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HUMAN PAPILLOMA VIRUS (HPV) AS A POTENTIAL CO-FACTOR IN BREAST CANCER - A RETROSPECTIVE CASE-CONTROL STUDY

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ABSTRACT

Human papillomavirus (HPV) is an intriguing yet contentious etiological factor in breast cancer, with growing evidences suggesting its role as early trigger in breast carcinogenesis. This retrospective case-control study compared the types and prevalence of HPV DNA in breast carcinoma tissues and non-malignant breast tissues to decipher a possible association between HPV infection and breast cancer. The study was conducted between 1st January, 2024 and 15th March, 2025 and included 230 tissue specimen preserved in formalin-fixed paraffin-embedded (FFPE) blocks in Azadi Teaching Hospital, Kirkuk General Hospital, and private laboratories in Kirkuk city, Iraq. Of these, 130 samples were diagnosed as breast cancer (cases), while 100 samples represented benign breast lesions (control). Clinical and pathological data, including tumour grade, tumour stage, hormone receptor status (ER and PR), and HER-2/neu status, were obtained from the medical records of participants. Molecular detection of HPV was performed using High- and Low-risk PapillomaStrip and PCR assays. HPV DNA was detected at a significantly higher rate in breast cancer cases as compared to controls (P < 0.0001). HPV type 32 was the most prevalent genotype, detected in 24.6% breast cancer samples as against 3% in control. HPV positivity was observed in patients with high-grade (grade III) and advanced-stage tumours. No significant associations were found between HPV positivity and tumour laterality, ER status, or PR status. However, HPV-positive cases showed significantly higher proportion of HER-2 positivity in comparison to HPV-negative cases. In conclusion, HPV infection was more prevalent in breast cancer patients, especially those with HER-2–positive, high-grade, and advanced-stage tumours, thereby suggesting a potential role for HPV in aggressive breast cancer progression.

Keywords: Breast cancer, hormone receptor status, human papilloma virus, molecular detection

INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy among women and remains a major public health concern. In Iraq, 855 breast cancer cases were reported in 2016, with approximately 35% occurring in women aged 45-54 years, revealing an early age onset as compared to the Western populations (Mohammed *et al.*, 2023). Although genetic predisposition, hormonal exposure, and life style factors are known contributors to breast carcinogenesis, the growing evidences suggest that infectious agents may also act as cofactors in tumour initiation and progression. Among these, oncogenic viruses such as human papillomavirus (HPV) and certain parasites like *Toxoplasma gondii* have been implicated in breast cancer development (Kalantari *et al.*, 2015; Mahmood *et al.*, 2021; Kudela *et al.*, 2022).

Breast cancer is classified on the basis of histopathological and molecular characteristics. Histologically, tumours are categorized as ductal carcinoma *in situ* (DCIS), invasive lobular carcinoma (ILC), or invasive ductal carcinoma (IDC), the latter accounting for 50-80% of diagnosed cases. Molecular characterization identified four principal subtypes: luminal A, luminal B, HER2-enriched, and triple-negative breast cancers (Islam *et al.,* 2020). Triple-negative tumours lack estrogen receptor (ER), progesterone receptor (PR), and HER2 expression and are associated with aggressive clinical behaviour and high recurrence rates. HER2-enriched tumours are characterized by amplification of HER2 oncogene, while luminal subtypes representing 60-70% cases express ER and PR, and generally exhibit a more favourable prognosis (Nascimento *et al.,* 2024; Abdulla *et al.,* 2024).

Epidemiologically, breast cancer predominantly affects older women in Europe and USA, whereas in Asia, including Iraq, it is more commonly diagnosed at a younger age. The geographic variation may be attributed to the differences in genetic susceptibility, reproductive patterns, environmental exposures, life-style, and healthcare access (Bahram Arif and Shareef Abdul-kareem, 2023). The oncogenic role of high-risk HPV in breast cancer remains controversial, however, several mechanistic pathways support its potential involvement. HPV oncoproteins E6 and E7 promote carcinogenesis through inactivation of tumour suppressor proteins p53 and retinoblastoma (Rb), resulting in genomic instability, impaired apoptosis, and uncontrolled cell proliferation. Persistent HPV infection may also induce chronic inflammation and alter DNA repair pathways, thereby facilitate malignant transformation of breast epithelial cells (El-Sheikh *et al.,* 2021). HPV has additionally been implicated in other chronic diseases, including neurodegenerative disorders, highlighting its broader pathogenic potential (Istifo *et al.,* 2024). Multiple studies have reported the presence of high-risk HPV genotypes, particularly types 16, 18, and 33, in breast cancer tissues but not in adjacent normal breast tissues (Khan *et al.,* 2008; Heng *et al.,* 2009). Furthermore, HPV prevalence is significantly higher in tumour tissues as compared to the surrounding non-malignant tissues, supporting a tumour-associated relationship (Gumus *et al.*, 2006). Advances in molecular diagnostics, especially quantitative real-time PCR (qRT-PCR) and sequence-based assays, have substantially improved the sensitivity and specificity of HPV detection in archived formalin-fixed paraffin-embedded (FFPE) tissues (Gao *et al.*, 2013; Abdulla *et al.*, 2024).

The association between HPV and breast cancer is biologically plausible, as the mammary ducts are anatomically exposed to the external environment through nipple, potentially enabling viral entry. Given that most breast cancers originate from ductal epithelial cells, it was hypothesised that HPV may be transmitted from genital tract to the breast, where it infects mammary duct epithelium and contributes to carcinogenesis (Sher *et al.*, 2020). A retrospective case-control study using archived FFPE tissues represents a robust methodological approach to study this association. The application of molecular techniques like PCR allows accurate detection and genotyping of HPV DNA in malignant and benign breast tissues while minimising the contamination and false-negative results. Moreover, integrating clinic-pathological parameters - including hormone receptor status, HER2 expression, tumour grade, and stage - facilitates evaluation of biological and prognostic relevance of HPV infection. Accordingly, the present study was aimed to compare the types and prevalence of HPV DNA in archived breast carcinoma tissues and non-malignant breast tissues to assess a possible association between HPV infection and breast cancer in local population.

MATERIALS AND METHODS

Study design and setting

The present retrospective case-control study was conducted between 1st January 2024 and 15th March 2025**.** A total of 230 tissue specimen preserved in formalin-fixed paraffin-embedded (FFPE) blocks were collected from the Pathology Departments of Azadi Teaching Hospital**,** Kirkuk General Hospital, and selected private laboratories in Kirkuk city, Iraq. Among these, 130 specimens were

histopathologically confirmed breast carcinoma cases**,** and 100 specimens were benign breast lesions **(**fibroadenoma and fibrocystic changes). The specimens were selected on the basis of the availability of sufficient tissue volume or adequate tumour content. The samples having insufficient tissue volume/inadequate tumour content were excluded. Also, the cases with missing clinical or pathological metadata (e.g., age, tumour type, block site, pathology reports) were ignored. The samples which did not meet assay specifications (PapillomaStrip kit specifications) or showed either insufficient DNA or absence of amplifiable DNA were excluded. The clinical and pathological data of patients, procured from their medical records, included HER2/neu status, hormone receptor status (ER and PR), tumor grade, and tumor stage**.** Prior approval for the study was obtained from the Ethics Committee of Kirkuk University, College of Medicine vide No. KU-EC/2023/145**,** and the study was conducted in accordance with the Declaration of Helsinki**.** Written informed consent was waived due to the retrospective nature of the study and the use of anonymized archival samples.

DNA extraction

Genomic DNA was extracted from FFPE tissue sections using the RIBO-prep nucleic acid isolation kit (catalogue No. K2-9-Et-100-CE), following the manufacturer's instructions. DNA quality and quantity were estimated to ensure the suitability for downstream PCR amplification.

HPV detection and genotyping

HPV detection was performed using high- and low-risk PapillomaStrip kits (Operon Immuno & Molecular Diagnostics, Zaragoza)**,** which combine PCR amplification of E6/E7 regions with reverse line-blot hybridization. Biotinylated PCR products hybridize to genotype-specific probes immobilized on membrane strips. HPV detection was achieved using a streptavidin-alkaline phosphatase conjugate and a chromogenic substrate, that produced visible bands on the strip corresponding to the specific HPV genotypes.

PCR amplification

PCR reactions were prepared using the enzyme-primer master mix provided in the kit (Hi-Media, India). Each run included a positive control (PC) and a no-template negative control (NTC). Thermocycling conditions consisted of initial denaturation, 35-40 amplification cycles, and a final extension, as specified by the manufacturer. Pre- and post-PCR areas were strictly separated to prevent contamination.

Hybridization and detection

The amplified products were denatured and hybridized to membrane strips for 45-60 min at controlled temperatures with gentle agitation. Stringent washes removed non-specific binding. Strips were then incubated with the streptavidin-enzyme conjugate, and a chromogenic substrate was added until bands developed. Assay validity required internal control bands and correct PC/NTC results. The HPV genotypes were identified by comparing the developed strips with the reference chart. The results were reported qualitatively, specifying the detected HPV genotypes and control performance, with kit version and lot number included.

Statistical analysis

Data were analysed using SPSS version 26. Categorical variables were compared using χ^2-test, and continuous variables were analysed using the unpaired t-test. A p-value < 0.05 was considered statistically significant, and $P < 0.01$ was considered highly significant.

RESULTS AND DISCUSSION

The present study revealed significant differences in mean ages of breast cancer cases and control ($P = 0.013$). The cases group had a mean age of 49.34 ± 11.40 years, consistent with prior studies in Qatar

Table 1: Mean ages and HPV positivity in breast cancer patients and control group

Variables	Breast cancer cases	Control	P-value
Mean age	49.34 ± 11.40	45.98 ± 8.90	0.013
HPV positivity [No. (%)]			
HPV –ve	98 (75.4)	97 (97)	<0.0001
HPV +ve	32 (24.6)	3 (3)	

Mean ages analysed by unpaired t-test & HPV positivity by χ^2 test

(Ngan *et al.*, 2015; Sher *et al.*, 2020) who reported a mean age of 44 years. The HPV prevalence was significantly higher in breast cancer patients than control. HPV 32 was the most frequently detected genotype, observed in 32 cases (24.6%) as compared to 3% in control group (Table 1). These findings align with Zhao *et al.* (2024) who reported HPV DNA in 10.5% breast cancer cases, predominantly high-risk genotypes.

Tumour grade and stage

Stratification by tumour grade showed that HPV positivity was highest in grade III tumours (19 cases, 30.6%) and lowest in grade I tumours (4 cases, 20%). Conversely, HPV negativity was most frequent in grade I tumours (16 cases, 80%) and least frequent in grade III tumours (43 cases, 69.4%) [Table 2]. The tumour stage analysis showed the highest HPV positivity in stage III tumours (16 cases, 30.8%) and the lowest in stage I tumours (3 cases, 20%), with the opposite trend observed for HPV-negative cases (P = 0.002). These results suggest a positive correlation between HPV infection and tumour aggressiveness, indicating that HPV may play a role in the progression and severity of breast cancer. These findings are in agreement with Eslamifar *et al.* (2015), Karachalios *et al.* (2023) and Khodabandehlou *et al.* (2023), who reported links between HPV infection, tumour aggressiveness, and inflammatory signalling.

Table 2: Correlation between high- and low-PapillomaStrip test results for HPV detection and the clinical and pathological characteristics of patients with breast cancer

Variables		Cases		P value (χ^2 test)
		HPV-ve	HPV+ve	
Tumor grade	I	16 (80.0)	4 (20.0)	<0.0001
	II	36 (75.0)	12 (25.0)	
	III	43 (69.4)	19 (30.6)	
Tumor stage	I	12 (80.0)	3 (20.0)	0.002
	II	45 (71.4)	18 (28.6)	
	III	36 (69.2)	16 (30.8)	
Tumour laterality	- Left	59 (71.1)	24 (28.9)	0.144
	- Right	39 (83.0)	8 (17.0)	
ER	+	45 (81.8)	48 (84.2)	0.804
	-	10 (18.2)	9 (15.8)	
PR	+	44 (45.4)	14 (42.4)	0.841
	-	53 (54.6)	19 (57.6)	
Her-2	+	24 (60.0)	71 (78.9)	0.023
	-	16 (40.0)	19 (21.1)	

+ = Present; - = Absent

Tumor laterality & hormone receptors

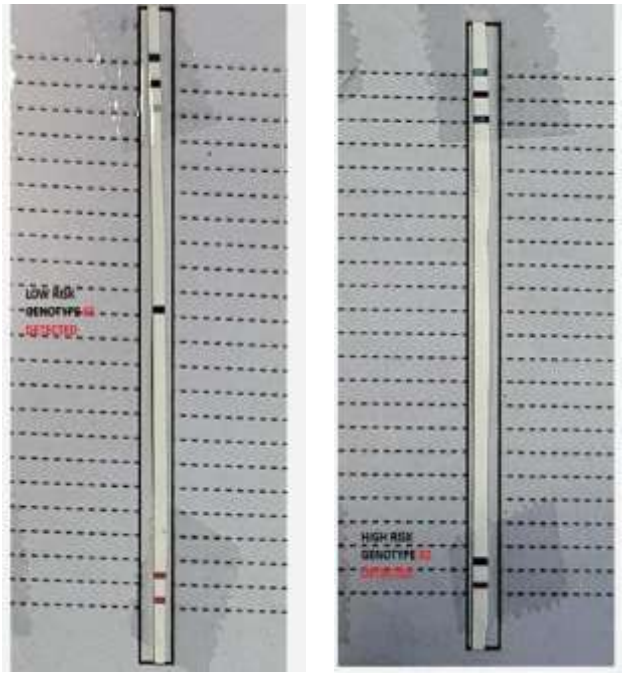
No significant associations were observed between HPV positivity and tumour laterality (P = 0.144), estrogen receptor (ER, P = 0.804), or progesterone receptor (PR, P = 0.841). While HPV-positive tumours were more frequently located in the left breast, this difference was not statistically significant (Boumba *et al.*, 2021; Elagali *et al.*, 2021).

HER2 expression

HER2 positivity was significantly higher in HPV-positive cases (78.9%) as compared to the HPV-negative cases (60%), whereas negative HER2 status followed the opposite pattern (P = 0.023). This suggests a potential interaction between HPV infection and HER2 expression, consistent with the idea that HPV may influence tumour biology and subtype susceptibility (Islam *et al.*, 2020).

HPV detection by PapillomaStrip assay and PCR

The HPV genotyping using the high- and low-PapillomaStrip assay detected low-risk HPV genotype 61 and high-risk HPV genotype 82 in breast cancer tissues (Fig. 1). The assay effectively distinguished HPV-positive from HPV-negative samples, and confirmed that the patients with breast cancer may have a variety of HPV genotypes (both low- and high-risk strains) in breast cancer tissues which may trigger or accelerate oncogenesis.



A **B**

Fig. 1: Detection of HPV by high- and low-PapillomaStrip. A) shows the detection of low risk genotype 61; and B) shows detection of high risk genotype 82

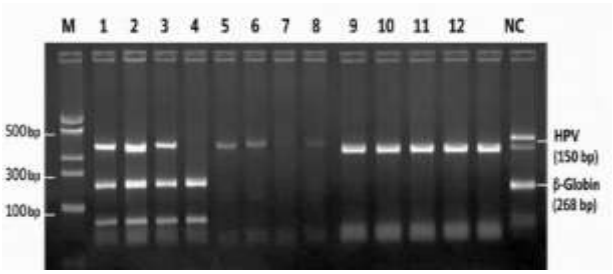


Fig. 2: PCR amplification of HPV (2% agarose gel); M: Maker; lane 1-12: Breast cancer samples; NC: Control

PCR-based detection was performed on all the samples to confirm the presence of HPV DNA. PCR results fully corroborated with the PapillomaStrip findings, confirming the HPV infection in the same patients identified by the strip assay. The combination of PapillomaStrip and PCR increased the detection reliability and provided strong evidence for HPV infection in breast cancer tissues (Table 3; Fig. 1 & 2).

Viruses are estimated to contribute to 15–20% of human cancers, and HPV has been proposed as a potential etiological factor in breast cancer. HPV infection may act as an early trigger in breast carcinogenesis, interacting with environmental factors over time via a "hit-and-run" mechanism. While our findings demonstrate significant associations between HPV infection, higher tumour grade, advanced stage, and HER2 positivity, causality cannot be established. PCR confirmation supports the reliability of our detection methods. Further longitudinal and mechanistic studies are required to determine whether HPV preferentially persist in aggressive tumour micro-environments or actively promotes tumour progression (Ren *et al.*, 2019). The precise mechanism by which HPV enters the breast is yet unknown (Kudela *et al.*, 2022).

Conclusions: The study demonstrated that breast cancer patients exhibited a significantly higher prevalence of HPV infection (24.6%) as compared to the control (3%) ($P < 0.0001$), and patients were generally older, with a mean age of 49.34 ± 11.40 years ($P = 0.013$). HPV positivity was the most frequently observed in high-grade tumours (grade III, 30.6%) and advanced-stage tumours (stage III, 30.8%) ($P = 0.002$), suggesting a relation-ship between HPV infection and tumour aggressiveness. No significant associations were identified between HPV status and PR, ER, or tumour laterality. However, HPV-positive tumours exhibited significantly higher HER2 expression (78.9%, $P = 0.023$), highlighting a potential link between HPV and HER2-positive breast cancer, supporting its role in tumour development and progression.

Table 3: PCR-confirmed HPV infection in breast cancer patients

HPV status	PCR-confirmed positive	PCR-confirmed negative	Total
HPV positive	32	0	32
HPV negative	0	98	98
Total	32	98	130

All PCR results fully matched PapillomaStrip assay results, confirming the HPV infection status

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