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Original article

Expression of CEA, CA125 and P16^{INK4A} in the Normal Breast, Fibroadenoma and Invasive Adenocarcinomas of the Breast

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ABSTRACT

Aims. *The incidence rate of breast cancer is higher in developed countries and it varies greatly with race and ethnicity. The p16^{INK4A}, a tumor suppressor gene, CA125, a gene involved in the arrest of the cell cycle and CEA, a cellular adhesion molecule are markers that have been expressed in breast cancer cases in previous studies, however this study was aimed at studying the various expression patterns of these markers at different stages of breast cancer starting from the normal breast, fibroadenoma down to the invasive adenocarcinoma of the breast.* ***Sample and method.*** *A total of 65 breast tissue blocks which included 15 normal breast tissue blocks, 25 fibroadenoma tissue blocks, and 25 breast tissue blocks with invasive adenocarcinoma diagnosis was provided by pathological archives of Obafemi Awolowo University Teaching Hospital Complex. These blocks were then used to make immunohistochemical stained sections which were analyzed using a digital camera and a LEICA research microscope after which photomicrographs were taken.* ***Results.*** *In p16, a positivity rate of 13% was shown in normal cases, 84% in fibroadenoma cases with moderate cytoplasm and nuclear staining and 92% in invasive adenocarcinoma cases with various degrees of positivity thereby indicating an overexpression in the invasive adenocarcinoma of the breast. In CA125, the normal cases showed a positivity rate of 60%, the fibroadenoma cases showed a positivity rate of 72% while the invasive adenocarcinoma cases had a positivity rate of 84% indicating a pattern of upregulation from the normal breast cases down to the invasive adenocarcinoma of the breast with cytoplasmic staining. CEA was also found to be overexpressed in the cases of invasive adenocarcinoma of the breast while showing a membranous staining with a positivity rate of 76% and a positivity rate of 60% in fibroadenoma cases while no positive reaction was observed in any of the normal breast cases thereby having a positivity rate of 0%. Although p16, CA125 and CEA appeared to be overexpressed in invasive adenocarcinoma of the breast, p16 was found to be the most reliable of them all.* ***Conclusion.*** *After carrying out this investigation, it was concluded that using these markers (p16, CA125 and CEA) singlehandedly to monitor the progression of breast cancer at the normal breast stage, fibroadenoma and invasive adenocarcinoma of the breast will not produce very reliable and specific results as compared to using all the markers at once. It is thereby advisable to use these three markers at the same time in order to reduce the chances of false positive or false negative results.*

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INTRODUCTION

Breast cancer is a malignant tumor that has developed from the cells of the breast and could result in changes from the normal breast sometimes to fibroadenoma before resulting in invasive malignant cancer [1]. Most breast cancers begin in the cells that line the ducts (ductal cancers). Some begin in the cells that line the lobules (lobular cancers), while a small number start in the other tissues [2]. Although cancer exists anywhere in the world, its incidence rate is higher in developed countries, and the incidence rate of breast cancer varies greatly with race and ethnicity. The incidence rate of breast cancer varies among different parts of the world [3]. The incidence of breast cancer in Nigeria has risen significantly. There has been a steady increase in the incidence of breast cancer in Nigeria from 15.3 per 100,000 in 1976 to 33.6 per 100,000 in 1992 to 52.0 and 64.6 per 100,000 in 2012 in Ibadan and Abuja respectively [4]. The p16 pathway

is a major pathway involved in control of the cell cycle and tumorigenesis. p16, a nuclear protein encoded by the $p16^{INK4a}$ gene, is a regulator of cell-cycle regulation [5]. Carcinoembryonic antigen (CEA) has been identified as one of the most significant and frequently expressed biological markers in breast cancer patients among the different biological markers discovered so far. CA125 is a repeating peptide epitope of the mucin MUC16, which promotes cancer cell proliferation and inhibits anti-cancer immune responses [6].

Although the progression of benign to malignant lesions of the breast may be studied through conventional analysis with hematoxylin and eosin, the best approach is to combine this hematoxylin and eosin analysis with the use of the immunohistochemical markers $p16^{INK4A}$, CA125, and CEA [7]. When hematoxylin and eosin staining reaction alone is carried out, early lesions which contain small globules may be missed unlike when using immunohistochemical markers which will appear visible. Hence, combining these two staining methods is best to avoid false positive or false negative staining results.

Previous studies have shown that p16, CEA and CA125 are associated with breast cancer. This study however shows the various expression patterns of these markers at different stages of breast cancer starting from the normal breast to fibroadenoma of the breast down to invasive adenocarcinoma of the breast. This investigation could really aid in present and future detection, diagnosis, monitoring and treatment of the progression of different stages of breast cancer as the use of these three markers at the same time could provide more sensitive, specific and reliable results.

MATERIALS AND METHODS

Tissue Blocks

Confirmed breast tissue blocks of normal, benign (fibroadenoma), and invasive adenocarcinoma were provided by pathological archives of ObafemiAwolowo University Teaching Hospital Complex. A total of 65 breast tissue blocks which included 15 normal breast tissue blocks, 25 benign breast tissue blocks, and 25 breast tissue blocks with invasive adenocarcinoma diagnosis was taken.

Methodology

Formalin-fixed and paraffin-embedded specimens were used in this study. Serial sections with four (4) micron thickness were cut, and the end sections were stained with H&E. Sections were then deparaffinized in xylene and rehydrated through a series of decreasing ethanol concentrations. The slides were pretreated with hydrogen peroxide (3%) for 10 minutes to remove the endogenous peroxidase, followed by antigen retrieval in the microwave for 15 minutes in 10 mM citrate buffer (pH 6.0). The primary antibodies were applied, followed by washing and incubation with the biotinylated secondary antibody for 30 minutes at room temperature. The slides were counterstained with haematoxylin and dehydrated in alcohol and xylene before mounting. Appropriate positive and negative controls were included with eachIHC run [8].

Photomicrography

All stained sections were analyzed using a digital camera and a LEICA research microscope (LEICA DM750, Switzerland) (LEICA ICC50). At different magnifications, digital photomicrographs of stained sections for histomorphology and immunohistochemistry on the tissue blocks studied were taken and recorded for morphological changes.

Immunostaining Assessment

The tumor's immunohistochemical (IHC) profile was assessed by staining one segment from a representative blockfor p16, CEA and CA125. IHC was thenperformed using the streptoavidin-biotin immunoperoxidase technique on 4 m thick parts from 10 percent formalin-fixed paraffin-embedded specimens (Dako-cytomation). Multiple slides were examined, and IHC staining was used on the ideal portion. The positive and negative controls were both run at the same time. Positive staining was characterized as strong brown nuclear immunoreactivity. The percentage of tumor cells that reacted with the antibody was later used to conduct the immunoquantification. To find areas with the most positive cells, each slide was examined at a magnification of 40 times. The proportion of positive cells to total cells was determined after these areas were examined at 400 magnifications. At least 500 cells were counted, and only the cells that were definitely positive for the desired marker was considered. The percentage of positive cells was graded as follows;

- 0% cells are stained = negative (-), grade 0
- 0.1% are stained = positive (+), grade 1
- 10.1 - 50% are stained = positive (++), grade 2
- 50.1 – 80% are stained = positive (+++), grade 3
- 80.1% - 100% are stained = positive (++++), grade 4 [9].

Data Analysis
Analysis of the data obtained from the study was carried out using Graph pad prism software program.

RESULTS

Table 1 showing the staining intensity of $p16^{INK4A}$ as measured by a semi-quantitative approach increasing from the normal breast to invasive adenocarcinoma. The positivity rate of the normal breast cases was 13% which is very low. While in fibroadenoma, a positivity rate of 84% was shown where nine (9) cases were weakly stained, and twelve (12) cases were moderately stained. In invasive adenocarcinoma there was a very high positivity rate of 92% in which 23 cases showed varying degrees of positivity.

Table 1a: Semi quantitative expression of $p16^{INK4A}$ in normal breast, fibroadenoma and invasive adenocarcinoma of the breast

	Total Cases	-	+	++	+++	Positivity rate
Normal	15	13	2			13
Fibroadenoma	25	4	9	12		84
Invasive adenocarcinoma	25	2	2	9	12	92

Table 1b: Expression of $p16^{INK4A}$ in normal breast, fibroadenoma and invasive adenocarcinoma of the breast

GROUPS	N	NEGATIVE (n %)	POSITIVE (n %)
Normal	15	13(87)	2 (13)
Fibroadenoma	25	4(16)	21(84)
Invasive adenocarcinoma	25	2`(8)	23(92)

Table 2 showing the negativity and positivity rate of the expression of p16 in normal, fibroadenoma and Invasive adenocarcinoma. A decrease in the negativity rate and an increase in the positivity rate from the normal breast to invasive adenocarcinoma is also shown.

Table 2a: Semi quantitative expression of CA125 in normal breast, fibroadenoma and invasive adenocarcinoma of the breast

	Total Cases	-	+	++	+++	Positivity rate
Normal	15	6	9			60
Fibroadenoma	25	7	18			72
Invasive adenocarcinoma	25	4	11	7	3	84

A table showing the staining intensity of CA125 as measured by a semi-quantitative approach increasing from the normal breast to invasive adenocarcinoma. Positivity rates of 60%, 72% and 84% were shown in the normal breast cases, fibroadenoma and invasive adenocarcinoma respectively.

Table 2b: Expression of CA125 in normal breast, fibroadenoma and invasive adenocarcinoma of the breast

Groups	N	Negative (N %)	Positive (N %)
Normal	15	6(40)	9(60)
Fibroadenoma	25	7(28)	18(72)
Invasive adenocarcinoma	25	4(16)	21(84)

A table showing the negativity and positivity percentile rates of the expression of CA125 in normal, fibroadenoma and Invasive adenocarcinoma. The negativity rate is shown to be gradually decreased from the normal breast to invasive adenocarcinoma while the there is an increase in the positivity rate when expressing the progression of breast cancer.

Table 3a: Semi quantitative expression of CEA in normal breast, fibroadenoma and invasive adenocarcinoma of the breast

	Total Cases	-	+	++	+++	Positivity rate
Normal	15	15				0
Fibroadenoma	25	10	15			60
Invasive adenocarcinoma	25	6	16	3		76

The staining strength of the expression pattern of CEA in normal, fibroadenoma and invasive adenocarcinoma is evaluated in this table. All normal breast cases showed no positivity rate. In fibroadenoma, a positivity rate of 60% was shown where a weak staining was expressed, and there was a positivity rate of 76% in invasive adenocarcinoma.

Table 3b: Expression of CEA in normal breast, fibroadenoma and invasive adenocarcinoma of the breast

Groups	N	Negative (N %)	Positive (N %)
Normal	15	15(100)	0(0)
Fibroadenoma	25	10(40)	15(60)
Invasive adenocarcinoma	25	6(24)	19(76)

The positivity and negativity rates of the expression pattern of CEA in normal, fibroadenoma and invasive adenocarcinoma is evaluated in this table. There is a gradual decrease in the negativity rate from the normal breast down to invasive adenocarcinoma while that of the positivity rate shows a gradual increase.

Table 4: Mean Percentage Reactivity of $P16^{INK4A}$, CA125 andCEA in normal breast, fibroadenoma and invasive adenocarcinoma of the breast

GROUPS	p16	CA 125	CEA
Normal	8	6	3
Fibroadenoma (Benign)	68	55	30
Invasive adenocarcinoma (Malignant)	96	70	50

This table shows the mean percentage reactivity of $p16^{INK4A}$, CA125 and CEA in normal, fibroadenoma and invasive adenocarcinoma of the breast. $p16^{INK4A}$is shown to be the one with the highest mean percentage reactivity in normal, fibroadenoma and even invasive adenocarcinoma of the breast thereby making it the most reliable.

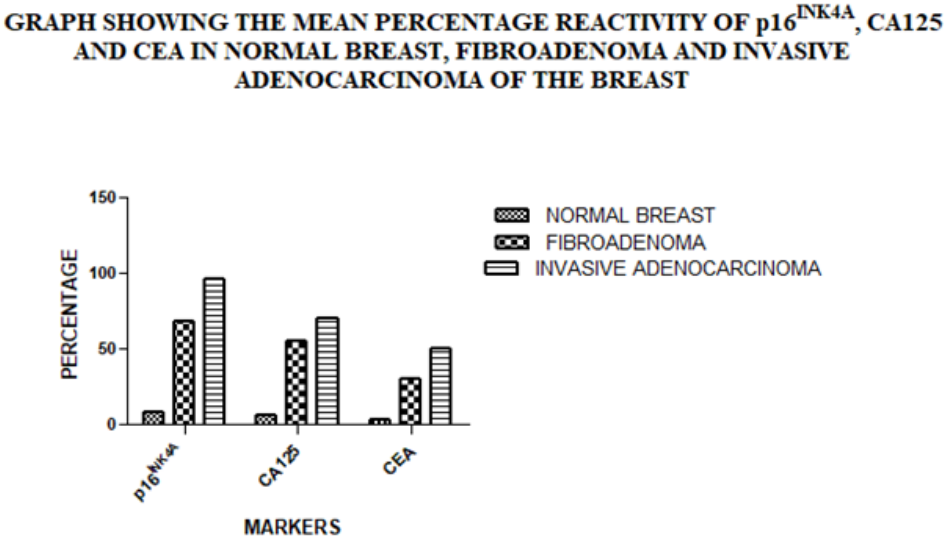

Figure 1. Comparative expression of IHC marker studied in Normal, Fibroadenoma and Invasive ductal Adenocarcinoma of the breast tissue

The above graph shows the percentage reactivity of the immunohistochemical markers p16, CA125 and CEA in normal, fibroadenoma and invasive adenocarcinoma of the breast. This graph also reveals that $p16^{INK4A}$ is the most reliable marker that can be used to study the progression of breast cancer as it has the highest percentage reactivity in normal breast, fibroadenoma and invasive adenocarcinoma of the breast compared to CA125 and CEA.

Micrographs Showing the Hematoxylin and Eosin Staining Reaction in Normal Breast, Fibroadenoma and Invasive Adenocarcinoma of The Breast.

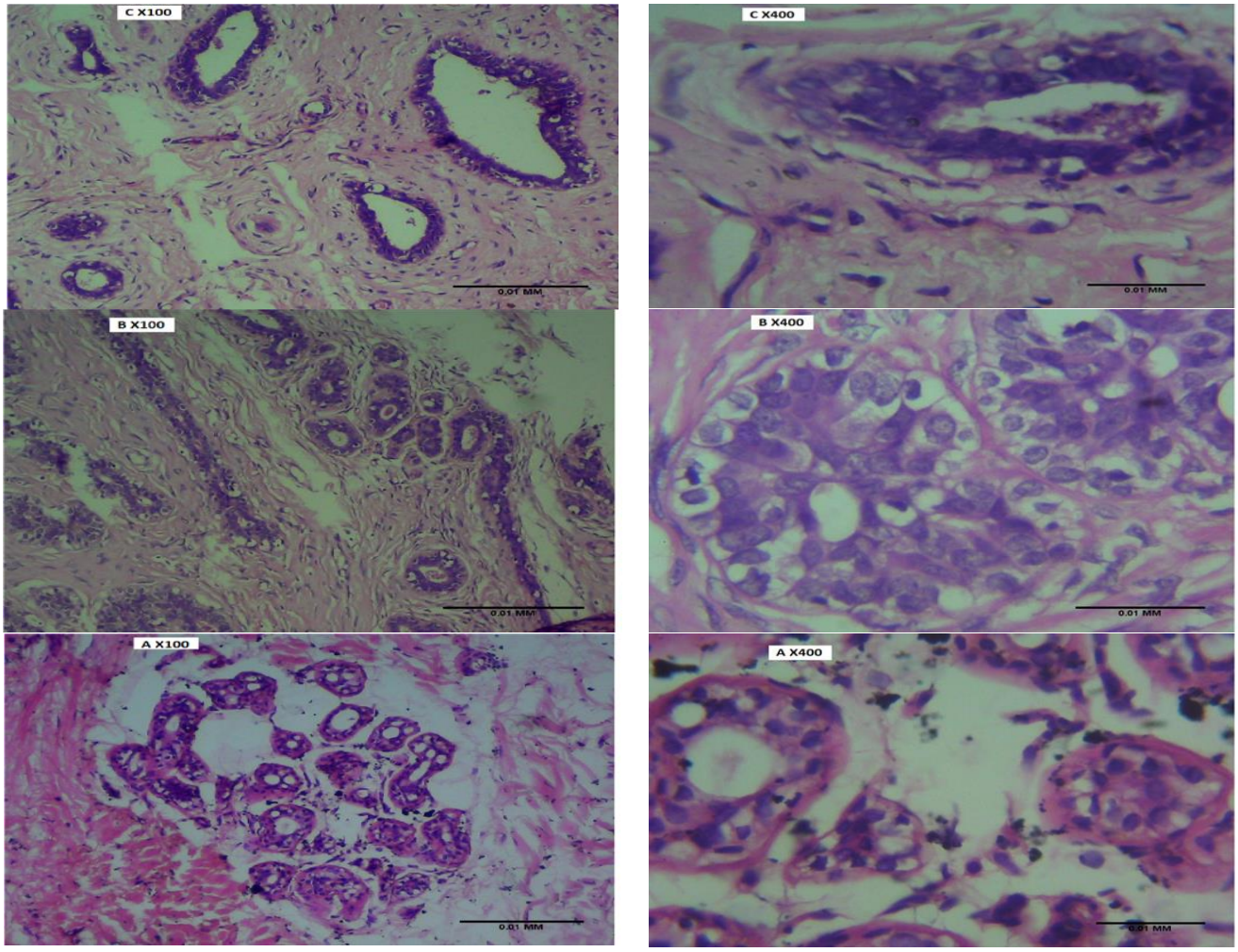

Figure H&E-stained micrograph of normal breast labelled C (x 100 and x400) showing the purple shaped nucleus and pink cytoplasm with well-defined features, in fibroadenomalabelled B (x100 and x400), the ducts were partially obliterated while in invasive adenocarcinoma labelled A (X100 and x400), the lumen appears really large and the ducts do not appear well defined.

Micrographs showing the immunohistochemical reaction of $P16^{INK4A}$in normal, fibroadenoma and invasive adenocarcinoma of the breast

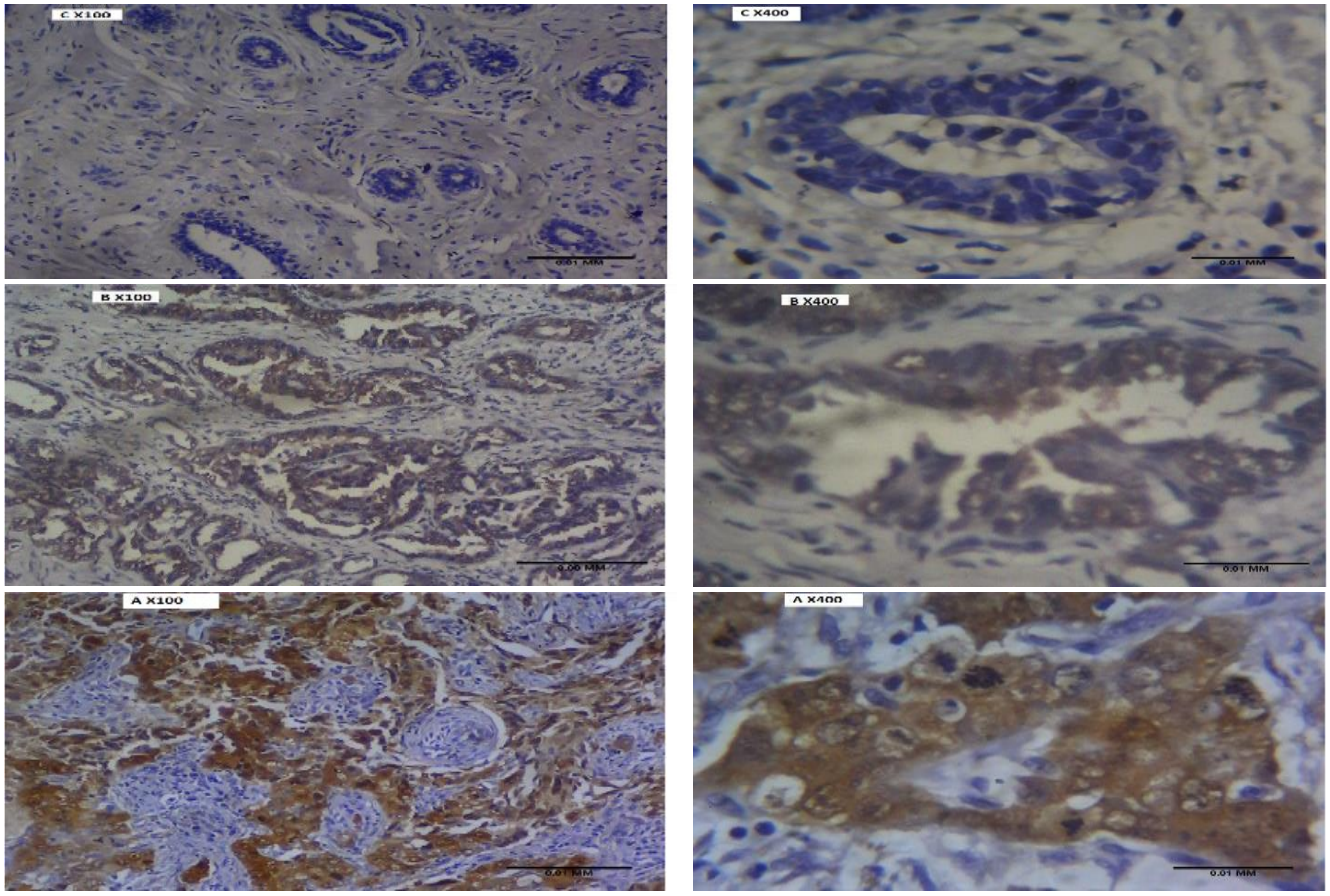

Figure 2: Micrographs showing immunohistochemistry-stained nuclear and cytoplasmic sections with $p16^{INK4A}$ of the normal breast labelled C (x 100 and x400) where no staining reaction was observed, fibroadenomalabelled B (x100 and x400) showing a moderate staining intensity and invasive adenocarcinoma labelled A (X100 and x400) which shows a very strong staining reaction and mild occlusion of the ducts.

Micrographs showing the immunohistochemical reaction of ca125 in normal, fibroadenoma and invasive adenocarcinoma of the breast

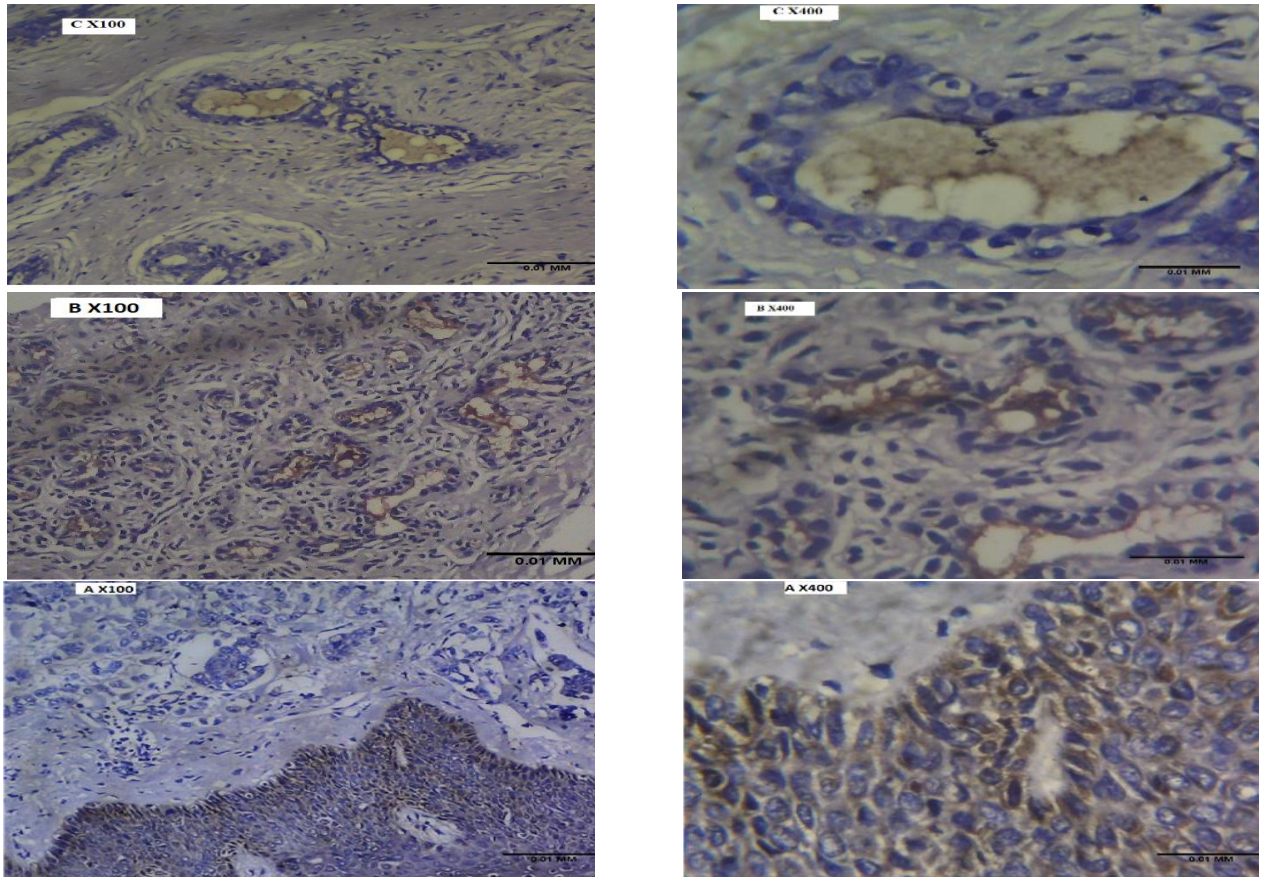

Figure 3: Micrographs showing immunohistochemistry-stained cytoplasmic sections with CA125 of the normal breast labelled C (x 100 and x400) with a mild staining intensity, fibroadenomalabelled B (x100 and x400) showing a moderate staining reaction, and invasive adenocarcinoma labelled A (X100 and x400) showing a quite severe staining reaction around the epithelial lining.

Micrographs showing the immunohistochemical reaction of CEA in normal, fibroadenoma and invasive adenocarcinoma of the breast

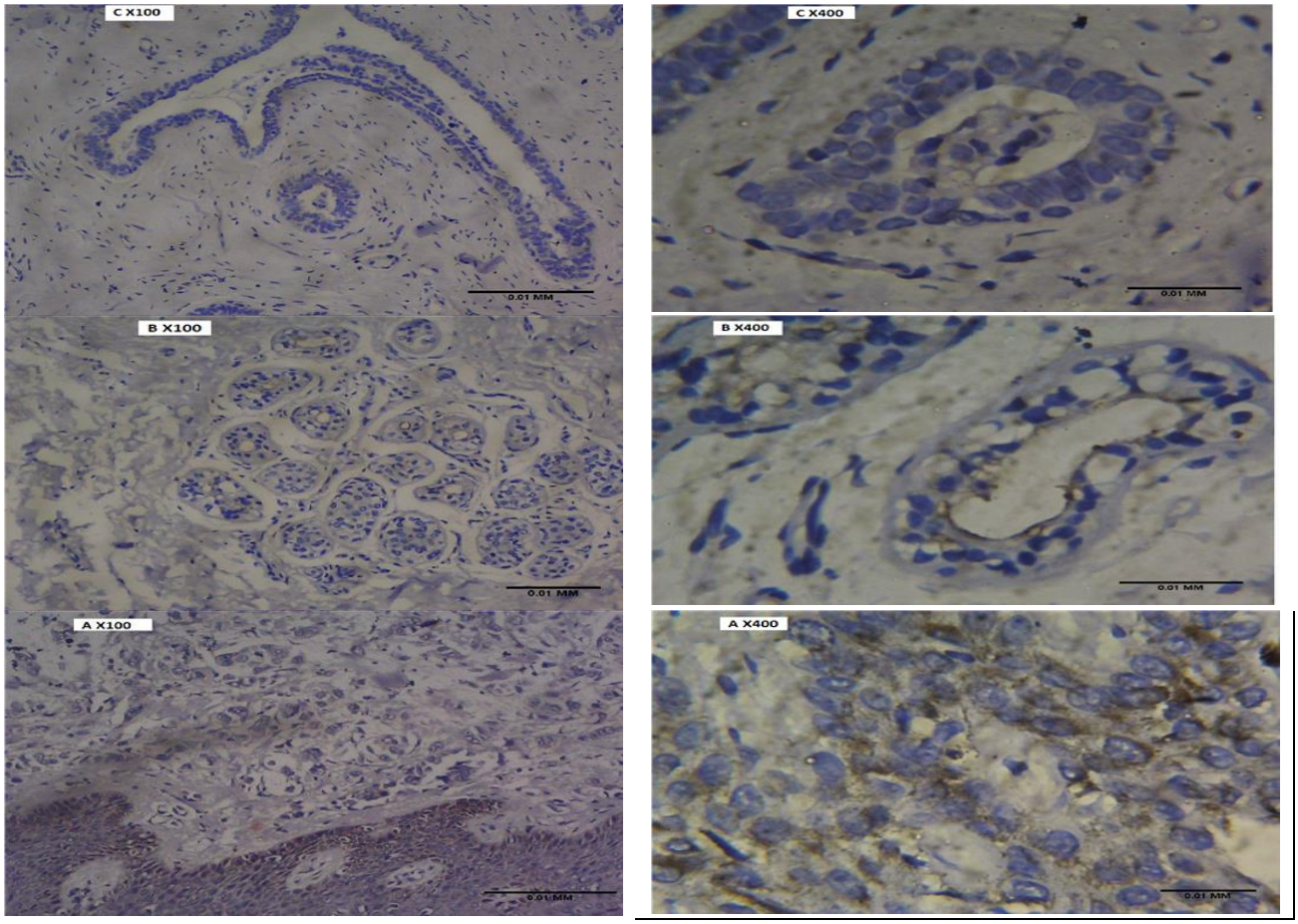

FIGURE 4: Membranous immunohistochemistry-stained sections of the normal breast labelled C (x 100 and x400) with no staining reaction at all, fibroadenomalabelled B (x100 and x400) showing a very mild staining intensity, and invasive adenocarcinoma labelled A (X100 and x400) with moderate staining.

DISCUSSION

Previous studies have shown that breast cancer is the most common cancer in women worldwide [10] and immunochemistry is known to be a major tool that has been useful in detecting and monitoring the progression of breast cancer. Hence this research was carried out and aimed at using certain immunohistochemical markers such as $p16^{INK4A,}$ Cancer Antigen 125 (CA125) and Carcinoembryonic Antigen (CEA) to determine the tissue distribution of the given markers in the normal breast, fibroadenoma and invasive adenocarcinoma of the breast. It was also carried out in order to be able to investigate the reliability of the used immunohistochemical markers and determine which of the markers is more reliable in studying and monitoring the progression of the normal breast to fibroadenoma and then invasive adenocarcinoma of the breast.

In this study, formalin-fixed and paraffin-embedded specimens were used to make immunohistochemical stained sections before being analyzed using a digital camera and a LEICA research microscope (LEICA DM750, Switzerland) (LEICA ICC50) after which photomicrographs were taken. In $p16^{INK4A}$, negative or low expression was seen in normal ductal epithelium, together with a progressive increase in benign lesions and carcinoma which aligns with the findings of [11]. He also researched and showed that in addition to p16's ability to slow down the cell cycle, this protein has also been implicated in other processes, such as apoptosis, cell invasion and angiogenesis, and these activities may be related to its

overexpression in cancer. In CA125 which is encoded by the MUC16 mucin gene, results showed that MUC16 was overexpressed in invasive adenocarcinoma whereas not expressed in non-neoplastic ducts as was also included in the findings of [12]. Studies have also shown that the stable knockdown of MUC16 in breast cancer cells (MDA MB 231 and HBL100) is known to have resulted in significant decrease in the rate of cell growth, tumorigenicity and increased apoptosis. His previous studies also revealed that the functional role(s) of MUC16 in breast cancer progression are not well understood. In the present study, we demonstrated that MUC16 expression is upregulated in breast cancer tissues and correlates with the stage of the disease. According to [13]. several members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) subfamily are involved in tumor progression and CEACAM19 was found to be overexpressed in breast cancer tissue specimens compared to normal tissue counterparts [14]. also stated in his findings that some researchers reported that deregulated overexpression of CEA markers is able to block cellular differentiation in large number of cell types; and CEA was found to be overexpressed in breast cancer tissue specimens in high grade tumors. These findings also correlated with the expression pattern of CEA gotten in this research.

The carrying out of immunohistochemical reactions to show the expression pattern of the immunohistochemical markers p16, CA125 and CEA in the normal breast, fibroadenoma and invasive adenocarcinoma of the breast was done successfully. From the entire hypothesis proposed in this research, the results proved that $p16^{INK4A}$ can be used as a marker to study the progression of normal to invasive adenocarcinoma of the breast. CA125 and CEA could also be used to study the progression of normal to invasive adenocarcinoma of the breast; however, they need to be used in conjunction with other markers before they can produce sensitive, specific and reliable results.

One of the limitations that should be acknowledged is the possibility of differences in some confounding factors, such as age, menopause status, body mass index (BMI), lifestyle and environment which may interfere with tumor marker levels. Another limitation is that as a heterogeneous disease, breast cancer may require combining multiple biomarkers to allow the detection of the different subtypes especially when CA125 and CEA are involved as they are not very reliable when studying breast cancer progression.

In this present study, it was expected of p16, CA125 and CEA to all be overexpressed in invasive adenocarcinoma. However, p16 aligned really well with the expectations as it produced a very strong overexpression in invasive adenocarcinoma, while the overexpression of CA125 and CEA in invasive adenocarcinoma was weak. This could be due to the fact that not every person with a particular type of cancer will have an elevated level of the corresponding tumor marker or the fact that many tumor markers may also be elevated in persons with noncancerous conditions or because of the biological variability in an individual patient's sample, as well as the huge range of biomarker concentrations in all patients compared [15]. Therefore, measurements of circulating tumor markers are usually combined with the results of other tests, such as biopsies or imaging, to diagnose cancer.

CONCLUSION

The present study reveals that even though all the immunohistochemical markers p16, CA125 and CEA can be used in the follow-up and study of the progression of breast cancer, the most reliable, sensitive and specific is p16. Hence, the findings gotten from this study agrees and support the use of the immunohistochemical marker p16 and the use of the immunohistochemical markers CA125 and CEA with other markers in monitoring the progression of breast cancer.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

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