Presence of Human Papillomavirus Type 16 Genome in Bladder Carcinoma in Situ of a Patient with Mild Immunodeficiency

Tadaichi Kitamura, Yoshiaki Yogo, Tetsuo Ueki, Shunichi Murakami, and Yoshio Aso

ABSTRACT

Using blot hybridization, we analyzed 10 bladder tumors (1 transitional cell carcinoma in situ, 1 adenocarcinoma, and 8 papillary tumors) for the presence of human papillomavirus (HPV) DNA. We detected HPV 16 DNA in a transitional cell carcinoma in situ, whereas no HPV DNA was found in the other bladder tumors. The patient, a 40-year-old female, who harbored HPV 16 DNA in the bladder tumor, had mild immunodeficiency and recently suffered from the bladder tumor, common warts on the right hand, Bowen’s disease of the vulva, and severe dysplasia of the vaginal wall. From each of these lesions, we detected the DNA of HPV 16 or an unclassified HPV. HPV DNAs existed in nonintegrated form in all lesions examined. To our knowledge, this is the first case in which a bladder tumor was shown to harbor HPV DNA. However, HPV does not seem to be regularly present in bladder tumor, because we could not detect HPV DNA from the most common bladder tumor, i.e., papillary tumor. Our demonstration of HPV 16 DNA in a transitional cell carcinoma in situ of the bladder suggests that HPV may be associated with some of the bladder tumors of this type.

INTRODUCTION

HPV\(^2\) has been implicated in the development of genital cancer (1, 2). Strong support for this is the demonstration of HPV genome in genital tumors by Southern blot hybridization. Thus, the genome of HPVs, mainly types 16 and 18, has frequently been detected in carcinomas of both the uterine cervix and lower genital tract (3-11). HPV DNA is also detectable in precancerous genital lesions which occasionally progress to invasive carcinomas (3, 5, 6, 9, 12-16).

On the other hand, there has been no report indicating the association of HPV with bladder tumors. However, since 1962, there have been several documented cases in which condyloma acuminatum involved the bladder (17-20). Because condyloma acuminatum is now known to be a typical lesion of HPV infection, the presence of these cases suggests that HPV infection, which would lead to the development of bladder cancer, may also occur in the bladder.

We recently encountered a case of transitional cell carcinoma in situ that developed in the bladder of a 40-year-old female (patient S. Y.) who had mild immunodeficiency. She had previously suffered from condyloma acuminatum of the vulva at age 18. Since then it frequently recurred. At age 29, anogenital Bowen’s disease developed and total anogenital skin, except for clitoris and vestibulum vaginae, was excised. At age 35, she was admitted to the local hospital due to left pleural effusion. Open lung biopsy revealed interstitial pneumonia which had probably been caused by viral infection. At this time, laboratory data suggested that she had mild immunodeficiency and anemia. Soon after this admission, she recognized vaginal bleeding after coitus. Biopsy of the uterine cervix showed squamous cell carcinoma in situ. She underwent total hysterectomy and bilateral oophorectomy. For about 5 years after this operation, she had not suffered from any serious disease.

Upon her first visit to our hospital, we found a group of common warts on the back of her right hand and skin transplantation in the anogenital region. Cystoscopic examination revealed a well-demarcated, white velvety lesion with slight elevation, spreading from the bladder neck to the left lateral wall. This velvety covered almost one-third of the entire bladder wall. Cold-cup biopsy of the vaginal wall demonstrated transitional cell carcinoma in situ (Fig. 1A). Biopsy of the right lateral wall, which was located to the opposite of the velvety lesion, showed normal bladder mucosa (Fig. 1B). A biopsy specimen was also taken from the pigmented skin of the vulva, which had been left at the time of anogenital skin resection, and from the vaginal wall. Histological examination revealed Bowen’s disease of the vulval skin (Fig. 2A) and severe dysplasia of the vaginal wall (Fig. 2B). Laboratory data confirmed the previous finding that the patient had mild immunodeficiency, as revealed by markedly decreased natural killer cell activity and reduced lymphocyte responsiveness to phytohemagglutinin (Table 1). She was also found to have mild aplastic anemia, since aspiration biopsy demonstrated hypocellular bone marrow (Table 1). The patient was negative for the antibody to human immunodeficiency virus. On October 25, 1987, she underwent total cystectomy, vulvectomy, resection of the anterior vaginal wall, and ileal conduit formation. Neoplasias and normal bladder mucosa were dissected from the surgically removed materials under aseptic conditions and were separated into two parts, one for DNA extraction and one for histological examination. The specimens for DNA extraction were immediately frozen at −80°C. Histological examination of the surgical specimens demonstrated pictures identical to those obtained with biopsies (Figs. 1 and 2).

Specimens from Other Patients. Bladder tumors derived from 9 other patients were either surgically removed (one invasive adenocarcinoma) or biopsied (8 noninvasive papillary tumors of grade I or II (24)). The specimens were processed as described above.

Detection of HPV DNA. For extraction of total DNA, tissues were minced with scissors and digested with 50 μg proteinase K/ml in 10 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% sodium dodecyl sulfate at 37°C for 16–18 h. The subsequent procedure was carried out as described previously (25).

Cloned HPV DNAs were obtained from Japanese Cancer Research Resources Bank. Their original developers were as follows: HPV 1 and HPV 2, Heilman et al. (26); HPV 6, de Villiers et al. (27); HPV 11,
Gissmann et al. (28); HPV 16, Dürst et al. (3); HPV 18, Boshart et al. (4). HPV DNAs were excised from the vectors with appropriate restriction enzymes and isolated by agarose gel electrophoresis and subsequent electrophoretic transfer. The HPV DNAs were labeled with [γ-32P]dCTP (Amer- sham International, plc.; 800 Ci/mmol) using a “random primed” DNA labeling system (Boehringer-Mannheim). The specific activity of the labeled DNAs was 2–6 × 10⁶ cpm/μg DNA.

Restriction enzymes were obtained from Toyobo Co., Ltd. Restriction enzyme digestion of tissue DNAs was carried out as recommended by the supplier. Restricted tissue DNAs were electrophoresed in a 0.6% horizontal agarose gel using 40 mM Tris-acetate-2 mM EDTA (pH 7.8) as the running buffer. The DNAs in the gel were transferred to a nitrocellulose filter by the method of Southern (29). The filter was prehybridized at 42°C for 4–6 h in 0.75 M NaCl, 0.075 M sodium citrate, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1 mg heat-denatured salmon testis DNA/ml, and either 20% (nonstringent conditions) or 50% (stringent conditions) formamide (4). Hybridization was performed in the same solution containing heat-denatured, 32P-labeled HPV DNA (final concentration, 5 ng/ml) at 42°C for 40–44 h. The filter was washed as described previously (4). Autoradiography was carried out with or without intensifying screens at –50°C for 1 or 2 days.

Two-dimensional gel electrophoresis was performed as described by Wettstein and Stevens (30). DNA extracted from the lesion of the bladder or vulva was digested with a restriction enzyme which did not cleave HPV 16 DNA (HindIII or Smal). The DNA was electrophoresed first in a 0.4% agarose gel at 20 V for 20–22 h and then in a 1% agarose gel at 33 V for 22–24 h in the second dimension. The DNA in the gel was transferred onto a nitrocellulose filter and hybridized with 32P-labeled HPV 16 DNA under stringent conditions as described above.

### RESULTS

Detection of HPV DNA from Various Lesions of Patient S. Y. Total DNA extracted from specimens of patient S. Y. was digested with BamHI that once cleaves HPV 16 DNA, fractionated by electrophoresis in a 0.6% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized with a mixed probe composed of 6 molecularly cloned HPV DNAs of types 1, 2, 6, 11, 16, and 18 under nonstringent or stringent conditions as described in “Materials and Methods.” After hybridization under nonstringent conditions, we detected a strong signal in each of three specimens from the lesion of the bladder (one biopsy and two different parts of the surgically removed bladder tumor) (Fig. 3A, Lanes 1–3) but did not in normal bladder mucosa (Lane 4). Under the same conditions, we also detected strong signals in the lesions of the vagina, vulva, and common warts (Fig. 3A, Lanes 5–7). These results indicate that DNA of some HPV type is present in the DNA from each lesion of patient S. Y. After hybridization under stringent conditions, we detected strong signals in the lesions of the bladder and vulva (Fig. 3B, Lanes 1–3 and 6), whereas very weak signals were observed in the lesion of the vagina and in the common warts (Lanes 5 and 7). Therefore, at least one HPV DNA contained in the mixed probe is identical with the HPV DNA present in the lesions of the bladder and vulva, but none is identical with the HPV DNA detected in the vaginal wall and common warts.

Typing of HPV. DNAs from lesions of patient S. Y. were digested with BamHI, electrophoresed in a 0.6% agarose gel, and blotted onto a nitrocellulose filter. We prepared six such

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Table 1 Pertinent laboratory data for immunodeficiency and anemia of patient S. Y.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow Nucleated cells</td>
<td>2.15 × 10⁶/mm³ (50 × 10⁶/mm³)</td>
</tr>
<tr>
<td>Peripheral blood RBC</td>
<td>341 × 10⁶/mm³, hemoglobin 11.1 g/dl, hematocrit 33.0%</td>
</tr>
<tr>
<td>Peripheral blood WBC</td>
<td>2500/mm³, with 8% band forms, 25% segmented, 56% lymphocytes, 4% monocytes, 7% eosinophils, 15.4 × 10⁶/mm³ platelets.</td>
</tr>
<tr>
<td>Lymphocytes T-cell</td>
<td>91% (66–89%), B cell 3% (4–13%).</td>
</tr>
<tr>
<td>Lymphocytes OKT3</td>
<td>89.0% (63–76%), OKT4 36.8% (31–45%), OKT8 39.7% (22–35%).</td>
</tr>
<tr>
<td>Natural killer cell activity</td>
<td>2% (18–40%), Phytohemagglutinin response 13,805 cpm (37,700–62,400 cpm).</td>
</tr>
<tr>
<td>Immunoglobulins IgG</td>
<td>1880 mg/dl (770–1550 mg/dl), IgA 149 mg/dl (76–376 mg/dl), IgM 316 mg/dl (47–217 mg/dl), IgE &lt;6 mg/dl (&lt;250 mg/dl).</td>
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* Numbers in parentheses, values within normal ranges. Lymphocytes and immunoglobulins were analyzed using the peripheral blood.
blots and hybridized each with one of the six HPV DNAs contained in the mixed probe under stringent conditions (data not shown). HPV DNAs in the lesions of the bladder and vulva hybridized only with type 16 DNA. HPV DNAs in the vaginal wall and common warts, in contrast, did not strongly hybridize with any of the probes, but weakly hybridized with type 16 DNA (vagina) or type 2 DNA (common warts). Under nonstringent conditions, HPV DNAs in the vagina and common warts hybridized with all probes but gave the most intense signal with type 16 DNA (vagina) or with type 2 DNA (common warts) (data not shown). These results strongly suggest that lesions of the bladder and vulva contained HPV 16 DNA and that the vaginal wall and common warts contained the DNA of HPVs related to types 16 and 2, respectively.

We also analyzed the HPV DNA present in lesions of the bladder and vulva using various restriction endonucleases which do not cleave or which cleave HPV 16 DNA once or several times (31). The numbers and sizes of HPV DNA fragments which were generated after each restriction enzyme digestion were consistent with those deduced from the complete nucleotide sequence of HPV 16 DNA (31) (data not shown). This result supports the above conclusion that the HPV DNA present in lesions of the bladder and vulva was type 16 DNA. Furthermore, we confirmed by similar restriction endonuclease analysis that the HPV DNA in the vaginal wall was different from that in common warts (data not shown), although we did not yet determine the types of these HPVs definitely.

Physical State of HPV DNA. The physical state of the HPV DNAs in various lesions of patient S. Y. was studied by two-dimensional gel electrophoresis (30). DNA from the lesion of the bladder or vulva was digested with HindIII which does not cleave HPV 16 DNA (31) and electrophoresed in a 0.4% gel in the first dimension and in a 1% gel in the second dimension. DNA in the gel was transferred to a nitrocellulose filter and hybridized with $^{32}$P-labeled HPV 16 DNA under stringent conditions. The results are shown in Fig. 4. A faint track in each autoradiogram indicates linear human cell DNA that weakly cross-hybridized with the probe HPV DNA. The majority of HPV-specific DNA was found as two separate spots migrating slower than linear cell DNA in the second dimension, thus representing circular HPV DNA (31). The spot migrating faster probably represents open-circular monomer of HPV 16 DNA, while that migrating slower represents open-circular dimer, as revealed by a one-dimensional gel electrophoresis in which HindIII-digested tumor DNAs were run in parallel with various open-circular recombinant DNAs containing EcoRI fragments of the varicella-zoster virus DNA (32) (data not shown). Circular molecules of dimeric HPV DNA appeared to be more abundant than those of monomeric HPV DNA, as judged from intensities of corresponding spots (Fig. 4). In addition, there are several faint spots migrating very slowly in electrophoresis in both dimensions; they may be oligomeric forms of circular viral DNA.

Fig. 4 also shows a few minor HPV-specific spots (arrows) comigrating with linear cell DNA. The sizes of these HPV DNAs approximately corresponded with the size of HPV 16 DNA (7.9 kilobase pairs) or with its multiples. We obtained the similar sizes of linear HPV DNAs, when HPV DNA in the bladder or vulva was analyzed using two-dimensional gel electrophoresis after digestion with SmaI, another uncut enzyme for HPV 16 DNA (31) (data not shown). We concluded that the linear HPV DNA molecules detected were not integrated into human cell DNA but were generated, probably by the action of a nuclease, from monomeric or oligomeric circular HPV DNA. Hence, in the lesions of the bladder and vulva, the majority of HPV 16 DNA molecules, if not all, exist in the nonintegrated form.

We carried out similar two-dimensional gel analysis of the HPV DNAs present in the vaginal wall and common warts. The results (data not shown) indicated that most of the HPV DNAs were also nonintegrated into cell DNA in these lesions. In contrast to lesions of the bladder and vulva, the majority of the free HPV DNA molecules were monomeric in the vaginal wall and common warts.

Quantity of HPV DNA. The quantity of the HPV 16 DNA in lesions of the bladder and vulva of patient S. Y. was determined with appropriate standards by agarose gel electrophoresis followed by blot hybridization. We estimated the amount of the HPV 16 DNA in each lesion of the bladder and vulva to be about 50 copies/diploid cell (data not shown). The precise amount of HPV DNAs in the vaginal wall and common warts could not be determined because we did not have homologous probes for quantitative detection of the HPV DNAs present in these lesions. However, using heterologous probes, we estimated the amount of HPV DNAs in the vaginal wall and common warts to be at least 50 copies/diploid cell (data not shown).

Failure in Detection of HPV DNA from Other Bladder Tu-
cinoma in situ of the bladder suggests that there may exist another type of carcinoma in situ for which HPV is a causative agent. By blot hybridization using the mixed HPV probe described above, we analyzed DNAs extracted from bladder tumors of 9 other patients: 1 adenocarcinoma and 8 papillary tumors. However, we could not detect a positive signal from any of these DNAs under stringent or nonstringent conditions (data not shown). This result implies that bladder tumors examined harbored neither the DNA of HPVs contained in the mixed probe (HPVs 1, 2, 6, 11, 16, and 18) nor the DNA related to these HPV DNAs.

DISCUSSION

In this report we have demonstrated the presence of HPV 16 DNA in a bladder carcinoma in situ of an immunodeficient patient. To our knowledge, this is the first case in which a bladder tumor was shown to harbor HPV DNA. The question arises as to whether HPV is regularly associated with bladder tumors. In previous studies by others, for example, Green et al. (33) analyzed at least 15 bladder tumors (the type of these neoplasias was not specified, but probably most of them were papillary tumors) for the presence of HPV DNA by reassociation kinetics with 32P-labeled HPV 1 or 2 DNA, but they did not detect HPV 1 or 2 DNA in any of these tumors. Recently, Kahn et al. (34) examined a few bladder papillomas for Southern blot hybridization using HPV 30 DNA as a probe; they found that these tumors were negative for HPV 30. In the current study, we analyzed 10 bladder tumors (1 carcinoma in situ, 1 adenocarcinoma, and 8 papillary tumors) under stringent or nonstringent conditions using a mixed probe composed of HPVs 1, 2, 6, 11, 16, and 18, but we failed to detect HPV DNA in these tumors, except for a carcinoma in situ. Although our data are too limited to determine a role of HPV in bladder tumor, HPV DNA does not seem to be generally associated with bladder tumor.

While papillary tumors comprise about 90% of all primary bladder tumors, primary carcinoma in situ represents only a small percentage. This could be the reason for rare detection of HPV DNA in bladder tumors, assuming that among various bladder tumors only carcinoma in situ harbors HPV genome. Historically, carcinoma in situ of the bladder was first recognized by Melicow and Hollowell (35) in 1952 as Bowen’s disease of the bladder epithelium. Much of the initial knowledge of this tumor came from studies with industrial workers exposed to a potent bladder carcinogen, p-aminodiphenyl (see Ref. 36 for review). Our demonstration of HPV 16 DNA in primary carcinoma in situ of the bladder suggests that there may exist another type of carcinoma in situ for which HPV is a causative agent. At present, we cannot rule out the possibility that papillary bladder tumors contain the DNA of some HPV which has a weak nucleic acid homology with HPV probes used in this and previous studies (33, 34). Since HPV is a remarkably heterogeneous group encompassing more than 45 types with distinct DNA sequences, it is conceivable that detection of a small amount of the DNA of some HPV type is difficult by cross-hybridization with a nonhomologous HPV probe. Under nonstringent conditions, which allow detection of nonhomologous HPV DNA, human cell DNA also cross-hybridizes with HPV DNA (see Fig. 3A, Lane 4) and would thereby interfere with detection of faint signals, if any, produced by hybridization between HPV DNA in a specimen and the HPV probe. It is known that the risk of developing cervical condyloma acuminatum as well as cervical carcinoma in situ is increased in immunosuppressed patients (37, 38). Consistent with this, patient S. Y. who had mild immunodeficiency was frequently infected with at least three different HPVs. At age 18, the patient suffered from genital condyloma acuminatum which has often recurred since then. For the past several years, she suffered from common warts and various urogenital neoplasias: carcinoma in situ of the uterine cervix; carcinoma in situ of the bladder; Bowen’s disease of the vulva; and severe dysplasia of the vaginal wall. From all of these lesions except cervical carcinoma in situ, for which a specimen was not available for DNA analysis, we detected HPV 16 DNA or the DNA of an HPV related to type 2 or to type 16. It seems reasonable to assume that we could detect HPV DNA in a bladder tumor owing to the high incidence of HPV infection in immunodeficient patients. In contrast to invasive cervical carcinomas, where HPV 16 DNA is generally integrated into the human cell genome (39–41), HPV DNA mostly exists as free, circular molecules in benign genital tumors (16, 40). In a transitional cell carcinoma in situ of the bladder, as described above, HPV 16 DNA existed largely as free molecules. Likewise, we detected only free HPV DNAs in genital intraepithelial neoplasias and common warts of the same patient. A remarkable feature of free HPV DNA detected in the bladder tumor is that more than one-half of the free DNA molecules were oligomeric (Fig. 4). In contrast, there were very few free oligomeric molecules in the intraepithelial lesion of the vagina or the common warts of patient S. Y. Oligomeric free HPV DNA detected may have not been derived from virions but rather from plasmid DNA which can replicate autonomously in tumor cells. We speculate that nonintegrated monomeric HPV DNA detected in the bladder carcinoma in situ may also represent plasmid DNA, although there is no evidence for this at present. It remains to be elucidated whether...
the expression of genetic information from the plasmid HPV DNA plays an important role in maintaining the malignancy of tumor cells.

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REFERENCES

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