionally, those with incomplete medical records based on the evaluated variables or paraffin blocks that were unsuitable for IHC examination were also excluded. There were final of 60 samples, selected through consecutive purposive sampling, comprising 30 subjects in the response group (+) and 30 subjects in the non-response group (-).

Subject's Medical Record Collection

The list of breast carcinoma subjects was obtained from the Cancer Patient Registration Data at the Surgical Oncology Division, Faculty of Medicine, Universitas Udayana/ Sanglah Hospital, Denpasar. Additional data were collected from subject's medical records.

Selected samples were those with histopathological diagnosis of infiltrating ductal carcinoma (IDC) and clinically categorized as stage III/LABC. Collected data included demographic information, clinical data, pathology data, chemotherapy administration records, and chemotherapy response data. All subjects received 3 cycles

of CAF/CEF combination NAC at 3-week intervals. Tumor response was assessed every 2–3 weeks after chemotherapy by measuring tumor diameter. Subjects were categorized into the case group (positive response) and the control group (negative response). Paraffin blocks from biopsy specimens with good quality were collected from Department of Anatomical Pathology, Faculty of Medicine, Universitas Udayana/Sanglah Hospital for the immunohistochemical (IHC) examination of $CD8^+$ and $Foxp3^+$.

Tumor grade was assessed by histopathological examination of biopsy or surgical tissue stained with hematoxylin and eosin (H&E). The grading followed the modified Bloom-Richardson system, which evaluates three components: tubule formation, nuclear pleomorphism, and mitotic count. Each component is scored from 1 to 3, and the total score classifies tumors into three grades. Low grade (Grade 1) includes well-differentiated tumors with a total score of 3 to 5, intermediate grade (Grade 2) includes moderately differentiated tumors with scores of 6 to 7, and high grade (Grade 3) consists of poorly differentiated tumors with scores of 8 to 9. For the purpose of analysis in this study, tumors were grouped into two categories: low grade, combining Grade 1 and Grade 2, and high grade, which corresponds to Grade 3. High-grade tumors generally exhibit more aggressive behavior, higher mitotic activity, and poorer differentiation compared to low-grade tumors.

IHC Examination for $CD8^+$ and $Foxp3^+$

IHC staining for $CD8^+$ and $Foxp3^+$ was performed following the standard protocol at the Anatomical Pathology Laboratory of Sanglah Hospital. Monoclonal antibodies used were CD8 (Clone RM9116-SO LV; Thermo Vision/LabVision, Fremont, CA) and Foxp3 (Flex RTU, Can. No. GA0263-U; DAKO, Carpinteria, CA, USA), with the DAKO IHC-Paraffin Protocol. After dewaxing and antigen retrieval using DAKO retrieval solution with microwave heating, slides were incubated overnight at room temperature with primary antibodies diluted 1:20 for $CD8^+$ and 1:100 for $Foxp3^+$ in 3% Normal Swine Serum. Detection was completed using a biotinylated secondary antibody, streptavidin, and DAB chromogen for visualization, followed by Mayer's hematoxylin counterstaining. Tonsil tissue served as the positive control. $CD8^+$ expression was identified on the cell membrane and cytoplasm, while $Foxp3^+$ expression was localized in the nuclei. Stained slides were examined under 400x magnification, and expression was quantified by averaging counts from ten high-power fields.

Data Analysis

The association between independent variables ($CD8^+$ and $Foxp3^+$) and the dependent variable (response to NAC) was tested using the Chi-square test. If the assumptions for the Chi-square test were not satisfied, Fisher's exact test was used as an alternative. The association's strength was measured using the Odds Ratio (OR) along with a 95% Confidence Interval (CI). A *p*-value of less than 0.05 was considered statistically significant. Potential confounding variables such as age, menopausal status, tumor grade, Her2neu status, and TILs were controlled by multivariate analysis using logistic regression.

Results

Subject Characteristics

The histopathological grade examination revealed that there were 4 subjects (6.7%) with grade I, 35 subjects (58.3%) with grade II, and 21 subjects (35.0%) with grade III carcinoma. Meanwhile, based on the clinical staging, 11 subjects (18.4%) were at stage IIIA, 44 subjects (73.3%) at stage IIIB, and 5 subjects (8.3%) at stage IIIC (Table 1).

In terms of the TIL status, 46 subjects (76.7%) were TIL-positive, while 14 subjects (23.3%) were TIL-negative. HER2/neu testing showed 52 (86.7%) subjects were negative and 8 (13.3%) subjects were positive. Estrogen receptor (ER) and progesterone receptor (PR) testing was only performed on 9 subjects, resulting in 5 positives and 4 negatives (Table 1).

Lymphovascular invasion (LVI) and mitotic activity index (MAI) could not be fully evaluated due to incomplete histopathological reports. The most common histopathological type was infiltrating ductal carcinoma (IDC) found in 58 subjects (96.7%), with 2 subjects (3.3%) diagnosed with mucinous adenocarcinoma.

$CD8^+$, $Foxp3^+$, and NAC Response

IHC examination of biopsy tissue showed 16 subjects (26.7%) had high $CD8^+$ expression and 44 subjects (73.3%) had low $CD8^+$ expression. For $Foxp3^+$, 46 subjects (76.7%) had low expression, while 14 subjects (23.3%) had high expression. After receiving three cycles of combination NAC (CAF/CEF), 30 subjects (50%) showed a positive response, and 30 subjects (50%) showed no response (Table 2). Among the 30 subjects who responded to NAC, 28 subjects (46.7%) experienced a partial response, and 2 subjects (3.3%) achieved a clinical complete response. No cases of pathological complete response were observed. In the non-

Table 1. Subject characteristics.

Variable	Positive Response (%)	Negative Response (%)	*p*-value
Mean Age (years), mean±SD	46.33±10.688	44.70±7.818	0.912
Menstrual Status, n (%)			
Premenopause	22 (73.3%)	25 (83.3%)	0.347
Menopause	8 (26.7%)	5 (16.7%)	
Clinical Stage, n (%)			
IIIA	6 (20%)	5 (16.7%)	1.000
IIIB	21 (70%)	23 (76.7%)	
IIIC	3 (10%)	2 (6.7%)	
HER2/neu Status, n (%)			
Positive	4 (13.3%)	4 (13.3%)	1.000
Negative	26 (86.7%)	26 (86.7%)	
TIL Status, n (%)			
Positive	24 (80%)	22 (80%)	0.542
Negative	6 (20%)	8 (20%)	
Tumor Grade, n (%)			
Low Grade	16 (53.3%)	23 (80%)	0.028
High Grade	14 (46.7%)	7 (20%)	

responsive group, 29 subjects (48.3%) had stable disease, and 1 subject (1.7%) experienced disease progression, with distant metastasis to the lungs. Representative IHC results of $CD8^+$ and $Foxp3^+$ lymphocutes expression were shown in Figure 1 and Figure 2, respectively.

Association Between $CD8^+$ and NAC Response

There was a significant association between high $CD8^+$ expression and a positive response to NAC (p=0.004). Subjects with high $CD8^+$ expression were 6.88 times more likely to respond to NAC compared to those with low $CD8^+$ expression (Table 3). High $CD8^+$ expression acts as a predictive factor for better NAC response.

Association Between Foxp3+ and NAC Response

Although breast cancer patients with low $Foxp3^+$ expression were 3.25 times more likely to respond to NAC compared to those with high $Foxp3^+$, the association was not statistically significant (p=0.067) (Table 4). Low $Foxp3^+$ expression showed a trend toward better response but was not a significant predictor.

Association Between NAC Response and Other Variables

Multivariate logistic regression analysis showed that high CD8+ expression, low Foxp3+ expression, and high tumor grade were significantly associated with a positive response to NAC ($p<0.05$) (Table 5). Other factors such as age, menstrual status, TIL status, clinical stage, and HER2/neu status were not significant predictive factors (data not shown).

Discussion

In this study, most of the subjects with stage III breast carcinoma were 50 years old or younger, with many under 35. The majority were also premenopausal. This is different from developed countries, where breast cancer tends to occur in older women, with most patients being over 50 and many over 65.(21) Early-onset breast cancer is often linked to internal factors, especially when diagnosis is delayed and the cancer is already in an advanced stage, leading to a poorer prognosis.(22) Risk factors for early-onset breast cancer include family history, early menarche, radiation exposure, and oral contraceptive use.(23) Breast cancer in young women is usually more aggressive, estrogen receptor-negative, poorly differentiated, and shows high p53 and Ki-67 expression.

All subjects in this study were married, and many had completed high school or higher education. However,

Table 2. IHC results for $CD8^+$, $Foxp3^+$, and NAC response.

Variable	Frequency	Proportion (%)
High $CD8^+$	16	26.7
Low $CD8^+$	44	73.3
High $Foxp3^+$	14	23.3
Low $Foxp3^+$	46	76.7
Positive NAC Response	30	50.0
Negative NAC Response	30	50.0

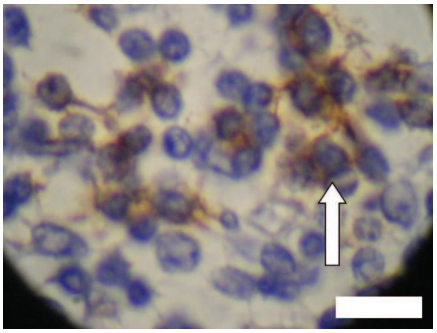

Figure 1. CD8+ T lymphocytes detected on the cell membrane of infiltrating immune cells in tumor tissue. Positive CD8+ expression appears as brown membrane staining, indicating localization on the cell surface. Immunohistochemical staining was performed using the CD8 RM9116-SO LV monoclonal antibody. White bar = 10 µm.

this did not ensure early medical consultation or treatment compliance. Most subjects first tried alternative treatments, which delayed proper diagnosis and treatment. Lack of health insurance also contributed to these delays. Health education and awareness about breast cancer need to be improved to promote earlier detection.

High-grade tumors are known to have active cell growth and good blood supply, even if there is tissue necrosis. These findings support previous studies.(24) Tumor grade is an important prognostic factor. Higher-grade tumors grow more aggressively and are more likely to recur than lower-grade tumors.(25) Some types of breast cancer, such as tubular, mucinous, and medullary carcinoma, are linked to better outcomes.

Most the subjects in this study were Her2/neu-negative, and Her2/neu overexpression was rare and not statistically significant. Some of those with overexpression responded better to CAF than Cyclophosphamide, Methotrexate, and Fluorouracil (CMF) chemotherapy, showing improved survival.(26) However, Her2/neu overexpression is also connected with more aggressive disease, especially in patients with lymph node involvement.

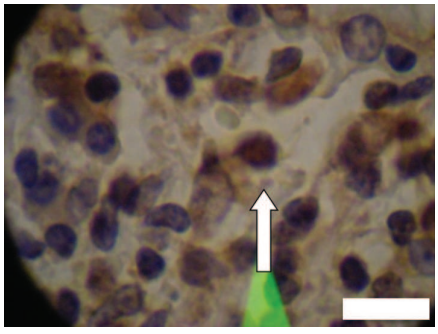

Figure 2. Foxp3+ regulatory T cells identified by nuclear localization in tumor-infiltrating lymphocytes. Positive Foxp3+ expression is indicated by brown staining confined to the cell nuclei. Immunohistochemical staining was performed using the DAKO monoclonal antibody for Foxp3. White bar = 10 µm.

Several factors such as age, menstrual status, tumor type, and tumor grade are believed to influence the response to NAC (NAC). However, most studies, including this one, found no significant relationship between these factors and treatment response.(27–29) These are considered non-regimen-dependent factors.

Although most patients showed positive TILs, they did not significantly affect the NAC response. TILs include various immune cells such as CD4+, CD8+, and Foxp3+. The ratio between CD8+ and Foxp3+ might be more meaningful. A higher number of Foxp3+ cells can suppress immune responses and help tumors avoid detection by the immune system.(30,31) The role of TILs in cancer prognosis has been studied in ovarian, colorectal, and lung cancer. The immune system is believed to support the effectiveness of NAC, especially when using agents like doxorubicin and paclitaxel.(32) After NAC, an increase in CD8+ cells has been seen in patients with complete clinical response. NAC may stimulate the immune system by reducing Foxp3+ cells and enhancing CD8+ activity.(33) In this study, CD8+ positivity was common. A portion of patients had high CD8+ expression, which was associated with a good response to NAC. CD8+ cells attack tumor cells, and NAC can enhance their effect. This agrees with other research showing better outcomes when CD8+ levels are high.

Chemotherapy and radiotherapy have been shown to lower Foxp3+ levels, while CD8+ levels remain unchanged. These treatments also increase immune-stimulating cytokines like interferon (IFN)-γ, interleukin (IL)-4, and IL-2, which are likely triggered by cyclophosphamide.(34) High CD8+ levels were also associated with cancer spread to lymph nodes. Some patients with high CD8+ later had recurrence or metastasis, suggesting that immune response is related to tumor behavior. CD8+ could be useful for predicting prognosis and lymph node involvement.(35)

Foxp3+ was found in several patients, but high levels were less common. Low Foxp3+ expression may suggest weaker immune suppression, although it was not statistically significant. Still, patients with lower Foxp3+ were more likely to respond to NAC (OR=3.250). Foxp3+ can reduce CD8+ activity. Therefore, patients with lower Foxp3+ may have better immune responses against tumors.(36) NAC reduces Foxp3+ and boosts CD8+ function, which may help explain the better outcomes in patients with low Foxp3+.(37)

Table 3. Association of $CD8^+$ expression with NAC response.

$CD8^+$ Status	Positive Response	Negative Response	Total
High $CD8^+$	13 (81.3%)	3 (18.8%)	16
Low $CD8^+$	17 (38.6%)	27 (61.4%)	44
Total	30	30	60

Chi-square (X^2) = 8.523
p-value = 0.004
Odds Ratio (OR) = 6.882
95% CI = 1.707–27.752

Table 4. Association of $Foxp3^+$ expression with NAC response.

$Foxp3^+$ Status	Positive Response	Negative Response	Total
Low $Foxp3^+$	26 (56.5%)	20 (43.5%)	46
High $Foxp3^+$	4 (28.6%)	10 (71.4%)	14
Total	30	30	60

Chi-square (X^2) = 3.354
p-value = 0.067
Odds Ratio (OR) = 3.250
95% CI = 0.888–11.889

Patients with low $Foxp3^+$ before and after NAC had better recurrence-free survival than those with high levels. This means $Foxp3^+$ could be used to predict treatment response. (38) In patients with a complete pathological response, $Foxp3^+$ decreased and $CD8^+$ increased after NAC.(39) Similar findings were seen in ovarian cancer, where NAC reduced $Foxp3^+$ and increased $CD8^+$ cells due to immune reactions triggered by dying tumor cells.(40)

In this study, logistic regression analysis results showed that patients with a combination of high $CD8^+$, low $Foxp3^+$, and high-grade tumors had a significantly better response to CAF or CEF chemotherapy. This combination of markers can help predict which patients are likely to benefit from NAC.

In this study, the tumor response was measured using calipers, which only provide two-dimensional data. Ideally, MRI should be used for more accurate measurement. The $Foxp3^+$ IHC test was done for the first time and required several antibody dilutions to get clear results. Further research is recommended to validate the predictive value of $CD8^+$ and $Foxp3^+$ expression for response to NAC in a larger, multi-center cohort using more advanced and quantitative immunological assays. Future studies should consider incorporating flow cytometry or real-time PCR for more precise measurement of immune markers. Additionally, longitudinal studies examining changes in $CD8^+$ and $Foxp3^+$ expression before and after NAC may help clarify their dynamic roles in treatment response. Advanced imaging modalities such as MRI should be utilized for more accurate assessment of tumor response. Given the observed immune involvement, it would also be beneficial to explore the potential synergistic effects of combining NAC with immunotherapeutic agents, particularly in patients with high $CD8^+$ and low $Foxp3^+$ profiles.

Conclusion

The results of this study showed that high $CD8^+$ T cell infiltration and high histopathological grade were significantly associated with a favorable response to NAC in stage III breast carcinoma. Although low $Foxp3^+$ expression showed a trend toward better response, the association was not statistically significant. The combination of high $CD8^+$, low $Foxp3^+$, and high-grade tumors may serve as a useful predictive marker for treatment response. These findings suggest potential biomarkers for optimizing neoadjuvant therapy strategies.

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Table 5. Logistic regression analysis for factors associated with NAC response.

Variable	B	SE	Wald	df	Sig. (p)	Exp(B)	95% CI for Exp(B)
High $CD8^+$	2.675	0.892	8.995	1	0.003	14.511	2.527–83.346
Low $Foxp3^+$	1.984	0.919	4.667	1	0.031	7.274	1.202–44.022
High Grade	1.544	0.667	5.355	1	0.021	4.684	1.267–17.321
Constant	-2.723	0.960	8.040	1	0.005	0.066	

Authors Contribution

INWTY and IWS contributed to the conceptualization and planning of the research. IWN and NPS conducted data acquisition and sample collection. IMJ and IWPSY performed data calculations and statistical analysis. INWTY drafted the manuscript. IBTWM contributed to data interpretation and critical revision. I.B.M.S. provided technical assistance and critical input. All authors read and approved the final manuscript.

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