The Aggressiveness of African breast cancer: An expose' on CD44⁺CD24^{-/low} breast cancer stem cell

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Abstract

Breast cancer (BC) in Africans and people of African descent is generally aggressive, with poorer prognosis and worse clinical outcomes. The molecular basis of this is however not entirely understood. The CD44⁺ / CD24^{-/low} BC stem cell is known for its tumourigenic potential, tumour aggressiveness and its association with poor prognosis. This study identifies the relationship between CD44⁺ / CD24^{-/low} BC stem cells and clinicopathological features of breast cancer in an African population.

Methodology A Ghanaian BC cohort (n= 222) was used to assess CD44 and CD24 expression. Tissue microarray was constructed from the cohort samples and Immunohistochemically stained with CD44 and CD24 antibodies. The associations between clinicopathological features and the expression of the individual markers and their combinations were analysed.

Results Of the total 222 breast cancer samples, 81.9 % were cytoplasmic CD24 positive and were associated with higher tumour grade (OR-3.623; $r=0.199$; $p=0.004$), gender (OR-9.514; $p=0.028$), clinical prognostic grading (OR-2.357 $r=0.162$; $p=0.027$) and Her2 positivity (OR-0.216; $r=-0.155$; $p=0.026$). CD44 was associated with higher tumour grade (OR-3.148; $r=0.145$; $p=0.037$), and increased mitotic count (OR-3.043, $r=0.173$; $p=0.028$). There was no association between CD44 expression and hormone receptor status. Together, CD44⁺/CD24^{-/low} staining was associated with higher tumour grade (OR-3.162; $r=0.166$; $p=0.018$), gender (OR- 12.0; $p=0.012$), and higher clinical prognostic staging (OR- 2.888; $r=0.186$; $p=0.011$). An inverse association of CD44⁺CD24⁺ was found with tumour grade (OR-0.220; $r=-0.246$; $p=0.000$), mitotic count (OR-0.406; $r=-0.190$; $p=0.017$) and clinical prognostic staging (OR-0.486; $r=-0.151$; $p=0.040$). There was no association between CD44⁺CD24⁺ and all the clinicopathological features.

Conclusion Combined, CD44⁺CD24^{-/low} was associated with poor prognosis and tumour aggression and may contribute to the tumour aggressiveness of African breast cancer. CD24 expression as a stand-alone marker was found to correlate with clinical and pathological indicators of tumour aggressiveness and poor prognosis.

Introduction

A plethora of data confirms the aggressiveness of breast cancers (BC) of African patients and of patients with African descent compared to their Caucasian counterparts.¹⁻³ The molecular basis for this disparity is however not fully understood. The expression of certain BC stem cells has however been suggested to be responsible for enhanced tumour aggressiveness. It is well established that breast cancers in Africans are more likely to be triple negative and basal like.⁴⁻⁷ Racial disparity in cancer risk, prevalence and clinical outcomes have also been very well documented.⁸⁻¹⁰ It is still unclear if breast cancer stem cells contribute to such racial disparity and to what extent they confer tumour aggressiveness remains largely unanswered. Knowledge on breast cancer stem cells in African breast cancers remains scanty.

Cancer stem cells are known to possess the intrinsic ability of self-renewal, have tumourigenic potential and drive tumour progression.¹¹ They are also known to be responsible for chemotherapy resistance and tumour recurrence.¹² The identification of these stem cells in tumours offers important evidences on tumorigenesis, therapeutic resistance and recurrence. These may also serve as important approaches for targeted therapies. Prognostication of tumours may also be based on presence or absence of specific cancer stem cells. Earlier studies have focused on elucidating the association that exists between cancer stem cells, prognostic markers, and response to adjuvant therapy. It has been reported that triple negativity and basal like phenotype (BLP) have poor prognosis and are associated with candidate stem cells CD44⁺/CD24⁻.^{4,13,14} Since the discovery of CD44⁺CD24^{-/low} phenotype breast stem cells with a tumourigenic potential by Al Hajj *et al*, various studies continue to elucidate its role in breast cancer.¹¹ Despite the intense research interest generated on this putative stem cell marker, its role remains puzzling. Do breast cancer stem cells contribute to such racial disparity and to what extent do they confer tumour aggressiveness? This study identifies the role of CD44⁺CD24^{-/low} breast cancer stem cell marker in contributing to the inherent aggressiveness of African breast cancers.

Materials And Methods

This is a hospital based retrospective study of breast biopsies and reports of patients (n=222) presenting with breast cancer at the departments of pathology, Korle-Bu Teaching Hospital, Accra and the Cape Coast Teaching Hospital, Cape Coast, Ghana from 2012 to 2018. The Korle-Bu Teaching Hospital department of pathology is the largest in Ghana, receiving specimens from the Korle-Bu Teaching Hospital, the largest referral hospital in Ghana and from other health facilities within the Greater Accra Region. The department also receives specimen from all other regions of Ghana. The Cape Coast Teaching Hospital's pathology department also receives specimen from mainly the Central and Western regions of Ghana. Clinical and demographic data regarding age, gender, and clinical information were obtained from the histopathology request forms and registry. Histopathology slides of selected cases within the study period (2012-2018) were reviewed by two pathologists (PKA & LDK). Archival blocks of primary breast carcinoma from the Departments of Pathology, Korle-Bu and Cape Coast Teaching Hospitals were retrieved. Additional clinical information and histopathological features were obtained from the histopathology reports of patients. The information included the mean age of presentation, duration of symptoms, tumour grade, (based on mitotic count, nuclear grade, tubule formation). All cases were reviewed histopathologically and classified according to the recent WHO classification for breast tumours and histopathological grading done in accordance with the Nottingham criteria¹⁵.

Tissue Microarray (TMA) Construction

Archival formalin fixed paraffin embedded (FFPE) blocks' and Hematoxylin and Eosin (H&E) stained slides were reviewed and areas of tumour selected. Areas of normal tissue, necrosis, and haemorrhage were ignored. Three cores 1mm each (2 peripheral tumour and 1 central) were punched out from the

representative selected areas and arrayed into a new recipient paraffin block using TMA Grand Master® (3D HISTECH®, Budapest, Hungary). Four micrometre thickness of TMA sections were cut and mounted on Superfrost slides.

Immunohistochemistry (IHC)

TMA were stained using CD24 Monoclonal antibody (SN3), Thermofisher and CD44 monoclonal antibodies (156-3C11), Thermofisher at dilutions of 1:200 and 1:750 respectively. Immunohistochemical antibody labelling was done using the NOVOLINK polymer detection system. To enhance tissue adhesion to the slide, tissue microarrays were pre-heated at 60°C on a hot plate for 20 minutes and cooled.

Deparaffinisation was done on tissue sections in xylene and rehydrated through a series of graded alcohols and rinsed in distilled water. Antigen retrieval was enhanced by boiling slides in citrate buffer (27ml of citrate in 123 ml disodium citrate and made up to 1.5L with ddH₂O) at pH-6.0 and microwaved at full power for 20 minutes. Endogenous peroxidase activity was blocked using the Peroxidase blocking reagent from the NOVOLINK® kit for 5 minutes and rinsed with PBS for 15 minutes. Protein blocking was done for 5 minutes to minimize nonspecific binding and rinsed thoroughly with PBS for 15 minutes. Primary antibodies were added in the following dilutions: CD24- 1:200 and CD44- 1:750 and incubated in a black box for 1 hour at room temperature. A thorough rinse was done for 15 minutes with PBS tween and then incubated with Post Primary Novolink reagent for 30 minutes in a black box. After a 15-minute thorough rinse, a Polymer was added and incubated for 30 minutes. Peroxidase was then developed by incubating 3,3'-diaminobenzidine chromogen solution (DAB) made up to in 1:20 dilution with DAB substrate buffer and incubated for 5 minutes. Counter staining with hematoxylin was done and incubated for 6 minutes. Dehydration and clearing were done using the Leica autostainer. Sections were then mounted with DPX. Breast cancer cases known to be positive for the markers being studied were used as positive controls. Evaluation of staining was done for both markers.

The semi-quantitative H scoring system was employed in scoring. The intensity of CD24 and CD44 expression were scored as 0 (no expression), 1 (weak), 2 (moderate) and 3 (strong). The total score was calculated as the percentage of positive cells multiplied by the intensity giving a range of 0-300. A cut point of $\leq 40\%$ score was designated as negative and $>40\%$ as positive in keeping with Ahmed *et al's* study¹⁶.

For Oestrogen and Progesterone receptor staining, positive expression was considered as nuclear immunoreactivity in $\geq 1\%$ of neoplastic cells. HER2 was analyzed at the time of diagnosis according to the American Society of Clinical Oncology (ASCO) / College of American Pathologists (CAP) protocols¹⁷.

The molecular subtypes were classified as Luminal A (ER+ and/or PR+ and Her2-), Luminal B (ER+ and/or PR+ and Her2+), Triple Negative (ER- PR- and Her2-) and Her2+ (ER- PR- and Her2+)¹⁷.

Statistical Analysis

IBM SPSS version 24.0 package program (SPSS inc., Chicago, IL, USA) was used in the statistical analysis. The association between the markers and clinicopathological features were done with cross tables using chi-square test and odd ratios. Correlations were done with Pearson’s correlation test. Statistical significance was put at the α level.

Results

A total of 222 breast carcinoma samples were used in the study. The study population were predominantly females with 219/222 (98.6%) being females and 3/222 (1.4%) males. The mean age of patients presenting with breast carcinoma in the cohort was 51.4 ± 12.5 . A total of 110 (49.5%) involved the right breast, while 107(48.2%) involved the left breast and only 1(0.5) were bilateral. There was no indication of laterality in 4 of the patients. Of the total, 177(79.7%) presented with solitary masses and 41(18.5%) with multiple masses with 4 missing data. Table 1 shows details of clinicopathological features of patients.

Table 1: Clinicopathological features of patients

Parameter	Frequency (%)
Age (n=220)*	
<50	108(49.1%)
≥50	112 (50.9%)
Grade (n=212)*	
I	24(11.3%)
II	84(39.6%)
III	104(49.1%)
Clinical prognostic staging (n=188)*	
IA	3(1.6%)
IB	9(4.8%)
IIA	28(14.9%)
IIB	27(14.4%)
IIIA	25(13.3%)
IIIB	48(25.5%)
IIIC	48(25.5%)
Nottingham Prog. Index (n=113)*	
Good NPI (<3.4)	3(2.7%)
Moderate NPI (3.41-5.4)	30(26.5%)
Poor NPI (>=5.41)	80(70.8%)
Lymph Node Stage(n=113)*	
1 (Negative)	26(23.0%)
2 (1-3 LN involved)	31(27.4%)
3 (> 3 LN involved)	56(49.6%)
Vascular Invasion (n=177)*	
Yes	118(66.7%)
No	59(33.3%)
Mitosis(n=164)*	
<10	58(35.4%)

11-20	63(38.4%)
>20	43(26.2%)
Tumour size, cm (n=196)*	
≤2	15(7.7%)
>2- <5	63(32.1%)
≥5	118 (60.2%)
Molecular subtypes (n=212)*	
Luminal A	75 (35.4%)
Luminal B	21 (9.9%)
Her2	22 (10.4%)
Triple Negative	94 (44.3%)

*missing data

Clinicopathological correlations

There was no significant association between age and the clinicopathological features. Apart from clinical prognostic stage ($p<0.001$), Nottingham Prognostic Index ($p<0.001$) and Mitosis count ($p<0.001$) which had significant association with tumour grade, all other clinicopathological parameters did not show any association. Clinical prognostic stage also showed significant association with all clinicopathological parameters (Grade $p<0.001$; NPI $p=0.004$; tumour size $p<0.001$; vascular invasion $p<0.001$; mitotic count $p<0.001$) except for weight and age.

Immunohistochemistry analysis

Of the total, 71(32.6%) and 74(33.8%) of patients expressed estrogen and progesterone receptors respectively. 38 (18.3%) patients expressed Her2 receptor. 94 (44.3%) were negative for all 3 receptors hence were triple negative.

Cytoplasmic CD24 expression and its association with clinicopathological features

Eighty-one-point one percent of tumours were cytoplasmic CD24 positive. Tumour grade ($p=0.004$) and clinical prognostic staging ($p=0.027$) were the only clinicopathological features which had significant association with CD24 cytoplasmic expression. Age did not have an association with CD24 cytoplasmic

expression (OR- 1.9, $p=0.064$). Grade 2 & 3 tumours had higher CD24 cytoplasmic expression with between 3 to 4-fold increase (OR-3.6, $p=0.004$). Females predominantly had higher CD24 cytoplasmic expression (OR- 9.5, $p=0.028$). Although marginally non-significant ($p=0.085$), it appeared tumours with >10 mitotic features per 10 high power field (/10hpf) had a 2-fold increased CD24 cytoplasmic expression compared with tumours <10/10hpf from the odd ratio. Clinical Prognostic staging (stage III and above) was associated with about 2-fold increased cytoplasmic CD24 expression compared with stage I&II tumours (OR- 2.3, $p=0.027$). Table 2 summarises the cytoplasmic expression of CD24 and its association with clinicopathological features. Figure 1 shows membranous staining of CD44 and homogenous cytoplasmic staining of CD24

Table 2: CD24 Cytoplasmic expression and its relationship with clinicopathological features

Parameters	CD24 Cytoplasmic expression			Significance	
	Positive(%)	Negative(%)	r (95%CI)	OR p value	
Patient's age					
<50	24(61.5)	79(45.1)	0.127 3.957)	1.9 (0.955-	0.064
≥50	15(38.4)	96(54.9)			
Grade					
1	9(37.5)	15(62.5)	0.199 3.623 (1.410-4.943)		0.004*
2/3	26(14.2)	157(85.8)			
Tumour size					
≤2	4(26.7)	11(73.3)	0.068 1.782 (0.531-5.974)		0.344
>2	30(16.9)	147(83.1)			
Vascular Invasion					
Yes	19(16.8)	94(83.2)	0.039 1.263 (0.516-3.090)		0.608
No	8(13.8)	50(86.2)			
LN stage					
Negative	5 (19.2)	21(80.8)	0.058 1.429(0.452-4.516)		0.542
Positive	12(14.3)	72(85.7)			
Mitosis					
≤10	14(24.6)	43(74.5)	0.0136 2.047 (0.896-4.673)		0.085
>10	14(13.7)	88(86.3)			
NPI					
Mod. To Good NPI (<3.4-5.4)	2(18.2)	27(81.8)	0.035 1.222 (0.416-3.590)		0.715
Poor NPI (>/=5.41)	12(15.4)	66(84.6)			

Clinical Prognostic Staging				
I&II	17(25.4)	50(74.6)	0.162 2.357(1.089-5.101)	0.027*
III	15(12.6)	104(87.4)		

*statistically significant p value at 95% CI, OR- Odd Ratio, r- Pearson's correlation coefficient, NPI- Nottingham Prognostic Index

Cytoplasmic CD24 expression and hormone receptor markers

Table 3 indicates the cytoplasmic CD24 expression pattern in relation to hormone receptor status. Only Her2 had a significant association with CD24 cytoplasmic expression. Her2 negative tumours had 5 times higher CD24 expression in comparison with Her2 positive tumours (OR- 4.626, $p=0.026$). There was no association between the molecular subtypes and CD24 expression (Table 3).

Table 3: Association between CD24 expression, Hormone receptor status and Her2 status

Marker	CD24 cytoplasmic expression			Significance	
	Negative(%) (95%CI)		Positive(%)	OR	^r <i>Value</i> <i>P</i>
ER					
Positive	13(18.6)	57(81.4)	1.076 (0.513-2.295)	0.013	0.845
Negative	25(17.5)	118(82.5)			
PR					
Positive	13(17.6)	61(82.4)	0.943 (0.452-1.965)	-0.011	0.875
Negative	26(18.4)	115(81.6)			
Her2					
Positive	2(5.3)	36(94.7)	0.216 (0.049-0.941)	-0.155	0.026*
Negative	34(20.5)	132(79.5)			
Subtypes					
Triple Neg	16(17.6)	75(82.4)	1.035 (0.502-2.132)	0.006	0.926
Others	20(17.1)	97(82.9)			
Molecular subtypes					
Luminal A	16(21.6)	58(78.4)	1.572(0.758-3.261)		0.222
Luminal B	2(9.1)	20(90.9)			
Her2+	2(9.1)	20(90.9)	0.447(0.100-2.004)		0.281
Triple Negative	16(17.6)	75(82.4)			
			1.035(0.502-2.132)		0.926

*statistically significant p value at 95% CI, OR- Odd Ratio, r- Pearson's correlation coefficient, ER- Estrogen receptor, PR- Progesterone receptor

Table 4: Association between CD44 and clinicopathological features

Parameters	CD44 Cytoplasmic expression		Significance		
	Negative(%)	Positive(%)	OR (95%CI) Value	r	p
Patient's age					
<50	10(47.6)	95(49.0)	0.947 (0.38-2.33) -0.008		0.906
≥50	11(52.4)	99(51.0)			
Grade					
1	5(25.0)	18(9.6)	3.148(1.027-9.674) 0.145		0.037*
2&3	15(75.0)	170(90.4)			
Tumour size					
≤2	1(5.0)	14(8.2)	0.590 (0.073-4.743) -0.036		0.616
>2	19(95.0)	157(91.8)			
Vascular Invasion					
Yes	15(78.9)	100(65.4)	1.988 (0.628-6.290) 0.090		0.235
No	4(21.1)	53(34.6)			
LN stage					
Negative	4(36.4)	22(22.4)	1.974 (0.529-7.367) 0.098		0.305
Positive	7(63.6)	76(77.6)			
Mitosis					
≤10	10(58.8)	46(31.9)	3.043 (1.089-8.503) 0.173		0.028*
>10	7(41.2)	98(68.1)			
NPI					
Mod. to Good NPI (<5.41)	6(54.5)	26(26.3)	3.369 (0.948-11.978) 0.187		0.050
Poor NPI (>/=5.41)	5(45.5)	73(73.7)			

Triple Negative status				
Triple Negative	8(8.5)	86(91.5)	0.814(0.318-2.081)	0.667
Others	12(10.3)	105(89.7)	-0.030	
Clinical Prognostic Staging				
I & II	6(9.1)	60(90.9)	0.831(0.300-2.298)	0.721
III	13(10.7)	108(89.3)	-0.026	

*statistically significant p value at 95% CI, OR- Odd Ratio, r- Pearson's correlation, NPI-Nottingham Prognostic index

CD24 nuclear staining

CD24 nuclear staining of 14% (30/212) was recorded in this study but did not have an association with any of the clinicopathological features and hormone receptor status.

Association between CD44 expression and clinicopathological features

Tumour grade and mitotic count per high power field were the only clinicopathological parameters significantly associated with CD44 expression. CD44 expression had a 3-fold increase in grades (2&3) tumours (OR-3.148, 95%CI 1.027-9.674, $p=0.037$). Similarly, a mitotic count > 10/10hpf was found to be associated with higher CD44 cytoplasmic positivity (3-fold increased odds) (OR-3.043 95%CI-1.089-8.503, $p=0.028$). Table 4 summarises the association between CD44 cytoplasmic expression and clinicopathological features.

Association between CD44 cytoplasmic expression and hormone receptor status

This study revealed no association between CD44 cytoplasmic expression and ER, PR & Her2 status. A summary of CD44 cytoplasmic expression and hormonal status can be found in table 5. Triple negative tumours predominantly expressed CD44 (45%).

Table 5: Association and correlation between cytoplasmic expression of CD44 and hormone receptor status

Marker	CD44 cytoplasmic expression			Significance	
	Negative (%)	Positive(%)	OR (95%CI)	r	<i>p value</i>
ER					
Positive	9(42.9)	61(31.3)	1.0	0.073	0.282
Negative	12(57.1)	134(68.7)	1.648 (0.659-4.117)		
PR					
Positive	8(38.1)	64(32.7)	1.0	0.034	0.615
Negative	13(61.9)	132(67.3)	1.269 (0.501-3.217)		
Her2					
Positive	4(20.0)	34(18.3)	1.0	0.013	0.850
Negative	16(80.0)	152(81.7)	1.118 (0.351-3.555)		
Molecular Subtypes					
Luminal A	8(40)	66(34.6)	1.0	-0.030	0.695
Luminal B	3(15)	18(9.4)	0.727 (1.175-3.026)		
Her2+	1(5)	21(11.0)	2.545 (0.301-21.550)		
Triple Neg	8(40)	86(45.0)	1.303 (0.465-3.654)		

*statistically significant p value at 95% CI, OR- Odd Ratio, r- Pearson's correlation

CD44/CD24 combined phenotypes

Predominantly, CD44⁺CD24⁺ was the most occurring combined phenotype (76%) with CD44⁺CD24⁻ as the second most common phenotype (15%). CD44⁻CD24⁻(3%) was however the least occurring phenotype Fig. 2.

Association between CD44/CD24 combined phenotypes and clinicopathological features

Figure 3 shows the various combinations of CD44 and CD24 using same cases as reference.

CD44⁺CD24^{-/low}

CD44⁺CD24^{-/low} was found to be significantly associated with tumour grade (OR-3.1, $p=0.018$), gender ($p=0.012$), and clinical prognostic staging (OR-2.8, $p=0.011$). In relation to age, a trend of CD44⁺CD24^{-/low} appeared to be predominant in the patients <50 years old although marginally non-significant (OR 2.0, $p=0.072$). Tumours with higher grades (grade 2&3) expressed more CD44⁺CD24^{-/low} (OR-3.1, $p=0.018$). Females have 12-fold increased risk of expressing CD44⁺CD24^{-/low} compared with males ($p=0.012$). CD44⁺CD24^{-/low} has almost a 3-fold increased cytoplasmic expression in clinical prognostic stage III compared with lower stages I&II ($p=0.011$, OR- 2.9)

CD44⁺CD24⁺

Significant association of CD44⁺CD24⁺ was found with tumour grade ($p<0.001$ $r=-0.246$), mitotic count ($p=0.017$ $r=-0.190$) and clinical prognostic staging ($p=0.040$ $r=-0.151$). Higher grade tumours (2&3) expressed less of CD44⁺CD24⁺ phenotype compared to the low grade tumours (OR-0.220, $p<0.001$). Less CD44⁺CD24⁺ expression was seen in the older age group (≥ 50 years) (OR 0.689, $p=0.248$). The odds of larger tumours (>2cm) expressing CD44⁺CD24⁺ phenotype was lesser (OR- 0.617, $p=0.398$ $r=-0.062$) likewise the odds of females presenting with this phenotype lesser than males (OR- 0.152 $p=0.081$). Tumours with <10 mitotic counts/10hpf expressed more CD44⁺CD24⁺ phenotypes compared to ≥ 10 mitotic counts/10hpf (OR-2.46 $p=0.017$). Clinical prognostic staging III tumours presented with less CD44⁺CD24⁺ phenotypic expression (OR-0.486, $p=0.040$, $r=-0.151$).

CD44⁻CD24^{-/low}

CD44⁻CD24^{-/low} is significantly associated with mitotic count ($p=0.011$ $r=0.202$) and Nottingham Prognostic Index (NPI) ($p=0.041$ $r=0.196$). The odds of expression of CD44⁻CD24^{-/low} is about 10 times higher in tumours with increased mitotic counts ($\geq 10/10$ hpf). The expression of CD44⁻CD24^{-/low} corresponded with increasing NPI (OR-7.86 $p=0.041$)

CD44⁻CD24⁺

There is no association between CD44⁻CD24⁺ and all the clinicopathological features. Table 6 summarises the CD44CD24 combinatorial phenotypes with clinicopathological features.

Table 6: Association between CD44/CD24 combination phenotypes and clinicopathological features

Parameter		%CD44+CD24-	%ofCD44+CD24+	%ofCD44-CD24-	%ofCD44-CD24+
Age	<50(n=101)	62.5(n=20)	45.6(n=73)	57.1(n=4)	33.3(n=4)
	≥ 50 (n=110)	37.5(n=12)	54.4(n=87)	42.9(n=3)	66.7(n=8)
	OR	2.016(0.930-4.372)	0.689(0.366-1.298)	1.471(0.321-6.738)	0.526(0.153-1.806)
	<i>p</i>	0.072	0.248	0.617	0.299
Grade	1(n=23)	24.1(n=7)	7.0(n=11)	33.3(n=2)	25.0(n=3)
	2&3(n=192)	75.9 (n=22)	93.0(n=157)	66.7(n=4)	75.0(n=9)
	OR	3.162(1.170-8.542)	0.220(0.09-0.539)	4.214(4.727-24.41)	2.867(0.717-11.466)
	<i>p</i>	0.018*	0.000*	0.083	0.121
Tumour size (cm)	≤2(n=15)	14.3(n=4)	7.0(n=10)	0.0(n=0)	8.3(n=1)
	>2(n=174)	85.7(n=24)	93.0(n=133)	100.0(n=6)	91.7(n=11)
	OR	2.273(0.669-7.720)	0.617(0.199-1.907)		1.058(0.127-8.806)
	<i>P</i>	0.178	0.398	0.465	0.958
Gender	Male(n=3)	6.3(n=2)	0.6(n=1)	0.0(n=0)	0.0(n=0)
	Female(n=210)	93.8(n=30)	99.4(n=161)	100.0(n=7)	100(n=12)
	OR	12.00(1.055-136.49)	0.152(0.014-1.714)		
	<i>p</i>	0.012*	0.081	0.748	0.670
Vascular Invasion	Present (n=112)	70.0 (n=14)	64.9(n=85)	71.4(n=5)	80.0(n=8)
	Absent (n=56)	30.0 (n=6)	35.1(n=46)	28.6(n=2)	20.0(n=2)
	OR	1.190(0.431-3.286)	0.684(0.305-1.537)	1.262(0.237-6.717)	2.077(0.426-10.124)
	<i>p</i>	0.736	0.357	0.785	0.356
Lymph Node stage	Negative(n=26)	23.1(n=3)	22.4(n=19)	50.0(n=2)	33.3(n=2)
	Positive (n=82)	76.9(n=10)	77.6(n=66)	50.0(n=2)	66.7(n=4)
	OR	0.939 (0.238-3.707)	0.658(0.236-1.833)	3.333(0.446-24.93)	1.625
	<i>P</i>	0.929	0.421	0.216	0.585
Mitosis	≤10(n=55)	40.9(n=9)	30.0(n=36)	83.3(n=5)	55.6(n=5)
	>10(n=102)	59.1(n=13)	70.0(n=84)	16.7(n=1)	44.4(n=4)

	OR	1.339 (0.533-3.366)	0.406(0.191-0.863)	10.100(1.149-88.77)	2.450(0.630-9.528)
	<i>p</i>	<i>0.533</i>	<i>0.017*</i>	<i>0.011*</i>	<i>0.184</i>
Nottingham Prognostic Index	<3.4-5.4 (n=32)	21.4 (n=3)	27.1(n=23)	75.0(n=3)	50.0(n=3)
	>5.4 (n=77)	78.6 (n=11)	72.9(n=62)	25.0(n=1)	50.0(n=3)
	OR	0.621(0.161-2.392)	0.618(0.238-1.607)	7.862(0.786-78.673)	2.552(0.487-13.379)
	<i>P</i>	<i>0.485</i>	<i>0.321</i>	<i>0.041*</i>	<i>0.253</i>
Triple Negative status	Present (n=91)	44.8(n=13)	45.0(n=72)	42.9(n=3)	27.3(n=3)
	Absent (n=116)	55.2(n=16)	55.0(n=88)	57.1(n=4)	72.7(n=8)
	OR	1.042(0.473-2.294)	1.206(0.623-2.334)	0.955(0.208-4.376)	0.460(0.119-1.787)
	<i>p value</i>	<i>0.919</i>	<i>0.579</i>	<i>0.952</i>	<i>0.252</i>
Clinical prognostic staging	I & II (n=66)	22.7(n=15)	68.2(n=45)	3.0(n=2)	6.1(n=4)
	III (n=198)	77.3(n=51)	31.8(n=21)	97.0(n=64)	93.9(n=62)
	OR	2.888(1.239-6.731)	0.486(0.243-0.973)	0.898(0.160-5.041)	1.032(0.291-3.665)
	<i>p value</i>	<i>0.011*</i>	<i>0.040*</i>	<i>0.903</i>	<i>0.961</i>

*statistically significant p value at 95% CI, OR- Odd Ratio

Association between CD44/CD24 combined phenotypic expression and hormonal status

No significant association is realised between CD44/CD24 combined phenotypes and hormonal status (Table 7).

Table 7: The association between CD44/CD24 combined phenotypic expression and hormonal status

Marker	Combinatorial phenotypes expression			
	CD44-/CD24-	CD44+/CD24 ^{-/low}	CD44+/CD24 ⁺	CD44-/CD24 ⁺
ER				
Positive	3(42.9)	10(32.3)	50(30.9)	6(50.0)
Negative	4(57.1)	21(67.7)	112(69.1)	6(50.0)
OR	1.580(0.344-7.261)	0.985(0.436-2.224)	0.728(0.376-1.411)	2.175(0.675-7.008)
<i>p value</i>	<i>0.554</i>	<i>0.970</i>	<i>0.376</i>	<i>0.184</i>
PR				
Positive	3(42.9)	10(31.3)	54(33.3)	5(41.7)
Negative	4(57.1)	22(68.8)	108(66.7)	7(58.3)
OR	1.489(0.324-6.840)	0.917(0.473-1.775)	0.872(0.389-1.957)	1.429(0.473-4.670)
<i>p value</i>	<i>0.607</i>	<i>0.796</i>	<i>0.741</i>	<i>0.553</i>
Her2				
Positive	0(0.0)	2(6.9)	32(20.6)	4(36.4)
Negative	7(100)	27(93.1)	123(79.4)	7(63.6)
OR		0.282(0.064-1.241)	1.778(0.694-4.555)	2.639(0.731-9.521)
<i>p value</i>	<i>0.195</i>	<i>0.076</i>	<i>0.226</i>	<i>0.126</i>
Subtypes				
Luminal A	4(57.1)	12(41.4)	53(33.1)	4(36.4)
	0(0.0)	2(6.9)	16(10.0)	3(27.3)
Luminal B	0(0.0)	2(6.9)	19(11.9)	1(9.1)
Her2+	3(42.9)	13(44.8)	72(45.0)	3(27.3)
Triple Neg				

*statistically significant p value at 95% CI, OR- Odd Ratio, ER- Estrogen Receptor, PR- Progesterone receptor

Discussion

Since the tumourigenicity of breast cancer stem cells (BCSC) was first demonstrated by Al-Haji et al in 2003¹¹, population based comparative ethnic and racial studies comparing the expression pattern of BCSC in different ethnic groups and races remain scanty. Although research evidence on cancer stem cells (CSC) are accumulating on Caucasian and Asian breast cancer populations, studies in African populations remain at the lowest ebb. There is well-documented evidence of racial difference of cancer risk, prevalence and clinical outcome⁸⁻¹⁰ with breast cancers of Africans and those of African descent presenting with relatively aggressive tumours¹⁸. A constellation of research evidence points to increased triple negative breast cancer in Africans.⁴⁻⁶

In keeping with literature¹⁻³, tumours from our cohort (African population) were aggressive as evidence by patients presenting with higher clinical prognostic stage (about 6 out of 10 having a clinical prognostic stage of III), higher tumour grade (9 out of 10 with tumour grade of II and above) and poor NPI (about 70% with NPI>5.4). Furthermore, over 90% of the tumours were >2cm in size with a total lymph node involvement of 77% at the time of presentation. The tumours also had a very high triple negative prevalence (44.3%) consistent with previous studies in African populations.⁴⁻⁷ To answer the question of whether breast cancer stem cells contribute to the inherent tumour aggressiveness in this cohort, we sort to find out the expression pattern of one of the well characterized breast cancer stem cell markers CD44⁺CD24^{-/low} across our cohort. The clinicopathological association of the expression of the individual markers (CD44 and CD24) as well as their combined phenotypes were studied.

We found a high cytoplasmic CD24 expression associated with tumour aggressiveness and poor prognosis comparable to earlier studies.^{16,19} A high prevalence of CD24⁺ tumours (about 80%) was recorded with significant associations with tumour grade, clinical prognostic staging and Her2 status in this study. However, Kapucuoglu *et al* found no association between the clinicopathological parameters and either CD24 and CD44 in a study involving 105 invasive ductal carcinoma samples.²⁰ Tumours without Her2 receptor amplification had between 4-5-fold increase in CD24 expression compared with tumours with positive Her2 receptor status in contradiction with the findings of Ahmed *et al*¹⁶. Their study rather reported a positive association with the reason for such discrepancy unknown. Although there was no significant association between lymph node involvement, mitotic count, Nottingham prognostic Index and Triple negative status, we realised a 1-2 fold increased expression of CD24 in tumours with lymph node involvement, a 2 fold increase in tumours with ≥ 10 mitotic counts per high power field and between 1-2 fold increase in tumours with poorer NPI (>5.41). Triple negative tumours had a slightly increase in CD24 expression compared with other molecular subtypes although statistically not significant, a trend similar to Ahmed *et al* who had significant association between CD24 negative tumours and luminal subtypes as well as CD24 positive tumours with triple negative subtypes.¹⁶

The high expression of CD24 in our cohort coupled with its association with tumour aggressiveness and poor prognosis makes it possibly an important target for immunotherapy. Although there was a comparable CD24 nuclear staining with Ahmed *et al*'s study (14% vs 14.5%) our cohort did not show any

significant association with the clinicopathological parameters in contradiction to Ahmed *et al*/ who recorded associations with tumour size, grade and lobular type tumour.¹⁶

Similar to the cytoplasmic CD24 expression, tumour grade and mitotic count were the only features significantly associated with CD44 expression with both recording 3-fold increased expression respectively. These associations suggest cytoplasmic CD44 staining is associated with higher grade breast cancer and thus may be proposed as a substitute for grading tumors where morphology is inconclusive. It may also be valuable if a decision needs to be made to treat a patient aggressively or otherwise based on equivocal Ki67 results or Grading/ staging. Although other parameters of tumour aggression such as lymph node involvement, Vascular invasion, Nottingham Prognostic Index, and triple negative status did not have significant association with CD44 expression, there is some evidence of correlation from this study. About 3-fold increase in CD44 expression was seen in tumours with increased mitotic index and a 2-fold increase was seen in tumours with lymph node involvement. However, in contrast with CD24, there was reduced expression of CD44 in triple negative breast tumours in our cohort likewise a reduced expression in higher stage clinical pathologic staged cancers (stage III). There were no significant associations with ER, PR and Her2 receptor status in contrast to Iris *et al*'s study on invasive ductal carcinoma of no special type which had significant associations with both CD24 and CD44.²¹ Invasive colloid carcinoma was the only histologic type that had an association with reduced CD44 expression (about 22-fold decrease in expression).

Similarly, the absence of either CD44 and CD24 expression was also associated with some features of tumour aggression and poor prognosis comparable to Giatromanolaki *et al*'s study.¹³ Tumours with CD44⁻CD24^{-/low} phenotype were 10 times more likely to have a mitotic count ≥ 10 and a 7-8 times likelihood of having a poor NPI > 5.41 .

In keeping with Ahmed *et al*'s study¹⁶, CD44⁺CD24⁺ was the most occurring combination phenotype (76%) however, a low CD44⁺CD24^{-/low} phenotype expression (6.1%) was recorded in their cohort as opposed to 15% in this current study. Their cohort rather expressed higher CD44⁻CD24⁺ phenotype (31.5% vs 5.6%).¹⁶ This disparity may be as a result of different molecular signatures between African and Caucasian breast cancers underlying the racial disparity in tumour prognosis, aggressiveness and clinical outcomes favoring the later. Their study was on a larger series of early primary invasive breast carcinoma (1046) as opposed to the more heterogenous and smaller cohort of advance breast carcinoma (222) in this current study. These factors may also explain the difference in the phenotypic expressions.

This study corroborates other studies that reported the involvement of CD44⁺CD24^{-/low} phenotype in tumour aggressiveness and poor prognosis.^{12,13,21,22} Its increased expression was significantly associated with higher tumour grade and higher clinical prognostic staging. Ahmed *et al*/ and Mylona *et al*/ however had the exact opposite conclusion with CD44⁺CD24^{-/low} cancers showing favourable outcomes.^{16,23} Several studies have already linked the CD44⁺CD24^{-/low} phenotype to triple negative breast cancer^{6,12-14,20,24-26} and hence it is not surprising that our cohort with a high triple negative prevalence records high CD44⁺CD24^{-/low}. This breast cancer stem cell marker may be related to tumour aggression in our cohort

despite its non-association with lymph node involvement. The high tumour aggressiveness of our cohort may favour hematological dissemination rather than lymphatic spread explaining this lymph node non-association.²¹

Conversely, the presence of the CD44⁺CD24⁺ phenotype was associated with good prognosis and less tumour aggression a finding contrary to an earlier Asian study¹². Our study reported a significant inverse association existing between high CD44⁺CD24⁺ expression and parameters such as tumour grade, mitotic count and clinical prognostic staging. Chen *et al*/however had no association with any of the clinicopathological parameters¹².

CD44⁺CD24⁺ phenotype on the other hand did not have any association with the clinicopathological features in keeping with Chen *et al*/¹². Ahmed *et al*/however recorded positive association between tumour grade, Nottingham prognostic index and CD44⁺CD24⁺ phenotype.¹⁶ The combination phenotypes similarly did not show any significant association with the hormone receptor status.

Conclusion

CD44⁺CD24^{-/low} stem cell marker is associated with poor tumour prognosis, and aggressiveness and may be partly responsible for the inherent African breast cancer aggressiveness. This study corroborates the clinicopathological significance and prognostic value of the putative stem cell CD44⁺CD24^{-/low} in breast cancer. It is to the best of our knowledge the first study of CD44⁺CD24^{-/low} in an African population. It is recommended that future studies look at the association between CD44⁺CD24^{-/low} and clinical outcomes such as Disease-Free Survival (DFS), and Overall Survival (OS) in African populations.

Declarations

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ETHICAL CONSIDERATION

This article does not contain any studies with human participants or animals by any of the authors. Archival blocks were used with no contact with human participants.

ETHICAL STATEMENT

This study was approved by Ethical Review Committee of the Cape Coast Teaching Hospital with review number CCTHERC/EC/2021/004.

CONSENT FOR PUBLICATION

'Not applicable'

AVAILABILITY OF DATA

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests

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AUTHOR CONTRIBUTIONS

EG: laboratory work, writing the manuscript, data analysis and interpretation. **LA:** reviewing the manuscript. **PKA and LDK:** contributed by double scoring and reviewing the manuscript. **MST:** Laboratory work and reviewing the manuscript. **GAR:** reviewing the manuscript. **AJ:** reviewing the manuscript. **WO:** reviewing the manuscript **AG:** reviewing the manuscript.

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Figures

Figure 1

Immunohistochemical expression of CD44 and CD24 a, b CD44 with homogenous membranous staining.
c,d CD24 homogenous cytoplasmic staining

Figure 2
Frequency distribution of CD44 CD24 combinatorial phenotypes

Figure 3

CD44 and CD24 expression in breast cancer using immunohistochemistry. a,b same case stained negative for CD44 in a and positive for CD24 in b. c,d The same case stained positive for both CD44 and CD24. e,f same case stained negative/low for both CD44 and CD24. g,h Same case stained positive of CD44 in g and negative for CD24 in h.