RESEARCH ARTICLE

Serum CD44 Variant 6 to CD44 Ratio and Vascular Endothelial Growth Factor Levels as Predictors of Metastasis in Luminal Subtype Breast Cancer

Ni Putu Yenny Kendarini[1,*], Ida Bagus Tjakra Wibawa Manuaba[2], I Wayan Sudarsa[3], Tjokorda Gde Bagus Mahadewa[4], Ni Nyoman Ayu Dewi[5], Prayuda[6], Putu Febry Krisna Pertiwi[7]

[1]Division of General Surgery, Balimed Hospital, Jl. Mahendradatta No.57 X, Denpasar, Bali 80119, Indonesia
[2]Division of Surgical Oncology, Prima Medika Hospital, Jl. Raya Sesetan No.10, Denpasar, Bali 80223, Indonesia
[3]Division of Surgical Oncology, Department of Surgery, Faculty of Medicine, Universitas Udayana/Prof. dr. I.G.N.G. Ngoerah General Hospital, Jl. P.B. Sudirman, Denpasar, Bali 80232, Indonesia
[4]Department of Neurosurgery, Faculty of Medicine, Universitas Udayana/Prof. dr. I.G.N.G. Ngoerah General Hospital, Jl. P.B. Sudirman, Denpasar, Bali 80232, Indonesia
[5]Department of Biochemistry, Faculty of Medicine, Universitas Udayana/Prof. dr. I.G.N.G. Ngoerah General Hospital, Jl. P.B. Sudirman, Denpasar, Bali 80232, Indonesia
[6]Division of Internal Medicine, Balimed Hospital, Jl. Mahendradatta No.57 X, Denpasar, Bali 80119, Indonesia
[7]Faculty of Medicine, Universitas Udayana, Jl. P.B. Sudirman, Denpasar, Bali 80232, Indonesia

*Corresponding author. Email: yennykendarinibedah@gmail.com

Received date: Feb 22, 2025; Revised date: May 7, 2025; Accepted date: May 9, 2025

Abstract

BACKGROUND: Metastasis is the leading cause of mortality in luminal subtype breast cancer. While cluster of differentiation (CD)44 has been widely studied, the prognostic relevance of its isoforms particularly the CD44v6/CD44s ratio remains unclear. This study evaluates CD44v6, CD44s, vascular endothelial growth factor (VEGF), and the CD44v6/CD44s ratio as potential prognostic biomarkers for metastasis in luminal breast cancer.

METHODS: This case-control study included 38 luminal subtype breast cancer patients (18 with metastasis, 20 without metastasis). Serum levels of CD44v6, CD44s, VEGF, and the CD44v6/CD44s ratio were measured using Enzyme-Linked Immunosorbent Assay (ELISA). Statistical analyses included ROC analysis to determine optimal cut-off points, logistic regression to assess risk factors, and correlation analysis for biomarker relationships.

RESULTS: A low CD44v6/CD44s ratio (<0.03) was identified as a significant independent factor for metastasis (adjusted OR 7.0, 95% CI: 1.2–40.6, p=0.03). While serum levels of CD44v6, CD44s, and VEGF were higher in the metastasis group, these individual markers showed a non-significant trend toward association with metastasis. A strong positive correlation was observed between CD44s and VEGF levels (r=0.7, p<0.01).

CONCLUSION: The CD44v6/CD44s ratio showed a significant association with metastasis and may have potential as a prognostic marker in luminal breast cancer. Further studies with larger sample sizes are needed to confirm these findings.

KEYWORDS: CD44v6, CD44s, VEGF, CD44v6/CD44s ratio, luminal breast cancer, metastasis

Indones Biomed J. 2025; 17(3): 263-72

Introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide, with metastasis being the primary cause of cancer-related mortality. Despite advances in early detection and treatment, approximately 5-10% of breast cancer cases present with metastasis at diagnosis.(1) The luminal subtype, characterized by hormone receptor positivity, accounts for the majority of breast cancer cases in Indonesia.(2) During 2015 to 2017, in geriatric clinics at Prof. Ngoerah General Hospital, it was found that 25% of breast cancer patients had experienced metastasis. The study revealed that 25% of breast cancer patients were diagnosed

at stage IV, indicating a substantial proportion present with advanced disease.(3) While luminal breast cancers are generally sensitive to hormonal therapy, their prognosis remains unpredictable due to the risk of micrometastases.

Liquid biopsy allows for early detection, even at the micrometastasis level, before macrometastasis occurs. This non-invasive and minimally invasive diagnostic methods are preferred over invasive procedures due to their proven advantages.(4) Plasma-based biomarkers have been investigated in luminal breast cancer subtypes to detect molecular and metabolic differences, including amino acid profiles.(5) This highlights the potential of circulating markers, such as cluster of differentiation (CD)44 isoforms and vascular endothelial growth factor (VEGF), as well as other non-traditional serum components, including mucins, which have shown promise in distinguishing malignant from benign breast lesions.(6) Biomarkers such as CD44 variants have emerged as potential tools for detecting early micrometastasis.(7) The human CD44 gene, located on chromosome 11p13, comprises 19 exons. Through alternative splicing of its pre-mRNA, different combinations of exons are included or excluded, resulting in structurally distinct protein isoforms. The standard isoform (CD44s) includes only the constant exons (1–5 and 16–20), whereas variant isoforms (CD44v) incorporate one or more variable exons (v1–v10), giving rise to diverse protein forms with different functional properties. CD44 is considered a marker associated with cancer stem cells (CSCs), indicating stemness, or the ability of a cell to self-renew and initiate tumor growth.(8) CD44v6, a variant isoform of CD44 produced through alternative splicing, is mainly expressed by epithelial and tumor cells. Its expression can be induced by inflammatory cytokines, hypoxia, and growth factors within the tumor microenvironment.(9) CD44v6 may enter the circulation via proteolytic shedding or extracellular vesicles. Among CD44 variants, CD44v6 has been most strongly associated with tumor aggressiveness, including higher histological grade, lymph node metastasis, and poor prognosis in breast cancer.(10) VEGF, a pro-angiogenic factor and prognostic marker in various cancers, including breast cancer.(11) VEGF is a key angiogenic factor that plays a crucial role in stimulating angiogenesis and tumor growth, together with VEGF receptors, activates signaling pathways essential for tumor-associated blood vessel formation.(12) In addition to CD44 and VEGF pathways, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ have been shown to induce apoptosis in breast cancer cells through upregulation of pro-apoptotic genes such as Bax and p53, and downregulation of anti-apoptotic gene B-cell lymphoma (Bcl)-2, highlighting the critical role of immune-mediated mechanisms in tumor progression.(13)

CD44 isoforms and VEGF play key roles in tumor progression, but their utility as circulating biomarkers for micrometastasis detection in luminal breast cancer remains unclear. Liquid biopsy offers a promising non-invasive tool, yet no specific serum biomarkers have been validated for this purpose. Although several studies have evaluated CD44 expression in breast cancer, most focused on either total CD44 or individual isoforms. The prognostic significance of the CD44v6/CD44s ratio, which may reflect a shift in isoform expression linked to tumor aggressiveness, has not been well studied. Previous research suggests CD44v6 is elevated in metastatic tumors, whereas CD44s may be more prevalent in less aggressive phenotypes. This study investigates CD44v6, CD44s, their ratio, and VEGF to explore their potential as prognostic biomarkers for metastasis in luminal breast cancer.

Methods

Study Design

This study employed a case-control design to investigate serum biomarkers associated with metastasis in luminal subtype breast cancer. The research was conducted at Prof. Ngoerah General Hospital Denpasar, Bali, and involved a total of 38 subjects, consisting of 18 patients with confirmed metastasis (cases) and 20 patients without metastasis (controls). This study was conducted following the ethical principles outlined in the Declaration of Helsinki and had been approved by the Ethics Committee of Prof. Dr. I.G.N.G. Ngoerah General Hospital (No. 2195/UN14.2.2.VII.14/LT/2022). All subejcts provided written informed consent before enrolling in the study. They were given comprehensive information regarding the study's objectives, procedures, and their right to withdraw at any point without consequences to their medical care. To ensure confidentiality, all patient identifiers were removed.

Inclusion and Exclusion Criteria

The inclusion criteria for cases were female patients aged 30-70 years diagnosed with luminal metastatic subtype breast cancer who developed metastases during therapy and received surgical, chemotherapy, and/or hormonal treatments. Control group patients were those diagnosed with stage II luminal subtype breast cancer according to the 8th edition merican Joint Committee on Cancer

(AJCC) tumor, nodes, and metastasis (TNM) criteria and had undergone surgery, followed by chemotherapy and/or hormonal therapy.(14) Case group defined as patients diagnosed with metastatic luminal subtype breast cancer, where, during the course of treatment, metastasis occurred, and the patient has undergone therapeutic modalities, including surgery, chemotherapy, targeted therapy, or hormonal therapy. Exclusion criteria included pregnancy, non-luminal subtypes, history of other cancers, unwillingness to participate, and hemolyzed blood samples.

Sample and Data Collection

Three mL of blood samples were collected from each subjects using standard venipuncture procedures. After centrifugation, the serum was aliquoted into 0.5 mL Eppendorf tubes and stored at −80°C until analysis. In addition to blood collection, relevant clinical and pathological data were obtained from the patients' medical records. Cancer stage was determined according to the 8th edition of the AJCC TNM classification system. Histopathological grading data was retrieved from the Pathology Anatomy Laboratory, based on standard histologic criteria assessing tubule formation, nuclear pleomorphism, and mitotic count. Estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) status, and Ki-67 index were previously assessed using immunohistochemistry (IHC) by the Pathology Anatomy Laboratory. ER and PR positivity were defined as ≥1% nuclear staining. HER2 status was scored as 0 to 3+; a score of 3+ was considered positive, and 2+ was followed by confirmatory testing if available. Ki-67 was reported as a percentage of positively stained tumor cell nuclei.

Breast cancer subtype was classified as luminal based on positive ER and/or PR status, with or without HER2 overexpression. The location of metastases was determined through radiology reports, including ultrasound (USG), computed tomography (CT), and/or magnetic resonance imaging (MRI). These findings were confirmed by clinicians based on imaging results and documented in the clinical records.

ELISA Analysis of CD44, CD44v6, and VEGF

Serum levels of CD44v6, CD44s, and VEGF were measured using sandwich Enzyme-Linked Immunosorbent Assay (ELISA) methods. The CD44v6 kit (Cat. No. E7578Hu; BT Lab, Jiaxing, China) with sensitivity of 0.12 ng/mL and a standard curve range of 0.25–16 ng/mL, the CD44s kit (Cat. No. E0239Hu; BT Lab) with a sensitivity of 2.5 ng/mL and a standard curve range of 5–1500 ng/mL, and the VEGF kit (Cat. No. E0080Hu; BT Lab) with a sensitivity of 10.42 pg/mL and a detection range of 20–6000 pg/mL were used according to the manufacturer's instructions. The plates were first pre-coated with capture antibodies, followed by sequential incubation with the sample, biotinylated detection antibodies, and streptavidin-HRP. Colorimetric detection was performed using substrate solution and measured at 450 nm. All samples were analyzed in duplicate. The intra-assay and inter-assay coefficients of variation (CV) for all kits were <8% and <10%, respectively.

Statistical Analysis

Data analysis included descriptive analysis, correlation tests, receiver operating characteristic (ROC) analysis, proportion difference tests, and logistic regression, all conducted using SPSS version 25.0 (IBM Corporation, Armonk, NY, USA). Descriptive analysis was used to characterize subjects by case and control groups, including variables such as breast cancer status (metastasis or no metastasis), age (≤40 years or >40 years), histopathological grading (Grade I, II, III), and CD44v6 expression (high or low). Correlations between CD44v6, CD44s, and VEGF were evaluated using Pearson's test for normally distributed data or Spearman's test for non-normal distributions. ROC analysis determined optimal cutoff points for CD44v6, CD44s, and VEGF by identifying the farthest point from the diagonal line on the ROC curve. Subjects were grouped based on metastatic status, and odds ratios (ORs) were calculated to assess associations between biomarkers and metastasis. Proportion difference tests compared risk factor distributions between case and control groups using cross-tabulations and Chi-square tests to calculate the OR or Crude OR. Logistic regression assessed independent associations of variables while controlling for confounders, with results reported as adjusted OR and significance set at $p \leq 0.05$. Correlation analysis was performed to evaluate the strength and direction of relationships between variables

Results

Baseline Characteristics

This study involved 38 subjects with luminal subtype breast cancer, including 18 cases (metastasis, clinical stage IV) and 20 controls (non-metastasis, clinical stage II). The mean age of participants was 50 years, and most tumors were classified as histopathological grade II. ER and PR positivity were prevalent, while HER2-negative status was dominant in both groups. Higher Ki67 levels (≥20%) were

observed more frequently in cases, with Luminal B subtype being more common. The detailed characteristics were presented in Table 1.

Cut-off Points for Study Variables

Before conducting bivariate analysis, the variables CD44v6, CD44s, VEGF, and the CD44v6/CD44s ratio were categorized based on cut-off points determined using ROC curves. Optimal cut-off points were identified using ROC analysis: 4 ng/mL for CD44v6, 119.2 ng/mL for CD44s, 340 ng/mL for VEGF, and 0.034 for CD44v6/CD44s ratio (Figure 1). The distribution of baseline characteristics and biomarker were provided in Table 2.

Serum CD44v6 Levels and the Risk of Metastasis

Subjects with low serum CD44v6 levels (<4 ng/mL) had a 2.4 times higher risk of metastasis compared to those with higher levels (≥4 ng/mL) (OR 2.4, 95% CI: 0.65–9.13, p=0.19). However, this result was not statistically significant.

Serum CD44s Levels and the Risk of Metastasis

Subjects with high serum CD44s levels (≥119.2 ng/mL) had a 2.4 times higher risk of metastasis compared to those with low levels (<119.2 ng/mL) (OR 2.4, 95% CI: 0.6–8.6, p=0.19). However, this result was not statistically significant.

Serum CD44v6/CD44s Ratio and the Risk of Metastasis

Participants with a low CD44v6/CD44s ratio (<0.03) had a significantly higher rate of metastasis (88.9%) compared to those with a high ratio (≥0.03), who had a metastasis rate of 11.1% (p<0.05). A low CD44v6/CD44s ratio was associated with a 6.5 times greater risk of metastasis compared to a

Table 1. Baseline haracteristics of study subjects.

Variable	Case (n=18)	Control (n=20)
Age (year), mean±SD	50.8±11.1	50.5±9.0
Grade, n (%)		
Grade 1	0	3 (15)
Grade 2	12 (66.7)	12 (60)
Grade 3	6 (33.3)	5 (25)
Estrogen Receptor, n (%)		
Positive	18 (100)	19 (95)
Negative	0	1 (5)
Progesterone Receptor, n (%)		
Positive	15 (83.3)	15 (75)
Negative	3 (16.7)	5 (25)
HER2, n (%)		
Positive	4 (22.2)	5 (25)
Negative	14 (77.8)	15 (75)
Ki67, n (%)		
<20	6 (33.3)	9 (45)
≥20	12 (66.7)	11 (55)
Subtype, n (%)		
Luminal A	3 (16.7)	7 (35)
Luminal B	15 (83.3)	13 (65)
Metastasis Location, n (%)		
Lung	7 (38.9)	
Bone	2 (11.1)	
Liver	2 (11.1)	
Lung and Bone	4 (22.2)	
Liver and Brain	1 (5.6)	
Lung and Liver	2 (11.1)	
CD44v6 (ng/mL), median (min-max)	3.9 (2.3-12)	4.3 (2.5-18.8)
CD44s (ng/mL), median (min-max)	127.3 (86.9-893.6)	116.4 (94.1-976.81)
VEGF (ng/mL), median (min-max)	431(270.9-800)	381(243-792.6)
CD44v6/CD44s ratio, mean±SD	0.0291±0.0066	0.0305±0.0083

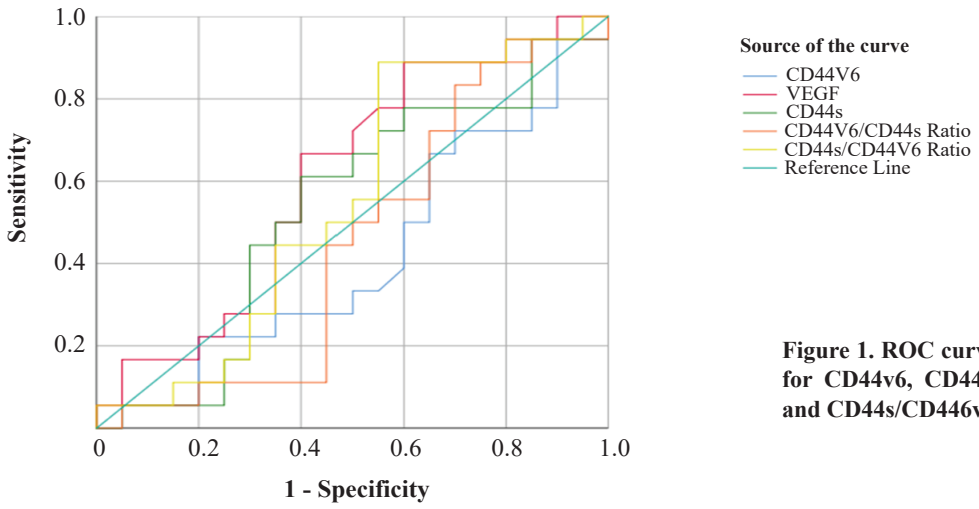

Figure 1. ROC curve to determine the cut-off points for CD44v6, CD44s, VEGF, CD44v6/CD44s ratio, and CD44s/CD446v ratio.

high ratio (OR 6.5, 95% CI: 1.2–36.2, p=0.02). These findings are statistically significant and reflect a real-world association, as indicated by the confidence interval not crossing 1. This study was the first ones to specifically identify the CD44v6/CD44s ratio as a significant risk factor for metastasis in breast cancer. The results suggested that the lower the CD44v6/CD44s ratio, the greater the risk of metastasis in luminal subtype breast cancer. This highlights the potential clinical relevance of the CD44v6/CD44s ratio as a biomarker for predicting metastasis.

Serum VEGF Levels and the Risk of Metastasis

Subjects with high serum VEGF levels (≥340 ng/mL) had a 4.3 times higher risk of metastasis compared to those with low VEGF levels (<340 ng/mL) (OR 4.3, 95% CI: 0.8–24.4, p=0.13). However, this result was not statistically significant.

Association of CD44v6, VEGF, CD44s, CD44v6/CD44s Ratio and Clinicopathological Characteristics

CD44v6 expression showed no statistically significant difference between tumor grade groups or ER status. However, a trend towards higher CD44v6 expression was observed in HER2-positive tumors compared to HER2-negative tumors. Similarly, CD44v6 expression was higher in Luminal B tumors compared to Luminal A, but this difference was not statistically significant (Table 3).

CD44s expression showed no significant association with tumor grade, ER status, PR status, or HER2 status. The expression of CD44s between Luminal A and Luminal B subtypes also showed no significant difference (Table 3).

A significant difference in CD44v6/CD44s ratio was observed between tumor stages (p=0.02), suggesting a

Table 2. Baseline characteristics and distribution of biomarker levels in study subjects.

Variable	n (%)
Stage	
Stage II	20 (52.6)
Stage IV	18 (47.4)
Grade	
Grade 1	3 (7.9)
Grade 2	24 (63.2)
Grade 3	11 (28.9)
Estrogen Receptor	
Positive	37 (97.4)
Negative	1 (2.6)
Progesterone Receptor	
Positive	30 (78.9)
Negative	8 (21.1)
HER2	
Positive	9 (23.7)
Negative	29 (76.3)
Ki67	
<20	15 (39.5)
≥20	23 (60.5)
Subtype	
Luminal A	10 (26.3)
Luminal B	28 (73.7)
CD44v6 (ng/mL)	
<4	21 (73.7)
≥4	17 (26.3)
CD44s (ng/mL)	
≥119.2	19 (50)
<119.2	19 (50)
VEGF (ng/mL)	
≥340	29 (76.3)
<340	9 (23.7)
CD44v6/CD44s ratio	
<0.034	27 (71.1)
≥0.034	11 (28.9)

Table 3. Association between CD44v6, VEGF, CD44s, and CD44v6/CD44s ratio with clinicopathological characteristics.

Characteristic	CD44v6		*p*-value	CD44s		*p*-value	CD44v6/CD44s Ratio		*p*-value	VEGF		*p*-value
	<4	≥4		≥119.2	<119.2		<0.03	≥0.03		≥340	< 340	
Stadium												
Std. 2	9	11	0.18	8	12	0.19	11	9	0.02*	13	7	0.08
Std. 4	12	6		11	7		9	2		16	2	
Grade												
Grade 1	2	1	0.71	1	2	0.51	2	1	0.77	2	1	0.39
Grade 2	14	10		11	13		18	6		17	7	
Grade 3	5	6		7	4		7	4		10	1	
ER Status												
Positive	20	17	0.36	19	18	0.31	27	10	0.11	28	9	0.57
Negative	1	0		0	1		0	1		1	0	
PR Status												
Positive	18	12	0.25	15	15	1.00	22	8	0.54	23	7	0.92
Negative	3	5		4	4		5	3		6	2	
HER2 Status												
Positive	3	6	0.13	6	3	0.25	7	2	0.61	7	2	0.90
Negative	18	11		13	16		20	9		22	7	
Ki67%												
<20%	10	5	0.25	6	9	0.31	11	4	0.80	12	3	0.66
≥20%	11	12		13	10		16	7		17	6	
Subtype												
Luminal A	5	5	0.69	5	5	1.00	7	3	0.93	7	3	0.58
Luminal B	16	12		14	14		20	8		22	6	

*Significant if $p<0.05$, analyzed with Chi-square tests.

potential role of this ratio in tumor progression. However, no significant differences were found in relation to tumor grade, ER status, PR status, HER2 status, or Ki67 levels. The CD44v6/CD44s ratio did not significantly differ between Luminal A and Luminal B subtypes (Table 3).

VEGF levels (≥340 *vs.* <340) were not significantly different across tumor grade, ER status, PR status, or HER2 status. A borderline association was noted with tumor stage, where VEGF expression was slightly higher in Stadium 2 tumors compared to Stadium 4, eventhough not statistically significant (Table 3).

These findings suggest that CD44v6/CD44s ratio may have prognostic value in tumor staging, whereas CD44v6 and VEGF show trends in association with HER2 status and tumor stage, respectively. However, no significant associations were observed with other clinicopathological parameters.

Multivariate Analysis

Following bivariate analysis of the independent and dependent variables, multivariate analysis was performed to evaluate the relationship between independent variables and confounders with the dependent variable. The analysis identified a low CD44v6/CD44s ratio (<0.03) as an independent risk factor for metastasis after controlling for the confounding effect of the breast cancer subtype (Table 4). The adjusted OR was 7.0, with a statistically significant result ($p=0.03$) and a confidence interval (CI) that did not cross 1. These findings indicate that patients with a low CD44v6/CD44s ratio have a 7-fold higher risk of developing metastasis compared to those with a higher ratio. This highlights the significant role of the CD44v6/CD44s ratio as a predictor of metastasis in luminal subtype breast cancer.

Correlations between CD44v6, CD44s, CD44v6/CD44s ratio and VEGF

The correlation analysis between CD44v6, CD44s, VEGF, and the CD44v6/CD44s ratio was assessed using Pearson's (r) and Spearman's (ρ) correlation tests, depending on data normality (Table 5).

A strong positive correlation was observed between CD44v6 and CD44s ($\rho=0.7$, $p<0.01$). Similarly, CD44s was strongly correlated with VEGF ($\rho=0.7$, $p<0.01$), indicating a

Table 4. Association of serum CD44v6, CD44s, VEGF levels, and CD44v6/CD44s ratio with metastasis risk in luminal subtype breast cancer.

Variable	Adjusted OR	95%CI	Adjusted P
Age	1.0	0.93 – 1.1	0.78
Subtype	0.3	0.06 – 1.7	0.19
Grade	0.7	0.12 – 4.3	0.72
VEGF	2.4	0.32 – 18	0.39
CD44v6/CD44s ratio	7.0	1.22 – 40.6	0.03*

*Significant if $p<0.05$, analyzed with logistic regression.

possible role for CD44s in angiogenesis-related processes. A moderate correlation was also found between CD44v6 and VEGF (ρ=0.4, p=0.01), further supporting the involvement of CD44 variants in tumor vascularization. Conversely, the CD44v6/CD44s ratio exhibited negative correlations with all tested markers. A moderate negative correlation was observed between the CD44v6/CD44s ratio and CD44s (r=-0.6, $p<0.01$), indicating that a higher CD44v6/CD44s ratio was associated with lower CD44s expression. Additionally, a weak to moderate negative correlation was found between the CD44v6/CD44s ratio and VEGF (r=-0.4, $p<0.01$), suggesting that tumors with a higher CD44v6/CD44s ratio may exhibit lower VEGF expression and possibly rely on non-angiogenic mechanisms for tumor progression. A similar weak negative correlation was noted between the CD44v6/CD44s ratio and CD44v6 (r=-0.4, p=0.02), which may indicate a shift in the balance of CD44 isoform expression.

No previous studies have specifically examined the correlation between CD44s and VEGF in luminal subtype breast cancer. This study identified a strong positive correlation between these biomarkers, suggesting a potential interaction in tumor progression. Higher VEGF levels were accompanied by an increase in CD44s levels, supporting their role in metastasis.

Discussion

The release of soluble CD44 is thought to regulate CD44 surface expression and is associated with lymphocytes.(15) Previous studies have shown a positive correlation between CD44 expression in primary tumors and its soluble form in serum.(16) In this study, it was found that low serum CD44v6 levels might indicate a risk of metastasis, though the association was not statistically significant. Contradictory findings in earlier studies report elevated serum CD44v6 levels in metastatic patients, potentially due to differences in sample distribution or the cut-off points used. For example, previous reported study identified elevated serum CD44v6 in cases with liver and bone metastasis, while our study primarily involved patients with lung metastasis.(17) Additionally, the low cut-off point of 4 ng/mL in this study differs from other studies reporting higher cut-off points.

High serum CD44s levels were associated with breast cancer metastasis in this study, though not statistically

Table 5. Correlations between CD44v6, CD44s, CD44v6/CD44s ratio and VEGF.

Compared Variables	r	ρ	*p*-value
CD44v6 and CD44s		0.7	0.00*
CD44v6 and VEGF		0.4	0.01*
CD44s and VEGF		0.7	0.00*
CD44v6/CD44s ratio and CD44v6	-0.4		0.02*
CD44v6/CD44s ratio and CD44s	-0.6		0.00*
CD44v6/CD44s ratio and VEGF	-0.4		0.00*

*Significant if $p<0.05$, r was analyzed with Pearson's, while ρ was analyzed with Spearman's Correlation test.

significant. While CD44s is known to act as a tumor suppressor in certain cancers like fibrosarcoma and prostate cancer, some studies have shown increased CD44s expression in higher-grade breast tumors.(18) CD44s expression increased significantly in grade 2 and 3 breast tumors compared to grade 1 tumors.(18) This context-dependent role of CD44s might explain its association with metastasis in this study.

This study is the first to identify the CD44v6/CD44s ratio as a statistically significant risk factor for metastasis in luminal subtype breast cancer (adjusted OR=7, $p<0.05$). The lower the ratio, the higher the risk of metastasis. The low CD44v6/CD44s ratio observed in this study can be explained through the isoform splicing mechanisms of CD44. In a study investigating isoform switching of CD44 during epithelial-mesenchymal transition (EMT) in human and mouse epithelial cells, as well as in breast cancer progression, it was shown that epithelial-mesenchymal transition (EMT) induces a shift in CD44 expression, transitioning from variant isoforms (CD44v) to the standard isoform (CD44s) in both *in vitro* and *in vivo* models.(18) This isoform switching to CD44s is critical for cells to undergo EMT and is essential for breast tumor formation. The transition is regulated by epithelial splicing regulatory protein 1 (ESRP1), a splicing factor that promotes alternative splicing of CD44 to produce CD44v isoforms. During EMT, ESRP1 mRNA levels significantly decrease, leading to the downregulation of CD44v and upregulation of CD44s. This finding establishes a strong link between isoform switching and EMT, highlighting the critical role of ESRP1 in regulating CD44 isoform expression and its impact on breast cancer development.(18) This mechanism supports the significance of the low CD44v6/CD44s ratio as a potential biomarker for breast cancer metastasis and its association with tumor progression.

Our finding shows high VEGF levels (≥340 ng/mL) were associated with a 4.3-fold increased risk of metastasis, but the result was not statistically significant. Several studies have confirmed that serum VEGF is associated with breast cancer, with higher concentrations generally detected in advanced-stage or metastatic disease compared to early-stage breast cancer.(19–23) Previous study demonstrated that higher VEGF levels correlate with poorer clinical outcomes and shorter progression-free survival in metastatic breast cancer patients.(23) The non-significant finding in this study may be due to the small sample size.

This study identified a strong positive correlation between CD44s and VEGF (r=0.7, $p<0.01$). Previous research supports this strong positive correlation, showing that CD44s enhances VEGF expression through the HIF-1α pathway, thereby promoting tumor angiogenesis.(24) CD44s also modulates cancer invasion and proliferation via the PI3K/AKT pathway, which can independently regulate VEGF secretion.(25,26) This interplay between CD44s and VEGF underscores their combined role in cancer progression and metastasis.

This study introduces two novel findings that contribute to the understanding of metastasis in luminal subtype breast cancer. First, it identifies the low CD44v6/CD44s ratio as a significant risk factor for metastasis, a discovery not previously reported in breast cancer research. This finding highlights the potential utility of the CD44v6/CD44s ratio as a biomarker for metastasis, particularly in luminal subtypes. Second, the study establishes a strong positive correlation between CD44s and VEGF levels in luminal breast cancer cases, a relationship that has not been documented in earlier studies.

Despite these novel contributions, this study has several limitations. The sample consisted of patients who had undergone surgery and no longer had primary tumors, contrasting with previous studies that included samples with primary tumors, which may explain differences in findings. Additionally, the study focused solely on two CD44 isoforms, CD44s and CD44v6, while other studies have investigated a broader range of CD44 isoforms for a more comprehensive analysis. Genomic Expression Profiling (GEP), a critical tool for prognostic evaluation, was not utilized in this study. The timing of sample collection, which occurred after surgery or adjuvant therapy, may have influenced the findings; a cohort study assessing variables before and after therapy could provide more clinically relevant insights. Furthermore, conventional diagnostic methods were used to determine metastasis, whereas advanced imaging techniques like positron emission tomography (PET) scans, unavailable at the research site, would have provided more accurate assessments.

Conclusion

The findings suggest that a lower CD44v6/CD44s ratio may be associated with an increased risk of metastasis, indicating its potential utility as a prognostic marker. A strong positive correlation between CD44s and VEGF further supports the interplay between stemness markers and angiogenic signaling in tumor progression. However, given the relatively small sample size and the absence of tissue validation or external cohort replication, these findings

should be interpreted with caution. Further studies with larger, independent populations and integrated molecular analyses are needed to validate the prognostic relevance of these markers and their possible role in guiding clinical management.

Acknowledgments

The authors would like to thank the Faculty of Medicine, Udayana University, and Prof. Ngoerah General Hospital, Denpasar, Bali, for providing the facilities and resources necessary for this study. We are also grateful to the laboratory staff for their assistance in histopathological preparations and technical support.

Authors Contribution

NPYK and TGBM were involved in concepting and planning the research, IBTWM and IWS performed the data acquisition/collection, NPYK and NNAD calculated the experimental data and performed the analysis, NPYK and IBTWM drafted the manuscript and designed the figures, IWS aided in interpreting the results. P contributed to literature review and final data verification. PFKP assisted in coordinating the project workflow and administrative tasks. All authors took part in giving critical revision of the manuscript.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68(6): 394–424.
2. Widiana IK, Irawan H. Clinical and subtypes of breast cancer in Indonesia. Asian Pacific J Cancer Care. 2020; 5(4): 281–5.
3. Dharmawan MS, Setiawan GB, Aryana IGNW. Karakteristik kanker payudara pada geriatri di rsup sanglah tahun 2015-2017. E-Jurnal Med Udayana. 2020; 9: 56–9.
4. Savitri M, Bintoro UY, Sedana MP, Diansyah MN, Romadhon PZ, Amrita PNA, *et al.* Circulating plasma miRNA-21 as a superior biomarker compared to CA 15-3: Assessment in healthy age matched subjects and different stage of breast cancer patients. Indones Biomed J. 2020; 12(2): 157–64.
5. Panigoro SS, Kurniawan A, Ramadhan, Sukartini N, Herqutanto, Paramita RI, *et al.* Amino acid profile of luminal A and B subtypes breast cancer. Indones Biomed J. 2023; 15(3): 269–76.
6. Abdihalim TS, Idris AAA. Mucin level as a potential biomarker for breast cancer diagnosis. Mol Cell Biomed Sci. 2022; 6(3): 117–20.
7. Engel P, Boumsell L, Balderas R, Bensussan A, Gattei V, Horejsi V, *et al.* CD Nomenclature 2015: Human leukocyte differentiation antigen workshops as a driving force in immunology. J Immunol. 2015; 195(10): 4555–63.
8. Prayogo AA, Wijaya AY, Hendrata WM, Looi SS, I'tishom R, Hakim L *et al.* Dedifferentiation of MCF-7 breast cancer continuous cell line, development of breast cancer stem cells (BCSCs) enriched culture and biomarker analysis. Indones Biomed J. 2020; 12(2): 115–23.
9. Porcellini S, Asperti C, Corna S, Cicoria E, Valtolina V, Stornaiuolo A, *et al.* CAR T cells redirected to CD44v6 control tumor growth in lung and ovary adenocarcinoma bearing mice. Front Immunol. 2020; 11: 99. doi: 10.3389/fimmu.2020.00099.
10. Qiao GL, Song LN, Deng ZF, Chen Y, Ma LJ. Prognostic value of CD44v6 expression in breast cancer: A meta-analysis. Onco Targets Ther. 2018; 11: 5451–7.
11. Perrot-Applanat M, Di Benedetto M. Autocrine functions of VEGF in breast tumor cells: Adhesion, survival, migration and invasion. Cell Adh Migr. 2012; 6(6): 547–53.
12. Guntarno NC, Rahaju AS, Kurniasari N. The role of MMP-9 and VEGF in the invasion state of bladder urothelial carcinoma. Indones Biomed J. 2021; 13(1): 61–7.
13. Widowati W, Jasaputra DK, Sumitro SB, Widodo MA, Afifah E, Rizal R, *et al.* Direct and indirect effect of TNFα and IFNγ toward apoptosis in breast cancer cells. Mol Cell Biomed Sci. 2018; 2(2): 60–9.
14. Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, *et al.* The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more 'personalized' approach to cancer staging. CA Cancer J Clin. 2017; 67(2): 93–9.
15. Ristamäki R, Joensuu H, Jalkanen S. Serum CD44 in non-Hodgkin's lymphoma. Leuk Lymphoma. 1999; 33(5-6): 433–40.
16. Baek JM, Jin Q, Ensor J, Boulbes DR, Esteva FJ. Serum CD44 levels and overall survival in patients with HER2-positive breast cancer. Breast Cancer Res Treat. 2011; 130(3): 1029–36.
17. Lackner C, Moser R, Bauernhofer T, Wilders-Truschnig M, Samonigg H, Berghold A, *et al.* Soluble CD44 v5 and v6 in serum of patients with breast cancer. Correlation with expression of CD44 v5 and v6 variants in primary tumors and location of distant metastasis. Breast Cancer Res Treat. 1998; 47(1): 29–40.
18. Brown RL, Reinke LM, Damerow MS, Perez D, Chodosh LA, Yang J, *et al.* CD44 splice isoform switching in human and mouse epithelium is essential for epithelial-mesenchymal transition and breast cancer progression. J Clin Invest. 2011; 121(3): 1064–74.
19. Salven P, Mänpää H, Orpana A, Alitalo K, Joensuu H. Serum vascular endothelial growth factor is often elevated in disseminated cancer. Clin cancer Res an Off J Am Assoc Cancer Res. 1997; 3(5): 647–51.
20. Salven P, Perhoniemi V, Tykkä H, Mäenpää H, Joensuu H. Serum VEGF levels in women with a benign breast tumor or breast cancer. Breast Cancer Res Treat. 1999; 53(2): 161–166.
21. Coskun U, Günel N, Toruner FB, Sancak B, Onuk E, Bayram O, *et al.* Serum leptin, prolactin and vascular endothelial growth factor (VEGF) levels in patients with breast cancer. Neoplasma. 2003; 50(1): 41–6.
22. Iovino F, Ferraraccio F, Orditura M, Antoniol G, Morgillo F, Cascone T, *et al.* Serum vascular endothelial growth factor (VEGF) levels correlate with tumor VEGF and p53 overexpression in endocrine positive primary breast cancer. Cancer Invest. 2008; 26(3): 250–5.
23. Banys-Paluchowski M, Witzel I, Riethdorf S, Pantel K, Rack B,

Janni W, *et al.* The clinical relevance of serum vascular endothelial growth factor (VEGF) in correlation to circulating tumor cells and other serum biomarkers in patients with metastatic breast cancer. Breast Cancer Res Treat. 2018; 172(1): 93–104.

24. Ryu D, Ryoo IG, Kwak MK. Overexpression of CD44 standard isoform upregulates HIF-1α signaling in hypoxic breast cancer cells. Biomol Ther. 2018; 26(5): 487–93.
25. Chen C, Zhao S, Karnad A, Freeman JW. The biology and role of CD44 in cancer progression: therapeutic implications. J Hematol Oncol. 2018; 11: 46. doi: 10.1186/s13045-018-0605-5.
26. Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci 2011; 4: 51. doi: 10.3389/fnmol.2011.00051.