

Human Papilloma Virus 16 Survey in Breast Epithelium of Women Using *In Situ* Hybridization Technique

Mahmoud Mishaal Mohamed*, Huda Mahdi Al-Khateeb**,
Khitam Razzak Kadhim Al-Khafaji***

ABSTRACT:

BACKGROUND:

There is increasing evidence that high-risk human papilloma virus (HPV) is involved in cancers other than cervical cancer. A number of reports have identified HPV DNA in breast tissue and breast cancer specimens, suggesting that the virus could play a role in the pathogenesis of this tumor.

OBJECTIVE:

The aim was directed towards the use of *In situ* molecular methods to localize the virus in breast tissue. In addition, this study investigated the prevalence of high-risk HPV infections in Iraqi women with and without ductal carcinoma (DC) of the breast.

PATIENTS AND METHODS:

29 cases of ductal carcinoma and 44 controls obtained from adjacent area to benign breast. Formalin fixed, paraffin embedded specimens were used by *In situ* hybridization technique for detection of HPV16 subtype. Data analysis was performed by SPSS 20 software using descriptive statistics and Chi-square tests.

RESULTS:

The HPV16 were identified in 69% and 27.3% of the ductal carcinoma and control breast tissue specimens respectively. Statistically, the difference between the normal and ductal carcinoma cases were highly significant ($P=0.001$).

CONCLUSION:

HPV16 *In situ* hybridization revealed statistically significant increase in DC (69%) as compared to controls (27.3%) and most of them were localized in the nuclei in integrative form. HPV16 were detected in skin and mammary tissue in both DC and control cases. This may indicate a role of HPV16 in the pathogenesis of DC.

KEY WORDS: human papillomavirus, breast cancer, ductal carcinoma .

INTRODUCTION:

Breast cancer, is the most common cancer and most common cause of death in middle aged women ⁽¹⁾, and the incidence of breast carcinoma has increased by more than 40% over 25 years ⁽²⁾. The aetiology of breast cancer remains unknown. Many risk factors have been associated with the pathogenesis of this disease, including family history, hormones, cigarette smoking and alcohol consumption ⁽³⁻⁷⁾. Hormones, cigarette smoking and family history

have also been demonstrated to enhance infections with papillomaviruses, mainly the high-risk human papillomavirus (HPV) types involved in the aetiology of cervical carcinoma ⁽⁸⁾.

High-risk human papillomaviruses (HPVs) are important risk factors for numerous human cancers including cervical, colorectal and head and neck; as roughly 96, 80 and 28% of these cancers are positive for high-risk HPVs, respectively ^(9,10,11). Furthermore, Human papilloma viruses are accepted as being carcinogenic in human cervical and anogenital cancers ⁽¹²⁾. Cervical cancer is caused by specific HPV infections ⁽¹³⁾. The role of human papillomavirus (HPV) in breast carcinogenesis is controversial ⁽¹⁴⁾. It has been reported that high risk human papilloma viruses (HPVs) are present in more than 50% of human breast cancers ⁽¹⁵⁻²³⁾. Moreover, HPVs were detected in normal breast tissue in patients with

*Department of Anatomy, Collage of Medicine/University of Baghdad.

**Department of Anatomy, Collage of Medicine/University of Baghdad.

***Department of Pathology, Collage of Medicine/University of Baghdad.

ductal carcinoma in situ and invasive ductal carcinoma ⁽²⁴⁾. Furthermore, HPVs were elicited in 3 days post partum breast milk sample [polymerase chain reaction (PCR) study] ⁽²⁵⁾. On the contrary, Hedau and coworkers ⁽²⁶⁾ didn't elicit HPVs in Breast cancer cases polymerase chain reaction (PCR study).

MATERIALS AND METHODS:

Patients were selected from those attending the operation room at "Baghdad teaching hospital-medical city complex" between January 2012 and

November 2012. A total of 73 patients were involved in this study were divided into two groups (Table 1). Their age were ranged 16-68 years. Breast surgeries (lumpectomy (Group I), and radical mastectomy (Group II)) were performed under general anesthesia. Specimens were obtained via sterile standard conditions. Laboratory procedures were done in collage of medicine – University of Baghdad. Mammary tissue & skin samples were taken from each patient. The apparently normal mammary tissue specimens were taken from adjacent area to the benign tumors, while the malignant mammary tissue specimens were taken from the mastectomized breasts.

Table 1: Tissue categorization.

Group	Disease	Number of patients
I	Apparently Normal breast tissue (control)	44(60.3%)
II	Breast tissue with ductal carcinoma	29(39.7%)
Total		73(100%)

All samples were fixed in 10% buffered neutral formalin for about 20 hours, and embedded in paraffin as blocks which sectioned into 5µm thick sections. Sections were mounted on charged slides. Then tissue sections were de-paraffinized. The DNA probe hybridization/detection system in situ kit were selected from Maxim bio (San Francisco, USA. Catalogue No. IH-60058(HPV-6010), IH-60001 (IHD-0050), and IH-60002 (IHD-0052). The procedure in accordance to the manufacturer instructions. 3 control slides were employed: First one (positive control) is previously known to be strongly positive for the targeted genes (HPV). The second positive one was prepared by adding 20µl of housekeeping gene instead of the detecting probe (to specify the Kit is still working). While the third, the negative control was used by adding 20µl of PBS instead of the diluted probe. The three control tissue sections were necessary to keep fidelity in terms of specificity and sensitivity. Proper use of

this hybridization/detection system gave an intense blue signal at the specific site of the hybridization probe in positive test tissue.

In situ hybridization scoring system for HPV-RNA according to Alizi *et al* (2012) ⁽²⁸⁾ was used in this study. This system calculates the percentages of epithelial cells with blue/black nuclear staining. Negative tissue sections were these of zero HPV-RNA expression cells. Quantification of different molecular markers in situ hybridization signal was evaluated under light microscopy and the counting of positive cells was performed at X100. Positive cells were counted in ten different fields of 100 cells for each sample and the average of positive cells of the ten fields was determined assigning cases to one of the three following percentage score categories: 1-25%, 26-50, and >50 were considered as low, intermediate, and high positive respectively, Table (2).

Table 2: In situ hybridization scoring system for HPV-RNA (Alizi *et al*, 2012).

Evaluation	Negative	Low	Intermediat	High
Percentage of stained cells	zero	1-25%	26-50%	>50%

Chi-square test was used to detect the significances between variables of our study. All the statistical analysis was done by SPSS program (version-20). P-value was considered significant when < 0.05, & highly significant when < 0.01.

RESULT:

The study show that HPV16 RNA found in 32/73 of cases. It represents 43.9% of the whole studied cases. The remaining 41 (56.1%) had no HPV16-RNA expression (Table 3). HPV 16 was expressed in the mammary tissue with integrative pattern.

HUMAN PAPILLOMA VIRUS IN BREAST EPITHELIUM

Table 3: HPV16-RNA expression in control and DC patients.

Cases	-ve	+ve HPV 16				Total	P-value
		low	intermediate	high	Total +ve		
control	32(72.7%)	4(33.3%)	8(66.7%)	0(0%)	12(27.3%)	44	0.001
DC*	9(31.0%)	13(65%)	6(30%)	1(5%)	20(69.0%)	29	
Total	41(56.1%)	17(53.1%)	14(43.8%)	1(3.1%)	32(43.9%)	73	

*DC= ductal carcinoma of the breast.

-ve= no HPV16-RNA expression.

Low= 1-25% of cells exhibit HPV16-RNA expression.

Intermediate= 26-50% of cells exhibit HPV16-RNA expression.

High= >50% of cells exhibit HPV16-RNA expression.

Of the total 44 control cases there was 32 (72.7%) have no HPV16-RNA expression. 27.3% (12/44) were found to be positively expressed for HPV16-RNA. Most of those revealed intermediate HPV16-RNA expression (Figure-2(A)). 33.3% exhibits low HPV16-RNA expression (Figure-2(B)). There was no high HPV16-RNA expression (Table -3).

On the other hand, out of 29 patients with ductal carcinoma of the breast, 9 (31%) patients were showed no HPV16-RNA expression. The

remaining 20(69%) patients exhibits a positive HPV16-RNA expression, majority of them (65%) show “low” HPV16-RNA expression. 6/20 (30%) and 1/20 (5%) revealing “intermediate” (Figure-3(A)) and “high” (Figure-3(B)) HPV16-RNA expression respectively (Table-3).

Statistically talking, there is a remarked difference between control and patients having ductal carcinoma presented by P=0.001 (highly significant) (Table-3).

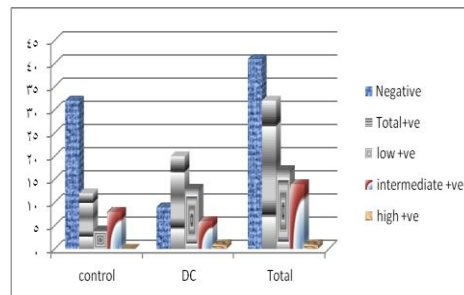
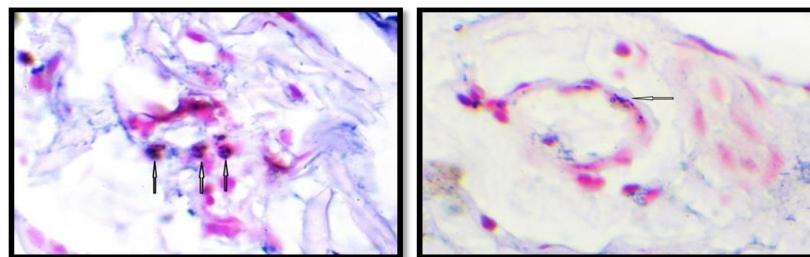


Figure 1: Histogram showing HPV16-RNA expression in each selected group.



(A)

(B)

Figure 2: Duct of two different control breast tissue shows A: “intermediate” and B: “low” positive HPV16-RNA expression by ISH, stained by BCIP/NBT (bluish purple, arrows) and counter stained by NFR (x200).

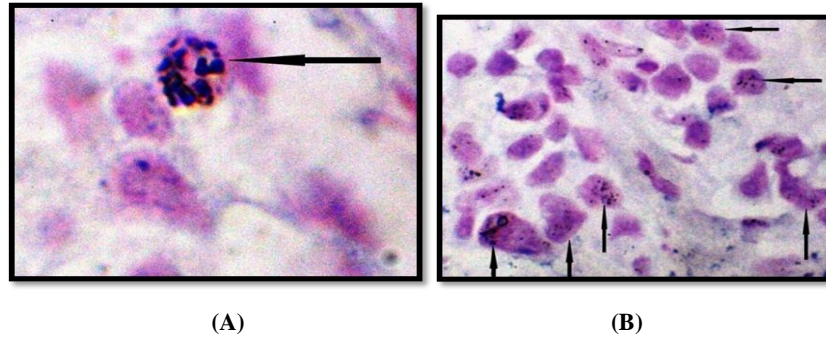


Figure 3: Two different patients with ductal carcinoma revealed A: “Intermediate” and B: “high” HPV16-RNA positive expression by ISH (bluish purple, black arrows), stained by BCIP/NBT (bluish purple) and counter stained by NFR (x1000 and x400, respectively).

DISCUSSION:

Human papilloma viruses belongs to the papilloma virus family, *Papillomaviridae*, capable of infecting humans and the most prevalent sexually transmitted viral infection. It is estimated that 80% of sexually active adults have been infected with at least one HPV type⁽²⁸⁾. HPV-16 and HPV-18 are the two most carcinogenic HPV types and are responsible for about 70% of cervical cancer⁽²⁹⁾.

In the present study, high percentages were found in the low score categories of HPV16. This may reflect a low reproduction (replication) rate of the virus⁽²⁷⁾ in mammary tissue.

There are few studies investigating the presence of HPV in the apparently normal breast tissue^(18,19,23,30,31,32,33). In the current study, HPV16 was expressed in 27.3% of the control cases. This result parallels that recorded by Lawson and colleagues⁽³³⁾, who records HPV16 in 22.2% of normal breast samples. Many of these investigations recorded absence of HPV16 in the apparently normal breast tissue samples^(18,23,32,34,35). Three researches^(18,30,31) disclose the presence of HPV16 in 15%, 5% and 5% of the apparently normal breast tissue samples respectively. On the other hands, the HPV16 detected in 69% of patients with DC of the breast. This outcome almost coincides with that conducted by with Damir *et al* (2004)⁽¹⁷⁾; De Leon and colleagues (2009)⁽²³⁾; He and colleagues (2009)⁽³¹⁾; Lawson and colleagues (2009a)⁽³³⁾; and Aceto and colleagues (2010)⁽³⁶⁾ who recorded HPV16 in 60%, 66.6%, 60%, 75% and 60% of the patients with breast carcinoma respectively. However, Khan *et al* (2008)⁽³⁷⁾ elicited higher percentage (92%) of patients with breast cancers.

Lower percentages were arrayed by other investigators^(16,25,32,38). Although it is well established that high-risk HPVs are the major causal agent for cervical cancer, involvement of the

virus in breast cancer is more controversial. This controversy may influenced by the technical limitations, different primer sets and/or detection probes, the epidemiology of HPV in different geographical area, different sexual behavioral patterns, differing incidences of ano-genital HPV infection and possibly different population genetics could play a role in these differing results^(35,39). Moreover, the viral load of HPV in breast cancer appears to be extremely low. In a study of HPV in breast cancer that had developed in Japanese women, which was estimate the viral load of HPV in cervical cancer was 4,000 folds greater than in breast cancer⁽³⁷⁾. This renders the detection of HPV in breast much more difficult and therefore may constitute to the absence of HPV in breast cancers reported by some investigators.

At present, about 130 HPV types were identified by their sequence of the gene encoding the major capsid protein L1 isolated from HPV associated diseases. Moreover, they can be also classified into high- and low-risk types depending upon their oncogenic potential. The high-risk HPV 16, 18, 31, 33, 35, 45 associated with ano-genital cancers and the precursor lesions (intraepithelial neoplasia), particularly of the cervix,. The most important players of cervical cancers are HPV16 and HPV18, found in 50–70% of cases⁽²⁹⁾. This may indicate oncogenic role of HPV16 in breast cancers. The presence of high-risk HPV in both malignant and apparently normal breast samples implies that a possible causal role in breast cancer carcinogenesis could not be ruled out⁽¹⁴⁾.

The oncogenic mechanisms by which HPV induces cervical cancer have been intensively studied⁽⁴⁰⁾. High-risk HPV encodes a series of proteins, designated as early (E1–E7) or late (L1 and L2). Key of cellular transformation are the E6 and E7

oncoproteins, which work in concert to disrupt cell-cycle regulation, inhibit apoptosis and stimulate cell-cycle progression by binding/inhibiting the p53 and p110RB tumor suppressor genes, respectively. Moreover, Xu and colleagues (1995)⁽⁴¹⁾ extensively study the programmed cell death (apoptosis) in normal human mammary epithelial cells, in cells immortalized with human papillomavirus, and in mammary carcinoma cell lines. He proposes that the HPV16 E6 protein modulates degradation not only of p53 but also of p21 and perhaps other proteins involved in apoptosis. In addition, Heng and coworkers, also show that the oncogenic characteristics of HPV associated breast cancer are very similar to HPV-associated cervical cancer. Furthermore, two researches from different geographical areas demonstrate the same High-risk HPV types; 16 and 18, present in both breast and cervical cancers of the same patient⁽³²⁾. These finding disclose strong relationship of the High-risk HPV as common factor influencing carcinogenesis in both cervical and breast cancers.

The statistical analysis of the present investigation (P=0.001), indicating high significance between the control and DC. A reflection for closing result, was reported by Khan and fellows (2008) (P=0.004 by Fisher's exact test), who investigates the morphologically apparently normal breast tissues adjacent to the malignancy.

Gathering the oncogenicity of HPV16 in cervical cancers with the statistical outcome of the current study may indicate role of HPV16 in breast cancers pathogenesis. Further investigation with larger sample size may needed to determine the exact role of HPV in ductal carcinoma of the breast. Consequently, vaccination programs may be beneficiary.

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