Survey of Human Papilloma Virus 18 in Breast Epithelium of Iraqi women Using In situ Hybridization Technique

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ABSTRACT:

BACKGROUND: There is an increasing evidence suggesting that high-risk human papilloma virus (HPV) was 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. Therefore, was directed towards the use of In situ molecular methods to localize the virus in mammary gland tissue. In addition, this study investigated the prevalence of high-risk HPV18 infections in Iraqi women with and without ductal carcinoma of the breast.

OBJECTIVE: Twenty night cases of ductal carcinoma patients and 44 controls obtained from adjacent area to benign breast lesions. Formalin fixed, paraffin embedded specimens were used by In situ hybridization technique for detection of HPV18 subtype. Data analysis was performed by SPSS 20 software using descriptive statistics and Chi-square tests.

RESULTS: The HPV18 were identified in 65.5% and 20.5% 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: Statistically, In situ hybridization revealed a significant increase of HPV18 in cases of ductal carcinoma (DC) (65.5%) when compared to their controls (20.5%). Most HPV were localized in the nuclei of integrative form. HPV18 were detected in skin and mammary tissue of both ductal carcinoma and control cases. This may indicates that HPV18 has a role in ductal carcinoma pathogenesis.

KEY WORDS: human papillomavirus, breast cancer

INTRODUCTION:
Breast cancer, is the most common cancer and most common cause of death in middle aged women (1), and its incidence of was 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 (3-8). Hormones, cigarette smoking and family history have also been demonstrated to enhance infections with papillomaviruses, mainly the types of high-risk human papillomavirus (HPV) that involved in the aetiology of cervical carcinoma (9).

High-risk human papillomaviruses (HPVs) are important risk factors for numerous human cancers including cervical, colorectal and head and neck; 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) were present in more than 50% of human breast cancers (15-23). Moreover, HPVs were
detected in normal breast tissue in patients with in situ and invasive ductal carcinoma (24). Furthermore, HPVs were elicited in milk of breast sample3 days post partum [polymerase chain reaction (PCR) study] (25). On the contrary, Hedau et al (2011) (26) didn’t elicit HPVs in cases of Breast cancer using polymerase chain reaction (PCR study).

MATERIALS AND METHODS:

Patients were selected from those who 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 and they were divided into two groups (Table-1). Their age were ranged 16-68 years.

Table 1: Tissue categorization.

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Apparently Normal breast tissue (control)</td>
<td>44(60.3%)</td>
</tr>
<tr>
<td>II</td>
<td>Breast tissue with ductal carcinoma</td>
<td>29(39.7%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>73(100%)</td>
</tr>
</tbody>
</table>

All samples were fixed in 10% buffered neutral formalin for about 20 hours, then embedded in paraffin (at 60 C˚) as blocks which sectioned into 5μm thick sections. These 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-60059(HPV-6011), IH-60001 (IHD-0050), and IH-60002 (IHD-0052). The procedure was used in accordance to the manufacturer instructions. Three 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 in positive test tissue gave an intense blue signal at the specific site of hybridization probe.

In situ hybridization scoring system for HPV-RNA according to Alizi et al (2012) (27) was used in this study. This system calculates the percentages of epithelial cells with blue/black nuclear staining. Negative tissue sections were those 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 by 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).

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Negative</th>
<th>Low</th>
<th>Intermediate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of stained cells</td>
<td>zero</td>
<td>1-25%</td>
<td>26-50%</td>
<td>&gt;50%</td>
</tr>
</tbody>
</table>

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.

RESULTS:

28 cases out of 73 (38.4%) cases were detected to have positive HPV18 expression in their breast
tissue. The remaining 45 cases (61.6%) were found to express negative HPV18 (Table-3) and (Figure-1).

Table 3: HPV18-RNA expression in control and DC patients.

<table>
<thead>
<tr>
<th>Cases</th>
<th>negative</th>
<th>positive HPV 18</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low</td>
<td>intermediate</td>
<td>high</td>
<td>Total +ve</td>
</tr>
<tr>
<td>Control</td>
<td>35(79.5%)</td>
<td>5(35.6%)</td>
<td>4(44.4%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>DC*</td>
<td>10(34.5%)</td>
<td>9(47.4%)</td>
<td>7(36.8%)</td>
<td>3(15.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>45(61.6%)</td>
<td>14(50%)</td>
<td>11(39.3%)</td>
<td>3(10.7%)</td>
</tr>
</tbody>
</table>

*DC= ductal carcinoma of the breast.

Figure 1: Histogram showing HPV18-RNA expression in each selected group.

The study detected that 79.5% (35/44) of control cases had negative HPV18-RNA expression. On other hand, 20.5% (9/44) of cases show positive HPV18-RNA expression. “Low” (Figure-2(A)) and “intermediate” (Figure-2(B)) HPV18-RNA expression nearly the same; representing 55.6% and 44.4% respectively. There was no high HPV18-RNA expression (Table-3) & (Figure-1).

In 34.5% of DC cases there was no HPV18-RNA expression. The remaining (65.5%) of DC patients showed positive HPV18-RNA expression. “Low”, intermediate (Figure-3(B)), and high (Figure-3(A)) HPV18-RNA expression were found to represents 47.4% (9/29), 36.8% (7/29), and 15.8% (3/29) respectively (Table-3) & (Figure-1). Highly significant P-value (equals to 0.001) indicates the differences between apparently normal and malignant cases (Table-3).
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Figure 2: Two different Control breast tissue revealed: (A) “low” & (B) “intermediate” positive HPV18-RNA expression by ISH, stained by BCIP/NBT (bluish purple, arrows) and counter stained by NFR (X1000).

Figure 3: Tow different patients with ductal carcinoma, the black arrows show: (A) “high” and (B) “intermediate” HPV18-RNA positive expression by ISH, stained by BCIP/NBT (bluish purple, black arrows) and counter stained by NFR (X1000).

DISCUSSION:

Human papillomaviruses belong to the papillomavirus family, *Papillomaviridae*. They were capable of infecting humans and they were the most prevalent cause of sexually transmitted viral infection. It is estimated that 80% of sexually active adults have been infected with at least one HPV type (28). Beside HPV16, the second most important player of cervical cancers was the HPV18, which was found in 7-20% of cases (29). Up to our best knowledge, this is the first study do scoring of HPV18 in the breast tissue all over the worlds. In the present study, high percentages were found in the low score categories of HPV18. This may reflect a low reproduction (replication) rate of the virus (27) in mammary tissue.

There were few studies investigating the presence of HPV in an apparently normal breast tissue (18,19,22,30,31,32,33). In the current study, HPV18 were reported in 20.5% (9/44) of the apparently normal breast tissue. This outcome almost coincides with that conducted by Heng et al (2009) (32); Lawson et al (2009) (33); and Glenn et al (2012) (34), who recorded 18%, 22.2% and 20% prevalence of HPV18 in control breast tissue samples respectively. However, Taiwanese and Iranian researchers (30,35) elicited lower percentage (5% & 7.8% ) respectively. On the contrary, many researchers failed to detect HPV18 in apparently normal breast samples (16,18,23).

In respect to ductal carcinoma patients, we observed HPV18 in 65.5% (19/29) of samples. This result parallels that recorded by Aceto et al (2010) (36); who elicited 60% HPV18 prevalence in breast cancer patients. Moreover, other researchers manifested HPV18 in 50% of breast cancer patients (12,34). Higher percentage was arrayed by Lawson et al (2009) (33), who demonstrate HPV18 in 75% of patients with Ductal carcinoma of the breast. Lower
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percentages were recorded previously (16,17,23,32,35,37). Although it is well established that HPVs are the major causal agent for cervical cancer, involvement of these viruses 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 anogenital 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 of Japanese women, it was estimated that viral load of HPV in cervical cancer was 4,000 folds greater than that in breast cancer (37). This renders the detection of HPV in the breast tissue more difficult and therefore may constitute to the absence of HPV in breast cancers reported by some investigators.

At present, about 130 types of HPV were identified by their sequence of the gene encoding the major capsid protein L1 isolated from HPV associated diseases. Moreover, HPVs can also be classified into high- and low-risk types depending upon their oncogenic potential. The high-risk HPV 16,18,31,33,35,45 associated with anogenital 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 HPV18 in breast cancers. The presence of high-risk HPV in both malignant and apparently normal breast samples implies their possible role in breast cancer carcinogenesis (14).

The oncogenic mechanisms by which HPV induces cervical cancer have been intensively studied (41). 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 (41). Moreover, Xu et al (1995) (40) extensively studied the programmed cell death (apoptosis) in normal human mammary epithelial cells, in cells immortalized with human papillomavirus, and in mammary carcinoma cell lines. He propose that HPV16 E6 protein modulates degradation not only of p53 but also of p21 and perhaps other proteins involved in apoptosis. Also, Heng and coworkers showed 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 a strong relationship of the High-risk HPV as a common factor in carcinogenesis of both cervical and breast cancers.

Statistical analysis of the current study disclosed (P-value = 0.001) high significance of HPV18 expression between the control and ductal carcinoma cases. This indicates a likely role of high-risk HPV18 in human breast cancer.

In addition to the oncogenicity of HPV18 in cervical cancers, the statistical outcome of the current study may indicate a role of HPV18 in DC pathogenesis. Further investigation with larger sample size and using different types of molecular techniques may needed to determine the exact role of HPV in pathogenesis of ductal carcinoma of the breast. Consequently, vaccination programs may be beneficiary.

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