Human papillomavirus detection in Moroccan patients with bladder cancer

Noâma Berrada1,2, Abderrahmane Al-Bouzidi3, Ahmed Ameur4, Mohammed Abbar4, Mohammed El-Mzibni1, Rabii Ameziane-El-Hassani1, Laïla Benbacer1, Meriem Khyatti5, Zineb Qmichou1, Saaïd Amzazi2#, Mohammed Attaleb1##

1Unité de Biologie et Recherche Médicale, Centre National de l’Energie, des Sciences et des Techniques Nucléaires, Rabat, Morocco
2Laboratoire de Biochimie et d’Immunoïologie, Département de Biologie, Faculté des Sciences, Université Mohammed V-Agdal, Rabat, Morocco
3Département d’Anatomopathologie, Hôpital Militaire d’Instruction Mohamed V, Rabat, Morocco
4Département d’Urologie, Pôle Reins-Uro, Hôpital Militaire d’Instruction Mohamed V, Rabat, Morocco
5Laboratoire d’Onco-virologie, Institut Pasteur du Maroc, Casablanca, Morocco

#Saaïd Amzazi and Mohammed Attaleb contributed equally to this paper.

Abstract
Introduction: Human papillomavirus (HPV) is associated with more human cancers than any other virus. Many studies have investigated the association between bladder cancer and HPV but the results remain controversial. The aim of the present study is to evaluate whether HPV have an etiological role in bladder carcinogenesis among Moroccan patients.

Methodology: Forty-eight fresh biopsies (43 bladder tumors and 5 non-tumor samples) were collected for this purpose. Nested PCR with the consensus MY09/MY11 and GP5+/GP6+ primers was performed to detect the presence of HPV L1 gene DNA.

Results: The results showed that 52.4% of bladder cancer patients were positive for HPV. Subsequent DNA sequencing of positive cases of HPV revealed the presence of HPV16 in 95.5% of bladder tumor samples. The occurrence of HPV infection varies according to clinicopathological features, but there is no significant correlation between the viral infection and tumor stage or grade. In addition, statistical analysis demonstrated that there is no association between age or sex and HPV infection.

Conclusion: Our data indicate for the first time that bladder tumors from Moroccan patients harbor HR-HPV genotypes, especially HPV16, and thereby suggest that this virus may play a causative role in bladder cancer.

Key words: bladder cancer; human papillomavirus; etiology


(Received 05 October 2012 – Accepted 11 November 2012)

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Introduction

Worldwide, bladder cancer (BC) is the seventh most common cancer, accounting for approximately 336,000 new cases each year [1,2]. In Morocco, according to the regional cancer register of Rabat, bladder cancer is the sixth most common cancer and its incidence is the highest in this region with an age-standardized incidence rate (ASR) of 11.3 per 100,000 persons [3]. The average age of occurrence of bladder cancer was 62.9 years in women and 63.8 years in men. Urothelial carcinoma (UC) was by far the most frequent histological type (70% in women and 82% in men) while squamous cell carcinoma (SCC) accounted for 10% of cases in women and 4.8% in men [4]. There are several known and potential risk factors for bladder cancer, with tobacco smoking, specific industrial chemicals, dietary nitrates, and arsenic among the most important ones [5,6].

The urinary bladder is lined with a transitional epithelium, formed by several layers of cells which change their shape according to the filling of the bladder. According to the venereal transmission of HPV and to their ability to transform epithelial cells, the male urethra is considered as a reservoir for the virus. In addition, the association of HPV with bladder carcinomas has attracted much attention because of the epithelial tropism of HPV and the proximity of the urethra and bladder [7]. The papillomaviruses are small double-stranded DNA viruses which infect squamous epithelia and display a very high selectivity for the specific epithelium infected [8,9]. More than 200 different HPV genotypes have been described, but
only 40 genotypes can infect the genital tract [10] and are associated with epithelial neoplasms ranging from benign common warts to malignant carcinoma of the uterine cervix [11]. According to their ability to transform epithelial cells, HPV genotypes are divided into low-risk and high-risk types. Low-risk types are associated with benign lesions such as warts, while infections with high-risk types progress to malignant lesions [9,11,12]. The oncogenicity of the HPV high-risk type is due to the activity of two oncoproteins, E6 and E7 [13].

Whereas an association of papillomavirus infection with urinary tumors seems very probable in cattle [14], controversial results have been published regarding the human urinary tract [15-16]. In recent years, different degrees of association between HPV and BC have been described in a number of molecular epidemiological studies. In a meta-analysis study performed by pooling data from 52 studies [17], an association between HPV and bladder cancer was shown, and the prevalence of HPV was estimated in 16.88% of the bladder cancer cases, most of whom were high-risk HPV types. Thereafter, the authors concluded that infection with high-risk HPV types, especially HPV16, may play a role in bladder cancer [17].

It seems that the association between HPV and bladder cancer varies with geographical location [17]. Some studies have investigated the association between HPV and bladder cancer in Africa. HPV was found to be rare in South Africa (1.1%) [18] and absent in Tunisia [7]. In contrast, a PCR-based study reported the detection of HPV DNA sequences in 49% cases of bladder cancer in Egypt [19].

Given the particular characteristics of BC in the Moroccan population in terms of incidence, sex and age distribution, and the high frequency of HPV (particularly HPV 16) in our country [20], we conducted a study using PCR techniques to evaluate the prevalence of HPV infection in bladder cancer in Morocco.

**Methodology**

**Cases and specimens**

During the course of one and a half years, 43 fresh frozen biopsies from urinary bladder cancer patients were collected at Urology department of the Military Hospital of Instruction Mohammed Vth in Rabat, Morocco. Tumor samples were collected by transurethral resection (TUR) or from cystectomy specimens. Each sample was divided into two portions: one portion was put in neutral buffered formalin and processed for routine histopathological examination in the Anatomopathology department at the same hospital, according to the World Health Organization (WHO) criteria [1] and TNM (tumor node metastasis) classification; the other portion was stored at -80°C immediately after surgical removal, until DNA extraction. In addition, five non-tumor samples (cystitis) were used as controls. The study was conducted under the local ethical rules and informed consent was obtained from all patients.

**DNA extraction**

Genomic DNA was extracted from fresh frozen tissue specimens using a standard technique of digestion with proteinase K in the presence of sodium dodecyl sulfate (SDS) at 37°C overnight, followed by phenol/chloroform extraction [21]. The tissue DNA was precipitated with 2/5 volumes of 7.4M ammonium acetate and 2 volumes of 100% ethanol, followed by incubation at -20°C and centrifugation at top speed (13,000 relative centrifugal force). DNA was then resuspended in sterile distilled water and stored at -20°C until use. To evaluate the efficiency of DNA extraction, all samples were amplified by PCR using PC04 and GH20 primers specific for human β-globin gene (Table 1).

**PCR quality control**

To avoid contamination leading to false positive results, all PCR-related work was performed in specialized zones within a PCR laboratory that undergoes UV purification at least once every 24 hours. Positive controls with DNA extracted from SiHa and Caski cell lines, as well as a negative control (PCR reagents mixture without template DNA) were included in every set of 10 clinical specimens for each PCR run. All negative controls were negative for β-globin and HPV assay. Positive controls containing the SiHa and Caski cell lines were always positive for β-globin and HPV DNA.

**HPV detection and typing by sequencing**

Nested PCR amplification of a conserved region of the HPV L1 gene DNA with the consensus MY09/MY11 and GP5+/GP6+ primers (Table 1), followed by genotyping with direct DNA sequencing [22-25], was used for HPV DNA detection. For primary PCR amplification, all specimens were first subjected to PCR amplification with HPV consensus primers MY09/MY11. PCR amplifications were performed in a 25 μL reaction mixture containing 10 pmol of each primer, 1.5 mM MgCl2 and 1U of
AmpliTaq Gold DNA polymerase (Applied Biosystems, Grand Island, NY, USA). DNA was amplified in a GeneAmp PCR System 9700 apparatus (Applied Biosystems, Grand Island, NY, USA, CITY, COUNTRY) with the following steps: an initial 10 minute denaturation at 94°C, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and a final elongation step of 7 minutes at 72°C.

For nested PCR, 2 μL of the MY09/MY11 PCR products were used as a template with a pair of GP5+/GP6+ general primers. PCR amplifications were realized in a 25 µL reaction mixture containing the same reagents at equal concentrations as the primary PCR. DNA amplification was performed using the above described steps of the first PCR.

After completion of the primary and the nested PCR runs, 10 μL of PCR products were mixed with 2 μL loading fluid for electrophoresis in 2% agarose gel containing ethidium bromide. The gel was examined under UV light. Visualization of a 450 bp PCR product band in the MY09/MY11 lane and/or a 150 bp band in the nested PCR lane on the agarose gel provided evidence of HPV DNA in the sample, pending genotyping with direct DNA sequencing as a means of final validation.

For DNA sequencing, the nested PCR products were purified by PCR purification ExoSaP-IT clean up system (USB Corporation, Cleveland, OH, USA) and sequenced directly using GP6+ primer as the sequencing primer and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA), according to manufacturer’s protocol, on an ABI 3130XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) in molecular and functional genomics platform (UATRS-CNRST, Rabat, Morocco). Nucleotide sequences were aligned and compared with those of known HPV types available through GenBank by using the online BLAST 2.0 software server (http://www.ncbi.nlm.nih.gov/blast).

**Statistical analysis**

The correlation between HPV infection and clinicopathological parameters was evaluated statistically using chi-square test by comparison between proportions. However, the t-test was used for comparison of age means. All these tests were performed by MedCalc software version 9 (MedCalc Software, Ostend, Belgium). The level of significance was set at 95% (α = 0.05) for all tests.

**Results**

**Histopathological data**

The demographic characteristics of the 48 patients showed that the mean age of patients was 65 with extreme ages at 32 and 86 years old. The pathological analysis, which was performed according to the WHO and TNM classification of malignant tumors, revealed that among the 48 cases, 42 cases were urothelial carcinomas (UC), 5 were bladder inflammatory cases, and one case was adenocarcinoma. The tumor staging revealed that among 40 UC cases, 9 were classified as Ta (22.5%), 24 as T1 (60%), 6 as T2 (15%) and only one case was staged as T4 (2.5%). The tumor grading showed that among 40 UC cases, 13 case classified as low grade (32.5%), whereas 27 were high grade (67.5%). The stage and the grade of two other UC cases were undetermined.

**Detection of HPV DNA**

The quality of tissue DNA was assessed by detecting a fragment of β-globin gene using a PCR-based technique. This confirmed the presence of amplifiable DNA for all cases and all DNA samples were adequate for further analysis. The results obtained using primers targeting HPV DNA are reported in Table 2. We demonstrated the presence of
HPV DNA in 52.4% of the UC cases (22/42) and in 100% of the inflammatory cases (5/5), while the one adenocarcinoma case was lacking in viral DNA.

The main type of HPV detected was the high-risk HPV 16, which was observed in both the UC and inflammatory cases positive for HPV. Only two cases exhibited a co-infection with the HPV31 type: one case in UC and the other in inflammatory lesions. Thus the single infection with HPV 16 was detected in 95.5% of UC cases (21/22) and in 80% of inflammatory cases (4/5).

The prevalence of cases positive for HPV varied according to tumor stage and grade; this distribution is detailed in Table 2. In the early stages (Ta-T1), the HPV DNA was detected in 19 cases whereas the viral DNA was present in 3 cases of advanced stage tumors (≥ T2). In terms of grade, the number of infected cases with low-grade HPV was 7, and the number of high-grade cases was 15. The statistical study showed no significant correlation between HPV infection and stage (p = 0.77) or grade (p = 0.82).

The mean age of patients positive for HPV, including inflammatory cases, was 67, and that of patients negative for HPV was 62.1 years. There was no significant correlation between HPV infection and age of patients (p = 0.15).

Although the difference in HPV prevalence observed between males (47.4%) and females (80%) was high, this difference was statistically not significant (p = 0.37) (Table 2).

**Discussion**

HPV is associated with more human cancers than any other virus, and it can be considered as one of the most important risk factors for human cancer [26]. It is well established that HPV shows a particular tropism for the epithelium of different mucosal sites, mainly of the oral and ano-genital regions. Several studies have recently suggested that HPV might be somehow implicated in the pathogenesis of bladder cancer [27-29] and studies determining the association between HPV and bladder cancer have recently been reviewed [17,30].

This possible relationship is based on the epithelial tropism of HPV and the anatomical proximity of the urethra, which is considered a reservoir for the virus, and the bladder [7,31]. In addition, a recent meta-analysis was conducted by a research group which has concluded that infection from high-risk HPV types, especially HPV16, may play a role in bladder carcinogenesis [17].

**Table 2. Distribution of human papillomavirus DNA among 43 bladder cancer cases according to sex and tumor characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>HPV+ (%)</th>
<th>HPV (%)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>38</td>
<td>18 (47.4)</td>
<td>20 (52.6)</td>
<td>0.37</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>4 (80)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta-T1</td>
<td>33</td>
<td>19 (57.6)</td>
<td>14 (42.4)</td>
<td>0.77</td>
</tr>
<tr>
<td>≥T2</td>
<td>7</td>
<td>3 (42.9)</td>
<td>4 (57.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>13</td>
<td>7 (53.8)</td>
<td>6 (46.1)</td>
<td>0.82</td>
</tr>
<tr>
<td>high</td>
<td>27</td>
<td>15 (55.6)</td>
<td>12 (44.4)</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Undetermined</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Control cases (Cystitis)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Comparison of HPV positive cases
The association between HPV and bladder cancer has been explored in some studies in Africa. HPV was observed at a low percentage in South Africa (1.1%) [18] and no evidence of HPV infection was detected by morphological examination and PCR in any case of bladder carcinoma from Tunisian patients [7]. In contrast, a PCR-based study has revealed the detection of HPV DNA sequences in 49% cases of bladder cancer from Egyptian patients [19]. In Morocco, the prevalence of HPV has not yet been clarified, which led to our decision to conduct a study to elucidate this point. By using nested PCR on 42 bladder cancer samples, we detected viral DNA in 52.4% of the cases. The high prevalence of HPV infection in the present study demonstrates an association between HPV and UC. These findings were very similar to those obtained from studies conducted in Egypt [19,28] and Italy [32]. In contrast, other studies reported no detection of HPV [7,26,33]. Several explanations of this variability have been proposed, including sampling problems, contamination, sensitivity of the detection systems, and geographic variation [34]. It seems that geographical location has an impact on the association between HPV and bladder cancer. Indeed, it has been reported that HPV was detected in urothelial carcinoma at the highest rates in Southeast Asia (Japan and Hong Kong, 31% and 81%, respectively) [35,36] and in Europe (Italy and Finland, 50% and 57% respectively) [32,37]. Mean values have been reported in Europe and in North America (Canada), with frequencies not exceeding 39% [38,39].

In our study, the typing assay revealed that the majority of tumor samples harbored HPV 16 and only two cases exhibited a co-infection with the HPV31 type (one UC and one inflammatory lesion). A PCR-based study by Khaled et al. reported that infection with HPV type 16 is the most frequent type (64.6%), whereas the other types are less frequent: HPV type 18 in 18.7%; HPV types 6/11 in 6.2%; and co-infection with HPV types 16 and 18 in 10.4% [19].

Human papillomavirus DNA was positive in all cases that were histologically diagnosed as severe chronic cystitis. Our findings were similar to those obtained from Egypt by Badawi et al., showing that 3 of 4 analyzed cystitis cases were HPV positive [28]; however, the difference observed in the HPV prevalence between males (47.4%) and females (80%) was high but statistically not significant (p = 0.37). Also, no significant correlation between HPV infection and stage (p = 0.77) or grade (p = 0.82) was observed. In contrast, several studies stated that high-risk HPV (HR-HPV) should be considered as cofactor in urothelial bladder cancer. Cai et al. have recently found a statistical significant difference in HR-HPV frequency between high-grade and low-grade urothelial bladder cancer cases [40]. Also, Moonen et al. found a higher infection rate in high-grade tumors and stated that the increase in infection rate in high-grade tumors suggests a relationship between tumor grade and high-risk HPV infection [29]. On the other hand, Tenti et al. found that the prevalence of HPV 16 and/or HPV 18 infection was significantly higher in low-grade than in high-grade tumors [39]. As suggested by Moonen et al., the relationship between high-grade urothelial bladder cancer and the presence of HR-HPV is likely related, to the fact that HR-HPV types stimulate degradation and deactivation of protein associated with the p53 tumor suppressor gene via the ubiquitin-dependent pathway [29].

Conclusion
This study is the first report from Morocco on HPV infection and urothelial carcinoma. Contrary to some previous reports, our findings, suggest that HPV may play a causative role in bladder cancer in our geographic area. However, further studies will be required to clarify the part played by HPV in bladder cancer and to confirm its role in predicting the evolution at least of a subset of bladder cancers, thus aiding the clinician in providing the most suitable treatment and follow-up strategy for individual patients.

Acknowledgements
We are grateful to all the staff of the Urology and Anatomopathology departments of Military Hospital of Instruction Mohammed V®. Special thanks to the staff of the molecular and functional genomics platform (UATRS-CNRST, Rabat, Morocco) for the sequencing data.

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**Corresponding author**
Dr. Mohammed Attaleb
Unité de Biologie et Recherches Médicales
Département des Sciences du Vivant
Centre National de l’Energie, des Sciences et Techniques Nucléaires (CNESTEN)
BP 1382 R., 10001 Rabat, Maroc
Telephone: +212537712751/212537712031
Fax: +21253771846
Email: attaleb_mohammed@yahoo.fr

**Conflict of interests:** No conflict of interests is declared.