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Correlation of CD8+ Expression, Foxp3+ Expression, and CD8+/Foxp3+ Ratio with Triple Negative Breast Cancer Stage in Sanglah General Hospital

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Abstract

BACKGROUND: Triple negative breast cancer (TNBC) is a breast cancer sub-type that lacks ER, PR and HER-2 expression. This type tends to be more aggressive than other types of breast cancer, with poor prognosis, distant metastases, higher recurrence rate, and lower overall survival. The TNBC is resistant to hormonal therapy, but generally very susceptible to chemotherapy. Expression of CD8+ and Foxp3+ were parts of the TIL, which often found in TNBC as an immune response to tumour antigens following antigens presenting cell (APC) stimulation.

AIM: This study was conducted to find out whether the expression of CD8+, Foxp3+ and CD8+/Foxp3+ ratio was associated with the stage of TNBC.

METHODS: This cross-sectional study was conducted from January 2014 until December 2016 at Sanglah Hospital with 46 research subjects. Two paraffin blocks were prepared for each sample to examine the CD8+ expression and Foxp3+ expression. Data were analysed using the Chi-Square test or Fisher's Exact tests as an alternative for bivariate analysis and logistic regression for multivariate analysis.

RESULTS: On bivariate analysis, we found a low of CD8+ expression in advanced stage ($p < 0.001$ with OR 3.5; CI 1.611-7.727). Expression Foxp3+ in advanced stage ($p = 0.482$; OR 0.8; CI 0.497-1.374), while the ratio of CD8+/Foxp3+ ($p = 0.213$; OR 2.2; CI 0.650-7.132). On multivariate analysis, a low of CD8+ expression (adjusted OR 16.5; CI 3.735-7.370; $p < 0.001$) was obtained.

CONCLUSION: Low expression of CD8+ was associated with the advanced stage of TNBC. The risk of becoming an advanced stage in TNBC patients with low CD8+ expression was 16.5 times higher than those with high of CD8+ expression. High expression of Foxp3+ was not associated with an advanced stage of TNBC. The low CD8+/Foxp3+ ratio was not associated with the advanced stage of TNBC.

Introduction

Triple-negative breast cancer (TNBC) is one of the subtypes of breast cancer that has a poor prognosis for free survival disease and shorter survival overall. The highest prevalence is in African-American women. Until now, there is no specific targeting therapy for TNBC. Significant overlap found in BRCA 1 associated breast cancer with TNBC phenotype [1].

TNBC is aggressive and has higher mortality due to metastases and has a high character of cell proliferation, poor cell differentiation, and in many cases, mutations in the TP53 tumour suppressor gene. TNBC often occurs at a younger age (< 40 years) [2]. TNBC recurrence increases in the first three years after diagnosis and decreases after eight years [3], and the mortality rate will increase within 5 years after diagnosed [4].

The poor prognosis of TNBC is related to tumour grading, lymph node status, tumour size and management [1]. TNBC sufferers also tend to have lower life expectancies but have a better chemotherapy response [5].

The potential mechanism that causes higher lymphocyte infiltration in TNBC because TNBC is a subtype of breast cancer that is immunogenic

because of genetic instability and increased mutation and expressing certain proteins [6]. An increase in tumour infiltrating lymphocytes (TIL) in TNBC is associated with increased disease-free and overall survival and complete pathologic response (pCR) on neoadjuvant therapy [6], [7].

High pathological complete response (pCR) values after neoadjuvant chemotherapy have been reported in patients with high levels of expression of TIL components such as CD3+, CD4+ and CD3+, CD8+, and Forkhead box protein 3 (Foxp3+). High pCR values in TNBC were also reported with a high CD8+/Foxp3+ (CFR) ratio [8].

Tumour infiltration by CD8+ can control tumour growth with cytotoxic effects on tumour cells. CD8+ expression is associated with a better prognosis if found in large quantities. Almost 20% of TNBC expresses strong TIL and if the amount of TIL is more in the tumour stroma is associated with a higher likelihood of healing in the early stages of TNBC. This presents that high CD8+ is associated with the early stages of TNBC sufferers [9].

There is a strong relationship between Foxp3+ with the development and progression of cancer. Some research evidence shows Foxp3+ effectively protects tumours from our body's immune response. Foxp3+ in tumours, ascites and peripheral blood of cancer patients was stated to be associated with a poor prognosis.[10] This presented that high Foxp3+ was associated with advanced stages of TNBC sufferers. The high ratio of CD8+/Foxp3+ T cells can predict that the clinical outcome is better in TNBC patients [11]. This can illustrate that the high ratio of CD8+/Foxp3+ T cells is associated with early stages in TNBC patients.

Many studies linking CD8+, Foxp3+ expression and CD8+/Foxp3+ ratio with complete pathological responses (pCR) to neoadjuvant therapy on TNBC patients made us want to know how the relationship of CD8+, Foxp3+, and CD8+/Foxp3+ expression to TNBC patients at Sanglah General Hospital Denpasar.

Methods

We conducted a cross-sectional study of 46 samples of TNBC patients (fulfilling inclusion and exclusion criteria) recorded from January 2014 to December 2016 at Sanglah General Hospital. Furthermore, the data is taken from the medical record to find out the age and clinical stage of TNBC sufferers as well as from the Anatomical Pathology report obtained from all TNBC patients who underwent a diagnostic procedure with biopsy or surgery. All operating specimens are then processed in paraffin blocks. The subsequent examination was performed by immunohistochemistry to determine CD8+ and Foxp3+ expressions.

The collected data were analysed using the Chi-Square test or Fisher's Exact test as an alternative for bivariate analysis and logistic regression for multivariate analysis.

Results

Characteristics of the 46 research subjects that we obtained can be seen in Table 1. The data on the subject of this study showed an age range between 31-75 years, with an average age of 47.6 years. The age grouping in this study was the early age group (< 40 years) and the advanced age group (> 40 years). From the table, it can be seen that the early age group is 13 (28.3%), and the elderly group is 33 (71.7%). Grading in this study was high grading groups as much as 28 (60.9%), low grading groups as many as 14 (30.4%) and unknown groups grading as many as 4 (8.7%). The clinical stage, which is the dependent variable in this study, is divided into 2, namely the advanced stage group (stage III and IV) and the early stage group (stage I and II). From the table, it was found that the advanced stage group was 26 (56.5%), and the early stage group was 20 (43.5%). For the low CD8+ expression group, there were 25 (54.3%), and the number of high CD8+ expressions was 21 (45.7%).

Whereas for the high Foxp3+ expression group, 32 (69.6%) and low Foxp3+ expressions were 14 (30.4%). The low CFR group (CD8+/Foxp3+) ratio was 39 (84.8%) while the high CFR group was 7 (15.2%). Based on the results of the bivariate analysis (Table 2), the low CD8+ expression group found at an advanced stage was 84.0% while in the high CD8+ expression group at an advanced stage as much as 23.8%. Based on statistical tests using the Chi-Square test showed that the relationship was significant ($p < 0.001$) with a 95% confidence interval of 1.611-7.727.

Table 1: An Overview of the characteristic of the research subjects

Variable	n = 46
Age (year), average ± SD	47.6 ± 11.5
≤ 40 years	13 (28.3)
> 40 years	33 (71.7)
Grading	
High	28 (60.9)
Low	14 (30.4)
Unknown	4 (8.7)
Stage	
Advanced (III-IV)	26 (56.5)
Early (I-II)	20 (43.5)
CD8+	
Low	25 (54.3)
High	21 (45.7)
Foxp3+	
High	32 (69.6)
Low	14 (30.4)
CFR (Rasio CD8+/Foxp3+)	
Low	39 (84.8)
High	7 (15.2)

The high Foxp3+ expression group was found at an advanced stage of 53.1% and 64.3% in the Foxp3+ low expression group. Based on statistical tests using the Fisher Exact test, the relationship was not significant (p = 0.482) with a 95% confidence interval of 0.497-1.374.

Table 2: Bivariate analysis of CD8+, Foxp3+, and CFR expressions (CD8+/Foxp3+ ratio) with clinical stage

Variable	Clinical Stage		OR	95%	P Value
	Advanced	Early			
CD8+					
Low	21 (84.0)	4 (16.0)	3.5	1.611-7.727	< 0.001[a]
High	5 (23.8)	16 (76.2)			
Foxp3+					
High	17 (53.1)	15 (46.9)	0.8	0.497-1.374	0.482[b]
Low	9 (64.3)	5 (35.7)			
CFR					
Low	24 (61.5)	14 (38.5)	2.2	0.650-7.132	0.213[b]
High	2 (28.6)	5 (71.4)			

[a]Uji Chi-Square; [b]Uji Fisher's Exact.

In the low CFR expression group at an advanced stage as much as 61.5% while in the high CFR expression group as much as 28.6%. But based on statistical tests using the Fisher Exact test, the relationship was not significant (p = 0.213) with a 95% confidence interval of 0.650-7.132.

Based on the bivariate analysis, it is necessary to continue the multivariate analysis to assess the pure effect of each variable. The variables included in the multivariate test are age variables and CD8+ expressions. Grading variables are not included because the data is missing so that it does not represent in its entirety (Table 3). Based on the table, there is a pure relationship between low CD8+ expression and advanced stage in TNBC patients with adjusted OR 16.5 after calculating age variables.

Table 3: Multivariable Analysis of CD8+ Interaction with Clinical Stages after controlling the age

Variable	*Adjusted* OR	95% CI	p-value
Low CD8+	16.5	3.735 – 7.370	< 0.001
Age	0.9	0.182 – 4.753	0.930

Discussion

The TNBC breast cancer subtype compared to other breast cancer subtypes is associated with younger age (< 40 years) when diagnosed [2], [12], [13]. The earlier the age of cancer patients the greater the influence of internal factors on the occurrence of malignancy compared to external factors, especially there is a delay in diagnosis and has been found in an advanced stage, the prognosis is getting worse.[14] In this study, the whole study subjects with TNBC were found to be older (> 40 years), namely 71.7% compared to young age (28.3%). Besides that, there were more advanced groups from the early stage group (56.5% vs 43.5%). This may be due to the delay of the patient in examining the health service centre.

In breast cancer, grading is a prognostic factor where high grading has more aggressive behaviour and poor prognosis; the recurrence rates are four times more than low grading [4]. The poor prognosis of TNBC is related to tumour grading, lymph node status, size tumour, and management [1]. In this study, grading was included as a confounding variable. The above is by this study where high grading was 60.9%; low grading was 30.4% while unknown grading was 8.7%.

From the characteristics of the research subjects above, the stadium obtained more groups than the early group stadiums. This means that TIL, in this case, is CD8+ with a lower expression more than high CD8+ expression. This is consistent with the results of a study that cites CD8+ low (54.3%) more than high CD8+ expression (45.7%). Meanwhile, for Foxp3+, according to the characteristics of the subject of this study, Foxp3+ groups (69.6%) were higher than Foxp3+ low expressions (30.4%). Likewise, from the CFR group (CD8+/Foxp3+ ratio), there was a lower CFR group (84.8%) more than the high CFR group (15.2%).

In the advanced stage, it was found that the low CD8+ expression group was 84.0% while the high CD8+ expression group was 23.8%. This shows that low CD8+ expression at an advanced stage is not capable of carrying out its function as surveillance by recognising and killing malignant cells that express peptides produced by mutant cell proteins or oncogenic viral proteins presented through MHC class I [15].

There is a pure relationship between low CD8+ expression and advanced stage (p < 0.001) in TNBC patients with adjusted OR 16.5 which means that the risk of TNBC patients with low CD8+ expression is 16.5 times higher than TNBC patients with high CD8+ expression. This is consistent with research that says that nearly 20% of TNBC expresses strong TIL and if the amount of TIL is more in the tumour stroma is associated with a higher likelihood of healing in the early stages of TNBC [9].

The distribution of Foxp3+ expression at an advanced stage showed that the high Foxp3+ expression (53.1%) was lower than Foxp3+ low expression (64.3%) and statistical analysis of Foxp3+ high expression with an advanced stage was not proven to have a significant relationship (p = 0.482). This is different from previous studies which stated that there was an increase in the appearance of CD4+, CD25+, Foxp3+ T Cell in malignancies such as the lungs, head and neck, ovary, gastrointestinal, and skin [10]. This shows that at an advanced stage, Foxp3 + expressions should be higher than low Foxp3+ expressions.

The causes of the absence of such

relationships include two hypotheses. First, as it is known that tumour progression is not only influenced by Foxp3 +, but there are also other influential factors such as CTLA4 and PD-1. T cell activity requires antigen recognition by TCR and the introduction of costimulatory, mainly B7 by CD8+. CTLA-4 resembles CD8+ receptor activity, is bound to B7 molecules. CTLA-4 has a greater affinity ability than CD8+ to B7 family receptors thus preventing B7 costimulatory in APC from CD8+ bond, then produces a signal that inhibits T cells which cause the non-working interaction of APC-complex T cells. CTLA-4 will cause no T cell sensitisation, T cells become anergic, and even T cell apoptosis can occur. Ultimately energy from T cells will correlate to the degree of differentiation and progression of cancer cells [15]. Other receptor blockers on CD8+ family are PD-1 (Programmed Cell Death 1). The use of PD-1 by other ligands leads to T cell inactivity. It is said that CTLA-4 main function is to control initial T cells that are active in lymphoid organs while PD-1 plays an important role in limiting the response to effector cell differentiation in peripheral tissues [15]. Second, in the early stages, higher Foxp3+ expressions were more found than Foxp3+ low expressions. This is probably because Foxp3+ circulates a lot in the peripheral circulation but is mostly in FoxP3+ naive T cell conditions, so it has not carried out its activities to inhibit CD8. This is by the results of the study that in the circulation of the Treg cell periphery, it is 5-10% naive CD4+ T cell [16].

In the advanced stage, the CFR group was low (61.5%) while in the CFR group it was high (28.6%), but the statistical test showed that the relationship was not significant (p = 0.213). This is because in the calculation of the CFR there are 2 independent variables, namely CD8+ and Foxp3+. The Foxp3 variable statistically did not show a significant relationship causing the results of the CFR to be meaningless.

In conclusion, low CD8+ expression is associated with advanced stages of TNBC sufferers. High Foxp3+ expression is not associated with advanced stages of TNBC sufferers. Low CD8+/Foxp3+ ratio is not associated with advanced stages of TNBC sufferers.

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