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The Potential of Differentiation-Related Gene-1 (*DRG1)* as a Biomarker for Metastasis of Estrogen Receptor-Positive Breast Cancer

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Authors' contributions

This work was carried out in collaboration among all authors. This work was collaboratively carried out among all the authors. Authors HB, BN, KTP conceived and designed the experiments; Authors HB, WN performed the experiments; Authors BN, KTP supervised the experiments; Authors HB, COO, ENM and YG analyzed the data, prepared figures and tables; Authors HB, ENM, COO and YG wrote the draft manuscript. All Authors read and approved the final version of the manuscript.

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ABSTRACT

Introduction: Breast cancer is major burden worldwide and the majority of breast cancers express estrogen receptors (ER) suggesting a high dependence on estrogen hormone. Age is among the major determinants of breast cancer development, however, although Western Kenya is one of the

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areas with high breast cancer cases, age distribution of ER-positive breast cancer in the sub-region remains largely undocumented. Differentiation-related gene-1 (*DRG1*) is a metastasis suppressor and thus a potential biomarker for predicting level of metastasis but its potential application in assessing extent of metastasis of ER positive breast cancer has not been fully explored. This study therefore investigated the age distribution and the potential of expression of *DRG1* in assessing metastasis of ER positive breast cancer.

Materials and Methods: Breast cancer tumour blocks archived in safe cabins in the histology laboratory section, Moi Teaching and Referral hospital, Eldoret, Kenya were used. Clinico-pathological parameters such as histology grade, tumor size, which are associated with metastatic cancer, were assessed using the archived clinico-pathological reports and/or histological analysis of the tumour blocks. Expression of DRG1 and Ki-67 proteins were determined using immunohistochemistry.

Results: ER positive breast cancer was predominant among women aged 40 and 50 years. No association was observed between immunohistochemical expression of *DRG1* and parameters such as histology grade, tumor size or expression of Ki-67 protein expressed DRG1 ($p > 0.05$).

Conclusion: The findings suggest that expression of DRG1 protein is not associated with parameters that indicate breast cancer metastasis. Thus, *DRG1* expression is not a potential biomarker candidate for ER positive breast cancer metastasis. However, since the small sample size was used, further research using larger prospective study is necessary to support the present findings.

Keywords: Differentiation related gene-1 (DRG1); estrogen receptor positive breast cancer; western kenya; age.

ABBREVIATIONS

CI : Confidence Interval;
ER : Estrogen Receptor;
DRG1 : Differentiation-Related Gene-1;
MTRH : Moi Teaching and Referral Hospital.

1. INTRODUCTION

Globally, breast cancer is the commonest malignancy and the leading cause of cancer-related deaths for women [1]. Developing countries are majorly affected and continue to report increased breast cancer incidences worldwide [2]. Annually, breast cancer accounts for 2553 deaths in Kenya, with majority of the deaths being attributed to late detection [3].

Two-thirds of breast cancers express estrogen receptor (ER) α and/or progesterone receptor, which are known to stimulate breast cancer growth [4]. Over, 52.5% of hormone receptor breast cancers express ER, which typically indicate a high degree of estrogen dependence for growth and survival [4]. About 50% of patients with metastatic ER-positive breast cancer exhibit nonresponse to first-line endocrine treatment due to primary, *de novo* resistance [5], or secondary acquired resistance [6] making ER positive breast cancer a major concern. Nevertheless, early cancer detection remains a major challenge in many developing countries, including Kenya [2]. Thus, more studies are required to better predict breast cancer metastasis level, which is an essential determinant of prognosis.

One of the critical factors that determines development of breast cancer is age [7,8]. It has also been shown that breast cancer incidences and death related cases increases with age. Among African women, breast cancer incidence peaks approximately 10–15 years earlier than peak incidence for western countries outside of the western Africa region [8]. Breast cancer is more aggressive among Chinese women aged 40 to 50 years [7]. In addition, African-American women are more predisposed to breast cancer than the Caucasians as from 18 years old [9]. Kenya has the highest risk of breast cancer among African countries [10]. Majority of breast cancers in western Kenya are ER positive [11]. Despite age being an important factor in breast cancer development and management, age distribution of ER breast cancer in western Kenya is largely undocumented yet, understanding the age distribution of ER breast cancer could inform on the type of therapeutic approaches to be used for breast management. This could lead to targeted procurement of equipment and therapeutic materials for the most affected age group leading to reduced mortality.

Breast cancer-related mortality is majorly linked to metastasis [12]. Differentiation-related gene-1

(*DRG1*) is a metastasis suppressor gene, which controls the metastasis spread without affecting growth of primary tumor [13]. It is a metastasis suppressor in breast cancer that affects the step of invasion through extracellular matrix. Studies have reported DRG1 as a potentially good biomarker for determining the level of metastasis in *in vitro* cell lines [13,14]. In most countries, only clinico-pathological characteristics are used to assess level of breast cancer metastasis. It is noteworthy that the potential role of immunohistochemical expression of DRG1 protein in determining metastasis level of ER breast cancer is largely unexplored. This information can reduce the cost of and improve the accuracy of predicting ER breast cancer metastasis level in resource strained facilities. Parameters, such as histology grade, lymph node metastasis, tumor size, expression of a proliferation markers, Ki-67 or survival rates, are predictors of metastases that may be used to predict metastatic cancer, including breast cancer [13,15].This study sought to better understand the age distribution of ER breast cancer, and the association between immunohistochemical expression of *DRG1* and the predictors of metastasis among ER positive breast cancer women.

2. MATERIALS AND METHODS

2.1 Biological Samples and Medical Data

This retrospective study targeted archived breast cancer tumour blocks (2012 – 2015) from female patients at Moi Teaching and Referral Hospital (MTRH), a primary academic hospital in western Kenya region [11]. Breast tissue samples had been collected previously during surgery or biopsy and were fixed and stored at the hospital as tumor blocks using standard procedures. Tissue blocks of normal cerebellum and normal breast tissue were used as control for *DRG1*, ER and Ki67 respectively. Inclusion criteria for breast tumor blocks were as follows: tissues obtained from women who were 18-55 years, ER-positive, HIV negative, had no history of any other types of tumour, and had not undergone chemotherapy, radiotherapy or any other cancer-related treatments. HIV status and clinico-pathological data, namely tumor size, survival rates and age, were obtained from the clinical records and pathology reports. The record officers and pathology department helped in providing the medical records and identification of tumour blocks, respectively.

2.2 Sample Processing

Upon identification of the tissue blocks meeting the inclusion criteria, the breast tumor blocks were retrieved from the safe cabins in the histology laboratory and placed on ice to cool. Then, tumor blocks were cut into 5 µm sections using rotary microtome (Lerts Leica, W. Nuhsbaum, Inc., McHenry, Illinois). The sections were put to float on distilled water at 25°C for easy selection of suitable section. A section was transferred to a glass slide and allowed to dry overnight at 25°C for histological grading and immunohistochemistry.

2.3 Histological Grading

Slides processed in the preceding section were deparaffinized in xylene (Agilent Technologies Inc., Glostrup, Denmark) for minutes and transferred into three baths of ascending (80, 95 and 100%) grades of ethanol (Agilent Technologies Inc) for 3 minutes each. The slides were rinsed in tap water followed by hematoxylin (Merck KGart, Darmstadt, Germany) application for 5 minutes. The slides were washed in tap water. Then, eosin (Loba Chemical DVT. Ltd, Mumbai, India) was applied for 1 minute followed by rinsing. Slides were then sequentially rinsed with 100% ethanol and xylene (Agilent Technologies Inc). Cover slips were placed, permount applied and dried overnight then viewed using Olympus BH-2 microscope (Olympus Inc., Tokyo, Japan) at ×400 to assess the grade of tumor. The slides were independently viewed and evaluated by two pathologists with specialty level training. Histological grading was based on degree of tubule or gland formation, nuclear pleomorphism, and mitotic count as previously described [16].

2.4 Immunohistochemistry and Immunoscoring

Immunohistochemistry was performed as previously described with some modifications on scoring rate [15]. Briefly, slide with 5 µm section was deparaffinized and hydrated. The sections were treated for 5 min with 100% methanol containing 3% hydrogen peroxide and incubated at 25°C for 10 minutes to block endogenous peroxidase activity. Non-specific binding was blocked by incubation in 1% normal swine serum (Dako) in phosphate-buffered saline. Following manufacturer's instructions, tissue sections were incubated with anti-ER (Cat no: GA084, Dako), anti-DRG1 (Cat no: HPA006881, Q92597,

Sigma-Aldrich Inc., St. Louis, Missouri, USA,) or anti-Ki-67 (MIB-1, Dako) antibody (1:100 dilution) at 25°C for 1 hour, followed by incubation for 30 min at 25°C with horseradish peroxidase (HRP)-conjugated secondary antibody (Agilent technologies Inc) following manufacturers' instructions. Immunostaining was performed using a DAB substrate (Dako), and counterstaining was performed with haematoxylin. Positive control and negative control specimens were included for each antibody set as a quality control measure. Color of antibody ER, DRG1 and Ki-67 were viewed and evaluated independently by two pathologists with specialty level training using Olympus BH-2 microscope (Olympus Inc) at ×400.

Immunoscoring was based on stain intensity and was used to categorize expression of DRG1, ER and Ki-67 in relation to their controls. The scores for DRG1 were 0 (negative), 1+, 2+ and 3+ as described previously [17]. Anti-DRG1 Rabbit polyclonal and monoclonal antibodies were used in the ratio 1:100 and staining intensity of ≥ 10% was considered positive as described previously [17]. For the ER receptor and Ki-67, scoring was defined as negative (< 10%) or positive (≥ 10%) based on the percentage of stained cells based on criteria described [15]. Histology grading was based on the average of tubule formation, mitotic count and nuclear pleomorphism following Nottingham grading system.

2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.03 (GraphPad Software Inc., California, USA). Association between variables was analyzed using chi-square test. Where applicable, data are presented as mean ± standard deviation (SD). A p-value ≤ 0.05 was considered significant.

3. RESULTS

3.1 Age Distribution of ER Positive Breast Cancer

Breast tumour blocks archived at MTRH and the corresponding medical records of 37 patients of African origin were used in this study. Among them, 16 either had inadequate samples or were ER negative, hence were excluded from this study (Table 1). The participants were aged between 26 and 55 years, with a mean age of 41.76 ± 7.71 years std dev. Majority of the ER-positive breast cancer women were aged 40 and 50 years (Fig. 1).

Table 1. Breast tumour blocks sample characteristics

Characteristic	Number (n)	Percentage (%)
ER receptor status		
Negative	2	5.4
Positive	***21***	***56.8***
Folded	1	2.7
No tissue	2	5.4
No tumor	8	22.2
Inadequate sample	3	8.1
Total (n)	37	

Note: Values in bold and italics depict the samples used in this study

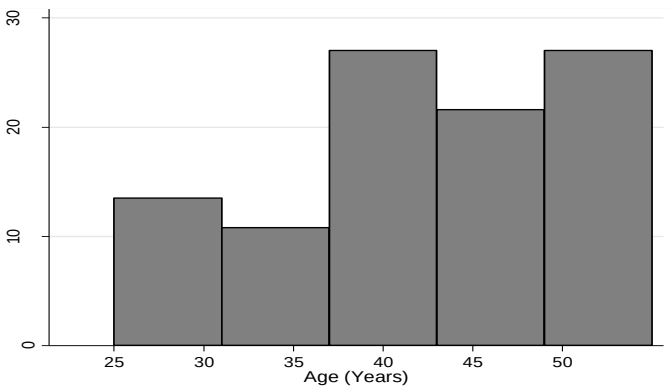

Fig. 1**. Age distribution of ER positive breast cancer**

3.2 Immunochemistry and Histology Images for the Tissues Stained for DRG1 and ER

Figs. 2, 3 and 4 show the representative immunochemistry and histology images for the tissues stained for DRG1 and ER. The magnification of all figures is ×400.

3.3 Association between DRG1 Expression and the Indicators of Cancer Metastasis

Majority of the ER-positive breast tumors in histology grade I and II expressed DRG1. However, there was no significant association between *DRG1* expression and histology grades ($p = 0.316$; Table 2). No significant association was observed between *DRG1* expression and ER positive breast tumor size ($p = 1.000$; Table 2). In addition, whereas most of the ER positive breast tumor blocks were both positive for DRG1 and Ki-67 protein, there was no statistically significant association between DRG1 expression and Ki-67 protein expression among the patients ($p = 0.387$; Table 2).

4. DISCUSSION

This study evaluated the age distribution of ER-positive breast cancer and expression of DRG1 protein as a potential biomarker for metastasis of ER-positive breast cancer. ER-positive breast cancer patients had a mean age of 41.76 ± 7.71 years with majority of them aged 40 and 50 years. The present finding is consistent with previous reports in which breast cancer incidence was shown to peak between the ages of 35 and 45 years among African females [8] and Chinese women between the age of 40 and 50 years [7]. However, the previous studies did not focus on ER-mediated breast cancer, hence, provided only a general age distribution of breast cancer.

Considering that ER is a nuclear receptor functioning as a transcriptional regulator that mediates the biological responses to the sex hormone, estrogen essential for reproduction [18], it is not clear why women aged 50 years had higher prevalence of ER-positive breast cancer. Additionally, ER negative breast cancers represent a more biologically heterogeneous disease than ER positive breast cancer [19] thus explaining why there were less younger age ER-positive cases as compared to the ER- negative.

Among women of the reproductive age (below 40 years) it is widely presumed that the increased risk of developing breast cancer is due to the ability of pregnancy-associated hormones to promote the further proliferation of an initiated target cell population. It is surprising however, that the majority of breast cancers that develop following pregnancy lack appreciable expression of either the estrogen or progesterone receptors and many are thus ER- negative. This important observation suggests that if hormones play a part in promoting breast cancer following pregnancy, they may not be doing so through by direct binding to hormone receptor molecules expressed by breast cancer cells. In this

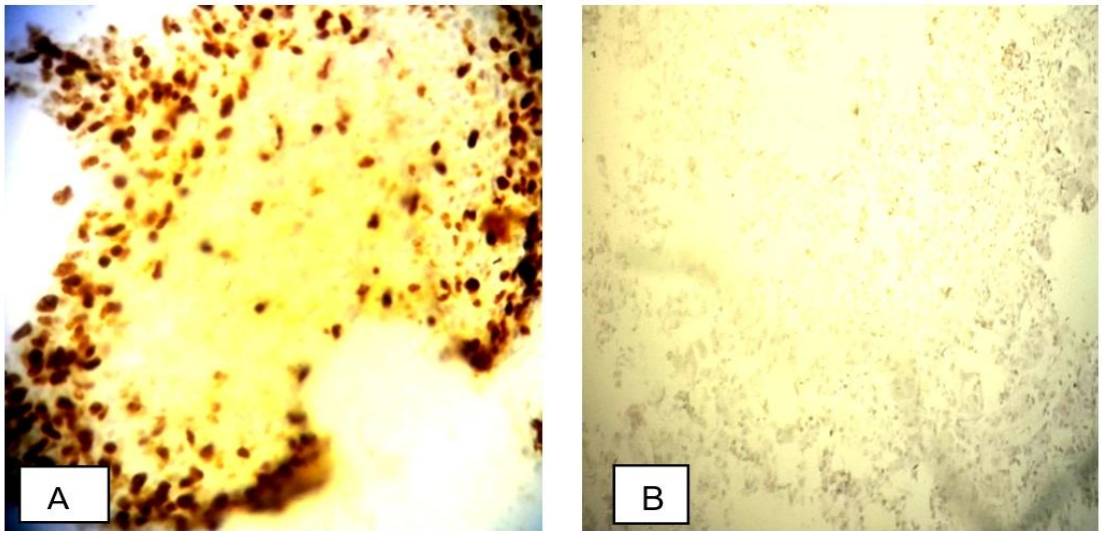

Fig. 2. Representative images for ER staining
Where: (A) ER-positive and (B) ER-negative

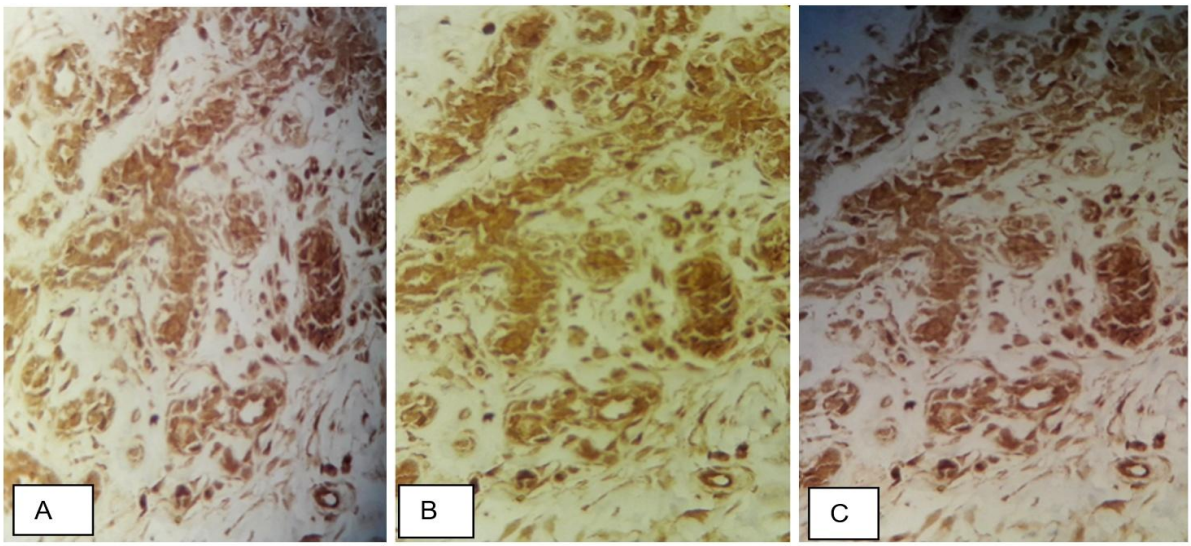

Fig. 3. Representative images for the *DRG1* Expression Intensity
Where: (A) DRG1 expression intensity 1+, (B) DRG1 expression intensity 2+, and (C) DRG1 expression intensity 3+

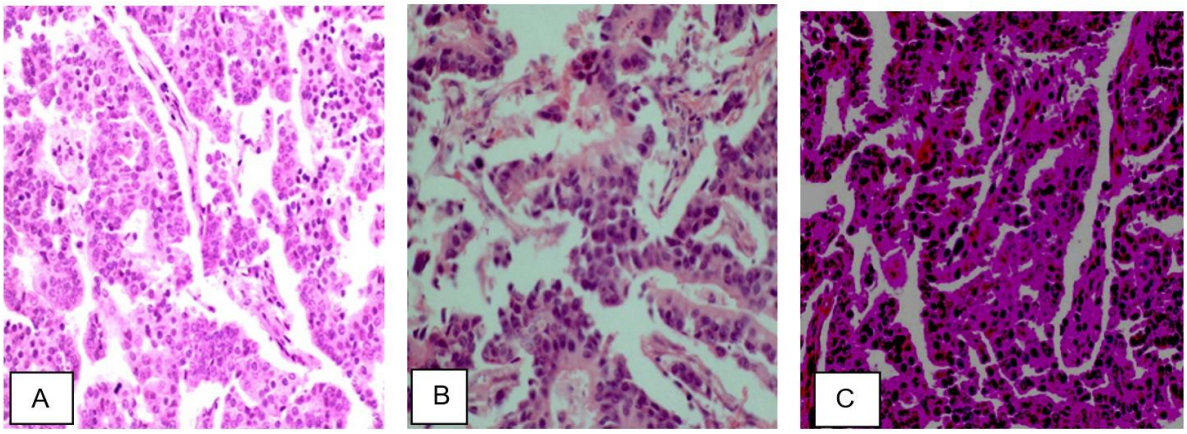

Figure 4. Representative images for the different histology grades
Where: (A) Histology Grade I, (B) Histology Grade II and (C) Histology Grade III

regard, there will therefore be less ER- positive breast cancer cases as opposed to ER- negative cases in this age group. This is partly explained by the study by Gupta and Kuperwasser that demonstrated that increasing the levels of circulating estrogens is sufficient to promote the formation and progression of ER-negative cancers [20]. This is supported by the observation that the effects of estrogen act via a systemic increase in host angiogenesis, in part through increased mobilization and recruitment of bone marrow stromal derived cells into sites of angiogenesis and to a growing tumor mass together suggesting that estrogen may promote the growth of ER-negative cancers by acting on cells distinct from the cancer cells to stimulate angiogenesis [20]. On the other hand, among ages 40 – 50 and above, most women are naturally entering or are in menopause and typically have reduced hormonal activity thus have less influence of these hormones on the non-target cells thereby leading to increased ER-positive cases. From this context therefore, we are in agreement to these explanation as some of the basic reasons for the contribution of the group age 40 to 50 to the observed higher cases of ER-positive breast cancer.

Furthermore, this study did not reveal association between DRG1 protein expression and the parameters indicative of cancer metastasis, such as histology grades, tumour size and Ki-67 protein expression among ER-positive breast cancer women. The present findings concur and

Table 2. Association between *DRG1* expression and the indicators of cancer metastasis among ER positive breast cancer

Parameter	Total (n = 21)	DRG1 I and II, n (%)	DRG1 III, n (%)	OR	95% CI	*p*-value
Histology grade						
I and II	17	8 (47)	9 (53)	2.667	0.229 - 31.09	0.603
III	4	1 (25)	3 (75)			
Tumour size						
< 5 cm	10	4 (40)	6 (60)	1.167	0.199 - 6.808	1.000
> 5 cm	11	4 (36)	7 (64)			
Ki-67 expression						
Positive	12	7 (58)	5 (42)	2.80	0.463 - 16.94	0.387
Negative	9	3 (33)	6 (67)			

partly agree with previous studies in which DRG1 was shown to have a correlation with lymph node metastasis, but not with tumour size or histology grades [13,14]. Considering that individual breast tumours exhibit great variations in clinical presentation in different ethnic populations [21] it is probable that the discrepancy between the present and previous studies with regards to lymph node metastasis could be linked to the different genetic backgrounds of the study populations.

From the results obtained, DRG1 appears not to be suitable marker for breast cancer metastasis. However, one of our study limitations is the relatively small sample size. This might have contributed to the lack of signification ssociation between the study variables. It is therefore difficult to entirely rule out the potential utility of DRG1 in breast cancer metastasis.

5. CONCLUSION

The present study showed that women of 40 – 50 years are the most affected by ER-positive breast cancer. Furthermore, no associations were observed between DRG1 and the parameters indicative of metastasis (histology grade, tumour size and Ki-67) that could be associated with metastasis of breast cancer. Further studies involving larger number of participants and targeting DRG1 and other potential molecular markers are warranted to in the fight against breast cancer as these will allow for earlier detection.

CONSENT

The study was exempted from consent since it used archived samples, hence was not in direct contact with the patients. All the tumor blocks were coded to conceal the identity of the patients from which tumor blocks were obtained. Only the investigators accessed patients' medical files.

ETHICAL APPROVAL

Ethical clearance to conduct this study was obtained from Institutional Research and Ethics Committee (IREC) of Moi University and MTRH, Eldoret, Kenya (Approval number: IREC 1203).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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