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Is There any Association between Urothelial Carcinoma of the Bladder and Human Papillomavirus? A Case-Control Study

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Keywords
Human papillomavirus · Urothelial carcinoma of the bladder · Polymerase chain reaction

Abstract
Objectives: Human papillomavirus (HPV) is a well-known oncogenic virus associated with anogenital carcinomas. Despite the anatomical proximity of the bladder and the anogenital region, the relationship between HPV and urothelial carcinoma of the bladder (UCB) is still a controversial issue. This study aimed to test the urethral swabs and first-void urine samples of patients with UCB for HPV-Deoxyribonucleic acid (DNA) using polymerase chain reaction (PCR) assay and to compare the results with a control group. ***Materials and Methods:*** Sixty-nine patients who were diagnosed with UCB between January and December 2018 were included in this case-control study. Sixty-nine patients who visited the urology outpatient clinic for non-oncological reasons within the study period were designated as the control group. Urethral swab and first-void morning urine samples were collected from each patient. HPV-DNA presence was investigated using a PCR kit that can detect a total of 22 HPV genotypes, of which 18 are high-risk and 3 are low-risk genotypes. ***Results:*** The mean age of the patients included in the study was 63.2 ± 12.6 years and the male to female ratio was 5.3. HPV-DNA was detected in 28.9% (20/69) of the patients in the case group and in 8.7% (6/69) of the patients in the control group. HPV-DNA positivity was significantly higher in the case group (OR 4.24; 95% CI 1.63–12.34). No statistically significant relationship was found between HPV-DNA positivity and tumor grade (p = 0.36). ***Conclusion:*** A statistically significant relationship exists between HPV infection and UCB, regardless of the tumor grade.

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Introduction

Human papillomaviruses (HPVs) are a group of double-stranded Deoxyribonucleic acid (DNA)-viruses, which are responsible for one of the most common sexually transmitted diseases. Most HPV infections are asymptomatic or subclinical. According to epidemiological studies, the global prevalence of HPV is 11.7% [1].

The socioeconomic burden HPV poses due to its oncogenic effect makes the virus a popular topic in medicine. Along with *Helicobacter pylori*, HPV is the most common cause of infection-related malignancies. Moreover, HPV is held responsible for 7–8% of all human malignancies [2].

To date, >200 types of HPV have been identified, and about 40 of these cause anogenital infections [3]. Unlike most viruses, HPVs are classified based on their DNA sequence homology instead of their antigenic structure. Thus, they are categorized according to their genotypes instead of their serotypes, and numbered in the order in which they are discovered [4]. On the other hand, based on their oncogenic potential, HPVs are classified as high-risk HPVs (types 16,18, 26, 31,35, 39, 45, 48, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) which are infamous for being the primary causes of carcinomas and low-risk HPVs (types 6, 11, 40, 42, 43, 44, 54,61, 70, 72, and 81), which evoke low-grade lesions such as condyloma accuminata [5, 6].

High-risk HPVs exert their oncogenic effect via the E6 and E7 oncoproteins. The disruption of the E2 protein during the replication of the virus inactivates the inhibitory effects of the virus on the E6 and E7 oncoproteins. Subsequently, the degradation of the tumor-suppressor p53 protein by E6 and the degradation of the RB1 protein of the tumor-suppressor retinoblastoma gene by E7 disrupt cell cycle control and causes a deficiency in DNA repair, leading to genomic instability and increasing the risk of malignant transformation [7].

Given the tropism of HPV for squamous epithelium, squamous cell carcinoma is the most common type of cancer related to HPV. The relationship between HPV and squamous cell carcinomas of the anogenital region has been defined clearly. An HPV infection is the underlying cause in 96% of cervical cancers, 36% of penile cancers, and 64% of anal cancers [8]. On the other hand, despite the close anatomical relationship between the bladder and the anogenital region, the association between bladder carcinoma and HPV has been a matter of debate for the last 30 years. There are 2 hypotheses regarding the association between HPV and bladder carcinoma. The first hypothesis is that the urethra acts as a conduit through which the sexually transmitted HPV can reach the bladder. In other words, the urethra connects the genital area and the urinary bladder, enabling viral migration. The second hypothesis is based on the innate epithelial tropism of HPV [9]. Several studies and meta-analyses have assessed the relationship between HPV infection and bladder carcinoma, but the topic still remains highly controversial. The ongoing debates on this topic arise from the variable clinical presentations of HPV and the methodological shortcomings of the previous studies. These shortcomings can be summarized as follows: the limited number of patients in published studies, the lack of studies on fresh samples, and the shortage of case-control studies [9, 10].

The aim of this study was to evaluate the presence of HPV-DNA in the urethral swab and urine samples collected from patients with urothelial carcinoma of the bladder (UCB) using the polymerase chain reaction (PCR) assay and to compare the results with a control group in order to assess the relationship between HPV infection and UCB.

Materials and Methods

Case Selection and Ethical Approval

Transurethral resection of bladder tumour candidates who were diagnosed with a primary or recurrent bladder tumor according to sonographic and/or cystoscopic evaluation between January and December 2018 were included in the study. Patients who underwent transurethral resection of bladder tumour at our clinic and were diagnosed with UCB with muscle invasion according to the pathology report, and for whom radical cystectomy was planned, were also included in the case group. Patients who were diagnosed with tumors other than UCB according to the pathology results and patients who had previously undergone radical cystectomy were excluded. In patients in the case group, extraordinary catheterization and cystoscopic intervention were not performed except routine practice. The demographic data, tumor grade, and smoking history of the patients in the case group were recorded. The same number of patients who visited the urology outpatient clinic for non-oncological reasons within the study period were designated as the control group.

The study was approved by the local Ethics Committee (Approval No. 005/2018) and written informed consent was received from all participants. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration.

Molecular Analysis

Urethral swab samples and first morning void urine (15 mL) samples were collected from all patients. Urethral swab samples were obtained using a cotton-tipped swab. All samples were stored at –80 °C until analysis.

DNA extraction from samples was done with the PREP-NA PLUS and PREP-GS PLUS extraction kits (DNA Technology®, Moscow, Russia) according to the manufacturer's recommenda-

Table 1. Patients and UCB profiles

	Study group	Control group	*p* value
Age, years, mean ± SD	63.2±12.6	62.1±12.9	0.469
Gender ratio, male/female, *n* (%)	5.3 (58/11)	5.9 (59/10)	0.813
TNM classificaton, *n*			
Ta	32		
T1	34		
T2	3		
Tumor grade, *n*			
High grade	37		
Low grade	32		

UCB, urothelial carcinoma of the bladder.

Table 2. Distribution of the HPV types detected in the case and control groups

Types of HPV	Case group, %	Control group, %
Type 16	11.1	
Type 18	11.1	
Type 26	3.7	
Type 39	11.1	
Type 45	3.7	
Type 51	7.4	14.3
Type 53	11.1	14.3
Type 56	3.7	
Type 59		14.3
Type 66	7.4	14.3
Type 68	7.4	14.3
Type 82	7.4	
Type 6	14.9	28.5
Total	100	100

HPV, human papillomavirus.

tions. In order to detect HPV-DNA, the samples were analyzed by DT 5′ Real-Time PCR, which is manufactured and programmed by the same company. All samples were tested for low-risk HPVs (Types 6, 11, and 44) and high-risk HPVs (Types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82).

Statistical Analysis

All statistical analyses were performed using the SPSS statistical software (SPSS for Windows, version 22.0, SPSS, Inc., Chicago, IL, USA) and Open Epi® Version 3.01 (Atlanta, GA, USA). Continuous variables were expressed as means and SD. Comparisons between groups were carried out using the Mann Whitney U test. The OR and the 95% CI for the association between HPV-DNA and UCB were calculated. The Chi-square test was used to determine the relationship between categorical variables. A p value <0.05 was considered statistically significant.

Results

The mean age of the 69 patients included in the study was 63.2 ± 12.6 years and male to female ratio was 5.3. No differences were found between the case and control groups in terms of age and gender (p = 0.469 and p = 0.813, respectively; Table 1). Of the case group, 28.9% (20/69) of the patients tested positive for HPV-DNA. This ratio was 8.7% (6/69) in the control group. HPV-DNA positivity was significantly higher in the case group compared to the control group. The OR was 4.24 (95% CI 1.63–12.34). In the case group, 53.6% (37/69) of the patients had high-grade UCB and 46.4% (32/69) of the patients had low-grade UCB. No statistically significant relationship was found between HPV-DNA positivity and tumor grade (p = 0.36). In the case group, 65.2% (45/69) of the patients had a history of smoking. No statistically significant relationship was found between smoking and HPV (p = 0.10). In the case group, high-risk and low-risk HPV types were identified in 90% (18/20) and 10% (2/20) of the patients respectively. Multiple types of HPV-DNA were detected in 4 patients in the case group and in 1 patient in the control group. The distribution of patients according to the HPV types determined in the case and control groups is presented in Table 2.

Discussion

Being the fourth most common malignancy in males and the eighth most common malignancy in females, bladder cancer causes approximately 170,000 deaths per year worldwide [11]. The main risk factors for bladder cancer are smoking, occupational exposure to chemicals

such as aromatic amines and hydrocarbons, age, ethnicity, and parasitic bladder infections in some geographic regions. In the last 3 decades, the incidence of bladder cancer has increased significantly. Despite all efforts, it is still hard to foresee the prognosis and to determine the optimal treatment method in patients with bladder cancer.

More than 90% of the patients with bladder cancer are diagnosed with urothelial carcinoma, and the remaining are diagnosed with squamous cell carcinoma or adenocarcinoma. At the moment of diagnosis, approximately 75% of patients with UCB have non-muscle invasive bladder cancer [12].

The most effective treatment for non-muscle invasive bladder cancer is intracavitary immunotherapy. Being a popular treatment method for nearly 40 years, intravesical Bacillus Calmette-Guerin (BCG) administration is one of the most successful applications of immunotherapy in oncology. Although the mechanism of this treatment is not fully elucidated, the administration of intravesical BCG is believed to increase the expression of cytokines in the urine and in the bladder wall, resulting in a massive local immune response characterized by the accumulation of granulocytes, mononuclear cells, and dendritic cells. The high amounts of interleukin-12 detected in the urine of patients who receive BCG therapy induce interferon gamma, which is a powerful promoter of T-helper cell response. Studies show that the therapeutic effect of BCG is regulated by T-helper cell immune response [13]. BCG therapy is much more effective than intracavitary chemotherapy in the treatment of stage T1 UCB in terms of recurrence and progression. On the other hand, the number of cases who are unresponsive BCG therapy is quite high. The high percentage of adjuvant intravesical BCG therapy failure and high recurrence rates constitute an important problem. The reasons behind responsiveness or non-responsiveness to BCG therapy have not been determined yet; also, it is not clear which patients should receive BCG therapy.

Immune response to HPV infection is usually delayed compared to other viral infections because HPV evades the host immune response. The expression of the HPV antigens depresses the innate immune response, facilitating the evasion of the virus from the immune system. Because the HPV infection does not induce necrosis, cytolysis, or inflammation, it does not promote the local release of proinflammatory cytokines [14]. The E6 and E7 oncoproteins inhibit the IFN response by interacting with various molecules of the signal transduction pathway [15]. In addition, HPV does not cause viremia. As a result, there are no danger signals that alert the immune system to develop an effective response to fight the infection [16]. Considering the fact that BCG therapy increases the local immune response, we hypothesize that in HPV-associated UCB, BCG therapy may promote an immune response, which the host is incapable of generating by itself. This immune response may also be effective in preventing the recurrence and progression of HPV-associated UCB. Further studies that compare the effects of BCG therapy on tumor progression and recurrence in HPV(+) and HPV(–) UCBs are needed to test this hypothesis. This way, the role of HPV as a prognostic factor in UCB may also be determined. It should also be kept in mind that HPV positivity is a good prognostic factor in cervical and anal carcinomas [17].

The relationship between HPV and bladder cancer has been investigated for about 3 decades. The first meta-analysis on this topic, which was a case-control meta-analysis by Wiwanitkit [18], consisted of only 5 studies and concluded that HPV is an important risk factor for bladder carcinoma. In the meta-analysis by Gutirrez, which was conducted in the following year, it was emphasized that patient outcomes could vary depending on the methods used and that it is difficult to reach a definitive conclusion due to the inadequacy of case-control studies. In the meta-analysis by Li et al. [19] which was conducted in 2011, the prevalence of HPV in patients with bladder cancer was found to be 16.88% and most HPVs were identified as high-risk HPV. In the 21-study meta-analysis by Jimenez-Pacheco et al. [20], a moderate relationship was found to exist between bladder cancer and HPV (OR 2.19; 95% CI 1.40–3.43).

In the majority of studies indicating that there is no relationship between HPV and UCB, HPV-DNA investigation has been performed in formalin-fixed, paraffin-embedded (FFPE) tissue specimens. It is noteworthy that HPV-DNA positivity rates were 0% in most of these studies. Formalin fixation and paraffin embedding is a standard method for preserving archived pathological specimens. FFPE tissue specimens can also be used as a source of DNA [21]. However, DNA extraction from an FFPE tissue sample poses a challenge. Formaldehyde, the active ingredient of formalin, causes cross-linking between nucleic acids and proteins, which leads to nucleic acid fragmentation during the fixation process [22]. In their meta-analysis, Li et al. [19] emphasized on this situation. They stated that the prevalence of HPV-DNA was found to be higher in studies using fresh tissue compared to studies using fixed tissue, and concluded

that it should be kept in mind that fixed tissues may yield false negative results [19]. In the epidemiological studies, the global prevalence of HPV was reported as 11.7% [1]. On the other hand, in 3 different studies conducted in 3 different geographic regions, the 125-patient study by Ben Selma et al. [23], the 108-patient series by Chang et al. [24], and the 108-patient series by Knowles [25] none of the FFPE tissue specimens tested positive for HPV-DNA. In our opinion, it is likely that these were false negative results.

Although in situ hybridization is a successful method for detecting HPV-DNA, PCR assay is accepted as the gold standard. By using multiple degenerate primer pairs in the amplification reaction, the PCR assay can be easily modified to identify most HPV types associated with anogenital infections. Given the above-mentioned drawbacks of using FFPE tissue for HPV-DNA detection, we preferred to analyze fresh samples. As recommended by the manufacturer, both urethral swab and first void urine samples were used to detect HPV-DNA because urethral swab and first void urine samples are easy to collect in both males and females.

In the past, PCR-based urine assay was not a preferred method to detect HPV infections because it had a low sensitivity. However, with the advances in PCR technology in recent years, the sensitivity of PCR-based assays for the detection of HPV-DNA in urine samples has increased [26]. In the study by Tanzi et al. [27], HPV-DNA detection by PCR-based urine assay was found to have a sensitivity of 98.6% and a specificity of 97.4% compared to cervical smear testing. Regarding the high concordance rate with the cervical smear results, the researchers concluded that a urine-based PCR assay is a suitable and effective screening tool. In the study by Cai et al. [28] where paired tumor tissue and urine samples were analyzed for the presence of HPV-DNA in patients with urothelial carcinoma, no differences were found between the frequencies of HPV-DNA presence in tissue and urine samples.

Tumor grade has an important role in the progression of bladder cancer. Previous studies have reported controversial results regarding the relationship between tumor grade and HPV. Tenti et al. [29] found a correlation between HPV-DNA and low-grade tumors, whereas Cai et al. [28] found a correlation between HPV-DNA and high-grade tumors. On the contrary, in our study, no statistically significant relationship was found between tumor grade and the presence of HPV-DNA. In light of these 3 different results, large-scale studies are needed to elucidate the relationship between HPV infection and tumor grade.

In this study, the mean age of the patients with UCB was 63.2 years and the male to female ratio was 5.3. Even though UCB may occur in all age groups, it mostly affects middle- and old-age patients. It is more common in males. Most epidemiological studies have reported that the prevalence of HPV among healthy males, who are regarded only as a reservoir for the virus, is as high as the prevalence of HPV among healthy women [30]. The prevalence of HPV has been reported to be 44.8% in the 20–24 age group [31] and 6.7% in patients over 60 years [32]. The high global prevalence of HPV in the young population despite preventive medical measures indicates that HPV-related cancers may increase further in the years to come. Given the fact that bladder cancer is frequently seen after the 6th decade, it should be kept in mind that the young population infected with HPV is a serious candidate for bladder cancer.

Another remarkable point in our study is the distribution of HPV types detected in patients with UCB. As is known, type 16 and type 18 are the most predominant high-risk types. HPV Types 16 and 18 are responsible for most HPV-related cervical, anal, and penile carcinomas [3, 16]. In previous studies examining the relationship between HPV and bladder cancer, it has been reported that HPV Types 16 and 18 are the 2 most frequently isolated types. On the other hand, paralleling the development of PCR technology, which has enabled the detection of a higher number of genotypes, a wider variety of high-risk genotypes have been detected in recent studies [28]. In our study, types 16 and 18 were detected only in 22.2% of the cases with UCB. We believe that this finding is important in terms of showing the diversity in high-risk HPV types. Future studies investigating a wider spectrum of HPV genotypes will shed more light on the relationship between HPV and UCB. Considering the results of this study, we think that determining which HPV types are associated with bladder cancer will be helpful in directing preventive medicine applications in terms of vaccine development.

There are some limitations in this study. Although the number of cases and results are statistically significant, a higher number will enhance the significance of the study.

Conclusion

This case-control study has shown that there is a significant relationship between UCB and HPV infection and this association is particularly between high-risk HPV genotypes, whereas there was no association be-

tween tumor grade and HPV infection. In future, follow-up studies are needed to reveal the prognostic effect of HPV infection on UCB.

Ethical Approval

The study protocol was approved by the local Ethics Committee.

Disclosure Statement

No potential conflict of interest was disclosed by the authors.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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