Molecular Detection of Human Papillomavirus Genotype-31 in Tissues from Patients with Prostate Cancer and Benign Prostatic Hyperplasia

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Abstract
Background: High oncogenic-risk genotypes of human Papillomavirus (HPV) infect a wide range of human cells, including prostate tissue that give rise to benign prostatic hyperplasia and prostatic adenocarcinomas. This study aimed to detect DNA of HPV genotype-31 using in situ hybridization technique in prostatic tissues from benign prostatic hyperplasia and prostatic adenocarcinomas, and elucidate the association between these HPV genotypes and prostatic carcinogenesis. Fifty (50) formalin-fixed, paraffin embedded prostatic tissue blocks were obtained, among them (25) tissue biopsies from prostatic carcinoma with different grades and (15) benign prostate hyperplastic tissue blocks as well as (10) apparently normal prostate tissue autopsies which were collected from the archives of Forensic Medicine Institute / Baghdad and used as prostate healthy control groups. Detection and genotyping of HPV was done by highly sensitive in situ hybridization technique. The signals of in situ hybridization reactions of HPV-31 in prostate cancer cases in the present study was 52% (13 / 25) whereas in BPH, HPV-31 was detected in 33.3% (5 / 15). Non HPV-31 was detected in the apparently healthy control group. The highest percentage (24%) of positive- HPV31- DNA ISH reactions was found in tissues of prostatic carcinoma showing moderate differentiation. Our results indicate that the oncogenic HPV-31 might contribute to the development of subset of prostate tumors.

Keywords: HPV-31; prostate cancer, benign prostatic hyperplasia, in situ hybridization.

1-Introduction:
Most common neoplasms of the male genital tract involve the prostate gland .( Mc Nicol and Dodd, 1990). Prostate cancer is the fifth common cancer in the world and the second in cancer mortality exceeded only by lung cancer1 (Bray etal,2000) Vr

Viral factors are the most important class of infectious agents associated with human cancers (Mao etal. 2003).
It was estimated that 17-20% of all worldwide incidence of cancers are attributable to a viral etiology(Clifford etal,2003).

Human papilloma virus is sexually transmitted in adults. Human papilloma viruses (HPVs) are regarded as specific epitheliotropic DNA viruses (Ferency and Jenson 1996). HPV can persistently infect prostate epithelium in non immunocomprised host (Scott etal, 2001).

To date, more than 200 types of HPVs have been reported, which are classified into low –oncogenic risk and high- oncogenic risk types according to their associations with malignant tumors (Munoz etal,2003).

High oncogenic risk HPV types may integrate into the host cell chromosome, here they interrupt the integration of E2 gene that regulates the transcription & expression of HPV-E6 & E7 oncoproteins. The E6 and E7 genes represent transforming genes and their products are responsible for the alteration of growth patterns of the infected cells as well as acting, at least in part, by interfering with host cell control of transcription and the cell cycle. (Rubin’s ,2008).

These oncoproteins inactivate the cellular tumor suppressor gene products of p53 and Rb, respectively(Satoshi etal,2008)(Ghittoni etal,2010).
It is clear that continued expression of these viral oncopgenes is necessary for histopathologic progression and the malignant phenotype of an HPV-associated tumors(Carole and Gillison 2006). Recent studies suggest that HPV infection may play a role in the development of oral cancers (Serth etal,1999). Head and neck cancers (Ibrahim etal 1992). esophageal cancers (Rotola et.al 1992) Lung cancers(Tu etal,1994), and colorectal cancers (Al-Ahdal et.al,1996).

In addition, other reports document the presence of HPV DNA in prostatic tissues( Al Jewari et.al,2007).

The involvement of oncogenic (HPVs) in the pathogenesis of prostate cancers is a subject of great controversy . (Serth etal,1999). However, molecular detection of HPV DNA was documented in 2.4% (through
53% and) up to 100% in prostate cancer and in 32 %- 93% of benign prostatic hyperplasia(Serth et.al.,1999).

So this study aims to assess the in situ hybridization expression of HPV-31 in BPH & prostate cancer and to elucidate the correlation of these high–risk oncogenic HPV-genotype with development of BPH & prostatic carcinogenesis.

2-Materials and methods
2-1.Patients and tissue samples: Fifty (50) formalin-fixed, paraffin embedded tissues were collected from prostate biopsies that were related to (25) prostatic carcinoma,(15) benign prostate hyperplasia and (10) apparently normal prostate .They were collected from records of pathological archives of Teaching Laboratories of Medical City Hospital and Forensic Medicine Institute / Baghdad during the period of November 2009 to April 2010. The age of these individuals ranged between 55-95 years.

The diagnosis of these tissue blocks were based on their accompanied records. A consultant pathologist reexamined all these cases to confirm the diagnosis following trimming process of these tissue blocks.

2-2.Methods
Detection of HPV by ISH kit (Maxim biotech Inc, USA) was performed on 4µm paraffin embedded tissue sections using a biotinylated long DNA probe for HPV-31(cat. No. IH-60058 and IH-60059, respectively). One section was mounted on ordinary glass slide and stained with haematoxylin and eosin, whereas other section was mounted on charged slide to be used for in situ hybridization for detecting HPV-31.

In situ hybridization procedure: - The slides were placed in 60˚C hot- air oven over night. The tissue sections were deparaffinized and treated by graded alcohols according to the standard methods. The slides were treated then with proteinase K solution.

One drop of the biotinylated long cDNA probe for HPV-16 and HPV-18was placed on each specified slides. Hybridization solutions was placed on the tissue section and placed in the oven at 95˚C for 8-10 minutes to denature the double stranded DNA. The slides were then placed in a humid chamber and incubated over night at 37˚C to allow hybridization of the probe to the target nucleic acid. The slides were soaked in protein block at 37˚C until the cover slips fell and then treated with conjugate one to 2 drops of conjugate (BCI P/NTB).

Positive control reactions were performed by replacing the probe with biotinylated house keeping gene probe. Negative control was obtained by omitting the probe from hybridization buffer. Then substrate was placed on tissue section at room temperature for 30 minutes or until color development was complete. Slides were then counterstained using nuclear fast red and sections were mounted with permanent mounting medium (DPX). Color development was monitored by viewing the slides under the microscope. A blue colored precipitate formed at the site of the probe in positive cells.

The in situ hybridization signal was evaluated under light microscope at oil emersion (X1000) for counting of positive cells . Positive cells were counted in ten different fields for each sample and the average of positive cells of the ten fields was determined as the scope of our research is to qualify the results as positive or negative HPV -31 ISH reactions. A scale zero was given to these results without detectable ISH reaction whereas the results pointing for >1% were evaluated as positive ISH reaction and without the need to include the scores 1-3 stated by (27), that are referring to low , intermediate, and high infection.

Statistical analysis was done by chi- square test, percentage ,range, mean and standard deviation .Correlation was considered significant when p<0.05.

3-Results
Table( 1) shows the positive results of HPV DNA-ISH detection ,where 52% ( 13 of total 25) malignant prostate tumors showed positive signals. The benign group revealed 33.3% positive signals which represented 5 out of 15 cases in this group. None of control group presented positive signals for HPV-ISH test.

The HPV DNA was detected in a higher percentage in the malignant prostate tumors group than their benign counterpart group. Statistically, highly significant differences (p<0.05) were found on comparing the results of these study groups.

4-Discussion
Since human papillomavirus (HPV) infection was first identified as a risk factor for cervical cancer, several studies have investigated HPV in relation to prostate cancer with mixed results (Strickler et at .,1998). When Taylor and colleagues (HYPERLINK "http://europepmc.org/abstract/MED/15988645%22"HYPERLINK http://europepmc.org/abstract/MED/159886 45"(Rosenblatt etal,2003). combined the results of ten of these studies, they observed a significant positive association between HPV and prostate cancer.

Many studies showed the association between HPV and PCa .These studies showed that HPV prevalence varies from 2% to 100% in PCa samples, The most reported types of HPVs in prostate cancers were
HPV types 16, 18, 33 and 31 (Anwar et al, 1992) and (Leiros et al, 2005).

The present results are much higher than the results of positivity of HPV31 in the examined prostatic cancerous tissues reported by Amitis et al, 2011 and (Leiros et al, 2005).

Where they found (4.3%, 12%, 32%), respectively.

It is possible that the tissues of prostate cancer with negative results by the present in situ hybridization study may not have an adequate copy numbers of this virus to permit its detection by ISH while it could show positive results on PCR (Al-Ahdal et al, 1996).

By analogy, it was found that the present findings of HPV 16 &31 in an equal proportions are consistent to those studies done in Iraq by Mohammed Ali (Mohammed-Al, 2001) and (Al-Azzawi, 2006) and (Bakir, 2006) and (Khashman, 2008) and (Al-Mahbobi, 2011) and (Al-Aizzi, 2011) and (Al-Allwany, 2013).

who found that the HPV 16, 18, 31 & 33as the prevalent types in their studied group of the cervical, laryngeal, esophageal, oral, prostatic, ovarian carcinomas and breast carcinoma, respectively.

In addition, the lower numbers of the included prostatic tissues in the present (as well as other studies) which were subjected for molecular testing as well as there was a shortage of knowledge regarding the prevalences of each HPV genotype in the general population of each communities and/or countries precluded any clear and definitive explanation regarding such differences and discrepancies in the reported results of positive percentages of HPV (this was noted even for those results that were reported by the same researcher and in the same patients of that specific country but at different, even short, time interval of achieving these studies). (Mc Nicol and Dodd, 1990).

Although human papillomavirus type16 and type 18 are known to play a role in the development of neoplastic disorders of the urogenital organs, the presence of HPV-16 and HPV-18 in prostatic tissues with benign hyperplasia has been a matter of controversy (Al-Jewari, 2007).

The present study was extended to include a set of benign prostatic hyperplasia tissues to be tested by ISH technique for these important highly oncogenic HPV genotypes.

By analogy, the results of this study were in disagreement with the findings of Ibrahim et al, 1992, who found 50% of HPV16 in BPH by using PCR method and also consistent with the findings of each (Ibrahim et al, 1992). who found 20% of HPV 18 in BPH) and (Al-Jewari, 2007) who found 30.8% of HPV-18 in BPH) by using PCR &Southern blot hybridization techniques.

However, our results are lower than those reported by (1) who found HPV16 and HPV18 in BPH in a percentage rate of (93.3% &20%) respectively; those reported by (Mc Nicol & Dodd, 1991) (60.7%) in BPH by using PCR method; and those reported by (Rotola et al, 1992) who found (82%) positivity of HPV-16 in BPH cases. On the other hand, some investigators have reported negative findings of HPV in BPH samples. In this respect, a pilot study by (Hisada et al, 2000) included a total of 10 BPH samples that were proved to be negative at for HPV by both PCR and in situ hybridization. Also, our obtained results are higher than (Mc Nicol & Dodd, 1991), who found HPV18 (5.4%); (Al-Jewari, 2007). who found (15.4%) for co-infection HPV16&HPV18 in their examined benign hyperplastic tissues.

The differences in the present obtained percentages are a reflection of low prevalence of HPV in our Iraqi patients and as reported by (Al-Jewari, 2007) that may constitutes a probable cause for the differences between all Iraqi studies and world-wide studies. (Robbins and Cotran, 2010). Therefore, other factors and agents might multifactorially or co-factorially played a role in initiation and promotion in prostate carcinogenesis of our country.

Although many researches tried to present evidences for liability of conversion of subset of BPH into PC, yet scientists have not confirmed the change of BPH to PC (Wilcox et al, 1998). Prostate cancer like that of cervical cancer is also preceded by precursor lesions called prostatic intraepithelial neoplasia (PIN) which are equally paralleled to CIN in cervical cancer (Strickler et al, 1998). In view of these facts & observations, and likewise that of HPV role in cervical carcinogenesis, the present results could fortify the possibility of changing PIN lesions to PC via the role of highly oncogenic risk HPV types in the course of prostatic carcinogenesis.

The detection of such high risk HPV types in BPH would not be interpreted as a chance phenomenon or left without giving a critical importance for the possibility of HPV in initiation or enhancing the conversion of a subset of BPH into the prostatic carcinogenesis to change into PIN and/or PC.

Small size of the studied samples compromised the statistical power of this study to detect the effects of these factors under consideration. In addition, the lack of detailed clinical information attached to those prostate tissue samples that were enrolled in this study has deprived the present study to reach to a solid impression for the real role of those mixed viral infections in prostate carcinogenesis and in turn raised a suggestion to compel an integrate team-work study, at molecular and virological levels to elucidate the role of these factors and many other agents in prostate carcinogenesis in this country. Also in the future, it will be interesting to design experimental studies to understand the synergistic effect of HPV with EBV and/or HSV mixed infections on prostate cancer.

In view of the clear variations in the results of HPV in BPH from the present study and many other
studies, more investigations should be carried out before a possible conclusion that the prostate may be a potential reservoir for the sexual transmission of high risk HPVs can be made.

Conclusions
The high percentage of high-oncogenic risk HPV31-associated PCa might reflect a crucial role for this important sexually –transmitted disease in the pathogenesis of PCa and BPH and their probable transforming role along the pivot of prostatic carcinogenesis.

References


Table (1) : Frequency distribution of HPV DNA signal scoring among the malignant prostate tumors , benign prostate tumors and healthy prostate tissues.

<table>
<thead>
<tr>
<th>HPV signal scoring</th>
<th>Malignant Prostate Tumors (n=20)</th>
<th>Benign Prostate tumors (n=15)</th>
<th>Normal Prostate Tissues (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Negative</td>
<td>12/25</td>
<td>48</td>
<td>10/15</td>
<td>66.7</td>
</tr>
<tr>
<td>Positive</td>
<td>13/25</td>
<td>52</td>
<td>5/15</td>
<td>33.3</td>
</tr>
<tr>
<td>Scoring</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6/13</td>
<td>46.2</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>II</td>
<td>5/13</td>
<td>38.5</td>
<td>1/5</td>
<td>20</td>
</tr>
<tr>
<td>III</td>
<td>2/13</td>
<td>15.3</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Mean Rank</td>
<td>95.6</td>
<td>67.1</td>
<td>55.5</td>
<td></td>
</tr>
</tbody>
</table>
(Table 2) shows the correlation of infection with high-risk HPV31 genotype and their scoring with prostate cancer grading. Thirteen cases out of 25 showed positive for HPV31-ISH reaction. Twelve percent (12%:3 cases out of 25) of prostate carcinoma that have well differentiated grade. Also, it was found that 24% (6 cases out of 25) and 16% (4 cases out of 25 cases) of prostate carcinoma that showed moderately differentiated grade and poorly differentiated grade, respectively. Statistically, no significant correlations between HPV31 infection and the breast cancer grade.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Count</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well</td>
<td></td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>% within grade</td>
<td>12%</td>
<td>16%</td>
<td>28%</td>
<td></td>
</tr>
<tr>
<td>% within HPV31</td>
<td>23.1%</td>
<td>0.00</td>
<td>23.1%</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td></td>
<td>6</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>% within grade</td>
<td>24%</td>
<td>12%</td>
<td>36%</td>
<td></td>
</tr>
<tr>
<td>% within HPV31</td>
<td>46.1%</td>
<td>0.00</td>
<td>46.1%</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td></td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>% within grade</td>
<td>16%</td>
<td>%20</td>
<td>36%</td>
<td></td>
</tr>
<tr>
<td>% within HPV31</td>
<td>30.8%</td>
<td>0.00</td>
<td>30.2%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>13</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>% within grade</td>
<td>52%</td>
<td>48%</td>
<td>100.0%</td>
<td></td>
</tr>
<tr>
<td>% within HPV31</td>
<td>100.0%</td>
<td>0.0%</td>
<td>100.0%</td>
<td></td>
</tr>
</tbody>
</table>
Figure (1): In situ hybridization results for HPV-31 DNA detection in prostatic adenocarcinomas; BCIP/NBT stained and counter stained by nuclear fast red; A. prostate tissue with negative ISH reaction for HPV-31 (40X). B. prostate cancer with positive ISH reaction for HPV-31 (40X).

The results of the in situ hybridization for HPV-31 DNA detection in prostatic adenocarcinomas show a positive reaction in B, indicating the presence of HPV-31 in prostate cancer tissue. In contrast, A shows negative ISH reaction for HPV-31 in prostate tissue.

The study evaluated the prevalence of HPV-31 in prostate cancer and compared it with normal prostate tissue. The findings indicate a significant association between HPV-31 and prostate cancer, highlighting the potential role of this virus in the development of prostate cancer.
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