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Human papillomavirus and non-muscle invasive urothelial bladder cancer: Potential relationship from a pilot study

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Abstract. The relationship between urothelial bladder cancer and high-risk human papillomaviruses (HR-HPV) is still a poorly understood entity, even if some studies have supposed a probably correlation. The aim of the present study was to assess the potential relationship between the presence of HR-HPV and non-muscle invasive urothelial bladder cancers (NMIBC). One hundred and thirty-seven subjects (78 patients affected by NMIBC and 59 controls) were recruited in this prospective study. HR-HPV DNA was evaluated both in urine and tumour tissues. Data from patients were compared with data from controls. The relationship between patients and controls, in terms of HR-HPV presence was performed. The relationship between all pathological data and HR-HPV presence in patient group was carried out. HR-HPV DNA in tissue was found in 27 of 78 (34.6%) tumour samples and in 6 of 59 (10.1%) specimens from TUR-P, with a statistically significant difference ($p=0.0009$; dF=1; $\chi^2=10.98$). HR-HPV DNA in urine was found in 36 of 78 (46.1%) samples obtained from patients, whereas in only 8 of 59 (13.5%) samples from controls ($p<0.0001$: dF=1; $\chi^2=16.37$). A statistical significant difference in terms of HR-HPV frequency between high-grade and low-grade urothelial bladder cancer, was found ($p=0.032$; RR=0.52 - 95% CI 0.27-0.93; OR=0.34 - 95% CI 0.13-0.90). In conclusion, this study highlights the correlation between urothelial bladder cancer and high-risk type HPV infection, suggesting the potential etiopathogenetic role of HR-HPV in urothelial bladder cancer development.

Introduction

Approximately 67,160 new bladder cancer cases are expected in the United States in 2007 with 13,750 estimated deaths (1). Moreover, the high recurrence rate and the percentage of adjuvant intravesical therapy failure contribute to bladder carcinoma becoming a social problem (2). In addition, following aspects of the urothelial bladder carcinoma natural history are partially unknown: i) whether a genetic factor exists which determines its predisposition (although there are several oncogenes involved), ii) which are the major risk factors involved in its genesis preventing us from putting into practice an effective prevention campaign. Among all etiopathogenetic factors and co-factors that contribute to urothelial bladder cancer development, we focused our attention on the role of human papillomavirus (HPV), which has been considered as a possible aetiologic agent (3). HPV is one of the most common sexually transmitted infections worldwide and it is associated with lesions ranging from benign cutaneous warts to malignancies, like cervical, penile or anal cancers (4). About 13 different HPV genotypes are associated with malignancy and are referred to as high-risk HPV (HR-HPV) (5,6). The oncogenic potential of HR-HPV types lies in the oncoproteins E6 and E7, which bind to and modulate a number of different gene products, in particular, the tumour suppressors p53 and pRb. These interactions lead to a disturbance of cell cycle control and a deficiency in DNA repair, resulting in genomic instability and an increased risk of malignant transformation (7-9). Even if, the probable relationship between HPV and urothelial bladder cancer is based on the HPV epithelial tropism and the physical closeness of the bladder with the mucosa that covers the urogenital tract, this relationship is not clear and, then, HPV prevalence in subjects with bladder carcinoma oscillates between 0 and 100% in previous studies (3). Moreover, Gutiérrez and co-workers, by using a meta-analysis study, stated that the relationship between bladder cancer and HPV depends on the method used and in the literature there are insufficient cases and samples compared to controls and all studies rely on a combination of various microbiological techniques in the same patient and sample, making it difficult to draw any definite conclusion (10). On the other hand, Jiménez Pacheco *et al* found a clear association between HPV and the bladder cancer (3), by means of a current literature revision. In our study, we have set forth to get further insights into the possible environmental factor that would act as a co-factor in the development of urothelial bladder cancer. The aim of the present study was to evaluate

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the presence of HR-HPV DNA in urine and tissue samples from urothelial bladder cancer patients, compared with a group of controls, in order to assess a potential relationship between HR-HPV and non-muscle invasive urothelial bladder cancer.

Materials and methods

Study design. All consecutive patients, who had undergone transurethral resection of a bladder tumour (TUR-BT) at the same urological unit between December 2005 and January 2007, were selected for this prospective pilot study. As a control group, 59 subjects were recruited from among all the patients attending the same urological clinic for other non-malignant urological diseases over the same period, matched for age, gender and risk factors. All controls are affected by bladder outlet obstruction due to benign prostatic hyperplasia and underwent transurethral resection of the prostate (TUR-P).

Eligibility for the study. Inclusion criteria were histologically demonstrated bladder tumours. Exclusion criteria were other neoplastic diseases, upper urinary tract tumours and urinary tract infections. Patients who had undergone surgery for either benign prostatic obstruction or bladder tumours were excluded, along with patients affected by major concomitant diseases. Only patients with non-muscle invasive bladder cancer (NMIBC) were enrolled. Patients with histologically documented carcinoma *in situ* were excluded. Moreover, all smoker subjects were excluded, in order to avoid confounding factors in the HR-HPV and bladder cancer relationship analysis. The local research ethics committee approved the present study. Written informed consent was obtained from all patients before surgery. The study was conducted in line with Good Clinical Practice guidelines, with the ethical principles laid down in the latest version of the Declaration of Helsinki and with the applicable laws and regulations of the Italy, where the study was conducted.

Sample collection and HPV DNA analysis. A sample of 150-300 ml of early morning, spontaneously voided urine was collected before surgery and sent for HPV DNA analysis. Tumour and tissue samples, obtained from surgery were used for pathological examination. From each control subject, a sample of apparently normal bladder mucosa was taken and sent for HPV DNA analysis. Then, a sample of bladder cancer from patients and a sample of apparently normal bladder mucosa from controls were analysed. Specimens were sent also for HPV DNA analysis without any clinical or pathological reference and analyses were, then, performed in blinded fashion. Each sample was sent for HPV DNA analysis to the Laboratory under refrigerated conditions and with RNAlater storage solutions (Qiagen GmbH, Hilden, Germany), in order to avoid any nucleic acids degradation. All biological analyses have been conducted according with our previous studies (11,12).

HPV DNA extraction and amplification. The DNA extraction and purification from all biologic materials was performed by DNeasy Tissue kit (Qiagen GmbH). Pellet from 200 μl of urine sample was subjected to DNA purification according to the manufacturer's instructions. In order to control for quality of DNA preparations, integrity and presence of an adequate quantity of DNA, each sample was checked by PCR for human ß-globin DNA. The PCR mix contained 2 μl of DNA samples, reaction buffer 1X, 0.8 U Taq polymerase (DyNazyme II DNA Polymerase, Finnzymes Oy), 10 pmol of each primer (primers BG4 and BG5), deoxynucleotide triphosphate 0.075 mM and water to a final volume of 25 μl. PCR conditions were as follows: 1 cycle at 95˚C for 4 min, then 30 cycles at 55˚C for 40 sec, 72˚C for 1 min and 92˚C for 30 sec. Amplification products, with the expected size of 408 base pair, were separated by gel electrophoresis on 1.5% agarose and observed under UV light after staining with ethidium bromide. The presence of HPV was investigated by Alpha Watch HPV (Alphagenics Diaco Biotechnologies Srl, Trieste, Italy), as previously described (11,12).

HPV genotyping. HPV genotyping has been carried out in accordance with the methods previously described (11,12). The test detects the following types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70, 71, 73, 74, 82. As HR-HPV we considered the following types, in according to Saslow and co-workers: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82 (13). Laboratory evaluation was performed by the same biologist (F.M.).

Histopathology. Tumour stage and grade were classified according to the 1998 WHO classification and the 1997 TNM classification of malignant tumours, as defined by the International Union Against Cancer (5th edition). Pathological evaluation was performed by a single genito-urinary pathologist (G.N.).

Statistical analysis. As null hypothesis, we assumed that there was no difference between patients and controls in terms of HR-HPV DNA presence in urine and tissue samples. Fisher's exact test and χ^2 test were used to assess the significance of all parameters, with $p<0.05$ accepted as significant. Moreover, the relationship between presence and typing of HPV infection and clinicopathologic features was studied using all analyzable samples. All statistical analyses were performed using StatPlus:mac 5.5.5 for Apple-Macintosh (Analyst Soft, 2001-2009, USA). The present study has been designed as an exploratory study in order to provide adequate, approximate information about variances and correlations needed for analysis of power and sample size for the future full-scale study. Therefore, the sample size calculations were not carried out.

Results

Eighty-three patients and 61 controls were enrolled. However, in 7 of 144 samples, presence of the internal control could not be detected and no amplifiable DNA was found, thus these patients were excluded from the analysis. Finally, 78 patients and 59 controls were analyzed. All patient and control characteristics, and clinical and laboratory data are described in Table I.

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Summary of clinical and pathological patient and ıta.

Patient and control characteristics		p-value
No. of patients	78	
No. of controls	59	
Median patients age (range)	73.9 (52-81)	0.49
Median controls age (range)	70.2 (58-76)	
Smokers (patients)	-	1
Smokers (controls)	-	
Gender (patients)		
Male	74 (94.8)	
Female	4 (5.2)	0.53
Gender (controls)		
Male	59 (100.0)	
Patient characteristics, pathological and clinical data		
No. of recurrence/year		
1	27 (34.6)	
2	33 (42.4)	
≥3	18 (23.0)	
No. of lesions		
1	35 (44.8)	
2	21 (27.0)	
≥3	22 (28.2)	
Diameter of lesion (if multiple, diameter of the largest) (cm)		
<3	62 (79.5)	
≥3	16 (20.5)	
Stage		
pTa	52 (66.6)	
pT1	26 (33.4)	
Grade		
Low grade	45 (57.6)	
High grade	33 (42.4)	
Previous intravesical therapy		
Chemotherapy	38 (48.7)	
BCG[a]	15 (19.3)	
No treatment	25 (32.0)	
Control clinical data		
Urological diseases		
TUR-P[b]	59 (100)	

The table shows the patient and control characteristics, clinical and laboratory data. [a]Bacillus Calmette-Guérin; [b]Transurethral resection of the prostate.

Table II. Distribution of HPV genotypes among patients and controls.

	Patients		Controls	
	Urine	Tissue	Urine	Tissue
HPV types				
6	4	5	4	3
11	4	3	2	1
16	**6**	**4**	**2**	**1**
18	**8**	**6**		**1**
26			1	
31	**4**	**3**		
33	**4**	**2**	**2**	**2**
35				
39	2			
40		1	1	
43		1		
44			1	
45	**5**	**5**		
51	**2**	**2**	**1**	
52				
53				
54	2			
56				
58	**4**	**3**		
59			**3**	**2**
66	3	4	4	6
68				
69				
70				
71				
73	**3**	**2**		
74				
82				

HPV DNA and genotyping results

Urine samples. The presence of HPV DNA was found in 51 out of 78 (65.3%) patients and in 21 out of 59 (35.5%) controls. HPV genotyping showed the presence of HR-HPV in 36 out of 51 patients (70.5%) and 8 out of 21 (38.0%) controls, with a statistically significant difference ($p<0.0001$: dF=1; $\chi^2=16.37$). In particular, in the patients group, the frequencies of HR-HPV types are as following: 16 (16.8%), 18 (22.2%), 31 (11.1%), 33 (11.1%), 45 (13.9%), 51 (5.5%), 58 (11.1%) and 73 (8.3%). Table II shows the HPV genotyping results among patients and controls.

Tissue samples. The presence of HPV DNA was reported in 41 of 78 (52.6%) tumour samples and in 16 of 59 (27.1%) specimens from TUR-P. Twenty-seven out of 41 (65.8%) patients were considered as HR-HPV, as showed in Table II. The HR-HPV type frequencies are as follows: 16 (14.8%), 18

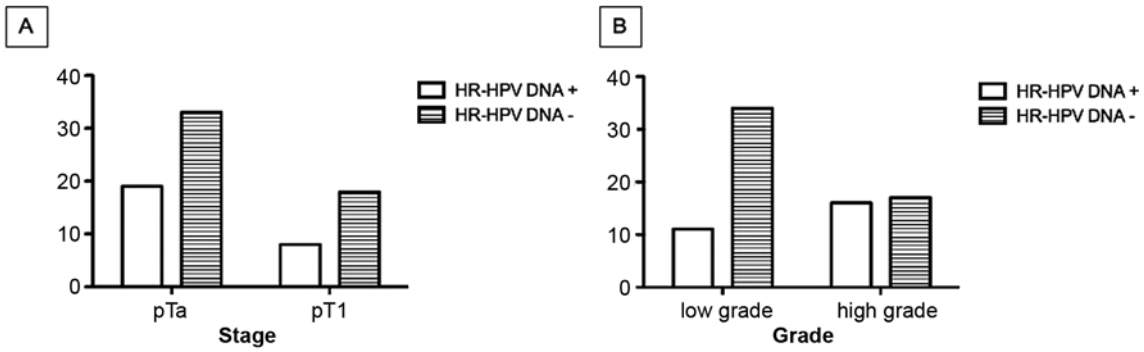

Figure 1. Distribution in stage (A) and grade (B) among all bladder cancer patients in accordance to high-risk human papillomavirus genotypes. HR-HPV$^+$, high-risk human papillomavirus-positive patients. HR-HPV$^-$, high-risk human papillomavirus-negative patients.

(22.2%), 31 (11.1%), 33 (7.4%), 45 (18.6%), 51 (7.4%), 58 (11.1%) and 73 (7.4%).

In the control group, only 6 out of 16 subjects (37.5%) were considered as HR-HPV. The statistical difference between the two groups was significant (p=0.0009; dF=1; χ^2=10.98).

Correlations between HR-HPV and clinicopathological factors in bladder cancer patients. No significant difference in terms of HR-HPV frequency between pTa and pT1 urothelial bladder cancer was found, in tissue sample analysis (p=0.80; RR=1.18 - 95% CI 0.60-2.34; OR=1.29 - 95% CI 0.47-3.35) (Fig. 1A). On the other hand, a statistically significant difference in terms of HR-HPV frequency between high-grade and low-grade urothelial bladder cancer, was found (p=0.032; RR=0.52 - 95% CI 0.27-0.93; OR=0.34 - 95% CI 0.13-0.90) (Fig. 1B).

No difference between the frequency of HR-HPV DNA detected in tissue and those detected in urine samples, both in patients and controls, was found (p=0.19; p=0.77).

Discussion

Tekin *et al*, recently, stated that HPV did not seem to be related to the aetiology of bladder tumour due to its low prevalence reported in several previous studies (14), highlighting the possibility, as observed in cervical cancer studies by PCR, that positive results may be caused by superficial contamination of the tissue with HPV (15). Furthermore, Tekin *et al* suggested that in order to eliminate the possibility of contamination, future studies must be designed by using *in situ* PCR techniques, including samples taken from tumour and normal bladder mucosa in the same patient (14). In the present study, in line with these suggestions, we analyzed samples taken both from tumour and urine, in order to eliminate the possibility of contamination and to demonstrate the feasibility and usefulness of HR-HPV DNA detected in the urine samples. However, in 137 subjects, we found a statistically significant difference in terms of HR-HPV DNA presence both in tumour and urine samples between patients and controls. The finding of HR-HPV DNA in tissue samples obtained from NMIBC patients is not a clear demonstration of the etiologic role of HR-HPV in urothelial bladder cancer development but should be considered a background to plan future studies which can demonstrate this association. The relationship between HR-HPV and bladder cancer is still unclear. Several authors stated that HR-HPV should be considered a co-factor in urothelial bladder cancer development. Therefore, Lopez-Beltran *et al* (16) and co-workers found a possible additional prognostic value of viral infection in bladder cancer, as reported also by Furihata *et al* (17) who found a significantly worse survival in HPV DNA positive patients. In addition, we found a statistical significant difference in terms of HR-HPV frequency between high-grade and low-grade urothelial bladder cancer. Several authors reported contrasting results. Moonen and co-workers have, recently, also found a higher infection rate in high-grade tumours (9). Moreover, the same authors stated that the increase in infection rate in high-grade tumours suggests a relation between tumour grade and high-risk HPV infection (9). On the other hand, Tenti and co-workers found that the prevalence of HPV 16 and/or HPV 18 infection was significantly higher in low-grade than in high-grade tumours (18). The relationship between high-grade urothelial bladder cancer and HR-HPV presence should be related, as suggested by Moonen *et al* (9), to the fact that HR-HPV types stimulate degradation and deactivation of protein associated with the p53 tumour suppressor gene via the ubiquitin-dependent pathway. In addition, Soulitzis *et al* reported HPV prevalence of 12% in a group of bladder cancer patients and an interesting correlation between HPV-18 and Arg/Arg genotype of the p53 codon 72 homozygosity (19). The same authors highlight the role of HPV as a significant factor in the development of a small percent of the tumours (19). However, several biases related to samples collection and laboratory analysis should be taken into account in interpretation. In bladder cancer group, we have found that HPV 18 was the most frequent type, both in tissue and in urine samples (22.2%). These results are in line with other previous reports (20). Moreover, we found HPV 45 both in tissue (18.6%) and in urine samples (13.9%). For this reason it could be considered a new emerging pathogen. Finally, the absence of statistical difference between HR-HPV DNA frequency in tumour and urine should be considered an important methodological finding due to the fact that, if the etiologic role of HR-HPV in urothelial bladder cancer development will be demonstrated, this test should be considered in the NMIBC screening. However, several points must be considered: should patients

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r developing urothelial bladder cancer and, for this reason, undergo other diagnostic exams, such as urinary cytology or ultrasonography? Should patients in follow-up for previous bladder cancer and positive for the presence of HR-HPV DNA undergo stricter follow-up? Should HR-HPV have a similar role in bladder carcinogenesis as in cervical cancer to plan a future-screening program? However, on the basis of high rate of HPV positivity in NMIBC cases, comparing with control group, the present report supports a probable etiologic role of HPV in bladder carcinogenesis.

In conclusion, the present study highlights the correlation between urothelial bladder cancer and high-risk type HPV infection, suggesting the etiologic role of HR-HPV in urothelial bladder cancer development. Moreover, we hypothesize the feasibility of HPV DNA detection in urine samples as a clinical marker in association with standard clinical factors for planning a more appropriate follow-up schedule in urothelial bladder cancer patients.

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