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HUMAN PAPILLOMAVIRUS TYPE 16 AND 18 INFECTION ASSOCIATION WITH IL-38 GENE EXPRESSION IN IRAQI WOMEN WITH BREAST CANCER

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ABSTRACT:
Introduction: Human papillomavirus (HPV) is implicated in the pathogenesis of several malignancies, and its possible role in breast cancer remains under investigation. This study aimed to explore the relationship between HPV infection and breast cancer and to assess its effect on the expression of interleukin-38 (IL-38).
Methodology: The study population consisted of 40 patients and 39 control participants. HPV detection was performed by ELISA technique, followed by PCR for confirmation, while gene expression analysis of IL-38 was carried out in a subset of seven patients using housekeeping gene normalization.
Results: ELISA results showed no positive cases among patients, whereas five control samples were positive. In contrast, PCR analysis was performed on 10 patients and 3 controls, the results showed that 8 of the patient samples were positive for both HPV types 18 and 16, while the controls had one positive for only HPV-16 and another sample was positive for both HPV-16 and -18. Gene expression analysis revealed altered expression of IL-38 among patients compared with controls, with most samples showing increased fold change.
Discussion: Overall, the results are consistent with the hypothesis of possible prior exposure or latent HPV infection and suggest that treatment-related immune suppression may warrant further investigation. Dysregulation of IL-38 expression may suggest an immune response in HPV-associated breast cancer. Further investigation into a larger sample size is necessary to fully understand the molecular relationship between HPV infection, cytokine regulation and breast cancer progression.

INTRODUCTION:

Breast cancer (BC) is the most common cancer among women worldwide (1). It is responsible for approximately 42,000 deaths annually in women. It ranks as the fifth leading cause of cancer-related mortality worldwide, making it the second most commonly diagnosed cancer globally (2). BC is a multifactorial disease with several established etiological factors, including late menopause (after 55 years of age), nulliparity, prolonged or increased estrogen exposure resulting from early menarche (before 12 years of age), high-dose ionizing radiation exposure, a high-fat diet, and regular alcohol intake. Furthermore, viral infections have also been proposed as potential causative agnets in the pathogenesis of BC (3). In the absence of specific primary prevention strategies for reducing breast cancer incidence, early detection and prompt treatment remain the major control optionsto improve survival and quality of life of the affected patients in low and middle income countries including Iraq (4). Emerging evidence has indicated a potential relationship between breast cancer and human papillomavirus (HPV) infection (5); nevertheless, although HPV DNA has been found in breast cancer cases, its aetiologic role in mammary carcinogenesis remains uncertain and has not been definitively proven (6).
Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease in both men and women worldwide. HPV is a member of the Papovaviridae family, it is a relatively small, non-enveloped virus, 55 nm in diameter (7). Different HPV types exhibit varying oncogenic potential. Some types are frequently identified in cervical cancer, whereas others are detected only rarely or are absent in large case series, forming the basis for their classification as high-risk or low-risk HPVs. In addition to cervical cancer, these HPV types have also been associated with malignancies affecting the anogenital region and the oropharynx (8). In 1991, it was first proposed that HPV might be implicated in breast cancer. The authors suggested that HPV types 16 and 18 could induce immortalization of normal mammary epithelial cells by inhibiting apoptosis (9). While most HPV strains are recognized as causative agents of cervical cancer, high-risk HPV types have also been reported in breast cancer (10). Followed by a report by Di Lonardo in 1992 suggesting a possible link between HPV infection and breast cancer through the detection of HPV-16 DNA in about 30% of breast and lymph node specimens, a growing body of research has since reported HPV DNA in breast cancer samples (11). These observations were later supported by 25 case-control studies, each of which found an increased prevalence of high-risk HPV in tissues of breast cancer in contrast to with normal and benign breast controls (12).

The immune system is vital for protecting the body against malignancy; however, immune dysregulation can contradictorily facilitate cancer progression (13). Although the inflammatory response is recognized as essential for effective antiviral immunity; however, during HPV infection, the virus can hijack cellular mechanisms and exploit the resulting hyper-inflammatory environment to promote tumor progression (14). Therefore, the starring role of the malignancy associated to cytokines has grabbed extensive consideration (15). ILs are considered as a pro-inflammatory, anti-inflammatory cytokines and are involved in viral infections as well as play an important roles in immune cell activation, differentiation, proliferation, maturation, migration and adhesion. Therefore, the main role of interleukins is to modulate growth, differentiation, and activation during inflammatory and immune response. Interleukins are grouped into 40 categories (IL-1 to IL40) each has an essential role in many viral infections (16). IL-38 is considered a natural inhibitor of inflammation, attenuating inflammatory responses during the early stages of disease (17). Although growing evidence highlights the immunoregulatory functions of this interleukin in inflammatory and autoimmune diseases, its role of IL-38 in HPV infection, breast cancer pathogenesis, and their potential as biomarker remain inadequately understood, particularly among Iraqi women and other populations with distinct epidemiological and environmental risk factors. Investigating their expression may provide insight into how HPV infection interacts with the host immune system and whether it contributes to immune dysregulation in breast cancer.

The present study investigated IL-38 gene expression in Iraqi women with breast cancer who were also positive for HPV-16 and/or HPV-18, with comparison to healthy controls. By examining IL-38 expression in this patient group, the study provides preliminary insight into alterations in IL-38-associated immune regulation in the context of concurrent breast cancer and high-risk HPV detection. These findings may contribute to a better understanding of the potential interplay between HPV infection, tumor-associated inflammation, and host immune responses, while further studies are needed to clarify the underlying mechanisms.

MATERIALS AND METHODS:

Sampling and Subjects:

Ethical approval for this study was obtained from both the hospital's Ethics Committee and the Ethics Committee of the College of Science, University of Baghdad. It was done on 40 Iraqi women patient (diagnosed with breast cancer) (females with age average 25-71 years), and 39 apparently healthy control (females with age average 23-69 years) were enrolled in the study; patients attending the Tumor Unit at Al-Imamain Al-Kadhimain Medical City in Baghdad for diagnosis and treatment between March and May 2026 were enrolled in the study. All participants provided informed consent during their clinical evaluation and routine healthcare procedures. Serological assessment was carried out using ELISA, whereas PCR was employed for molecular analysis. Peripheral blood samples (5 mL) were collected from each patient and separated into two fractions. Approximately 2 mL was dispensed into an EDTA-containing tube for ELISA analysis, whereas the remaining 3 mL was transferred into a TRIzol tube for RNA extraction for the interleukin-38 test and tubes placed in deep freeze -20˚C until used).

ELISA procedure:

The Sandwich ELISA was used for the quantitative detection of target analytes in plasma samples. Following collection in EDTA tubes, blood samples were centrifuged to obtain plasma, which served as the specimen for ELISA. Serial dilutions were carried out using the standard diluent to prepare the calibration standards. Standards and samples were loaded in duplicate into a 96-well plate. The assay was performed according to the manufacturer's instructions. A volume of 10 µL of each sample was transferred into the sample wells together with 40 µL of sample diluent. The plate was incubated for 40 minutes at 37°C, and washed five times to remove any unbound materials. All wells, except the blank, were then treated with HRP conjugate, and the plate was subsequently incubated again before undergoing an additional washing step. Subsequently, chromogen A and chromogen B were introduced. Blank-corrected absorbance values were used for analysis, and final concentrations were corrected for the five-fold sample dilution.

HPV detection by PCR:

Primers:

Table 1: The designed sequences of primer for PCR detection of HPV-18 and -16 based on the E7 and E6 gene, respectively.

Target gene	Product size bp	Forward 5' ⟶ 3'	Reverse 5' ⟶ 3'
HPV-16	119	TCAAAAGCCACTGTGTCCTGA	CGTGTTCTTGATGATCTGCAA

HPV-18	495	CGAGCACGACAGGAACGTCT	TCGTTTTCTTCCTCTGAGTCCCTT

Procedure:
For PCR amplification, 12.5 µL of PCR premix, 2 µL of template DNA, and 1 µL of each primer were combined in a PCR tube. The remaining volume was completed with sterile deionized water, after which the reaction mixture was mixed well. The PCR tubes were transferred to the thermocycler, where amplification was carried out in a thermocycler under standard cycling conditions. Followed by performing agarose gel electrophoresis according to the protocol described by Green and Sambrook (2019). PCR products were separated by electrophoresis on a 1.5% gel in 1× TBE buffer and run at 150 V for 30 minutes. After separation, the gel was stained with ethidium bromide, and the DNA bands were examined under UV illumination.

IL-38 gene expression:

Primers:

Table 2: The primer sequences for IL-38 gene expression.

Target gene	**Primer name**	**Forward 5' 3'→**	**Reverse 5' 3'→**
IL-38	173	TCACCTTCTTCCAGAGCAGC	CCTGTCTCCCTACCAGCTCT
GADPH	90	GCCTTCTCCATGGTGGTGAA	GCACAGTCAAGGCCGAGAAT

Procedure:
The samples were selected based on ELISA and PCR. RNA was extracted using GENEzol™ TriRNA Pure Kit and cDNA was synthesized using RevertAid Reverse Transcriptase. Quantitative real-time PCR was performed using gene-specific primers. Gene expression levels were normalized to GAPDH, and relative expression was calculated using the 2^-ΔΔCt method as described by Livak and Schmittgen (2001).

RESULTS:

HPV detection by ELISA:
The test showed negative results for all patients; however all five positive results belonged to the control group. The main wavelength was set at 450, and the cut-off was set at (NC+0.15). The same pattern was observed for both HPV-16 and HPV-18, as shown in Tables 3 and 4.

Table 3: shows ELISA results for HPV-16.

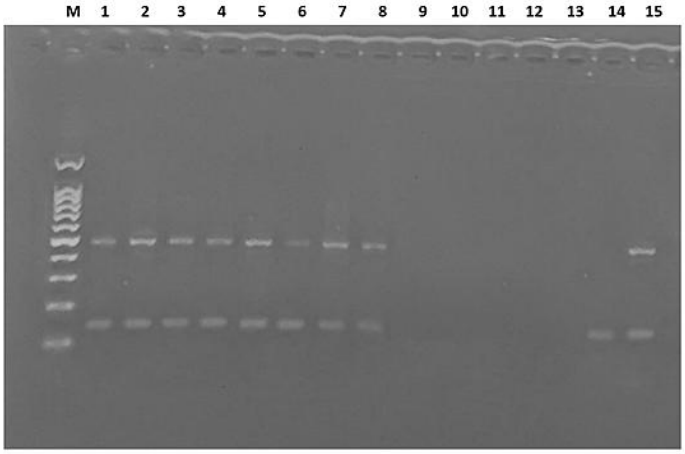
group	**N.**	**Positive, n (%)**	**Negative, n (%)**
control	39	5 (12.8)	34 (87.2)
patients	40	0 (0.0)	40 (100.0)
total	79	5 (6.3)	74 (93.7)

Table 4: shows ELISA results for HPV-18.

group	**N.**	**Positive, n (%)**	**Negative, n (%)**
control	39	5 (12.8)	34 (87.2)
patients	40	0 (0.0)	40 (100.0)
total	79	5 (6.3)	74 (93.7)

HPV detection by PCR:
PCR analysis was performed on 15 selected samples; 12 patients and 3 controls. The selection was based on the availability of the remaining samples after ELISA testing; the control group included two HPV-positive samples and one HPV-negative sample. The results showed 8 (66.7%) of the patients were positive for both HPV-16 and -18 types and 4 (33.3%) were negatives for both genotypes; and among the three control samples, one was negative for both HPV types, one was positive only for HPV-18, and one was positive for both HPV-16 and HPV-18. Agarose

gel electrophoresis demonstrated the expected amplicon sizes of 199 bp and 495 bp, confirming successful amplification (Figure 1).



IL-38 gene expression measurement by qPCR:

Relative gene expression analysis showed altered expression patterns of the IL-38 cytokine compared with controls. The number of samples exhibiting increased IL levels was higher than the number exhibiting decreased ILs levels as shown in Fig. 2A and B. Sample 15 exhibited the highest fold increase in the expression of IL-38 (fold change= 14.97). In contrast, sample 5 demonstrated reduced expression (fold change = 0.89), all shown in table 5.

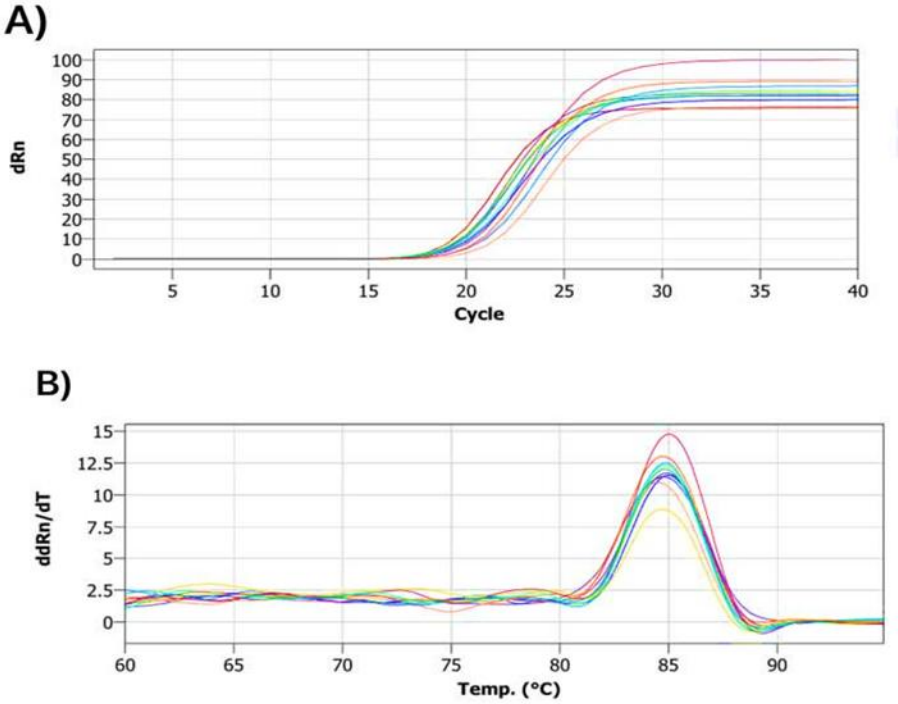


Figure 2: A) Ct values for IL-38 gene. B) Melt curve for IL-38 gene.

Table 5: IL-38 gene expression results.

sample	IL-38 Fold Change
1	1.24
2	0.89
3	0.64
4	0.39
5	4.42
6	1.32
7	14.9

DISCUSSION:

The incidence and mortality of breast cancer among women continue to increase globally, making it the leading cause of death in relation to cancer in females. These observations suggest the importance of investigating novel causative factors that may contribute to breast cancer pathogenesis (18). The link between breast cancer and HPV continues to be a topic of considerable debate. This suspicious role of HPV in human breast cancer is based on the identification of HPV of high oncogenic risk (16, 18, 31, 33, and 35) in breast cancer tissues and in the immortalization of the human breast normal cells (19). For example, Khodabandehlou, N. *et al*., identified the presence of DNA of HPV in 48.6% of BC samples vs. 16.1% in controls. Similar observations have been documented in a number of previous studies (20). Detection of HPV is often challenging because viral loads in clinical specimens, particularly paraffin-embedded tissues, are frequently very low. In addition, variations in the diagnostic methods used—including in situ hybridization (ISH), next-generation sequencing (NGS), nested PCR, polymerase chain reaction (PCR), quantitative real-time PCR (RT-qPCR)—may contribute to differences in detection rates (21).

The association of cervical cancer and HPV infection with is well established, but its involvement in BC remains unresolved. Therefore, the current study was done to investigate the association between HPV infection and its role as a risk factor or as an etiologic pathogen in breast cancer patients and how does the HPV infection in this case alter IL-38 expression. These finding showed differences between patients and controls in HPV detection and the interleukin gene expression, which suggest that the HPV might be involved in the disease progress through mechanisms that involve some immune manipulation.

The Inconsistency between the PCR and ELISA findings observed in the present study may be explained by the different biological targets detected by each assay. PCR detects HPV DNA directly and is therefore capable of identifying current infections even when viral load is low or the infection is latent. In contrast, ELISA relies on the detection of host antibodies, which may be absent or below the detection threshold despite the presence of viral DNA. Newly detected HPV infection may result from multiple mechanisms, including recent acquisition, reactivation of latent infection, reinfection, viral deposition following recent sexual contact, renewed detection of a persistent low-level infection, or autoinoculation (22). The virus titer may have been too low for the ELISA to detect, which would explain our results from the ELISA test in the patients group. The contrast between serological and molecular findings may reflect differences in the stage of infection, antibody persistence, or assay sensitivity. HPV Serology is not an entirely reliable test for detecting prior infections. Not everyone will have a positive antibody response to HPV, and responses can be quite poor. Antibody levels against HPV can also reduce. The sensitivity of serology assays can also be affected by the poor humoral immunity to HPV16/18 E6 and E7 proteins seen in patients with cancerous lesions (23).Research has shown that recently acquired HPVs reduces with age. Antibody levels can drop so that previous infection is hard to establish. Antibody levels can drop so that previous infection is hard to establish. Compared to many common viral infections, high risk HPV (hrHPV) does not trigger a strong humoral immune response (24). Therefore previous infection could be underestimated. Antibodies to specific types of HPV were detected in only around 60% of women with cervical HPV infections. Antibody levels may also decrease over time to the point where they cannot be detected. False positives may also be caused if HPV is restricted to non-genital areas (25). There appears to be a growing amount of evidence to suggest that HPV can remain in the basal epithelium cells of the cervix in a latent or low grade chronic stage, despite not being detectable using conventional methods of diagnosis. It may also be detected again if immune surveillance decreases (22). This would explain why there appears to be a discrepancy between PCR and ELISA results in this study due to latent infection and reactivation.

Although acquiring a new HPV infection is most commonly associated with exposure to a new sexual partner, particularly among older women, this explanation may be less applicable in our study population. Sociocultural factors, including religious prohibitions against sexual intercourse outside marriage and conservative social norms, may limit the likelihood of new sexual partnerships and, consequently, reduce the probability of a newly acquired infection.

The positive ELISA results observed in some control samples may reflect previous asymptomatic exposure to HPV and the persistence of detectable antibodies following natural infection (26). Additional explanations include antibody cross-reactivity (27) and variations in immune response (28). In contrast, the PCR for the control, which were positive in the ELISA test, one sample showed positive results for HPV-18 only. The other sample showed negative for both and only one sample showed consistent results with the ELISA test. Consequently, this difference in the results between serology and molecular test indicates the importance of using complementary detection methods when studying HPV in cancer patients.

The present study demonstrated an overall increase in the gene expression of IL-38 in breast cancer patients compared with controls, although a small number of samples exhibited reduced expression. It should be noted that gene expression analysis was performed exclusively in HPV-positive breast cancer patients and compared with healthy controls. Therefore, this increase in IL-38 may be due to the inflammatory effects of breast cancer and the immune modulation caused by HPV. Due to being an anti-inflammatory cytokine, its expression may be a way to

reduce excessive inflammation. While IL-38 is known to act as an anti-inflammatory cytokine, recent evidence has suggested that the action of IL-38 in cancers can depend on context (29). While suppressing IL-38 has been shown to cause increased immune cell infiltration, it can also lead to development of tumor specific immune memory and reduce tumour progression. Further research is required to understand the complex interactions between IL-38 and the tumor microenvironment in differing cancers (30). There was clear variation in the expression of IL-38 between patients, this can be due to differences in viral persistence, genetic susceptibility, tumour stage and individual immune response. Differences in cytokine expression have been seen in previous studies looking at cytokine expression in breast cancer. Persistent infections with HPV can cause constant immune stimulation, therefore causing changes in cytokine expression. Our results support the hypothesis that HPV may be able to become latent within the body and can be reactivated if there is immune suppression (31). HPV clearance can occur through full eradication of the virus, with or without development of protective immunity, or reduced replication below detection levels, meaning it is latent (24). Although we cannot prove this hypothesis using our data, our results do suggest further research should be done to investigate the link between HPV latency, immune alterations caused by the treatments and progression of breast cancer.

The function of IL-38 in cancer is controversial. In several studies it has been shown that an upregulation of IL-38 leads to immunosuppression and a weak antitumor immune response. However, some studies have shown that IL-38 may have a protective role in suppressing too much inflammation in tissues (29). This shows that the function of IL-38 may depend on the local immune environment and may have pro or anti-inflammatory functions in tumor development. Limitations of this study include. The amount of samples used in the PCR analysis and gene expression analysis was small meaning there weren't enough samples to carry out proper statistical analysis. Therefore, the results are mainly based on the fold changes seen. This means that they cannot be generalised. Finally, as it was a cross-sectional study, no causal relationships could be established.

Recently there is an increasing interest in studying the role of IL-38 in breast cancer. However, little is known about the effect of IL-38 gene expression in HPV-associated breast cancer especially in Iraqi women. Hence, exploring the relationship between HPV infection and IL-38 expression in breast cancer patients can be considered as a novel area which will contribute to better understanding the role of virus-induced immune modulation in breast cancer. Further studies on larger samples along with HPV detection using tissue samples and more detailed analysis on cytokine expression are also needed. In conclusion, our results demonstrated that HPV infection might be associated with breast cancer. In addition, IL-38 was expressed abnormally which may indicate immune modulation by HPV and tumor. Moreover, molecular and serological results were inconsistent indicating the importance of combining different methods for diagnosing HPV infection in breast cancer. Despite the need to elucidate the biological importance of these findings, this work provided a good starting point for future studies regarding the role of HPV and immune biomarkers in breast cancer progression.

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