ABSTRACT

**Background:** Triple Negative Breast Cancer (TNBC) is one of the breast cancer subtypes with an aggressive clinical course and has a limited therapeutic strategy. Until now, conventional chemotherapy is still used as standard therapy, although it still has an inadequate therapeutic response. One of the chemoresistance mechanisms is likely due to the immunosuppressive effect of CD73-derived adenosine. This study aims to evaluate the high CD73 expression as a negative predictor of clinical neoadjuvant chemotherapy response in TNBC.

**Methods:** This study was a retrospective case-control study. A total of 46 TNBC patients who received neoadjuvant chemotherapy at Prof Dr. I.G.N.G Ngoerah's Hospital were enrolled. Twenty-three patients with positive neoadjuvant chemotherapy responded as the control group and 23 with negative neoadjuvant chemotherapy responded as the case group. Evaluation of CD73 expression was carried out by immunohistochemistry from a biopsy taken before the patient underwent neoadjuvant chemotherapy. CD73 expression was categorized into low and high based on staining intensity and percentage of the stained tumor cells. Data were analyzed using SPSS version 25.0 for Windows.

**Results:** Analysis showed a significant relationship between CD73 expression and clinical neoadjuvant chemotherapy response (OR=4.41; 95% CI=1.26-15.41; p=0.017). Multivariate analysis showed CD73 expression (AOR=4.88; 95% CI=1.08-21.99; p=0.039) and T stage (AOR=18.82; 95% CI=1.83-193.00; p=0.013) simultaneously affect clinical neoadjuvant chemotherapy response.

**Conclusion:** It can be concluded that high expression of CD73 and T stage is an independent predictor of negative clinical neoadjuvant chemotherapy response in TNBC patients.

**Keywords:** TNBC, chemotherapy, CD73, Immunohistochemistry.


INTRODUCTION

Triple Negative Breast Cancer (TNBC) covers 15–20% of all breast cancer cases and generally occurs in premenopausal women.\(^3\) TNBC tends to be more aggressive and has limited therapeutic options; thus, it is often diagnosed at an advanced stage.\(^2\) Currently, treatment of TNBC is still very challenging, where chemotherapy is still the main therapeutic option due to the absence of targeted therapy.\(^3\) Despite the more aggressive behavior, approximately 30–40% of TNBC patients obtained pathological Complete Response (pCR) after administration of neoadjuvant chemotherapy. This pCR rate is higher compared to patients with positive hormone receptors, yet strangely TNBC patients have a worse prognosis, and the therapeutic response is far from satisfactory. These findings show that TNBC is heterogeneous.\(^6\) This heterogeneity is causing more challenging and less effective therapy, so some biomarkers are needed to predict chemotherapy responses in TNBC patients.\(^7\)

The Cluster of Differentiation 73 (CD73) is a key enzyme for purine metabolism in the tumor microenvironment (TME) which produces adenosine both in classical and alternative pathways.\(^3,8\) CD73 expression is upregulated in breast cancer, and the CD73-derived adenosine contributes to the immune suppression effect that allows the development and metastasis.\(^9\) Adenosines produced by CD73 activity reduce the anti-tumor immune response expected after chemotherapy administration to induce immunogenic tumor cell death. This is thought to play a role in chemotherapy resistance.\(^10,12\) CD73 is also a potential targeted therapy. Several inhibitor therapies targeting CD73, as well as adenosine A2A receptor antagonists, are currently still in phase I clinical trials in patients with solid tumors, including TNBC.\(^2,9,13\)

Based on those mentioned above, the present study aimed to prove that high expression of CD73 is associated with negative clinical neoadjuvant chemotherapy response in TNBC patients.
METHODS
This was a retrospective case-control study. The subject of this study was TNBC patients who underwent neoadjuvant chemotherapy at RSUP Prof. Dr. I.G.N.G Ngoerah Denpasar from January 1, 2016, to December 31, 2021. There were 77 TNBC patients who underwent neoadjuvant chemotherapy, and the initial biopsy specimens were archived in the Anatomic Pathology Laboratory of Prof. Dr. I.G.N.G. Ngoerah hospital with complete clinical data availability. Specimens containing insufficient tumor masses, TNBC patients with recurrent invasive breast carcinoma diagnoses, and TNBC patients who underwent radiation therapy before assessing clinical neoadjuvant chemotherapy response were excluded. There were 23 TNBC patients that met the inclusion and exclusion criteria. Twenty-three patients from the total 28 control groups that met the inclusion and exclusion criteria were consecutively included in this study.

Clinicopathological data such as age, menopausal status, clinical neoadjuvant chemotherapy response, and clinical T stage were obtained from the medical records of RSUP Prof. Dr. I.G.N.G Ngoerah Denpasar and the Indonesian Association of Surgical Oncology Specialists (PERABOI) cancer registration data. Slides were re-evaluated to confirm the histopathology types and grade. Formalin-fixed-paraffin embedded tissue from the patients obtained was then cut and stained with CD73 immunohistochemistry.

CD73 expression was assessed by immunohistochemistry using the anti-CD73 rabbit monoclonal antibody clone D7F94 with 1:100 dilution. Immunohistochemistry was performed with Leica Bondmax Autostainer. Heat antigen retrieval was performed at 100 °C for 30 minutes in EDTA (pH 9.0). Section from the lung adenocarcinoma specimen was used as the positive control.

CD73 expression was evaluated based on intensity staining and the percentage of neoplastic cells.\(^9\) The intensity of the staining was given a score of zero if it was no staining, a score of 1 for weak intensity, 2 for moderate intensity, and 3 for strong intensity. The total score of the CD73 expression was obtained by multiplying the intensity score and the percentage of cells stained. The median value was used as a cut-off. CD73 expression with a total score more than the median value was considered a high expression; meanwhile total score less than the median value was considered a low expression. Immunohistochemistry evaluation was done by 2 researchers (SK and NPS) independently without neoadjuvant chemotherapy response information.

The clinical neoadjuvant chemotherapy response assessed tumor size before and after 3 series of neoadjuvant chemotherapy. Tumors were measured by surgeons in the Surgical Oncology Department bi-dimensionally using a calliper and measuring tape. Measurements were recorded at baseline before chemotherapy was started and in the third week after the third chemotherapy cycle. Clinical neoadjuvant chemotherapy response was categorized into a positive clinical response which consists of clinical Complete Response (cCR) and clinical Partial Response (cPR), and negative clinical response, which consists of clinical Stable Disease (cSD) and clinical Progressive Disease (cPD).\(^14\) Positive responder was the control group, while the negative responder was the case group. The clinical response of neoadjuvant chemotherapy was obtained from the medical records of Prof Dr. I.G.N.G Ngoerah Central General Hospital, Denpasar, and cancer registration data from PERABOI.

A descriptive analysis of the clinicopathological characteristics was done. The Chi-Square test was used in bivariate analysis to compare the proportion of CD73 expression in case and control groups and to assess whether CD73 expression is a predictive factor on clinical neoadjuvant chemotherapy response. In addition, bivariate analysis was also performed between each clinicopathological characteristic and clinical neoadjuvant chemotherapy response. The p-value (α) < 0.05 is considered significant. A multivariate logistic regression test was carried out on CD73 expression by the Saphiro-Wilk test, and a p-value of <0.001 was obtained. It was concluded that CD73 expression data were not normally distributed; hence the median value was used as cut off. The median expression of CD73 in this study was 30. CD73 expression is considered low if the total score is ≤ 30 and high expression if the total score is >30. The assessment was carried out in the area with the strongest intensity and percentage. Two pathologists interpreted without knowing the patients’ clinical neoadjuvant chemotherapy response. If there is a difference of opinion, a discussion is held to make a mutual agreement. Low CD73 expression was seen in 26 (56.5%) cases, while high CD73 expression was in 20 (43.5%) cases. Most cases with high CD73 expression (70%) showed a negative clinical neoadjuvant chemotherapy response after controlling other demographic confounder variables analytically. The regression equation formed is then carried out for calibration and discrimination tests to assess the regression quality. Data were processed using the Statistical Package for the Social Sciences (SPSS) version 25.0 program for Windows.

RESULTS
In this study, there were 46 patients included, 23 patients from the case group and 23 from the control group. Clinicopathological characteristics are summarized in Table 1. Several characteristics were assessed, including age, menopausal status, grade, histopathological type, and clinical T stage. The average age of the study patients was 48.8 ± 10.12 years, ranging from 30 to 72 years. The majority of the patients, 37 (80.4%) were over 40 years old and most of the patients 30 (65.2%) were in the premenopausal category. Based on the grade parameters, the largest distribution of cases was grade 3 (high grade), with as many as 36 (78.3%) cases, and high clinical T stage (T3-T4) as many as 36 cases (78.3%). Based on histopathological type, 40 cases (87.0%) were invasive breast carcinoma of no special type, and 6 other cases were special types (including invasive lobular carcinoma, mucinous carcinoma, and metaplastic carcinoma) (Table 1).

A data normality test was carried out on CD73 expression by the Saphiro-Wilk test, and a p-value of <0.001 was obtained. It was concluded that CD73 expression data were not normally distributed; hence the median value was used as cut off. The median expression of CD73 in this study was 30. CD73 expression is considered low if the total score is ≤ 30 and high expression if the total score is >30. The assessment was carried out in the area with the strongest intensity and percentage. Two pathologists interpreted without knowing the patients’ clinical neoadjuvant chemotherapy response. If there is a difference of opinion, a discussion is held to make a mutual agreement. Low CD73 expression was seen in 26 (56.5%) cases, while high CD73 expression was in 20 (43.5%) cases. Most cases with high CD73 expression (70%) showed a negative clinical neoadjuvant
Table 1. Clinicopathological Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n=46)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 40</td>
<td>9</td>
<td>19.60</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>37</td>
<td>80.40</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopause</td>
<td>30</td>
<td>65.22</td>
</tr>
<tr>
<td>Postmenopause</td>
<td>16</td>
<td>34.78</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1-2</td>
<td>10</td>
<td>21.70</td>
</tr>
<tr>
<td>Grade 3</td>
<td>36</td>
<td>78.30</td>
</tr>
<tr>
<td>Histopathology type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive Breast Carcinoma of no special type</td>
<td>40</td>
<td>87.00</td>
</tr>
<tr>
<td>Invasive Breast Carcinoma, special types</td>
<td>6</td>
<td>13.00</td>
</tr>
<tr>
<td>Clinical T Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>10</td>
<td>21.70</td>
</tr>
<tr>
<td>T3-T4</td>
<td>36</td>
<td>78.30</td>
</tr>
</tbody>
</table>

Figure 1. CD73 immunohistochemistry with various staining intensities. (A) Strong (score +3); (B) Moderate (score +2); (C) Weak (score +1); (D) Negative (score 0).

Table 2. Relationship between CD73 Expression and Clinical Neoadjuvant Chemotherapy Response

<table>
<thead>
<tr>
<th>CD73 Expression</th>
<th>Chemotherapy Response (n=46)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n=23)</td>
<td>Positive (n=23)</td>
<td></td>
</tr>
<tr>
<td>Low, n (%)</td>
<td>9 (34.6)</td>
<td>17 (65.4)</td>
<td>4.41 (1.26-15.41)</td>
</tr>
<tr>
<td>High, n (%)</td>
<td>14 (70.0)</td>
<td>6 (30.0)</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant if p-value less than 0.05

The Chi-square test was used to determine the relationship between CD73 expression and the clinical neoadjuvant chemotherapy response. The result is presented in Table 2. Bivariate analysis showed a significant relationship between CD73 expression and the clinical neoadjuvant chemotherapy response in TNBC (OR 4.41; 95% CI 1.26-15.41; p=0.017). The relationship between clinicopathological characteristics and the clinical neoadjuvant chemotherapy response in TNBC is presented in Table 3. There was no significant association between age, menopausal status, grade, histopathological types, and clinical neoadjuvant chemotherapy response in TNBC. However, there was an association between the clinical T stage and clinical neoadjuvant chemotherapy response (OR 14.14; CI 95% 1.612-124.10; p=0.004).

Furthermore, a multivariate analysis was carried out on variables that, in the bivariate analysis, had a p-value < 0.25, namely the CD73 expression, grade, histopathological type, and clinical T stage. A logistic regression test using the Backward LR method was used, and the result was presented in Table 4. Multivariate analysis showed CD73 expression (AOR 4.88, CI 95% 1.08-21.99, p=0.039) and T stage (AOR 18.82, CI 95% 1.83-193.0, p=0.013) simultaneously affect clinical neoadjuvant chemotherapy response. The regression equation was obtained: Y= −2.843+ 2.935 (clinical T Stage) + 1.585 (CD73 expression).

This multivariate analysis model was tested with Hosmer Lameshow’s test to determine whether the model formed was good. Hosmer Lameshow’s significance value in this study was 0.278, which shows that the regression equation has good calibration. The Nagelkerke R square value is 0.436, which indicates that the ability of CD73 expression and clinical T stage to explain the clinical neoadjuvant chemotherapy response is 43.6%.

DISCUSSION

Breast cancer is the most common cancer and the leading cause of cancer death in women worldwide. Data from Global Cancer Statistics showed that in 2020, breast cancer incidence replaced lung cancer in the top position. The estimated...
of No Special Type (IBC-NST). Other (95%) is an Invasive Breast Carcinoma was in the fourth decade. 

In this study, 87% of cases were diagnosed as IBC-NST, and the rest were diagnosed as special types. The distribution of grades of the cases in this study was mostly high grade (Grade 3), as much as 78.3%, and the rest were low grade (Grades 1 and 2). The behavior of TNBC, according to the literature, tends to be more aggressive and has limited therapeutic options, so it is often diagnosed at an advanced stage. In this study, 36 patients (78.3%) were at a high stage (T3-T4).

Chemotherapy is still the main therapeutic option in TNBC. Despite having more aggressive behavior, around 30-40% of patients obtained pCR after receiving neoadjuvant chemotherapy. The pCR rate is higher compared to the positive predictive value of pCR is approximately 30-40%. The pCR rate is higher compared to the positive predictive value of pCR is approximately 30-40%. The pCR rate is higher compared to the positive predictive value of pCR is approximately 30-40%. The pCR rate is higher compared to the positive predictive value of pCR is approximately 30-40%. The pCR rate is higher compared to the positive predictive value of pCR is approximately 30-40%.
production of cytokines such as IL-4 and IL-10.\textsuperscript{9,23} It is increasingly known that to win against cancer cells, besides developing a strategy to kill the cancer cells efficiently, it is also imperative to stimulate the immune response to control the residual tumor cells, the so-called “immunogenic tumor cell death”. Chemotherapy generally exerts an effect through several mechanisms, such as inhibiting DNA replication, triggering DNA damage and apoptosis, and inhibiting important enzymes necessary for DNA synthesis and mitotic activity. Cancer cell death triggered by the effects of chemotherapy will release massive ATP. ATP through stimulation of P2X7 receptors in dendritic cells will initiate a cascade that ends in the production of IFNγ by CD8+ T cells, indispensable to cause optimal immunogenic tumor cell death. The adenosine produced by CD73 activity, in addition to causing immune suppression, also quickly converts ATP back into adenosine. This leads to sterilization of the anti-tumor immune response expected after chemotherapy administration. This is thought to play a role in chemotherapy resistance.\textsuperscript{10-12} Several similar studies have been conducted by researchers from other countries but are still limited. To date, researchers have not found other studies analyzing the relationship between CD73 expression and Indonesia's clinical neoadjuvant chemotherapy response. A study conducted by Loi\textsuperscript{24} evaluates the relationship between CD73 gene expression and pCR. In 59 TNBC samples receiving neoadjuvant chemotherapy, it was observed that low CD73 gene expression was significantly associated with higher pCR rates ($p=0.00$; CI 95% 0.68-1.00). Chemotherapy agents rely on activating anti-tumour CD8+ T cells to improve their efficacy. This phenomenon also depends on the accumulation of extracellular ATP. Increased CD73 gene expression in tumor cells suppresses the anti-tumor immune response through the Adenosine A2A receptor. In addition, CD73 also inhibits the anti-tumor immune response after chemotherapy administration. This is thought to play a role in chemotherapy resistance. A study conducted by Cerbelli evaluated the relationship of CD73 expression to neoadjuvant chemotherapy responses in 61 TNBC samples.\textsuperscript{3} The median value of CD73 expression in the study by Cerbelli\textsuperscript{3} was 40 and pCR was found in 53% of cases with low CD73 expression and 21% in cases with high CD73 expression ($p=0.011$, OR 4.34, CI 95% 1.39-13.52) in univariate analysis and ($p=0.014$, OR 95 CI% 0.07-0.74) in multivariate analysis.

In this study, most cases with high CD73 expression (70%) showed a negative clinical neoadjuvant chemotherapy response. In contrast, most patients with low CD73 expression (65.4%) showed a positive clinical chemotherapy response. Bivariate analysis shows a significant relationship between CD73 expression and the clinical neoadjuvant chemotherapy response in TNBC (OR 4.41; CI 95% 1.26-15.41; $p=0.017$). Bivariate analysis of clinicopathological characteristics and clinical neoadjuvant chemotherapy response showed an association between the clinical T stage on the clinical neoadjuvant chemotherapy response (OR 14.14; 95 CI% 1.612-124.10; $p=0.004$).

Multivariate analysis showed CD73 expression (AOR 4.88, CI 95% 1.08-21.99, $p=0.039$) and T stage (AOR 18.82, CI 95% 1.83-193.0, $p=0.013$) simultaneously affect clinical neoadjuvant chemotherapy response. The regression equation was obtained: $Y = -2.843 + 2.935$ (clinical T Stage) + 1.585 (CD73 expression). Although tumor size is often considered when patients are about to receive neoadjuvant chemotherapy, the exact role of tumor size (T stage) in influencing pCR is not yet known for certain. When clinicians discuss pCR potential in their patients, they refer only to biological subtypes or hormone receptor status. However, data directly comparing the relative risk of tumor size and hormone receptor status to pCR is still very limited. Although the biological properties of breast cancer may be most explainable through hormone receptor status, it turns out that the size of the tumor also gives an additional picture in explaining the biological properties of breast cancer. Tumor size is almost always considered just as a sign that cancer has been around for a longer time and therefore is usually less responsive to the administration of chemotherapy, but more than that, the size of the tumor may also explain the biological properties of cancer that are not reflected from the hormone receptor status.\textsuperscript{25}

A previous study showed that the size of the tumor independently corresponded to the pCR after neoadjuvant chemotherapy after controlling the status of hormone receptors. Stage T3 (OR 0.64; CI 09% 0.59-0.70) corresponds to a lower pCR rate than stage T1. However, stage T 2 (OR 0.95; CI 95% 0.89-1.02) is not associated with the pCR rate. Nevertheless, the status of hormone receptors is more strongly associated with the pCR rate than tumor size.\textsuperscript{25} Other studies also showed a similar result, where low T-stage (T1-T2) is independently a predictor of higher pCR compared to high T-stage (T3-4) ($p<0.001$; OR 3.15).\textsuperscript{26-28}

This was a retrospective study with a limited number of patients. Further research with prospective and multicentre research methods with a larger sample size is needed to prove the consistency of this study’s results. Other research can be carried out to show the relationship between CD73 expression and adenosine concentrations, to investigate further mechanisms of chemotherapy response in breast carcinoma.

**CONCLUSION**

Based on the results of this study, it can be concluded that high CD73 expression is a negative predictor for clinical neoadjuvant chemotherapy response in TNBC patients. Increased expression of CD73 had a 4.41 times greater risk of experiencing a negative clinical chemotherapy response than TNBC patients with low CD73 expression (CI 95% 1.26-15.41).

**CONFLICT OF INTEREST**

The author stated that there was no conflict of interest regarding the publication of this study.

**ETHICS CONSIDERATION**

The study protocol has been approved by the Research Ethics Commission of the Faculty of Medicine, Universitas Udayana / RSUP Prof. Dr. I.G.N.G Ngoreah Denpasar, with an ethics eligibility letter.
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AUTHOR CONTRIBUTIONS
All authors have made the same contribution in writing this research article from the beginning of data collection, analysis of research data, and results reporting.

REFERENCE