TITLE:

BREAST CANCER STEM CELLS IN AFRICA: A FALLOW RESEARCH GROUND

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Keywords: Cancer Stem Cells; Aldehyde Dehydrogenase-1(ALDH1), B cell specific Moloney murine leukemia virus integration site 1 (BMI1); PROM1; CD24; CD44

INTRODUCTION

The dynamic crosstalk between the heterogeneous sub-populations of breast cancer cells, immune cells and their complex interactions with the tumour microenvironment confer significant plasticity to breast cancer. This is largely responsible for the difficulty in understanding the mechanisms by which tumour initiation, progression, metastasis and treatment failure occurs. Although our understanding of breast cancers has generally increased over the past decades owing to advancement of several molecular and genetic techniques, the disease remains puzzling due to several vital gaps in knowledge.

To explain how tumour initiation and progression occurs, several models have been proposed including the clonal evolution theory[1,2] and the genetic alteration theory[23]. While the former postulates mutations in a single or few cells leading to uncontrolled and unlimited proliferation of cells, the latter proposes the significant accumulation of genetic alterations leading to either gain or loss in function mutations of proto-oncogenes and tumour suppressor genes respectively[1-3]. This is responsible for the characteristic hallmarks of cancer[4,5]. It has also been shown that several subpopulations of cells in different stages of the cell life cycle exist in breast cancer[6,7]. Several models have been propounded as well in an attempt explain the cellular heterogeneity of breast carcinoma but none currently explain this inherent complexity[7-9]. Prominent among them are the hierarchical and the stochastic models[9]. The evidence of a few cells with stem like properties having the propensity to self-renew and also differentiate into mature non-stem cell cancer progenies in the tumour gives support to proponents of the hierarchical stem cell theory[8,10]. According to this model, cellular heterogeneity within a tumour result from the presence or absence of different cells in varying levels of the cell cycle possessing tumour initiation potential. These cells are referred to as *cancer stem cells*[10]. This model proves to be an elaboration of the oversimplified clonal evolution theory. The stochastic model however proposes the resulting effect of immune and environmental remodelling together with the intrinsic gene regulatory signals as being responsible for the different tumour sub-populations and not the variation in tumour initiating potential[11,12]. Owing to the inability of these models to adequately explain the heterogeneity of breast cancer, further research is required. Research into understanding the heterogeneity of breast cancer, on the disease initiation, progression, metastasis and how it confers poor outcomes has led to a relatively new research niche in Cancer Stem Cells (CSC).

Cancer Stem Cells (CSC)

Several studies have referred to CSC with diverse terminologies including stem-like cancer cell, tumour-initiating cell, tumourigenic cell, side population cell, and clonogenic stem-like cell. Although CSC are a minor subset of the tumour subpopulations, their importance in the tumour microenvironment is however very crucial[13]. Since these cells have the properties of self-renewal, proliferation and differentiation, they are found at the crossroads of important tumour events such as tumour initiation and progression. CSC having significant mutational events can initiate tumourigenesis and produce clone for tumour progression. They can also differentiate into other tumour sub-populations increasing the tumour heterogeneity. CSC are notorious for entering quiescence thereby evading therapeutic agents aimed at targeting rapidly proliferating cells leading to relapse and treatment failure[14-16]. Among the putative CSC markers are CD44+/CD24+/low, Aldehyde dehydrogenase 1 (ALDH1), BMI1, and CD133.

$CD44^{+}/CD24^{-/low}$

One of the most well studied putative stem cell markers for breast cancer is the transmembrane glycoprotein CD44. It is linked with aggressive behaviour in breast cancer in several studies[17-19]. The involvement of CD44 in tumourigenesis, proliferation, cellular adhesion, motility and metastases has been identified[18-20]. The high expression of this glycoprotein coupled with the absence or low expression of the glycosylated mucin-type protein CD24 are expressed in a number of haematological and other solid malignancies[19-21]. The role of the $CD44^{+}/CD24^{-/low}$ phenotype as a CSC marker in breast cancer remains largely puzzling and is under intense investigation. While some earlier works significantly associate this phenotype with poor breast cancer prognosis such as shorter recurrence free and overall survival[22-26], others however report the contrary[27,28]. According to Al hajj and colleagues, $CD44^{+}/CD24^{-/low}$ cells have tumour initiating potential and became successful tumour xenografts when transferred into the mammary fat pads of immunodeficient mice[17]. A study in primary breast tumours after radiation and chemotherapy, $CD44^{+}/CD24^{-/low}$ cells demonstrate intrinsic resistance to chemotherapy[29]. It is of critical essence that that additional studies focus on the expression of the putative breast CSC markers to enhance our understanding of tumourigenesis and treatment failure.

Aldehyde Dehydrogenase 1 (ALDH1)

Aldehyde dehydrogenase 1 (ALDH1) has also emerged a well-recognised CSC marker. ALDH1 is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes. Several studies on ALDH1 in breast cancer have indicated its association with poor prognosis[30-34]. ALDH1 expression in breast cancer ranges from as low as 8.4% to as high as 95%[35-38] with the wide range attributable to sample selection and varied cut off points employed by various investigators[34]. The addition of ALDH1 has greatly enriched tumourigenic capacity of $CD44^{+}/CD24^{-/low}$ as evidence suggests associations between the $CD44^{+}/CD24^{-/low}$ / $ALDH1^{+}$ phenotype and basal –like phenotype, high histologic grade and poor prognosis in breast cancer[24]. One in vitro study identified this combined phenotype as having the strongest ability of self-renewal, invasion, proliferation and tumourigenicity[39]. $CD44^{+}/CD24^{-/low}/ALDH1^{+}$ phenotype is thought to be more reliable in characterising BCSC than individual phenotypes[40]. The importance of $CD44^{+}/CD24^{-/low}/ALDH1^{high}$ in chemotherapy and radiation resistance has also been elucidated in recent studies suggesting a crucial role of ALDH1 in treatment response[41]. $ALDH^{+}$ cells show a more hybrid Epidermal Mesenchymal morphology with different spatiotemporal localisation compared to the more mesenchymal like CD44+/CD24-. ALDH+ cells are more centrally localised towards the stromal part of the tumour while CD44+/CD24- cells are distributed around the invasive end of the tumours[42].

CD133 (Prominin-1)

The Cluster of Differentiation 133 (CD133) otherwise known as Prominin-1 (PROM1) has recently been identified as a specific CSC marker [43-47]. A constellation of studies has identified its overexpression in several solid cancers including brain, prostate, colon, hepatocellular, ovarian, colon and lung contributing to adverse clinical outcomes including shorter survival, tumour progression and recurrence [48-51]. Prominin-1 is a pentaspan transmembrane glycoprotein located in membrane appendages such as microvilli and in the apical surface of some epithelial cells[52]. It plays a crucial role in stem cell migration and asymmetric division[53]. PROM1 gene is located on chromosome 4 in humans with a 60% homology with that located on chromosome 5 in mice[54] and has been identified as an important CSC marker in triple negative breast cancers[52,55,56]. In vitro studies of $CD133^{+}$ in *BRCA*1 associated breast cancer cell lines have identified characteristics including the ability to form spheroids, expression of stem cell genes and higher proliferative output, as well as the ability to reconstitute tumours with as few as 100 cells in *in vivo* studies[57]. Also, *BRCA1* breast tumours with the $CD44^{+}CD24^{-/low}$ CSC marker characteristics has is an association with CD133[55]. In triple negative breast tumours, CD133 is

associated vasculogenic mimicry known to significantly impact tumour relapse [52,57,58]. This gives a strong indication that its expression could help in prognostication and determination of appropriate treatment[58,59].

B cell specific Moloney murine leukemia virus integration site 1 (BMI1)

B cell specific Moloney murine leukemia virus integration site 1 (BMI1) is transcriptional repressor of the Polycomb group (PcG) of transcription factors. The gene is located on the short arm of chromosome 10. BMI1 gene comprises of 10 exons and 9 introns encoding a 326 amino acids protein of approximately 36.8kDa[60]. The N terminal end of BMI1 protein contains a conserved ring finger domain and a central helix-turn-helix-turn helix-turn motif (H-T-H-T) notable for inducing telomerase activity[61,62]. The nuclear localisation signals of BMI1; KRRR and KRMK are conserved with high expression in a variety of tissues including brain, thymus, kidney, lungs, blood and bone marrow.

BMI1 has been linked with several cancers with properties of self-renewal, proliferation, Epithelial and Mesenchymal Transition, and chemoresistance[60,63]. Evidence also exist in BMI1 acting as an epigenetic modifier protein involved in the maintenance of CSCs[64,65]. Via the activation of telomerase, BMI1 also inhibits cellular senescence, evade apoptosis to increase cell longevity[60,66]. This phenomenon plays a key CSC property in chemoresistance. The expression of BMI1 has mainly been characterised in haematopoietic malignancies[67-69] but other solid tumours such as lung, prostate, liver, medulloblastoma, neuroblastoma, colon and nasopharyngeal carcinomas have also been identified[70-76].

The role of BMI1 in mammary carcinogenesis has been established in a number of studies and currently under intense research[31,77,78]. While some studies conclude an association with favourable prognosis[79-81], others report the contrary[67,82]. According to Arnes *et al*, there was no association between BMI1 positivity and basal like features and low Ki67 expression in breast carcinoma[79]. BMI1 expression has also been associated with relapse free survival and overall survival in univariate analysis[81]. Same study also concluded BMI1 expression as an independent prognostic for overall survival especially in ER positive breast cancer[81]. Conversely, in a univariate and multivariate analysis conducted, Kim *et al* concluded that BMI1 may be involved in tumour progression and metastasis since its overexpression correlated with axillary lymph node metastasis and positive estrogen receptor status[80]. The high expression of BMI1 has also been found to correlate with markers of poor clinical outcomes such as high expression p53 and the absence of progesterone receptor. With such conflicting evidences in the role of BMI1 in breast cancer, further research is warranted to further throw more light on the role of BMI1 in breast cancer.

The role of BMI1 varies in different cancer types. The overexpression of BMI1 plays a vital role in self-renewal, cancer cell proliferation, invasion/metastasis, chemoresistance and survival. The self-renewal CSC regulatory role is encountered in type 1 neuroblastoma through an expression dependent specific lineage commitment[83]. In neuroblastoma, BMI1 knockdown in progenitor cells suppressed proliferation and disease development[84]. BMI1 expressing leukaemia Stem Cells induced leukaemia in irradiated mice whereas cells lacking BMI1 expression did not[85]. Clinical outcomes of patients who received adjuvant therapy in Non-Small Cell Lung Cancer (NSCLC) were better in BMI1 negative tumours compared to BMI1 positive tumours[60]. In Hepatocellular carcinoma, low expression of BMI1 correlates with reduction of tumour invasiveness[86]. A high expression of BMI1 in gastric cancer enhanced tumour migration and invasiveness[87]. The role of BMI1 in inducing Epithelial-Mesenchymal Transition is revealed in endometrial carcinoma[88]. BMI1 overexpression is associated with insensitivity to conventional chemotherapy and radiotherapy. This has been well documented in haematological malignancies and breast malignancies[77,85,89,90].

Table 1: The role of cancer stem cells in different types of cancers

CSC marker	Role	Cancer types	References
BMI-1	Self-Renewal	Neuroblastoma	83
		Leukemia	90
	Proliferation	Neuroblastoma	84
		Breast cancer	77
		AML	85
	Invasion	Gastric carcinoma	87
		HCC	87
		Squamous cell carcinoma	91
		Lung adenocarcinoma	92
	Metastasis	Gastric ca,	
		Lung ca	92
		Breast Cancer	89
		Endometria Ca	
		HCC	87
	Survival	Non-Small Cell Lung Carcinoma,	60,93
		Lung ca	
		Breast cancer	67,82,94
		Non-Hodgkin B cell lymphoma	68
		Nasal pharyngeal carcinoma	
		Squamous cell Carcinoma	91
		Bladder Cancer	95
	Chemo/Radio resistance	Gastric ca	
		Breast cancer	96
		Ovarian carcinoma	64,97
		HCC	98
		B cell lymphoma	99
		Melanoma	100

		Nasopharyngeal carcinoma	[101]
	EMT	Endometrial Ca Lung squamous cell carcinoma	[88] [102]

Clinicopathological significance of Cancer Stem Cells

Prognostication

The identification of CSC in tumours offer important evidence on tumourigenesis, tumour progression, metastasis, therapeutic resistance and recurrence. These may serve as important targets for diagnostic and targeted therapies. Prognostication of patients may also be based on presence or absence of specific CSC in tumours. Earlier researchers have focused on elucidating the association that exist between CSC, prognostic markers and response to adjuvant therapy. Basal like phenotype (BLP) in breast cancer with basal cell markers having poor prognosis are associated with candidate CSC markers CD44+/CD24-[24,31,103,104] . This phenotype has also been implicated in malignant relapse following conventional therapies[105]. Although there are a few conflicting results, ALDH1 expression is generally associated with

poor clinical outcome and resistance to chemotherapy due to its high expression in Triple Negative Breast Cancer (TNBC)[32].

Tumourigenicity of Cancer Stem Cells

It is evident that different breast CSC have different levels of tumourigenic potential. Greater tumourigenicity has been realised in tumours expressing multiple breast CSC markers. For instance, a high tumourigenic capacity is realised in tumours expressing combined CD44+/CD24-$^{/low}$/ALDH1^{+} when compared with tumours without either of these markers according to Da Cruz *et al*. The high tumourigenicity of this CSC phenotype is evident by the ability to form tumours from as low as 20 cells[106]. Another *in vitro* study also identified this combined phenotype as having the strongest ability of self-renewal, invasion, proliferation and tumourigenicity[39]. Although there has been some significant correlation of some breast CSC with clinicopathological features there are those that have shown otherwise[104]. Conflicting associations are similarly observed with respect to BMI1 in other studies[31]. These studies have put CSC in the spotlight not only in breast cancer but in other haematological and non-haematological cancers as well[107]. It is imperative that additional studies into the correlation of CSC between clinicopathological features and clinical outcomes are conducted to expatiate their tumourigenic potential.

Cancer Stem Cells and treatment failure/relapse

Treatment failure and tumour recurrence remains a major challenge in breast cancer therapy. In recent years, chemoresistance has strongly been linked to some candidate stem cells through a number of mechanisms. Among these include ABC transporter expression which actively pump-out chemotherapeutic compounds from tumour cells. By extrusion of the agent, the tumour evades the therapeutic action. The enzyme Aldehyde dehydrogenase1 ALDH1 converts aldehydes into carboxylic acids through oxidation. It is an important mechanism by which CSC cause treatment failure. B-cell lymphoma-2 (BCL2) related chemoresistance also impairs the ability of affected cells to release proapoptotic proteins such as cytochrome C enhancing cell immortalisation[108]. Others include enhanced DNA damage response and activation of key signalling pathways. Another property of CSC is their ability to enter a state of dormancy evading chemotherapeutic agents aimed at actively proliferating cells[9]. Knowledge into the

chemoresistance properties of CSCs is valuable in overcoming the challenges inherent in most breast cancer treatment modalities to improve clinical outcomes for patients. The ability of cancer regimens to target quiescent CSC and eliminate these cells from the tumour is paramount in overcoming treatment failure and tumour recurrence.

Compelling evidence in CSC in the past few decades have given clear indication that therapeutically targeting CSC in combination with traditional chemotherapy and radiotherapy has the potential of making cancer therapy more potent[109,110]. The potential for the therapeutic use of CSC is now made unequivocal through the inhibition of CSC function, CSC eradication, reversal of resistance and induction of CSC differentiation. This relatively new cancer research niche has the great promise of unearthing more cancer therapeutic regimen and to potentiate the effectiveness of conventional treatments directed against tumour bulk.

Racial Heterogeneity and Cancer Stem Cells

Although research evidence on CSC is currently being conducted in Caucasian breast cancer populations, studies in African populations remain significantly low. Compared to their Caucasian counterparts, breast cancers of African origin are relatively aggressive[111-113]. A plethora of research has given evidence of increased triple negative breast cancer and breast CSC in Africans and people of African ancestry[31,32,114]. The aggressiveness of breast tumours of African origin, the late reporting stage, the high therapeutic resistance, increased recurrences, increasing morbidity and mortality and poor overall survival compounded by prevailing poor socio-economic indicators such as poverty, and lack of knowledge have made breast cancer treatment challenging in Africa. This underscores the need for intensified molecular research to understand the peculiarities of the clinicopathological phenotype in relation to the profile of CSC of tumours in African population. This will help in the development of novel therapeutic strategies to target these aggressive cancers and to decrease morbidity and mortality in Africans.

There is well-documented evidence of racial difference of cancer risk, prevalence and clinical outcome[115-117]. Although there is complex racial heterogeneity, an interesting trend of increasing poor outcomes appears to exist with increasing level of African ancestry[116,118]. Though African-Americans have lower risk of breast cancer compared with Caucasians, African-Americans have significantly higher cancer related deaths in the USA[117]. This

population also record higher prevalence of ER-negative, TNBC and early onset breast cancer[115,119,120].

Since the tumourigenicity of BCSC was first demonstrated by Alhaji *et al* in 2003, population based comparative ethnic and racial studies comparing the expression pattern of BCSC in different ethnic groups and races remain scanty. Therefore, a study comparing the breast CSC profile in Caucasians, Western sub-Saharan African descents, native Africans is plausible. Here we review Breast CSC studies in African Populations and elucidate their differences with races.

A study in Uganda revealed a high prevalence (48%) of ALDH1 associated with aggressive tumours (TNBC and HER2) in 192 breast cancer cases[31]. Similar studies conducted in Ghana also reported comparable findings of 42%[32] and 45%[33] ALDH1 expression in 104 and 147 breast carcinoma patients respectively. Conversely, less than 30% of ALDH1 expression is reported in a number of European and White American tumours[114]. In all studies, ALDH1 was significantly associated with TNBC. In Asian populations, a range of 40% to 65% ALDH1 positivity is found all of which were associated with poor prognosis comparable to their African counterparts[121-126]. Similarly, a high tumour grade was associated with ALDH1 positivity[121,122]. Conflicting reports have however characterised ALDH1 expression metastasis to axillary lymph nodes[123-125] and tumour size[30,124]. ALDH1 is associated with poor clinical outcomes such as shorter relapse free survival and overall survival[122,123]. None of the African studies however associated ALDH1 with clinical outcome. There has not been any report of $CD44^{+}/CD24^{-/low}$ / $ALDH1^{+}$ CSC phenotype study in an African cohort.

In a study which compared African-American and Hispanic/Latina, women with a high $CD44^{+}/CD24^{-/low}$ expression was reported in TNBC in both populations[127]. The increased incidence of $CD44^{+}/CD24^{-/low}$ was associated significantly with disease free survival in univariate analysis but was however not an independent predictor when subjected to a multivariate analysis[127]. A plethora of studies have compared racial and ethnic disparities in cancer, but non comparing CSC in African populations.

Most CSC studies conducted in African populations focused only on ALDH1 except for Nalwoga *et al*[31]. None of these studies have therefore elucidated the relationship between ALDH1 and other stem cell markers such as $CD44^{+}/CD24^{-/low}$, BMI1, and CD133. While ALDH1 is associated with poor prognosis, a meta-analytical studies by Zhou *et al*[128], Li *et al* [40] suggest a combination of $CD44^{+}$ $CD24^{-/low}$ and ALDH1 as a better CSC buttressing the

assertion that ALDH1 activity does not universally select for the most clonogenic cells in certain breast cancer cell lines[41].

CONCLUSION

Breast cancer heterogeneity is evidenced by various histological subtypes, with variable clinical presentations and diverse molecular signatures. The use of a single biomarker as CSC marker is not adequate. It is therefore imperative that additional research focuses on combinations of biomarkers that can reliably select BCSC phenotypes.

Most African studies have not yet associated CSC with clinical outcomes such as resistance to chemotherapy, and overall survival. Breast cancer patients in Africa usually present with higher stage and grade and mostly require neo-adjuvant chemotherapy to downstage the tumour before surgery and subsequent therapy. It is important that studies into the significance of CSC before and after neo-adjuvant therapy and correlated with prognostic parameters and clinical outcomes are conducted. Studies into the clinicopathological significance of other recognised BCSC markers such as CD133, and BMI1 remain a fallow research area in African populations. Long term follow-up studies are strongly recommended in African cohorts to identify the relationship between these breast cancer stem cell markers, prognosis and clinical outcomes. The relationship between various histological subtypes and the various BCSC is similarly not well established in African cohorts. A study comparing Invasive Ductal Carcinoma-NOS with Invasive Ductal Carcinoma-SHT is also yet to be conducted in an African population.

Although poor socioeconomic status remains a significant risk factor for poor breast cancer outcomes in African populations[129,130], it does not exhaustively explain why breast cancers of Africans and those of African ancestry are aggressive and exhibit such adverse clinical outcomes. Underlying molecular and genetic signatures are of great importance, particularly the significance of CSC in contributing to such disparity between Africans and other races. Several studies are currently underway to explain this observation. Comparing CSC in racial and ethnic population studies is of outmost importance in the identification the similarities and differences that exist among races to enable the development of effective personalised treatment regimens that take cognisance of such racial and ethnic disparity.

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