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The role of human papillomavirus and P16 protein in Iraqi women infected with breast cancer

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ABSTRACT

Numerous research investigations have connected the emergence of a few human cancers to the human papillomavirus (HPV). Breast cancer is one of the most prevalent types of cancer in women everywhere; via rates of both death and incidence continuously growing. There may be a connection between HPV infection and a higher risk of breast cancer, according to recent studies. Evaluation of human papillomavirus 16 &18 prevalence in breast cancer tissue samples and its relationship with p16 protein expression level. In the present study, 80 formalin-fixed, paraffin-embedded breast tissue samples were examined. The samples included 40 pathological tissue samples containing breast cancer; besides 40 normal tissues samples that did not show any phenotypic changes as a control group. To detect the presence of human papillomavirus (HPV) types 16 and 18, the highly sensitive In situ Hybridization (ISH) technique was used, while Immunohistochemistry (IHC) was used to analyze the gene expression level of p16 protein in breast cancer samples, as well as normal samples used as a control group.HPV-16 and HPV-18 positive reactions were detected using CISH in 37.5% of breast cancer samples (15 out of 40). On the contrary, no positive HPV-16 and HPV-18 reactions were detected in healthy breast tissues in the control group. The consequences also showed that 47.5% of breast cancer cases (19 out of 40) were positive for p16 protein reactions using IHC. The statistical analysis revealed a highly significant difference in the expression rates of p16 protein among breast cancer grade groups, with a P value of less than 0. 001.The results of the present study reveal the prominent role of HPV types 16 and 18 in promoting the development of breast cancer in Iraqi women. In addition, the expression of p16 gene can be considered a potential prognostic indicator for this disease.

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Introduction

Breast cancer is the most widespread cancer in the world, accounting for about 12% of all cancer cases, followed by lung cancer at 11.5%. In women, it is the most common type, accounting for 25.4% of cases. In 2020, more than 2 million women were diagnosed with breast cancer, resulting in 685,000 deaths worldwide (Santos et al, 2024). The Potential cancer risk factors include tobacco use, exposure to toxins and ionizing radiation, viral infections, genetic predisposition, thyroid hormone imbalances, and weakened immunity (Cuthrell and Tzenios 2023).

Various investigators have studied the connection between HPV and breast cancer and have achieved important findings in this area. HPV infection has been classified as a major risk factor for cervical cancer

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(Brantley and Tamimi, 2024). In addition, a study by Majid and colleagues indicated a strong positive association between HPV infection and an increased risk of breast cancer development (Majeed et al ,2023). HPV types that infect mucosal and cutaneous sites are classified into two categories: low-risk (LR) and high-risk (HR), based on their carcinogenic potential. The high-risk types most associated with malignant tumors include types 16, 18, 31, 33, and 45 (Abedien et al, 2023).

The p16 protein, also known as p16INK4A, is encoded by the CDKN2A gene located on chromosome 9 (9p21.3). Its main purpose is to inhibit the activity of cyclin-related kinases (CDK4 and CDK6), which are necessary for the phosphorylation-mediated activation of pRb (AL- Lebawy, 2018, Wong et al, 2020). Certain genes, like CDKN2A, which controls the cell division cycle, require the transcription factor E2F, which is released when pRb is active (Chahoud et al., 2022).

High-risk HPV strains produce the oncoproteins E6 and E7, which enable human keratinocytes to divide endlessly. The tumour suppressor proteins p53 and pRb are deactivated to achieve this. It's interesting to note that certain oncoproteins, particularly E6 and E7, continue to function in cancer cells permanently, so enhancing their malignancy. However, there is a noticeable decline in the characteristics of cancer cells when these proteins' activity is decreased (Skelin and Tomaić, 2023) Studies have shown overexpression of p16 in certain types of tumors and premalignant lesions, especially those associated with high-risk HPV infection (Hongo et al, 2022).Given the association of p16 with cancer progression in a variety of malignancies, so in this present study, we measured the expression of p16 in 40 patients with breast cancer.

Our aim is to detect HPV-16 and 18 and analyze the expressions of p16 protein in patients with breast cancer at different grades to reveal its role in the diagnosis and treatment decision for patients with breast cancer.

Materials and Methods

This study was intended as a retrospective study, and included 80 formalin-fixed, paraffin-embedded breast tissue samples. These samples included 40 breast cancer tissue samples at different stages, along with 40 apparently normal tissue samples (fibrosis adenoma) that served as negative controls. These samples were diagnosed based on the accompanying records and then subjected to additional review by a consultant pathologist to confirm the diagnosis.

HPV types 16 and 18 were distinguished using a CISH kit provided by Zyto Vision (Bremerhaven, Germany). This technique was applied to 4 μm thick paraffin-embedded tissue sections, using a digoxigenin-labeled oligonucleotide probe targeting the DNA of the two viruses. Expression of p16 protein was also assessed using the Immunohistochemistry system (Abcam, UK), which is based on monoclonal antibodies specially designed for this purpose (Mohammed *et al*, 2018).

For data analysis, the chi-square tests along with T-test were utilized to determine the statistical significance between the studied variables. All analyses were achieved via SPSS version 17, and consequences were considered statistically significant when the P value was less than 0.05.

Results

Age distribution of breast-cancer patients and control group

This study incorporated archival samples of breast cancer patients, whose ages ranged between 36 and 80 years, with a mean age of 57.45 ± 10.034 years. The control group, which included apparently healthy individuals, ranged between 37 and 75 years, with a mean age of 45.9 ± 10.628 years. The statistical results showed a significant difference in ages between the two groups ($P<0.01$), as shown in Table (1). The age group most affected by breast cancer was women aged 56 years and above, who constituted 47.5% of cases (19 cases out of 40). This was followed by the age group between 46 and 55 years, with 45% (18 cases). The least affected group was between 36 and 45 years, which represented only 7.5% (3 cases), according to the data shown in Table (2).

Screening- HPV16&18-CISH test

The results of applying the in situ hybridization (ISH) technique to detect the DNA of human papillomavirus HPV16 and HPV18 in the tissues of breast cancer patients showed that 15 cases out of 40 patients (37.5%) showed a positive reaction to in situ hybridization (Table 3, Fig. 1). The prevalence of HPV16 alone was 17.5%, while the mixed infection between HPV16 and HPV18 was 15%. The total prevalence of HPV16 (either alone or in mixed infection) was 32.5%**.** As for HPV18, its prevalence alone was 5%, while the percentage of mixed infection with HPV16 was about 15%, bringing the total prevalence of HPV18 (either alone or mixed) to 20%**.**

Positive HPV16&18 - CISH signal Scoring

The results of HPV16&18-CISH test showed that the percentage of high-scoring breast cancer was 5% (two cases), while the percentage of intermediate- scoring was 17.5% (seven cases), whereas the percentage of low-scoring was 15% (six cases), as shown in table 4 and figure 1.

Table 1 Mean and range of ages (in Years) of the study groups

Studied groups	No	Mean Age / Year	Std. Deviation	Std. Error	Range Min.	Range Max.	T. test	LSD test (P-value)
Breast carcinoma	40	57.45	10.034	3.115	36	80	6.698	P=0.00 HS
Control	40	45.90	10.628	3.230	37	75		
Total	80							

Note: P= Control **Vs** breast carcinoma

Table 2 Statistical analysis of age group distribution based on histological diagnosis of the study groups

Age groups/Year		Studied groups Breast carcinoma	control	Pearson Chi-Square (P-value)
36-45	No.	3	2	P=0.421
	%	7.5 %	5 %	Non sign.
46-55	No.	18	18	(P>0.05)
	%	45 %	45%	
≥56	No.	19	20	
	%	47.5 %	50%	
Total	No.	40	40	
	%	100%	100%	

***** Non-Significant differences using Pearson Chi- square test at **P>**0.05 level.

Table 3 Results of in situ hybridization for detecting HPV16 &18–CISH intissues with breast carcinoma

Result		HPV16	HPV18	HPV16&18	Total
Positive	No.	7	2	6	15
	%	17.5	5	15	37.5

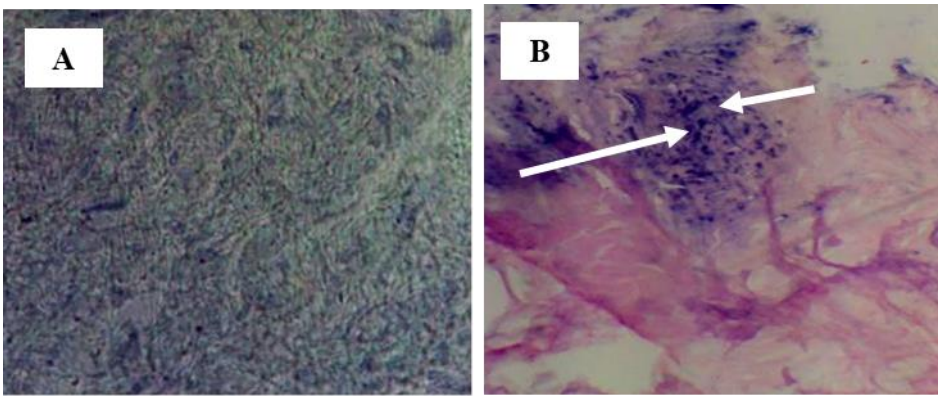

Fig 1. In situ hybridization detection of human papillomavirus 16 and 18 (HPV16/18) DNA in breast tumors. BCIP/NBT staining and nuclear fast red counterstaining were used. A. shows a breast cancer with a negative ISH reaction for HPV-16/18 (400X magnification). B. shows a breast cancer with a positive ISH reaction for HPV-16/18 (400X magnification).

Positive HPV16&18-CISH signal intensity

The results of table 5 and figure 1 demonstrated that 17.5% of breast cancer cases showed strong signal intensity in the HPV16&18-CISH test (seven cases), while the medium intensity was in 12.5% of cases (five cases), and the weak intensity was in 7.5% of cases (three cases).

Histological grading of breast carcinoma

The study showed that the highest percentage of histological grades in breast cancer cases was grade II (42%: 17 cases), followed by grade I (23%: 9 cases), while grade III was the least common (35%: 14 cases).As for the prevalence of HPV16 and HPV18, the highest percentage was in grade III, appearing in (53.3%: 8 cases) out of 14 HPV-positive samples, followed by grade II and then grade I. The results showed statistically significant differences (P<0.05) in the number of HPV-positive samples between the different histological grades, as shown in figure 2.

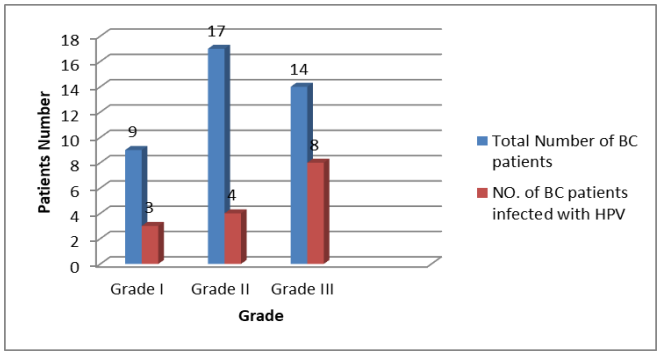

Fig 2. Frequency of histological grades of the studied breast carcinoma.

Signaling of P16-IHC score breast cancer

The results of detection of P16 protein expression using immunohistochemical (P16-IHC) technique were illustrated in Table (6). Out of all breast cancer cases only47.5% (19 out of 40 cases) showed positive signals. Among the positive cases, 36.8% (7 out of 19 cases) were recorded in low and intermediate histological grades, followed by 26.3% (5 out of 19 cases) in high histological grades, as shown in Figure (3). Statistical analysis showed highly significant dissimilarity between negative, low, intermediate and high cases, at a significance level of 1% (P<0.01).

P16-IHC intensity signal in breast carcinoma

Table (7) shows the consequences of the P16-IHC test, where 47.5 % of breast cancer cases (19 out of 40) showed positive signals. Among these cases, 47.5 % (9 out of 19) showed strong signal intensity, followed by 36.8% (7 out of 19) with medium signal intensity, and 15.7% (3 out of 19) with weak signal intensity, as shown in Figure (3). Statistically, significant differences were observed between negative cases and cases with weak, medium and strong intensity, where the statistical significance was very high (P<0.01) in the breast cancer group.

Table 4 Distribution of HPV signals (16 and 18) associated with breast cancer using chromogenic in situ hybridization (CISH) technique.

Scoring of HPV16&18		Breast carcinoma
Negative	No	25
	%	62.5 %
Low	No.	6
	%	15%
Moderate	No.	7
	%	17.5%
High	No.	2
	%	5 %
Total	No.	40
	%	100%

Table 5 HPV16&18 signal intensity distribution related to breast carcinoma using CISH technique

HPV 16&18 intensity		Breast carcinoma	
Negative	No.	25	
	%	62.5%	
Weak	No.	3	
	%	7.5%	
Moderate	No.	5	
	%	12.5%	
Strong	No.	7	
	%	17.5%	
Total	No.	40	
	%	100.0%	100.0%

Table 6 Percentage of P16-IHC score signaling in breast carcinoma

P16 signal scoring		Breast carcinoma No.	Breast carcinoma %	P-value
Negative		21	52.5	
Positive		19	47.5	
Scoring	Low	7	36.8	χ^2test P=0.00 Highly sign. (P<0.01)
	Moderate	7	36.8	
	High	5	26.3	

Table 7 The percentage of P16-IHC Intensity signaling in breast carcinoma.

P16 signal intensity		Breast carcinoma No.	Breast carcinoma %	P-value
Negative		21	52.5	
Positive		19	47.5	
Intensity	Weak	3	15.7	χ^2test P=0.00 Highly sign. (P<0.01)
	Moderate	7	36.8	
	Strong	9	47.5	

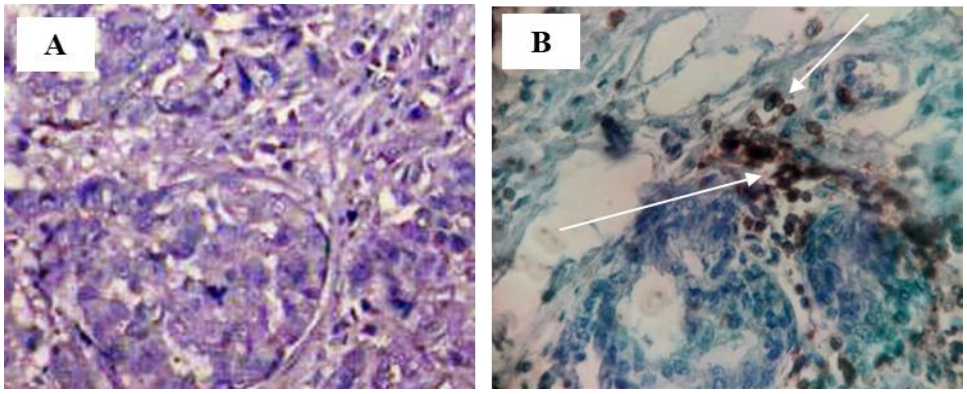

Fig 3. Immunohistochemical inspection to assess p16 gene expression in breast cancers using DAB chromogen stain (brown) and Mayer's hematoxylin counterstain (blue): A, Breast cancer with negative IHC reaction (400X magnification). B, Breast cancer with positive IHC reaction (400X magnification).

Association between P16 expression and grade of breast carcinoma

Figure 4 expresses the association of P16 expression and grade of breast carcinoma. The results exposed that P16 expression augmented as the grade of breast carcinoma augmented. The maximum ratio and of number P16 expression was observed in grade III (42.1%: 8) whereas the lowest percentage and of number P16 expression was observed in grade I (26.3%: 5).

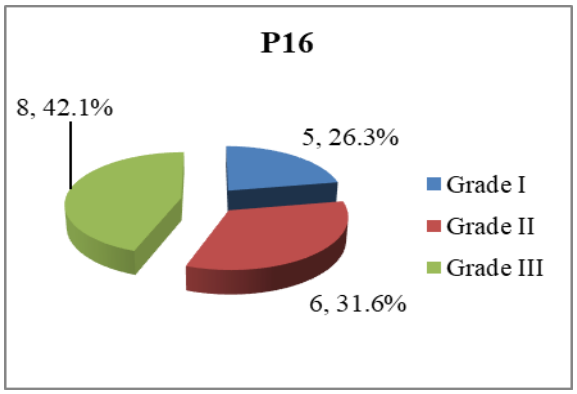

Fig 4. Association between P16 expression and grade of breast carcinoma.

Discussion

Cancer is a principal cause of global death, and breast cancer is the most communal malignancy among women, making it the leading cause of malignant death. Breast cancer incidence rates have been steadily increasing worldwide (Mohamed *et al*, 2023). The World Health Organization estimates that 15.4% of cancer cases are attributable to infections, of which 9.9% are caused by viruses. The International Agency for Research on Cancer (IARC) has identified eleven human carcinogens, including viruses responsible for four of the most common infection-related cancers, including human papillomavirus (HPV), which is estimated to cause approximately 640,000 cases annually. Research shows that viruses play a pivotal role in tumor development, contributing to many of the biological processes involved in cancer formation and contributing to many of its hallmarks (Zapatka *et al*, 2020).

Tables 1 and 2 show that a patient's age increases the risk of developing breast cancer. This outcome is backed by the data collected of Garrido-Castro *et al*. (2020) and Karachalios *et al.* (2023), where the mean age of onset was 53 and 51.34 years, respectively. Our findings are also in line with those of Javan Biparva *et al.* (2023), who found that the risk of breast cancer rises significantly with age. This increase may be caused by a number of genetic and biochemical factors associated with ageing.

The risk of breast cancer rises with prolonged exposure to cumulative hormones, including oestrogen and progesterone. Following menopause, these hormone levels shift. It might encourage the growth of tumours. Weakened immune system: As we age, our immune systems deteriorate, which makes it more challenging to identify and eliminate cancer cells early on. Viral infections: As people age, they are more likely to contract viral infections, which can cause cancer in a number of ways. Together, these factors suggest that aging increases the risk of breast cancer in women.

Table (3) showed that HPV-16 and HPV-18 were confirmed in breast cancer cases, which is in great agreement with the findings of Haghighi *et al*. (2023), who showed that HPV (16 and 18) genotypes are the most common HPV genotypes. The present outcomes also showed that the prevalence of HPV-16 was higher than that of HPV-18, which is consistent with several previous studies that have shown wide variation in the frequency of HPV infection in different regions, including British Columbia, where prevalences ranged from 1.6% to 86.2%, with a higher prevalence of the high-risk HPV-16 subtype (Islam *et al*, 2020, El-Sheikh *et al*, 2021, Zapana *et al*, 2023, Karachalios *et al*, 2023).

Figure (2) shows that the largest proportion of breast cancer patients were stage II, followed by stage III, while stage I was the least common. These results are steady with the outcomes of Dolatkhah *et al*. (2020) and Ignatov *et al*. (2024), who indicated that patients often approach the healthcare system at late stages, after low-grade breast cancer has transformed into more aggressive types.In the context of British Columbia tissues, HPV genomes were detected in a high proportion in stage III (53.3%), which are consistent with the study of Haghighi *et al.* (2023), who showed that most HPV genomes were detected in stage III breast cancer tissues (54.4%).These results propose that HPV could contribute to the progress of breast cancer through direct and indirect mechanisms. One of the new points highlighted by the current investigation is that HPV gene products may be included in chemotherapy resistance by disorderly cellular gene products such as p16, which may enhance tumor progression and make it more difficult to treat.

As shown in Tables (6 and 7) and Figures (3 and 4), the percentage of P16 protein positivity in breast cancer patient tissues indicates a close relationship between HPV16 and HPV18 infection and the expression level of P16. The results also showed a clear correlation between the expression level of P16 protein and the tumor grade in breast cancer, which are stable with the studies of Samman *et al.* (2024), Rezaei *et al.* (2023), and Mohammadizadeh and Nasri (2023).This is attributed to the fact that p16 is a tumor suppressor gene that plays a key role in cell cycle regulation by inhibiting the activity of cyclin-dependent kinases (CDK4 and CDK6), which impedes the function of the RB gene responsible for cell cycle progression from G1 to S phase.

Uncontrolled cell proliferation results from a breakdown in cell cycle regulation caused by p16 inactivation, which does not stop RB phosphorylation. Moreover, HPV infection triggers the production of oncoproteins like E7, which interfere with the RB pathway and lead to an overexpression of p16. This gene has been found to be overexpressed in some lesions and carcinomas, especially those associated with high-risk HPV infection. These results support the role of p16 as a measurable prognostic marker and predictor of the association between HPV infection and breast cancer (Wong *et al*., 2020, Hongo *et al*., 2022).

Conclusion

The results of the study showed that HPV 16 and 18 were found in a sizable portion of breast cancer tissues, indicating that they might contribute to the growth of tumours. The study also demonstrated how crucial the p16 protein is to the onset of the P16 protein may be detected by immunohistochemistry, which could be a helpful first screening method for assessing the prognosis of patients with breast cancer. To ascertain the predictive significance

of the p16 protein level in breast cancer and its function in prevention and treatment, more research in a variety of populations is needed.

Ethical approval
Non applicable.

Conflict of interests
All authors declare that they have no conflict of interests.

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