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Relevance of the MicroRNA (Mirna) Processor DICER Expression in the biological behaviour and pathological response of Nigerian breast cancer tissues

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Abstract

Background: Breast cancer (BC) among Nigerians is characterised by high grade, triple negative, basal-like phenotype tumours with high proliferation indices and poor prognosis. The loss of Dicer expression has been speculated to play a key role in BC with similar features among the women in the Western countries.
Objectives: To demonstrate the role of the Dicer expression in relation to pathological response in BC, in order to determine the biological behaviour and its prognostic significance in BC among Nigerian women using immunohistochemistry and Tissue microarray (TMA).
Methods: This study investigated the immune profiles of the Dicer in 241 tissue microarray of breast cancer tissue of Nigerian women and correlated the protein expression with the pathological response and the other biomarker expressions to determine the functional significance in Nigerian women.
Results: Protein expression of Dicer as compared with other biomarkers expression showed there was significant association between the loss of Dicer expression and the down-regulators of Breast Cancer Associated Gene-1 (BRCA1), metastasis tumour antigen-1(MTA 1) ($p = 0.004$), Inhibitor differentiation-4 (ID4) ($p = 0.002$), ubiquitin conjugating enzyme-9 (UBC9) ($p = 0.008$) and protein inhibitor of activated signal transducer gamma PIASγ ($p = 0.002$). Other relevant Homologous repair pathway markers included poly (ADP-ribose) polymerase-1(PARP1) ($p < 0.001$) and RAD51 ($p < 0.001$), cell cycle regulator protein-27(p27) ($p = 0.024$), the proliferation kinetic protein (Ki-67) ($p = 0.003$) and epidermal growth factor receptor (EGFR) expression ($p = 0.013$). Survival analysis also showed that there was no significant correlation between tumours negative for Dicer and patient outcome.
Conclusion: This study demonstrated that the loss of Dicer is associated with intermediate to higher grade tumour, discrepant MI/Ki-67 expression, p27 loss, homologous recombination response dysregulation, high EGFR and Ki-67 expression. Therefore, Dicer expression appears to play a major role in the biology of BC among Nigerian women. A targeted therapy on Dicer expression would enhance the management of BC among Nigerian women.

Keywords: *Breast cancer, Dicer, Homologous recombination response dysregulation, Pathological response, Nigerian women.*

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Introduction

Dicer is a cytoplasmic microRNA (miRNA)-processing endoribonuclease III, which is encoded by the *DICER I* gene on chromosome 14q32.13. [1] Specifically, Dicer cleaves pre-

miRNA (which is about 70 nucleotides long), into mature double-strand (ds) miRNA fragments of approximately 22-30 nucleotides. [1, 2] The ds-miRNA unwinds and the single strands of the duplex are incorporated into the multi-protein RNA-induced silencing complex (RISC) where they bind to complementary regions of their target mRNAs. This leads to mRNA cleavage or translational repression and gene repression. [2, 3] DICER is involved in the regulation of gene expression in the course of miRNA processing. The expression of Dicer itself is regulated, in this way, by the miRNA 222 and 221. [4]

The role of Dicer in carcinogenesis appears to be cancer-specific. [5-9] For example, Dicer expression is mainly reduced in cancers of the lungs and ovary where the reduction of expression is associated with adverse clinicopathological and prognostic characteristics. On the other hand, Dicer over-expression has been reported in prostate and colorectal cancer and acute myeloid leukaemia, in which it is associated with adverse features among Caucasians. [5-9]

In breast cancer (BC), many studies carried out on Western women showed the loss of Dicer expression in primary tumours. [3, 10-11] Furthermore, this loss of Dicer expression has been associated, in some of these studies, with high tumour grades, hormone receptor status and breast cancer subtypes, the epithelial-mesenchymal transition (EMT), lymph node metastasis, and poor survival characteristics among women in the western countries. [3, 10-12] However, the pattern, clinicopathological and prognostic significance of Dicer expression in Nigerian BC cases have not been investigated. BC among Nigerians is characterised by high grade, oestrogen receptor (ER)-negative, basal-like phenotype, Breast cancer associated gene-1 (BRCA-1) negative tumours with high proliferation indices and poor prognosis. [13-14] This hypothesised that Nigerian BC cases will be characterised by a high rate of loss of Dicer expression, and this expression pattern will be associated with ER-negative, basal-like, BRCA-1negative BC with high pathological grade and poor prognosis.

The objective of this study was to demonstrate the role of Dicer expression in relation to pathological response and prognosis in BC among Nigerian women.

Methods

Subjects

Two hundred and forty-one (241) formalin-fixed paraffin embedded (FFPE) BC tissues from women who presented at the Histopathology Laboratory of the Olabisi Onabanjo University Teaching Hospital, Sagamu, and the Histopathology Specialist Laboratory, Idi-Araba, Lagos, both in south-west Nigeria between January 2002 and December 2008 were studied. The data obtained from the hospital records included clinical history (age and menopausal status), patient outcome (survival and recurrence) and treatment data. Tumour characteristics, including, tumour type, histological grade, tumour size, lymph node status and vascular invasion were also included in this study. The subjects were followed up for at least 60 months. This study was approved by the Medical Advisory Committee, Olabisi Onabanjo University Teaching Hospital, Sagamu, Nigeria. The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria, recommended by McShane *et al* [15] were adopted for the study.

Tissue Microarray Array Construction

Tissue microarrays (TMA) were constructed as previously described. [12] Briefly, breast tumour cores were taken from each FFPE donor tissue block marked for the most representative points of the tumour (both peripherally and centrally). A precision instrument (ALPHELYS MiniCore®) was used to take representative cores of tissue (0.6mm diameter) from each sample, which was arrayed into recipient paraffin blocks.

Immunohistochemistry

Four micrometres (4µm) sections of TMA were immunohistochemically stained for the biomarkers of interest as previously described. [13, 16] The standard StreptAvidin–Biotin complex method as previously described by Agboola *et al*, was used for the detection of tissue markers. [13] All, except epidermal growth factor receptor-2 (c-erbB2) and EGFR, required antigen retrieval

which was performed by microwaving the slides at 800W for 10 minutes and subsequently at 560W for 10 minutes in 1M Sodium Citrate pH 6.0. The microwaving was immediately followed by cooling in running water. The primary antibody for the biomarkers (anti–Dicer; Ab14601, Abcam Plc, UK) diluted at 1:300, was incubated for 60 minutes at room temperature.

Diaminobenzidinetetrahydrochloride (DAB) solution was incubated for 10 minutes, after which copper sulphate solution (0.5% Copper Sulphate in 0.8% Sodium Chloride) was applied to the slides and incubated for 10 minutes each. Counterstaining with haematoxylin was done for 2-3 minutes, followed by rinsing in tap water. The slides were dehydrated by immersing them in three alcohol baths for 10 seconds and cleared in two xylene baths followed by the application of coverslips. Negative and positive controls respectively were performed by omitting the primary antibody and including control tissues (human fibroma) as specified by the manufacturer of the antibody. The sources of other primary antibodies, positive controls, and dilution methods used in this study had been previously described. [13,16]

Immunohistochemical Scoring

The immunoreactivity of Dicer was assessed using the percentage of positive cells. The cases were scored without the knowledge of the clinicopathological parameters or patient outcome. TMAs were scored independently twice by one of the researchers (AA). The means of the scores were calculated to obtain the final scores. 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 a positive expression for Dicer. The scores for the other biomarkers were determined as previously described. [13,16]

The American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for HER2 Testing in Breast Cancer was used for the assessment of c-erbB2 (HER2). [17] Equivocal (2+) cases were confirmed by chromogenic *in-situ* hybridisation (CISH) as previously described. [18] The Nielsen's method was used for molecular classification. [19] This comprised 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 Dicer expression, pathological parameters and other biomarkers. The Kaplan–Meier survival method and the log-rank test were used for survival curves. A two-sided p-value of <0.05 was considered significant.

Results

The majority of the subjects were less than 50years (158/241; 65.5%) and premenopausal (166/241; 68.9%). The tumour characteristics included invasive ductal carcinoma without any special histological type (210/241; 87.1%), larger size greater than 2cm (220/241; 91.3%), higher tumour grade II (145/241; 60.2%) and III (91/241; 37.8%) compared to grade I(5/241; 2.0%) and metastasis either through blood vessels (180/241; 74.7%) or through lymphatic channels (219/241; 90.9%). All the patients received both chemotherapy and hormonal therapy. Only a few of them had additional radiotherapy (n=45; 18.7%).

Using a cut-off of >1%, out of 241 BC, Dicer expression was lost in 188 (78. 01%) and positive in 53 (21.99%) cases.

Dicer expression and tumour grade and mitotic count There was a significant association between the loss of Dicer expression and tumour grade in the cohort studied. The loss of Dicer was significantly more common in grades II and III BCs compared to grade I (p = 0.001). However, there was no association between Dicer expression and the other clinicopathological indices. (Table I)

Dicer expression and biomarkers of cellular pathways and molecular subtypes of breast cancer

Table II shows the relationship between Dicer expression and other biomarkers after removing uninformative cores. There was significant direct association between loss of Dicer expression and biomarkers of DNA repair pathways, including the down-regulators of Breast cancer associated gene 1(BRCA1), metastasis tumour antigen 1(MTA 1) (p = 0.004), Inhibitor differentiation 4 (ID4) (p = 0.002), ubiquitin conjugating enzyme 9 (UBC9) (p = 0.008) and protein inhibitor of activated signal transducer gamma PIASγ (p = 0.002). Others included the Homologous repair pathway markers, poly (ADP-ribose) polymerase 1(PARP1) (p < 0.001) and RAD51 (p < 0.001), cell cycle regulator protein 27(p27) (0.024), the proliferation kinetic protein (Ki-67) (p = 0.003) and epidermal growth factor receptor (EGFR) expression (p = 0.013). However, this study found no associations between Dicer expression and BRCA1, Placenta-cadherin (P-cadherin), Epithelial –cadherin (E-cadherin), Estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor (HER2) and cytokeratin 5/6 (CK 5/6).

Table I: Relationship between DICER expression and clinicopathological parameters of Breast cancer cases

Parameters		*DICER*		*X²*	*p-value*
		Negative n = 188 (%)	Positive n = 53 (%)		
Age	< 50 years	124 (66.0)	34 (64.2)	0.06	0.80
	≥50 years	64 (34.0)	19 (35.8)		
Tumour size	<2cm	15 (8.0)	6 (11.3)	0.58	0.44
	≥2cm	173 (92.0)	47 (88.7)		
Menopause	Pre-	126 (67.0)	40 (75.5)	1.377	0.24
	Post-	62 (33.0)	13 (24.5)		
Vascular invasion	Positive	135 (71.8)	45 (84.9)	3.751	0.05
	Negative	53 (28.2)	8 (15.1)		
Tumour grade	1	3 (1.6)	2 (3.8)	14.360	0.01
	2	25 (66.5)	20 (37.7)		
	3	60 (31.9)	31 (58.5)		
Lymph node	Negative	17 (9.0)	5 (9.4)	0.008	0.93
	Positive	171 (91.0)	48 (90.6)		
Tumour type	Ductal NST	163 (86.7)	47 (88.7)	6.976	0.53
	Medullary NST	13 (6.9)	0 (0.0)		
	Tubular	2 (1.1)	1 (1.9)		
	Lobular	2 (1.1)	2 (3.8)		
	Mucinous	2 (1.1)	1 (1.9)		
	Tubular Mixed	2 (1.1)	1 (1.9)		
	Lobular Mixed	2 (1.1)	1 (1.9)		
	Mixed NST	1 (0.5)	0 (0.0)		
	Others	1 (0.5	0 (0.0)		

NST – No Special Type

Table IIa: Relationship between DICER and other biomarkers in breast cancer

Biomarkers		*DICER-Positive*	*DICER-Negative*	*X²*	*P-value*
E_CAD		N = 133	N = 40		
	Negative	97 (72.9)	27 (67.5)	0.447	0.50
	Positive	36 (27.1)	13 (32.5)		
UBC9		N =130	N = 45		
	Negative	58 (44.6)	10 (22.2)	7.056	0.008
	Positive	72 (55.4)	35 (77.8)		
Triple Negative BC		N = 135	N = 45		
	Negative	68 (50.4)	19 (42.2)	0.897	0.34
	Positive	67 (49.6)	26 (57.8)		
PAISγ		N = 151	N = 46		
	Negative	67 (44.4)	9 (19.6)	9.156	0.002
	Positive	84 (55.6)	37 (80.4)		
BRCA-1		N = 150	N = 42		
	Negative	126 (84.0)	34 (81.0)	0.219	0.63
	Positive	24 (16.0)	8 (19.0)		
PARP1		N = 138	N = 46		
	Negative	74 (53.6)	11 (23.9)	12.252	< 0.001
	Positive	64 (46.4)	35 (76.1)		
MTA 1		N = 172	N = 47		
	Negative	96 (55.8)	15 (31.9)	8.435	0.004
	Positive	76 (44.2)	32 (68.1)		
ID4		N = 151	N = 47		
	Negative	56 (37.1)	6 (12.8)	9.857	0.002
	Positive	95 (62.9)	41 (87.2)		
EGFR		N = 143	N = 42		
	Negative	98 (68.5)	20 (47.6)	6.146	0.01
	Positive	45 (31.5)	22 (52.4)		

E_CAD - Epithelial Cadherin; UBC9 - Ubiquitin conjugating enzyme-9; PIASγ - Protein Inhibitor Activated Signal Transducer-Gamma; BRCA1 - Breast Cancer Associated Gene-1; PARP1 - Poly (ADP Ribose) Polymerase-1; MTA1 - Metastasis Tumour Antigen-1; ID4 - Inhibitor Deifferentiation-4; EGFR - Epidermal Growth Factor Receptor.

Table IIb: Relationship between DICER and other biomarkers in breast cancer

Biomarkers		*DICER-Positive*	*DICER-Negative*	X^2	*P-value*
CK56		N = 155	N = 50		
	Negative	91 (58.7)	23 (46.0)	2.474	0.11
	Positive	64 (41.3)	27 (54.0)		
ER		N = 173	N = 51		
	Negative	128 (74.0)	43 (84.3)	2.325	0.12
	Positive	45 (26.0)	8 (15.7)		
PGR		N = 141	N = 44		
	Negative	104 (73.8)	38 (86.4)	2.986	0.08
	Positive	37 (26.2)	6 (13.6)		
HER_2		N = 160	N =49		
	Negative	133 (83.1)	38 (77.6)	0.783	0.37
	Positive	27 (16.9)	11 (22.4)		
P27		N = 168	N = 45		
	Negative	129 (76.8)	27 (60.0)	5.102	0.02
	Positive	39 (23.2)	18 (40.0)		
KI_67		N = 160	N = 51		
	Negative	35 (21.9)	2 (3.9)	8.602	0.003
	Positive	125 (78.1)	49 (96.1)		
RAD51		N = 150	N = 47		
	Negative	85 (56.7)	12 (25.5)	13.880	0.001
	Positive	65 (43.3)	35 (74.5)		
P_CAD		N = 142	N = 39		
	Negative	65 (45.8)	13 (33.3)	1.931	0.16
	Positive	77 (54.2)	26 (66.7)		

CK56 – Cytokeratin5/6; ER – Estrogen Receptor; PGR – Progesterone Receptor; HER_2 – Epidermal Growth Factor Receptor - 2; P27 – Cell Cycle Protein 27; KI_67 – Cell Proliferative Kinetics - 67; RAD51 –Recombinase-51; P_CAD – Placental Cadherin.

Prognostic significance of Dicer expression in Nigerian breast cancer cases

Out of the 241 cases, 111 (46.1%) died while 21 (8.6%) survived. The remaining 109 (45.3%) were lost to follow-up. Univariate analysis showed no prognostic significance for Dicer expression in BC tissues of Nigerian women. There was no association between Dicer expression and breast cancer-specific survival (BCSS) (p = 0.979) or disease-free interval (DFI) (p = 0.331) as shown in Figure 1.

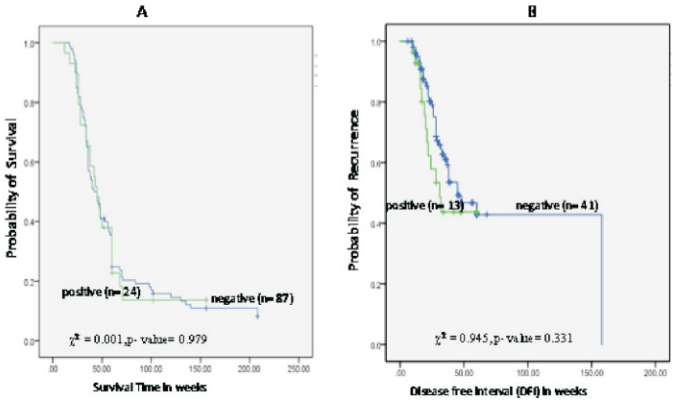

Figure 1: Dicer expression in relation to (A) BCSS and (B) recurrence differences respectively (B) between positive and negative expression in Nigeria BC

Discussion

To the best of the authors' knowledge, this is the first study investigating the pathological and prognostic significance of Dicer expression in Nigerian BC cases. This study found that 78.01% of Nigerian BC cases showed loss of Dicer expression. This rate of Dicer loss was comparable to reports from other Western populations. [3, 10-12]

The loss of Dicer in tumours have been attributed to the following mechanisms: point mutations in *DICER* and hypermethylation of *DICER* promoter region, [5] degradation of *DICER* transcripts and post-translational repression of by miRNA, especially miR-221/222, miR-103/107, let-7, miR-29a and miR-192, [4] reduced export of *DICER* from miRNA through competition with other miRNAs, [20] copy number loss of *DICER* [21] and transcriptional repression of Dicer by p53, p63 and p73. [22]

The expression pattern of Dicer and p27 in the present cohort suggested that Dicer loss may be due to repression by micro-RNA in line with *in-vitro* knockdown of *DICER* resulting in the up-regulation of p27. [23] This would imply a negative relationship between p27 and Dicer; it has been shown that both Dicer and p27 are negatively regulated by miR-221/222. [4] This means that with miR-221/222 in the picture, a direct correlation of expression between Dicer and p27, such as observed in the present study may arise.

In this study, the loss of Dicer expression was associated with intermediate and higher grade

tumours compared to low grade. These findings were in agreement with previous reports by Khoshnaw *et al* and Dedes *et al* in which loss of Dicer was associated with high grade, poorly differentiated tumours with loss of tubule formation. [12]

The finding of an indirect relationship between Dicer expression and the Homologous Recombination pathway components (RAD51 and PARP1) was in keeping with recent observation that Dicer regulates the Homologous Recombination pathway. [24] Dicer-dependent RNA products are essential for the formation of DNA damage response foci which is an essential part of the DNA damage repair mechanisms. [24, 25] In the absence of Dicer, there is deficient formation of the repair foci at the DNA damage sites. [24] The loss of RAD51 would prevent the formation of BRCA1-RAD51-BARD1 repair complex at the DNA damage response site. [25] This is important when there is over-expression of the BRCA1 down-regulators: MTA1, ID4, UBC9 and PIASγ. [26] In the presence of BRCA1 down-regulation, the BRCA1-RAD51-BARD1 repair complex is unavailable. [25] The implication of this development would be loss of HR repair. Dicer also leads to increased NHEJ efficiency. [23] The loss of Dicer expression in the present cohort of patients may, therefore, partly explain the relationship between HRR pathway loss, NHEJ up-regulation and genomic instability.

Research works studying the role of Dicer in cancer metastases have shown that the loss of Dicer is associated with cancer cell metastases and the epithelial mesenchymal transition. [10, 11] Further, Khoshnaw *et al* and Caffrey *et al* showed that Dicer loss was associated with nodal and distant metastases. [10, 11] The work by Martello *et al*, revealed that the miRNA 103/107 down-regulated Dicer expression and induced a mesenchymal morphology and metastatic potentials in epithelial breast cancer cells which were indicative of an EMT. [29] Although we found no statistically significant association between Dicer expression and nodal metastases in the present study, the subjects were characterised by delayed clinical presentation. The evidence included tumour size greater than 2cm and lymph node involvement which was almost uniformly node-positive (>90%) .This observation may have precluded any meaningful statistical analysis in this regard.

In the present study, we found no associations between the hormonal receptors (ER, PR) and Dicer expression and there was no association between Dicer expression and HER2 receptors. This contrasts with the findings in other studies conducted in the Western countries, in which Dicer expression was associated with the molecular subtypes of breast cancer. [3, 10-12] Specifically, these studies have found the loss of Dicer to be associated directly with ER-negative, HER2-positive and triple-negative subtypes of breast cancer. The difference in the results might be due to differences in the study population used. However, we did find that the loss of Dicer expression was positively associated with EGFR expression. The present study and some previous ones have shown that EGFR expression is characteristic of the basal-like subtype of BC which is the commonest subtype among Nigerian women. [13, 14] This observation might be due to differences in ethnicity since BC is heterogeneous, in terms of behaviour, in different races. The present also showed that Dicer loss was associated with high expression of the proliferation marker, Ki-67, which is also characteristic of the basal-like BC subtype. [13, 14] Caffrey *et al* have similarly shown that Dicer loss was associated with high Ki-67 expression. [13]

Furthermore, it was observed that Dicer loss was associated with Ki-67 expression. Discrepant mitotic index and Ki-67 index (low MI/high Ki-67 or high MI/low Ki-67) have been observed, in two BC studies among Caucasian women, to be associated with survival .[30] In the more recent study, it was found that patients whose tumours have discrepant MI/Ki-67 had survival characteristics which were intermediate between those whose tumours had high MI/high Ki-67 and those with low Mi/low Ki-67. [30] The present study, however, did not find any direct prognostic value for Dicer, as univariate analysis did not reveal any association between Dicer expression and BCSS or DFI. Although this is in contrast to some studies which reported associations between Dicer loss and disease-free survival in BC sub-groups such as ER-positive, HER2-positive, lymph node-negative and in subjects who received chemotherapy. [10-12] The study also agreed with other studies in which no prognostic value was found for Dicer expression. [3, 12] Although the strength of this study was the ability to

demonstrate the importance of the Dicer expression among Nigerian women, its area of limitation was the sample size used compared to previous studies conducted in the Western world. Therefore, there is a need to validate the findings in the present study using larger samples across the country in the future.

Conclusion

Nigerian BC cases were characterised by loss of Dicer expression and this loss of expression was associated with intermediate grade tumour, discrepant MI/Ki-67 expression, p27 loss, homologous recombination response dysregulation, high EGFR and Ki-67 expression. The loss of Dicer expression might have contributed towards the aggressive nature of BC as observed among Nigerian women. Further studies on the development of a novel therapy targeted at Dicer expression and its consequences may improve survival in BC among Nigerian women.

Contribution of Authors: AA conceived the study, performed the immunohistochemistry study and drafted the manuscript. SB, BA, EH and IV participated in data collection, analysis and interpretation as well as drafting of the manuscript. NC performed the optimisation of specimens and prepared the slides. GA, EI and RE participated in specimen analysis and confirmation of diagnosis. All the authors made substantial contributions to the intellectual contents of the manuscript.

Conflict of Interest: None

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