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RESEARCH ARTICLE

P53, KI67, BRCA1 AND BRCA 2 GENES OVER EXPRESSION IN PARAFFIN EMBEDDED PRIMARY BREAST CANCER TISSUES OF AN INDIGENOUS BLACK POPULATION: A 5YEARS RETROSPECTIVE STUDY

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ABSTRACT

Molecular markers have been extensively investigated with a view to providing early, precise and accurate information on breast tumor outcome and predict response to treatment. We aim at comparing overexpression of certain tumour markers in patient below and above 50years of age. Archived breast tissue specimens were retrieved from the department of histopathology and subjected to routine pathological examinations and receptor status assessed by immunostain. The p53, Ki-67, BRCA1 and BRCA2 over expression were reported as dark golden-brown precipitate in 35%, 70%, 75% and 55% respectively out of the 20 cases that were malignant within the period of study. The p53 overexpression was more frequently seen in patients below 50years (85.7%), compared with those over 50 years old (14.3%).The immunohistochemical over expression of p53 shows high degree of receptor negativity for Her 2, progesterone and estrogen compared with positive receptor status. The Ki-67 immunostain shows more reactivity in patient below 50 (71.4%) compared with those above 50 years (28.6%). Ki-67 over expression was seen in 57.1%, 57.1%, and 35.7% for estrogen, progesterone, and Her2+ respectively compared with 42.9%, 42.9% and 64.3% with majority of the tumour been grade 3(71.4%).Also the immunostain ratio of BRCA 1: BRCA 2 shows a corresponding reactivity for estrogen positivity of 67.7**:**40.0%, progesterone 80.0**:**30.0% and Her2+ of 26.7**:**20.0% versus corresponding negativity of 33.3**:**60.0%, 20.0**:**70.0%, 73.3**:**80.0% for estrogen, progesterone and Her2 though not statistically significant at $p<0.05$.Tumor suppressor gene and proliferative tendencies is more expressed in breast cancer patients below 50 years suggesting good prognosis and response to treatment than those above 50years.

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INTRODUCTION

The p53 gene is a tumor suppressor gene that encodes a nuclear protein involved in adjusting cell proliferation and usually undetectable by immunohistochemical evaluation due to its short life (Ryan, 2011). Thus p53 over expression through routine immunohistochemical staining identifies the tumors with mutations of the p53- gene, thus the analysis of p53 status using immunohistochemical methods has been proven to be a powerful and independent prognostic factor in breast cancer as is associated with aggravating prognostic factors, like high histology grading, increased cell proliferation rate and aggressive clinical behavior (Maiuri *et al*., 2010). It used as a predictive marker to patients respond to chemotherapy. The loss and (or) alteration of p53 protein due to gene rearrangements can cause an imbalance in cell cycle through replicating errors and genetic accumulations. When cell repair fails p53 induces self-destruction of the cell through apoptosis (Stuckey, 2011). Therefore, immunohistochemical methods or demonstration which is based on the accumulation of p53 cellular proteins becomes necessary in our study population. Furthermore Ki-67 is a nuclear protein found in the G1-phase of cell cycle and it is considered a useful marker of cell proliferation. Many studies have found a link between the percentage of positive Ki-67 cells and the clinical evaluation, suggesting that measuring of Ki-67 expression can be useful in

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classification of breast cancer patients into two categories; good prognosis and bad prognosis (Dickson *et al*., 2005, Fitzgibbons, 2001) as it done in this study. Though not having phase specificity, it's expressed in all active phases of the cell cycle not expressed in the G0- phase. Finally an increase in Ki-67-expression indicates an increase in mitotic cell activity and proliferation (Stuckey, 2011). In order to monitor the proliferative activity of the breast cancer in our study population, ki67 became a gene of important to study. Finally BRCA1 and BRCA2 are the two most commonly mutated tumor suppressor genes associated with early onset and familial forms of breast cancer (Solanke and Adebamowo, 1996) Tumors associated with germ line BRCA1 mutations are more likely to be ER negative, PR negative, HER2/neu negative (triple-negative) whereas tumors in BRCA2 mutation carriers are usually ER positive and amenable to treatment with hormonal therapies (Oluwole *et al*., 1987, Madukwe and Oboma, 2016) and given the clinical implications of these mutations and the promise of prevention in further reducing morbidity and mortality associated with breast cancer, the American Society of Clinical Oncology has promoted integration of genetic testing as part of a comprehensive risk assessment and prevention plan for breast cancer. Several studies have reported low or undetectable frequencies of BRCA1/2 mutations in African American cohorts (Bassett, 1995). Previous study has reported a 1.3% prevalence of BRCA1 mutations in African American breast cancer patients, but 16.7% in African American patients selected for extremely early age of onset (<35 years old) (Hulka and Moorman, 2001). Also Buchok *et al*., (2002) in a work evidence of halotype insufficiency in human cells containing a germ line mutation in BRCA 1 and BRCA 2 in 2002, attributed inherited breast cancer (5-10%) to 2 germ line mutations, BRCA 1 and BRCA 2 which are inherited in an autosomal dominant fashion with varying penetrance. This finding reveals that family history of people who had first degree relatives; mother and sister that had breast cancer, were at greater risk of developing breast cancer. BRCA1 mutation is however not a significant independent prognostic factor (Ijaduola and smith, 1998, Nwajana and Oboma, 2016).To determine whether these results reflect the frequency and spectrum of BRCA1/2 mutations in an African population without non-African genetic admixture, we have performed complete BRCA1/2 immunohistochemical analysis on 20 paraffin embedded archived breast cancer tissue at the department of histopathology in Niger delta University Okolobiri, Bayelsa state, Nigeria

MATERIAL AND METHODS

Research Material

The breast tissues were fixed in formalin and initially processed through the usual technique of paraffin enclosure at the histopathology Laboratory in Niger Delta University Teaching Hospital Okolobiri being brought to paraffin block. Afterwards, an immunohistochemistry assessment was carried out in all the malignant cases (20 cases of invasive ductal carcinoma). In the morphology study the prepared materials were stained with Heamatoxylin–Eosin, the staining technique being done by going through the staining procedure for heamatoxylin and eosin. Results of heamatoxyl in and eosin staining shows nuclei, stained in dark blue; pink-red cytoplasm; Pale pink collagen fibers; Elastic and reticular fibers do not come out. The histological subtype of invasive breast carcinoma and Nottingham histological grade were defined using the routine (H + E) morphological evaluation.

Immunohistochemistry Methods/Protocol

The method use is the Avidin Biotin Complex (ABC) method also referred to the Avidin biotin imuunoperoxidase method. The entire antibodies used for this work are manufactured by Novocastra products and the antibody dilution factor of 1:100 dilutions was used for all the antibody markers. We took 4micron meter thick sections and applied on polylysine treated slides and then dried at laboratory temperature for 12 hours. The work procedure implies the following sequences of deparaffinising, alcohol rehydrating, endogenous peroxidase inhibition, incubation with peroxide in 3% distilled water, incubation with the primary antibody (the negative control), in the optimal solution, incubation with the secondary biotinylated species specific antibody (serum) for the primary antibody, incubation with the horse peroxidase, chromogen developing (DAB – 3,3'-diaminobenzidine) in the dark, counterstained with Mayer's Heamatoxylin, for 30 seconds, alcohol dehydrating with increasing concentrations, xylol clarification, then mounting with Canada Balm. The result visualized by investigating the antigens, with the DAB chromogen, that determines a brown solution at their levels (negative nuclei are stained in light blue with Heamatoxylin). To validate the results of immunostaining, we use reagents and control-tissue for each antibody used both external positive control and external negative control.

Methods of interpreting results

Ki-67 immunostaining cells were only considered positive when the cells that had an undoubtedly positive nuclear staining, while the cells where the staining not clear were considered negative. We then calculated an index of Ki67 staining by comparing the number of positive cells (nuclei), with the total number of cells (positive and negative), multiplying the result by 100 percent. There must be at least 100 nuclei for each case being interpreted as positive the ones that were brown to black. A Ki-67 index of 0–15% was considered low, between 16–30% was medium and a 31–100% index as high The reaction for p53 was considered positive (the over expression of p53-protein) when over 10% of tumor cells had a clear nuclear staining, no matter the intensity. The percentage of positive cells, with p53 protein accumulation, was calculated by determining the number of positive nuclei in ten different microscopic fields likewise BRCA 1 and BRCA 2.

RESULTS

In this present study, p53, Ki-67, BRCA1 and BRCA2 over expression according to the manufacturer's guidelines were reported as dark golden-brown precipitate. The percentage expressions were 35%, 70%, 75% and 55% respectively out of the 20 malignant cases (all ductal carcinoma) studied. P53 over

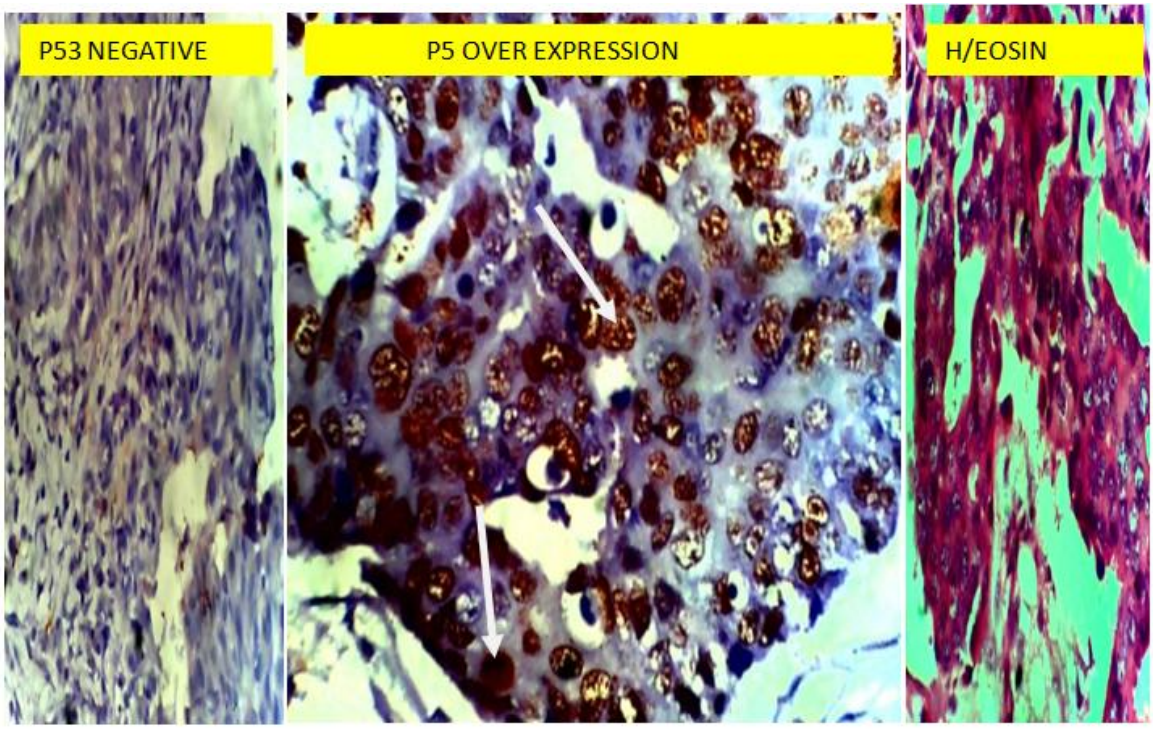
expression was more frequently encountered in patients under 50years (85.7%), compared with patients over 50yeras (14.3%). The immunohistochemical over expression of p53 was seen in 42.9% of HER2 positive (score 2+ and 3+), ER+(28.6%), PR+(42.9%), compared with 57.1%, 71.4% and 57.1% in the cases that were negative for HER2 negative (score 0 and 1+), estrogen receptor and progesterone receptor respectively. The over expression of p53 (corresponding to a high value of p53 protein, IHC determined) was more frequent in HER2-negative carcinomas compared to HER2-positive (57.1% *vs*.42.9%). Also 71.4% (cases) that were p53-positive tumour had a proliferation index of over 30%, and 28.6% had a proliferation index of over 15%. The analysis of immunostaining in Ki-67 shows more reactivity in patient below 50 (71.4%) compared with those more than 50 years (28.6%). Ki67 over expression was seen in 57.1%, 57.1%, and 35.7% for estrogen, progesterone, and Her2+ respectively compared with 42.9%, 42.9% and 64.3% with majority of the tumour grade 3(71.4%). Also the immunostain ratios of BRCA 1: BRCA 2 shows a corresponding reactivity for estrogen positivity of 67.7%**:**40.0%, progesterone 80.0%**:**30.0% and Her2+ of 26.7%**:**20.0% versus corresponding negativity of 33.3%**:**60.0%, 20.0%**:**70.0%, 73.3%**:**80.0% for estrogen, progesterone and Her2+ respectively with variation in Ki-67 proliferation index of 60.0**:**20.0 for 15% compared with 30% index of 40.0**:**80.0.

Table 1. Tumour markers parade among participants with carcinoma of the breast

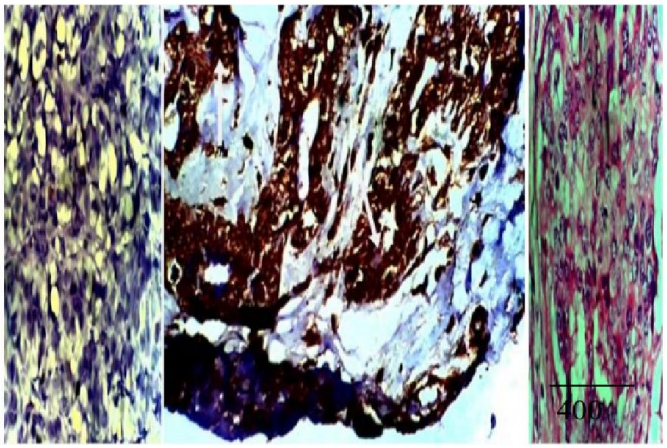
N=20	%P53(7/20)	%Ki67(14/20)	%Brca1(15/20)	%Brca2(11/20)	χ^2	C
Age (years)<50	6(85.7)	10(71.4)	10(66.7)	8(80.0)		
Age (years)>50	1(14.3)	04(28.6)	05(33.3)	02(20.0)	1.15	NS
HT						
Ductal ca.	07(100.0)	14(100.0)	15(100.0)	10(100.0)	NA	
Lobular ca.	0(0.0)	0(0.0)	0(0.0)	0(0.0)		
HG						
G1/2	2(28.6)	4(28.6)	9(60.0)	02(20.0)	5.28	NS
G3	5(71.4)	10(71.4)	6(40.0)	08(80.0)		
ER						
ER positive	02(28.6)	10(71.4)	10(66.7)	04(40.0)	5.23	NS
ER negative	05(71.4)	04(28.6)	05(33.3)	06(60.0)		
PR						
PR positive	03(42.9)	08(57.1)	12(80.0)	03(30.0)	6.76	NS
PR negative	04(57.1)	06(42.9)	03(20.0)	07(70.0)		
Her2						
HER2 positive	02(28.5)	05(35.7)	04(26.7)	02(20.0)	1.31	NS
HER2 negative	05(71.4)	09(64.3)	11(73.3)	08(80.0)		
P. index						
>15%	02(28.6)	-	9(60.0)	2(20.0)	3.2	NS
>30%	05(71.4)	-	8(40.0)	08(80.0)		

PR=Progesterone receptor, ER= Estrogen receptor, P. index=proliferation index, HG= Histology grade, HT= Histology type, Her2= Epidermal growth factor 2.NS=Negative correlation, p. value>0.05.

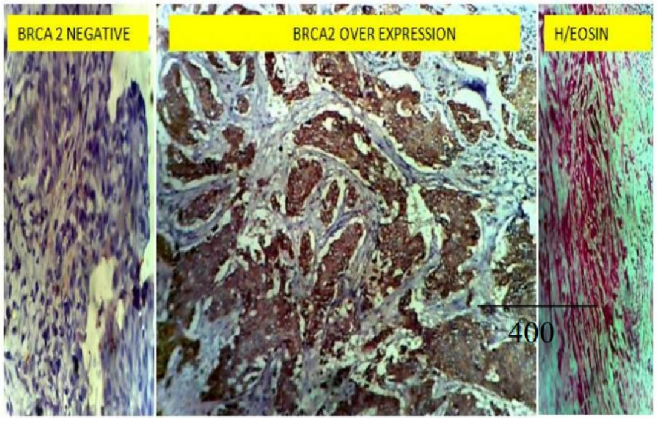
Photomicrography



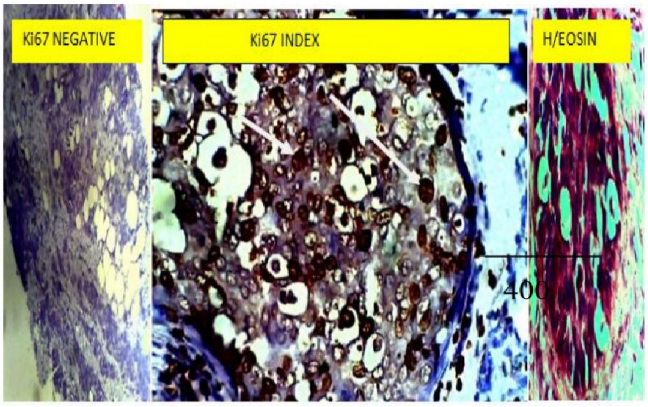
p53 over expression ×400 admixed a negative slide right and heamatoxylin and eosin left in ductal carcinoma of the breast. The brownish deposits show positivity



BRCA1 over expression ×400 admixed a negative slide right and heamatoxylin and eosin left in ductal carcinoma of the breast. The brownish deposits show positivity



BRCA2 over expression ×400 admixed a negative slide right and heamatoxylin and eosin left in ductal carcinoma of the breast. The brownish deposits show positivity



Ki-67 cytoplasmic staining with accentuated membrane staining, ×400 admixed a negative slides left and heamatoxylin and eosin right X400

DISCUSSION

The p53 gene play the role of "genome guardian" that is to monitor the DNA integrity during cell division. The protein product of normal allele of the p53-gene negatively regulates the growth and cell proliferation, blocking cells in G1 cell cycle phase (Bull *et al*., 2004). The loss or altering of p53-protein, due to gene rearranging, can cause the unbalancing of cell growth through replicating errors and genetic accumulations. If the DNA is altered, p53 blocks replication, favoring the activation of genome repairing systems (Song *et al*., 2005). When cell repair fails; p53 induces destruction through apoptosis and when mutated, corresponding protein products exhibit altered regulation properties with the mutant proteins having the ability to neutralize normal protein. In addition, some mutant forms could manifest new properties responsible with the growth of their oncogenic behavior (Gasco *et al*., 2002). In breast carcinoma, the mutations of p53 are associated with a more aggressive behavior and with a lower survival rate. Still, the frequency of the p53 mutations is lower in breast carcinoma in comparison with other solid tumors. In this study, the over expression of p53 protein was encountered in 42% of cases studied , the result is well correlated with the data from other studies that varies between 16% and 48% percent of positive p53 cases in invasive mammary carcinoma (Bartley and Ross, 2001, Al-moundhri *et al*., 2003).

However the over expression of p53 (immunohistochemical) does not reflect accurately the appearance of p53 mutations, given that the existing antibodies are determined by both wild and mutant types of p53 gene, this wild type possibly accumulating in some tumors as a response to DNA-alteration. The tumor suppressor gene (p53) over expression in this study is frequently encountered in patients under 50 years compared with the patients over 50 years (85.7% *vs.* 14.3%) though not statistically significant. Bartley and Ross (2001) have reported in a study that, p53 positivity was detected in five out of seven patients under 43years, Al-Moundhri *et al.* (2003) has reported that p53 over expression tends to appear in patients younger than 40years and pre-menopause patients, and Pietiläinen *et al.* (1995) also stated that p53 nuclear positivity is significantly correlated to age factors with the highest percentage of the cases seen in group of patients under 50-year-old. Despite the fact that p53 mutation differ from p53 accumulation as demonstrated in immunostain, it is therefore important to know that the apoptosis signals is reduced in breast cancer occurring between 40 and 50 years of age thereby halting gene surveillance.

Moreso the p53 immunopositivity was correlated with the lack of estrogen and progesterone receptors: 71.4% had no receptor expression for estrogens *vs.* 28.6% having the immunoexpression of estrogen receptors in previous studies compared with 61.9% vs. 38.1% estrogen receptor status as documented in the present study. Also 57.19% cases with p53-overexpression were negative for PR, while 42.9% cases were PR positive though not statistically significant. This present scenario is in agreement with previous study documenting 57.6% vs.45.42% of progesterone. All studies done before have noticed that mammary tumors with increased p53 immunoexpression or genetically determined mutations of p53 were much more often progesterone and estrogen receptor negative with most studies finding significant statistical differences (Lacroixm *et al*., 2006, Feki and Imimger- Finger, 2004) While the coexpression of p53 and HER2 were seen in 28.5% of all carcinoma cases that were included in this study

reflecting the rarity of the double genetic defect. Finally analyzing the risk of recurrences and mortality in patients that had p53 mutations and HER2 amplification determined by genetic methods has proven that there is a significant risk of recurrences of the disease in patients that have mutations of the p53-gene associated with HER2, positive status compared with the patients that have only one of these alterations (Bull *et al*., 2004). Still, considering the facts mentioned above; testing for any existing p53 mutations in patients with HER2+ breast tumors would be useful (immunohistochemistry) to identify certain sub-group of patients that have more aggressive tumors that could later benefit from a more aggressive treatment.

Ki-67

Ki67 is considered a useful marker of cell proliferation with increase indicating an increase of cell mitotic activity and cell proliferation (Taylor *et al*., 2003). The immunohistochemistry expression of Ki-67 has a good correlation with the growth fraction and does not seem to express itself during the DNA repair process; also it correlates with other measurement of proliferation including the S-phase and bromodeoxyuridine uptake (Tan *et al*., 2005). In breast cancer Ki-67 is used to stratify patients into categories with a favorable and unfavorable prognosis and its correlation with the clinical response to chemotherapy (Archer *et al*., 2003) although the optimal value of the cutoff that makes the distinction between high proliferation and low proliferation activity in a clinically relevant manner when it is IHC determined in breast cancers has not been universally established and thus the independent significance is modest and does not merit measurement in most routine clinical scenarios. However its implication as a pharmacodynamic intermediate marker of the effectiveness of medical therapy holds great promise to rapid evaluation of new drugs. In the present study ki-67 proliferation was more common in breast cancers presenting at ages below 50years than those above 50 years (71.4% vs. 28.6%). The immunostain for ki-67 in estrogen and progesterone receptors positive tumor is more frequent (71.4% vs. 57.1% than in the corresponding negative tumors (28.6% vs. 42.7%). From this study, it therefore implies that estrogen and progesterone receptor positive tumors exhibits higher proliferative ability than their negative counterpart thus the need to incorporate ki-67 evaluations in receptor positive tumors in our study population.

BRCA1 has distinctive morphology and immunohistochemical phenotype and thus combining morphologic and immunohistochemical stain can be used to predict the risk of young patient harboring a germline mutation though BRCA 2 phenotype is currently not fully established. In this study BRCA 1 over express immunostain positive tumors exhibited higher degree of estrogen receptor, progesterone and epidermal growth factor negativity (60%, 70% and 80%) and it is in line with previous studies that stated that breast cancer in patients with BRCA1 germline mutation are more of negative for estrogen , progesterone and Her 2 and more likely to be positive for p53 protein compared with control in contrast to BRCA2 tumors that do not show a significance difference of any of the three proteins compared with control(Sunil *et al* 2002).

Conclusion

Tumors suppressor gene and proliferative markers are more expressed in breast cancer patients below 50 years suggesting poor prognosis and response to treatment than those above 50years. These findings suggest that biomarker analysis in the pathology laboratory is critical for the effective management of women with breast cancer in Bayelsa State.

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