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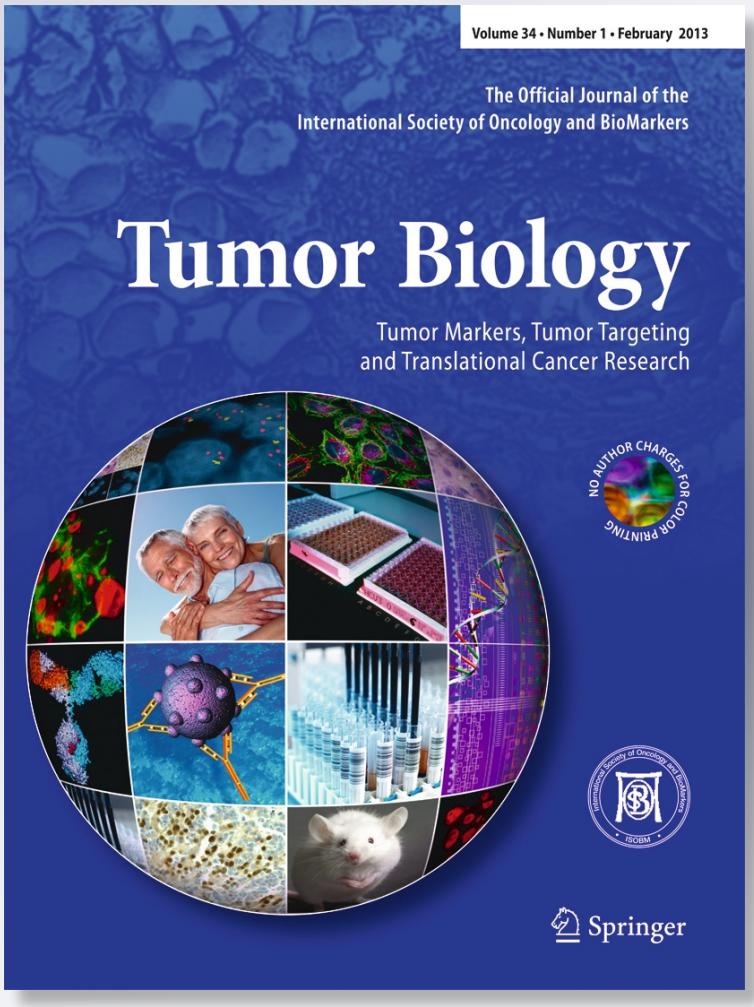

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RESEARCH ARTICLE

Association of human herpes, papilloma and polyoma virus families with bladder cancer

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Abstract The aim of the present study was to assess the possible etiologic role of human papillomavirus (HPV), human herpes virus (HHV) and the human polyoma virus families (BKV and JCV) in the tumourigenesis of bladder cancer. Thirty biopsy specimens from patients with different grades and stages of bladder cancer, who underwent transurethral bladder cancer resection, and 30 normal bladder mucosa specimens were analysed using polymerase chain reaction (PCR) for the detection of the above three virus family members. The presence of HPV was determined in all specimens with nested PCR and real-time quantitative PCR. All cancerous specimens, including the control group, were found to be negative both by PCR and real-time qPCR for the presence of HPV DNA, whilst all samples examined by PCR tested negative for the presence of HSV-1,2 Varicella zoster virus and HSV-7 DNA. Cytomegalovirus, HHV-6 and HHV-8 exhibited similar incidence in sample positivity in both cancerous and healthy tissues. EBV showed a higher prevalence in bladder cancer specimens compared to healthy tissue (p=0.048), whilst BKV and JCV were detected only in tumour samples. The presence of EBV in a significant proportion of bladder tumours indicates the etiological role of this virus in cancer tumourigenesis.

Keywords Herpes virus · Papilloma virus · Polyoma virus · PCR · Bladder cancer

Abbreviations

HPV	Human papillomavirus
HHV	Human herpes virus
HPyV	Human polyoma virus
VZV	Varicella zoster virus
CMV	Cytomegalovirus
EBV	Epstein–Barr virus
HSV	Herpes simplex virus
HHV	Human herpes virus
PCR	Polymerase chain reaction
RT qPCR	Real-time quantitative polymerase chain reaction
TCC	Transitional cell carcinoma
SCC	Squamous cell carcinoma
NPC	Nasopharyngeal carcinoma

Background

Vesical neoplasms account for nearly 2 % of all cancers and are the second most common malignancies of the genitourinary tract. Males are affected twice as often as females. More than 90 % of these tumours are transitional cell carcinomas (TCCs), whilst the remaining 10 % are squamous cell carcinomas (SCCs) or adenocarcinomas [1]. Most TCCs (over 80 %) do not invade the bladder wall at the time of diagnosis. The etiology of TCC remains unknown, although there is strong association with chronic cigarette smoking and exposure to chemicals prevalent in dye, rubber, leather,

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Georgios I Panagiotakis and Danae Papadogianni have equally contributed to this work.

G. I. Panagiotakis (✉) · D. Papadogianni · M. N. Chatziioannou · I. Lasithiotaki · D. A. Spandidos
Laboratory of Clinical Virology, Medical School, University of Crete,
P.O. Box 2208, Heraklion 71003 Crete, Greece
e-mail: gpanagiotakis@hotmail.com

D. Delakas
Department of Urology, Asklipeiion Hospital,
Voula, Athens, Greece

paint and other industrial components [2]. Common use of artificial sweeteners such as cyclamates and saccharin has also been implicated.

Human papillomavirus (HPV) is a small double-stranded DNA virus that comprise a family of more than 130 genotypes [3]. HPV has been implicated in several human cancers, particularly in tumours of the cervix, the anogenital region, the skin, as well as the head and neck. It has been strongly suggested that HPV could also play a role in the human urinary bladder tumourigenesis since HPV causes condylomata acuminata [4] and urothelial malignancies have been reported in association with extensive urethral and bladder condylomata [5, 6].

The herpes virus superfamily contains eight different types of viruses, and many of them have already been detected in human TCC specimens [7]. Herpes simplex virus (HSV-1, HSV-2) can potentially infect any site of the body, but infections of the skin, genital, oral mucosa, eyes, anus, rectum and the central nervous system are more common. It has been suggested that HSV-1 has the ability to transform cells in vitro, possibly creating an oncogenic potential in vivo [8], whilst HSV-2 had been suggested as a possible cofactor in the development of cancer of the uterine cervix [9, 10]. Varicella zoster virus (VZV or HHV-3) is responsible for skin lesions and manifestations of the central nervous system. Epstein–Barr virus (EBV or HHV-4) is associated with a great number of malignancies including Burkitt's lymphoma, undifferentiated NPC, B cell lymphoma in immunodeficient patients and Hodgkin's disease. EBV has been found to replicate in the cervical epithelium, and genomic sequences have been detected in cervical biopsies from patients with invasive carcinoma, thus suggesting the possible role of EBV in genital carcinoma [11, 12]. EBV DNA has also been detected in the urethra of men with gonorrhea, raising questions concerning the spread of this virus to the urogenital tract [13]. Human cytomegalovirus (CMV or HHV-5) causes mononucleosis-like disease, neonatal disease, systemic infection in immunocompromised patients and central nervous system disease, and it has been associated with nephrogenic adenoma [14]. Human herpes virus type 6 (HHV-6) has been detected in a series of patients with bladder cancer in the Mediterranean [15]. Human herpes virus type 8 (HHV-8), also known as Kaposi's sarcoma-associated herpes virus, mainly infects immunodepressed patients [16].

Human polyoma virus (HPyV) is a small, non-enveloped, double-stranded DNA virus. BK polyoma virus (BKV) and JC polyoma virus (JCV) infections occur in childhood, and BKV's role in urothelial carcinoma has been suggested in immunocompetent individuals [17, 18].

The aim of the present study was to assess the possible etiologic role of HPV, EBV, CMV, VZV, HSV, human herpes virus (HHV-6–8) and human polyoma viruses (BKV and JCV) in bladder cancer. We suggest that a deeper understanding of viral infection in bladder cancer may lead to targeted diagnostic procedures and possibly new therapeutic methods.

Materials and methods

Study population

A total of 30 bladder carcinomas were obtained from patients diagnosed with primary urothelial cancer who underwent surgical transurethral resection at the Department of Urology, Asklipeiion General Hospital, Voula, Athens, Greece, between May 2006 and January 2007. Twenty-four (80 %) of the tumours were firstly diagnosed at the time of the resection, whereas six (20 %) of them were recurrent primary carcinomas. Each tumour sample was matched to a non-cancerous specimen derived from normal adjacent bladder tissue. Following surgical resection, all tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until DNA extraction. The median age at surgical resection was 75.5 years (range, 44–86 years). All tumour specimens were histopathologically examined using haematoxylin and eosin-stained slides in order to determine the tumour stage and grade. Tumour grade was determined according to the 1973 WHO and the 2004 WHO/International Society of Urological Pathology classifications. Tumour stage was assessed according to the 2002 American Joint Committee on Cancer staging system. Normal adjacent tissue samples had no contaminating tumour cells, as confirmed by histopathologic examination. The clinicopathological parameters of the study population are listed in Table 1. The Bioethics Committee of the Medical School of the University of Crete approved the current study protocol. Written informed consent was obtained from all the patients or their relatives.

DNA extraction

Tissue specimens were transferred to sterile DNase/RNase-free Eppendorf tubes, where 400 μl lysis buffer (buffer AL, cat. no. 19075, Qiagen®) and Proteinase K to a final concentration of 50–100 μg/ml were added. Samples were incubated for 2–4 days at 60 °C until tissues were lysed. Proteinase K (20 mg) was added to the samples every 8 h. DNA isolation was performed using the standard phenol/chloroform protocol, followed by precipitation with absolute ethanol, washing with 70 % ethanol and resuspension in 50 μl sterile bidistilled water. DNA quantification and determination of DNA purity were assessed by measuring its 260-nm absorbance and its 260:280-nm absorbance ratio, respectively, on a UV spectrophotometer. DNA samples

Table 1 Clinicopathological parameters of the study population

	Bladder cancer (%)
Cases, *n*	30
Age, mean ± SD (years)	72.2±10.6
Gender	
Male	27 (90.0)
Female	3 (10.0)
Stage	
pTa-pT1	18 (60.0)
pT2-pT3	12 (40.0)
Grade (WHO 1973)	
II	10 (33.3)
III	20 (66.7)
Tumour type	
Papillary	23 (76.7)
Non-papillary	7 (23.3)
Recurrence	
Primary	24 (80.0)
Recurrent	6 (20.0)
Smoking habits	
Current smokers	18 (60.0)
Former smokers	8 (26.7)
Non-smokers	4 (13.3)
Occupational exposure[a]	
Yes	19 (63.3)
No	11 (36.7)

[a]To chemicals, paints, pesticides, petroleum, ink, etc.

were stored at −20 °C until polymerase chain reaction (PCR) amplification.

PCR amplification reactions

Both tumour and normal samples were examined for the presence of HPV, HSV-1, HSV-2, CMV, EBV, VZV, HHV-6, HHV-7, HHV-8 and human polyoma viruses BKV and JCV DNA by PCR using the respective set of primers. The primer sequences used have been described previously [19–22] and are listed in Table 2. All samples were examined with the amplification of a 110-bp region of the b2-globuline gene, which served as the housekeeping gene in order to assess the presence of amplifiable DNA in all samples. The first-round PCR products from the EBV and HHV-6 reactions were used as templates for nested PCR using an additional set of inner primers (Table 2). The PCR conditions for human papilloma viruses, herpes viruses and β2m, as previously described [20], are shown in Table 2. Extreme care was taken during the process of DNA extraction and PCR amplification to avoid potential contaminations, whilst a negative control was always included in each reaction.

To ensure that our PCR assay was sensitive enough to detect relatively low levels of viral DNA, as described elsewhere [23], a serial dilution assay was employed. Ten microlitres of PCR, real-time qPCR and restriction fragment length polymorphism analysis products was electrophoresed on agarose gels stained with ethidium bromide (agarose concentration varying from 2 to 3 %, depending on the PCR product size) and photographed on a UV light transilluminator.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reactions (qPCR) for the detection of HPV DNA were performed in a final volume of 20 μl containing 1 μl DNA from tumour and normal samples, 2× KAPA™ SYBR® FAST qPCR Kit Master Mix (containing $MgCl_2$ at a final concentration of 2.5 mM and Rox passive reference dye; Kapa Biosystems, Boston, MA) and 200 nM of each primer ($GP5^+/GP6^+$). After the initial denaturation at 95 °C for 3 min, product amplification was performed for 40 cycles comprising denaturation at 95 °C for 3 s, annealing at 45 °C for 20 s and elongation at 72 °C for 10 s, followed by a melt curve analysis in which the temperature was increased from 60 to 95 °C at a linear rate of 0.2 °C/s. Fluorescent data were collected both during annealing and extension, with two measurements at the annealing step and one measurement at the extension step, and at all times during the melt curve analysis. Real-time qPCR experiments were performed on the Mx3000P real-time qPCR thermocycler using the software version 4.01 (Stratagene, La Jolla, CA).

The analytical sensitivity and specificity of all the assays (PCR, nested-PCR, RT qPCR) as well as the linear dynamic range of detection have been previously described [23].

Statistical analysis

The results were analysed through standard statistical methods applied in case–control studies. The correlation of each of the studied characteristics with viral infection was evaluated using Pearson's χ^2 analysis or Fisher's exact test, where indicated (expected frequencies, <5). Numerical values were expressed as the mean ± SEM, and the differences between means were compared by the two-tailed, unpaired Student's *t* test. Probability values (*p* values) <0.05 were considered statistically significant. The analyses were performed using SPSS software, version 11.5 (SPSS. Chicago, IL).

Table 2 Primer sequences, annealing temperatures and PCR product lengths

Virus	Sequence (5′–3′)	Annealing T_m (°C)	PCR product (bp)
HPV	TTGTTACTGTGGTAGATAC CTTATACTAAATGTCAAATAAAAAG	43×10 40×30	150
HSV-1,2	CAGTACGGCCCCGAGTTCGTGA TTGTAGTGGGCGTGGTAGATG	64	478
CMV	GTCACCAAGGCCACGACGTT TCTGCCAGGACATCTTTCTC	57	167
EBV	Outer primers		
	AGCACCCCCACATATCTCTTCTT CGAGTCATCTACGGGGACACGGA	65	197
	Inner primers		
	GGAGAAGGTCTTCTCGGCCTC TTCAGAGAGCGAGACCCTGC	69	102
VZV	TTGTAGTGGGCGTGGTAGATG ATGTCCGTACAACATCAACT	60	267
HHV-6	Outer primers		
	GACAATCACATGCCTGGATAATG TGTAAGCGTGTGGTAATGGACTAA	58	178
	Inner primers		
	GTTAAATTGATAGTACTTACGTG ATCAAAATATAAAGAGCACAGCA	53	70
HHV-7	GGAAATAGGATCTTTTCAAATTC GTTACTTTCAAAAATGTTTGTCCC	59	122
HHV-8	Outer primers		
	ACTACGACAACCCTCAAAATAG AGTAGAATATCATCCTGTGCG	45	319
	Inner primers		
	TCGCATGGAGGACCTAGTCAATAA GGTTGTAGTCATTCTCGTCCAGGG	55	116
HPyV	AGTGGATGGGCAGCCTATGTA TCATATCTGGGTCCCCTGGA	60	97
β2-m	TCCAACATCAACATCTTGGT TCCCCCAAATTCTAAGCAGA	55	102

Results

Human papillomavirus DNA was detected neither in tumour nor in normal samples by PCR using the $GP5^+/GP6^+$ primers. Following PCR analysis as well as further validation by real-time qPCR analysis (primer sequences shown in Table 2), all samples were examined for the presence of HPV DNA; no tumour or normal sample was found to be positive (Electronic supplementary material (ESM) Additional File 1). Moreover, all tumour and normal specimens were examined for the presence of herpes and polyoma virus DNAs by PCR using the respective set of primers for each virus (Table 2).

Cytomegalovirus DNA was detected in 22 tumour samples (73.3 %) and 23 normal samples (76.7 %; Figs. 1 and 2). EBV DNA was detected in 15 (50.0 %) tumour samples versus 4 (13.3 %) normal samples (p=0.0048; Figs. 1 and 2). HHV-6 was detected in 11 tumour samples (36.7 %) and 11 normal samples (36.7 %; Fig. 1). Paired samples were highest for CMV DNA presence (17 pairs) followed by HHV-6 DNA presence (three pairs) and EBV DNA presence (one pair), with statistical significance for CMV versus HHV-6-positive pairs (p=0.0003) as well as for CMV versus EBV-positive pairs (p<0.0001; Table 3).

HHV-8 DNA was detected in one tumour sample (3.3 %) and two normal samples (6.7 %; Fig. 2). Four tumour samples (13.3 %) were positive for HPyV DNA (Fig. 1). Seven samples (11.7 %) were negative for all viruses included in the study, four of which were tumour samples (13.3 %) and three of which were normal samples (10.0 %). No sample pair was found positive for HSV-1, HSV-2, VZV, HHV-7, HHV-8 and HPyV (ESM Additional File 2). One sample pair (3.3 %) was negative for all viruses included in the study. The cumulative results of the PCR analyses for both tumour and normal samples are demonstrated in Tables 3 and 4.

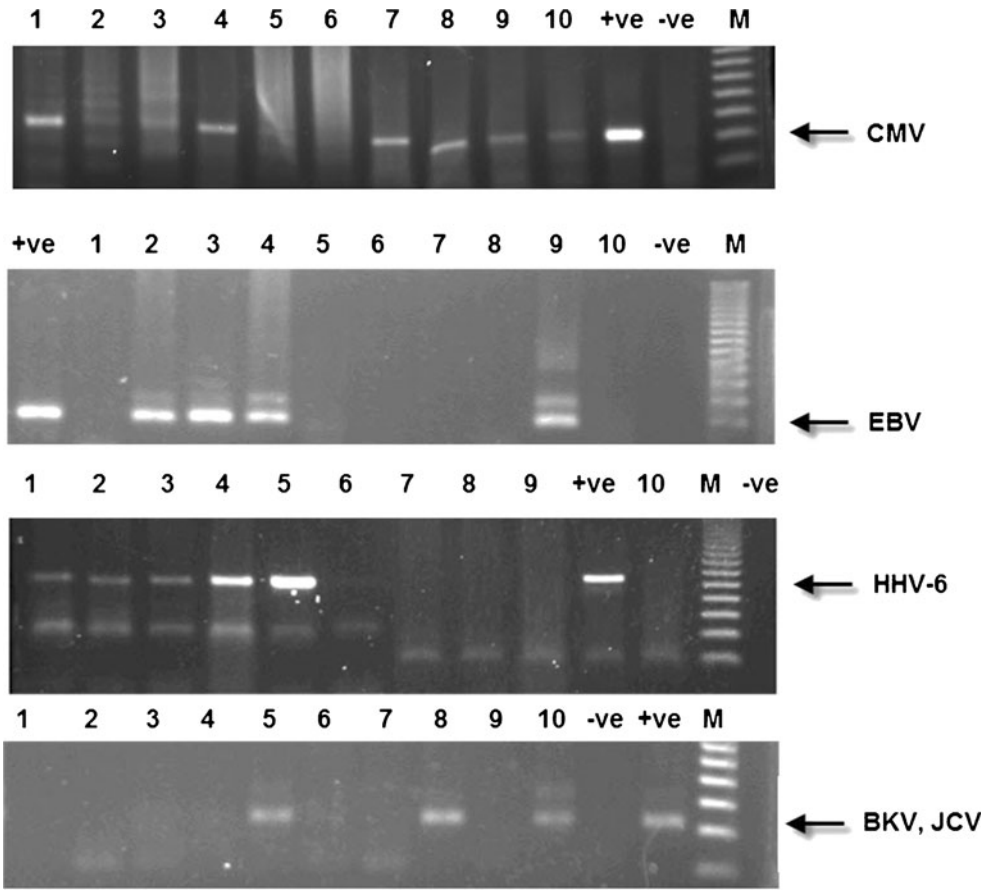

Fig. 1 Detection of CMV, EBV, HHV-6 and polyoma virus DNA using PCR. Samples 1, 3, 5, 7, 9 (tumour) and 2, 4, 6, 8, 10 (healthy specimens)

The simultaneous presence of two or more viruses was detected in 29 samples (48.3 %), 17 of which were tumour samples (56.7 %) and 11 of which were normal samples (36.7 %). The DNAs of two, three or four viruses were detected in 20 (33.3 %), 7 (11.7 %) and 4 (3.3 %) samples, respectively. CMV and HHV-6 co-infection was most predominant in this study (detected in two tumour and seven normal samples), followed by CMV and EBV co-infection (five tumour and two normal samples; Table 3).

Discussion

The incidence of bladder cancer has highly increased during the last decades, whilst the possible role of the viruses has already been examined with some positive results. Bladder cancer incidence rates in Greece have not been officially submitted to the WHO database. The present study contains only 10 % females, which is in accordance with the only study to date, representing the local frequencies for the

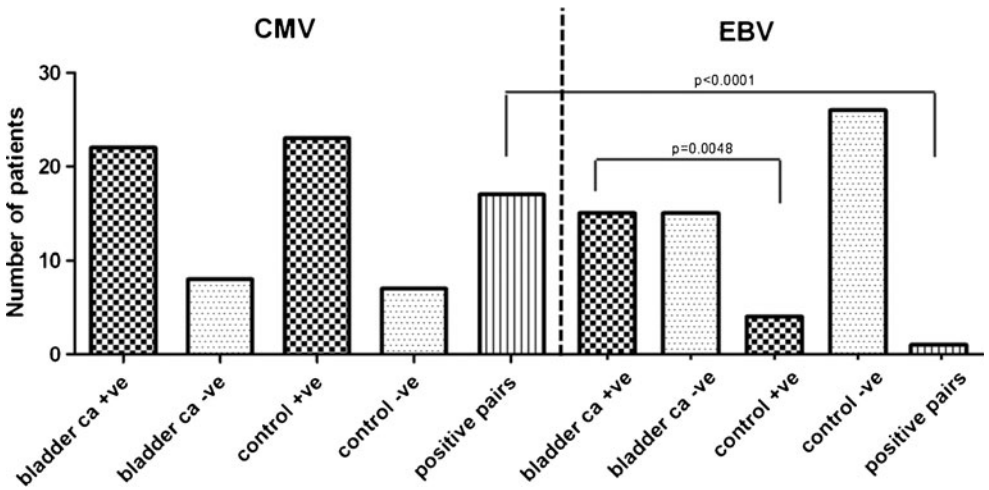

Fig. 2 Graphical representation of CMV and EBV DNA presence in bladder tissue and healthy specimens

Table 3 Cumulative results of the detection of human papilloma, herpes and polyoma viruses in tumour and normal bladder specimens

Virus	Tumour samples (%)	Normal samples (%)	Paired samples (%)	Total virus presence (%)
HPV	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/60 (0.0)
CMV	22/30 (73.3)	23/30 (76.7)	17/30 (56.7)	45/60 (75)
EBV	15/30 (50.0)	4/30 (13.3)	1/30 (3.3)	19/60 (31.7)
HSV	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/60 (0.0)
VZV	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/60 (0.0)
HHV-6	11/30 (36.7)	11/30 (36.7)	3/30 (10.0)	22/60 (36.7)
HHV-7	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/60 (0.0)
HHV-8	1/30 (3.3)	2/30 (6.7)	0/30 (0.0)	3/60 (5.0)
HPyV	4/30 (13.3)	0/30 (0.0)	0/30 (0.0)	4/60 (6.7)

disease [24]. To the best of our knowledge, there is no referred examination combining the herpes virus, HPV and HPyV virus families in bladder cancer with the use of PCR.

Our study was designed to investigate the potential role of DNA viruses HHV and HPyV in urinary bladder cancer. It is of interest that nearly 50 % of the samples were infected by more than one virus. EBV is a human DNA virus already linked to a range of human tumours, whilst CMV and HSV-2 have been demonstrated to display an oncogenic potential in vitro and have been proposed as cofactors for the development of cervical cancer [25]. There are no reports concerning the prevalence of HHV and HPyV genomes in bladder neoplasms. In the present study, CMV DNA was detected in the majority of tumour and normal samples, without any positive correlation of infection and carcinogenesis, as was the case for HHV-6 DNA presence. It is very likely that CMV and HHV-6 infections do not play a critical role in the development and progression of urinary bladder cancer. Notably, CMV infection of both the tumour and healthy sites of the urinary bladder showed a higher prevalence than HHV-6 paired positivity, suggesting a tropism of CMV for the urothelium that has not yet been reported. Meanwhile, EBV DNA presence was abundant in tumour samples compared to normal tissue, with statistical significance (p=0.0048). This finding could promote the hypothesis that EBV, already an etiopathological factor in human cancer, may be involved in the initial cancerous stimuli or in the progression of tumourigenesis in bladder cancer. It has already been suggested that EBV DNA presence in urothelial carcinoma positively correlates with poor differentiation status in a study that provided similar EBV DNA presence to ours [26].

A small yet notable percentage of samples examined (10 %) contained CMV and HHV-6 (6.7 %) and CMV and EBV (3.3 %) sequences, raising the possibility of a synergism between these viruses in urinary bladder carcinogenesis. Such synergism has already been suggested in other human tumours, as in epithelial malignancies of the anogenital tract. In particular, CMV and a HSV-2 have already been implicated in the etiology of human cancer mainly in cervical carcinoma, where their presence was found together with high-risk HPV sequences by different groups [27].

The remaining human herpes virus (HSV-1, HSV-2, VZV, HHV-7, HHV-8) DNA presence or absence analysis did not reveal evidence regarding their involvement in human bladder carcinogenesis, although co-infection with other DNA viruses may facilitate genetic alterations that promote malignant phenotypes.

BKV and JCV infection accounted for four tumour samples in this study, suggesting the possible implication of these HPyV in bladder tumourigenesis. However, recent research [28] shows that HPyV infection prevalence in TCC is similar to controls, whilst another study disproved the causality of BKV DNA presence in most TCC of the bladder [29]. HPyV involvement in TCC may be elucidated by gender and smoking criteria. In our study, all positive BKV and JCV samples came from males, and the majority (75 %) had a positive smoking history. Moreover, the viruses' detection in only tumour-derived samples only suggests that bladder cancer cells are susceptible to polyoma infections compared to healthy cells of the bladder [30], whilst the role of polyoma viruses in bladder cancer remains to be elucidated.

The present study did not detect a correlation between the presence of HPV DNA and bladder cancer using PCR and also the sensitive technique real-time qPCR, with the same general primers for HPV ($GP5^+/GP6^+$). It is the first time that real-time qPCR is used for detecting HPV DNA in patients with bladder cancer. Previous studies of normal and neoplastic tissues of the bladder for the presence of HPV DNA using paraffin-embedded specimens and in vitro gene amplification by PCR showed that the urinary bladder in both genders is another site where infection with the common genital tract HPV may carry a risk of malignant transformation [31]. A similar study [32] analyzing patients with TCC using PCR and probes for 6, 11, 16, 18 and 31 HPV DNA detected HPV DNA in all tumours. Despite these findings, a previous study using PCR techniques showed that human papillomavirus infection is rare in TCC of the bladder [33], whilst another study using in situ hybridization indicated that high-

Table 4 Results of the detection of human papilloma, herpes and polyoma viruses in tumour and normal bladder specimens

Patient no.	HPV PCR	HPV qPCR	CMV	EBV	VZV	HSV-1,2	HHV-6	HHV-7	HHV-8	BKV, JCV
1 tumour	−	−	+	+	−	−	−	−	−	−
1 control	−	−	+	+	−	−	−	−	−	−
2 tumour	−	−	+	+	−	−	+	−	−	−
2 control	−	−	+	−	−	−	−	−	−	−
3 tumour	−	−	+	+	−	−	+	−	−	−
3 control	−	−	+	−	−	−	−	−	−	−
4 tumour	−	−	+	+	−	−	−	−	−	+
4 control	−	−	−	−	−	−	−	−	−	−
5 tumour	−	−	+	+	−	−	−	−	−	−
5 control	−	−	−	−	−	−	−	−	−	−
6 tumour	−	−	−	−	−	−	−	−	+	−
6 control	−	−	−	−	−	−	−	−	−	−
7 tumour	−	−	+	+	−	−	−	−	−	−
7 control	−	−	+	−	−	−	−	−	−	−
8 tumour	−	−	−	−	−	−	−	−	−	−
8 control	−	−	+	−	−	−	−	−	−	−
9 tumour	−	−	−	−	−	−	+	−	−	−
9 control	−	−	+	−	−	−	−	−	−	−
10 tumour	−	−	+	−	−	−	+	−	−	+
10 control	−	−	+	−	−	−	+	−	−	−
11 tumour	−	−	−	−	−	−	−	−	−	−
11 control	−	−	+	−	−	−	+	−	−	−
12 tumour	−	−	−	+	−	−	+	−	−	−
12 control	−	−	−	−	−	−	+	−	−	−
13 tumour	−	−	+	−	−	−	−	−	−	−
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14 tumour	−	−	−	−	−	−	−	−	−	−
14 control	−	−	+	+	−	−	−	−	−	−
15 tumour	−	−	−	+	−	−	−	−	−	−
15 control	−	−	+	−	−	−	+	−	−	−
16 tumour	−	−	+	−	−	−	−	−	−	−
16 control	−	−	+	+	−	−	+	−	−	−
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23 tumour	−	−	+	−	−	−	+	−	−	−
23 control	−	−	+	−	−	−	−	−	−	−
24 tumour	−	−	+	−	−	−	−	−	−	−
24 control	−	−	+	−	−	−	−	−	−	−
25 tumour	−	−	+	−	−	−	−	−	−	+

Table 4 (continued)

Patient no.	HPV PCR	HPV qPCR	CMV	EBV	VZV	HSV-1,2	HHV-6	HHV-7	HHV-8	BKV, JCV
25 control	−	−	−	+	−	−	+	−	−	−
26 tumour	−	−	+	+	−	−	+	−	−	+
26 control	−	−	+	−	−	−	+	−	+	−
27 tumour	−	−	+	+	−	−	+	−	−	−
27 control	−	−	+	−	−	−	−	−	−	−
28 tumour	−	−	+	−	−	−	+	−	−	−
28 control	−	−	−	−	−	−	−	−	−	−
29 tumour	−	−	+	+	−	−	+	−	−	−
29 control	−	−	+	−	−	−	−	−	−	−
30 tumour	−	−	+	+	−	−	−	−	−	−
30 control	−	−	−	−	−	−	+	−	−	−

risk HPV is likely to be a causative agent of some low-grade bladder carcinomas that develop in younger patients [34]. A recent meta-analysis suggests that infection of high-risk HPV types, especially HPV-16, may play a role in bladder carcinogenesis, whilst the authors suggest a moderate geographic variation in HPV prevalence [35]. In this meta-analysis, no Greek study was used, which could answer for the fact that our study deviates from this suggestion, taking into account the different genetic backgrounds, environmental risk factors, and other ethnic and cultural differences. A very recent study in urinary bladder in squamous cell carcinoma, supporting our results, suggests that HPV does not play a role in the development of SCC of the urinary bladder or urothelial carcinoma with squamous differentiation [36]. Moreover, in a series of Tunisian patients, an association between HPV and bladder cancer was proven by the detection of HPV DNA in patients with anogenital cancer, but not in patients with bladder cancer [37]. These results are in accordance with the results of the present study. Interestingly, an Egyptian study [38] showed a significant association of HPV-16, HPV-18, and HPV-52 with bladder cancer, suggesting the hypothesis of a viral synergistic action in bladder carcinogenesis. In addition, such HPV types were associated with TCC tumours of grade 2 stage III, with schistosomal infection and recurrence tendency [38]. It is of note that HPV DNA presence in bladder washes has been positively related to the severity of the clinical outcomes of bladder cancer [39]; however, this pilot study's suggestion does not take in to account the incorporation of the HPV genome, which was the aim of our study. Thus, the authors feel that any comparison between the two would lack value.

A possible correlation between infection with HPV and the development of TCC of the bladder is therefore not unreasonable; that HPV infection of the bladder occurs is certainly true, as condylomata, which are characteristic of HPV infection, have been reported in the bladder. There may be a geographical variation in the incidence of HPV infection of the urinary tract, although evidence for this is lacking. A hypothetical protective factor could be the Mediterranean diet, which is rich in antioxidative nutrients (a diet followed by Greek individuals), in contrast with the Egyptian diet deriving from a Middle Eastern cultural background. This must be proven in the future with other clinical studies.

Conclusion

The absence of HPV DNA in all bladder samples provides evidence that HPV may not be associated with bladder cancer pathogenesis in the Greek population. CMV and HHV-6 showed a similar incidence in both bladder cancer and bladder healthy urothelium, which suggests a tissue-specific sensitivity for these viruses. The presence of EBV in a significant proportion of bladder tumours indicates an etiological role of this virus in cancer tumourigenesis.

Competing interests The authors declare they have no competing interests.

References

1. Pashos CL, Botteman MF, Laskin BL, Redaelli A. Bladder cancer: epidemiology, diagnosis, and management. Cancer Pract. 2002;10:311–22.
2. Kaufman DS, Shipley WU, Feldman AS. Bladder cancer. Lancet. 2009;374:239–49.
3. Mammas IN, Sourvinos G, Michael C, Spandidos DA. Human papilloma virus in hyperplastic tonsillar and adenoid tissues in children. Pediatr Infect Dis J. 2006;25:1158–62.
4. Zderic SA, Carpiniello VL, Malloy TR, Rando RF. Urological applications of human papillomavirus typing using deoxyribonucleic acid probes for the diagnosis and treatment of genital condyloma. J Urol. 1989;141:63–5.

5. Libby JM, Frankel JM, Scardino PT. Condyloma acuminatum of the bladder and associated urothelial malignancy. J Urol. 1985;134:134–6.
6. Mevorach RA, Cos LR, di Sant'Agnese PA, Stoler M. Human papillomavirus type 6 in grade I transitional cell carcinoma of the urethra. J Urol. 1990;143:126–8.
7. Di Paolo JA, Woodworth CD, Popescu NC, Koval DL, Lopez JV, Doniger J. HSV-2-induced tumorigenicity in HPV-16 immortalized human genital keratinocytes. Virology. 1990;177:777–9.
8. Hildesheim A, Mann V, Brinton LA, Szklo M, Reeves WC, Rawls WE. Herpes simplex virus type 2: a possible interaction with human papilloma virus types 16/18 in the development of invasive cervical cancer. Int J Cancer. 1991;49:335–40.
9. Kjaer SK, De Villiers EM, Çaglayan H, Svare E, Haugaard BJ, Engholm G, et al. Human papillomavirus, herpes simplex virus and other potential risk factors for cervical cancer in a high-risk area (Greenland) and a low-risk area (Denmark)—a second look. Br J Cancer. 1993;67:830–7.
10. Schmauz R, Okong P, De Villiers E-M, Dennin R, Brade L, Lwanga SK, et al. Multiple infections in cases of cervical cancer from a high-incidence area in tropical Africa. Int J Cancer. 1989;43:805–9.
11. Landers RJ, O'Leary JJ, Crowley M, Annis P, Burke L, O'Brien D, et al. Epstein–Barr virus in normal, premalignant lesions of the uterine cervix. J Clin Pathol. 1993;46:931–5.
12. Wong KY, Colins RJ, Srivastava G, Pittaluga S, Cheung ANY, Wong LC. Epstein–Barr virus in carcinoma of the cervix. Intern J Gynec Pathol. 1993;12:224–7.
13. Israele V, Shirley P, Sixbey JW. Excretion of the Epstein–Barr virus from the genital tract of men. J Infect Dis. 1991;163:1341–3.
14. Hung SY, Tseng HH, Chung HM. Nephrogenic adenoma associated with cytomegalovirus infection of the ureter in a renal transplant patient: presentation as ureteral obstruction. Transpl Int. 2001;14:111–4.
15. Escudero AL, Luque RJ, Quintero A, Alvarez-Kindelan J, Requena M, Montironi R, et al. Association of human herpesvirus type 6 DNA with human bladder cancer. Cancer Lett. 2005;230:20–4.
16. Russo JJ, Bohenzky RA, Chien MC, Chen J, Yan M, Maddalena D, et al. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci USA. 1996;93:14862–7.
17. Major EO. Human polyomavirus. In: Knipe D, Howley PM, editors. Fields virology, 4th edition, vol. 2 (edited by Lipincott). Philadelphia: Williams & Wilkins; 2001. p. 2175–96.
18. Weinreb DB, Desman GT, Amolat-Apiado MJM, Burstein DE, Jr Godbold JH, Johnson EM. Polyoma virus infection is a prominent risk factor for bladder carcinoma in immunocompetent individuals. Diagn Cytopathol. 2006;34:201–3.
19. De Roda Husman A-M, Walboomers JMM, van den Brule AJC, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol. 1995;76:1057–62.
20. Neofytou E, Sourvinos G, Asmarianaki M, Spandidos DA, Makrigiannakis A. Prevalence of human herpes virus types 1–7 in the semen of men attending an infertility clinic and correlation with semen parameters. Fertil Steril. 2009;91:2487–94.
21. Balis V, Sourvinos G, Soulitzis N, Giannikaki E, Sofras F, Spandidos DA. Prevalence of BK virus and human papillomavirus in human prostate cancer. Int J Biol Markers. 2007;22:245–51.
22. Dokianakis DN, Sourvinos G, Sakkas S, Athanasiadou E, Spandidos DA. Detection of HPV and ras gene mutations in cervical smears from female genital lesions. Oncol Rep. 1998;5:1195–8.
23. Panagiotakis SH, Soufla G, Baritaki S, Sourvinos G, Passam A, Zagoreos I, et al. Concurrent CMV and EBV DNAemia is significantly correlated with a delay in the response to HAART in treatment-naive HIV type 1-positive patients. AIDS Res Hum Retrovir. 2007;23:10–8.
24. Baltogiannis D, Tasos A, Voutsinas G, Dauaher H, Chambilomatis P, Giannakopoulos X. Bladder cancer mortality in the Epirus district population (Greece). Int Urol Nephrol. 2001;32:377–9.
25. Koffa M, Koumantakis E, Ergazaki M, Tsatsanis C, Spandidos DA. Association of herpesvirus infection with the development of genital cancer. Int J Cancer. 1995;63:58–62.
26. Chuang KL, Pang ST, Liao SK, Wu CT, Chang YH, Chuang HC, et al. Epstein–Barr virus DNA load in tumour tissues correlates with poor differentiation status in non-muscle invasive urothelial carcinomas. BJU Int. 2011;107:150–4.
27. Gazzaniga P, Vercillo R, Gradilone A, Silvestri I, Gandini O, Napolitano M, et al. Prevalence of papillomavirus, Epstein–Barr virus, cytomegalovirus, and herpes simplex virus type 2 in urinary bladder cancer. J Med Virol. 1998;55:262–7.
28. Polesel J, Gheit T, Talamini R, Shahzad N, Lenardon O, Sylla B, et al. Urinary human polyomavirus and papillomavirus infection and bladder cancer risk. Br J Cancer. 2012;106:222–6.
29. Rollison DE, Sexton WJ, Rodriguez AR, Kang LC, Daniel R, Shah KV. Lack of BK virus DNA sequences in most transitional-cell carcinomas of the bladder. Int J Cancer. 2007;120:1248–51.
30. Loghavi S, Bose S. Polyomavirus infection and urothelial carcinoma. Diagn Cytopathol. 2011;39:531–5.
31. Anwar K, Naiki H, Nakakuki K, Inuzuka M. High frequency of human papillomavirus infection in carcinoma of the urinary bladder. Cancer. 1992;70:1967–73.
32. Boucher NR, Scholefield JH, Anderson JB. The aetiological significance of human papillomavirus in bladder cancer. Br J Urol. 1996;78:866–9.
33. Chang F, Lipponen P, Tervahauta A, Syrjänen S, Syrjänen K. Transitional cell carcinoma of the bladder: failure to demonstrate human papillomavirus deoxyribonucleic acid by in situ hybridization and polymerase chain reaction. J Urol. 1994;152:1429–33.
34. Shigehara K, Sasagawa T, Kawaguchi S, Nakashima T, Shimamura M, Maeda Y, et al. Etiologic role of human papillomavirus infection in bladder carcinoma. Cancer. 2011;117:2067–76.
35. Li N, Yang L, Zhang Y, Zhao P, Zheng T, Dai M. Human papillomavirus infection and bladder cancer risk: a meta-analysis. J Infect Dis. 2011;204:217–23.
36. Alexander RE, Hu Y, Kum JB, Montironi R, Lopez-Beltran A, Maclennan GT, Idrees MT, Emerson RE, Ulbright TM, Grignon DG, Eble JN, Cheng L. p16 expression is not associated with human papillomavirus in urinary bladder squamous cell carcinoma. Mod Pathol. 2012 Jun 8. doi:10.1038/modpathol.2012.103.
37. Ben Selma W, Ziadi S, Ben Gacem R, Amara K, Ksiaa F, Hachana M, et al. Investigation of human papillomavirus in bladder cancer in a series of Tunisian patients. Pathol Res Pract. 2010;15:740–3.
38. Badawi H, Ahmed H, Ismail A, Diab M, Moubarak M, Badawy A, et al. Role of human papillomavirus types 16, 18, and 52 in recurrent cystitis and urinary bladder cancer among Egyptian patients. Medsc J Med. 2008;10:232.
39. Moonen PM, Bakkers JM, Kiemeney LA, Schalken JA, Melchers WJ, Witjes JA. Human papilloma virus DNA and p53 mutation analysis on bladder washes in relation to clinical outcome of bladder cancer. Eur Urol. 2007;52:464–8.