Human Papillomavirus in Transitional Cell Carcinoma of the Urinary Bladder

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ABSTRACT
The presence of human papillomavirus (HPV) was detected in transitional cell carcinoma of the urinary bladder. PCR amplification of DNA from 71 tumors, using consensus primers for a fragment of the L1 gene, detected 6 strongly positive tumors (through ethidium bromide staining of a gel) and 22 moderately positive tumors (through Southern blotting of the amplified DNA) for a total of 28 (39%) of the tumors. The presence of HPV was correlated with grade but not stage of the tumors. Typing of HPV was performed on 31 tumors: all positive tumors contained HPV 16 DNA except for one Ta tumor which contained HPV 11 DNA. Our data also showed a large variability in the sensitivity of HPV DNA detection, depending on sample fixation, DNA preparation, and amplification conditions, which may explain in large part the discrepancies reported in the literature on the association of HPV to bladder cancer. Because of the low HPV DNA copy number observed in bladder tumors, our results suggest that HPV should ideally be tested on fresh or frozen tumor material, that SDS detergent should be avoided for the preparation of DNA, and that the amplification conditions are critical for optimal detection.

INTRODUCTION
Bladder cancer is currently the fifth most common cancer in North America and its incidence appears to be increasing. Chemical carcinogenesis is known to be involved in its etiology and cigarette smoking probably accounts for 25–60% of all bladder cancers in industrialized countries (1). However certain characteristics of the disease such as multicentricity, high level of recurrence, and a papillary mode of growth for most tumors may suggest an infectious origin.

The infectious agent most often considered to be involved in tumorigenesis in humans is HPV4. An association has been reported between bladder cancer and bovine papillomavirus type 2 in cattle fed a diet including bracken fern (a known source of carcinogens; Ref. 2). Numerous reports have also been published in recent years on a possible association between HPV and human bladder cancer. The presence of HPV was tested by immunostaining for the HPV structural antigen by in situ hybridization, Southern blotting, or PCR. Some groups, using in situ hybridization or Southern blotting, have reported the presence of HPV in 10–30% of bladder tumors (3–6). Other groups, using the PCR technique, detected HPV DNA in a much higher proportion of bladder tumors (>50%; Refs. 7 and 8) and even in 33% of 21 normal urinary bladder specimens (8). Finally, other studies, also using PCR or in situ hybridization in one instance, reported a much lower level (<1% of tumors) or even an absence of HPV in bladder tumors (9–14). We report here the detection, by PCR, of HPV DNA in a significant proportion of 71 bladder tumors but not in urothelium from 8 normal individuals. Considering the numerous contradictory reports on the presence of HPV in bladder tumors, we have also attempted to identify technical problems associated with HPV detection by PCR amplification.

MATERIALS AND METHODS
Tissues, Cells, and Plasmids. Bladder tumors were obtained by transurethral resection or cystectomy, frozen in liquid nitrogen, embedded in optimal cutting temperature compound (Tissue Tek; Miles, McLean, VA) and stored at −80°C for future use. Normal urothelia were obtained from cadaver organ donors with no history of bladder tumors. Urothelial cells were obtained by gently scraping off the mucosa of the bladder specimen with a sterile scalp knife blade, in PBS, centrifuged, and stored at −80°C in MEM (GIBCO-BRL, Burlington, Ontario) containing 20% FCS and 10% DMSO. Cervical carcinoma cell lines containing HPV 16 (SiHa and Caski) or HPV 18 (HeLa and Me180) were obtained from the American Type Culture Collection, grown in MEM, and used as positive controls. Plasmids containing the cloned genome of HPV 6, 11, 16, and 18 in pBR322 were obtained from Dr. Harald zur Hausen (Heidelberg, Germany).

DNA Preparation. Frozen 20-μm tissue sections were prepared from the bladder tumors. To prevent cross-contamination between the different samples, the cryostat blade was washed with a sterile gauze soaked in ethanol between each tumor sampling. The first sections made were discarded. The sections were washed in PBS to remove optimal cutting temperature compound prior to DNA extraction. Cultured and normal urothelial cells were also washed in PBS to remove culture media. DNA was prepared for PCR by treatment with proteinase K in the presence of 0.45% NP40 and 0.45% Tween 20 as described (15). The DNA preparations were stored frozen at −80°C. Alternatively, DNA was extracted from cervical carcinoma cell lines and from human lymphocytes by treatment with proteinase K in the presence of 1% SDS (12). DNA was also prepared from formaldehyde-fixed cervical carcinoma cells and
from paraffin-embedded, formaldehyde-fixed cervical carcinoma cells. Prior to proteinase K digestion, paraffin was removed by two successive washes with xylene followed by one ethanol wash (16). DNA concentrations were determined by the fluorometric diaminobenzoic acid assay.

**DNA Amplification.** PCR amplification of a conserved 454-base pair fragment of the L1 gene was carried out using as primers degenerate 20-base pair oligonucleotides (15), recognizing conserved sequences present in many different genital HPV types. Another 20-base pair oligonucleotide complementary to an internal conserved region of the 454-base pair fragment was used as a consensus probe while oligonucleotides complementary to nonconserved regions were used as type-specific probes for HPV 6, 11, 16, 18, 33 (15). All oligonucleotides were synthesized on a Applied Biosystems 391 DNA synthesizer.

Amplification was carried out on 500 ng genomic DNA in 100 μl 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.25 mM MgCl2, 200 μM dATP, dGTP, dCTP, and dTTP, 100 μg/ml gelatin containing 2.5 units Taq DNA polymerase (GIBCO-BRL) and 100 pmol of each primer. Thirty amplification cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) were performed. The amplification products were analyzed either by acrylamide gel electrophoresis and ethidium bromide staining, or agarose gel electrophoresis and ethidium bromide staining.

**RESULTS**

**Optimization of PCR Conditions.** The high sensitivity of the PCR technology requires meticulous procedures to avoid cross-contamination and false-positive reactions. For this reason, DNA preparation of normal bladder and tumor specimens and setting up of amplification reactions were carried out in one location while preparation of DNA from the positive controls and manipulation of amplified DNA were carried out elsewhere. Throughout this study, autoclavable micropipets and plugged tips were used to prevent aerosol carry-over through pipetting. The different solutions used for the setting up of amplification reactions were stored in small aliquots and a new one of each was used for an amplification. A no-DNA control tube was always included and consistently found to be negative even upon long exposures of Southern blots.

The quality of the DNA preparations was ascertained in another study where p53 exons 5 to 9 have been successfully amplified from the DNA samples we also used for HPV amplification (data not shown). However we noticed that the concomitant use of two pairs of primers, one for HPV/L1 and one for a p53 exon as an internal standard, in one PCR assay selectively decreased the HPV amplification. We thus never use internal standards.

Considering the discrepancies in the reported data on the presence of HPV in bladder tumors, we tested the effect of various experimental conditions on the detection of HPV DNA in cervix carcinoma cell lines. We compared our usual method of DNA extraction, using NP40 and Tween 20 as detergents, to one using SDS. We extracted DNA from fresh cells, frozen cells, formaldehyde-fixed cells, paraffin-embedded, formaldehyde-fixed cells, and propidium iodide-stained cells (with the idea of using cells after flow cytometry analysis as a source of material). Amplification of HPV/L1 DNA was then carried out on all of these samples. Some of the results obtained using HeLa cell DNA are presented in Fig. 1. Freezing the cells prior to DNA extraction had no effect on the detection of HPV DNA (Fig. 1, Lane 3). The use of SDS for DNA preparation markedly decreased the amplification (Fig. 1, Lane 2). Furthermore, mixing positive control DNA preparations with negative control lymphocyte DNA prepared in SDS also interfered with the amplification confirming the sensitivity of DNA amplification to the presence of traces of SDS. Prior fixation or pre-staining of HeLa cells with propidium iodide practically abolished the HPV amplification in the test illustrated in Fig. 1 (Lanes 4–6). We also varied MgCl2 and primer concentrations in the PCR assay. We found that while 1 mM MgCl2 was sufficient to detect HPV DNA in cervix carcinoma DNA such as HeLa or Caski DNA, 1.25 mM was necessary for DNA containing less HPV DNA (data not shown). Higher concentrations of MgCl2 did not increase amplification of the HPV-specific band and were thus avoided to minimize the possibility of non-specific amplification. We also observed that increasing the quantity of each primer used from 50 to 100 pmol had a dramatic effect on the detection of HPV DNA in bladder tumors as well as in high copy number cell lines such as HeLa or Caski (data not shown). Thus, throughout this study, only frozen tumors were used and all of the bladder tissue DNA extractions were performed with NP40 and Tween 20 as detergents. Amplification of HPV/L1 DNA was carried out in 1.25 mM MgCl2 using 100 pmol of each primer. Subsequent to these tests, we also observed a strong decrease in the level of HPV detection in DNA samples stored for prolonged periods at −80°C and subjected to a few thaw-freeze procedures (Fig. 3, Rows 4 and 5).

**Detection of HPV/L1 DNA through PCR Amplification.** HPV/L1 DNA was amplified from 71 human bladder tumor and 8 normal urothelium DNA samples. One tenth (10 μl; 2 μl for the positive controls) of the amplification reaction was electrophoresed on agarose gels which were stained with ethidium bromide. Six amplified DNA samples showed an approximately 450-base pair band similar to that observed in positive control data not shown).

**Detection of HPV/L1 DNA through PCR Amplification.**
cervical carcinoma cell lines (HeLa, SiHa, Me180, and Caski). Southern blot and hybridization to a consensus HPV/L1 DNA probe confirmed the HPV identity of this amplified DNA (Fig. 2). An additional 22 tumors, negative by ethidium bromide staining, were found to be HPV positive by Southern blot. By contrast, the eight normal urothelial DNA samples were negative, both by ethidium bromide staining or by Southern blotting of the PCR products (Fig. 2). The intensity of hybridization of amplified bladder tumor DNA was at most equal to that of SiHa DNA, although five times less DNA was electrophoresed for the positive controls. Since the SiHa cell line contains one HPV 16 copy per genome, this observation suggests that bladder tumor cells are heterogeneous with respect to their HPV content and that some cells do not contain any HPV DNA.
There was no correlation between the presence of HPV DNA and tumor stage (Table 1), although three of six strongly positive tumors (detected by ethidium bromide staining) were papillary superficial (Ta). Overall, there was also no correlation of the presence of HPV DNA and tumor stage (Table 1), although three of six strongly positive HPV tumors with known clinical information were from patients with multiple tumors. The panel of tumors analyzed contained 46 primary tumors and 24 recurrences. HPV DNA was present in tumors with known clinical information were from patients with between the presence of HPV DNA and tumor multicentricity. This association of HPV DNA with primary tumors did not reach statistical significance using the Mann-Whitney test. There was no correlation between the presence of HPV DNA and tumor stage (Table 1), although three of six strongly positive HPV tumors with known clinical information were from patients with multiple tumors. The panel of tumors analyzed contained 46 primary tumors and 24 recurrences. HPV DNA was present in tumors with known clinical information were from patients with between the presence of HPV DNA and tumor multicentricity. This association of HPV DNA with primary tumors did not reach statistical significance using the Mann-Whitney test.

Typing of HPV DNA. Typing of HPV DNA was performed through four approaches: dot blot hybridization with L1-type-specific probes, sequencing, restriction enzyme typing, and HPV 16/E6 specific amplification.

Forty-five positive and negative amplified bladder tumor DNA samples and the eight amplified normal urothelial DNA samples were dotted on nitrocellulose and hybridized to the L1 probes specific for HPV types 6, 11, 16, 18, and 33 (Fig. 3). Of these, 29 (Fig. 3, Rows 1–3 and 6) were amplified from the same bladder tumor DNA as used in Fig. 2. Sixteen were amplified from 6-month-old DNA specimens, some of which had previously given positive signals with the consensus probe. All of the amplified DNA from the 29 fresh specimens, hybridizing with the consensus probe, also hybridized to the HPV 16 probe. As expected, SiHa DNA hybridized to the HPV 16 probe and HeLa DNA to the HPV 18 probe. However, the older DNA samples were all negative (Fig. 3, Rows 4 and 5), as were the normal urothelial DNA samples (Fig. 3, Row 8).

Restriction enzyme typing was performed on the six ethidium bromide-positive samples by digesting the L1-amplified DNA samples with Rsal, Ddel, Hinfl, or PstI and comparing the pattern of digestion on acrylamide gels with that obtained from DNA amplified from cloned HPV DNA types 6, 11, 16, or 18. Results obtained with two samples are shown in Fig. 4 along with digestions of DNA amplified from cloned HPV 6 and 16. In all cases, the pattern of digestion was identical to that of HPV 16.

Sequencing confirmed the presence of HPV 16 in three tumors already tested by dot blot or restriction enzyme typing but found HPV 11 DNA in another Ta tumor. Finally, amplification of a DNA fragment from the HPV 16 E6 gene using specific primers was also carried out on five of the six ethidium bromide-positive tumor DNA samples and all were found to be positive (data not shown). In summary, a total of 31 amplified bladder tumor DNA samples were tested for HPV type and all of the positive ones were found to contain HPV 16 DNA except for one Ta tumor which contained HPV 11 DNA detected through DNA sequencing.

### DISCUSSION

Taking meticulous precautions to avoid contamination and after optimization of the DNA extraction and amplification conditions, we detected HPV L1 DNA by PCR in 28 (39%) of 71 frozen specimens of transitional cell carcinoma. In a parallel immunohistochemistry study, carried out on a different group of bladder tumors, 15 (28%) of 53 tumors were found to express the HPV structural antigen. Considering that not all HPV DNA-containing tumors necessarily express the HPV structural antigen, this proportion is consistent with what we observed by PCR.

Comparison with SiHa cells suggested the presence of HPV DNA at less than one copy per cell in most positive samples. The presence of normal cells in the tissue samples could be an explanation for this observation. However, the tissue sections used for DNA preparation contained more than 70% tumor cells as estimated from hematoxylin and eosin-stained tumor sections. For papillary tumors (Ta, T1) the proportion was even higher. Thus, while the proportion of HPV-positive bladder tumors is significant, the HPV copy number appears to be rather low.

The proportion of HPV-positive tumors found in the present study is similar to the 10–30% rate reported by other groups using *in situ* hybridization or Southern blotting (3–6). The slightly lower rate of HPV DNA-positive specimens reported in the latter studies compared to our own may be explained by the lower sensitivity of the techniques used compared to the PCR-based assay used in the present study. Other studies have reported either a much higher proportion of HPV-positive

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**Table 1**  
PCR amplification of HPV/L1 DNA from bladder tumor and normal urothelium DNA

<table>
<thead>
<tr>
<th>Specimen tested</th>
<th>No. tested</th>
<th>No. HPV positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta</td>
<td>20</td>
<td>3 (15%) 5 (25%)</td>
</tr>
<tr>
<td>T1</td>
<td>23</td>
<td>1 (4%) 6 (26%)</td>
</tr>
<tr>
<td>≥T2</td>
<td>27</td>
<td>2 (7%) 11 (41%)</td>
</tr>
<tr>
<td>Tis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Normal urothelium</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

*++*, detected by ethidium bromide staining of a gel; +, additional cases detected by hybridization with a consensus probe.

**Table 2**  
PCR amplification of HPV/L1 DNA from bladder tumor DNA

<table>
<thead>
<tr>
<th>Tumor grade</th>
<th>No. tested</th>
<th>No. HPV positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>3 (10%) 8 (26%)</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>3 (10%) 12 (44%)</td>
</tr>
</tbody>
</table>

*++*, detected by ethidium bromide staining of a gel; +, additional cases detected by hybridization with a consensus probe.

H. LaRue, C. Dufour, M. Simoneau, and Y. Fradet, unpublished data.
bladder tumors or an almost complete absence of HPV from bladder tumors. With a technique as sensitive as PCR, cross-contamination of samples is always a possibility to explain false positives. However, it remains an unlikely explanation for our results as our no-DNA control was consistently negative as were our eight normal urothelial specimens. Moreover, using the same technology, the same primers and the same probes, no HPV DNA was detected in a series of colon cancer samples tested in our laboratory (19). On the other hand, it has been our experience that the sensitivity of detection is largely dependent on a series of technical factors and that negative results can be explained by tissue fixation, DNA preparation, or amplification conditions. In our hands, use of SDS-prepared DNA or of 50 pmol of primers rather than 100 pmol could prevent the detection of HPV in some tumors. Notwithstanding these difficulties, some of the observed divergences could still reflect differences in patient populations.

Dot blot hybridization to L1-type-specific HPV probes, restriction enzyme typing, sequencing, and HPV 16 E6 amplification all identified HPV type 16 DNA except for one Ta tumor where HPV 11 DNA was found. Other Ta tumors, however, were found to contain HPV 16 DNA, so no conclusion can be drawn on the association between a nontransforming HPV type and low stage Ta tumors. The identification of HPV 16 in all other HPV-infected tumors is consistent with the involvement of HPV16 in over 50% of genital infections and in a large proportion of anogenital cancers (20).

The presence of HPV DNA correlated with increasing grade of the tumors (Table 2). Moreover, the six ethidium bromide-positive tumors were all grade 2 or 3. This preliminary observation could suggest that HPV-positive and HPV-negative tumors have distinct clinical courses. The presence of HPV DNA may be associated with multiple tumors, at least for the strongly HPV-positive ones, although the association was not as clear as with grade. These two observations could indicate an association of HPV with increased risk of tumor recurrence. A recent study analyzing the presence of HPV DNA in tumors by in situ hybridization similarly suggested an association between the presence of HPV and a poor prognosis in bladder cancer (6). However, analysis of a greater number of tumor DNA specimens will be necessary in order to validate these observations and to assess the possible value of HPV as a prognostic factor in bladder cancer.
The presence of HPV in a significant proportion of bladder tumors and the association of HPV with higher grade tumors and possibly with multicentricity may suggest an etiological role for these viruses in bladder cancer. A contribution of HPV in the initiation of tumorigenesis in bladder cancer has recently been proposed by Reznikoff et al. (21) in one of two model pathways to uroepithelial tumorigenesis based on in vitro transformation of cultured normal human urothelial cells.

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