RESEARCH ARTICLE

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Correlation of *CD44* Protein Expression with Larger Tumor Size and Advanced Stage of Breast Cancer Patients

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Abstract

Objectives: This study aimed to determine the expression of *CD44* in breast cancer and its association with patients' clinicopathological data, particularly in Bali. **Material and Methods:** This was a cross-sectional study in the Integrated Biomedical Laboratory and Biochemistry Laboratory of the Faculty of Medicine Udayana University, during January-December 2022, which lasted 12 months with 46 samples. **Results:** The study showed that the age range of our subjects was 38-86 years old, with the majority of the parity being less than three. The mean *CD44* expression in all samples was 1177.83 ± 268.47 ng/mL. Based on the clinicopathological data, there was a significant difference in *CD44* expression based on the patient's menstrual status (p=0.016), tumor size (p=0.003), and stage (p=0.002). Based on the analysis using the chi-square test with a cut-off *CD44* expression of 85.81, significant results (p=0.001) were obtained on the association of *CD44* expression with tumor size. The cut-off value with the stage of breast cancer was 99.66 and showed a significant association between *CD44* expression advanced stage (p = 0.001) in breast cancer patients. Other variables showed insignificant results. **Conclusion:** *CD44* protein expression in breast cancer patients is between 35.47-1407.83 ng/mL. This study showed a significant association between *CD44* expression with tumor size and the advanced stage of breast cancer patients.

Keywords: Bali- Breast Cancer- *CD44*- Clinicopathology

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Introduction

Breast cancer is one of the leading causes of cancer worldwide. According to the American Cancer Society, breast cancer is the second leading cause of cancer mortality in female patients after lung cancer. In addition, with an average risk of 12%, this cancer may affect one in eight women in America. In 2017, it was projected that there would be around 40,610 cancer-related deaths, 252,710 invasive cases, and 63,410 non-invasive (CIS) cases diagnosed [1]. According to the World Health Organization (WHO) 2018, the mortality rate of breast cancer is 627,000, or approximately 15% of deaths of all types of cancers that affect women. In the Asia Pacific, Indonesia reaches third place with the highest prevalence of breast cancer (12%) after China (46%) and Japan (14%). The prevalence of breast cancer in Indonesia is 41.7%, with a mortality rate is 22% [2].

Breast cancer is a highly complex and heterogeneous disease that has diverse clinical and biological behaviors. It also has distinct responses to any treatment that can be classified into certain subtypes according to histopathological types and molecular profiles [3]. Tumor heterogeneity in breast carcinoma is related to the presence of populations of heterogeneous cells within a single patient (intratumor heterogeneity) or between different patients (inter-tumor heterogeneity), resulting in the clinical manifestations of the disease. Although the understanding of breast cancer heterogeneity has increased significantly, there are still several obstacles standing in the way of reaching a better diagnosis, treatment, and prognosis of breast cancer disease [4].

CD44 is a complex transmembrane adhesion glycoprotein found in several molecular forms, such as the standard and variant isoforms. *CD44* is a molecule that is specifically located on chromosome 11p13. Hyaluronic acids and the main components of the extracellular matrix are intrinsically linked to *CD44*. Moreover, *CD44* can interact with other cell surface receptors to facilitate the activation of distinct signaling pathways that control cell migration, survival, cell invasion, and the epithelial-mesenchymal transition (EMT). *CD44* has

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also been revealed to play a role in cellular signaling and cell-cell communication by creating complexes between extracellular components and intracellular cytoskeletal elements. In addition, *CD44* regulates various cell behaviors, such as cell survival, proliferation, differentiation, and motility, and it has been linked to sensing changes in the extracellular matrix and the cellular microenvironment. Moreover, the previous study also stated that the expression of *CD44* correlated with tumor grade and recurrence in breast cancer patients. It is also reported that *CD44* may promote cancer metastasis [4].

The evidence of *CD44* genes related to clinicopathological aspects in breast cancer was shown in some previous studies. The *CD44* expression was found to correlate with HER-2 negative patients [5]. In addition, increased *CD44* expression is associated with tumor grade and distant metastasis [6]. However, in a previous study, it was found that there was no correlation was found between *CD44*-positive / CD24-negative with the size of the tumor, nodal status, and metastasis, but it was related to the overall stage [7]. With these variable findings, there is a need to conduct a study associated with *CD44* expression as well as its association with clinicopathological aspects of breast cancer. The studies conducted in Indonesia are scarce and need to be evaluated considering the diversity of ethnicities and demographics of the country.

Our present study takes place in Bali, considering the scarcity of the study that is related to the topic and the increasing number of breast cancers from time to time. Therefore, it is necessary to investigate the *CD44* expression and its association with clinicopathological aspects of breast cancer patients in Bali. This study was expected to encourage further breast cancer research to support patients' prognoses

Materials and Methods

Study design

This study was conducted using a cross-sectional study in the Integrated Biomedical Laboratory and Biochemistry Laboratory of the Faculty of Medicine Udayana University, during January-December 2022.

Eligibility Criteria of Participants

A consecutive sampling technique was used as a sampling method in this study with 46 patients who met the criteria to be the subject of this study. In this study, the inclusion criteria were (1) patients diagnosed with breast cancer who underwent a complete blood count in the clinical pathology laboratory of Prof. dr. IGNG Ngoerah General Hospital, (2) had complete medical record data, and (3) willing to participate in the study through informed consent. Exclusion criteria were (1) pregnant women and nursing mothers, (2) patients with a history of other malignancies, (3) patients with impaired liver or kidney function, and (4) taking multivitamins.

Blood Sampling

A peripheral blood sample (5 ml) was collected from the brachial vein of the patient. It was taken during a routine blood examination at Prof. dr. IGNG Ngoerah General Hospital Clinical Pathology Laboratory. The blood tube was stored in a cool box and transposed to the Faculty of Medicine's biochemical laboratory, Universitas Udayana. As soon as the blood was collected, serum was prepared by centrifugation at 4000×g for 10 min at 4°C. The resulting supernatant was stored in an Eppendorf tube at -80 °C before the immunoassay.

ELISA Procedure

ELISA procedure began with preparing a sample in blood serum and reagent preparation. This procedure used the Human *CD44* ELISA KIT from the Bioassay Technology Laboratory per the manufacturer's protocol (Korain Biotech, China). The expression of *CD44* protein was measured in only one group (patients with breast cancer) without subject control. In each well plate, 50 µl of standard solution was added into the standard wells, as well as 40 µl of serum in the sample wells and 10 µl of anti-*CD44* antibody to each well. 50 µl of streptavidin-HRP was added to the sample and standard wells. The mixtures were homogenated, covered with a sealer cover, and incubated at 37oC for 60 minutes. 50 µl of substrate solution-A and 50 µl of substrate solution-B were then added to all wells. The plate was then covered with a sealer cover and incubated at 37oC for 10 minutes in a dark room. Subsequently, 50 µl of stop solution was added to each well, and the color was expected to change from blue to yellow immediately. The ELISA plate was then read by using a microplate reader at 450 nm.

Statistical analysis

All numerical data in this study, including *CD44* expression, were analyzed using the Shapiro-Wilk normality test. As the result showed that the data was not normally distributed, this study used a non-parametric test (Mann-Whitney). All nominal data were analyzed using the Chi-square test. The p-value <0.05 was considered significant. All analysis tests used SPSS software for Windows version 25.0

Results

This study's youngest and oldest ages were 38 years and 86 years, respectively, with a mean age of 54.24 years. Based on parity, the result of this analysis was obtained with the majority <3 Children (87.0%). Also, 56.5% of breast cancer patients were postmenopausal, and 43.5% were premenopausal. In clinicopathological aspects of breast cancer patients in Prof. dr. IGNG Ngoerah General Hospital, it was found that most of the patients (78.3%) had a tumor size ≥ 5 cm, 47.8% with N1 regional lymph node metastases, and 65.2% of patients without distant metastases (M0). The patients' primary tumor was equal, with 50% in dextra mammae and 50% in sinistra mammae. Based on Karnofsky's score, 87% of the patients had scores ≥90%, and the rest (13%) with <90%.

In this study, the breast cancer stage was divided into four groups, with most of the patients had stage III (45.7%) and stage IV (34.8%). The histopathological grade was divided into three groups: 43.5% of the patients were in grade III, 37% were in grade II, and the rest were in grade

Table 1. Characteristics of Breast Cancer Patients

Characteristic	Number of Samples (n=46)	
	Frequency (n)	Percentage (%)
Age		
Mean (Years Old)	54.24±10.67	
Minimum	38	
Maximum	86	
Parity		
< 3 (low parity)	40	87.0
≥ 3 (high parity)	6	13.0
Menstrual status		
Pre-menopause	20	43.5
Post-menopause	26	56.5
Tumor Size		
<5 cm	10	21.7
≥ 5 cm	36	78.3
Lymph Node Metastasis		
N0	14	30.4
N1	22	47.8
N2	7	15.2
N3	3	6.5
Distant Metastasis		
M0	30	65.2
M1	15	32.6
Primary Tumor Location		
Dextra	23	50.0
Sinistra	23	50.0
Karnofsky Score		
< 90%	6	13.0
≥ 90%	40	87.0
Stage		
I	3	6.5
II	6	13.0
III	21	45.7
IV	16	34.8
Histopathological Grade		
Grade 1	9	19.6
Grade 2	17	37.0
Grade 3	20	43.5
ER status		
Negative	16	
Positive	30	
PR status		
Negative	23	
Positive	23	
HER-2 status		
Negative	22	
Positive	24	
Ki67		
Low	9	19.6
High	37	80.4
Histopathological Subtype		
Luminal A	9	19.6
Luminal B	14	30.4
Her2	9	19.6
TNBC	6	13.0
Luminal-*HER2*	8	17.4
CD44		
Mean±SD	177.83±268.47	
Median	81.42	
Range (Min-Max)	35.47-1407.83	

I. The estrogen receptor (ER), progesterone receptor (PR), and *HER2* status of breast cancer patients in this study were divided into two groups, which are positive and negative. Most breast cancer patients have positive ER status (65.2%). Meanwhile, the patients' PR status was equal in the positive (50%) and negative (50%) groups. At *HER2* status, 52.2% of patients were *HER2* positive. About 80.4% of patients had high *Ki67*, and 19.6% had low *Ki67*. Based on histological subtypes, 30.4% of patients with luminal-B were followed by luminal-A and *HER2* subtypes with 19.6% each, then luminal- *HER2* and Triple Negative Breast Cancer (TNBC) subtypes were 17.4% and 13%, respectively.

CD44 ELISA results showed that the mean *CD44* expression of all breast cancer patients was 177.83±268.47 ng/mL, with a median expression of 81.42 ng/mL. The lowest value of *CD44* expression was 35.47 ng/mL. Meanwhile, the highest value was 1407.83 ng/mL, as seen in Table 1.

Based on the mean difference test in Table 2, there was a significant difference in *CD44* expression based on the patient's menstrual status, tumor size, and stage. We continued the analysis using ROC curve analysis to find the cut-off value of *CD44*. The cut-off value of *CD44* in tumor size was 85.81 (Table 3; Figure 1). The other fact, the cut-off value of *CD44* in the tumor stage was 99.66 (Table 4; Figure 2).

Based on the cut-off value *CD44* expression (85.81 ng/ml; obtained from ROC analysis), we classified the group into "high" and "low" categories. Analyzing the association between these categories with tumor size revealed that *CD44* expression was significantly associated with tumor size with Odds Ratio (OR) 18.0 (p=0.001) (Table 5) which indicate higher risk of having larger tumor in breast cancer patients with high *CD44* expression. Using similar method, we classify the group into "high" and "low", then analyzed its association with tumor stage (Early vs. Advanced). Accordingly, we also found significant association between *CD44* expression to tumor stage (OR: 21.6 (p:0.001*)) with higher *CD44* expression has higher risk of having advanced tumor (Table 6). Meanwhile, other variables did not show significant results.

Table 2. Association of *CD44* Expression with the Clinicopathological Aspect of Breast Cancer Patients

CD44 Expression	Mean diff (CI95%)	P-value
Age		
<52 years old	158.07 (12.79-303.35)	0.078
≥ 52 years old		
Parity		
< 3 (low parity)	81.27 ((-157)-319.55)	0.636
≥ 3 (high parity)		
Menstrual Status		
Pre-Menopause	67.04 ((-311.03) – (-35.25))	0.016*
Post-Menopause		
Tumor Size		
<5 cm	91.72 (25.58-395.28)	0.003*
≥ 5 cm		
Lymph Node Metastasis		
Without metastasis	174.10 ((-78.08)-426.29)	0.17
With metastasis		
Distant Metastasis		
Without metastasis	30.33 ((-163.63)-224.31)	0.068
With metastasis		
Primary Tumor Location		
Dextra	21.43 (139.79-182.94)	0.606
Sinistra		
Karnofsky Score		
< 90%	124.61 (111.93-361.15)	0.234
≥ 90%		
Stage		
Early (I-II)	239.778 (49.90-429.655)	0.002*
Advance (III-IV)		
Histopathological Grade		
Grade I-II	130.61 (11.43-272.67)	0.352
Grade III		
Histopathological Subtype		
Luminal	83.51 (-86.70-253.73)	0.87
Non-Luminal		
ER Status		
Negative	36.96 ((-132.05)-205.98)	0.721
Positive		
PR Status		
Negative	9.85 ((-171.18)-151.47)	0.435
Positive		
HER-2		
Negative	6.90 ((-154.58)-168.40)	0.218
Positive		
Ki-67		
Low	87.12 ((-288.76)-114.52)	0.198
High		

Discussion

This study is one of the few studies that rarely addresses *CD44* plasma serum in breast cancer. Several other studies that have been published mostly used stem cells and tissues. *CD44* is a non-kinase transmembrane glycoprotein expressed on embryonic stem cells and various cell types, including connective tissue and bone marrow [8]. This study showed a proportion difference in *CD44* expression with the patient's age. More severe disease phenotypes are frequently correlated with *CD44* expression. Both younger and older individuals have increased *CD44* expression; however, other research suggests that age may not be a relevant factor in *CD44* expression levels. On the other hand, advanced tumor stages may have higher *CD44* expression, which may be associated with older age groups [9].

There was a significant relationship between *CD44* expression and menstrual status. Based on menstrual status, menopause is the last menstrual cycle experienced by a woman with clinical manifestations of menstruation cessation. This study found a significant difference in the proportion of *CD44* expression between the premenopausal and postmenopausal groups. *CD44* is expressed mainly in endometrial stromal cells during the secretion phase. Meanwhile, glandular epithelial cells do not express *CD44*. Expression of *CD44* during the secretion phase indicates that the molecule was involved in implanting a fertilized ovum. *CD44* also functions in the early stage of the adhesive contact between the endometrium and ovum. Thus, the absence or decrease of *CD44* expression in the endometrium in this phase may cause infertility or early abortion [10].

This study showed a significant relationship between *CD44* expression and tumor size but not significant with regional lymph node metastasis and distant metastasis. Higher *CD44* expression was associated with tumor size [11]. *CD44* plays a crucial part in tumor development, invasion, and metastasis. Research indicates that higher levels of *CD44* expression are linked to more extensive tumors because *CD44* enhances the characteristics of cancer stem cells (CSCs) that facilitate tumor development, invasion, and metastasis. Through improving survival pathways, *CD44*-positive CSCs in breast cancer encourage cell proliferation and tumor growth [9, 12].

This study also found a significant association between *CD44* and the stage of breast cancer. Studies show that *CD44* is clinically correlated with the stage of breast cancers, implying that higher levels of *CD44* are frequently associated with more aggressive tumors with a higher potential for metastasis, especially in patients with triple-negative breast cancer (TNBC); in other words, higher levels of *CD44* expression are linked to later stages of breast cancer [3]. Compared to previous stages (I and II), stage III and IV cancers frequently have higher levels of *CD44*, which may indicate a function for *CD44* in the metastasis and development of the tumor [12].

There was no significant relationship between *CD44* expression and breast cancer patients' ER, PR, and *HER2* status. Breast cancer with ER+ has the best prognosis, with a low incidence rate in the first five years. In contrast,

Table 3. The AUC, Cut-off Value, Sensitivity, and Specificity for *CD44* for Tumor Size of Breast Cancer

Parameter	AUC	95%CI	Cut-off value	Sensitivity	Specificity	p-value
CD44	0.814	0.66-0.96	85.81	90%	66.70%	0.003*

*significant (p<0.05)

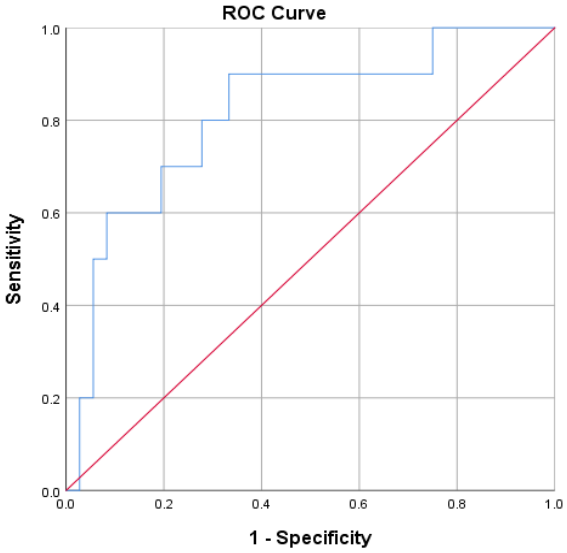

Figure 1. The ROC Curve Analysis of *CD44* for Tumor Size

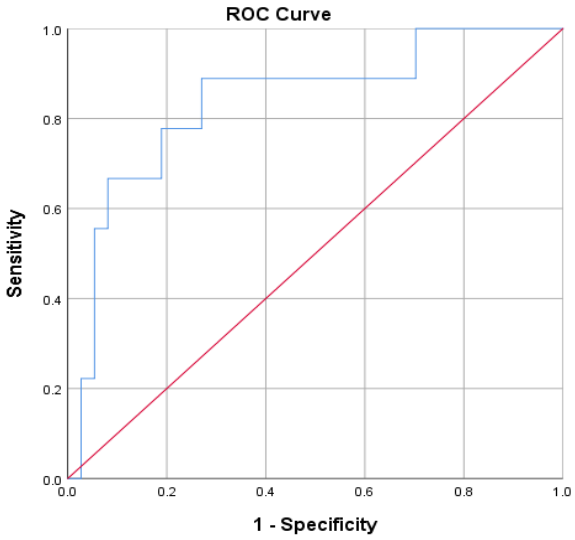

Figure 2. The ROC Curve Analysis of *CD44* based on the Stage of Breast Cancer

Table 4. The AUC, Cut-off Value, Sensitivity, and Specificity for *CD44* for Cancer-Stage

Parameter	AUC	95%CI	Cut-off value	Sensitivity	Specificity	p-value
CD44	0.838	0.68-0.99	99.66	88.90%	73%	0.002*

*significant (p<0.05)

TNBC breast cancer showed the worst prognosis, and nearly all metastases occur within the first five years, with the incidence rate increasing in the first one or two years [13]. However, this study showed no significant association between *CD44* expression with primary tumor and metastatic location.

This study showed the difference between the proportion of *CD44* expression between the *Ki67* group was 20.8% but no significant correlation. This result is similar to that conducted by Farida, [14], which used 44 samples of breast cancer patients at Dr. Moh. Hoesin Palembang Hospital [14]. The functional status of patients was assessed on an 11-point scale ranging from all points filled up completely (100%) until cancer patients died (0%) [15]. It was found that there was no significant relationship between *CD44* expression and Karnofsky score in breast cancer patients.

Despite our findings, there are some limitations in this study. This study uses a small sample size, therefore, further studies with a larger sample are needed to represent more representative results. Additional research is required in order to evaluate the more specific *CD44* variant and its relation to the clinicopathological condition and prognosis of breast cancer patients.

In conclusion, based on the study results, it can be concluded that the *CD44* protein expression in breast cancer patients is between 35.47 and 1407.83 ng/mL. This study showed a significant relationship between *CD44* expression with tumor size and the stage of breast cancer patients. Therefore, *CD44* expression may be beneficial to predict the prognosis of breast cancer patients.

Author Contribution Statement

All authors contributed to the research processes, including data analysis, drafting, and revising the paper. The authors gave final approval of the published version of the study.

Table 5. The Chi-Square Analysis of the Association between *CD44* with Tumor Size

Variable	Tumor size		OR (p-value)
	≥5 cm	<5 cm	
CD44			
High (≥85.81 ng/ml)	24 (96.0)	1 (4.0)	18.0 (0.001*)
Low (<85.81ng/ml)	12 (57.1)	9 (42.9)	

Table 6. The Chi-Square Analysis of the association between *CD44* with Stage

Variable	Stage of tumor		OR (p-value)
	Advance	Early	
CD44			
High (≥99.66 ng/ml)	27 (96.4)	1 (3.6)	21.6 (0.001*)
Low (<99.66 ng/ml)	10 (55.6)	8 (44.4)	

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Funding Statement

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Ethical Declaration

The Research Ethics Committee of Udayana University approved this study. Letter of Exemption Number 1483/UN.14.2.2/VII.14/LT/2020.

Data Availability

The corresponding author will provide the datasets used and/or analyzed during the current work upon reasonable request.

Conflict of Interest

All authors declare there was no conflict of interest regarding this study.

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