Prognostic Significance of p16 Protein Expression in Breast Cancer

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Abstract. *Background/Aim: Breast cancer is the most common cancer in Sudan. The p16 protein plays a vital role in the regulation of the cell cycle. Patients and Methods: This study analysed the protein expression of p16 in 202 paraffin blocks from Sudanese women with breast cancer using immunohistochemistry. Results: This study included 168 (83.2%), 16 (7.9%), and 18 (8.9%) patients with invasive ductal carcinoma, invasive lobular carcinoma, and papillary carcinoma, respectively. There were 95 cases (47.0%) with grade III, 70 cases (34.6%) with grade II, and 23 cases (11.4%) with grade I breast cancer. The hormone receptor status was available for 119 of the cases, and 31 (15.3%), 25 (12.4%), and 63 (31.2%) cases were positive for oestrogen, progesterone, and HER2 receptors, respectively. Conclusion: p16 protein expression was associated with high histologic grade, lymph node metastasis, and poor prognosis. p16 protein expression may potentially be used as a prognostic marker.*

Breast cancer is one of the most common causes of cancer-associated death among women worldwide, and most of the death rates are rising in underdeveloped countries (1, 2). Breast cancer is the most common cancer in Sudan with an incidence rate of 25.1 per 100,000 people (3). For patients identified with breast cancer and distant metastasis, management is usually aimed at improving survival (4). Alterations in breast cancer susceptibility genes *BRCA1* and *BRCA2* (5), sex-steroid hormones, and way-of-life origins

have been strongly associated with the development of breast cancer, but the mechanisms of breast carcinogenesis are still not clearly understood.

Deregulation of the cell cycle is an important feature of cancer. One of the proteins involved in the cell cycle is p16 (6). p16 oncoprotein is consistently expressed in cancer and can inactivate the retinoblastoma (Rb) tumour suppressor (7). p16 acts as a tumour suppressor, limiting cell proliferation. Changes in p16 can lead to down-regulation of genes, which may increase the incidence of epithelial cell cancer (8, 9).

Previous studies have shown that the expression of p16 correlates with clinicopathological factors in breast cancer (10). Some report a weak association between immunohistochemical expression of p16 and invasive ductal breast cancer, but another study reported that it is the main mechanism of cell-cycle deregulation in invasive breast cancer (11). The aim of this study was to analyse p16 protein expression among Sudanese women with breast cancer using immunohistochemistry.

Patients and Methods

Breast cancer patient population and sample collection. Paraffin wax blocks were selected from patients that were previously diagnosed with breast neoplasia at the Radiation and Isotopes Centre Khartoum (RICK) in Khartoum State, Republic of Sudan, from May 2017 to June 2018. Clinicopathological data of the patients were collected from the records of RICK. A total of 202 breast cancer patients (n=202), aged 18-85 years, were recruited for analysis of p16 protein expression. The study received ethical approval from the ethical committees of the Faculty of Laboratory Sciences, Al Neelain University.

Immunohistochemical reaction. From each tissue block, two 4- μ m-thick sections were cut and mounted on two microscope slides: one coated with amino-propyl triethoxysilane for immunohistochemistry analysis and the other coated in routinely used adhesive media. All sections were incubated in an oven at 60°C for one hour, and then sections were de-waxed with xylene and hydrated through descending alcohol (100%, 90%, 70%, and 50% ethanol and distilled water). To confirm the diagnosis, one section from each

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case was stained with haematoxylin and eosin as described by Fischer *et al.* (12).

For immunohistochemical staining of p16, slides were incubated overnight at 4°C with p16 primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1:400. After primary antibody exposure, the slides were washed and treated with biotinylated antibody for 30 min. Antigen visualization was achieved using ENVISION FLEX (DAKO, Carpinteria, CA, USA) for 30 min, followed by diaminobenzidine chromogen (DAKO Liquid DAB+, K3468). Slides were counter-stained with haematoxylin. Finally, sections were dehydrated in alcohol, cleared in xylene, and mounted in Distyrene, a Plasticizer, and Xylene (D.P.X.). All sections were stained in the same batch to eliminate inter-batch variation.

A negative control was prepared from the same tissue block but incubated in phosphate buffer solution instead of the primary antibody. The positive control section for p16 immunohistochemistry was cervical squamous cell carcinoma, which has high p16 expression. The analysis was performed according to the novel recommendations of the College of American Pathologists (CAP) for Human papilloma virus analysis in head and neck carcinomas used in routine clinical practice (13). The p16 immunohistochemistry results were interpreted as follows: high p16 expression: tumours with ≥70% nuclear and cytoplasmic staining; moderate p16 expression: tumours with 30-70% nuclear and cytoplasmic staining; low p16 expression: tumours with 10-30% nuclear and cytoplasmic staining, which is considered as abnormal positive p16 expression; and p16 negative: tumours with 1-10% nuclear and cytoplasmic staining, considered as normal negative p16 expression.

Statistical analyses. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) version 21 (SPSS Inc. IBM, IL USA, 2012). Qualitative variables such as positive p16 expression and clinicopathological features of the patients were expressed as frequencies and percentages. The correlation between p16 expression of breast carcinomas and clinicopathological features was analysed using a chi-squared test (χ^2). A *p*-value <0.05 was considered significant.

Results

Study group. This study included 202 female breast-cancer cases, including 168 (83.2%), 16 (7.9%), and 18 (8.9%) patients with invasive ductal carcinoma, invasive lobular carcinoma, and papillary carcinoma, respectively. There were 95 cases (47.0%) with grade III breast cancer, 70 cases (34.6%) with grade II, and 23 cases (11.4%) with grade I. Axillary lymph node metastasis was presented in 57 (28.2%) cases. The hormone receptor status was available for 119 of the cases, and 31 (15.3%), 25 (12.4%), and 63 (31.2%) patients were positive for oestrogen, progesterone, and HER2 receptors, respectively. Of the 202 samples, 63 (31.2%) were positive for p16. The mean age and standard deviation (SD) of the patients was 48.14±14.4 years with a range of 18 to 85 years. The clinicopathological characteristics of the study group are summarized in Table I.

Table I. Clinical, pathological and geographical features of breast cancer patients.

Characteristics	BC (n=202)
Age (mean±SD)	48.14 (±14.4)
≤45 n (%) years	101 (50.0)
≥46 n (%) years	101 (50.0)
Histologic type, n (%)	
Invasive ductal carcinoma	168 (83.2)
Invasive lobular carcinoma	16 (7.9)
Papillary carcinoma	18 (8.9)
p16 immunohistochemistry n (%)	
p16 positive N (%)	63 (31.2)
Histological grade n (%)	
0	10 (4.9)
I	23 (11.4)
II	70 (34.6)
III	95 (47.0)
Missing	4(2.1)
Lymph node metastasis (+), n (%)	57 (28.2)
Hormone receptors	
Oestrogen receptor (+), n (%)	96 (47.5)
Progesterone receptor (+), n (%)	71 (35.1)
Her2 status (+), n (%)	41 (20.3)

p16 immunohistochemistry. In regard to p16 immunohistochemistry results, negative expression was found in 139 (68.8%) cases, while 63 (31.2%) cases showed moderate and marked nuclear immune reactivity (Figure 1). p16 expression was statistically associated with worse clinicopathological characteristics, such as high histologic grade ($p=0.011$) and lymph node metastasis ($p=0.003$). No association was found between p16 expression and breast cancer types, different hormone receptors, and patient age groups ($p=0.436$, 0.375, 0.185, 0.323 and 0.693, respectively; Table II and Table III).

Discussion

There is limited information on the efficacy and prognostic significance of p16 in breast cancer. The significance of p16 expression has been recognized in a variety of human cancers but has not been well studied in breast cancer. To the best of our knowledge, this is the first study to offer extensive clinical data on p16 expression in breast cancer among Sudanese women.

p16 is a cell cycle regulator that blocks the action of cyclin-dependent kinases, which inhibit the retinoblastoma gene (*RB*). The *RB* gene guides the progression of the cell cycle from the G1 phase to the S phase. Changes in the p16 gene and its expression have been investigated in many cancers (14).

The assessment of p16 expression in breast cancer in this study showed a negative reaction in 139 (68.8) cases, while 63 (31.2%) cases showed moderate and marked nuclear immunoreactivity. Previous immunohistochemistry studies

Figure 1. Microscopical images showing p16 immunohistochemical expression. (A) Hematoxylin and eosin-stained section of invasive ductal breast cancer. (B) Immunohistochemical staining for high p16 expression. (C) Positive control of immunohistochemical staining for p16. (D) Negative control of immunohistochemical staining for p16.

Table II. Immunohistochemical expression of p16, cancer types, lymph node metastasis and hormone receptors.

	p16+	p16-	p-Value
Invasive ductal carcinoma N (%)	53 (31.5)	115 (68.5)	0.436
Invasive lobular carcinoma N (%)	3 (18.8)	13 (81.2)	
Papillary carcinoma N (%)	7 (38.9)	11 (61.1)	
LN+ N (%)	26 (41.2)	31(22.3)	0.003
LN- N (%)	37 (58.8)	108 (77.7)	
ER+ N (%)	31(49.2%)	65 (46.8%)	0.375
ER- N (%)	32 (50.8%)	74 (53.2%)	
PR+ N (%)	25 (39.7%)	46 (33.0%)	0.185
PR- N (%)	38 (27.3%)	93(67.0%)	
HER2+ N (%)	14 (22.2%)	27 (19.5%)	0.323
HER2- N (%)	49 (77.8%)	112 (80.5%)	

LN: Lymph node; N: number; ER: oestrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

on breast cancers reported incidences of p16 positivity of 21 to 51% (15, 16), which is comparable to our result. Slight differences between different immunohistochemistry studies may exist due to usage of different antibodies, laboratory procedures, and grading standards.

The present study found that p16 expression correlated with tumour grade and lymph node metastasis ($p=0.011$, $p=0.003$), but not with other demographic and clinical features. This finding is in agreement with previous studies, which found associations between strong p16 expression and various features of breast cancer, such as high grade and lymph node metastasis (17-22).

Table III. Immunohistochemical expression of p16, tumour grades, and age groups.

	p16+	p16-	p-Value
Tumour grades			
0	1 (10.0)	9 (90.0)	0.011
I	7 (41.2)	16 (58.8)	
II	12 (17.1)	58 (82.9)	
III	42 (44.2)	53 (55.8)	
Age groups years			
18-25	3 (4.8)	7 (5.0)	0.693
26-35	9 (14.3)	24 (17.3)	
36-45	23 (36.5)	35 (25.2)	
46-55	12 (19.0)	27 (19.4)	
56-65	9 (14.3)	27 (19.4)	
≥66	7 (11.1)	19 (13.7)	

In this study, p16 was not significantly expressed in patients under the age of 55 years with a peak at 36-45 years. This result is in agreement with that of Harbhajanka *et al.* (23). Our findings are also consistent with the Carolina breast cancer study, where Carey *et al.* (24) reported that pre-menopausal African-American women are most likely to present with breast cancer compared to pre-menopausal women.

p16 expression has been shown to increase with age in numerous types of cancers (25-27), but the correlation between p16 expression and age in breast neoplasia has not been well described. Nevertheless, cells in which p16 expression is sustained for prolonged periods of time are known to express proteins that encourage tumour growth (28, 29).

Of the cases examined, 47.5% were ER-positive, 35.1% were PR-positive, and 20.3% were HER2-positive. Furthermore, 52.5% of cases were ER-negative, 64.8% were PR-negative, and 79.7% were HER2-negative. The incidences of ER, PR, and HER2-positive tumours in the Western population are 70%, 54%, and 18.3%, respectively (30). Awadelkarim *et al.* reported much higher incidence of ER and PR positivity and a lower incidence of HER2 positivity (64.0%, 67.0%, and 17.5%, respectively) in Sudanese patients (31). Furthermore, Sengal *et al.* reported results that are similar to our results (45% ER, 38% PR, and 45.9% HER2) (32).

The difference in the number of positive cases in Sudan may be due to erroneous staining and the use of diverse antibodies with different specificity and sensitivity. The duration and methods of fixation also differ from one laboratory to another. Furthermore, there may also be genetic differences. Hormone receptors play a significant role in adaptable growth and variation of ordinary breast tissue. They are recognized as prognostic factors, with a positive receptor grade associated with a minor rate of cell proliferation and well differentiated tumours (33).

Limitations

A limitation of this study was that molecular testing of p16 was not achieved. Consequently, we recommend molecular testing of p16 for future studies to identify mutations and their association with over-expression of this marker.

Conclusion

In this study, high p16 protein expression was associated with high histologic grade, lymph node metastasis, and poor prognosis. p16 protein expression may be used as a prognostic marker. The reverse relationship between hormone receptors and robust p16 expression might suggest that durable p16 expression is connected to a more aggressive cancer. Nevertheless, larger studies will be essential to validate this finding.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in relation to this study.

Authors' Contributions

AH and MS conducted the study designed and laboratory work. EE and MS drafted and revised the manuscript. EE conducted the statistical analyses; all Authors read and approved the final manuscript.

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