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Low frequency of human papillomavirus infection in initial papillary bladder tumors

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Abstract The involvement of human papillomavirus (HPV) in bladder cancer remains controversial. We previously reported detection of L1-HPV DNA in 39% of bladder cancers of mixed grade and stage. To clarify the possible etiologic role of HPV we studied, using the same technique, a more homogeneous group of initial low-stage tumors. We investigated a total of 187 newly diagnosed superficial papillary bladder tumors for the presence of L1-HPV DNA by the polymerase chain reaction method and hybridization with specific probes for HPV 6, 11, 16, 18, 33. HPV DNA was detected in 16 (8.5%) of the 187 specimens tested, although in a low copy number compared with SiHa cervical cancer cells used as control. HPV type 16 was observed in eight tumors while HPV type 6 and type 11 were each observed in three tumors. Two tumor specimens contained two types of HPV: one tumor hybridized with type 6 and 16 and the other with type 11 and 18. This low rate of HPV detection (8.5%) in initial tumors does not favor a prominent role for HPV in bladder carcinogenesis.

Key words Human papillomavirus · Bladder cancer · DNA · Polymerase chain reaction

Introduction

Human papillomaviruses are involved in several urogenital epithelial malignancies including cervical and anal carcinomas, and they may play a role in carcinogenesis [32]. Numerous studies have described the presence of human papillomavirus (HPV) in bladder cancer, as shown by the detection of HPV antigens or HPV deoxyribonucleic acid (DNA) sequences [2, 7, 10, 15, 17, 19, 21, 31, 36, 38, 40]. However, the exact incidence of HPV DNA in bladder cancer remains controversial [21, 28], the detection rates varying from 2.5% to 100% in the literature [10, 13, 15]. Moreover, HPV DNA has also been detected in urine samples from patients with condylomata acuminata [27]. Meanwhile, other studies failed to detect any evidence of HPV infection in bladder cancer [3, 5, 9, 18, 22, 34, 35], which prompted us to investigate further the role of HPV in transitional cell carcinoma (TCC) of the bladder.

In our previous study we reported the presence of HPV DNA detected by the polymerase chain reaction (PCR) method in 39% (28/71) of the bladder tumors analyzed, albeit in a low copy number. The tumors analyzed were of different stages and grades and comprised invasive and recurrent cases. A correlation was found between the presence of HPV DNA and high-grade tumors, while no correlation was observed with tumor stage. Moreover, we then observed that the sensitivity of detection depended on a series of technical factors that could explain the contradictory reports in the literature [19]. A recent study supports these observations and it now seems obvious that the sensitivity and characteristics of the PCR systems used for the detection of HPV within clinical samples should be considered when comparing data between studies [30]. In the present study, our hypothesis was that if HPV has an etiologic role in bladder cancer then viral DNA should be detected at higher frequency in initial tumors of early stage. We thus attempted to clarify the involvement of HPV in the development of bladder cancer by investigating the incidence of HPV DNA in a series of 187 newly diagnosed stage Ta/T1 TCC of the bladder using the PCR analysis then used in the first study on frozen tissues. We report here the detection of HPV DNA in only 16 (8.5%) of the tumors analyzed and in a low copy number.

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Materials and methods

Tissues and cells

Bladder carcinoma specimens were collected over a 3-year period from 1990 to 1992; a total of 187 Ta/T1 first primary tumors of different grades were used in this study (Table 1). All tumor specimens were obtained by transurethral resection and a cell suspension was prepared for each tumor and stored frozen at −80°C in MEM (GIBCO-BRL, Burlington, Ontario) containing 20% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO) for future use. Cervical carcinoma cell lines containing HPV 16 (SiHa and Caski) or HPV 18 (HeLa and Me180) were obtained from the American Type Cell Culture Collection (Rockville, Md.), grown in MEM and used as positive controls.

DNA extraction and amplification

For each tumor, DNA extraction was carried out from 3×10^5 cells, as previously described [19, 25]. PCR amplification of a 454 base pair fragment of the L1 gene was carried out using the consensus primers MY11 and MY09, as previously described [25]. Amplification products were analyzed either by acrylamide gel electrophoresis and ethidium bromide staining, Southern blot or dot-blot hybridization, as previously described [19]. All necessary precautions were taken to avoid cross-contamination at all stages of extraction, amplification and post-PCR manipulations by using autoclavable micropipets and plugged tips, and working in different locations.

Amplification of a fragment of the HPV 16 E6 gene, electrophoresis and hybridization to a HPV 16 specific probe were carried out as described [26].

Results

The PCR amplicon of 16 (8.5%) of the 187 newly diagnosed cases tested showed positive signals after Southern blotting and hybridization to a consensus HPV L1 probe (Fig. 1a). However, no signal was detected on polyacrylamide gels stained with ethidium bromide in any of the tumor specimens analyzed. The intensity of the hybridization signal of amplified tumor DNA was always very weak compared with that of SiHa DNA, even though gels were loaded with 5 times more tumor DNA than SiHa DNA. Taking into consideration that SiHa cell line has only one HPV 16 copy per cell, this observation suggests that bladder tumors contained less than one copy of HPV DNA per cell in all positive samples.

Typing of HPV DNA was performed by two approaches: dot-blot hybridization with L1 type-specific probes and HPV 16 E6 gene-specific amplification. All 16 tumor DNA samples positive with the L1 consensus probe were dotted and hybridized to L1 probes specific for HPV type 6, 11, 16, 18 and 33. Half these samples hybridized with the HPV 16 probe (8/16), while three samples hybridized with the HPV 6 probe and three others with the HPV 18 probe. Two tumor specimens contained more than one type of HPV; one specimen hybridized with the HPV 6 and 16 probes while the other hybridized with the HPV 11 and 18 probes (Table 2). Amplification of the HPV 16 E6 gene was also conducted on the 16 L1-HPV positive tumor DNA samples and a few negatives. Results confirmed the presence of HPV 16 in eight of the nine tumors positive for HPV 16 in dot-blot hybridization (Fig. 1b); the tumor that failed to amplify HPV 16 E6 gene was the one found to contain HPV 6 and HPV 16 DNA. All other HPV-positive or HPV-negative DNA samples failed to amplify the HPV 16 E6 gene.

No correlation was observed between the presence of HPV DNA and tumor stage and grade (Table 3), although most of the positive tumors were noninfiltrating papillary superficial tumors (Ta) and of grade 2 or 3. Although HPV DNA was found more frequently in single tumors smaller than 3 cm in diameter, no significant association was found between the presence of HPV DNA and the size or number of tumors, tumor recurrence or sex of the patient.

Table 1 Distribution of histologic grades and clinical stages of the 187 cases of transitional cell carcinoma included in this study (*NA* not available)

Stage	Grade			
	1	2	3	NA
Ta	38	76	8	0
T1	2	20	15	0
NA	1	2	1	24

Discussion

The etiology of transitional cell carcinoma of the bladder, a common cancer in industrialized countries, is still not well understood. The etiologic role of HPV is well documented in genital cancers but its involvement in bladder cancer remains controversial [28, 41]. HPV has been detected in condylomatous bladder lesions from immunocompetent and immunosuppressed patients and in a TCC in situ of an immunodepressed patient with vulvar Bowen's disease [4, 11, 17, 27]. A number of studies, however, have failed to demonstrate an association of HPV with TCC of the bladder, while others have reported the detection of HPV DNA in 20–80% of bladder tumors analyzed.

We previously reported that the sensitivity of detection of HPV is largely dependent on a series of technical factors such as tissue fixation, DNA preparation and amplification conditions [19]. A recent study by Qu et al. [30] has supported these observations and described other factors that should be taken into consideration such as the source of the clinical material, the size of the PCR product, the spectrum of HPV types amplified and the ability to amplify multiple HPV types. The studies summarized in Table 4 illustrate the discrepancies in the results among studies using fixed or frozen tissues and a variety of techniques for HPV detection. Few studies have used normal urothelium from individuals with no history of bladder cancer as a negative control. In two

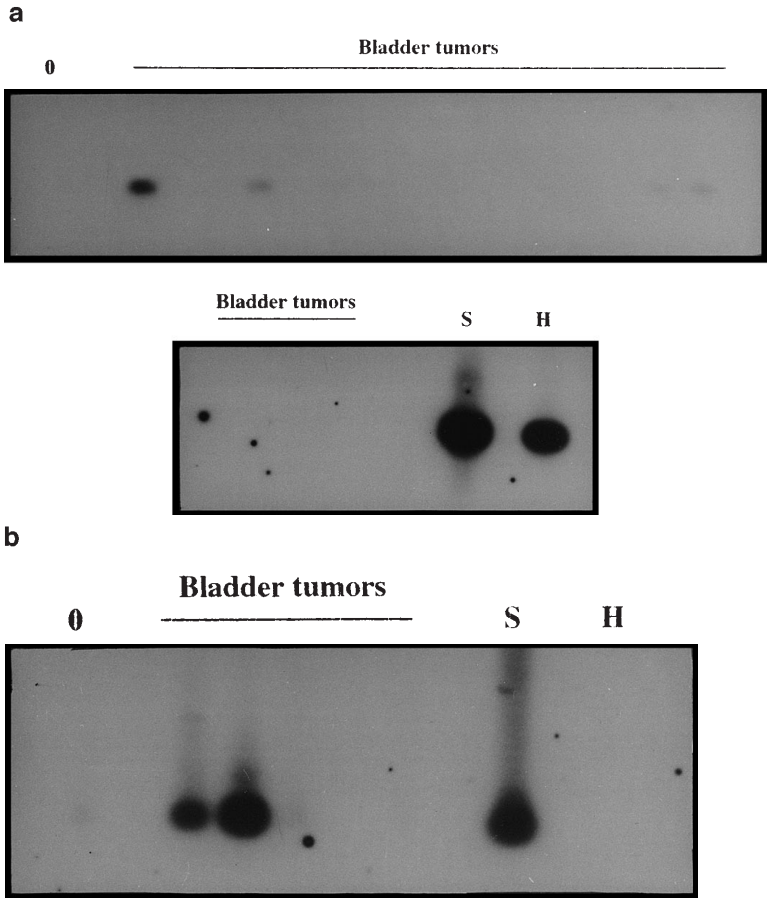

Fig. 1 Southern blot of polymerase chain reaction products from an amplification carried out on bladder tumor DNA using HPV L1 consensus primers (**a**) or HPV 16 E6 primers (**b**). *0* no-DNA control, *S* SiHa DNA, *H* HeLa DNA. Of 19 bladder tumor samples, four DNA samples show a positive signal with the L1 consensus probe (**a**) while three of five tumor DNA samples show a positive signal with the HPV 16 E6 specific probe (**b**)

Table 2 Human papillomavirus (HPV) typing of tumor DNA samples positive with the L1 consensus probe by dot-blot hybridization and HPV 16 E6 specific amplification (*NA* not available, *PCR* polymerase chain reaction)

Caseno.	Tumor stage	Tumor grade	HPV type					PCR HPV 16 E6
			6	11	16	18	33	
1	Ta	2	+	–	–	–	–	–
2	T1	2	–	+	–	+	–	–
3	Ta	2	–	–	–	+	–	–
4	Ta	2	–	–	+	–	–	+
5	Ta	2	+	–	+	–	–	–
6	Ta	3	–	–	–	+	–	–
7	Ta	3	+	–	–	–	–	–
8	Ta	1	–	–	+	–	–	+
9	Ta	1	–	–	+	–	–	+
10	Ta	1	–	–	+	–	–	+
11	Ta	2	+	–	–	–	–	–
12	NA	NA	–	–	+	–	–	+
13	Ta	1	–	–	+	–	–	+
14	Ta	2	–	–	+	–	–	+
15	Ta	2	–	–	+	–	–	+
16	Ta	2	–	–	–	+	–	–

Table 3 PCR amplification of HPV L1 DNA from bladder tumor DNA (*NA* not available)

	No. tested	No. HPV positive (%)
Tumor stage		
Ta	122	14 (11.5)
T1	37	1 (2.7)
NA	28	1 (3.6)
Tumor grade		
1	41	4 (9.8)
2	98	9 (9.2)
3	24	2 (8.3)
NA	24	1 (4.2)

studies, a high rate (33% and >70%) of HPV DNA detection was observed in normal bladder tissue specimens, raising concerns over the significance of the positive results observed in their cancer specimens [2, 33]. On the other hand, in our previous study none of eight urothelial specimens from cadaver kidney donors expressed HPV while 39% of tumors had detectable L1-HPV by PCR. One could expect that the highly sensitive technique of PCR should reveal a higher prevalence of HPV DNA compared with immunohistochemistry and in situ hybridization. Nevertheless, eight studies using the PCR technique failed to detect the presence of HPV DNA, while seven studies detected HPV DNA in less than 5% of cases analyzed (Table 4). Two sets of primers are widely used for the amplification of the L1 region: MY09/11 and GP5/6. Qu et al. [30] observed that the MY09/11 pair is more sensitive in cervical cancer samples infected with multiple HPV types than

Table 4 Summary of studies on HPV detection in bladder cancer (*IHC* immunohistochemistry, *ISH* in situ hybridization, *URR* upstream regulatory region, *PS* present study)

Technique	No. of studies		Prevalence	Total cases	% positive (range)	References
	Positive	Negative				
Fixed tissues						
IHC	2		7/50, 19/110[a]	26/160	16.3 (14.0–17.3)	[7, 38]
ISH		3	0/22[a], 0/31, 0/108[a]	0/161	0	[9, 16, 22]
	7		1/93[a], 4/76[a], 1/10[a], 12/100, 24/110[a], 28/90, 27/47	97/526	18.4 (1.1–57.0)	[6, 12, 14, 15, 21, 35, 38]
Southern blot		1	0/55	0/55	0	[5]
PCR, L1		3	0/25, 0/93[a], 0/108[a]	0/226	0	[9, 34, 35]
	3		1/42[a,b], 1/36, 26/79	28/157	17.8 (2.4–32.9)	[24, 28, 39]
PCR, E6		1	0/93[a]	0/93	0	[35]
	4		1/42[a,b], 7/76[a], 23/46, 39/48	70/212	33.0 (2.4–81.0)	[1, 2, 21, 24]
PCR, E7	1		2/75	2/75	2.7	[29]
PCR, E1	1		20/59[a]	20/59	33.9	[38]
PCR, URR	1		2/10[a]	2/10	20.0	[14]
PCR, type spec.	2		1/22[a,b], 6/20	7/42	16.7 (4.5–30.0)	[8, 16]
Total	21	8		207/1256	16.5	
Frozen tissues						
Southern blot		2	0/42[a], 0/57[a]	0/99	0	[3, 18]
	3		1/22, 1/10[c], 4/21	6/53	11.3 (4.5–19.0)	[17, 36, 40]
PCR, L1		2	0/14, 0/57[a]	0/71	0	[3, 37]
	4		1/44, 16/187, 6/23[d], 28/71	51/325	15.7 (2.3–39.4)	[10], PS, [19, 23]
PCR, L1 + E1		2	0/8[d], 0/100[a]	0/108	0	[18, 34]
PCR, E6	1		20/26	20/26	76.9	[33]
Total	8	6		77/583	13.2	

[a] Samples analyzed by two or more techniques; [b] squamous cell carcinoma; [c] patient with mild immunodeficiency; [d] fresh tissue

the GP5/6 pair. Three studies have used this GP5/6 primer set in bladder tumors: HPV DNA was not detected in two studies and only 2.4% of specimens were positive in the third (Table 4) [18, 24, 37]. Studies in bladder cancer frequently reported the presence of multiple HPV types in bladder tumors. The use of the primer set GP5/6 could prevent the detection of HPV in these tumors. In our hands, the use of fixed tissue practically abolished HPV detection by PCR amplification. However, surprisingly, the overall frequency of HPV detection in bladder tumor studies was 16.5% in fixed tissue and 13.2% in frozen tissue (Table 4). The E1 and L1 regions of HPV are good amplification targets for several reasons. First, these regions are well conserved among different types of HPV and secondly they are consistently present in HPV-associated lesions even when the viral genome is integrated into the host chromosome [34]. Nevertheless, only four groups have observed the presence of HPV DNA E1 or L1 region in more than 10% of cases studied (Table 4) [19, 23, 38, 39]. These observations suggest that HPV is not very frequent in bladder cancer and do not favor a role for HPV in bladder carcinogenesis.

In the present work we tested very early bladder tumors (initial Ta/T1), under the assumption that if HPV plays an etiologic role in the development of bladder cancer these tumors would be an ideal source of material to demonstrate it. In a series of 187 TCC of the bladder at initial diagnosis, we detected HPV L1 DNA by PCR in only 16 (8.5%) patients, a frequency significantly lower than the 39% we previously reported using the same technique on a distinct group of tumors representing a mix of recurrent low-stage and more advanced bladder cancers. Furthermore the comparison with SiHa cells suggested that the presence of HPV DNA was less than one copy per cell in all the positive specimens, a result which is consistent with our previous study. Dot-blot hybridization with HPV L1 type-specific probes and HPV 16 E6 amplification identified HPV type 6, 11, 16 and 18 in the positive specimens and two tumors were co-infected with more than one type. The low HPV detection rate in the present study, 8.5% of tumors from patients at initial diagnosis, and in previous studies (Table 4), suggests that the presence of HPV in bladder is coincidental rather than pathogenic. Moreover, Shibutani et al. [36] observed that the HPV DNA in bladder tumors was mainly in episomal form, which supports the latter hypothesis. In conclusion, on the basis of all these observations it appears unlikely that HPV has a significant role in the development of human bladder cancer.

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