Original paper

Expression of p16 protein in breast cancer

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Breast cancer is the most common cancer and a leading cause of death in women in Saudi Arabia. P16 is a tumour suppressor gene that plays a crucial role in regulating cell cycle. Several studies have investigated the significance of p16 expression in various cancer types. However, the significance of p16 in breast cancer remains controversial and insufficiently studied.

The present study aims to examine the association between p16 expression and clinicopathological factors in breast cancer using immunohistochemistry staining.

The study utilised 475 prospectively collected tissue samples from 475 women with breast cancer in Saudi Arabia.

Nuclear and cytoplasmic immunohistochemical staining of p16 was observed in 338 (71%) of the cases and showed significant direct associations with adverse tumour features, including high tumour grade ($p < 0.0001$), negative oestrogen receptor/progesterone receptor status ($p < 0.001$), and lymph node metastasis ($p = 0.02$).

Our study revealed a significant association between p16 protein expression and the established negative prognostic parameters in breast carcinoma including tumour grade, lymph node metastasis, and oestrogen receptor and progesterone receptor status.

Key words: p16, breast carcinoma, immunohistochemistry, prognosis.

Introduction

Breast cancer is the most prevalent cancer and a leading cause of death in Saudi Arabia and worldwide [1, 2]. Despite significant efforts in breast cancer research, there remains a need to identify additional markers that can enhance our understanding of the characteristics and progression of different types of breast carcinoma.

P16 is a crucial regulatory protein in the cell cycle. It acts as a tumour suppressor gene located on chromosome *9p21* and functions as a cyclin-dependent kinase inhibitor. Normally, p16 inhibits the phosphorylation of the retinoblastoma gene (*RB*), and therefore the phosphorylation status of *RB* serves as an indicator of p16 expression [3]. The significance of p16 protein expression is well-established in various types of human cancers. However, its role in breast cancer remains controversial [4]. In this study, we aim to investigate the role of p16 in breast carcinoma, considering various prognostic parameters such as tumour stage, tumour grade, nodal metastasis, and lymphovascular invasion. To the best of our knowledge, this is the first study conducted in Saudi Arabia to evaluate the expression of p16 through immunohistochemistry (IHC) in breast carcinoma.

Material and methods

This study utilised 475 prospectively collected tissue samples from women who underwent breast cancer treatment at King Abdulaziz University Hospital (KAUH) in Jeddah, Saudi Arabia, from January 1996 to December 2012. The samples were archived

at the pathology department of KAUH, and the study protocol received approval from KAUH's institutional review board.

Ethical approval and consent to participate

This retrospective study has received approval from the Research Committee of the Biomedical Ethics Unit at our institution (Reference No. 77-21) without the requirement of consent. The study adheres to the principles outlined in the Declaration of Helsinki.

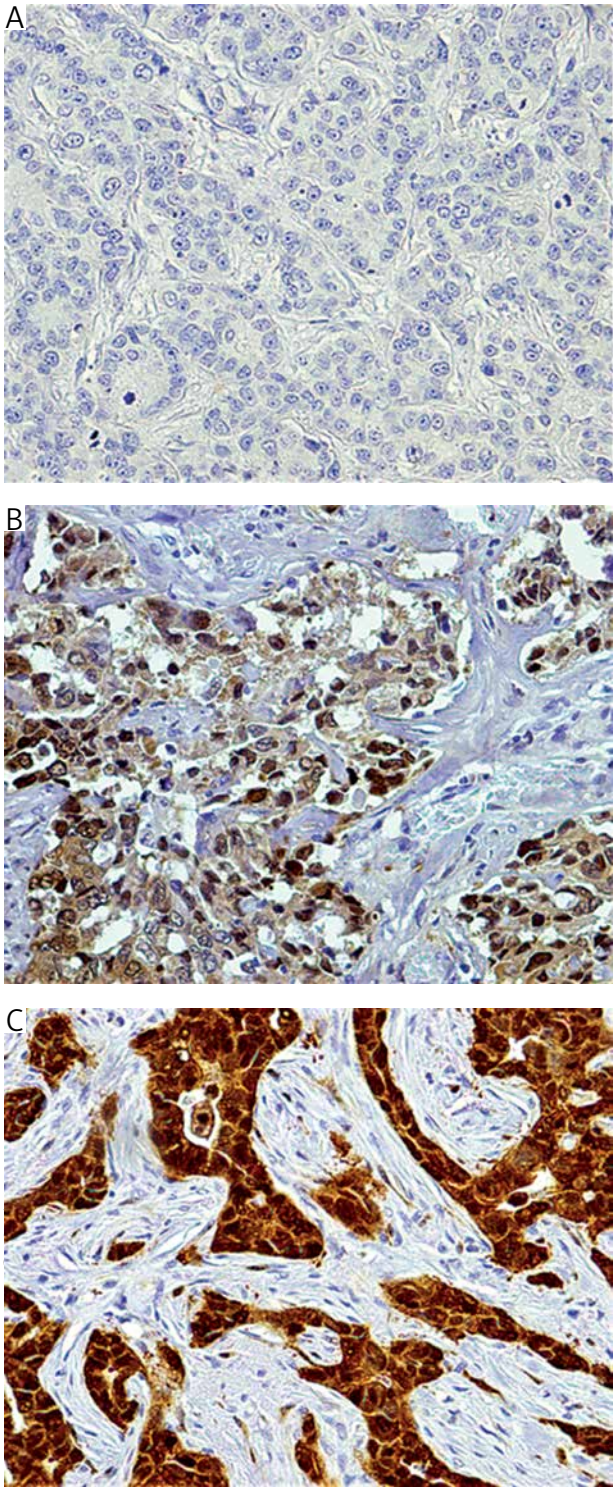

Fig. 1. Cytoplasmic and nuclear expression of p16 in breast cancer. A) Negative stain (20×). B) Low positive stain (20×). C) High positive stain (20×)

Tissue microarray production

A tissue microarray was constructed from 475 breast carcinoma samples. Two cores, 1.5 mm in diameter were selected from each sample and assembled in paraffin blocks using an automated tissue microarray (TMA) instrument (TMA Master 1.14 SP3 from Histech Ltd., Budapest, Hungary). The tissue microarray blocks were sliced and then stained by p16 immunohistochemical staining.

Immunohistochemistry staining protocol

The recipient blocks were constructed using a special protocol. The blocks were then cut at 4 mm thickness and mounted on special slides with a positive charge (Leica Microsystem plus slides). Deparaffinisation of the sections was carried out using xylene, followed by automated rehydration using an immunostainer (BenchMark XT, Ventana Medical Systems Inc., Tucson, AZ, USA). A pretreatment step of 60 min was performed using a Prediluted CC1 cell conditioning solution. Anti-human rabbit anti-p16 polyclonal antibody (Spring™ Bioscience; Cat # E3284) was incubated for 20 minutes at 37°C, following the manufacturer's instructions from the Ventana I-view DAB detection kit. Subsequently, the slides were washed, counterstained with Mayer's haematoxylin, and then mounted. Positive control was established using placenta tissue.

The cases were meticulously examined and reviewed by 2 pathologists using a semiquantitative method. Positive cases were identified when tumour cells exhibited nuclear and/or cytoplasmic staining in more than 5% of the examined area. The scoring for p16 immunopositivity was categorised as follows: a score of 0 was assigned for negative staining, while a score of 1 indicated low immunopositive staining (+). High immunopositive staining was classified as scores 2 (++) and 3 (+++) (Fig. 1).

Study variables

Trained pathologists at KAUH utilised standardised protocols to determine tumour size, grade, invasion, lymph node metastasis, histological type, and the statuses of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Distant metastases were identified through clinical and pathological correlation.

Statistical analysis

The descriptive statistics for continuous variables are presented as means (SD), while frequencies

Table I. Characteristics of breast cancer patients overall and by p16 immunostaining intensity

Parameters*	Overall sample	Intensity of p16 staining			
		No staining, 137 (29)	Low, 139 (29)	High, 199 (42)	*p*-value
Age (years), mean ±SD	50.8 ±13.3	54.7 ±14.2	50.9 ±12.7	48.1 ±12.3	< 0.0001
Tumour size [cm]					0.85
< 3	155 (37.7)	47 (39.5)	47 (39.5)	61 (35.3)	
3–6	196 (47.7)	55 (46.2)	53 (44.5)	88 (50.9)	
> 6	60 (14.6)	17 (14.3)	19 (16.0)	24 (13.9)	
Tumour grade					< 0.0001
Grade 1	70 (17.1)	23 (19.2)	22 (18.3)	25 (14.7)	
Grade 2	207 (50.5)	67 (55.8)	73 (60.8)	67 (39.4)	
Grade 3	133 (32.4)	30 (25.0)	25 (20.8)	78 (45.9)	
Invasive tumor	427 (97.0)	125 (98.4)	127 (96.9)	175 (96.2)	0.51
Vascular invasion	141 (42.1)	35 (38.9)	44 (45.4)	62 (41.9)	0.67
Number of LN with metastasis					0.02
No LN metastasis	152 (37.3)	34 (27.9)	44 (37.6)	74 (44.0)	
1–3	113 (27.8)	43 (35.2)	36 (30.8)	34 (20.2)	
> 3	142 (34.9)	45 (36.9)	37 (31.6)	60 (35.7)	
Distant metastasis	68 (14.3)	20 (14.6)	19 (13.7)	29 (14.6)	0.97
Histological type					0.97
Ductal	427 (90.3)	123 (89.8)	125 (90.6)	179 (90.4)	
Others	46 (9.7)	14 (10.2)	13 (9.4)	19 (9.6)	
ER positive	257 (67.5)	84 (75.0)	88 (77.2)	85 (54.8)	< 0.0001
PR positive	233 (61.0)	73 (65.2)	82 (71.9)	78 (50.0)	0.0007
HER2 positive	134 (37.1)	37 (34.3)	41 (39.0)	56 (37.8)	0.75
Died of disease	47 (9.9)	17 (12.4)	12 (8.6)	18 (9.0)	0.50
Recurrence	64 (25.6)	23 (32.9)	16 (21.6)	25 (23.6)	0.25

ER – oestrogen receptors, HER2 – human epidermal growth factor receptor 2, LN – lymph node, PR – progesterone receptors
** Frequencies with percentages are reported unless otherwise specified*

with percentages are used for categorical variables. The study sample was divided into 3 categories based on the intensity of p16 staining: no staining, low staining, and high staining. To compare descriptive statistics among the levels of p16 staining, analysis of variance was used for normally distributed variables, and the χ^2 test was employed for frequency distributions of categorical variables. Ordinal logistic regression was applied to assess the direction and magnitude of associations between p16 staining (no, low, and high) and age (10-year age interval), tumour grade, and ER, PR, and HER2 statuses. The score test was used to examine the proportional odds assumption. In cases where the score test was significant, multinomial logistic regression was used, with "no staining" as the reference level. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC), with a significance level of 0.05.

Results

The mean ±SD age of the study participants at the time of diagnosis was 50.8 ±13.3 years. Out of the 475 breast cancer tissue samples, p16 staining was found to be high in 199 (42%) of the cases, low in 139 (29%), and not detected in 137 (29%) of the cases.

Table I presents the clinicopathological characteristics of the patients. There was a significant difference in tumour grade among the various p16 staining intensities ($p < 0.0001$), indicating that higher tumour grades were more likely to exhibit high staining. Positive ER and PR statuses were associated with no and lower p16 staining intensity, respectively, with both p-values < 0.001. However, no relationship was found between HER2 status and p16 staining intensity. The number of lymph nodes with metas-

Table II. Odds ratios of the association between histopathological characteristics and p16 immunostaining level

Parameters	Ordinal logistic regression		Multinomial logistic regression (reference = no staining)			
	OR (95% CI)	p-value	Low, p16 staining		High, p16 staining	
			OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age (*per* 10 years older)	0.74 (0.65, 0.85)	0.0001				
Tumour grade (reference = grade 1)*			1		1	
Grade 2			1.14 (0.58, 2.23)	0.70	0.92 (0.48, 1.78)	0.80
Grade 3			0.87 (0.40, 1.92)	0.73	2.39 (1.18, 4.84)	0.02
Positive ER*			1.13 (0.61, 2.08)	0.70	0.41 (0.24, 0.69)	0.001
Positive PR*			1.37 (0.78, 2.41)	0.28	0.53 (0.32, 0.88)	0.01
Positive HER2	1.11 (0.75, 1.65)	0.61				

ER – oestrogen receptors, HER2 – human epidermal growth factor receptor 2, LN – lymph node, PR – progesterone receptors
** Ordinal logistic regression models with significant score tests*

tasis demonstrated a significant association with p16 staining intensity ($p = 0.02$). No other clinicopathological characteristics exhibited a significant association with p16 staining intensity.

Table II presents the direction and magnitude of the associations between the characteristics and the predicted p16 staining intensity. With every 10-year increase in age, the odds ratio (OR) for a higher p16 staining intensity was 0.74 (95% CI: 0.65–0.85; $p = 0.0001$). In comparison to no detectable p16 staining and a tumour grade of 1, a tumour grade of 3 was more likely to exhibit high p16 staining, with an OR (95% CI) of 2.39 (1.18, 4.84) ($p = 0.02$). Positive ER and PR statuses were less likely to be highly stained with p16 when compared to no staining, with ORs (95% CI) of 0.41 (0.24, 0.69) and 0.53 (0.32, 0.88), respectively. Both associations had *p*-values less than 0.01.

Discussion

The significance of p16 expression is well-known in multiple types of tumours. However, the effect of p16 in breast carcinoma has not been well-studied [4]. P16 (p16INK4a) is a tumour suppressor gene that plays a regulatory role in the cell cycle by blocking the action of cyclin-dependent kinases (CDK4 and CDK6) and inhibiting the action of the *RB* gene, which leads to a halt in cell cycle progression from G1 to S phase [3, 4]. Inactivation of p16 leads to a loss of cell cycle regulation due to the absence of *RB* phosphorylation inhibition. This phenomenon is observed in many types of cancers, including high-grade carcinomas of the oropharynx, cervix, and genitourinary tracts [3].

Breast cancer is a heterogeneous tumour that exhibits different characteristics and responses to the available treatment options [5]. Triple-negative breast cancer (TNBC), which accounts for 20% of breast cancers worldwide [6, 7], is characterised by the absence of the ER, PR, and HER2. Triple-negative breast cancer is considered an aggressive type of breast cancer with a poor prognosis due to its resistance to hormone-related drugs such as tamoxifen and trastuzumab, as well as its unpredictable response to chemotherapy [8–11]. This reality necessitates ongoing research to identify predictive markers that can lead to the development of more optimistic treatment strategies [8].

Immunohistochemistry is a convenient and practical method for studying gene expression in tumours. However, interpreting p16 immunohistochemical staining is not straightforward due to the possibility of both cytoplasmic and nuclear staining. Milde-Langosch *et al*. [12] investigated p16 expression in breast cancer samples using Western blotting and IHC. By comparing the results of the 2 methods, they suggested that both nuclear and cytoplasmic immunostaining in neoplastic cells should be considered as true p16 expression, which serves as a negative prognostic indicator in mammary carcinoma [12].

Our study investigated the p16 IHC stain in 475 cases of breast carcinoma from women diagnosed and treated at KAUH in Jeddah, Saudi Arabia, between January 1996 and December 2012. We observed low-positive p16 staining in 139 cases (29%) and high-positive staining in 199 cases (42%), while 137 cases (29%) were negative for p16. We observed a significant correlation between p16 positivity and high-grade tumours that were negative for ER and PR. Additionally, we found a strong relationship between the intensity of p16 staining in high-grade tumours and the number of lymph node metastases. No other parameters showed significant correlations.

Lebok *et al*. [13] reported a direct association between p16 overexpression and deletion of its gene *9p21* with worse prognosis and aggressive phenotypes of breast cancer. They investigated a tissue mi-

croarray with over 2000 breast cancer samples using fluorescence *in situ* hybridisation (FISH) and IHC for *9p21* deletion and p16 expression. Strong p16 staining was found to be associated with high stage ($p = 0.0003$), high grade ($p < 0.0001$), negative ER and PR status ($p < 0.0001$ each), and short overall survival ($p = 0.0038$). Deletion of *9p21* was also associated with high grade ($p < 0.0001$), positive lymph node metastasis ($p = 0.0063$), negative ER/PR status ($p \leq 0.0006$), and positive HER2 status ($p = 0.0078$). Furthermore, they demonstrated that p16 expression was lacking in samples with homozygous *9p21* deletions but present in samples with heterozygous deletions or normal copy numbers. Despite these differences, both alterations were associated with poor prognosis and aggressive phenotypes [13].

P16 exhibited significantly higher expression in the basal subgroup [5, 6, 8, 14, 15]. In a study conducted in Egypt, p16 was expressed in 16 out of 20 (80%) cases of basal-like cancers, which were found to have a lower disease-free survival rate [5]. Additionally, Shan *et al*. [15] examined 400 cases of breast cancer, including ductal carcinoma *in situ* (DCIS), invasive ductal carcinoma, luminal-A, luminal-B, HER2, and triple-negative subtypes, and compared them with 50 normal case controls. They observed that luminal-A DCIS exhibited the lowest level of p16 expression. Moreover, they demonstrated that DCIS with high expression of p16 were more likely to progress into invasive breast cancers. These findings suggest that p16 expression varies throughout tumour development and thus can serve as a measurable prognostic factor for breast cancer [15].

Salih *et al*. [4] analysed p16 IHC expression in over 200 breast cancer cases. They discovered a correlation between p16 expression, tumour grade ($p = 0.011$), and lymph node metastasis ($p = 0.003$). Similarly, Golmohammadi *et al*. [16] examined a total of 100 breast cancer specimens, observing p16 overexpression in 82% of breast cancer cases, with no overexpression detected in normal samples. They also found significant associations between p16 overexpression and high-grade tumours and tumour stage ($p < 0.001$, $p < 0.46$, respectively) [16]. These findings align with our results and with other studies, which have also reported connections between strong p16 expression and high-grade breast cancer, such as TNBC [17–21].

Conclusions

Our study revealed the correlation between p16 protein expression and the established negative prognostic parameters in breast carcinoma. We found significant association between p16 expression level and tumour grade, lymph node metastasis, and ER and PR status. These findings suggest a role of p16 in breast carcinoma progression and biological behaviour.

Additional future studies are needed to further evaluate its relevance in developing targeted therapies for breast carcinoma.

The authors declare no conflict of interest.

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