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Relationship between tumor infiltrating lymphocyte CD8+ stromal and intratumoral with grading, Estrogen Receptors (ER) and Progesterone Receptors (PR) expression in invasive breast carcinoma of no special type at Dr. Saiful Anwar General Hospital Malang

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Abstract

Invasive breast carcinoma of no special type (IBC of NST) is a group of malignant epithelial neoplasms of the breast glands with the highest incidence of cancer in women. Tumor infiltrating lymphocytes (TIL) CD8+ is considered a group of T cells that play a role in the reactivity of specific immunity against cancer cells. TIL CD8+ group is a component that plays a role in specific adaptive immunity. TIL CD8+ infiltration in stromal tumor is related to a favorable prognosis and can predict the therapy outcome in patients. This study aims to determine the relationship between TIL CD8+ stromal and intratumoral with grading, ER and PR expression in the IBC of NST. The design of this study is analytical observational using 44 paraffin block samples in patients with IBC of NST at the Anatomical Pathology Department of Dr. Saiful Anwar Malang General Hospital by measuring TIL CD8+ expression on stromal and intratumoral, associated with grading and ER and PR expression. The results of this research showed that there is no significant relationship between histopathological grading with TIL (TIL CD8+ stromal p = 0.264, TIL CD8+ intratumoral p = 0.820), ER expression with TIL (TIL CD8+ stromal p = 0,297, TIL CD8+ intratumoral p = 0,145) and PR Expressions with TIL (TIL CD8+ stromal p = 0.240, TIL CD8+ intratumoral p = 0,003). In conclusion, there is no relationship between TIL CD8+ stromal and intratumoral with grading, expression of ER and PR in patients with IBC of NST.

Keywords: Invasive breast carcinoma of no special type (IBC of NST); Tumor infiltrating lymphocytes (TIL) CD8+ stromal and intratumoral; Grading; ER and PR expression

1. Introduction

Invasive breast carcinoma of no special type (IBC of NST) is a group of malignant epithelial neoplasm of the breast glands, which is one of the causes of mortality in women. In 2018, 2.1 million new cases of breast cancer are diagnosed, with a mortality rate of 627,000 worldwide. In the case of IBC of NST, there is abnormal proliferation of malignant cells that infiltrate the basal membrane and stroma. [1,2]

Breast carcinoma lymphocyte infiltration is important in determining whether the tumor is aggressive or not. High tumor infiltrating lymphocyte (TIL) indicates a better prognosis for tumor. Cytotoxic CD8+ T lymphocyte cells (TIL CD8+) is an important component of tumor-specific cellular adaptive immunity that attacks tumor cells by presenting tumor-related antigen peptides against the class I main histocompatibility complex on their surface. Subsequently, TIL CD8+ produces interferon gamma after interact with the tumor target. The mechanism of successive formation of

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interferon gamma occurs through cell cycle inhibition, apoptosis, angiostasis, and induction of tumoricidal activity of macrophage. [4,5,8]

ER: Estrogen Receptor; PR: Progesterone Receptor

Estrogen receptors is a nuclear transcription factor that, when activated by the hormone estrogen, stimulates the growth of normal epithelial cells. Proliferation may also be activated in the cells of invasive breast cancers expressing ER which of course is detrimental. ER is a strong predictive factor for response to hormonal therapies. PR is also routinely assessed by immunohistochemistry in invasive breast cancer. ER regulates the expression of PR, so the presence of PR usually indicates that the ER pathway is intact and functional.[3]

Many studies have examined the relationship between the expression of Tumor Infiltrating Lymphocytes and clinicopathology in breast cancer of both luminal and Triple Negative Breast Cancer (TNBC) subtypes. [6,7,9] However, the results obtained are still very varied and remain controversial. Ghebeh et al.[14] showed that high CD4+ and CD8+ lymphocyte infiltration has been associated with positive lymphnode status and worse overall survival rate, but another study by Macchetti et al. [15] found otherwise.

In this study, we want to determine the relationship between TIL CD8+ stromal and TIL CD8+ intratumoral with grading, ER, PR expression in patients with IBC of NST.

2. Material and methods

2.1. Research Design

The design of this study is analytical observational study carried out at the Anatomical Pathology Department in Dr. Saiful Anwar Malang General Hospital using the IBC of NST paraffin block from January 2018 to March 2020. This study used primary and secondary data. Primary data are obtained from CD8+ expression by immunohistochemical method, while secondary data obtained from tissue preparations from patients who had been examined for ER and PR. Sample selection is carried out by random sampling in groups with ER and PR expressions with a total sample of 44 cases. The independent variables are histopathological grading and the expression of stromal and intratumoral TILs in the cases group. The dependent variables are ER expressions and PR expressions. The inclusion criteria of the study are preparations with a histopathological diagnosis of IBC of NST that has been smeared ERr and PR with the immunohistochemical method, preparations derived from biopsy specimens and/or IBC of NST surgery or mastectomy, paraffin blocks are still stored and/or available in the Anatomical Pathology Department of Dr. Saiful Anwar Malang General Hospital with complete medical record data.

If the paraffin block and preparations from chemotherapy post neoadjuvant is damaged, it is excluded from the study. This research has been declared to have passed the ethics review by the Health Research Ethics Committee of Dr. Saiful Anwar Malang General Hospital (Number: 400/053/ K.3/302/2021).

2.2. CD8+ starters by Immunohistochemical Method

This study is conducted on paraffin blocks of IBC of NST that have been smeared with ER and PR by immunohistochemistry methods. The paraffin block has been cut and pasted at poly-L-lysine coated slide and then deparaffined in xylol and stratified alcohol with a decreased concentration, rinsed with flowing water, then soaked in Biocare peroxide block for 5 minutes. The preparation is put into a staining jar containing the Diva solution, then the staining jar containing this preparation is put in a decloacking chamber until reaching 95 ^{o}C for 40 minutes. Then the preparation is removed from the decloacking chamber and waited to cool down, for 20 minutes, then rinsed with aquabides and soaked with PBS for 5 minutes. The preparation is dried and then dripped with primary antibodies to cover the entire surface of the preparation and incubated for 30 minutes at room temperature. Then the preparation rinsed with PBS and then dripped with polymer and incubated for 30 minutes at room temperature. The preparation is then rinsed with PBS and then dripped with Biocare DAB chromogen and incubated for 5 minutes at room temperature. The preparation is then rinsed in flowing water and counter staining is also done using hematoxylin for 2 minutes then continued with lithium carbonate for 30 seconds and rinsed by flowing water. The preparation is then soaked in alcohol with a stratified concentration for 3 minutes. The preparation is then soaked in xylol solution for clearing, then the preparation is dried and covered with enthelan and cover slip.

2.3. Expression Evaluation CD8+ stromal and intratumoral

Assessment of CD8+ T lymphocyte expression at the membrane and cytoplasm of the cell is carried out semiquantitatively with a binocular light microscope at a low magnification (40x) to select areas with high density. TIL CD8+ calculation based on the criteria namely in stromal tumors (defined as T lymphocyte cells around the stroma of tumor cells) and intratumorally (defined as T lymphocyte cells in the group of tumor cells). Furthermore, each of these areas is calculated the total amount of TIL CD8+ from an average of five field of views at a magnification of 400x (HPF), on the stromal area of the tumor (the area outside of the tumor cell island) and intratumorally, regardless to the intensity of the smearing using the application of cell counting and divided by five to determine the average value. Furthermore, the two categories of stromal and intratumoral are categorized into three groups, namely group A (low TIL) = 0% -≤10% stroma/intratumoral TIL; group B (intermediate TIL) = >10% -≤40% stroma/intratumoral TIL; and group C (high TIL) = >40% - ≥90% stroma/intertumoral TIL.

2.4. ER PR Expression

ER expression is said to be positive if ≥ 1% positive staining tumour cells. PR expression is said to be positive if ≥ 1% positive staining tumour cells. [3]

2.5. Grading

Histopathologic grading was performed based on the gradation of changes in the shape and cells characteristic and tissue architecture obtained from biopsy preparations. The method used is semi-quantitative where the grade is divided into three groups: grade I, grade II, and grade III. Grade assessment was calculated from the total score of tubule formation, nuclear pleomorphism, and number of mitoses. In tubule formation, tubules >75% are worth 1 point, 10-75% are worth 2 point, and <10% are worth 3 point. Core pleomorphism is assessed if mild is worth 1 point, moderate 2 point, and heavy 3 point. Mitotic count <11 are worth 1 point, 12-23 are worth 2 point, and >24 are worth 3 point. The three criteria are summed. Grade 1 with scores 3-5, grade 2 with scores 6-7, and grade 3 with scores 8-9. [3,10]

2.6. Data Analysis

Data analysis using SPSS is used to determine the relationship between TIL CD8+, ER and PR expressions using Spearman's correlation test. Statistical tests are meaningful if p value < 0.05.

3. Results

3.1. Characteristics of Research Samples

This study consisted of 44 samples. Based on the histopathological grading, the highest number is grade 2 as many as 21 cases (47.7%) and grade 3 as many as 21 cases (47.7%). For CD8 stromal out of 44 patient samples, the most TILs is in the intermediate category (68.2%), 11.4% classified as Low and 20.5% in the high category. As for intratumoral CD8 out of 44 patient samples, the most TILs is in the Low category (86.4%), 13.6% are classified as intermediate, and there are no samples with the High category. For ER, 53,5% are classified as positive, and for PR, 45% are classified as positive. (Table 1)

3.2. Relationship between TIL CD8+ expression with Histopathological Grading

The results showed that there is no relationship between TIL CD8+ stromal expressions ($r = 0.172$; $p = 0.264 > 0.05$) and intratumoral ($r = 0.035$; $p = 0.820 > 0.05$) with histopathological grading (Table 2). Figure 2 showed the TIL CD8+ stromal and TIL CD8+ intratumoral expression in Invasive Breast Carcinoma of No Special Type based on histopathological grading. (Table 2)

3.3. Relationship between ER expression and stromal CD8

The results of the correlation test between ER and the CD8 stromal category (low, intermediate, high) obtained a spearman correlation coefficient value of 0.145 with a p value of 0.352 ($p>0.05$), so it can be concluded that there is no significant relationship between ER and the stromal CD8 category (low, intermediate, high). In other words, positive or negative ER is not related to the stromal CD8 category (low, intermediate, high). (Table 3)

3.4. Relationship between PR expression and stromal CD8

The results of correlation testing between PR and the stromal CD8 category (low intermediate, high) obtained a spearman correlation coefficient value of 0.003 with a p value of 0.987 ($p>0.05$), so it can be concluded that there is no

significant relationship between PR and the stromal CD8 category (low, intermediate, high). In other words, positive or negative PR is not related to the stromal CD8 category (Low, Intermediate, high). (Table 3)

Table 1 Demographic Characteristic

Demographic Characteristic	Frequency	Percentage
Usia (tahun) mean±Standar dev.	52.80	±11.14
30-39 thn	4	9.1%
40-49 thn	16	36.4%
50-59 thn	13	29.5%
60-69 thn	7	15.9%
70-79 thn	4	9.1%
Grade		
1	2	4.5%
2	21	47.7%
3	21	47.7%
CD8 stromal (%) mean± SD	28,34	±16.48
Low	5	11.4%
Intermediate	30	68.2%
High	9	20.5%
CD8 intratumoral (%) mean± SD	4,40	±3.54
Low	38	86.4%
Intermediate	6	13.6%
High	0	0.0%

Table 2 Relationship between histopathological grading and TIL CD8+ stromal expression and TIL CD8+ intratumoral expression

TIL CD8+ Stromal Expression		TIL CD8+ Intratumoral Expression
Grading	r = 0.172	r = 0.035
	p = 0.264	p = 0.820
	N = 44	N = 44

Table 3 Relationship of estrogen and progesterone expression with stromal TILs

	Low (n=5)		Intermediate (n=30)		High (n=9)		Correlation Coefficient Value	P value
	Freq	%	Freq	%	Freq	%		
ER								
(-)	4	80,0%	12	41,4%	4	44,4%	0.145	0.352
(+)	1	20,0%	17	58,6%	5	55,6%		
PR								
(-)	4	80,0%	13	46,4%	5	71,4%	0.003	0.987
(+)	1	20,0%	15	53,6%	2	28,6%		

Table 4 Relationship of estrogen and progesterone expression with intratumoral TILs

	Low (n=38)		Intermediate (n=6)		High (n=0)		Correlation Coefficient Value	P value
	Frek	%	Frek	%	Frek	%		
ER								
(-)	10	32,3%	4	66,7%	0	0%	-0,163	0,297
(+)	21	67,7%	2	33,3%	0	0%		
PR								
(-)	18	51,4%	4	80,0%	0	0%	-0,190	0,240
(+)	17	48,6%	1	20,0%	0	0%		

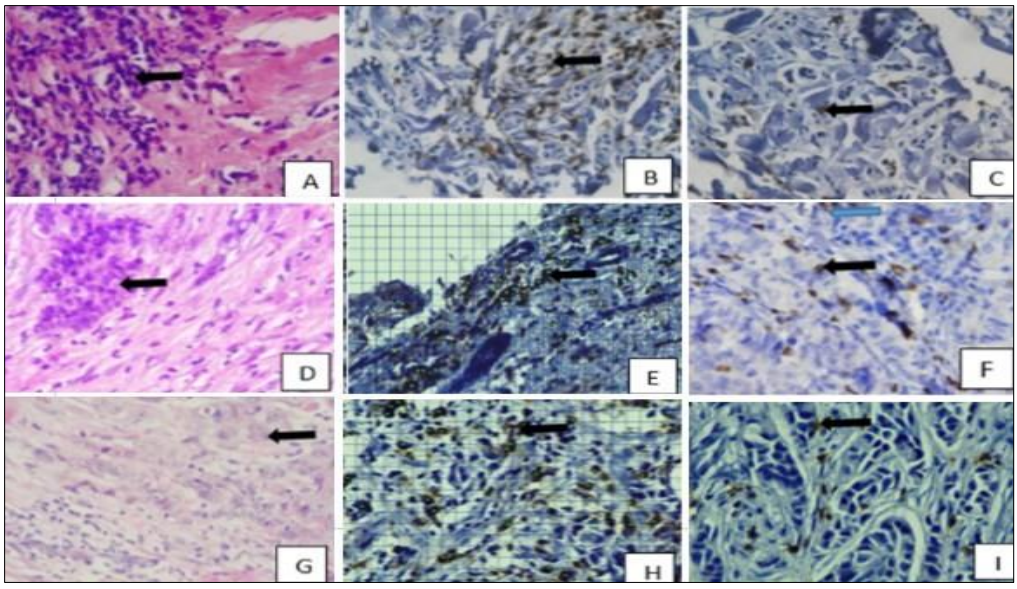

Figure 1 Histopathological image of Invasive Breast Carcinoma of No Special Type based on histopathological grading with TIL CD8+ stromal and TIL CD8+ intratumoral expression. Description: (A). IBC of NST grade 1 (black arrow) (HE, 400x), (B). Distribution of TIL CD8+ stromal expressions on IBC of NST grade 1 (black arrow) (CPI, 400x) (C). Distribution of TIL CD8+ intratumoral expressions in the IBC of NST grade 2 (black arrow) (CPI, 400x), (D). IBC of NST grade 2 (black arrow) (HE, 400x), (E). Distribution of TIL CD8+ stromal expressions on IBC of NST grade 2 (black arrow) (CPI, 400x), (F). Distribution of TIL CD8+ intratumoral expressions on the IBC of NST grade 2 (black arrow)

(CPI, 400x), (G). IBC of NST grade 3 (black arrow) (HE, 400x), (H). Distribution of TIL CD8+ stromal expressions on IBC of NST grade 3 (black arrow) (CPI, 400x), (I). Distribution of TIL CD8+ intratumoral expressions at IBC of NST grade 3 (black arrow) (CPI, 400x)

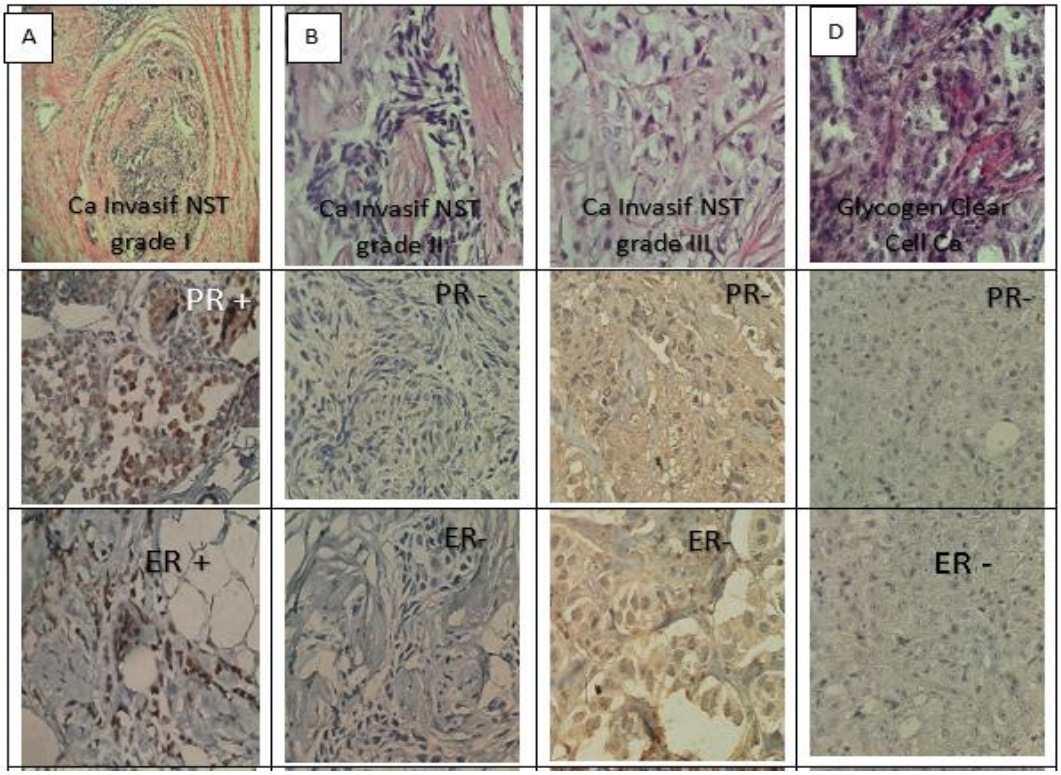

Figure 2 Histopathological findings based on grade compared with immunohistochemical findings. A. *Invasive carcinoma of No Special Type grade I* (40x,HE ; *Hematoxylin-Eosin*), *ER(+)* (100x HE) , *PR(+)* (100x HE), *HER2 equivocal* (100x HE), B. *Invasive carcinoma of No Special Type grade II, ER(-)* (100x HE), *PR(-)*(100x HE), *HER2(-) (*100x HE). C. *Invasive carcinoma of No Special Type grade III, ER(-)* (400x HE), *PR(-)* (400x HE), *HER2(+)* (400x HE) D. *Glycogen Clear Cell carcinoma ER(-)* (400x HE), *PR(-)* (400x HE), *HER2(+)* (400x HE)

3.5. Relationship between ER and intratumoral CD8 expression

The results of the correlation test between ER and the inner CD8 category (low, intermediate, high) obtained a spearman correlation coefficient value of -0.163 with a p value of 0.297 (p>0.05), so it can be concluded that there is no significant relationship between ER and the intratumoral CD8 category (low, intermediate, high). In other words, positive or negative ER is not related to the intratumoral CD8 category (low and intermediate). (Table 4)

3.6. Relationship between PR and intratumoral CD8 expression

The results of the correlation test between PR and the intratumoral CD8 category (low, intermediate, high) obtained a spearman correlation coefficient value of -0.190 with a p value of 0.240 (p>0.05), so it can be concluded that there is no significant relationship between PR and the inner CD8 category (low, intermediate, high). In other words, positive or negative PR is not related to the inner CD8 category (low and intermediate). (Table 4)

4. Discussion

4.1. Relationship between TIL CD8+ stromal and intratumoral Excretion with Histopathology Grading

In this study, there is no relationship between grade 1, grade 2, and grade 3 with the TIL CD8+ stromal and intratumoral expression category (low, inter- mediate, high). This results are supported by other studies which showed that there is no clinicopathological relationship (histopathological grading, stage and involvement of lymph node) in breast, ovarian, and pancreatic cancers. [8] This reflects the clinical quantification of TIL is less relevant to this type of cancer. There is a difference between the types of cancer in the anti-immune ability which means it efficiently protects the cancer cells from CD8+ cytotoxic T cells, but instead there is a significant relationship between the density of TIL CD8+ in the

colorectal cancer and gastric cancer which is more favorable with longer survival and reduced incidence rate of distant metastases. [8] In a research of the relationship between TIL expression and clinicopathology in triple negative breast cancer (TNBC) showed that high TIL expression is associated with smaller tumor size, but is not significantly associated with grading or staging. [9,13]

The clinical significance of TIL in breast cancer remains controversial. The results of previous studies showed that high CD4+ and CD8+ lymphocyte infiltration is associated with the status of positive lymph nodes infiltrated by tumor cells related with worse survival. But other studies have found the opposite, they found that high immune cell infiltration is associated with better survival. This could be due to no standard consensus available to analyze TIL calculations because TIL can be calculated from stromal and intratumoral. [11,12]

4.2. Relationship of stromal and intratumoral CD8+ TIL expression with ER and PR expression

The correlation test between ER and PR expression with TILs CD8+ stromal (low, intermediate, high) and TILs CD8+ intratumoral (low, intermediate, high) showed no significant relationship. This is similar to other studies that showed no relationship with breast cancer expressing ER and PR and no significant relationship between TIL stromal and intratumoral hot spot locations. This could be due to non-immunogenic tumor cells and a larger sample is needed to see the correlation. [6,7]

Many studies have linked TILs to the molecular subtype status of the breast. [6,7] Luminal A is the least malignant subtype of breast cancer and the prognosis tends to be good. In contrast, TNBC is the most malignant subtype and the prognosis of patients tends to be poor despite resection. The variability in TILs can also be explained from the above description. Luminal A subtype is the subtype with the lowest mutation burden and overall still has the same molecular pattern as normal luminal cells. [7,16,17] In contrast, Triple Negative Breast Cancer (TNBC) has twice the mutation burden of Luminal A so that it has more neoantigens that can induce anticancer immune responses. [7,16,17] In addition, genomic instability is also often found in TNBC which further increases the mutation rate and apoptosis which increases the exposure of neoantigens to antigen-presenting cells (APCs). [7,16,18]

The more neoantigens that are produced by the mutations experienced by the cancer, the greater the immune response [7,19]. The high TIL in TNBC can also be explained because TNBC has a higher mutation rate compared to non-TNBC tumors. TNBC has a specialized immune microenvironment. TNBC subtypes were found to have the highest FOXP3+ regulatory T cells (Treg) compared to other BC types. Theoretically, the greater the relationship of Tumor Infiltrating Lymphocytes expression, [20] the greater the mutational burden of the cancer, the more neoantigens it has that are produced by the mutations experienced by the cancer, so subsequently the greater the immune response generated [7,19]

Immunogenicity of tumor cells is also an important consideration so that further examination to determine the immunonegenity of tumor cells and immunohistochemical detection of the different lymphocyte subtypes (including cytotoxic and regulatory T cells, or B/plasma cells) would have been more informative. [21]

5. Conclusion

This study concluded that there is no association between TIL CD8+ expression with grading and expression of ER and PR in patients with invasive breast carcinoma (Invasive Breast Carcinoma of No Special Type).

Suggestion

There is a need for further research with a larger number of samples for each histopathological grading group equipped with other prognostic factors and further research is needed to see the host immune response as a regulator of tumor cells. Immunogenicity of tumor cells is also an important consideration so that further examination to determine the immunonegenity of tumor cells.

A complete immunohistochemistry panel including HER2 and Ki67 status is needed, so that tumors can be classified by molecular subtype. Examination of other lymphocyte subtypes (including cytotoxic and regulatory T cells, or B/plasma cells) would have been more informative.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors. Studies using paraffin blocks do not require ethical approval, in accordance with the regulation implemented by Research Ethics Committee of Saiful Anwar General Hospital.

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