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ORIGINAL ARTICLE

Tumour cell membrane laminin expression is associated with basal-like phenotype and poor survival in Nigerian breast cancer

AGBOOLA AOJ, EBILI HO, IYAWE VO, BANJO AAF, SALAMI BS*, RAKHA EA**, NOLAN C**, ELLIS IO**, GREEN AR**

*Department of Morbid Anatomy and Histopathology and *Department of Surgery, Olabisi Onabanjo University, Nigeria, and **Division of Cancer and Stem Cells, Nottingham University Hospitals and University of Nottingham, United Kingdom*

Abstract

Introduction: Laminin is a glycoprotein with diverse functions in carcinogenesis including cell proliferation, invasion, metastases and epithelial-mesenchymal transition (EMT). In breast cancer (BC) laminin expression is speculated to be associated with unfavourable clinicopathological and molecular characteristics. We hypothesize that laminin expression would contributed to the aggressive nature of basal like and triple negative BC phenotype observed in Black women. *Methods:* The expression of laminin was determined in a well-characterised Nigerian cohort of 255 BC using tissue microarray and immunohistochemistry. Laminin expression was compared with clinical, pathological and survival characteristics. *Results:* Laminin was expressed in 146 (57.3%) cases and significantly correlated with younger age at diagnosis (p=0.005), premenopausal status (p=0.003), expression of EGFR (p=0.002), ID4 and MTA1, basal cytokeratin 5/6, p53, and triple negative tumours (all p<0.001). In addition, there was an inverse association of laminin expression with E-cadherin (p=0.03), ER and PgR (all p<0.001) and a trend with BRCA1 (p=0.05). Univariate survival analysis showed tumours positive for laminin had significantly poorer breast cancer specific survival (BCSS, p=0.009) and disease free interval (p=0.03), but not associated in Cox multivariate analysis. *Conclusion:* This study demonstrates that laminin expression may have important roles in the aggressive nature observed in the basal-like and triple negative molecular subtype of Nigerian BC women.

Key words: Laminin, basal-like breast cancer, poor survival, Nigerian women

INTRODUCTION

Laminin is a heterotrimeric glycoprotein with diverse functions during embryonic development and in mature tissues.[1-3] This extracellular matrix protein is involved in the mediation of attachment, migration and organization of cells into tissues during embryosis.[2] It also has a role in cellular differentiation and cell survival, and in supporting the growth of embryonic stem cells.[4] In mature tissue, laminin is a component of the epithelial and vascular basement membrane where it is involved in the maintenance of cell adhesion and cohesion.[3,5] It is secreted by both epithelial and stromal cells and binds to integrin receptors on the cell surfaces.[5-7]

In addition to its physiological roles, laminin expression has been implicated in tumour progression. Cancer cells express laminin on their cell membranes during invasion to evade anoikis (apoptosis which is triggered by cell detachment from the basement membrane).[8] Indeed, laminin expression has been implicated in the hallmarks of carcinogenesis; including cell proliferation, invasion, metastases and the epithelial-mesenchymal transition (EMT).[9-17]

Many studies have attempted to determine the clinicopathological and prognostic significance of laminin expression in cancers. For example, tumour basement membrane laminin predicts prognostic outcome after curative resection of pancreatic head cancer.[18] Also, expression of laminin in glial tumours is associated with tumour grade, tumour recurrence and overall survival of patients.[19] The expression of γ2 chain of laminin is

Address for correspondence: Dr Agboola A.O.J, Department of Morbid Anatomy and Histopathology, Faculty of Basic Medical Sciences, Olabisi Onabanjo University, Nigeria. Tel: +2348031115113, Email: johndeji2001@yahoo.co.uk

associated with the infiltrative pattern of tumour invasion in mucinous ovarian neoplasm.[20]

The role of laminin in BC remains inconclusive. Although some studies found laminin expression to be associated with less aggressive and slowly proliferating tumour cells,[21] most studies investigating the prognostic significance of laminin expression in BC report an inverse association between survival and laminin expression,[22-24] while one study reported no significant association with patients' outcome.[25]

The laminin isoform investigated in this study is known as laminin N (anti –human laminin (LAM -89, Abcam 49726, Abcam Ltd, Cambridge, UK). It is also a glycoprotein with similar functions to other isoforms; such as mediating the attachment, migration and organization of cells into tissue during embryonic development by interacting with other extracellular matrix components. This isoform has been found to be expressed in skin, heart, lung, reproductive tract and breast.[25]

Nigerian BC has a high mortality rate of 22.8/100,000 persons/year.[26] It was speculated that by 2020, about 50 million women will be at the risk of developing BC.[27] BC in black women has early onset occurrence and an association with basal like phenotype, triple negative and BRCA1 deficiency.[28,29] The majority of these tumours are resistant to conventional chemotherapy and therefore there is a need to identify more biomarkers for better management of black BC.[30] Laminin is speculated to be associated with basal-like phenotype and BRCA1 deficiency.[22,25,28] This biomarker may therefore be useful in identification of basal like phenotype that are commonly diagnosed in black BC. Thus, the aims of this study were to determine the roles of laminin expression in a series of Nigerian BC cases.

MATERIALS AND METHODS

Patients

This study is based on tumours from 255 women who presented at the Olabisi Onabanjo University Teaching Hospital, Sagamu and Histopathology Specialist laboratory, Idi-Araba Lagos, Nigeria from January 2002 to December 2008. Clinical history and tumour characteristics were available on these samples and included; age at presentation, menopausal status, tumour histological subtype, tumour histological grade, tumour size, lymph node status and presence or absence of vascular invasion. Patient outcome and treatment data were retrieved from the patient's records. Breast cancer specific survival (BCSS) was defined as the interval (months) from the date of diagnosis to time of death due to BC. Disease free interval (DFI) was identified as the time between the date of primary treatment to the first locoregional recurrence or distant metastasis. All patients were treated according to standard practice. Patients were followed up for at least 60 months (260 weeks).

The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria, recommended by McShane *et al* were followed.[31] This study was approved by the Medical Advisory Committee, Olabisi Onabanjo University Teaching Hospital and by the Nottingham Research Ethics Committee 2 under the title of "Development of a molecular genetics classification of breast cancer."

Tissue microarray array construction

Tissue microarrays (TMA) were constructed from whole tissue blocks as previously described.[28] Breast tumour cores of 0.6mm in diameter were extracted from each FFPE donor tissue block marked for the most representative points of tumour (both peripherally and centrally) and inserted sequentially into recipient paraffin blocks using a precision instrument (ALPHELYS MiniCore®).

Immunohistochemistry

4µm thick TMA sections were immuno-histochemically stained for laminin using Novocastra Novolink Polymer Detection Systems (Leica Microsystems, Newcastle, UK). Paraffin sections were dewaxed in xylene and three (3) graded alcohol baths. The antigen retrieval was performed using protenaise K. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide solution. The primary antibody for the biomarkers (Ab49726, Abcam Ltd, Cambridge, UK) with a dilution of 1:50 was incubated for a period of 60 minutes at room temperature. Diaminobenzidinetetrahydrochloride (DAB) solution (Novolink DAB Substrate buffer plus Novacastra DAB Novolink, Newcastle, UK) used as chromogen was applied to the slides and incubated for 10mins and counter-stained with haematoxylin for 2-3 minutes, followed by rinsing in tap water. Slides were de-hydrated by immersing in three alcohol baths for 10 seconds and cleared in two xylene baths followed by application of coverslip.

Negative and positive controls were performed by omitting the primary antibody in BC samples and including control tissues (kidney tissue) as specified by the antibody supplier respectively. IHC for other biomarkers used for the analysis was also available as previously described for ER, PgR, HER2, CK5/6, CK14, EGFR, BRCA1, E-cadherin, P-cadherin, p53, ID4, MTA1[28,32-34] (Table 1)

Immunohistochemical scoring

The immunoreactivity was assessed using the percentage of positive cells. The cases were scored for estimation of molecular subtyping, without knowledge of the clinico-pathological parameters or patient outcome. TMAs were scored independently twice with a wash out period of a week by one observer (JA). The mean of the scores were calculated to reach a final score. A proportion of these were counter scored by an observer (AG) to ensure reproducibility. The biomarker was dichotomised into groups according to the median of frequency distributions of the percentage of the staining. Tumour with staining >1% was considered as showing positive expression for laminin. The score of the other biomarkers are shown in Table 1. [28,32-34]

For c-erbB2 (HER2), the American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for HER2 Testing in Breast Cancer was used for assessment.[35] Equivocal (2+) cases were confirmed by chromogenic in-situ hybridisation (CISH) as previously described.[36] For molecular classification, Nielsen's method was used.[37] This comprises of Luminal A (ER, PR positive and HER2 negative), Luminal B (ER, PR HER2 positive), Basal (ER, PR, HER2 negative and CK5/6 and or EGFR positive), HER2 (ER negative and HER2 positive) and an unclassified group (ER, PR, HER2 CK5/6 and EGFR negative).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software. Chi-squared analyses were used for inter-relationships between the laminin expression, clinicopathological parameters and other biomarkers. The Kaplan–Meier survival method and the log-rank test were used for survival curves. Multivariate analyses using Cox proportional hazard regression models were performed and from the model both the risk factor and 95% confidence intervals were generated. A two-sided p-value of <0.05 was considered significant.

RESULTS

Laminin expression was localised in the cell membrane, cytoplasm and extracellular matrix. In addition to supplier instruction, normal basal cell staining were taken as an important internal positive control (Figure 1). Using the cut-off point of >1%, 146 of 255 (57.3%) cases were considered positive for laminin expression.

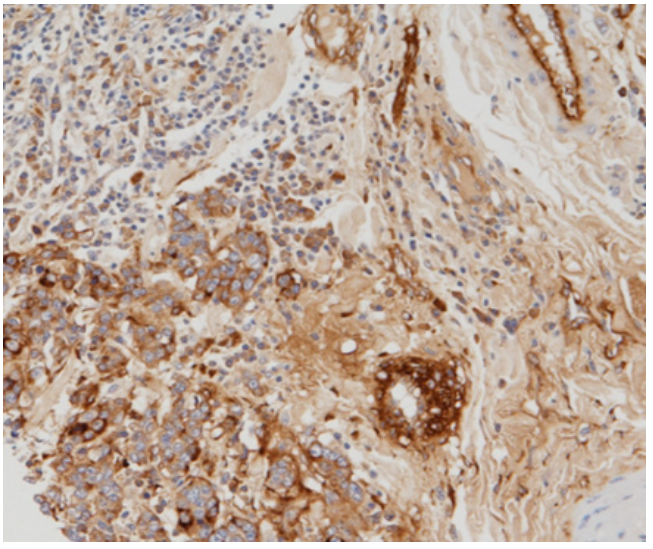

FIG. 1: Laminin staining showing localisation of protein in the cell membrances of breast cancer cells

TABLE 1: Sources, dilution, distribution, cut-offs point and pre-treatment used for revalidation

Antibody	Clone	Source	Dilution	Distribution	Scoring System	Cut-offs	Pre-treatment	Positive control	Negative control
BRCA1	Ab-1 (MS110)	Calbiochem	1:150	Nuclear	% of positive cells	<25% (negative)	Antigen retrieval Microwave	MCF 7 cells (human breast adenocarcinoma cell line)	Omitting the antibody
Ck5/6	M7237	Dako-cytomation	1:60	Cytoplasm	% of positive cells	≥10% (positive)	Antigen retrieval Microwave	Known case of CK56 BC	Omitting the antibody
Ck14	LL002	Novocastra	1:40	Cytoplasm	% of positive cells	≥10% (positive)	Antigen retrieval Microwave	Known case of CK14 breast cancer	Omitting the antibody
E – cadherin	NCH-38	Dako-Cytomation	1:100	Cytoplasm and membrane	% of positive cells	≥100 H score (positive)	Antigen retrieval Microwave	Normal gastric mucosa	Omitting the antibody
EGFR	31G7	Novocastra	1:30	Membrane	% of positive cells	≥10% (positive)	Not required	Myoepithelial cells of normal duct in normal mammary gland	Omitting the antibody
erbB2	Polyclonal	Dako-Cytomation	1:100	Membrane			Not required	Known case of erbB2 strong BC expression	Omitting the antibody
ER	1D5	Dako-Cytomation	1:200	Nuclear	% of positive cells	≥0(positive)	Antigen retrieval Microwave	Normal breast acini	Omitting the antibody
ID4	Ab77345	Abcam	1:100	Nuclear	% of positive cells	≥50% (positive)	Antigen retrieval Microwave	Human colon cancer	Omitting the antibody
LamininN	Ab49726	Abcam	1:50	Cytoplasm/ memembrane	% of positive cells	≥1% (positive)	Antigen retrieval Proteinase K	Kidney tissue	Omitting the antibody
MTA 1	Ab84136	Abcam	1:100	Nuclear	% of positive cells	≥1% (positive)	Antigen retrieval microwave	Human gastric adeno-carcinoma	Omitting the antibody
P-cadherin	NCL-P-cad	Novocastra	1:200	Cytoplasm	% of positive cells	≥5% (positive)	Antigen retrieval Microwave	Known case of P-cadherin strong BC expression	Omitting the antibody
PgR	PgR	Dako-Cytomation	1:150	Nuclear	% of positive cells	≥0 (positive)	Antigen retrieval Microwave	Normal breast acini	Omitting the antibody
p53	DO7	Novocastra	1:50	Nuclear	% of positive cells	>10% (negative)	Antigen retrieval Microwave	Normal breast acini	Omitting the antibody

Clinicopathological significance of laminin expression in Nigerian breast cancer

The relationships between expression of laminin and clinicopathological variables are summarised in Table 2. There was a significant correlation between age at diagnosis, menopausal status and tumour with positive laminin expression, where the majority of tumour with positive laminin expression were from premenopausal women (p=0.003) and patients diagnosed at a younger age (p=0.005). There was no significant association with other clinicopathological parameters.

The relationship of laminin expression with other biomarkers in Nigerian breast cancer

The relationship between laminin and other biomarkers expression are shown in Table 3. There was a positive correlation between laminin and other BRCA1 down regulators, where more than 70% of the tumours that were positive for laminin were also positive for Helix-loop-helix protein inhibitor of differentiation 4 (ID4) and Metastasis Tumour Antigen1 (MTA1) biomarkers (p<0.001). However there was only a trend between laminin expression and reduced BRCA1 expression (p=0.05). With respect to the expression of steroid hormone receptors, laminin-positive tumours were significantly inversely correlated with ER and PgR with majority of these tumours either showing absence or low level of ER and PgR (p<0.001). Comparing laminin expression with HER family receptor expression, a significant association was observed with EGFR expression (p=0.02), but not with HER-2 expression.

Furthermore, there was a positive correlation between laminin expression and basal cytokeratin CK5/6, basal like phenotype, triple negative and p53 (all p<0.001). There was an inverse association between laminin with E-cadherin (p=0.03). There was no significant correlation between laminin expression and P-cadherin (Table 3).

Prognostic significance of laminin expression in Nigerian breast cancer

Univariate survival analysis showed tumours positive for laminin had significantly poorer BCSS (p=0.009) and DFI (p=0.03) (Figure 2), but not in Cox multivariate analysis (Table 4).

DISCUSSION

The previous studies on different laminin isoforms are grouped together for the purpose of discussion in this study. Laminin expression in BC from Nigerian women was higher (57.3%) than those studies conducted among the Western women in which 17.9%,[25] 28%,[38] 31%,[39] 53.8% [40] and 55% [24] of the tumours were observed to have laminin protein expression. The difference in the rate of expression of laminin in these studies could be explained by the fact that the basal phenotype of breast cancer, which significantly showed a higher rate of laminin expression than other breast cancer subtypes, has a high prevalence in the Nigerian BC population, which is an indication that laminin expression might have played important roles in BC in Nigeria women and by extension black population. We are the first authors to study the expression of laminin in BCs in a unique cohort of black women from Nigeria.

Most of the clinicopathological and molecular characteristics of BC in Western women are associated with luminal A phenotype, less aggressive and associated with favourable outcome.[28] The findings in this study of a significant association between laminin expression and unfavourable clinicopathological and molecular characteristics of BC are in concurrence with few Western women BC that exhibit basal like phenotype and aggressive tumour features.[22,25,28,29,41-43] For example, Martignone *et al* showed significant associations between laminin expression and young age at diagnosis and premenopausal status.[22] The association of laminin expression with triple-negativity, CK 5/6, EGFR, p53 expression is in line with the basal phenotype of most of the laminin-positive BC.[25, 28, 29, 42, 43]

In this study, laminin was found to be associated with poor patient outcome in our BC cohort and this is similar to the prognostic value of laminin expression in other BC populations.[22-24] Our findings are also in agreement with those of other studies which investigated and found laminin expression to be associated with adverse clinical, pathological and prognostic features in other cancers including of pancreatic, malignant glioma and malignant ovarian epithelial tumour.[18-20] However, our findings are at variance with the results from a study which found that laminin expression was associated with less aggressive breast tumour cells[21] and also with the study by Rodriguez-Panilla *et al* that reported no association between prognosis and the expression of laminin in BC.[25] The difference in the results might be a reflection

TABLE 2: Relationship between laminin expression and clinicopathological variables

Variables	Laminin			
	Negative (%)	Positive (%)	χ^2 value	p-value
Age (years)				
≤50	59 (54.1)	104 (71.2)	**8.67**	**0.005**
>50	50 (45.9)	42 (28.8)		
Lymph node involvement				
Negative	10 (9.2)	11 (7.5)	0.03	0.63
Positive	99 (90.8)	135 (92.5)		
Menopausal				
Pre	62 (56.9)	109 (74.7)	**11.29**	**0.003**
Post	47 (43.1)	37 (25.3)		
Mitotic figure				
Low (≤4)	78 (71.6)	88 (60.3)		
Medium (5-9)	17 (15.6)	31 (21.2)	3.27	0.17
High (>10)	14 (12.8)	27 (18.5)		
Nuclear pleomorphism				
Small uniform cells	0 (0.0)	1 (0.7)		
Moderate increase in size	44 (40.4)	43 (29.5)	4.27	0.14
Marked variation	65 (59.6)	102 (69.9)		
Tubule formation				
> 75 %	2 (1.8)	1 (0.7)		
10 -75 %	3 (2.8)	10 (6.8)	2.60	0.24
< 10 %	104 (95.4)	135 (92.5)		
Tumour grade				
1	3 (2.8)	3 (2.1)	3.41	0.15
2	76 (69.7)	86 (58.9)		
3	30 (27.5)	57 (39.0)		
Tumour type				
Invasive ductal	93 (85.3)	129 (88.4)		
Tubular mixed	8 (7.3)	7 (4.8)		
Lobular mixed	2 (1.8)	2 (1.4)		
Classical lobular	2 (1.8)	1 (0.7)		
Mucinous	1 (0.9)	2 (1.4)	4.89	0.72
Medullary	2 (1.8)	3 (2.1)		
Tubular	0 (0.0)	2 (1.4)		
Tubulolobular	1 (0.0)	0 (0.0)		
Tumour Size (cm)				
≤2	8 (7.3)	14 (9.6)	0.23	0.5
>2	101 (92.7)	132 (90.4)		
Vascular invasion				
Negative	29 (26.6)	41 (28.1)	0.03	0.79
Positive	80 (73.4)	105 (71.9)		

TABLE 3: Relationship between laminin and other biomarkers expression in Nigerian breast cancer

Variables	Laminin exprerssion			
	Negative (%)	Positive (%)	χ^2 value	p-value
BRCA1				
Negative	61 (76.3)	108 (87.1)	4.03	0.05
Positive	19 (23.7)	16 (12.9)		
ID4				
Negative	41 (66.1)	35 (24.5)	**37.50**	**<0.001**
Positive	21 (33.9)	108 (75.5)		
MTA1				
Negative	60 (83.3)	48 (33.1)	**28.55**	**<0.001**
Positive	12 (16.7)	97 (66.9)		
Ck5/6				
Negative	74 (80.4)	51 (41.1)	**31.69**	**<0.001**
Positve	18 (19.6)	73 (58.9)		
CK14				
Negative	46 (62.2)	56 (49.6)	3.11	0.09
Positive	28 (37.8)	57 (50.4)		
E-Cadherin				
Negative	57 (78.1)	72 (63.7)	**4.30**	**0.03**
Positive	16 (21.9)	41 (36.3)		
P-Cadherin				
Negative	41 (51.2)	43 (38.4)	5.31	0.07
Positive	39 (48.8)	69 (61.6)		
p53				
Negative	34 (49.3)	24 (19.8)	**19.09**	**<0.001**
Positive	35 (50.7)	97 (80.2)		
EGFR				
Negative	57 (75.0)	73 (58.9)	**8.07**	**0.02**
Positive	19 (25.0)	51 (41.1)		
HER-2				
Negative	73 (83.0)	110 (84.0)	0.01	0.84
Positive	15 (17.0)	21 (16.0)		
ER				
Negative	60 (63.2)	115 (84.6)	**14.07**	**<0.001**
Positive	35 (36.8)	21 (15.4)		
PgR				
Negative	47 (61.0)	105 (89.7)	**26.09**	**<0.001**
Positive	30 (39.0)	12 (10.3)		
Classification				
Basal	8 (17.0)	60 (65.2)		
HER-2	10 (21.3)	14 (15.2)		
LuminalA	24 (51.1)	15 (16.3)	**39.80**	**<0.001**
LuminalB	5 (10.6)	3 (3.3)		
Triple Negative				
Negative	47 (54.0)	26 (26.0)	**14.18**	**<0.001**
Positive	40 (46.0)	74 (74.0)		

TABLE 4: Cox multivariate analysis of probability of DFI and BCSS in Nigerian breast cancer series with laminin expression

Variables	Laminin expression							
	DFI				BCSS			
	p-value	Hazard ratio	95% CI		p-value	Hazard ratio	95% CI	
			Lower	Upper			Lower	Upper
Laminin	0.57	1.20	0.63	2.28	0.16	1.33	0.88	2.02
Grade	<0.001	4.44	2.34	8.40	<0.001	2.47	1.65	3.69
Lymph node	0.07	0.16	0.02	1.20	0.93	0.96	0.45	2.06
Tumour size	0.29	1.59	0.66	3.801	0.35	1.36	0.70	2.64

of the ethnic population used in the studies. Most of the previous studies were conducted among the Caucasians and these further give credence to the difference that we have reported between ethnic Nationality in relation to BC development and patient outcome among black and white women.[28]

The association of laminin expression with the E-cadherin loss in this study is also in keeping with the basal phenotype of the laminin-positive tumours. In the study by Sarrio *et al* the cadherin switch and laminin expression were shown to be associated with the basal phenotype of BC.[41] Thus, this might probably explain in part the aggressive and metastatic characteristic of BC among the black women.

Although this study only observed a trend between laminin expression and BRCA1 loss, there was positive correlation with BRCA1 down-regulators MTA1 and ID4, evidence that

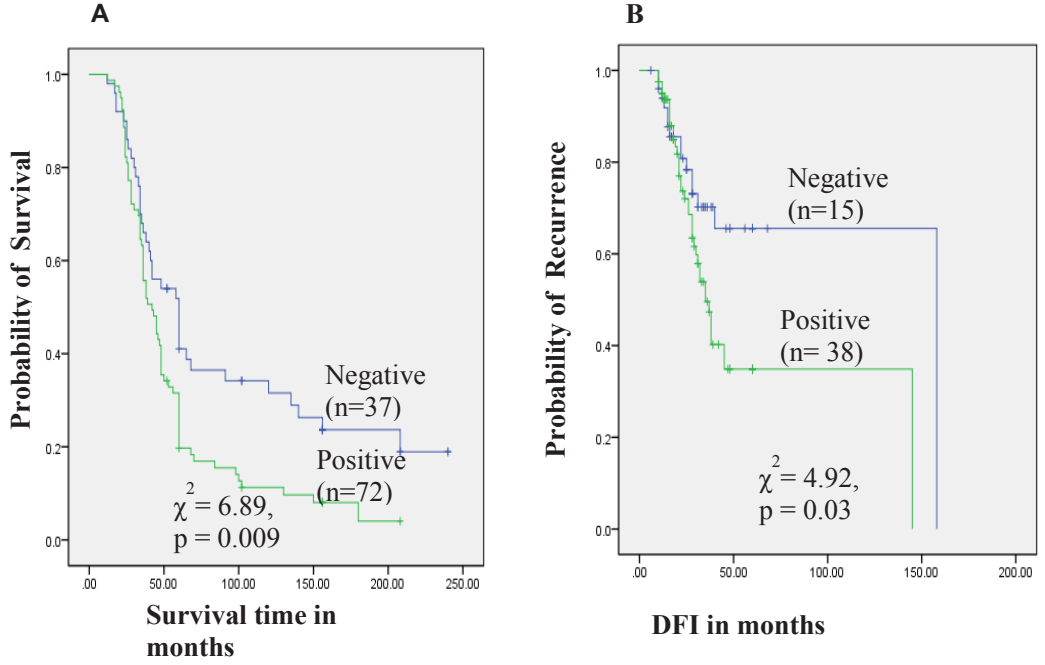

FIG. 2: The relationship between Laminin expression and (A) Breast Cancer Specific Survival, (B) Disease Free Interval.

laminin expression could be associated with the pathway of BRCA1 down-regulation in these tumours.[44,45] Interestingly, laminin-rich extracellular matrix has been shown to down-regulate BRCA1 expression in breast epithelial cells.[46,47] Incidentally, we have determined the expression of BRCA1 and its down-regulators in Nigerian BC cases treated with the CMF/AC regimen by using retrospective studies and observed that they were associated with basal like phenotype, triple negative adverse clinicopathological features and poor outcome.[28] Others have also shown that BRCA1 loss is characteristic of the basal-like BC.[34,47,48] BRCA1, a DNA-repair protein has been shown to be predictive of resistance to DNA-damaging agents such as cyclophosphamide and cisplatin.[49] Combinations of chemotherapeutic agents have long been used for BC management in Nigerian BC.[30] However, following varying degrees of initial responses, resistance develops to the chemotherapeutic regimens; Cyclophosphamide, Methotrexate and 5-Fluorouracil (CMF) and Adriamycin and Cyclophosphamide (AC).

In recent times, biomarkers are increasingly being shown to predict response/resistance to chemotherapeutic agents.[50] A study has proposed targeting novel drug on laminin.[51] Therefore, targeting laminin expression in Nigerian BC that are commonly associated with basal-like phenotype, BRCA1 deficiency and triple negative BC might enhance better management.

In conclusion, this study has demonstrated that laminin expression is associated with the basal-like molecular subtype of BC and with adverse prognosis in the Nigerian BC cohort. Therefore, a novel laminin targeted therapy in Nigerian BC might enhance better management.

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