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Content

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REVIEW ARTICLE

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Total and Intratumoral CD8+ T Cell Expressions are Correlated with Miller Payne Grading and WHO Clinical Response of Neoadjuvant Chemotherapy

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Abstract

BACKGROUND: Chemotherapy has reported to stimulate immune system through direct activation of cluster of differentiation (CD)8+ T cells. Neoadjuvant chemotherapy (NAC) is known to improve the clinical response of locally advanced breast cancer (LABC) patients. However, the immune response-related factor evaluation of NAC in LABC patients has not been routinely performed. Therefore, current study was conducted to evaluate the correlation of NAC-induced CD8+ T cell with chemotherapy response based on Miller Payne grading and World Health Organization (WHO) criteria.

METHODS: LABC patients were recruited and data regarding age, gender, tumor, nodal stages, histopathological grade, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67 were obtained. Biopsy and mastectomy tissues were collected and processed for hematoxylin-eosin and CD8 immunohistochemical staining. CD8+ T cell expression in peritumoral and intratumoral areas were documented and measured. Clinical responses based on Miller Payne grading and WHO were analyzed and correlated with CD8+ T cell expression.

RESULTS: There were more subjects with high expression of total (80%), intratumoral (82.5%) and peritumoral (65%) CD8+ T cell expressions. The total (p=0.013) and intratumoral (p=0.015) CD8+ T cell expression, but not peritumoral CD8+ T cell expression, were significantly correlated with Miller Payne Grading. The total (p=0.009) and intratumoral (p=0.001) CD8+ T cell expressions were also significantly correlated with WHO clinical response.

CONCLUSION: Total and intratumoral CD8+ T cell expressions are correlated with Miller Payne grading and WHO clinical response of NAC. Therefore, total and intratumoral CD8+ T cell expressions could be suggested as a predictive marker for clinical response of NAC.

KEYWORDS: breast cancer, neoadjuvant chemotherapy, CD8, clinical response, Miller Payne, intratumoral, peritumoral

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Introduction

Based on data of Global Cancer Observatory in 2020, breast cancer is the most prevalent type of cancer among female in the world, with incidence of more than two million cases annually, and predicted to keep increasing each year.(1-4) Breast cancer is the most prevalent cause of death in women globally, responsible for 15% mortality rate worldwide, whereas Indonesia is ranked as the country with highest mortality rate due to breast cancer in South East Asia.(5-8) In Indonesia, approximately 57.1% locally advanced breast cancer (LABC) patients seek for treatment. LABC is an invasive breast cancer limited to the breast and regional lymph nodes.(9,10) Conventionally, the standard chemotherapy were done after the surgery. Neoadjuvant chemotherapy (NAC) is proven to be more beneficial by increasing breast conservation rates in the resectable breast cancer cases. With NAC, micro-metastasis can be eradicated, therefore can prevent metastasis. For LABC patients, NAC can improve clinical response up to 70-90%.(11,12)

Conventional Chemotherapy agent has been reported to stimulate immune system to attack cancer cells through direct activation of cluster of differentiation (CD)8^+ T cells that could significantly eliminate tumor cells. T cells have an important role to produce interferon gamma which has cytotoxic effects by inhibiting cell cycles as well as inducing apoptosis and tumoricidal activity. Earlier studies showed that high number of $CD8^+$ T cell was independently correlated with pathological complete response.(13,14)

Precise assessment of certain chemotherapy response can be evaluated through microscopic examination of the residual tumor on surgical resection after chemotherapy. Current evaluation of breast cancer prognosis is limited to biological tumor characteristics such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER)2, and Ki67 expressions. However, clinical response to chemotherapy does not always correlated with those markers, thus additional factors should be considered.(13) Since immune response has been reported to play an essential role in chemotherapy response, assessment of immune response-related factor such as $CD8^+$ T cell (15), could be suggested. However, immune response-related factor evaluation is not routinely performed since it has not been well-established. Therefore current study was conducted to evaluate the correlation of NAC-induced $CD8^+$ T cell with chemotherapy response based on Miller Payne grading and world health organization (WHO).

Methods

Subject Selection and Criteria

LABC patients of Department of Surgery, Faculty of Medicine, Universitas Indonesia and Dr. Cipto Mangunkusumo National Central General Hospital from September 2015 to February 2022, were selected and included for this study based on inclusion and exclusion criteria. The inclusion criteria were LABC patients with age of >18 years old, who received full dose of NAC with anthracycline- or taxane-based regimen, prior to mastectomy. Meanwhile, the exclusion criteria were the patients with bilateral or recurrent breast cancer, different/change/inadequate of therapeutic regimen, incomplete medical record and unavailable paraffin block. The protocol of this study was approved by the Ethical Committee of Faculty of Medicine Universitas Indonesia (No. KET-131/UN2.F1/ETIK/PPM.00.02/2022).

Data and Sample Collection

Subject-related data were collected from medical record for information of age, gender, tumor and nodal stages, histopathological grade, as well as immunohistochemical examinations of ER, PR, HER2 and Ki67. Histopathological grade was examined by anatomic pathologist based on haematoxylin-eosin features and divided into 3 categories; grade 1: well differentiated, grade 2: moderately differentiated and grade 3: poorly differentiated. Meanwhile, immunohistochemical examinations of ER, PR, HER2 and Ki67 were carried out with standard immunohistochemical staining procedures in Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia and Dr. Cipto Mangunkusumo National Central General Hospital, with following primary antibodies: anti-ER (Leica Biosystems, Wetzlar, Germany), anti-PR (Leica Biosystems), anti-HER2 (Diagnostic BioSystems, Pleasanton, CA, USA) and anti-Ki67 (Diagnostic BioSystems) antibodies, respectively.

For CD8 immunohistochemical detection, paraffin blocks of biopsy samples were collected, sliced in 4 μm and processed for immunohistochemical staining. Meanwhile for Miller Payne grading, paraffin blocks of mastectomy samples were collected, sliced in 4 μm and processed for hematoxylin-eosin staining.

CD8 Immunohistochemical Staining and Evaluation

Sliced tissues were placed on coated slides, heated, deparaffinized, rehydrated, blocked with 3% H_2O_2, antigen

retrieved with Tris EDTA pH 9.0 and blocked with protein blocking buffer. CD8 (SP16) rabbit monoclonal antibody (Cell Marque, Rocklin, CA, USA) with dilution of 1:200 was used as the primary antibody. Then Starr Trek universal HRP detection system (Biocare Medical, Pacheco, CA. USA) was applied, followed by 3,3′-diaminobenzidine tetrahydrochloride. Counterstaining was performed with hematoxylin. The slide was then dehydrated and coverslipped with Entellan. For positive control, tonsil tissue was used.

Five fields of each sample were randomly selected under a microscope (BX51, Olympus, Tokyo, Japan) with 400x magnification. $CD8^+$ T cell expression in peritumoral and intratumoral areas of each sample were captured and measured by ImageJ (USA National Institutes of Health, Bethesda, MA, USA). Intratumoral and peritumoral areas were defined as inside and outside areas of the tumor stroma, respectively. Then, the results were divided into two groups, low and high expression, based on each's group cut-off.

Miller Payne Grading

Based on the hematoxylin-eosin histopathological features, samples were graded with Miller Payne Grading (15), by 2 calibrated observers, an anatomic pathologist and a surgical oncologist with <10% inter-observer difference. In this study, Miller Payne grading was categorized into two groups, grade 1 was considered as no response, while grade 2-5 were considered as response group.

WHO Clinical Response

WHO clinical response was categorized based on tumor diameter changes, according to WHO criteria. Progression response: >25% increase in tumor size and/or the appearance of new lesion in other site. Stable response: <50% decrease or ≤25% increase in tumor size. Partial response: ≤50% decrease in in tumor size at least for 4 weeks, no appearance of new lesion or disease progression. Complete response: disappearance of the disease during two different observations conducted not less than 4 weeks apart.(16) In this study, subject chemotherapy responses were collected, analyzed based on the WHO Criteria, and divided into two groups. The partial and complete response were considered as response group, while the progression and stable were considered as no response.

Statistical Analysis

Data analysis was done with SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). The cut-offs of intratumoral, peritumoral, and total expression were calculated by area under curve (AUC) analysis and Youden's Index. Fisher Exact and Mann-Whitney tests were used to analyze independent variables and outcomes, with significancy of $p<0.05$.

Results

Forty LABC subjects were selected. Majority of the subjects were aged ≥40 years old (70%), T4 (87.5%), N0 (42.5%) & N1 (42.5%), invasive histopathological appearance with no special type (82.5%), histopathological grade 2 (60%), luminal B (42.5%), treated with anthracycline-based NAC (60%), ER positive (92.5%), PR positive (55%), HER2 negative (70%) and high Ki67 (67.5%) (Table 1).

Immunohistochemical expression of $CD8^+$ T cell was detected clearly in tonsil tissue (Figure 1A) and breast cancer biopsy (Figure 1B). Based on the AUC analysis and Youden's Index, cut-off for total $CD8^+$ T cell expression was 23.8, with sensitivity of 86.5% and specificity of 100%; cut-off for intratumoral was 6.4, with sensitivity of 89.2% and specificity of 100%; cut-off for peritumoral expression was 14.3, with sensitivity of 67.6% and specificity of 66.7%. By applying the cut-offs, the total, intratumoral and peritumoral immunohistochemical expressions were categorized into low or high expression. Current results showed that there were more subjects with high expression of total (80%), intratumoral (82.5%) and peritumoral (65%) $CD8^+$ T cell expressions (Table 2). Based on Miller Payne grading (Figure 2), mostly subjects were categorized as response (92.5%) (Table 2). Meanwhile based on WHO clinical response, 87.5% of the subjects were categorized as response.

Based on Fisher Exact test, although there was no correlation between total $CD8^+$ T cell expression with age, histopathological grade, immunohistochemical subtype, ER, PR, HER2 and Ki67 (Table 3), the total $CD8^+$ T cell expression was significantly correlated with Miller Payne Grading ($p=0.013$) (Table 4). Intratumoral $CD8^+$ T cell expression, but not peritumoral $CD8^+$ T cell expression, was significantly correlated with Miller Payne Grading ($p=0.015$) as well. When the subject distribution was analyzed, the total ($p=0.006$) and intratumoral ($p=0.004$) $CD8^+$ T cell expressions were significantly correlated with Miller Payne Grading (Table 5).

Clinical responses based on WHO showed similar results with the ones based on Miller Payne Grading. The

Table 1. Subject characteristics (n=40).

Characteristics	n (%)
Age	
≤ 40 years old	12 (30)
≥ 40 years old	28 (70)
Tumor	
T2	1 (2.5)
T3	4 (10)
T4	35 (87.5)
Node	
N0	17 (42.5)
N1	17 (42.5)
N2	3 (7.5)
N3	3 (7.5)
Histopathological Appearance	
Invasive NST	33 (82.5)
Lobular	3 (7.5)
Others	4 (10)
Histopathological Grade	
Grade 1	3 (7.5)
Grade 2	24 (60)
Grade 3	13 (32.5)
Immunohistochemical Subtype	
Luminal A	8 (20)
Luminal B	17 (42.5)
Luminal B & HER2	12 (30)
Triple negative breast cancer	3 (7.5)
NAC	
Taxane-based	16 (40)
Anthracycline-based	24 (60)
ER	
Negative	3 (7.5)
Positive	37 (92.5)
PR	
Negative	18 (45)
Positive	22 (55)
HER2	
Negative	28 (70)
Positive	12 (30)
Ki67	
Low	13 (32.5)
High	27 (67.5)

NST: no special type; NAC: neoadjuvant chemotherapy; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

total (p=0.009) and intratumoral (p=0.001) CD8+ T cell expressions were significantly correlated with WHO clinical response (Table 6). In regards of subject distribution, the total (p=0.003) and intratumoral (p=0.000) CD8+ T cell expressions were significantly correlated with WHO clinical response as well (Table 7).

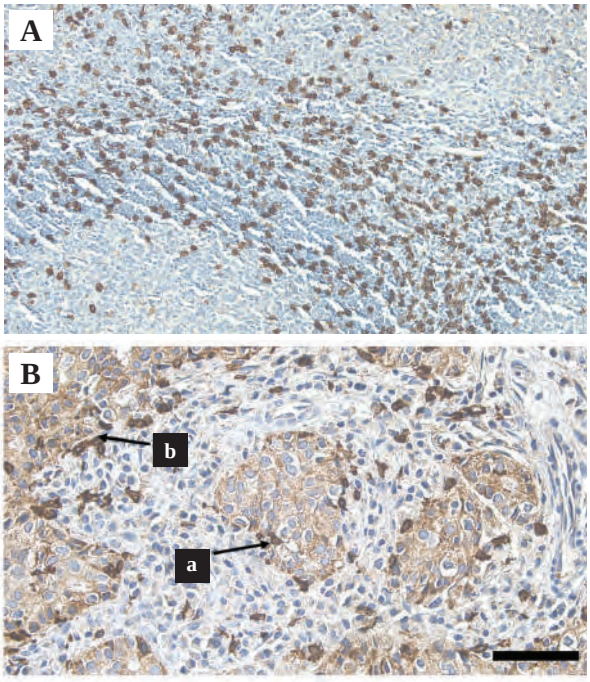

Figure 1. Immunohistochemical expression of CD8. A: tonsil tissue; B: breast cancer biopsy. CD8+ T cells were observed in intratumoral (a) and peritumoral areas (b). Black bar: 100µm.

Discussion

Earlier breast cancer study in Indonesia reported that higher prevalent of female patients in the age of ≥40 than those in the age of <40 (68.9% *vs.* 31.1%). In addition, women in

Table 2. Total, intratumoral and peritumoral CD8+ T cell expression, Miller Payne grading and clinical response subject distribution (n=40).

Characteristics	n (%)
Total CD8+ T Cell Expression	
Low	8 (20)
High	32 (80)
Intratumoral CD8+ T Cell Expression	
Low	7 (17.5)
High	33 (82.5)
Peritumoral CD8+ T Cell Expression	
Low	14 (35)
High	26 (65)
Miller Payne Grading	
No response	3 (7.5)
Response	37 (92.5)
WHO Clinical Response	
No response	5 (12.5)
Response	35 (87.5)

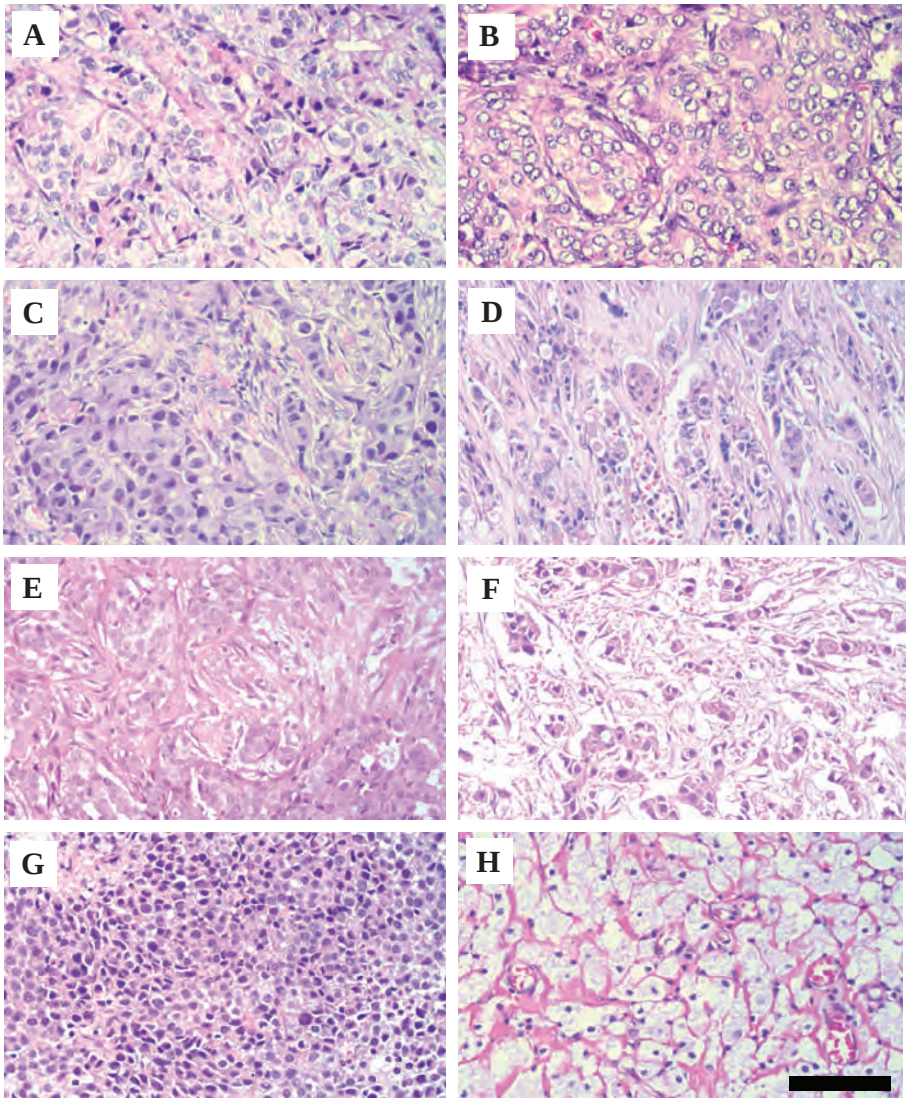

Figure 2. The histopathological expression of biopsy and mastectomy tissue based on Miller Payne grading. Grade 1, from biopsy (a) and mastectomy tissue (b); Grade 2, from biopsy (c) and mastectomy tissue (d); Grade 3, from biopsy (e) and mastectomy tissue (f); Grade 5, from biopsy (g) and mastectomy tissue (h). Black bar: 100 μm.

the age of ≥40 were reported to have an increase of breast cancer risk up to 13.3 times.(17) In the current study, similar population number was included, 70% of the subjects were aged ≥40. Based on the histopathological appearance, most samples of the current study were categorized as invasive carcinoma with no special type (NST) (82.5%), which also has been reported as the most common histopathological appearance of breast cancer in previous reports.(18,19) From the subject characteristics data, majority of subjects had luminal B type (42.5%), which is in accordance with the breast cancer registry data in Indonesia.(18)

In the current study, there was no correlation between $CD8^+$ T cell expression with age, histopathological appearance, histopathological grade, immunohistochemical subtypes, ER, PR, HER2 and Ki67. Factors related to the $CD8^+$ T cell immune profile were found to be multifactorial, including tumor genetics, germline genetics, microbiomes and pharmacological agents.(20,21) However, there were studies reported that $CD8^+$ T cell expression was correlated with higher histopathological grade, triple negative breast cancer subtype, ER negative, tumor grade and size.(22,23)

In the current study, the total $CD8^+$ T cell expressions was significantly correlated with Miller Payne grading and WHO clinical response. This result is in accordance with previous report showing that tumor infiltrating lymphocytes (TIL) was associated with NAC response.(24) In addition, in the current study, intratumoral $CD8^+$ T cell expressions was significantly correlated with Miller Payne grading and WHO clinical response as well. These results supported the recent report suggesting that intratumoral $CD8^+$ was the potential prognostic marker in breast cancer patient, instead

Table 3. Subject characteristics *vs.* total CD8+ expression T cell.

Characteristics	Total CD8+ Expression T Cell		*p- value
	Low n (%)	High n (%)	
Age			
≤40 years old	2 (5)	10 (25)	0.548
>40 years old	6 (15)	22 (55)	
Histopathological Grade			
Low grade	6 (15)	21 (52.5)	0.479
High grade	2 (5)	11 (27.5)	
Immunohistochemical Subtype			
Luminal	8 (20)	29 (72.5)	0.502
Non-Luminal	0 (0)	3 (7.5)	
ER			
Negative	0 (0)	3 (7.5)	0.502
Positive	8 (20)	29 (72.5)	
PR			
Negative	6 (15)	12 (30)	0.065
Positive	2 (5)	20 (50)	
HER2			
Negative	6 (15)	22 (55)	0.548
Positive	2 (5)	10 (25)	
Ki67			
Low	3 (7.5)	10 (25)	0.521
High	5 (12.5)	22 (55)	

*Tested with Fisher Exact test; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

Table 4. Total, intratumoral and peritumoral CD8+ T cell expression *vs.* Miller Payne Grading (no response (n=3) and response (n=37)).

Characteristics	Miller Payne Grading		*p- value
	No Response (Mean±SD)	Response (Mean±SD)	
Total CD8+ T cell expression	15.80±7.27	40.57±20.98	0.013*
Intratumoral CD8+ T cell expression	3.93±2.88	18.56±12.18	0.015*
Peritumoral CD8+ T cell expression	11.86±5.31	22.00±14.08	0.248

*Tested with Mann-Whitney test, significant at $p<0.05$

Table 5. Subject distribution of total, intratumoral and peritumoral low/high CD8+ T cell expression *vs.* Miller Payne Grading (no response (n=3) and response (n=37)).

Characteristics	Miller Payne Grading		*p- value
	No Response n (%)	Response n (%)	
Total CD8+ T Cell Expression			
Low	3 (7.5)	5 (12.5)	0.006*
High	0 (0)	32 (80)	
Intratumoral CD8+ T Cell Expression			
Low	3 (7.5)	4 (10)	0.004*
High	0 (0)	33 (82.5)	
Peritumoral CD8+ T Cell Expression			
Low	2 (5)	12 (30)	0.276
High	1 (2.5)	25 (62.6)	

*Tested with Fisher Exact test, significant at $p<0.05$

Table 6. Total, intratumoral and peritumoral CD8+ T cell expression *vs.* WHO clinical response (no response (n=5) and response (n=35)).

Characteristics	WHO Clinical Response		*p*- value
	No Response (Mean±SD)	Response (Mean±SD)	
Total CD8+ T cell expression	18.92±10.25	41.54±21.01	0.009*
Intratumoral CD8+ T cell expression	4±2.05	19.39±12.00	0.001*
Peritumoral CD8+ T cell expression	14.92±9.77	22.14±14.22	0.357

*Tested with Mann-Whitney test, significant at $p<0.05$

of peritumoral expression.(25) In addition, another study from Indonesia reported that CD8+ might be a predictive factor for clinical response of NAC in breast cancer patients. (13) However, there were also reports suggesting that NAC in breast cancer patients were related with CD8+ T cell expression in both intratumoral and tumor parenchyma, high CD8+ T cell expression in both areas could result in good clinical response.(21) Taken together, current study has strengthened the importance of total and intratumoral CD8+ T cell expressions for achieving good NAC clinical response based on both Miller Payne and WHO. Nevertheless, further long-term observational study with more numbers of study subjects should be conducted.

Conclusion

Total and intratumoral CD8+ T cell expressions are correlated with Miller Payne grading and WHO clinical response of NAC. Therefore, total and intratumoral CD8+ T cell expressions could be suggested as a predictive marker for clinical response of NAC.

Authors Contribution

SSP, SCM, and PR were involved in concepting and planning the research. SSP, SCM, and HH performed the data acquisition/collection. SSP, SCM, HH, and FS conducted the data analysis and interpreted the results. SSP, SCM, and FS edited the manuscript. AK, DJP, PR, and FS designed the figures and tables. All authors took parts in giving critical revision of the manuscript.

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Table 7. Subject distribution of total, intratumoral and peritumoral low/high CD8+ T cell expression *vs.* WHO clinical response (no response (n=5) and response (n=35)).

Characteristics	WHO Clinical Response		*p*- value
	No Response n (%)	Response n (%)	
Total CD8+ T Cell Expression			
Low	4 (10)	4 (10)	0.003*
High	1 (2.5)	31 (77.5)	
Intratumoral CD8+ T Cell Expression			
Low	5 (12.5)	2 (5)	0.000*
High	0 (0)	33 (82.5)	
Peritumoral CD8+ T Cell Expression			
Low	3 (7.5)	11 (27.5)	0.222
High	2 (5)	24 (60)	

*Tested with Fisher Exact test, significant at $p<0.05$

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RESEARCH ARTICLE

Total and Intratumoral CD8+ T Cell Expressions are Correlated with Miller Payne Grading and WHO Clinical Response of Neoadjuvant Chemotherapy

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Abstract

BACKGROUND: Chemotherapy has reported to stimulate immune system through direct activation of cluster of differentiation (CD)8+ T cells. Neoadjuvant chemotherapy (NAC) is known to improve the clinical response of locally advanced breast cancer (LABC) patients. However, the immune response-related factor evaluation of NAC in LABC patients has not been routinely performed. Therefore, current study was conducted to evaluate the correlation of NAC-induced CD8+ T cell with chemotherapy response based on Miller Payne grading and World Health Organization (WHO) criteria.

METHODS: LABC patients were recruited and data regarding age, gender, tumor, nodal stages, histopathological grade, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67 were obtained. Biopsy and mastectomy tissues were collected and processed for hematoxylin-eosin and CD8 immunohistochemical staining. CD8+ T cell expression in peritumoral and intratumoral areas were documented and measured. Clinical responses based on Miller Payne grading and WHO were analyzed and correlated with CD8+ T cell expression.

RESULTS: There were more subjects with high expression of total (80%), intratumoral (82.5%) and peritumoral (65%) CD8+ T cell expressions. The total (p=0.013) and intratumoral (p=0.015) CD8+ T cell expression, but not peritumoral CD8+ T cell expression, were significantly correlated with Miller Payne Grading. The total (p=0.009) and intratumoral (p=0.001) CD8+ T cell expressions were also significantly correlated with WHO clinical response.

CONCLUSION: Total and intratumoral CD8+ T cell expressions are correlated with Miller Payne grading and WHO clinical response of NAC. Therefore, total and intratumoral CD8+ T cell expressions could be suggested as a predictive marker for clinical response of NAC.

KEYWORDS: breast cancer, neoadjuvant chemotherapy, CD8, clinical response, Miller Payne, intratumoral, peritumoral

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RESULTS: There were more subjects with high expression of total (80%), intratumoral (82.5%) and peritumoral (65%) CD8+ T cell expressions. The total (p=0.013) and intratumoral (p=0.015) CD8+ T cell expression, but not peritumoral CD8+ T cell expression, were significantly correlated with Miller Payne Grading. The total (p=0.009) and intratumoral (p=0.001) CD8+ T cell expressions were also significantly correlated with WHO clinical response.

CONCLUSION: Total and intratumoral CD8+ T cell expressions are correlated with Miller Payne grading and WHO clinical response of NAC. Therefore, total and intratumoral CD8+ T cell expressions could be suggested as a predictive marker for clinical response of NAC.

KEYWORDS: breast cancer, neoadjuvant chemotherapy, CD8, clinical response, Miller Payne, intratumoral, peritumoral

Indones Biomed J. 2023; 15(2): 171-8

Introduction

Based on data of Global Cancer Observatory in 2020, breast cancer is the most prevalent type of cancer among female in the world, with incidence of more than two million cases annually, and predicted to keep increasing each year.(1-4) Breast cancer is the most prevalent cause of death in women globally, responsible for 15% mortality rate worldwide, whereas Indonesia is ranked as the country with highest mortality rate due to breast cancer in South East Asia.(5-8) In Indonesia, approximately 57.1% locally advanced breast cancer (LABC) patients seek for treatment. LABC is an invasive breast cancer limited to the breast and regional lymph nodes.(9,10) Conventionally, the standard chemotherapy were done after the surgery. Neoadjuvant chemotherapy (NAC) is proven to be more beneficial by increasing breast conservation rates in the resectable breast cancer cases. With NAC, micro-metastasis can be eradicated, therefore can prevent metastasis. For LABC patients, NAC can improve clinical response up to 70-90%.(11,12)

Conventional Chemotherapy agent has been reported to stimulate immune system to attack cancer cells through direct activation of cluster of differentiation (CD)8$^+$ T cells that could significantly eliminate tumor cells. T cells have an important role to produce interferon gamma which has cytotoxic effects by inhibiting cell cycles as well as inducing apoptosis and tumoricidal activity. Earlier studies showed that high number of CD8$^+$ T cell was independently correlated with pathological complete response.(13,14)

Precise assessment of certain chemotherapy response can be evaluated through microscopic examination of the residual tumor on surgical resection after chemotherapy. Current evaluation of breast cancer prognosis is limited to biological tumor characteristics such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER)2, and Ki67 expressions. However, clinical response to chemotherapy does not always correlated with those markers, thus additional factors should be considered.(13) Since immune response has been reported to play an essential role in chemotherapy response, assessment of immune response-related factor such as CD8$^+$ T cell (15), could be suggested. However, immune response-related factor evaluation is not routinely performed since it has not been well-established. Therefore current study was conducted to evaluate the correlation of NAC-induced CD8$^+$ T cell with chemotherapy response based on Miller Payne grading and world health organization (WHO).

Methods

Subject Selection and Criteria

LABC patients of Department of Surgery, Faculty of Medicine, Universitas Indonesia and Dr. Cipto Mangunkusumo National Central General Hospital from September 2015 to February 2022, were selected and included for this study based on inclusion and exclusion criteria. The inclusion criteria were LABC patients with age of >18 years old, who received full dose of NAC with anthracycline- or taxane-based regimen, prior to mastectomy. Meanwhile, the exclusion criteria were the patients with bilateral or recurrent breast cancer, different/change/inadequate of therapeutic regimen, incomplete medical record and unavailable paraffin block. The protocol of this study was approved by the Ethical Committee of Faculty of Medicine Universitas Indonesia (No. KET-131/UN2.F1/ETIK/PPM.00.02/2022).

Data and Sample Collection

Subject-related data were collected from medical record for information of age, gender, tumor and nodal stages, histopathological grade, as well as immunohistochemical examinations of ER, PR, HER2 and Ki67. Histopathological grade was examined by anatomic pathologist based on haematoxylin-eosin features and divided into 3 categories; grade 1: well differentiated, grade 2: moderately differentiated and grade 3: poorly differentiated. Meanwhile, immunohistochemical examinations of ER, PR, HER2 and Ki67 were carried out with standard immunohistochemical staining procedures in Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia and Dr. Cipto Mangunkusumo National Central General Hospital, with following primary antibodies: anti-ER (Leica Biosystems, Wetzlar, Germany), anti-PR (Leica Biosystems), anti-HER2 (Diagnostic BioSystems, Pleasanton, CA, USA) and anti-Ki67 (Diagnostic BioSystems) antibodies, respectively.

For CD8 immunohistochemical detection, paraffin blocks of biopsy samples were collected, sliced in 4 µm and processed for immunohistochemical staining. Meanwhile for Miller Payne grading, paraffin blocks of mastectomy samples were collected, sliced in 4 µm and processed for hematoxylin-eosin staining.

CD8 Immunohistochemical Staining and Evaluation

Sliced tissues were placed on coated slides, heated, deparaffinized, rehydrated, blocked with 3% H_2O_2, antigen

retrieved with Tris EDTA pH 9.0 and blocked with protein blocking buffer. CD8 (SP16) rabbit monoclonal antibody (Cell Marque, Rocklin, CA, USA) with dilution of 1:200 was used as the primary antibody. Then Starr Trek universal HRP detection system (Biocare Medical, Pacheco, CA. USA) was applied, followed by 3,3′-diaminobenzidine tetrahydrochloride. Counterstaining was performed with hematoxylin. The slide was then dehydrated and coverslipped with Entellan. For positive control, tonsil tissue was used.

Five fields of each sample were randomly selected under a microscope (BX51, Olympus, Tokyo, Japan) with 400x magnification. $CD8^+$ T cell expression in peritumoral and intratumoral areas of each sample were captured and measured by ImageJ (USA National Institutes of Health, Bethesda, MA, USA). Intratumoral and peritumoral areas were defined as inside and outside areas of the tumor stroma, respectively. Then, the results were divided into two groups, low and high expression, based on each's group cut-off.

Miller Payne Grading

Based on the hematoxylin-eosin histopathological features, samples were graded with Miller Payne Grading (15), by 2 calibrated observers, an anatomic pathologist and a surgical oncologist with <10% inter-observer difference. In this study, Miller Payne grading was categorized into two groups, grade 1 was considered as no response, while grade 2-5 were considered as response group.

WHO Clinical Response

WHO clinical response was categorized based on tumor diameter changes, according to WHO criteria. Progression response: >25% increase in tumor size and/or the appearance of new lesion in other site. Stable response: <50% decrease or ≤25% increase in tumor size. Partial response: ≤50% decrease in in tumor size at least for 4 weeks, no appearance of new lesion or disease progression. Complete response: disappearance of the disease during two different observations conducted not less than 4 weeks apart.(16) In this study, subject chemotherapy responses were collected, analyzed based on the WHO Criteria, and divided into two groups. The partial and complete response were considered as response group, while the progression and stable were considered as no response.

Statistical Analysis

Data analysis was done with SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). The cut-offs of intratumoral, peritumoral, and total expression were calculated by area under curve (AUC) analysis and Youden's Index. Fisher Exact and Mann-Whitney tests were used to analyze independent variables and outcomes, with significancy of $p<0.05$.

Results

Forty LABC subjects were selected. Majority of the subjects were aged ≥40 years old (70%), T4 (87.5%), N0 (42.5%) & N1 (42.5%), invasive histopathological appearance with no special type (82.5%), histopathological grade 2 (60%), luminal B (42.5%), treated with anthracycline-based NAC (60%), ER positive (92.5%), PR positive (55%), HER2 negative (70%) and high Ki67 (67.5%) (Table 1).

Immunohistochemical expression of $CD8^+$ T cell was detected clearly in tonsil tissue (Figure 1A) and breast cancer biopsy (Figure 1B). Based on the AUC analysis and Youden's Index, cut-off for total $CD8^+$ T cell expression was 23.8, with sensitivity of 86.5% and specificity of 100%; cut-off for intratumoral was 6.4, with sensitivity of 89.2% and specificity of 100%; cut-off for peritumoral expression was 14.3, with sensitivity of 67.6% and specificity of 66.7%. By applying the cut-offs, the total, intratumoral and peritumoral immunohistochemical expressions were categorized into low or high expression. Current results showed that there were more subjects with high expression of total (80%), intratumoral (82.5%) and peritumoral (65%) $CD8^+$ T cell expressions (Table 2). Based on Miller Payne grading (Figure 2), mostly subjects were categorized as response (92.5%) (Table 2). Meanwhile based on WHO clinical response, 87.5% of the subjects were categorized as response.

Based on Fisher Exact test, although there was no correlation between total $CD8^+$ T cell expression with age, histopathological grade, immunohistochemical subtype, ER, PR, HER2 and Ki67 (Table 3), the total $CD8^+$ T cell expression was significantly correlated with Miller Payne Grading (p=0.013) (Table 4). Intratumoral $CD8^+$ T cell expression, but not peritumoral $CD8^+$ T cell expression, was significantly correlated with Miller Payne Grading (p=0.015) as well. When the subject distribution was analyzed, the total (p=0.006) and intratumoral (p=0.004) $CD8^+$ T cell expressions were significantly correlated with Miller Payne Grading (Table 5).

Clinical responses based on WHO showed similar results with the ones based on Miller Payne Grading. The

Table 1. Subject characteristics (n=40).

Characteristics	n (%)
Age	
≤ 40 years old	12 (30)
≥ 40 years old	28 (70)
Tumor	
T2	1 (2.5)
T3	4 (10)
T4	35 (87.5)
Node	
N0	17 (42.5)
N1	17 (42.5)
N2	3 (7.5)
N3	3 (7.5)
Histopathological Appearance	
Invasive NST	33 (82.5)
Lobular	3 (7.5)
Others	4 (10)
Histopathological Grade	
Grade 1	3 (7.5)
Grade 2	24 (60)
Grade 3	13 (32.5)
Immunohistochemical Subtype	
Luminal A	8 (20)
Luminal B	17 (42.5)
Luminal B & HER2	12 (30)
Triple negative breast cancer	3 (7.5)
NAC	
Taxane-based	16 (40)
Anthracycline-based	24 (60)
ER	
Negative	3 (7.5)
Positive	37 (92.5)
PR	
Negative	18 (45)
Positive	22 (55)
HER2	
Negative	28 (70)
Positive	12 (30)
Ki67	
Low	13 (32.5)
High	27 (67.5)

NST: no special type; NAC: neoadjuvant chemotherapy; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

total (p=0.009) and intratumoral (p=0.001) CD8+ T cell expressions were significantly correlated with WHO clinical response (Table 6). In regards of subject distribution, the total (p=0.003) and intratumoral (p=0.000) CD8+ T cell expressions were significantly correlated with WHO clinical response as well (Table 7).

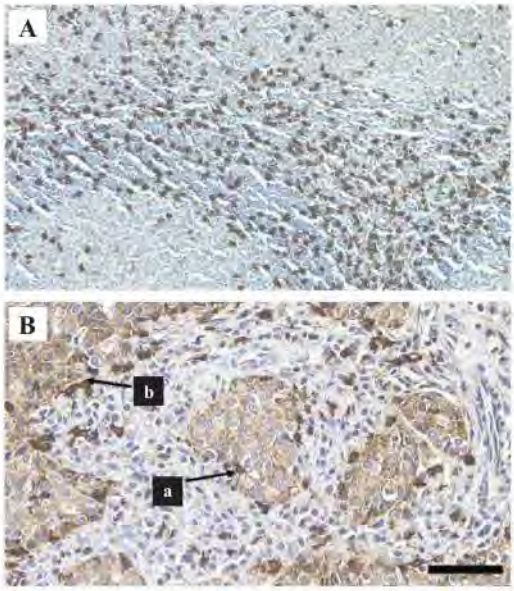

Figure 1. Immunohistochemical expression of CD8. A: tonsil tissue; B: breast cancer biopsy. CD8+ T cells were observed in intratumoral (a) and peritumoral areas (b). Black bar: 100µm.

Discussion

Earlier breast cancer study in Indonesia reported that higher prevalent of female patients in the age of ≥40 than those in the age of <40 (68.9% *vs.* 31.1%). In addition, women in

Table 2. Total, intratumoral and peritumoral CD8+ T cell expression, Miller Payne grading and clinical response subject distribution (n=40).

Characteristics	n (%)
Total CD8+ T Cell Expression	
Low	8 (20)
High	32 (80)
Intratumoral CD8+ T Cell Expression	
Low	7 (17.5)
High	33 (82.5)
Peritumoral CD8+ T Cell Expression	
Low	14 (35)
High	26 (65)
Miller Payne Grading	
No response	3 (7.5)
Response	37 (92.5)
WHO Clinical Response	
No response	5 (12.5)
Response	35 (87.5)

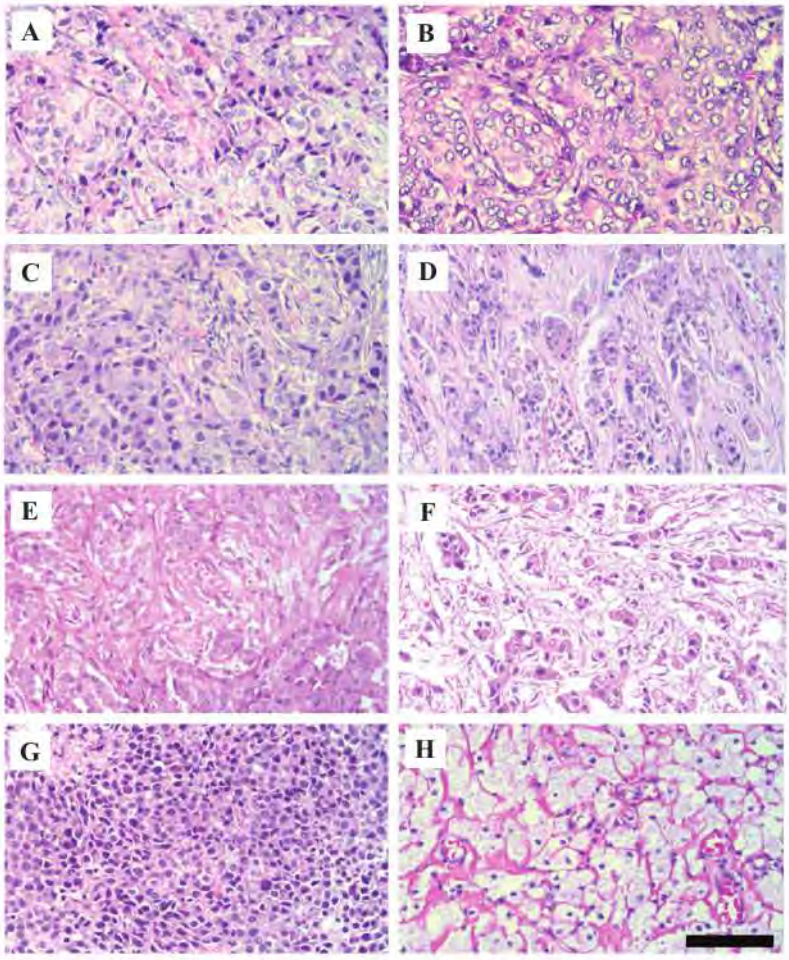

Figure 2. The histopathological expression of biopsy and mastectomy tissue based on Miller Payne grading. Grade 1, from biopsy (a) and mastectomy tissue (b); Grade 2, from biopsy (c) and mastectomy tissue (d); Grade 3, from biopsy (e) and mastectomy tissue (f); Grade 5, from biopsy (g) and mastectomy tissue (h). Black bar: 100 µm.

the age of ≥40 were reported to have an increase of breast cancer risk up to 13.3 times.(17) In the current study, similar population number was included, 70% of the subjects were aged ≥40. Based on the histopathological appearance, most samples of the current study were categorized as invasive carcinoma with no special type (NST) (82.5%), which also has been reported as the most common histopathological appearance of breast cancer in previous reports.(18,19) From the subject characteristics data, majority of subjects had luminal B type (42.5%), which is in accordance with the breast cancer registry data in Indonesia.(18)

In the current study, there was no correlation between $CD8^+$ T cell expression with age, histopathological appearance, histopathological grade, immunohistochemical subtypes, ER, PR, HER2 and Ki67. Factors related to the $CD8^+$ T cell immune profile were found to be multifactorial, including tumor genetics, germline genetics, microbiomes and pharmacological agents.(20,21) However, there were studies reported that $CD8^+$ T cell expression was correlated with higher histopathological grade, triple negative breast cancer subtype, ER negative, tumor grade and size.(22,23)

In the current study, the total $CD8^+$ T cell expressions was significantly correlated with Miller Payne grading and WHO clinical response. This result is in accordance with previous report showing that tumor infiltrating lymphocytes (TIL) was associated with NAC response.(24) In addition, in the current study, intratumoral $CD8^+$ T cell expressions was significantly correlated with Miller Payne grading and WHO clinical response as well. These results supported the recent report suggesting that intratumoral $CD8^+$ was the potential prognostic marker in breast cancer patient, instead

Table 3. Subject characteristics *vs.* total CD8$^+$ expression T cell.

Characteristics	Total CD8$^+$ Expression T Cell		*p*- value
	Low n (%)	High n (%)	
Age			
≤40 years old	2 (5)	10 (25)	0.548
>40 years old	6 (15)	22 (55)	
Histopathological Grade			
Low grade	6 (15)	21 (52.5)	0.479
High grade	2 (5)	11 (27.5)	
Immunohistochemical Subtype			
Luminal	8 (20)	29 (72.5)	0.502
Non-Luminal	0 (0)	3 (7.5)	
ER			
Negative	0 (0)	3 (7.5)	0.502
Positive	8 (20)	29 (72.5)	
PR			
Negative	6 (15)	12 (30)	0.065
Positive	2 (5)	20 (50)	
HER2			
Negative	6 (15)	22 (55)	0.548
Positive	2 (5)	10 (25)	
Ki67			
Low	3 (7.5)	10 (25)	0.521
High	5 (12.5)	22 (55)	

*Tested with Fisher Exact test; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

Table 4. Total, intratumoral and peritumoral CD8$^+$ T cell expression *vs.* Miller Payne Grading (no response (n=3) and response (n=37)).

Characteristics	Miller Payne Grading		*p*- value
	No Response (Mean±SD)	Response (Mean±SD)	
Total CD8$^+$ T cell expression	15.80±7.27	40.57±20.98	0.013*
Intratumoral CD8$^+$ T cell expression	3.93±2.88	18.56±12.18	0.015*
Peritumoral CD8$^+$ T cell expression	11.86±5.31	22.00±14.08	0.248

*Tested with Mann-Whitney test, significant at $p<0.05$

Table 5. Subject distribution of total, intratumoral and peritumoral low/high CD8$^+$ T cell expression *vs.* Miller Payne Grading (no response (n=3) and response (n=37)).

Characteristics	Miller Payne Grading		*p*- value
	No Response n (%)	Response n (%)	
Total CD8$^+$ T Cell Expression			
Low	3 (7.5)	5 (12.5)	0.006*
High	0 (0)	32 (80)	
Intratumoral CD8$^+$ T Cell Expression			
Low	3 (7.5)	4 (10)	0.004*
High	0 (0)	33 (82.5)	
Peritumoral CD8$^+$ T Cell Expression			
Low	2 (5)	12 (30)	0.276
High	1 (2.5)	25 (62.6)	

*Tested with Fisher Exact test, significant at $p<0.05$

Table 6. Total, intratumoral and peritumoral CD8⁺ T cell expression *vs.* WHO clinical response (no response (n=5) and response (n=35)).

Characteristics	WHO Clinical Response		*p*-value
	No Response (Mean±SD)	Response (Mean±SD)	
Total CD8⁺ T cell expression	18.92±10.25	41.54±21.01	0.009*
Intratumoral CD8⁺ T cell expression	4±2.05	19.39±12.00	0.001*
Peritumoral CD8⁺ T cell expression	14.92±9.77	22.14±14.22	0.357

*Tested with Mann-Whitney test, significant at $p<0.05$

of peritumoral expression.(25) In addition, another study from Indonesia reported that CD8⁺ might be a predictive factor for clinical response of NAC in breast cancer patients. (13) However, there were also reports suggesting that NAC in breast cancer patients were related with CD8⁺ T cell expression in both intratumoral and tumor parenchyma, high CD8⁺ T cell expression in both areas could result in good clinical response.(21) Taken together, current study has strengthened the importance of total and intratumoral CD8⁺ T cell expressions for achieving good NAC clinical response based on both Miller Payne and WHO. Nevertheless, further long-term observational study with more numbers of study subjects should be conducted.

Conclusion

Total and intratumoral CD8+ T cell expressions are correlated with Miller Payne grading and WHO clinical response of NAC. Therefore, total and intratumoral CD8+ T cell expressions could be suggested as a predictive marker for clinical response of NAC.

Authors Contribution

SSP, SCM, and PR were involved in concepting and planning the research. SSP, SCM, and HH performed the data acquisition/collection. SSP, SCM, HH, and FS conducted the data analysis and interpreted the results. SSP, SCM, and FS edited the manuscript. AK, DJP, PR, and FS designed the figures and tables. All authors took parts in giving critical revision of the manuscript.

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Table 7. Subject distribution of total, intratumoral and peritumoral low/high CD8⁺ T cell expression *vs.* WHO clinical response (no response (n=5) and response (n=35)).

Characteristics	WHO Clinical Response		*p*-value
	No Response n (%)	Response n (%)	
Total CD8⁺ T Cell Expression			
Low	4 (10)	4 (10)	0.003*
High	1 (2.5)	31 (77.5)	
Intratumoral CD8⁺ T Cell Expression			
Low	5 (12.5)	2 (5)	0.000*
High	0 (0)	33 (82.5)	
Peritumoral CD8⁺ T Cell Expression			
Low	3 (7.5)	11 (27.5)	0.222
High	2 (5)	24 (60)	

*Tested with Fisher Exact test, significant at $p<0.05$

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GRADEMARK REPORT

FINAL GRADE

/15

GENERAL COMMENTS

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UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2022191 Editor Decision Round 1 - Resubmit for Review

Secretariat of InaBJ <secretariatinabj@gmail.com> Tue, Nov 22, 2022 at 9:28 AM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Expression of CD8+ Lymphocytes as a Predictor for Miller Payne's Pathological Response to Neoadjuvant Chemotherapy in Locally Advanced Breast Cancer**".

Our decision is: **Resubmit for Review.**

Find the file attached to see detailed comments from reviewers. Please make sure you read all the comments and revise the manuscript based on the suggestions given.

Revise this manuscript thoroughly before **December 2, 2022**. Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes.

When you are done, you can upload it in: https://inabj.org/index.php/ibj/author/submissionReview/2110, or simply send us an email of your revised manuscript and response letter.

Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,

--
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Manuscript Review Form

Reviewer	:	Reviewer 1
Manuscript #	:	M2022191
Manuscript Title	:	Expression of CD8+ Lymphocytes as a Predictor for Miller Payne's Pathological Response to Neoadjuvant Chemotherapy in Locally Advanced Breast Cancer

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?		√
	Notes: The novelty in this manuscript is not clear since high CD8+ lymphocytes expression has been known to be associated with pathological complete response in other studies and no statement about the novelty by define this CD8+ expression in this study		
2.	Are the title and abstract of the manuscript appropriate?		√
	Notes: No, the title needs revision according to the study main result.		
3	Do the title and abstract reflect the study result/content?		√
	Notes: The title has not reflected the study content. The background in the abstract has not explain about the importance of the study. The method in the abstract was not reflected the main variable (no CD8+ T cell expression explained, but hematoxylin eosin that was not for CD8+ cell expression).		
4.	Is the significance of the study well explained at the Background?		√
	Notes: No, the importance and scientific reasoning to use CD8+ as predictive factor for LABC in this study have not been explained. The problem and research question that trigger the author to conduct this study have not stated clearly		
5.	Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists?		√
	Notes: 1. Study design: There is major error in the methodology in this study. The author aimed to predict the pathological response after therapy, however, the study design was cross		

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	sectional study. Predictor study should use cohort or case control study. There should be a follow up time to be able to predict the outcome. 2. Inclusion criteria: The description of study population that included in this study is not clear related with age limit, the disease staging, location and severity, diagnosis category, pretreatment and after requirement and other requirement that will clarify the representative study population for predictor study. No information regarding the treatment and control study in this predictor study. 3. The research flow has not been mentioned clearly in this study from the patient recruitment, diagnosis, laboratory procedure until post therapy analysis. 4. For the laboratory test: a. How did the technical procedure to define the expression of CD8+ T cell in parafilm block? b. What are the operational definitions to define intratumoral, peritumoral and total expression? c. How did the cut off for high and low CD8+ expression define? What is the cut off for high and low CD8+ expression? e. How does the author validate that the specimens for CD8+ expression before NAC and after mastectomy are from the same area in the tumor and from the same sample origin?		
6.	Are the results, ideas, and data presented in this manuscript important enough for publication?		√
	Notes: The results, ideas, and data presented in this study are not in line with the aim of the study which is predicting the pathological response. The study tends to be overclaimed and using inappropriate statistical analysis to claim the evidence. The ROC analysis, sensitivity and specificity data should be for diagnostic study but were used in predictor study, so this is analysis error. The association between variables that shown by the independent t-test is for descriptive study. If there are significant results, it is shown that there were an association between those variables but not strong enough to claim that these significant results can be used to predict the outcome.		
7.	Are all figures and tables necessarily presented?		√
	Notes: The data presented in the results as figures and tables are basically for descriptive study, not predictor study.		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?		√
	Notes: Since there are major problem since research design, the major problem follow till discussion		
9.	Are the conclusions and interpretations valid and supported by the data?		√
	Notes: The author is overclaimed the study results and conclusion. The descriptive results were used to claim predictive results.		
10.	Is the manuscript clear, comprehensible, and written in a good English		√

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	structure?		
	Notes: The English structure is acceptable but the writing is not clear enough and not yet comprehensive		

Specific Reviewer's Comments and Suggestions:

(These comments may be in addition to or in lieu of reviewer comments inserted into the text of the manuscript. Use as many lines as needed.)

This study can be a good study if the study use a proper study design, research methodology, in line with study aim, proper statistical analysis and proper evidence to claim the study results as predictor study.

Reviewer's Recommendation (Please tick only one option)	
Accept Submission (No significant alterations suggested)	
Revisions Required (Suggest changes to the manuscript as specified in this review)	
Resubmit for Review (Major revisions should be made and suggestions as specified in this review must be addressed. Revised manuscript should be resubmitted to the reviewer for further review)	√
Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	

Further Reviewer's Comments Regarding Disposition of the Manuscript:

Date and Sign:
November 20th, 2022

Reviewer 1

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Manuscript Review Form

Reviewer	:	Reviewer 2
Manuscript #	:	M2022191
Manuscript Title	:	**Expression of CD8+ Lymphocytes as a Predictor for Miller Payne's Pathological Response to Neoadjuvant Chemotherapy in Locally Advanced Breast Cancer**

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?	**x**	
	Notes:		
2.	Are the title and abstract of the manuscript appropriate?	x	
	Notes:		
3	Do the title and abstract reflect the study result/content?	x	
	Notes:		
4.	Is the significance of the study well explained at the Background?		x
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5.	Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists?	x	
	Notes:		

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6.	Are the results, ideas, and data presented in this manuscript important enough for publication?	x	
	Notes:		
7.	Are all figures and tables necessarily presented?	x	
	Notes:		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?	x	
	Notes:		
9.	Are the conclusions and interpretations valid and supported by the data?	x	
	Notes:		
10.	Is the manuscript clear, comprehensible, and written in a good English structure?	x	
	Notes:		

Specific Reviewer's Comments and Suggestions:

(These comments may be in addition to or in lieu of reviewer comments inserted into the text of the manuscript. Use as many lines as needed.)

Attached in the manuscript

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Reviewer's Recommendation (Please tick only one option)	√
Accept Submission (No significant alterations suggested)	
Revisions Required (Suggest changes to the manuscript as specified in this review)	√
Resubmit for Review (Major revisions should be made and suggestions as specified in this review must be addressed. Revised manuscript should be resubmitted to the reviewer for further review)	
Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	

Further Reviewer's Comments Regarding Disposition of the Manuscript:

This is a good written manuscript, and everything is appropriate.

The study will give quote benefit for the clinicians, and the benefit of this study (compare to current method) is better to be explain clearly in the manuscript.

Date and Sign:
Nov 11, 2022

Reviewer 2

Expression of CD8+ Lymphocytes as a Predictor for Miller Payne's Pathological Response to Neoadjuvant Chemotherapy in Locally Advanced Breast Cancer

*please re-check and revise the highlighted words.

ABSTRACT

Background: More than half breast cancer cases in Indonesia were diagnosed as locally advanced breast cancer (LABC). Neoadjuvant chemotherapy (NAC) is considered as the standard treatment for LABC, which could be further evaluated through clinical and Miller Payne pathological response. This study aims to evaluate CD8+ T cells expression as a predictive factor of NAC using Miller-Payne pathological grading and its association with clinicopathological factors.

Method: This is a cross-sectional study involving LABC patients. Demographic and tumor characteristics of LABC patients were taken from medical record. Haematoxylin and eosin staining was done for biopsy sample before and after NAC to evaluate CD8+ T cells expression intratumoral, peritumoral, trotal and Miller Payne grading. Data were further analysed by Image J imaging analysis and SPSS v.20.

Result: There were 40 LABC patients included in the study, majority were aged above 40 years old (n=28;70%) and had luminal B subtype (n=12;30%). Majority of the patients had high total CD8+ expression (n=32;80%). There were no association between CD8+ expression and clinicopathological factors. Mean difference of total (p=0.013) and intratumoral (p=0.015) CD8+ expression based on Miller Payne grading were statistically significant. Total (p=0.037) and intratumoral (p=0.002) expression were associated with clinical response of NAC.

Conclusion: Total expression and intratumoral of CD8+ expression were associated with Miller payne pathological grading and clinical response of NAC chemotherapy in LABC patients.

Comment [AM1]: Rather than say majority, it will be better to just state the number, as this is a quantitative study.

Comment [AM2]: Check my comment on the conclusion in the end of this manuscript

Keywords: Locally advanced breast cancer, neoadjuvant chemotherapy, CD8+ T cell, Miller Payne pathological response, clinical response,

INTRODUCTION

Data from Global Cancer Observatory (GLOBOCAN) from 2020 reported that breast cancer is the first most prevalent type of cancer among female in the world, with incidence of more than two million cases in women annually, and predicted to keep increasing each year. It is also the most prevalent cause of cancer death in Indonesia. [1-3] In Indonesia, approximately 57.1% breast cancer patients seek for treatment in locally advanced stage. Locally advanced breast cancer (LABC) is an invasive breast cancer limited to the breast and regional lymph nodes.[4,5]

Neoadjuvant chemotherapy (NAC), is the standard treatment for LABC patients which can improve clinical response up to 70-90%.[6,7] In addition to clinical response, pathological response could also be used to evaluate NAC effectiveness, using Miller payne methods. Miller Payne score grading could be used to predict survival rate, higher grades predict better outcome.[8,9]

Tumor-infiltrating lymphocytes (TIL) is the stimulated immune system in response to cancer cells, which consists of mononuclear cells, including T cells (CD4+, CD3+, CD8+), B cells, macrophages, and other immune cells in peritumoral and intratumoral region.[10,11] Recently, more researches proved that TIL played important role in tumorigenesis.

Conventional chemotherapy agents can stimulate immune system to attack cancer cells through direct activation of TIL that could significantly eliminate tumor cells, which is T cells CD8+. It has important role to produce interferon gamma which has cytotoxic effects and to attack tumor cells by inhibiting cell cycles, apoptosis, and inducing tumoricidal activity from macrophages. Earlier studies showed that high T-cell CD8+ was independently associated with

pathological complete response.[12,13] Prior studies also reported that clinical pathology factors that associated with chemotherapy response after NAC, includes tumor grading, molecular subtype, chemotherapy regimen, and CD8+.[10] The aim of the study is to evaluate CD8+ T cells expression as a predictive factor of NAC in LABC patients using Miller Payne pathological grading and the association between CD8+ T cells and breast cancer clinicopathological factors.

Comment [AM3]: Please insert a brief introduction the term when NAC is given and how is the relationship between NAC and conventional therapy?

Comment [AM4]: Can you explain briefly on the benefit using CD8 as the NAC respond predictor compare to current method?

METHODS

Design Study and Sampling

A cross-sectional study is conducted in Department of Surgery, Faculty of Medicine, Universitas Indonesia (FKUI) and Cipto Mangunkusumo Hospital (RSCM) in January to June, 2022. Breast cancer patients from September 2015 to February 2022, were included in this study. Data were collected from patient medial record through consecutive sampling. The inclusion criteria were breast cancer patients who receive full dose NAC before mastectomy were done at RSCM. Anthracycline-based full dose regimen was given for six cycles, while taxane-based regimen was given for six or eight cycles (four cycles of anthracycline and four cycles of taxane). Exclusion criteria were patients with bilateral breast cancer, recurrent breast cancer, patients with changes of regimen during their cycles, not receiving full-dose regimen, prior neoadjuvant hormonal therapy, incomplete medical record, and unavailable paraffin block. This study had been approved Faculty of Medicine Universitas Indonesia Ethical Committee (KET-131/UN2.F1/ETIK/PPM.00.02/2022)

Data Collection

Baseline patient and tumor characteristics data included age, gender, stage, tumor stage, nodal stage, tumor grade, estrogen receptor (ER) status, progesteron (PR) status, Human epidermal

growth factor receptor-2 (HER2) status, Ki67 expression. Clinical response of NAC was categorized based on tumor diameter changes, according to World Health Organization (WHO) criteria. Progression response was defined as a 25% or more increase in total tumor size/and or the appearance of new lesion in other site. Stable response, or no change, was defined as less than 50% decrease of tumor size or increase of tumor size not more than 25%. Partial response was defined as 50% or more decrease in size (volume) at least for four weeks, no appearance of new lesion or disease progression. The disappearance of the disease during two different observations conducted not less than four weeks apart was classified as complete response.[14] In this study, clinical response of NAC was further divided into two groups, partial and complete response were considered as response group, while progression and stable were considered as no response.

Paraffin block samples include biopsy sample before NAC and mastectomy sample after NAC, from Pathology Anatomy Department RSCM. Haematoxylin and eosin (HE) staining was done to the paraffin block with biopsy sample before NAC to evaluate whether it was eligible for further immunohistochemistry evaluation assessing CD8+ T cells expression. A tonsil tissue sample was used as positive control sample. The evaluation of CD8+ T cells was measured on intratumoral, peritumoral and total expression, which was the sum of both area. Then, the results were divided into two groups, low and high expression, based on each's groups cut-offs..

Paraffin block with mastectomy sample post NAC was used to evaluate pathologic response with Miller Payne grading ranged from one to five and described as follows. Grade 1 shows no change to individual malignant cells and no reduction in tumor cellularity; grade 2 shows minimal reduction up to 30% loss of tumour cells; grade 3 shows significant tumor cells reduction after chemotherapy, estimated 30-90% reduction; grade 4 shows minimal tumor cells,

more than 90% loss of tumor cells; grade 5 shows complete response, no appearance of residual tumor cells.[13] In this study, Miller Payne grading was categorized into two groups, grade 1 was considered as no response, while grade 2-5 considered as response group. Few images were captured in different five field of views as the representative area from each sample by Olympus Bx51 microscope with 400x magnification. There were two observers that consists of a surgical oncologist and an anatomical pathologist, to assess the Miller Payne grading, with intraclass coefficient correlation of 91,2% (good), which means interobserver result difference is under 10%. The evaluation of IHC staining was further analysed by Image J image analysis through blinding. Intratumoral, peritumoral, and total expression of CD68+ T cells were reported in semiquantitative results.

Data Analysis

Data analysis were done with SPSS version 20.0. Univariate analysis was used to report demographic characteristics of the subjects. Initially, the cut-offs of intratumoral, peritumoral, and total expression were calculated by receiver operating characteristic (ROC) curve analysis and Youden's Index. Chi-square and Fisher Exact test were used to analyze independent variables and outcome, with $p<0.05$ means statistically significant. Association between CD8+ T cell expression and Miller Payne grading was evaluated with either independent T-test or Mann-Whitney U, considering the data distribution.

RESULTS

There were 40 LABC patients fulfilled the inclusion and exclusion criteria then included to the study analysis. Majority of the subjects were aged above 40 years old (70%), had T4 tumor (87.5%), histopathological grade results in grade 2 (60%), diagnosed as luminal B breast cancer

Comment [AM5]: Please add the min,max,means and SD value in the table when possible, so the reader can get a more complete description on the subjects.

(42.5%), estrogen receptor (ER) positive (92.5%), progesterone receptor (PR) positive (55%), HER2 negative (70%), and received anthracycline based chemotherapy (60%). More details in demographic data were shown in Table 1.

Based on the ROC curve analysis, cut-off for intratumoral CD8+ T cell expression is 6.4, with sensitivity of 89.2% and specificity of 100%; for peritumoral expression is 14.3, with sensitivity of 89.2% and specificity of 100%; and for total expression is 23.8, with sensitivity of 86.5% and specificity 100%. Figure 1 shows the intratumoral and peritumoral of CD8+ T-cell expression. Expression of CD8+ T cell in intratumoral and peritumoral region were found high in 80% (n=32) patients, and 65% (n=26) patients, respectively. Overall, 80% (n=32) patients had high expression of CD8+ T cell. Table 2 explains CD8+ expression regarding chemotherapy response distribution. Based on bivariate analysis, there were no association between CD8+ T cell expression with clinicopathological factors, such as age, histopathology grade, ER, PR status, Ki67, HER2, and breast cancer subtypes. (Table 3) The mean difference in total expression of CD8+ T cell (p=0.013) and intratumoral CD8+ expression (p=0.015) based on Miller Payne grading were found statistically significant. Higher means were found in response group (Table 4). Figure 2 shows the Miller payne grading images under the microscope from biopsy and after NAC from mastectomy samples. Fisher exact analysis showed that CD8+ T cell total expression (p=0.037) and intratumoral expression (p=0.002) had association with clinical response of NAC (Table 5).

DISCUSSION

Study reported that breast cancer patients in Indonesia was the most prevalent in 40-49 years old group. However, in western countries, breast cancers were found mostly after menopause. Earlier

study in Indonesia found higher prevalent of breast cancer in women age 40 and above group rather than below 40 years old (68.9% vs 31.1%), and they had increased risk of breast cancer up to 13.3 times.[17] This finding was similar to this study whereas 70% of the patients were aged 40 and above.

Based on the histopathology type, this study reports invasive carcinoma of no special type (NST) as the most prevalent one with 82.5% cases. In fact, invasive carcinoma NST is the most common histopathology morphology of breast cancer, and earlier studies reported up to 80% cases of invasive carcinoma of NST in LABC patients. [18,19]

On the other hand, the majority of patients had luminal B type (42.5%), based on the IHC subtype. This finding is consistent breast cancer registry data and a retrospective study in Indonesia.[18] Study from Vietnam also reported the percentage of luminal B subtype in 56.5% cases. [20] It was known that IHC subtypes variety might be explained by differences in age, race, and ethnicity.[21] Less than 10% of the subjects had triple negative breast cancer, in which it is a rare subtype of breast cancer that associated with poor response in chemotherapy.[22,23] According to the prior studies, it was concluded that South East Asia was dominated by luminal B subtypes.

This study found that there was no correlation between CD8+ T cell expression and clinicopathological factors, such as age, histopathological grading, immunohistochemistry subtypes (ER, PR, HER2, Ki67), luminal and non-luminal in LABC patients. Factors related to the CD8+ T cell immune profile were found to be multifactorial, including tumor genetics, germline genetics, microbiomes, infection agents, sun exposure, and pharmacological agents.[24,25] However, other studies found that CD8+ T cell expression was associated with higher histopathological grade, triple negative breast cancer subtype, ER negative status, tumor grading,

higher tumor size, and necrosis tumor.[16,26] It was explained that more mutation in tumor results in higher immune response thus increasing CD8+ T cell expression.

The association between CD8+ T cell expression and Miller Payne pathological grading was also supported by other study where TIL could be a positive predictive factor of NAC. Higher CD8+ T cell expression indicates higher stratification of Miller Payne grading.[27] In addition, total and intratumoral CD8+ T cell expression were also found have significant association with both pathological grading and clinical response after NAC. Recent study found that CD8+ intratumoral was the potential prognostic marker in breast cancer patient, instead of peritumoral expression.[28] While other study from Indonesia reported that CD8+ might be a predictive factor for clinical response of NAC in breast cancer patients.[12] From biological aspects, peritumoral expression of CD8+ T cell could protect the host from cancer progression since TIL function had not been impaired. If CD8+ could be found intratumoral, means that the immune system could counter immune escaping from the cancer cells. Chen D, et al. also reported that NAC in breast cancer patients were related to tumor phenotypes which categorized into three immune profiles. Immune-inflamed phenotype marked CD8+ T cell expression both in intratumoral and tumor parenchyma, hence this phenotype results in good clinical response of anti-cancer therapy. However, immune-excluded phenotype were limited in peritumoral expression and immune-dessert phenotype showed lack of expression in intratumoral, peritumoral even tumor stroma. These phenotypes showed limited and no clinical response from anti-cancer therapy, respectively.[23]

Difference in results from this study may be explained by limited sample size, and other predisposing factors that related to CD8+ T cell expression that were not observed. Limitation in this study includes sample size and measurement on the scale bar that couldn't be done directly

Comment [AM6]: Can you add more explanation on this? Why the peritumoral expression doesn't give quite significancy, based on the mechanism.

under the microscope. Further study evaluating CD8+ T cell association with clinical and pathological response should be done to add scientific evidences, with more sample size and other related factors that may affect its expression or comparing to other evaluation system of NAC response, like residual cancer burden methods which assessing based on tumor cells and lymph nodes.

CONCLUSION

There were no association between CD8+ T cell expression and clinicopathological factors and molecular subtypes. Total expression and intratumoral expression of CD8+ T cell were associated with pathological and clinical response of NAC chemotherapy. Thus, CD8+ T cell could be a potential predictive marker of NAC response.

Comment [AM7]: I am disagree with the total expression, since the significant result came from the intratumoral expression.

Comment [AM8]: Thus this need to be clarify as the intratumoral CD8 only.

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285 **Tables and Figures**

286

287 **Table 1. Demographic characteristics of the subjects**

Characteristics	N (%) (N=40)
Age	
≤ 40 years old	12 (30)
> 40 years old	28 (70)
Tumor	
T2	1 (2.5)
T3	4 (10)
T4	35 (87.5)
Nodul	
N0	17 (42.5)
N1	17 (42.5)
N2	3 (7.5)
N3	3 (7.5)
Histopathology	
Invasive NST	33 (82.5)
Lobular	3 (7.5)
Others	4 (10)
Histopathology grade	
Grade 1	3 (7.5)
Grade 2	24 (60)
Grade 3	13 (32.5)
Immunohistochemistry subtype	
Luminal A	8 (20)
Luminal B	17 (42.5)
Luminal B 2 type	12 (30)
Triple negative breast cancer	3 (7.5)
HER2 type	0 (0)
Chemotherapy	
Taxane-based	16 (40)
Anthracycline-based	24 (60)
Estrogen receptor	
Negative	3 (7.5)
Positive	37 (92.5)
Progesteron receptor	
Negative	18 (45)
Positive	22 (55)

HER2 receptor	
Negative	28 (70)
Positive	12 (30)
Ki67 status	
Low	13 (32.5)
High	27 (67.5)

NST, no special type; HER2, human epidermal growth factor receptor 2.

Table 2. CD8+ expression and chemotherapy response distribution

Characteristics	N (%) (N=40)
Total CD8+ expression	
Low	8 (20)
High	32 (80)
Intratumoral CD8+ expression	
Low	7 (17.5)
High	33 (82.5)
Peritumoral CD8+ expression	
Low	14 (35)
High	26 (65)
Miller Payne Grading	
Grade 1	3 (7.5)
Grade 2	15 (37.5)
Grade 3	18 (45)
Grade 4	2 (5)
Grade 5	2 (5)
Chemotherapy clinical response	
Stable	4 (10)
Partial	35 (82.5)
Complete	1 (2.5)

Table 3. Association between clinicopathology parameters and CD8+ T cell expression

Clincopathology parameters	Total CD8+ T Cell expression				*p-value*
	Low (N=8)		High (N=32)		
	N	%	N	%	
Age					
≤40 years old	2	16.7	10	83.3	0.548
>40 years old	6	21.4	22	78.6	
Histopathology grade					
Low grade	6	22.2	21	77.8	0.479
High grade	2	15.4	11	84.6	
Estrogen receptor status					
Negative	0	0	3	100	0.502
Positive	8	21.6	29	78.4	
Progesterone status					
Negative	6	33.3	12	66.7	0.110
Positive	2	9.1	20	90.9	
Ki67					
Low	3	23.1	10	76.9	0.521
High	5	18.5	22	81.5	
HER2					
Negative	6	21.4	22	78.6	0.548
Positive	2	16.7	10	83.3	
IHC subtypes					
Luminal	8	21.6	29	78.4	0.502
Non-Luminal	0	0	3	100	

HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry

Table 4. Mean difference of CD8+ expression based on Miller Payne Grading

CD68+ expression	Miller Payne Grading		*p-value*
	Response (N=37)	No Response (N=3)	
	Mean	Mean	
Total	21.81	4.33	0.013*
Intratumoral	21.78	4.67	0.015*
Peritumoral	21.11	13.00	0.248

***Mann-Whitney test, significant at p-value<0.05*

Table 5. Association of CD8+ expression and chemotherapy clinical response

CD8+ expression	Clinical response				*p-value*
	No response (N=4)		Response (N=36)		
	N	**%**	**N**	**%**	
Total					
Low	3	37.5	5	62.5	0.02*
High	1	3.1	31	96.9	
Intratumoral					
Low	3	42.9	4	57.1	0.013*
High	1	3	32	97	
Peritumoral					
Low	3	21.4	11	78.6	0.115
High	1	15.4	25	84.6	

***Fisher exact test, significant at p-value<0.05*

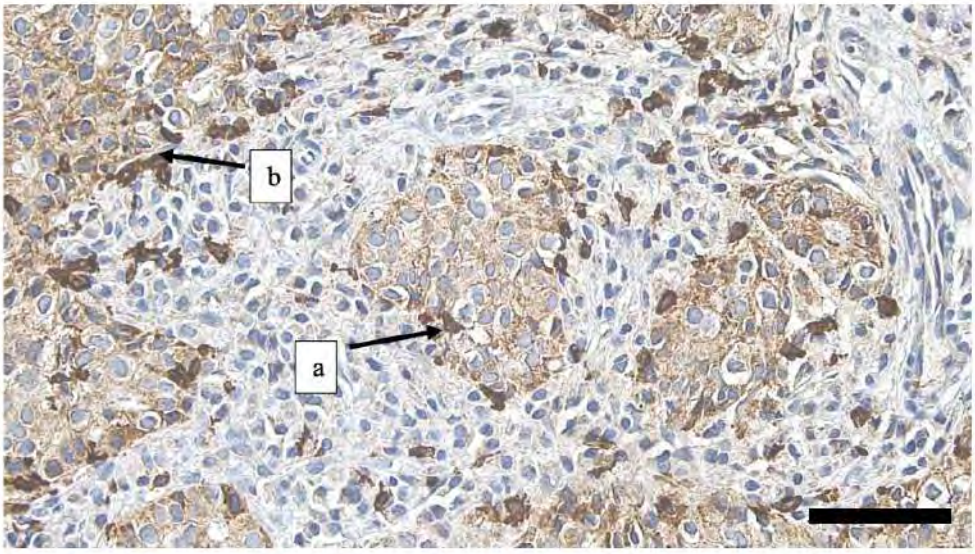

Figure 1. On a biopsy sample of breast cancer with 400x magnification showing (a) Intratumoral and (b) peritumoral expression of CD8+ T cell. Black bar: 100μm

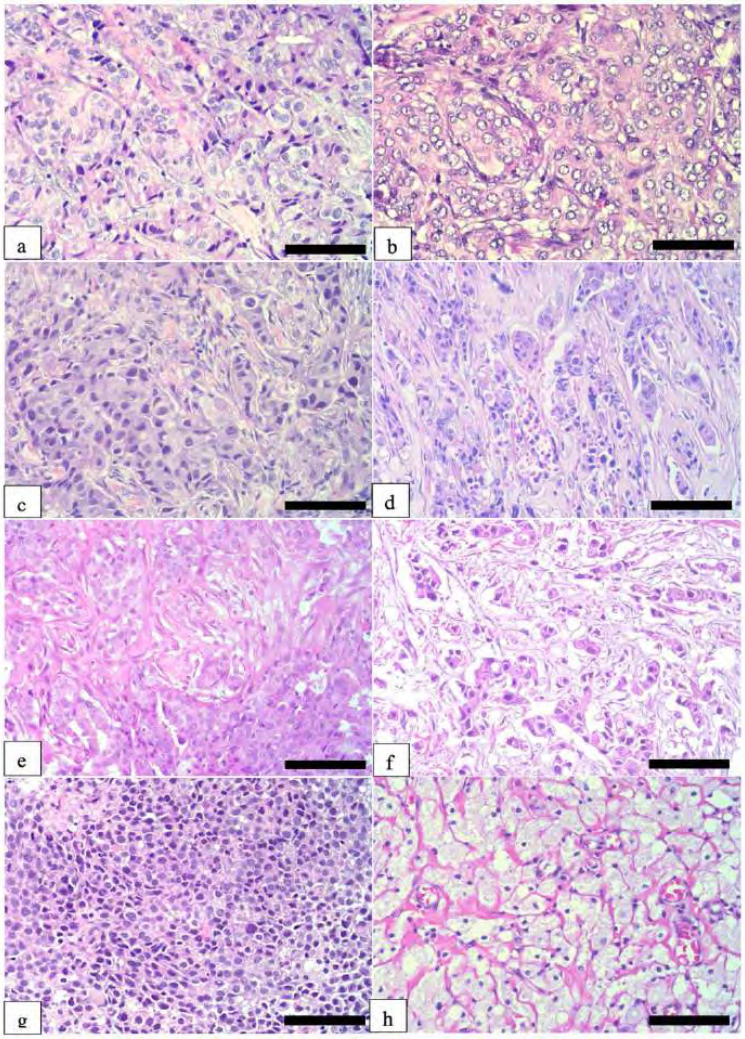

Figure 2. Pathological chemotherapy response based on Miller Payne grading. (a) Grade 1, from biopsy sample; (b) Grade 1, from mastectomy sample; (c) Grade 2, from biopsy sample; (d) Grade 2, from mastectomy sample; (e) Grade 3, from biopsy sample; (f) Grade 3, from mastectomy sample; (g) Grade 5, from biopsy sample; (h) Grade 5, from mastectomy sample. Black bar: 100 μm

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Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2022191 Editor Decision Round 1 - Resubmit for Review

Ferry Sandra <ferry@trisakti.ac.id> Fri, Dec 16, 2022 at 7:13 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Dear Secretariat of The Indonesian Biomedical Journal,

I sincerely apologize for the late response. Thank you for the review results. Please find the revision of manuscript M2022191 as well as the response letter addressing the reviewer's and editor's question.

Thank you.

Regards,
Ferry Sandra
[Quoted text hidden]
--
Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

2 attachments

Round 1 Revision from Author.docx
5160K

Round 1 Response Form from Author.xlsx
16K

The association of CD8+ Lymphocytes expression and Miller Payne's Pathological Response Grading to Neoadjuvant Chemotherapy in Indonesian Locally Advanced Breast Cancer Patients

ABSTRACT

Background: Immune response has an essential role regarding chemotherapy response, which also influence clinical and pathological response. One of the tumor-infiltrating lymphocytes is CD8+ lymphocyte. However, immune expression evaluation has not well-established and routinely done. Hence, this study aims to evaluate the association between CD8+ T cells and Miller-Payne pathological grading and clinicopathological factors.

Method: This is a cross-sectional study involving LABC patients. Demographic and tumor characteristics of LABC patients were taken from medical record. Biopsy sample before and after NAC to evaluate the association between CD8+ T cells expression intratumoral, peritumoral, total with Miller Payne grading and clinicopathological factors. Data were further analysed by Image J imaging analysis and SPSS v.20.

Result: There were 40 LABC patients included in the study, 70% patients were aged above 40 years old (n=28;70%) and had luminal B subtype (n=12;30%). A total of 32 patients (80%) had high total CD8+ expression. No association was found between CD8+ expression and clinicopathological factors. There were association between total (p=0.013) and intratumoral (p=0.015) CD8+ expression based on Miller Payne grading which was statistically significant. Total (p=0.037) and intratumoral (p=0.002) expression were associated with clinical response of NAC.

Comment [i1]: R1 #2: The background in the abstract has not explain about the importance of the study. The method in the abstract was not reflected the main variable (no CD8+ T cell expression explained, but hematoxylin eosin that was not for CD8+ cell expression).

Comment [i2]: Note from editor: This still has not been revised by the author! The reviewer ask the author to:
1. Please add explanation about the importance of the study in the Background of Abstract
2. HE is used for the staining, not to calculate the CD8+ T cell expression, please revise.

Conclusion: Total expression and intratumoral of CD8+ expression were associated with Miller payne pathological grading and clinical response of NAC chemotherapy in LABC patients.

Keywords: Locally advanced breast cancer, neoadjuvant chemotherapy, CD8+ T cell, Miller Payne pathological response, clinical response

INTRODUCTION

Data from Global Cancer Observatory (GLOBOCAN) in 2020 reported that breast cancer is the first most prevalent type of cancer among female in the world, with incidence of more than two million cases in women annually, and predicted to keep increasing each year.[1-4] Breast cancer also known as the most prevalent cause of death in women globally, responsible for 15% mortality rate worldwide, whereas Indonesia ranked as a country with the highest mortality rate in South East Asia region.[5,6] In Indonesia, approximately 57.1% breast cancer patients seek for treatment in locally advanced stage. Locally advanced breast cancer (LABC) is an invasive breast cancer limited to the breast and regional lymph nodes.[7,8] Traditionally, the standard chemotherapy were done after the surgery. Neoadjuvant chemotherapy is proven to be more beneficial by increasing breast conservation rates in resectable breast cancer cases. The goal is also to eradicate mircometastasis disease and prevent distant metastasis. Neoadjuvant chemotherapy (NAC), is the standard treatment for LABC patients which can improve clinical response up to 70-90%.[9,10] In addition to clinical response, pathological response could also be used to evaluate NAC effectiveness, using Miller Payne methods. Miller Payne score grading could be used to predict survival rate, higher grades predict better outcome.[11,12]

Tumor-infiltrating lymphocytes (TIL) is the stimulated immune system in response to cancer cells, which consists of mononuclear cells, including T cells (CD4+, CD3+, CD8+), B

Comment [i3]: R1 #3: The importance and scientific reasoning to use CD8+ as predictive factor for LABC in this study have not been explained well in the Background. The problem and research question that trigger the author to conduct this study have also not been stated clearly.

Comment [i4]: Note from editor: Your revision is not relevant to the question asked. Please state the novelty of using CD8+ as predictor or marker.

cells, macrophages, and other immune cells in peritumoral and intratumoral region.[13,14] Recently, more researches proved that TIL played important role in tumorigenesis. Conventional chemotherapy agents can stimulate immune system to attack cancer cells through direct activation of TIL that could significantly eliminate tumor cells, which is T cells CD8+. It has important role to produce interferon gamma which has cytotoxic effects and to attack tumor cells by inhibiting cell cycles, apoptosis, and inducing tumoricidal activity from macrophages. The current evaluation of breast cancer prognosis is limited to biological tumor characteristics such as hormonal receptors, human epidermal growth factor receptor 2 (HER2), and Ki67 expressions. However, immune response have an essential role regarding chemotherapy response, yet its evaluation is not routinely done since it has not been well-established. Besides, a precise assessment of certain chemotherapy response can only be evaluated through microscopic examination of the residual tumor on surgical resection after chemotherapy. Clinical response to chemotherapy does not well correlated to the pathological response, thus the evaluation of pathological response is necessary.[15] Earlier studies showed that high T-cell CD8+ was independently associated with pathological complete response.[15,16] Prior studies also reported that clinical pathology factors that associated with chemotherapy response after NAC, includes tumor grading, molecular subtype, chemotherapy regimen, and CD8+.[13] Thus, in this study we evaluate CD8+ and its association with chemotherapy response and Miller Payne pathological grading also clinicopathological factors in LABC patients after receiving NAC. This study may added the scientific value of the association between CD8+ expression and pathological response, especially study from Indonesia regarding this topic is very limited.

METHODS

Design Study and Sampling

Comment [i5]: R2 #3: Please insert a brief introduction the term when NAC is given and how is the relationship between NAC and conventional therapy?

Comment [DAH6]: Added on line 39-42

Comment [i7]: R1 #5: The research flow has not been mentioned clearly in this study from the patient recruitment, diagnosis, laboratory procedure until post therapy analysis.

Comment [i8]: Note from editor: Please state the line number that you have conduct the revision? Because we don’f find much revision in the Methods section.

Comment [i9]: R1 #6: There is major error in the methodology in this study. The author aimed to predict the pathological response after therapy, however, the study design was cross sectional study. Predictor study should use cohort or case control study. There should be a follow up time to be able to predict the outcome.

Comment [i10]: Note from editor: Author have agree that this manuscript is a cross-sectional study, hence can not be to detiermine a predictor. However, there are still some part in the manuscript that mention 'predictor', please read your manuscript thoroughly and revise all related statement.

A cross-sectional study is conducted in Department of Surgery, Faculty of Medicine, Universitas Indonesia (FKUI) and Cipto Mangunkusumo Hospital (RSCM) in January to June, 2022. Breast cancer patients from September 2015 to February 2022, were included in this study. Data were collected from patient medial record through consecutive sampling. The inclusion criteria were breast cancer patients aged 18 years old above, who receive full dose NAC before mastectomy were done at RSCM. Anthracycline-based full dose regimen was given for six cycles, while taxane-based regimen was given for six or eight cycles (four cycles of anthracycline and four cycles of taxane). Exclusion criteria were patients with bilateral breast cancer, recurrent breast cancer, patients with changes of regimen during their cycles, not receiving full-dose regimen, prior neoadjuvant hormonal therapy, incomplete medical record, and unavailable paraffin block. This study had been approved Faculty of Medicine Universitas Indonesia Ethical Committee (No. KET-131/UN2.F1/ETIK/PPM.00.02/2022)

Data Collection

Data collection were done through medical record by taking data of baseline and tumor characteristics of subjects included age, gender, stage, tumor stage, nodal stage, tumor grade, estrogen receptor (ER) status, progesteron (PR) status, Human epidermal growth factor receptor-2 (HER2) status, Ki67 expression. Then, immunohistochemistry evaluation were done by evaluating the available paraffin blocks. Paraffin block samples include biopsy tissue before NAC and mastectomy sample after NAC, from Pathology Anatomy Department RSCM.

Immunohistochemistry procedures

Firstly, from the paraffin block, an unstained slide is made with a microtome to cut the tissue with 4µm and put in the coated slide. Each slide was coded with sample number and dried in

Comment [i11]: R1 #8: For the laboratory tests:
a. How did the technical procedure to define the expression of CD8+ T cell in parafilm block?
b. What are the operational definitions to define intratumoral, peritumoral and total expression?
c. How did the cut off for high and low CD8+ expression define? What is the cut off for high and low CD8+ expression?
d. How does the author validate that the specimens for CD8+ expression before NAC and after mastectomy are from the same area in the tumor and from the same sample origin?

37^{0}C. Then, the slide was heated on the slide warmer for paraffin impregnation as long as 60 minutes in 56,5-60^{0}C. Deparaffinization by xylol was done three times (xylol I, II, III) for 5 minutes each. The slides were next rehydrated with ethanol, alcohol 96%, and alcohol 70% for.5 minutes each, respectively, and washed under running water. Each slide was given blocking endogen peroxide with H2O2 3% in ethanol for 30 minutes to prevent false positive results, then washed under running water for 2 minutes. Antigen retrieval procedure was done as pre-treatment with Tris EDTA pH 9,0 inside decloaking chamber with 96^{0}C for 20 minutes. Slide was dried, cooled, and washed by Phosphate Buffer Saline (PBS) pH 7.4 for 3 minutes to prevent cell lysis. The tissue was marked with Pap-pen. Blocking was done by protein block for 30 minutes to prevent non-specific antibody binding. Thereafter, protein block was removed, and slide was washed with PBS for 3 minutes. Slides were incubated with primary antibodies, which is Anti-CD8 antibody (*Cell Marque SP16*) with dilution of 1:200 and washed with PBS for 3 minutes. Then, secondary antibody was dripped (*Star trek universal HRP detection*) for 30 minutes and rewashed with PBS for other 3 minutes. The slide was incubated with mixed solution of 1 drop (50µl) of Diamino Benzidine Tetrahydrochloride (DAB) chromogen and 1ml DAB buffer substrate (Polymer) for 10-20 seconds until looked brown and washed under the running water for 5 minutes. Counterstained helped to visualize and localize the target, and was done with Hematoxylin Mayer for a minute, then washed under the running water for five minutes. Bluing was done with lithium carbonate 5% for 10 seconds and washed under the running water for other five minutes. The slide was dehydrated to remove the fluid content from the tissue by alcohol 70%, alcohol 96%, and ethanol, respectively, for 5 minutes each. Clearing was done by dipping the sample into xylol I, II, and III, for 5 minutes each to remove the dehydrant substance. Slide was covered by Entellan and deck glass. Both negative and positive

control were always included during the procedures. Positive control was taken from tonsil tissue. A tonsil tissue sample was used as positive control sample.

Pathology Anatomy Evaluation

Few images were captured in different five field of views as the representative area from peritumoral and intratumoral area from each sample by Olympus Bx51 microscope with 400x magnification. The evaluation of CD8+ T cells was measured on intratumoral, peritumoral and total expression, which was the sum of both area. Intratumoral and peritumoral expression defines as CD8+ expression that were seen inside and outside the tumor stroma, respectively. While total expression defines as the sum of intratumoral and peritumoral expression. Then, the results were divided into two groups, low and high expression, based on each's groups cut-offs.

Paraffin block with mastectomy sample post NAC was used to evaluate pathologic response with Miller Payne grading ranged from one to five and described as follows. Grade 1 shows no change to individual malignant cells and no reduction in tumor cellularity; grade 2 shows minimal reduction up to 30% loss of tumour cells; grade 3 shows significant tumor cells reduction after chemotherapy, estimated 30-90% reduction; grade 4 shows minimal tumor cells, more than 90% loss of tumor cells; grade 5 shows complete response, no appearance of residual tumor cells.[13] In this study, Miller Payne grading was categorized into two groups, grade 1 was considered as no response, while grade 2-5 considered as response group. To assess the pathological response, it's not always have been taken from the same origin location of pre-NAC biopsy, since as long as it is in the tumor of the breast, it represents the pathological response. There were two observers that consists of a surgical oncologist and an anatomical pathologist, to assess the Miller Payne grading, with intraclass coefficient correlation of 91,2% (good), which

means interobserver result difference is under 10%. The evaluation of IHC staining was further analysed by Image J image analysis through blinding. Intratumoral, peritumoral, and total expression of CD8+ T cells were reported in semiquantitative results.

Clinical response of chemotherapy

Clinical response of NAC was categorized based on tumor diameter changes, according to World Health Organization (WHO) criteria. Progression response was defined as a 25% or more increase in total tumor size/and or the appearance of new lesion in other site. Stable response, or no change, was defined as less than 50% decrease of tumor size or increase of tumor size not more than 25%. Partial response was defined as 50% or more decrease in size (volume) at least for four weeks, no appearance of new lesion or disease progression. The disappearance of the disease during two different observations conducted not less than four weeks apart was classified as complete response.[14] In this study, clinical response of NAC was further divided into two groups, partial and complete response were considered as response group, while progression and stable were considered as no response.

Data Analysis

Data analysis were done with SPSS version 20.0. Univariate analysis was used to report demographic characteristics of the subjects. Initially, the cut-offs of intratumoral, peritumoral, and total expression were calculated by area under curve (AUC) analysis and Youden's Index. Chi-square and Fisher Exact test were used to analyze independent variables and outcome, with $p<0.05$ means statistically significant. Association between CD8+ T cell expression and Miller

Payne grading was evaluated with either independent T-test or Mann-Whitney U, considering the data distribution

RESULTS

There were 40 LABC patients fulfilled the inclusion and exclusion criteria then included to the study analysis. Majority of the subjects were aged above 40 years old (70%), had T4 tumor (87.5%), histopathological grade results in grade 2 (60%), diagnosed as luminal B breast cancer (42.5%), estrogen receptor (ER) positive (92.5%), progesterone receptor (PR) positive (55%), HER2 negative (70%), and received anthracycline based chemotherapy (60%). More details in demographic data were shown in Table 1.

Based on the AUC analysis, cut-off for intratumoral CD8+ T cell expression is 23.8, with sensitivity of 86.5% and specificity 100% (Figure 3); for intratumoral is 6.4, with sensitivity of 89.2% and specificity of 100% (Figure 4); for peritumoral expression is 14.3, with sensitivity of 89.2% and specificity of 100% (Figure 5). Figure 1 shows the intratumoral and peritumoral of CD8+ T-cell expression. Expression of CD8+ T cell in intratumoral and peritumoral region were found high in 80% (n=32) patients, and 65% (n=26) patients, respectively. Overall, 80% (n=32) patients had high expression of CD8+ T cell. Table 2 explains CD8+ expression regarding chemotherapy response distribution. Based on bivariate analysis, there were no association between CD8+ T cell expression with clinicopathological factors, such as age, histopathology grade, ER, PR status, Ki67, HER2, and breast cancer subtypes. (Table 3) The mean difference in total expression of CD8+ T cell (p=0.013) and intratumoral CD8+ expression (p=0.015) based on Miller Payne grading were found statistically significant. Higher means were found in response group (Table 4). Figure 2 shows the Miller payne grading images under the microscope from

Comment [i12]: R1# 9: The results, ideas, and data presented in this study are not in line with the aim of the study which is predicting the pathological response. The study tends to be overclaimed and using inappropriate statistical analysis to claim the evidence. The ROC analysis, sensitivity and specificity data should be for diagnostic study but were used in predictor study, so this is analysis error. The association between variables that shown by the independent t-test is for descriptive study. If there are significant results, it is shown that there were an association between those variables but not strong enough to claim that these significant results can be used to predict the outcome.

The data presented in the results as figures and tables are basically for descriptive study, not predictor study.

Comment [i13]: Note from editor: Please make sure you write the Results and data presentation according to the aim and study design (cross-sectional) of this study.

biopsy and after NAC from mastectomy samples. Fisher exact analysis showed that CD8+ T cell total expression (p=0.037) and intratumoral expression (p=0.002) had association with clinical response of NAC (Table 5).

DISCUSSION

Study reported that breast cancer patients in Indonesia was the most prevalent in 40-49 years old group. However, in western countries, breast cancers were found mostly after menopause. Earlier study in Indonesia found higher prevalent of breast cancer in women age 40 and above group rather than below 40 years old (68.9% vs 31.1%), and they had increased risk of breast cancer up to 13.3 times.[17] This finding was similar to this study whereas 70% of the patients were aged 40 and above. Based on the histopathology type, this study reports invasive carcinoma of no special type (NST) as the most prevalent one with 82.5% cases. In fact, invasive carcinoma NST is the most common histopathology morphology of breast cancer, and earlier studies reported up to 80% cases of invasive carcinoma of NST in LABC patients. [21,22]

On the other hand, the majority of patients had luminal B type (42.5%), based on the IHC subtype. This finding is consistent breast cancer registry data and a retrospective study in Indonesia.[21] Study from Vietnam also reported the percentage of luminal B subtype in 56.5% cases. [23] It was known that IHC subtypes variety might be explained by differences in age, race, and ethnicity.[24] Less than 10% of the subjects had triple negative breast cancer, in which it is a rare subtype of breast cancer that associated with poor response in chemotherapy.[25,26] According to the prior studies, it was concluded that South East Asia was dominated by luminal B subtypes.

This study found that there was no correlation between CD8+ T cell expression and clinicopathological factors, such as age, histopathological grading, immunohistochemistry

Comment [i14]: R1 #10: Since there are major problem since research design, the major problem follow till discussion.

Comment [i15]: Note from editor: Please make sure you write the Discussion in line with the aim and study design (cross-sectional) of this study.

subtypes (ER, PR, HER2, Ki67), luminal and non-luminal in LABC patients. Factors related to the CD8+ T cell immune profile were found to be multifactorial, including tumor genetics, germline genetics, microbiomes, infection agents, sun exposure, and pharmacological agents.[27,28] However, other studies found that CD8+ T cell expression was associated with higher histopathological grade, triple negative breast cancer subtype, ER negative status, tumor grading, higher tumor size, and necrosis tumor.[19,29] It was explained that more mutation in tumor results in higher immune response thus increasing CD8+ T cell expression.

The association between CD8+ T cell expression and Miller Payne pathological grading was also supported by other study where TIL was associated with NAC response that reported higher CD8+ T cell expression indicates higher stratification of Miller Payne grading.[30] In addition, total and intratumoral CD8+ T cell expression were also found have significant association with both pathological grading and clinical response after NAC. Recent study found that CD8+ intratumoral was the potential prognostic marker in breast cancer patient, instead of peritumoral expression.[31] While other study from Indonesia reported that CD8+ might be a predictive factor for clinical response of NAC in breast cancer patients.[15] From biological aspects, peritumoral expression of CD8+ T cell could protect the host from cancer progression since TIL function had not been impaired. If CD8+ could be found intratumoral, means that the immune system could counter immune escaping from the cancer cells. Chen D, et al. also reported that NAC in breast cancer patients were related to tumor phenotypes which categorized into three immune profiles. Immune-inflamed phenotype marked CD8+ T cell expression both in intratumoral and tumor parenchyma, hence this phenotype results in good clinical response of anti-cancer therapy. However, immune-excluded phenotype were limited in peritumoral expression and immune-dessert phenotype showed lack of expression in intratumoral,

Comment [i16]: R2 #7: Can you add more explanation on this? Why the peritumoral expression doesn't give quite significancy, based on the mechanism.

peritumoral even tumor stroma. These phenotypes showed limited and no clinical response from anti-cancer therapy, respectively.[26] The mentioned biological characteristics of the tumor might be the underlying reason that peritumoral expression of CD8+ did not associated with pathological response of NAC.

Difference in results from this study may be explained by limited sample size, and other predisposing factors that related to CD8+ T cell expression that were not observed. Limitation in this study includes sample size and measurement on the scale bar that couldn't be done directly under the microscope. Further study evaluating CD8+ T cell association with clinical and pathological response should be done to add scientific evidences, with more sample size, control group, and other related factors that may affect its expression or comparing to other evaluation system of NAC response, like residual cancer burden methods which assessing based on tumor cells and lymph nodes.

CONCLUSION

There were no association between CD8+ T cell expression and clinicopathological factors and molecular subtypes. Total expression and intratumoral expression of CD8+ T cell were associated with pathological and clinical response of NAC chemotherapy.

Comment [i17]: R1 #11: The author is overclaimed the study results and conclusion. The descriptive results were used to claim predictive results.

Comment [i18]: Note from editor: Please write it accordingly with the aim and study design (cross-sectional) of this study.

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344 **Tables and Figures**

345

346 **Table 1. Demographic characteristics of the subjects**

Characteristics	N (%) (N=40)
Age	
≤ 40 years old	12 (30)
> 40 years old	28 (70)
Tumor	
T2	1 (2.5)
T3	4 (10)
T4	35 (87.5)
Nodul	
N0	17 (42.5)
N1	17 (42.5)
N2	3 (7.5)
N3	3 (7.5)
Histopathology	
Invasive NST	33 (82.5)
Lobular	3 (7.5)
Others	4 (10)
Histopathology grade	
Grade 1	3 (7.5)
Grade 2	24 (60)
Grade 3	13 (32.5)
Immunohistochemistry subtype	
Luminal A	8 (20)
Luminal B	17 (42.5)
Luminal B 2 type	12 (30)
Triple negative breast cancer	3 (7.5)
HER2 type	0 (0)
Chemotherapy	
Taxane-based	16 (40)
Anthracycline-based	24 (60)
Estrogen receptor	
Negative	3 (7.5)
Positive	37 (92.5)
Progesteron receptor	
Negative	18 (45)
Positive	22 (55)

HER2 receptor	
Negative	28 (70)
Positive	12 (30)
Ki67 status	
Low	13 (32.5)
High	27 (67.5)

NST, no special type; HER2, human epidermal growth factor receptor 2.

Table 2. CD8+ expression and chemotherapy response distribution

Characteristics	N (%) (N=40)
Total CD8+ expression	
Low	8 (20)
High	32 (80)
Intratumoral CD8+ expression	
Low	7 (17.5)
High	33 (82.5)
Peritumoral CD8+ expression	
Low	14 (35)
High	26 (65)
Miller Payne Grading	
Grade 1	3 (7.5)
Grade 2	15 (37.5)
Grade 3	18 (45)
Grade 4	2 (5)
Grade 5	2 (5)
Chemotherapy clinical response	
Stable	4 (10)
Partial	35 (82.5)
Complete	1 (2.5)

Table 3. Association between clinicopathology parameters and CD8+ T cell expression

Clincopathology parameters	Total CD8+ T Cell expression				*p-value*
	Low (N=8)		High (N=32)		
	N	%	N	%	
Age					
≤40 years old	2	16.7	10	83.3	0.548
>40 years old	6	21.4	22	78.6	
Histopathology grade					
Low grade	6	22.2	21	77.8	0.479
High grade	2	15.4	11	84.6	
Estrogen receptor status					
Negative	0	0	3	100	0.502
Positive	8	21.6	29	78.4	
Progesterone status					
Negative	6	33.3	12	66.7	0.110
Positive	2	9.1	20	90.9	
Ki67					
Low	3	23.1	10	76.9	0.521
High	5	18.5	22	81.5	
HER2					
Negative	6	21.4	22	78.6	0.548
Positive	2	16.7	10	83.3	
IHC subtypes					
Luminal	8	21.6	29	78.4	0.502
Non-Luminal	0	0	3	100	

HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry

Table 4. CD8+ expression based on Miller Payne Grading

CD68+ expression	Miller-Payne Grading										p-value
	Response (N=37)					Non-Response (N=3)					
	Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max	
Total	40.17	21.13	14.60	9.20	23.60	15.80	7.27	32.80	9.4-	107.40	0.013*
Intratumoral	24.75	42.51	5.60	0.60	5.60	3.93	2.88	16.00	2.40	266.00	0.015*
Peritumoral	22.42	14.31	9.00	8.60	18.00	11.86	5.31	20.00	3.80	58.40	0.248

***Mann-Whitney test, significant at p-value<0.05*

Table 5. Association of CD8+ expression and chemotherapy clinical response

CD8+ expression	Clinical response				*p-value*
	No response (N=4)		Response (N=36)		
	N	%	N	%	
Total					
Low	3	37.5	5	62.5	0.02*
High	1	3.1	31	96.9	
Intratumoral					
Low	3	42.9	4	57.1	0.013*
High	1	3	32	97	
Peritumoral					
Low	3	21.4	11	78.6	0.115
High	1	15.4	25	84.6	

Fisher exact test, significant at p-value<0.05

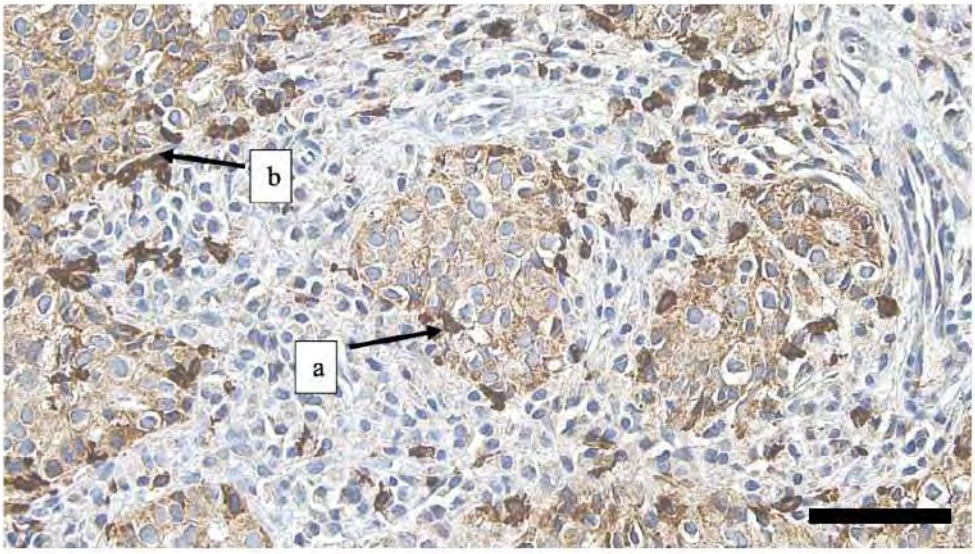

Figure 1. On a biopsy sample of breast cancer with 400x magnification showing (a) Intratumoral and (b) peritumoral expression of CD8+ T cell. Black bar: 100μm

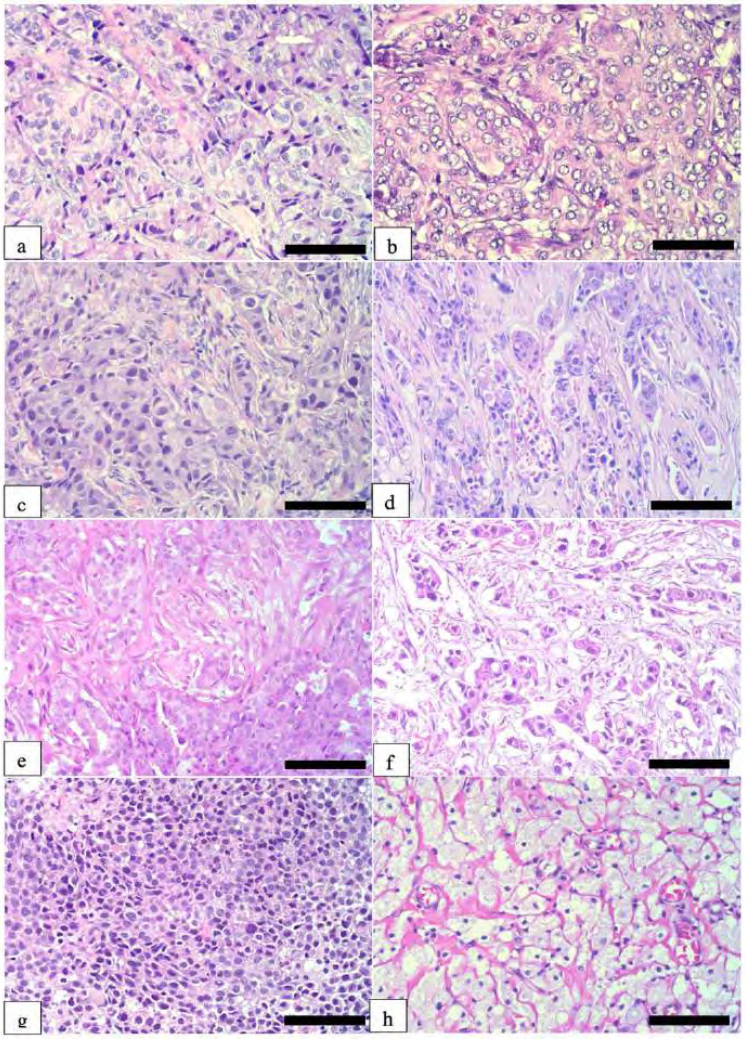

Figure 2. Pathological chemotherapy response based on Miller Payne grading. (a) Grade 1, from biopsy sample; (b) Grade 1, from mastectomy sample; (c) Grade 2, from biopsy sample; (d) Grade 2, from mastectomy sample; (e) Grade 3, from biopsy sample; (f) Grade 3, from mastectomy sample; (g) Grade 5, from biopsy sample; (h) Grade 5, from mastectomy sample. Black bar: 100 μm

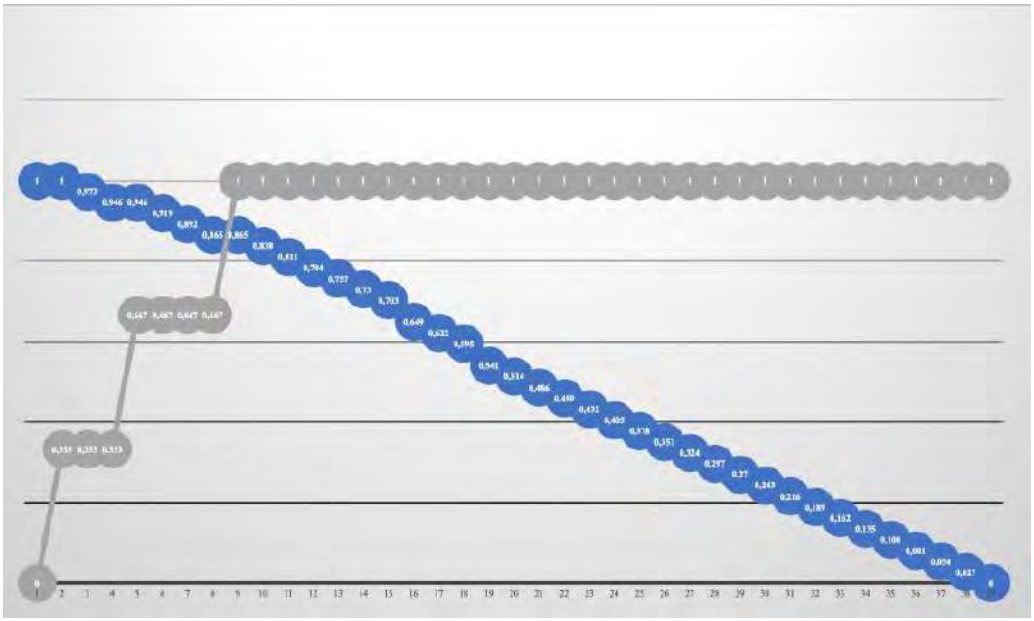

Figure 3. Cut-off for total CD8+ T cell lymphocyte

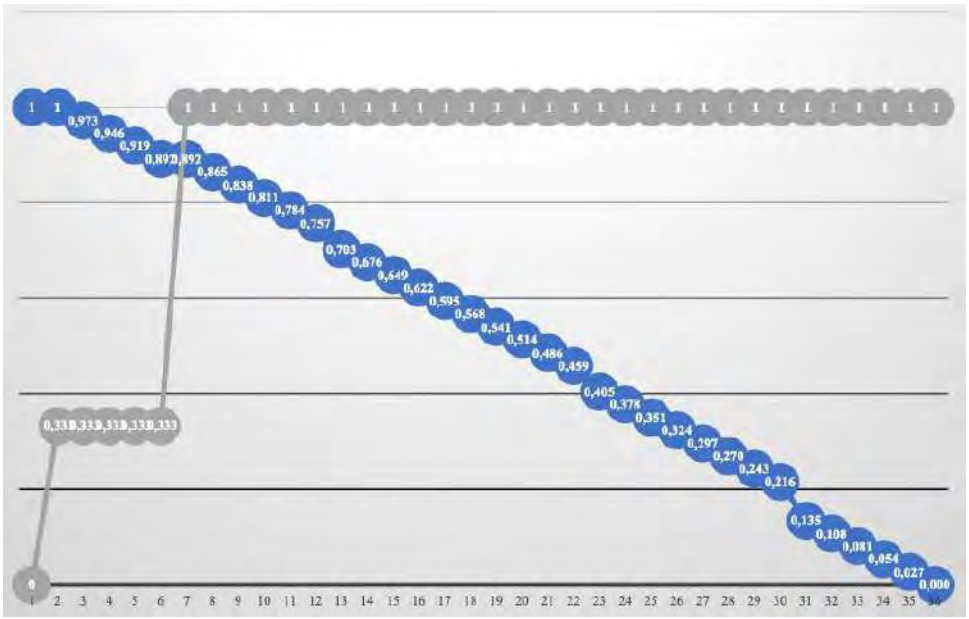

Figure 4. Cut-off for intratumoral CD8+ T cell lymphocyte

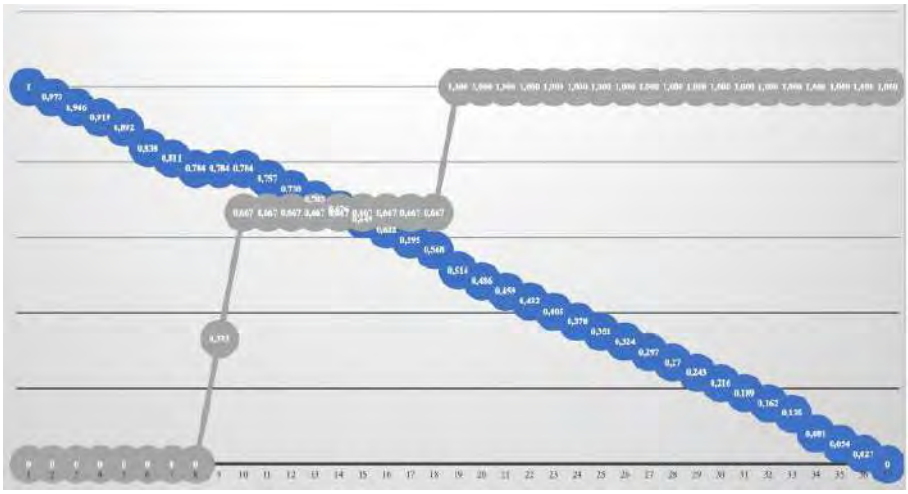

Figure 5. Cut-off for peritumoral CD8+ T-cell lymphocyte

Response Form for Reviewer's Comments

Corresponding Author : Ferry Sandra
Manuscript Code : M2022191
Manuscript Ttile : Expression of CD8+ Lymphocytes as a Predictor for Miller Payne's Pathological Response to Neoadjuvant Chemotherapy in Locally Advanced Breast Cancer

No. (Reviewer and comments number code)	Comments (Comments/question from reviewer or editor)	Author's Response (Please write your response regarding the comment here)	Line Number (Please write the line number of the said revision)	Note from Editor
R1 #1	The title needs revision according to the study main result, since the current title has not reflected the study content.	Thank you for the suggestions, we have discussed to change the title into: "The association of CD8+ Lymphocytes expression and Miller Payne's Pathological Response Grading to Neoadjuvant Chemotherapy in Indonesian Locally Advanced Breast Cancer"	1-2	√
R1 #2	The background in the abstract has not explain about the importance of the study. The method in the abstract was not reflected the main variable (no CD8+ T cell expression explained, but hematoxylin eosin that was not for CD8+ cell expression).	Thank you for the advices, we have updated the background to emphasize the importance of the study.	5-10	This still has not been revised by the author! The reviewer ask the author to: 1. Please add explanation about the importance of the study in the Background of Abstract 2. HE is used for the staining, not to calculate the CD8+ T cell expression, please revise.
R1 #3	The importance and scientific reasoning to use CD8+ as predictive factor for LABC in this study have not been explained well in the Background. The problem and research question that trigger the author to conduct this study have also not been stated clearly.	We have added more information in the background. The novelty of using CD8+ is currently there is no established immune marker for chemotherapy response. Thus, this our study may add scientific value of CD8+ as an immune marker for NAC response and as a cornerstore for further study concerning this finding.	51-58, 60-66	Your revision is not relevant to the question asked. Please state the novelty of using CD8+ as predictor or marker.
R1 #4	The novelty in this manuscript is not clear since high CD8+ lymphocytes expression has been known to be associated with pathological complete response in other studies and no statement about the novelty by define this CD8+ expression in this study.	This study may added the scientific value of the association between CD8+ expression and pathological response, especially study from Indonesia regarding this topic is very limited.	64-66	You have answer it in the response form but have not add it in the manuscript. You can add the statement in your manuscript.
R1 #5	The research flow has not been mentioned clearly in this study from the patient recruitment, diagnosis, laboratory procedure until post therapy analysis.	We have added more tailored details of the procedures.	89-115	Please state the line number that you have conduct the revision? Because we don't find much revision in the Methods section.
R1 #6	There is major error in the methodology in this study. The author aimed to predict the pathological response after therapy, however, the study design was cross sectional study. Predictor study should use cohort or case control study. There should be a follow up time to be able to predict the outcome.	Thank you for the suggestions, we agree that this is a cross sectional study thus we changed the title of the manuscript. We have deleted all the sentences related to the predictor study.	Done	Author have agree that this manuscript is a cross-sectional study, hence can not be to detiermine a predictor. However, there are still some part in the manuscript that mention 'predictor', please read your manuscript thoroughly and revise all related statement.

R1 #7	Inclusion criteria: The description of study population that included in this study is not clear related with age limit, the disease staging, location and severity, diagnosis category, pretreatment and after requirement and other requirement that will clarify the representative study population for predictor study. No information regarding the treatment and control study in this predictor study.	The inclusion criteria were breast cancer patients aged 18 years old above, diagnosed with any stages of breast cancer. There is no control in this study since this is a cross sectional study and we have considered that it is not a predictor study. Thank you for you correction.	69	√
R1 #8	For the laboratory tests: a. How did the technical procedure to define the expression of CD8+ T cell in parafilm block? b. What are the operational definitions to define intratumoral, peritumoral and total expression? c. How did the cut off for high and low CD8+ expression define? What is the cut off for high and low CD8+ expression? d. How does the author validate that the specimens for CD8+ expression before NAC and after mastectomy are from the same area in the tumor and from the same sample origin?	Thank you for the advices, additional information regarding the laboratory tests is added to the manuscript. a. Detailed information regarding the procedural technique is added to the manuscript. b. The intratumoral expression defines as CD8+ expression that were seen inside the tumor stroma, while the peritumoral expression were those outside the tumor stroma. Total expression defines as the sum of peritumoral and intratumoral expression. We have added the description in the manuscript. c. The cut off for high and low CD8 expression were defined using AUC analysis. The analysis is added to the manuscript. d. To assess the pathological response, it's not always have been taken from the same place of origin, since as long as it is in the tumor of the breast, it represents the pathological response.	a. 89-115 b. 121-123 c. 390-395 d. 132-134	Please state the line number that you have conduct the revision? Because we don'f find much revision in the Methods section. And to make each step easier to understand, please divided this subsection into some subsection.
R1 #9	The results, ideas, and data presented in this study are not in line with the aim of the study which is predicting the pathological response. The study tends to be overclaimed and using inappropriate statistical analysis to claim the evidence. The ROC analysis, sensitivity and specificity data should be for diagnostic study but were used in predictor study, so this is analysis error. The association between variables that shown by the independent t-test is for descriptive study. If there are significant results, it is shown that there were an association between those variables but not strong enough to claim that these significant results can be used to predict the outcome. The data presented in the results as figures and tables are basically for descriptive study, not predictor study.	We have revised our manuscript as it is not a predictive study, but rather limited to analyze the association between the CD8+ expression and Miller Payne grading. Thank you for your valuable advice.	Done	Please make sure you write the Results and data presentation according to the aim and study design (cross-sectional) of this study.
R1 #10	Since there are major problem since research design, the major problem follow till discussion.	We have revised our manuscript as it is not a predictive study, but rather limited to analyze the association between the CD8+ expressiong and Miller Payne grading. Thank you for your valuable advice.	Done	Please make sure you write the Discussion in line with the aim and study design (cross-sectional) of this study.
R1 #11	The author is overclaimed the study results and conclusion. The descriptive results were used to claim predictive results.	We have rephrase the sentences so it won't be overclaim.	Done	Please write it accordingly with the aim and study design (cross-sectional) of this study.
R2 #1	Rather than say majority, it will be better to just state the number, as this is a quantitative study.	It has been revised as adviced.	16	√
R2 #2	I am disagree with the total expression, since the significant result came from the intratumoral expression. Please also check the main Conclusion.	Based on the chi-square analysis, the total and intratumoral expression had p-value <0.05.	no revision needed	√
R2 #3	Please insert a brief introduction the term when NAC is given and how is the relationship between NAC and conventional therapy?	We have added further introduction about NAC term.	33-36	√
R2 #4	Can you explain briefly on the benefit using CD8 as the NAC respond predictor compare to current method?	The current evaluation of breast cancer prognosis is limited to biological tumor characteristics such as hormonal receptors, HER2, and Ki67 expressions. However, immune response have an essential role regarding chemotherapy response, yet its evaluation is not yet routinely done since it has not been well-established. Besides, a precise assessment of certain chemotherapy response can only be evaluated through microscopic examination of the residual tumor on surgical resection after chemotherapy. Clinical response to chemotherapy does not well correlated to the pathological response, thus the evaluation of pathological response is necessary	51-58	You have answer it in the response form but have not add it in the manuscript. You can add the statement in your manuscript.

R2 #5	You mean CD8+?	Sorry for the mispelled, we have corrected it. It is CD8+.	109	√
R2 #6	Please add the min,max,means and SD value in the table when possible, so the reader can get a more complete description on the subjects.	We have added into the table.	314	√
R2 #7	Can you add more explanation on this? Why the peritumoral expression doesn't give quite significancy, based on the mechanism.	The tumor biological characteristics that might underlying the finding of the insignificance of CD8+ peritumoral expression.	228-230	We find that you don't more explanation about this. Please add explanation. You may want to add the statement you have write in the response form to your manuscript.
R2 #8	I am disagree with the total expression, since the significant result came from the intratumoral expression.	Based on the chi-square analysis, the total and intratumoral expression had p-value <0.05.	no revision needed	√
R2 #9	Thus this need to be clarify as the intratumoral CD8 only.	Agree, thank you for the correction.	193	√
SE #1	Please also add these references: 1. https://cellbiopharm.com/ojs/index.php/MCBS/article/view/238/102 2. https://cellbiopharm.com/ojs/index.php/MCBS/article/view/21/19 3. https://inabj.org/index.php/ibj/article/view/1674/552 4. https://inabj.org/index.php/ibj/article/view/977/473	Done	Reff	√
SE #2	This sentence is unfinished. Please complete it.	We have revised the sentence.	228-230	

Note : R1 = Reviewer 1
R2 = Reviewer 2
SE = Section Editor

UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2022191 Editor Decision Round 2 - Resubmit for Review

Secretariat of InaBJ <secretariatinabj@gmail.com> Tue, Jan 10, 2023 at 1:32 PM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**The association of CD8+ Lymphocytes expression and Miller Payne's Pathological Response Grading to Neoadjuvant Chemotherapy in Indonesian Locally Advanced Breast Cancer Patients**".

Our decision is: **Resubmit for Review.**

Find the file attached to see detailed comments from reviewers. Please make sure you read all the comments and revise the manuscript based on the suggestions given.

Revise this manuscript thoroughly before **January 24, 2023**. Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes.

When you are done, you can upload it in: https://inabj.org/index.php/ibj/author/submissionReview/2110, or simply send us an email of your revised manuscript and response letter.

Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,

--
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Manuscript Review Form

Reviewer	:	Reviewer 1
Manuscript #	:	M2022191
Manuscript Title	:	The association of CD8+ Lymphocytes expression and Miller Payne's Pathological Response Grading to Neoadjuvant Chemotherapy in Indonesian Locally Advanced Breast Cancer Patients

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?		√
	Notes: The author statement on novelty of this study was still not specific enough since the association between CD8+ T cell with MP Pathological Response Grading had been known in other studies in other population. Please specify the novelty in target population in this study!		
2.	Are the title and abstract of the manuscript appropriate?		√
	Notes: Please revise the title that represent the study results and findings. Author can check the title style of other publication that had been accepted in this journal to get the idea.		
3	Do the title and abstract reflect the study result/content?		√
	Notes: The title has not reflected the study results. The abstract method has not yet reflected the research flow, importance methods and techniques to evaluate the study objective and inclusion-exclusion criteria. Abstract should be able to represent the whole study content, as the window show of this study.		
4.	Is the significance of the study well explained at the Background?	√	
	Notes: It is clearer than the first manuscript but still need more specific remark on the target population.		
5.	Are the research study methods technically correct, accurate, and complete		√

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	enough to be reproduced/cited by other scientists?		
	Notes: The study design was stated as cross-sectional study, however, the data obtained from medical record means it was secondary data and the parameter measurement was also performed from already stored paraffin block. It gave impression of retrospective study. There is unclear information how the researcher handle the study population and the study flow since define the study population, getting the informed consent from the study subject, when and how was the specimen collection conducted, when the treatment was performed and when the evaluation was conducted. Is there any informed consent from the patient for this study? Table 4. The data presented in this table should be checked for its normal distribution or not then the author can choose to show the data as mean ±SD or median (min-max)		
6.	Are the results, ideas, and data presented in this manuscript important enough for publication?		√
	Notes: This study has problem on the research methodology and the way of researcher write the study to show a comprehensive information to the reader.		
7.	Are all figures and tables necessarily presented?		√
	Notes: Table 1. The table title is not appropriate. Demographic data is actually related with population data. The data presented in table 1 is more appropriate to be baseline characteristic data, rather than demography data. Table 4. The data presented in this table should be checked for its normal distribution or not then the author can choose to show the data as mean ±SD or median (min-max) Figure 1 and 2. What is the title for the x and y axis in this figure and what is this figure represented? If this is ROC curve, it should show a proper legend and notes for ROC curve. Please check other publication to learn how to present a ROC curve in journal properly.		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?		√
	Notes: Partially.		
9.	Are the conclusions and interpretations valid and supported by the data?		√
	Notes:		
10.	Is the manuscript clear, comprehensible, and written in a good English		√

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	structure?		
	Notes:		

Specific Reviewer's Comments and Suggestions:
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Reviewer's Recommendation (Please tick only one option)	
Accept Submission (No significant alterations suggested)	
Revisions Required (Suggest changes to the manuscript as specified in this review)	
Resubmit for Review (Major revisions should be made and suggestions as specified in this review must be addressed. Revised manuscript should be resubmitted to the reviewer for further review)	
Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	√

Further Reviewer's Comments Regarding Disposition of the Manuscript:

Date and Sign:
January 10th, 2023

Reviewer 1

UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2022191 Editor Decision Round 2 - Resubmit for Review

Ferry Sandra <ferry@trisakti.ac.id> Tue, Mar 28, 2023 at 7:29 AM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Dear Secretariat of The Indonesian Biomedical Journal,

Apologies for the delayed response; major revisions took time. Thank you for the review. Enclosed are the revised version of the manuscript M2022191. All the comments from the reviewer have been addressed in the manuscript.

Thank you.

Regards,
Ferry Sandra
[Quoted text hidden]
--
Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

Round 2 Revision from Author.docx
6425K

Total and Intratumoral $CD8^+$ T Cell Expressions are Correlated with Miller Payne Grading and WHO Clinical Response of Neoadjuvant Chemotherapy

ABSTRACT

Background: Chemotherapy has reported to stimulate immune system through direct activation of cluster of differentiation (CD)8^+ T cells. Neoadjuvant chemotherapy (NAC) is known to improve the clinical response of locally advanced breast cancer (LABC) patients. However, the immune response-related factor evaluation of NAC in LABC patients has not been routinely performed. Therefore, current study was conducted to evaluate the correlation of NAC-induced $CD8^+$ T cell with chemotherapy response based on Miller Payne grading and World Health Organization (WHO) criteria.

Method: LABC patients were recruited and data regarding age, gender, tumor, nodal stages, histopathological grade, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67 were obtained. Biopsy and mastectomy tissues were collected and processed for hematoxylin-eosin and CD8 immunohistochemical staining. $CD8^+$ T cell expression in peritumoral and intratumoral areas were documented and measured. Clinical responses based on Miller Payne grading and WHO were analyzed and correlated with $CD8^+$ T cell expression.

Result: There were more subjects with high expression of total (80%), intratumoral (82.5%) and peritumoral (65%) $CD8^+$ T cell expressions. The total (p=0.013) and intratumoral (p=0.015) $CD8^+$ T cell expression, but not peritumoral CD8+ T cell expression, were significantly correlated with Miller Payne Grading. The total (p=0.009) and intratumoral

(*p*=0.001) CD8+ T cell expressions were also significantly correlated with WHO clinical response.

Conclusion: Total and intratumoral $CD8^+$ T cell expressions are correlated with Miller Payne grading and WHO clinical response of NAC. Therefore, total and intratumoral $CD8^+$ T cell expressions could be suggested as a predictive marker for clinical response of NAC.

Keywords: breast cancer, neoadjuvant chemotherapy, CD8, clinical response, Miller Payne, intratumoral, peritumoral

INTRODUCTION

Based on data of Global Cancer Observatory in 2020, breast cancer is the most prevalent type of cancer among female in the world, with incidence of more than two million cases annually, and predicted to keep increasing each year.(1-4) Breast cancer is the most prevalent cause of death in women globally, responsible for 15% mortality rate worldwide, whereas Indonesia is ranked as the country with highest mortality rate due to breast cancer in South East Asia.(5-8) In Indonesia, approximately 57.1% locally advanced breast cancer (LABC) patients seek for treatment. LABC is an invasive breast cancer limited to the breast and regional lymph nodes.(9,10) Conventionally, the standard chemotherapy were done after the surgery. Neoadjuvant chemotherapy (NAC) is proven to be more beneficial by increasing breast conservation rates in the resectable breast cancer cases. With NAC, micro-metastasis can be eradicated, therefore can prevent metastasis. For LABC patients, NAC can improve clinical response up to 70-90%.(11,12)

Conventional Chemotherapy agent has been reported to stimulate immune system to attack cancer cells through direct activation of cluster of differentiation (CD)8^+ T cells that could

significantly eliminate tumor cells. T cells have an important role to produce interferon gamma which has cytotoxic effects by inhibiting cell cycles as well as inducing apoptosis and tumoricidal activity. Earlier studies showed that high number of $CD8^+$ T cell was independently correlated with pathological complete response.(13,14)

Precise assessment of certain chemotherapy response can be evaluated through microscopic examination of the residual tumor on surgical resection after chemotherapy. Current evaluation of breast cancer prognosis is limited to biological tumor characteristics such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER)2, and Ki67 expressions. However, clinical response to chemotherapy does not always correlated with those markers, thus additional factors should be considered.(13) Since immune response has been reported to play an essential role in chemotherapy response, assessment of immune response-related factor such as $CD8^+$ T cell (15), could be suggested. However, immune response-related factor evaluation is not routinely performed since it has not been well-established. Therefore current study was conducted to evaluate the correlation of NAC-induced $CD8^+$ T cell with chemotherapy response based on Miller Payne grading and world health organization (WHO).

METHODS

Subject Selection and Criteria

LABC patients of Department of Surgery, Faculty of Medicine, Universitas Indonesia and Dr. Cipto Mangunkusumo National Central General Hospital from September 2015 to February 2022, were selected and included for this study based on inclusion and exclusion criteria. The inclusion criteria were LABC patients with age of >18 years old, who received full dose of NAC

with anthracycline- or taxane-based regimen, prior to mastectomy. Meanwhile, the exclusion criteria were the patients with bilateral or recurrent breast cancer, different/change/inadequate of therapeutic regimen, incomplete medical record and unavailable paraffin block. The protocol of this study was approved by the Ethical Committee of Faculty of Medicine Universitas Indonesia (No. KET-131/UN2.F1/ETIK/PPM.00.02/2022).

Data and Sample Collection

Subject-related data were collected from medical record for information of age, gender, tumor and nodal stages, histopathological grade, as well as immunohistochemical examinations of ER, PR, HER2 and Ki67. Histopathological grade was examined by anatomic pathologist based on haematoxylin-eosin features and divided into 3 categories; grade 1: well differentiated, grade 2: moderately differentiated and grade 3: poorly differentiated. Meanwhile, immunohistochemical examinations of ER, PR, HER2 and Ki67 were carried out with standard immunohistochemical staining procedures in Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia and Dr. Cipto Mangunkusumo National Central General Hospital, with following primary antibodies: anti-ER (Leica Biosystems, Wetzlar, Germany), anti-PR (Leica Biosystems), anti-HER2 (Diagnostic BioSystems, Pleasanton, CA, USA) and anti-Ki67 (Diagnostic BioSystems) antibodies, respectively.

For CD8 immunohistochemical detection, paraffin blocks of biopsy samples were collected, sliced in 4 μm and processed for immunohistochemical staining. Meanwhile for Miller Payne grading, paraffin blocks of mastectomy samples were collected, sliced in 4 μm and processed for hematoxylin-eosin staining.

CD8 Immunohistochemical Staining and Evaluation

Sliced tissues were placed on coated slides, heated, deparaffinized, rehydrated, blocked with 3% H_2O_2, antigen retrieved with Tris EDTA pH 9.0 and blocked with protein blocking buffer. CD8 (SP16) rabbit monoclonal antibody (Cell Marque, Rocklin, CA, USA) with dilution of 1:200 was used as the primary antibody. Then Starr Trek universal HRP detection system (Biocare Medical, Pacheco, CA. USA) was applied, followed by 3,3′-diaminobenzidine tetrahydrochloride. Counterstaining was performed with hematoxylin. The slide was then dehydrated and coverslipped with Entellan. For positive control, tonsil tissue was used.

Five fields of each sample were randomly selected under a microscope (BX51, Olympus, Tokyo, Japan) with 400x magnification. $CD8^+$ T cell expression in peritumoral and intratumoral areas of each sample were captured and measured by ImageJ (U. S. National Institutes of Health, Bethesda, MA, USA). Intratumoral and peritumoral areas were defined as inside and outside areas of the tumor stroma, respectively. Then, the results were divided into two groups, low and high expression, based on each's group cut-off.

Miller Payne Grading

Based on the hematoxylin-eosin histopathological features, samples were graded with Miller Payne Grading (15), by 2 calibrated observers, an anatomic pathologist and a surgical oncologist with <10% inter-observer difference. In this study, Miller Payne grading was categorized into two groups, grade 1 was considered as no response, while grade 2-5 were considered as response group.

WHO Clinical Response

WHO clinical response was categorized based on tumor diameter changes, according to WHO criteria. Progression response: >25% increase in tumor size and/or the appearance of new lesion in other site. Stable response: <50% decrease or ≤25% increase in tumor size. Partial response:

≥50% decrease in in tumor size at least for 4 weeks, no appearance of new lesion or disease progression. Complete response: disappearance of the disease during two different observations conducted not less than 4 weeks apart.(16) In this study, subject chemotherapy responses were collected, analyzed based on the WHO Criteria, and divided into two groups. The partial and complete response were considered as response group, while the progression and stable were considered as no response.

Statistical Analysis

Data analysis was done with SPSS version 20.0. (IBM Corporation, Armonk, NY, USA). The cut-offs of intratumoral, peritumoral, and total expression were calculated by area under curve (AUC) analysis and Youden's Index. Fisher Exact and Mann-Whitney tests were used to analyze independent variables and outcomes, with significancy of $p<0.05$.

RESULTS

Forty LABC subjects were selected. Majority of the subjects were aged ≥40 years old (70%), T4 (87.5%), N0 (42.5%) & N1 (42.5%), invasive histopathological appearance with no special type (82.5%), histopathological grade 2 (60%), luminal B (42.5%), treated with anthracycline-based NAC (60%), ER positive (92.5%), PR positive (55%), HER2 negative (70%) and high Ki67 (67.5%) (Table 1).

Immunohistochemical expression of $CD8^+$ T cell was detected clearly in tonsil tissue (Figure 1A) and breast cancer biopsy (Figure 1B). Based on the AUC analysis and Youden's Index, cut-off for total $CD8^+$ T cell expression was 23.8, with sensitivity of 86.5% and specificity of 100%; cut-off for intratumoral was 6.4, with sensitivity of 89.2% and specificity of 100%; cut-off for peritumoral expression was 14.3, with sensitivity of 67.6% and specificity of

66.7%. By applying the cut-offs, the total, intratumoral and peritumoral immunohistochemical expressions were categorized into low or high expression. Current results showed that there were more subjects with high expression of total (80%), intratumoral (82.5%) and peritumoral (65%) $CD8^+$ T cell expressions (Table 2). Based on Miller Payne grading (Figure 2), mostly subjects were categorized as response (92.5%) (Table 2). Meanwhile based on WHO clinical response, 87.5% of the subjects were categorized as response.

Based on Fisher Exact test, although there was no correlation between total $CD8^+$ T cell expression with age, histopathological grade, immunohistochemical subtype, ER, PR, HER2 and Ki67 (Table 3), the total $CD8^+$ T cell expression was significantly correlated with Miller Payne Grading (p=0.013) (Table 4). Intratumoral $CD8^+$ T cell expression, but not peritumoral $CD8^+$ T cell expression, was significantly correlated with Miller Payne Grading (p=0.015) as well. When the subject distribution was analyzed, the total (p=0.006) and intratumoral (p=0.004) $CD8^+$ T cell expressions were significantly correlated with Miller Payne Grading (Table 5).

Clinical responses based on WHO showed similar results with the ones based on Miller Payne Grading. The total (p=0.009) and intratumoral (p=0.001) $CD8^+$ T cell expressions were significantly correlated with WHO clinical response (Table 6). In regards of subject distribution, the total (p=0.003) and intratumoral (p=0.000) $CD8^+$ T cell expressions were significantly correlated with WHO clinical response as well (Table 7).

DISCUSSION

Earlier breast cancer study in Indonesia reported that higher prevalent of female patients in the age of ≥40 than those in the age of <40 (68.9% vs 31.1%). In addition, women in the age of ≥40 were reported to have an increase of breast cancer risk up to 13.3 times.(17) In the current study,

similar population number was included, 70% of the subjects were aged ≥40. Based on the histopathological appearance, most samples of the current study were categorized as invasive carcinoma with no special type (NST) (82.5%), which also has been reported as the most common histopathological appearance of breast cancer in previous reports.(18,19) From the subject characteristics data, majority of subjects had luminal B type (42.5%), which is in accordance with the breast cancer registry data in Indonesia.(18)

In the current study, there was no correlation between $CD8^+$ T cell expression with age, histopathological appearance, histopathological grade, immunohistochemical subtypes, ER, PR, HER2 and Ki67. Factors related to the CD8+ T cell immune profile were found to be multifactorial, including tumor genetics, germline genetics, microbiomes and pharmacological agents.(20,21) However, there were studies reported that $CD8^+$ T cell expression was correlated with higher histopathological grade, triple negative breast cancer subtype, ER negative, tumor grade and size.(22,23)

In the current study, the total $CD8^+$ T cell expressions was significantly correlated with Miller Payne grading and WHO clinical response. This result is in accordance with previous report showing that tumor infiltrating lymphocytes (TIL) was associated with NAC response.(24) In addition, in the current study, intratumoral $CD8^+$ T cell expressions was significantly correlated with Miller Payne grading and WHO clinical response as well. These results supported the recent report suggesting that intratumoral $CD8^+$ was the potential prognostic marker in breast cancer patient, instead of peritumoral expression.(25) In addition, another study from Indonesia reported that $CD8^+$ might be a predictive factor for clinical response of NAC in breast cancer patients.(13) However, there were also reports suggesting that NAC in breast cancer patients were related with $CD8^+$ T cell expression in both intratumoral and tumor

parenchyma, high $CD8^+$ T cell expression in both areas could result in good clinical response.(21) Taken together, current study has strengthened the importance of total and intratumoral $CD8^+$ T cell expressions for achieving good NAC clinical response based on both Miller Payne and WHO. Nevertheless, further long-term observational study with more numbers of study subjects should be conducted.

CONCLUSION

Total and intratumoral $CD8^+$ T cell expressions are correlated with Miller Payne grading and WHO clinical response of NAC. Therefore, total and intratumoral $CD8^+$ T cell expressions could be suggested as a predictive marker for clinical response of NAC.

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Tables and Figures

Table 1. Subject characteristics (n=40)

Characteristics	n (%)
Age	
≤ 40 years old	12 (30)
≥ 40 years old	28 (70)
Tumor	
T2	1 (2.5)
T3	4 (10)
T4	35 (87.5)
Node	
N0	17 (42.5)
N1	17 (42.5)
N2	3 (7.5)
N3	3 (7.5)
Histopathological appearance	
Invasive NST	33 (82.5)
Lobular	3 (7.5)
Others	4 (10)
Histopathological grade	
Grade 1	3 (7.5)
Grade 2	24 (60)
Grade 3	13 (32.5)
Immunohistochemical subtype	
Luminal A	8 (20)
Luminal B	17 (42.5)
Luminal B & Her-2	12 (30)
Triple negative breast cancer	3 (7.5)
NAC	
Taxane-based	16 (40)
Anthracycline-based	24 (60)
ER	
Negative	3 (7.5)
Positive	37 (92.5)
PR	
Negative	18 (45)
Positive	22 (55)
HER2	

Negative	28 (70)
Positive	12 (30)
Ki67	
Low	13 (32.5)
High	27 (67.5)

NST: no special type; NAC: neoadjuvant chemotherapy; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

Table 2. Total, intratumoral and peritumoral CD8$^+$ T cell expression, Miller Payne grading and clinical response subject distribution (n=40)

Characteristics	n (%)
Total CD8$^+$ T cell expression	
Low	8 (20)
High	32 (80)
Intratumoral CD8$^+$ T cell expression	
Low	7 (17.5)
High	33 (82.5)
Peritumoral CD8$^+$ T cell expression	
Low	14 (35)
High	26 (65)
Miller Payne grading	
No response	3 (7.5)
Response	37 (92.5)
WHO Clinical response	
No response	5 (12.5)
Response	35 (87.5)

Table 3. Subject characteristics *vs.* total CD8$^+$ expression T cell

Characteristics	Total $CD8^+$ expression T cell		*p
	Low n (%)	High n (%)	
Age			
≤40 years old	2 (5)	10 (25)	0.548
>40 years old	6 (15)	22 (55)	
Histopathological grade			
Low grade	6 (15)	21 (52.5)	0.479
High grade	2 (5)	11 (27.5)	
Immunohistochemical subtype			
Luminal	8 (20)	29 (72.5)	0.502
Non-Luminal	0	3 (7.5)	
ER			
Negative	0 (0)	3 (7.5)	0.502
Positive	8 (20)	29 (72.5)	
PR			
Negative	6 (15)	12 (30)	0.065
Positive	2 (5)	20 (50)	
HER2			
Negative	6 (15)	22 (55)	0.548
Positive	2 (5)	10 (25)	
Ki67			
Low	3 (7.5)	10 (25)	0.521
High	5 (12.5)	22 (55)	

*Fisher Exact test; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2

Table 4. Total, intratumoral and peritumoral $CD8^+$ T cell expression *vs.* Miller Payne Grading (no response (n=3) and response (n=37))

Characteristics	Miller Payne Grading		*p*
	No Response (Mean±SD)	Response (Mean±SD)	
Total $CD8^+$ T cell expression	15.80±7.27	40.57±20.98	0.013*
Intratumoral $CD8^+$ T cell expression	3.93±2.88	18.56±12.18	0.015*
Peritumoral $CD8^+$ T cell expression	11.86±5.31	22.00±14.08	0.248

*****Mann-Whitney test, significant at $p<0.05$

Table 5. Subject distribution of total, intratumoral and peritumoral low/high $CD8^+$ T cell expression *vs.* Miller Payne Grading (no response (n=3) and response (n=37))

Characteristics	Miller Payne Grading		*p*
	No Response n (%)	Response n (%)	
Total $CD8^+$ T cell expression			
Low	3 (7.5)	5 (12.5)	0.006*
High	0 (0)	32 (80)	
Intratumoral $CD8^+$ T cell expression			
Low	3 (7.5)	4 (10)	0.004*
High	0 (0)	33 (82.5)	
Peritumoral $CD8^+$ T cell expression			
Low	2 (5)	12 (30)	0.276
High	1 (2.5)	25 (62.6)	

* Fisher Exact test t, significant at $p<0.05$

Table. 6 Total, intratumoral and peritumoral CD8$^+$ T cell expression *vs.* WHO clinical response (no response (n=5) and response (n=35))

Characteristics	WHO Clinical Response		*p*
	No Response (Mean±SD)	Response (Mean±SD)	
Total CD8$^+$ T cell expression	18.92±10.25	41.54±21.01	0.009*
Intratumoral CD8$^+$ T cell expression	4±2.05	19.39±12.00	0.001*
Peritumoral CD8$^+$ T cell expression	14.92±9.77	22.14±14.22	0.357

*****Mann-Whitney test, significant at $p<0.05$

Table 7. Subject distribution of total, intratumoral and peritumoral low/high CD8$^+$ T cell expression *vs.* WHO clinical response (no response (n=5) and response (n=35))

Characteristics	WHO Clinical Response		*p*
	No Response n (%)	Response n (%)	
Total CD8$^+$ T cell expression			
Low	4 (10)	4 (10)	0.003*
High	1 (2.5)	31 (77.5)	
Intratumoral CD8$^+$ T cell expression			
Low	5 (12.5)	2 (5)	0.000*
High	0 (0)	33 (82.5)	
Peritumoral CD8$^+$ T cell expression			
Low	3 (7.5)	11 (27.5)	0.222
High	2 (5)	24 (60)	

*****Fisher Exact test, significant at $p<0.05$

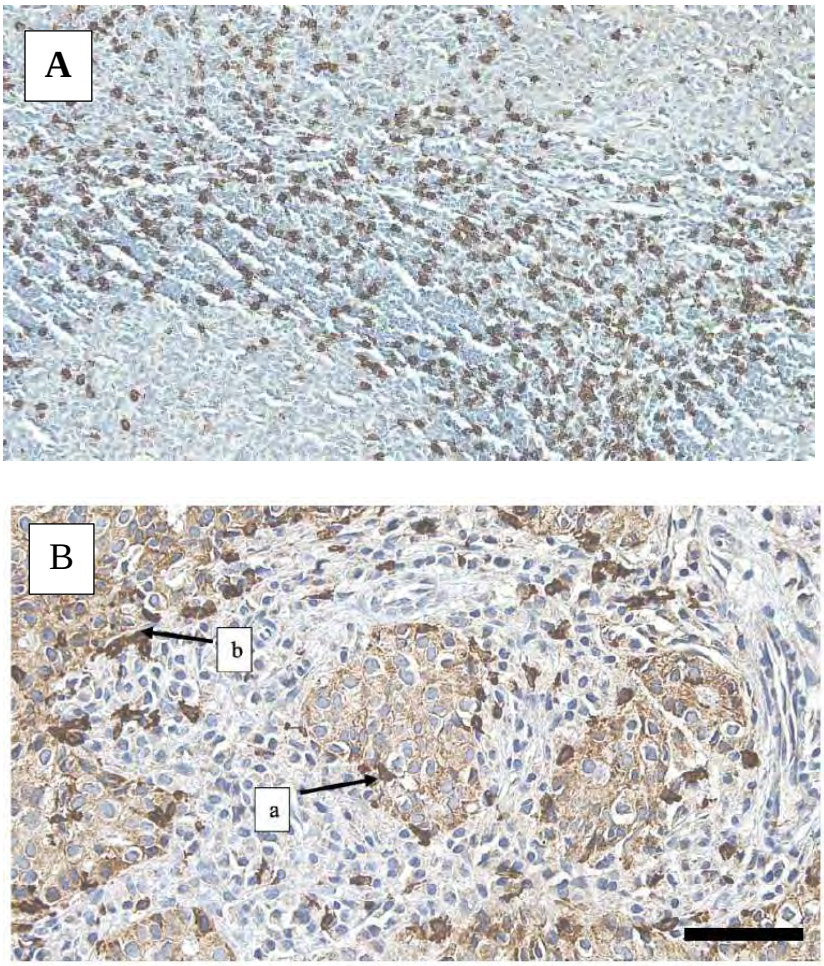

Figure 1. Immunohistochemical expression of CD8. A: tonsil tissue; B: breast cancer biopsy. $CD8^+$ T cells were observed in intratumoral (a) and peritumoral areas (b)**.** Black bar: 100μm.

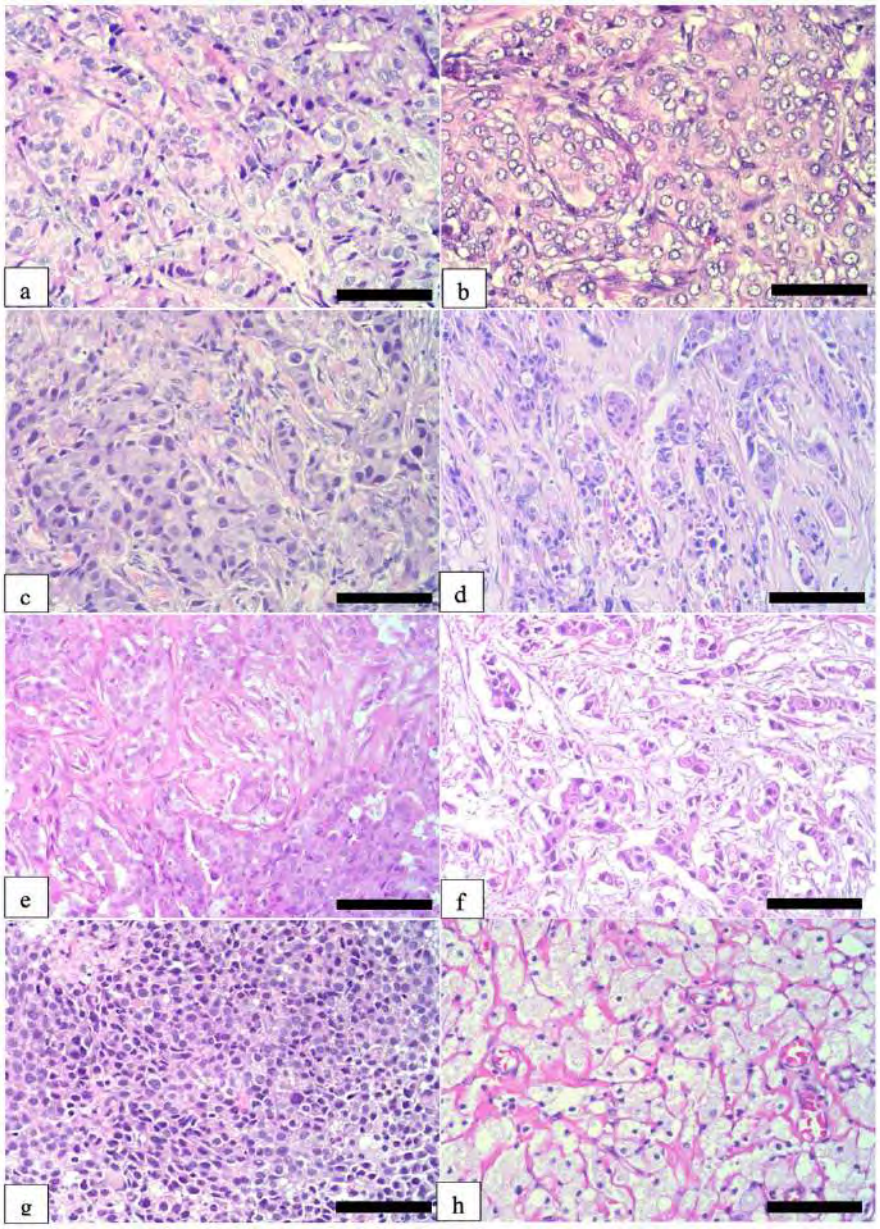

Figure 2. Histopathological expression of biopsy and mastectomy tissue based on Miller Payne grading. Grade 1, from biopsy (a) and mastectomy tissue (b); Grade 2, from biopsy (c) and mastectomy tissue (d); Grade 3, from biopsy (e) and mastectomy tissue (f); Grade 5, from biopsy (g) and mastectomy tissue (h). Black bar: 100 μm.

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[InaBJ] M2022191 Editor Decision - Manuscript Accepted

Secretariat of InaBJ <secretariatinabj@gmail.com> Tue, Mar 28, 2023 at 12:15 PM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Total and Intratumoral CD8+ T Cell Expressions are Correlated with Miller Payne Grading and WHO Clinical Response of Neoadjuvant Chemotherapy**."

Our decision is to: **Accept Manuscript.**

Your manuscript will be sent to our publisher for typesetting and you should receive the proofreading in due course.

Congratulations on your interesting research, and thank you for allowing us to publish this valuable material. Please let us know once you have read this email. We wish you a nice day.

Best Regards,

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