Detection of Human Papillomavirus DNA in Anal Intraepithelial Neoplasia and Anal Cancer

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ABSTRACT

Forty anal paraffin-embedded tissue specimens from 24 subjects were studied for the presence of human papillomavirus (HPV) types 6, 11, 16, 18, 31, and 33, herpes simplex virus (HSV), Epstein-Barr virus, and cytomegalovirus DNA by using the polymerase chain reaction. These tissues ranged from histologically normal to invasive squamous cell carcinoma. HPV DNA was detected in the invasive anal cancer tissues of 11 of 13 subjects. HPV types were segregated by histopathological severity, with HPV 16 associated exclusively with high grade anal intraepithelial neoplasia and invasive cancer. HPV types 6 and 11 were associated with condyloma and low grade anal intraepithelial neoplasia. HPV DNA in situ hybridization studies confirmed the presence of HPV DNA in the invasive cancer tissues of 6 of 12 subjects. HPV DNA in these tissues was highly focal and primarily associated with invasive cell nests that demonstrated the greatest degree of squamous differentiation. HSV DNA was detected only in association with advanced disease, being found in the cancer tissues of 5 of 13 subjects, and in 3 of 4 subjects with high grade anal intraepithelial neoplasia, but was not detected by in situ hybridization. Epstein-Barr virus and cytomegalovirus DNA were not detected in the 40 tissue specimens. We conclude that HPV infection may play an important role in the pathogenesis of anal cancer. The association between HSV infection and high grade anal disease suggests that HSV infection may also play a role in disease progression.

INTRODUCTION

Recent studies have shown an increase in the incidence of anorectal cancer among homosexual men in the United States (1). Several lines of evidence point to a role for human papillomavirus in the pathogenesis of this disease, including an epidemiological association between anal cancer and a history of genital warts (2, 3), as well as the close proximity of anal cancer to anal condyloma (4–6). Furthermore, HPV (1) DNA has been detected in the tissue of most subjects with anal intraepithelial neoplasia, which likely represents an invasive anal cancer precursor (7–11), and HPV RNA has been detected in anal cancer (12). However, DNA in situ hybridization studies have shown that only a small proportion of invasive anal cancer tissues contain HPV DNA (9–11), and the role of HPV in the development of anal cancer is therefore not clear.

In addition to HPV, other viruses may play a role in the development of anal cancer. Among subjects without a history of genital warts, a history of seropositivity for HSV type 2 has been found to be associated with anal cancer (3, 13). HSV infection may therefore be a cofactor, as has been suggested for cervical cancer (14). The role of other members of the herpes-virus group that can infect epithelial cells has not been thoroughly investigated. EBV has been shown to infect the cervical epithelium (15), and like HSV, may be a potential cofactor in the development of cervical intraepithelial neoplasia and cancer. Likewise, a role for CMV, which has been detected in cervical cancer (16) and is associated with colonic adenocarcinoma and colitis in both immunocompetent and immunodeficient individuals, has not yet been investigated in anal cancer (17–19).

The purposes of this study were 2-fold: (a) to use polymerase chain reaction to detect HPV, HSV, EBV, and CMV DNA to determine if these viruses may play a role in the pathogenesis of anal intraepithelial neoplasia and cancer, and (b) to determine the relationship between HPV type and severity of associated anal histopathology by using both in situ hybridization and polymerase chain reaction.

MATERIALS AND METHODS

Subject Selection. Eighteen subjects with anal lesions were identified by the San Francisco Bay Area SEER program, and 6 were identified from Veterans’ Administration medical records. All subjects were diagnosed with anal cancer, anal intraepithelial neoplasia, or condyloma between 1979 and 1985 at participating institutions in the counties of Alameda, Contra Costa, Marin, San Francisco, San Mateo, or Los Angeles. Epidemiological data were obtained from the 18 subjects identified by the SEER program. Details of subject selection have been published (2).

Anal tissues obtained at the time of anal biopsy or surgery were formalin fixed and embedded in paraffin. Tissue blocks were sent to the Northern California Cancer Center, where all identifying patient information was removed. Forty tissue blocks from the 24 subjects were studied at the University of California, San Francisco. One tissue block per subject was available for study from 14 subjects, 2 blocks per subject were obtained for study from 7 subjects, 3 blocks per subject from 2 subjects, and 6 blocks were obtained from the remaining subject. Seven μm tissue sections were cut from each block and each section was placed in an individual 500-μl Eppendorf tube for polymerase chain reaction studies. Four μm sections from each block were cut and placed on glass slides for routine histopathology, as well as in situ hybridization studies when sufficient material was available. Slides for routine histopathology were stained with hematoxylin and eosin, and were submitted for assessment to two different pathologists, both of whom were unaware of the results of the HPV DNA hybridization studies.

Polymerase Chain Reaction. Polymerase chain reaction studies were performed without knowledge of histopathological assessment and in situ hybridization results. Each of the 40 anal tissue sections cut from paraffin blocks were studied by using a modification of the method of Shibata et al. (20). Primers were synthesized (Operon, Inc., Alameda, CA) and selected to amplify a portion of the E6 region specific to each of HPV 16, 18, 31, and 33, as well as a portion common to HPV 6 and 11. HSV DNA was amplified with primers from a region of the thymidine kinase gene common to HSV types 1 and 2; EBV DNA was...
amplified with primers from a region of the genome encoding the Epstein-Barr nuclear antigen 2 common to the B95-8 (type A) and AG 876 (type B) strains; and CMV DNA was amplified with primers from a portion of the L-S junction region of the genome common to the Towne and AD 169 strains. The primer and probe sequences used for each virus are listed in Table 1 (21–33).

In order to perform the polymerase chain reaction on the 7-μm tissue sections cut from each paraffin block, the paraffin was removed by suspending the section in 500 μl of xylene in its original Eppendorf tube. After centrifugation, xylene was removed and the tissue was resuspended in 95% ethanol. The tissue was centrifuged and washed once more in 95% ethanol. After centrifugation and removal of the ethanol, the tissue was desiccated, resuspended in 100 μl of buffer containing 2.5 mM MgCl₂, 0.5% Tween 20, 0.5% Nonidet P-40, 50 mM KCl, 10 mM Tris, pH 8.3, 0.01% gelatin (Sigma, St. Louis, MO), and protease K (Sigma), at a concentration of 100 μg/ml, and digested overnight at 37°C. A portion consisting of one-twentieth of the protease K digests from each tissue section was added to each of 3 mixtures containing: 800 μM deoxynucleotide triphosphates (United States Biochemical Corp., Cleveland, OH), 50 units/ml AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 1 mM of each of the primers for α-hemoglobin, HPV 6/11, and CMV; primers for HPV 16, HPV 18, and HSV; primers for HPV 31, 33, and EBV. Positive controls consisted of the amplification of human α-hemoglobin DNA from each tissue, as well as DNA of each of the target viruses. Negative controls were used to minimize the possibility of false positives due to contamination from one specimen to another in the laboratory, and consisted of amplification of each mixture with all components of the reaction except target DNA. Fifty cycles of amplification were performed. The sensitivity of detection was determined to be less than 1000 gene copies for each reaction.

Oligonucleotide probes specific for each of the target DNAs were synthesized (Operon, Inc.) and were end labeled with 32P. The specificity of the primers and probe for each of the target DNAs was confirmed by dot blot and Southern blot analysis: probes for a given HPV DNA type only detected the amplification product of that type, and did not detect the amplification product of the other HPV types. Similarly, there was no cross-reactivity between any of the herpes virus probes and any of the HPV amplification products, nor any between HPV probes and the herpesvirus amplification products.

One-seventh of each of the amplicon amplification mixtures was probed separately for all three potential target DNA sequences in a dot blot format. After amplification of the entire set of specimens was completed, positive reactions were confirmed at least once by repeating the amplification in a separate digestion mixture with the appropriate primer sets, and the size of the amplification product confirmed by polyacrylamide gel electrophoresis.

**HPV DNA in Situ Hybridization.** HPV DNA in situ hybridization was performed on at least one block from 18 of 24 subjects, using the ViraType In-situ method (Life Technologies, Inc., Gaithersburg, MD), insufficient tissue being available from the remaining 6 subjects. In situ hybridization was performed according to the manufacturer's recommendations on three separate tissue sections from each tissue block, with probe mixtures corresponding to the following groups: HPV 6 and 11; HPV 16 and 18; HPV 31, 33, and 35. The results were interpreted by two separate reviewers (J. G. and J. S. G.) who were unaware of the results of polymerase chain reaction studies. Each tissue section was analyzed for the presence and the location of HPV DNA positivity within each grade of pathological abnormality in the block.

**HSV DNA in Situ Hybridization.** HSV in situ hybridization was performed on a section of each of the tissues shown to contain HSV DNA by the polymerase chain reaction method, using the HSV Pathogène method (Enzo Diagnostics, Inc., New York, NY); this method detects both HSV type 1 and HSV type 2, using Bam-F and PDG 305 probes, respectively (34). In situ hybridization was performed according to the manufacturer's recommendations. This method is thought to be capable of detecting approximately 150 viral genome copies per cell. Positive controls consisted of in situ hybridization of Vero cells infected with HSV 2 DNA, and negative controls consisted of uninfected Vero cells. Each tissue section was analyzed for the presence and the location of HSV DNA positivity within each grade of pathological abnormality in the block.

### RESULTS

#### Subject History.

The 18 subjects identified by the SEER program ranged in age from 32 to 74 years, and were interviewed at home (2). Each was diagnosed as having either anal intraepithelial neoplasia grade 3 or invasive anal cancer between 1979 and 1985. Nine subjects were women who were between the ages of 32 and 63 years (mean age of 46.7 years); four subjects were men who identified themselves as heterosexual, and who were 43 to 74 years old (mean age of 59.3 years); five subjects were men who identified themselves as homosexual and who ranged in age from 42 to 70 years (mean age of 48.6 years). Anal intercourse had been practiced by 4 of the 9 women, by 3 of 5 homosexual men, and by none of the heterosexual men. All study subjects except 2 women, reported a history of smoking. Ten of 18 reported a history of anogenital warts, 11 reported a history of hemorrhoids, and 5 reported a history of anal fissures or fistulas. None of the 18 subjects reported a history of chlamydia or genital herpes infection; 3 of 18 reported a history of syphilis, and 7 of 18 reported a history of gonorrhea. Incomplete data were available for the 6 patients identified through Veterans' Administration records.

**Histopathology.** Complete agreement between the two pathologists occurred in 35 of 40 tissue blocks, with the remainder differing only by one grade of pathology. In cases of disagreement, the most advanced diagnosis was recorded. The results of histopathological assessment are presented in Table 2. More than one grade of histopathological abnormality was present within some tissue blocks, most often representing coexisting anal intraepithelial neoplasia grade 3 and invasive cancer. In

| Table 1 Primer and probe sequences used for polymerase chain reaction studies |
|-----------------------------|-----------------------------|-----------------------------|
| **HPV 16 (from the E6 ORF)** | Primer 1 = nucleotides 328–348 | Primer 2 = nucleotides 411–429 |
| **HPV 18 (from the E6 ORF)** | Primer 1 = nucleotides 328–349 | Primer 2 = nucleotides 417–436 |
| **HPV 6/11 (from the E6 ORF)** | Primer 1 = nucleotides 29–52 | Primer 2 = nucleotides 151–174 |
| **HPV 31 (from the E6 ORF)** | Primer 1 = nucleotides 125–142 | Primer 2 = nucleotides 215–233 |
| **HPV 33 (from the E6 ORF)** | Primer 1 = nucleotides 180–202 | Primer 2 = nucleotides 159–182 |
| **EBV (from the EBNA 2 ORF of B95-8)** | Primer 1 = nucleotides 4–19 | Primer 2 = nucleotides 324–243 |
| **CMV (from the L-S junction region of strain AD 169)** | Primer 1 = nucleotides 1706–1726 | Primer 2 = nucleotides 1943–1963 |
| **HSV (from the thymidine kinase gene of HSV-1)** | Primer 1 = nucleotides 324–346 | Primer 2 = nucleotides 665–682 |
| **Human hemoglobin (from α gene)** | Primer 1 = nucleotides 6979–6998 | Primer 2 = nucleotides 7154–7178 |

* ORF, open reading frame; EBNA, Epstein-Barr nuclear antigen.
cause the blocks determined to be HPV DNA positive, using polymerase chain reaction, contained only invasive cancer with subjects because of in situ hybridization confirmation, or be below the limit of sensitivity of in situ hybridization could in the overlying epithelium by in situ hybridization; the presence using polymerase chain reaction, HPV DNA was detected only in invasive cancer component of the tissue block of 9 of 11 (82%) HPV DNA positivity could be definitively ascribed to the hybridization and agarose gel electrophoresis (Figs. 1 and 2). Studied. Of these, 19 tissue blocks (83%) from 11 of 13 subjects detection studies are presented in Table 2. Twenty-three blocks tissue blocks studied. Results of the HPV and HSV DNA human tissue and the integrity of the amplification reaction. detected in each of the sections, confirming the presence of tissue obtained from the same subject. addition, variation in histopathology was noted in different tissue blocks obtained from the same subject. Polymerase Chain Reaction. Human α-hemoglobin DNA was detected in each of the sections, confirming the presence of human tissue and the integrity of the amplification reaction. However, no EBV or CMV DNA was detected in any of the tissue blocks studied. Results of the HPV and HSV DNA detection studies are presented in Table 2. Twenty-three blocks from 13 different subjects that contained invasive cancer were studied. Of these, 19 tissue blocks (83%) from 11 of 13 subjects with cancer (85%) contained HPV DNA, as shown by dot blot hybridization and agarose gel electrophoresis (Figs. 1 and 2). HPV DNA positivity could be definitively ascribed to the invasive cancer component of the tissue block of 9 of 11 (82%) subjects because of in situ hybridization confirmation, or because the blocks determined to be HPV DNA positive, using polymerase chain reaction, contained only invasive cancer with no overlying epithelium. In the two other subjects whose invasive cancer tissues were shown to be positive for HPV DNA, using polymerase chain reaction, HPV DNA was detected only in the overlying epithelium by in situ hybridization; the presence of HPV DNA in the invasive cancer portion of the tissue at a level below the limit of sensitivity of in situ hybridization could therefore be neither confirmed nor excluded. The results of HPV DNA hybridization by histopathological grade are presented in Table 3. Of the 11 subjects whose invasive cancer tissues were positive for HPV DNA, HPV 16 was...
detected in 10; the cancer tissue blocks of 6 subjects contained HPV 16 DNA alone, while the remainder contained mixed infection with other HPV types. HPV 16 DNA was not detected in tissues containing low grade anal intraepithelial neoplasia and condyloma, in contrast to HPV 6 or 11, which were detected in 6 of 7 tissues in this group.

HSV DNA was detected by dot blot hybridization and agarose gel electrophoresis in tissue blocks that contained invasive cancer from 5 of 13 subjects (38%), and in tissue blocks that contained anal intraepithelial neoplasia grade 3 from 3 of 4 subjects (75%) (Fig. 3). HSV DNA was never detected in the absence of HPV DNA in the blocks that contained invasive cancer. In contrast to high grade anal intraepithelial neoplasia and anal cancer, HSV DNA was not detected in low grade anal intraepithelial neoplasia or condyloma.

In most cases, polymerase chain reaction studies of multiple tissue blocks from the same subject yielded similar results, particularly when the blocks contained tissues of similar pathological grades. However, in one block that contained invasive cancer, HPV 6/11, 31, and 16 DNA were detected, whereas HPV 6/11 and 31 DNA, as well as HSV DNA, were detected in a second cancer tissue block from the same subject. Studies of blocks from the same subject that contained different pathological grades of disease revealed a wider range of results; in one subject, HSV DNA and HPV 16 DNA were detected in a block that contained anal intraepithelial neoplasia grade 2 and microinvasive carcinoma. A second tissue block from the same subject that contained epithelial ulceration without intraepithelial neoplasia or cancer, yielded only HSV DNA, while a third block contained only inflammatory tissue, and was negative for all viral DNA. Similarly, one block from a subject that contained invasive cancer was positive for HPV 31 DNA, whereas a second block from the same patient that contained lymph nodes and fibrous tissue without tumor was negative for viral DNA.

**In Situ Hybridization.** HPV DNA was detected in the invasive cancerous tissue of 6 of 12 (50%) subjects for whom tissue was available (Figs. 4–6). Cancerous tissues from 4 subjects were negative for HPV DNA, and in 2 subjects, HPV DNA was detected in surface epithelial tissue adjacent to invasive cancer, but not within the invasive portion itself. Within invasive tissues, the pattern of HPV DNA positivity was irregular, and in most blocks, the most intense signal was present in areas of greatest squamous differentiation. In each block that contained anal intraepithelial neoplasia adjacent to HPV DNA-positive invasive cancer cells, HPV DNA was detected in the former as well (Fig. 7).

The HPV type identified by *in situ* hybridization was confirmed in each tissue block by polymerase chain reaction, with the exception of one subject whose block contained condyloma. In this block, HPV 6/11 DNA was detected by *in situ* hybridization, but not by polymerase chain reaction. In several tissue blocks, however, HPV DNA was identified by polymerase chain reaction, but not by *in situ* hybridization (Table 2). Despite a
The association between HPV infection and cancer of the cervix, vulva, vagina, and penis is now well established (35). Of the greater than 60 HPV types, infection with HPV types 16 and 18 are most closely associated with cervical cancer and high grade intraepithelial neoplasia; types 31, 33, and 35 are associated with an intermediate risk of cancer, and types 6 and 11 are usually associated with low grade intraepithelial neoplasia and condyloma (36-42). Whereas several lines of evidence suggest that HPV may play a role in the pathogenesis of anal cancer as well, in situ hybridization studies have revealed the presence of HPV DNA in only a small proportion of anal cancer tissues. In one study, HPV DNA was detected in 21% of subjects with invasive squamous cell cancers (9), and the HPV DNA was primarily localized to the upper one-third of the adjacent epithelium. In another study using in situ hybridization with genomic HPV 6, 11, 16, and 18 DNA, HPV infection was not detected in any of 13 subjects with anal carcinoma (10).

In our study, using the highly sensitive polymerase chain reaction technique, the presence of HPV DNA was demonstrated in the anal cancer tissues of 11 of 13 subjects (85%), with HPV DNA positivity demonstrable in the invasive tissue itself in 9 of 11 subjects (82%). These results strongly support a role for HPV in the pathogenesis of anal cancer. In contrast, studies of the same tissues using in situ hybridization revealed the presence of HPV DNA in the invasive component of the tissue blocks in only 50% of subjects, suggesting that the failure to detect HPV DNA in this study and in previous studies may have been due to lower sensitivity of in situ hybridization when compared to polymerase chain reaction. As in the cervix, HPV 16 was the predominant HPV type associated with high grade anal intraepithelial neoplasia and anal cancer, whereas types 6 and 11 were associated with low grade anal intraepithelial neoplasia and anal condyloma.

Infection with multiple HPV types was common among subjects with anal cancer and anal intraepithelial neoplasia grade 3. These data are consistent with HPV DNA hybridization studies of anal swab material obtained from homosexual men practicing anal intercourse, indicating a 20% rate of multiple infection among HPV DNA-positive subjects (8). The high rate of infection with two or more HPV types in this population may reflect a large number of sexual consorts and potential exposures, and it is unclear whether multiple infection is associated with a higher risk of malignancy or a more rapid rate of progression. In the tissues of three subjects, different HPV types were detected in different blocks, despite similar histopathology. In part, this may be explained by the observation that in situ HPV DNA positivity was highly focal within a given block, suggesting that sampling variation may occur when tissues are divided into blocks for histopathological examination. In contrast, contamination from one specimen to another is an unlikely explanation, given the use of negative controls, and the segregation of HPV types by histopathological grade.

In our study, using polymerase chain reaction, no EBV DNA or CMV DNA were found. It is therefore unlikely that these viruses play a significant role in the development of either anal intraepithelial neoplasia or anal cancer. However, HSV DNA was detected in the tissues of 5 of 13 subjects with anal cancer and 3 of 4 subjects with high grade anal intraepithelial neoplasia, respectively. Similar to HPV 16, it appeared to be associated with high grade lesions, with no detection of HSV DNA in the tissues of any of the subjects with low grade and intraepithelial neoplasia or condyloma. The association between HSV DNA detection and high grade disease suggests that HSV infection could play a role in disease progression. However, we were unable to confirm the polymerase chain reaction results by in situ hybridization, suggesting that the DNA was present in low copy number. Of note, the number of tissues examined in this study was small, and confirmation of this hypothesis will require the study of a larger number of tissues; previous studies of the role of HSV in the pathogenesis of cervical cancer have failed to yield consistent results (43, 44), and similarly, the role of HSV infection in the pathogenesis of anal cancer remains unclear at this time.

In addition to viral infection, other factors may be important in the development of anal cancer. Smoking has been shown to be a cofactor in both cervical cancer and in anal cancer (2, 3, 13); 16 of 18 subjects in this study for whom data were available reported a history of smoking. Immunosuppression has also been shown to be an important risk factor for the development of anogenital cancer (45), and the recent increase in anal cancer...
and anal intraepithelial neoplasia among homosexual men suggests that this in part may be due to concurrent HIV-associated immunosuppression (1, 46). Moreover, the high rate of anal intraepithelial neoplasia found among male homosexuals with severe HIV-associated immunosuppression suggests that the rate of anal cancer may increase further as improvements in medical therapy permit a longer survival time (8). While the HIV status of our study subjects at the time the tissues were obtained is not known, it is possible that several may have been HIV positive at the time anal cancer was diagnosed.

In conclusion, this study confirms that HPV DNA can be detected in the anal cancer tissues of most subjects using polymerase chain reaction, suggesting that as in cervical cancer, HPV may play an important role in its pathogenesis. Segregation of HPV types of histopathological classification was similar to that described in the cervix, with HPV 16 most strongly associated with anal cancer. HSV DNA was detected in the tissues of over one-third of subjects with high grade anal intraepithelial neoplasia and cancer, suggesting that it may also play a role in the pathogenesis of anal intraepithelial neoplasia and anal cancer. In contrast, EBV and CMV do not appear to play a significant role. Larger laboratory and epidemiological studies will be needed to elucidate the role of HPV and HSV in the development of anal cancer, as well as the role of other potential cofactors such as smoking and immunosuppression.

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