Colorectal Papillomavirus Infection in Patients with Colorectal Cancer
Sohrab Bodaghi, Koji Yamanegi, Shu-Yuan Xiao, Maria Da Costa, Joel M. Palefsky, and Zhi-Ming Zheng

**Abstract**

**Purpose:** Infection with human papillomaviruses (HPV) is associated with the development of cervical cancer, but whether HPVs have a role in colorectal cancer remains controversial.

**Experimental Designs:** To determine the relationship between HPV and colorectal cancer, we did a retrospective, controlled study using tumor and tumor-adjacent colorectal tissues dissected from patients with colorectal cancer, as well as colorectal tissues from control individuals with no cancer. The samples were processed in a blinded fashion for nested PCR and in situ PCR detection of HPV DNAs. The PCR products were gel-purified and sequenced for HPV genotyping.

**Results:** We found that colorectal tissues from 28 of 55 (51%) patients with colorectal cancer were positive for HPV DNA. Colorectal tissues from all 10 control individuals were negative for HPV DNA (P = 0.0034). Of the 107 usable (GAPDH +) samples collected as paired colorectal tissues (tumor and tumor-adjacent tissues) from the patients, 38 (36%) had HPV16 (n = 31), HPV18 (n = 5), or HPV45 (n = 2), with HPV DNA in both tumor and tumor-adjacent tissues of 10 paired samples, 13 in only the tumor, and 5 in only tumor-adjacent tissues. **In situ** PCR detection of the tumor tissues confirmed the presence of HPV DNA in tumor cells.

**Conclusion:** Our results suggest that colorectal HPV infection is common in patients with colorectal cancer, albeit at a low DNA copy number, with HPV16 being the most prevalent type. HPV infection may play a role in colorectal carcinogenesis.

**Materials and Methods**

Sample acquisition and preparation. Colorectal tissues were obtained from 55 patients with colorectal cancer as paired tissue specimens by dissection of tumor and tumor-adjacent tissues. The first set of paired colorectal tissues, collected from 45 patients in the Baltimore area, was provided by Dr. Curtis Harris at the National Cancer Institute. The second set of paired colorectal tissues was collected from 10 patients in the Houston area by one of the authors (S.Y. Xiao). In the second set of tissues, the tumor-adjacent tissues were dissected from an area ~20 cm away from the tumor lesions. Normal colorectal tissues dissected from 10 individuals who died accidentally were also provided by Dr. C. Harris and were used as negative controls. Of the 55 patients with colorectal cancer, 53 had adenocarcinoma in various colon locations, 1 had rectal squamous carcinoma, and 1 had descending colon adenoma; 32 were male and 23 female; 22 were Black, 30 White, 2 Asian, and 1 Hispanic; and their ages ranged from 35 to 82. Among the 10 controls without colorectal cancer, 4 were Black, 5 White, and 1 Asian, and they ranged in age from 21 to 75. Specific locations of the cancers and their adjacent tissues are described in Table 1.
All specimens were frozen immediately following the sample dissections and were kept at −70°C until further analysis. About 100 mg of each specimen was randomly coded and screened in a blinded manner for the presence of HPV DNAs. Each colorectal tissue was homogenized in an Eppendorf tube in 1 mL TRIzol solution (Invitrogen, Carlsbad, CA) using an electric homogenizer (Omni International, Marietta, GA) with a separate disposable probe for each tissue. The isolated DNA was dissolved in 200 μL of 8 mmol/L NaOH and adjusted to pH 7.0 with 1 mol/L HEPES. Various precautions were taken to minimize sample-to-sample cross-contamination, including the presence of GAPDH DNA and not analyzed.

**Table 1. Colorectal HPV infection in patients with colorectal cancer**

<table>
<thead>
<tr>
<th>Colorectal tissues</th>
<th>Paired samples</th>
<th>Total</th>
<th>HPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecum and ascending N</td>
<td>14</td>
<td>3 (21)*</td>
<td></td>
</tr>
<tr>
<td>Transverse N</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Descending N</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Sigmoid N</td>
<td>13</td>
<td>4 (31)</td>
<td></td>
</tr>
<tr>
<td>Rectum N</td>
<td>18</td>
<td>7 (39)</td>
<td></td>
</tr>
<tr>
<td>Unspecified N</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>38 (36)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Data in the table are the combined results of two sets of samples. Abbreviations: N, tumor-adjacent tissues; T, tumor tissues.

*Two samples had poor DNA quality with no detection of GAPDH DNA and were not analyzed.

†One sample had poor DNA quality with no detection of GAPDH DNA and was not analyzed.

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**HPV DNA detection.** HPV DNA was first amplified with pooled HPV L1 consensus primers, PGMY09/11 (20). This pooled primer set contains 5′-primers and 13′-primers and amplifies more than 25 types of anogenital HPVs. This was followed by nested PCR using an internal primer set, GP5/6 (21, 22). Each PCR reaction was carried out in a total volume of 50 μL containing 5 μL purified DNA, 1× AmpliTaq Gold PCR buffer [10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl], 2.5 mmol/L MgCl2, 200 μmol/L of each deoxynucleotide triphosphate, 100 nmol/L of pooled PGMY09/11 primers, and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA). PCR was carried out by activation of AmpliTaq Gold DNA polymerase for 9 minutes at 94°C and followed by 40 cycles of 30 seconds each at 94°C, 55°C, and 72°C, with a final extension of 7 minutes at 72°C. One microliter of the first-run PCR reaction was used as a template for the nested PCR. The conditions for the nested PCR (40 cycles) were identical to the first-run PCR with the exception of the use of 100 nmol/L of the GP5/6 primers and annealing at 40°C for 30 seconds. The amplified products with the expected size (141 bp; Fig. 1) were gel-purified and sequenced. The individual sequence was then used in a BLAST search against Genbank HPV sequences (National Center for Biotechnology Information).

Detection of HPV16 E6 (16E6), HPV18 E6 (18E6), and HPV45 E6 (45E6) DNA was also carried out by nested PCR, using primers named by the location of their 5′-ends in each virus genome. The first-run PCR primer pairs for 16E6 were Pr80 (5′-CTGACTCGAG/TTTATGCACCAAAAGAGAAC-3′) and Pr625 (5′-GATCAGTTGTCTCTGGTTGC-3′); for 18E6, Pr79 (5′-CTGACTCGAG/AGATGTGAGAAACACACCAC-3′) and Pr749 (5′-CTCGTCGGGCTGGTAAATGT-3′); and for 45E6, Pr113 (5′-TGACGATCCAAAGCAACG-3′) and Pr563 (5′-CCTACGTCTGCGAAGTCT-3′). First-run PCR was followed by reamplification using nested primer pairs for 16E6, Pr106 (5′-GTTTCAGGACCCCCAGGAC-3′) and Pr562 (5′-GTACGTTGGTCTCGGTTGC-3′); for 18E6, Pr121 (5′-ATCCAACACGGCGACCCTAC-3′) and Pr528 (5′-AGCACGAATGGCACTGGC-3′); and for 45E6, Pr131 (5′-ACCCTACAAGCTACCAGAT-3′) and Pr545 (5′-TCCTTGCGGCTCTGGTC-3′). The final PCR yielded a 456-bp

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**Fig. 1.** Schematic diagram of HPV16 genome as a representative for relative positions of primers and probes used in this study. The line in the middle of the figure represents a linear form of the virus genome for better presentation of head-tail junctions, early (E) and late (L) polyadenylation sites. Above the line, the genomic organization of HPV16 open reading frames with nucleotide (nt) positions of the start and stop codons numbered above each open reading frame. Below the bracket line, the diagrams of PCR and nested PCR amplifications for E6, E2, and L1 genes as well as L1 probe position described in this study. Illustrations are not to scale.
product for 16E6 (Fig. 1), a 407-bp product for 18E6, and a 414-bp product for 45E6.

All 16E6-positive samples were also examined by nested PCR for the presence of an intact E2 open reading frame to evaluate the integration of HPV16 DNA into the host genome. This was done using the HPV16E2 (16E2) primer set Pr3385 (5′-TATTAGGCAGCATTGGC-3′) and Pr3840 (5′-AATCTACGTAAGACCTGTAATAG-3′) for first amplification and another primer set, Pr3439 (5′-CTTGGGCACAAAGAAGAACC-3′) and Pr3790 (5′-TGGTCAGCTTGGATCATTAC-3′), for nested amplification. The final nested PCR yielded a 351-bp product for 16E2 (Fig. 1).

Under these PCR and nested PCR conditions, the detection efficiency for plasmid HPV DNA by nested PCR was limited to 1 to 10 fg of DNA (equivalent to ~130-1,300 HPV genome copies), depending on the type of HPV and the primers used (data not shown).

Dot-blot hybridization. Dot-blot hybridization was done only for the nested L1 PCR reaction and has been described elsewhere (23). A biotinylated HPV16 L1 probe (nucleotides 6664-6684; Fig. 1) was used for the hybridization at concentration of 1 pmol/mL hybridization solution.

Validation of PCR reactions. Human GAPDH DNA in each DNA sample was screened by PCR amplification using a sense primer, Pr6732 (5′-GTGATCAATGGAAATCCCATACC-3′), in combination with an antisense primer, Pr7207 (5′-TAAATGGACATCATTAGGGA/CCCTTCAGTTGCAAGGTG-3′). This primer set amplifies a 496-bp product and provides an indication of good DNA quality for each sample. Colorectal DNA samples from which GAPDH DNA could not be amplified were dropped from further study.

For HPV amplification from colorectal DNA samples, two water controls were also included for both first-run and nested PCR. If either of the two water controls yielded a false positive in the nested PCR, the controls were also included for both first-run and nested PCR. If either of these controls yielded a false positive in the nested PCR, the sample was screened by PCR amplification using a sense primer, Pr6732 (5′-GTGATCAATGGAAATCCCATACC-3′), in combination with an antisense primer, Pr7207 (5′-TAAATGGACATCATTAGGGA/CCCTTCAGTTGCAAGGTG-3′). This primer set amplifies a 496-bp product and provides an indication of good DNA quality for each sample. Colorectal DNA samples from which GAPDH DNA could not be amplified were dropped from further study.

For HPV amplification from colorectal DNA samples, two water controls were also included for both first-run and nested PCR. If either of the two water controls yielded a false positive in the nested PCR, the whole set of PCR and nested PCR reactions were started over. A sample was considered to be HPV-positive if PCR products of the expected sizes were detected for both L1 and E6 and were further confirmed by DNA sequencing.

In situ PCR. The randomly labeled colorectal tissues, which had been fixed in 10% buffered formalin for 16 to 18 hours at room temperature, were embedded in paraffin and then cut at 7 μm. The sections were placed on silane-coated slides (Labsco, Louisville, KY) and stored at 4°C until use.

The sections were deparaffinized in xylene twice for 10 minutes each and rehydrated twice for 5 minutes in each graded ethanol before being put into distilled water. The sections were then digested with 0.8% pepsin (DAKO, Carpenteria, CA) in 0.2 M HCl for 5 minutes at 37°C and rinsed in diethyl pyrocarbonate water before being subjected to a hot-start PCR amplification using AmpliTaq Gold DNA polymerase (24, 25). The PCR amplification was done on the slide in 50 μL of reaction solution containing 1× AmpliTaq Gold PCR buffer, 4 mmol/L MgCl₂, 200 μmol/L each dATP, dCTP, and dGTP, 60 μmol/L dTTP, 40 pmol/L digoxigenin-11-dUTP solution (Roche, Indianapolis, IN), 400 nmol/L of each primer 16E6 Pr106 (sense) and 16E6 Pr562 (antisense), 10 units of AmpliTaq Gold DNA polymerase, and 28 μL of water, and covered with Hybaid SureSeal (Hybaid, Franklin, MA). The slide was placed in aluminum foil on the sample block of a thermal cycler that was filled with mineral oil. After first denaturation at 95°C for 10 minutes, the section underwent amplification for 30 cycles (95°C for 1 minute, 72°C for 2 minutes, and 55°C for 2 minutes). After PCR amplification, the sections were washed in stringent wash solution (DAKO) at 50°C for 60 minutes. Detection of digoxigenin-11-dUTP incorporated into the PCR product was done with an alkaline phosphatase–conjugated digoxigenin-11-dUTP antibody (DAKO) and visualized in a chromogen solution containing nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (DAKO). Nuclear fast red was used for counterstaining. A positive reaction was defined as the presence of a purple-blue precipitate in cell nuclei.

Statistics. A two-tailed Fisher’s exact test was used for the analysis. A two-tailed McNemar’s exact test was used for the analysis of paired categorical data.

In this study, colorectal tissues from 55 patients were dissected as paired samples of the tumor itself and of the noncancerous neighboring tissues. Single tissues were obtained from descending or rectosigmoid regions of 10 control individuals with no cancer. All samples were randomly coded and processed in a blinded manner for screening; first for the presence of HPV DNA using L1 consensus primers and then using type-specific E6 primers (Fig. 2) after direct sequencing of the L1 PCR products. Sample codes were decoded after completion of the screening and sequencing. We found that 51% (28 of 55) of the patients with colorectal cancer were positive for HPV DNA in their colorectal tissues, with no relation to patient race, sex, or age, whereas none of the 10 controls had HPV DNA in their tissues (P = 0.0034). Among the 28 patients with HPV DNA in their colorectal tissues, 27 had adenocarcinoma in various colon locations and 1 had rectal squamous carcinoma.

HPV DNA was found in 42% (23 of 55, P = 0.011) of the tumor tissues from the patients and 29% (15 of 52, P = 0.1) of the tumor-adjacent tissues, as compared with none of the controls. Ten paired samples contained HPV DNA in both tumor and tumor-adjacent tissues, 13 only in the tumor, and 5 only in tumor-adjacent tissues from the rectosigmoid area. There was a weak trend for HPV DNA to be detected in the tumor tissues more than the tumor-adjacent tissues (P = 0.14 by McNemar’s exact test), but the sample size was too small to detect statistical significance. Among the 38 HPV-positive tissues obtained from the 55 patients, 31 had HPV16, 5 had HPV18, and 2 had HPV45. Among the 10 patients who had HPV DNA in both the tumor and tumor-adjacent tissues, 8 had HPV16, 1 had HPV18, and 1 had mixed rectal HPV16/18 infection with HPV16 DNA in the tumor tissue and HPV18 DNA in the tumor-adjacent tissue. Tissues taken from cecal and ascending colon cancers had similar HPV detection rates as those taken from the rectosigmoid locations (Table 1).

Sequence analysis of all HPV16 isolates from colorectal tissue showed that they were European-derived HPV16 prototype viruses with a T or G at position 350, an A at position 6695, and a G at 6721. Among the eight paired samples that were HPV16 DNA-positive both for the tumor tissue and the tumor-adjacent tissue, two paired samples had additional variations in the
The samples were found to be 16E2-positive (episomal DNA; HPV16 E2 gene (16E2) by nested PCR, and 32% (10 of 31) of positive samples were examined for the presence of the intact DNA has integrated (27). Thirty-one of the HPV16 DNA-amplified by our nested PCR.

Indirect, amplification of the E2 region indicates the presence of episomal HPV DNA in the cells; otherwise, it is assumed that the DNA has integrated relatively frequently in the tumor tissues. However, interpretation is limited by the small sample size, and the fact that some tissues might contain both episomal and integrated forms, as has been reported (26). Direct in situ PCR amplification with an 16E6-specific primer set was further done on three tumor tissues and two tumor-adjacent tissue sections. Each of the three tumor tissue sections gave strong in situ amplification signals, which consistently appeared as blue-purple deposits in the nuclei of tumor epithelial cells (Fig. 5).

Discussion

Colorectal cancer is the third most common type of cancer in the U.S., with an estimated 147,500 new cases in 2003, and is the second leading cause of cancer mortality, accounting for an estimated 57,100 deaths in 2003 (28). The precise etiology of colorectal cancer is not known. Although there is substantial evidence implicating high-risk HPV infection in anal cancer (29), a rare neoplasm in the anus, such implication in colorectal cancer remains controversial. In the present study, we have shown that HPV infection,
particularly with HPV16, is common in colorectal cancer tissues and tissues adjacent to the cancers. The HPV16 DNAs detected from these tissues were all European-derived subtypes. Although additional sequence variations were observed at various positions in these subtypes, these seemed to be more common in the viral DNAs detected from tumor tissues. In two sets of paired tissues, both the tumor and tumor-adjacent tissues contained European-derived HPV16 DNAs, but the additional nucleotide substitutions appeared only in the HPV DNAs detected from tumor tissues, indicating that the viral DNAs in the tumor were different from the ones in the tumor-adjacent tissues in those individuals. In addition, it remains to be investigated whether the patients with HPV infections also had predisposing genetic factors or some form of genetic instability, which has been associated with colorectal cancer (30). Our finding that HPV DNA-positive cells are most common in tumor lesions suggests that HPV might play a role in the pathogenesis of colorectal cancer. However, this presumption is confined by our sample size and needs further confirmation. In a previous study, HPV DNA was found in 27% of colorectal adenomas, 31% of invasive colorectal carcinomas, and 69% of colorectal carcinomas in situ by in situ hybridization using pooled probes for HPV6, 11, 16, 18, 31, 33, and 35 (12). In a separate study, HPV DNA was detected by HPV type–specific E6 PCR in 30% of adenomas and in 53% of carcinomas, with HPV16 as the predominant type (14).

HPV16 was the most prevalent type in colorectal tissues in this report (31 of 38 positive samples, 82%). A recent study reported a high frequency of colorectal HPV18 infection in patients with colorectal cancer in Taiwan (15); however, other studies have shown that colorectal HPV16 infection is common in Taiwanese patients with colorectal cancer (14). We found that colorectal HPV18 infection was much less prevalent in our patients (present in only 5 of 38 samples, <13%), one of whom had dual infection with HPV16 and 18. In addition, two patients had HPV45 in their tumor tissues. The finding that HPV infection in the tumor tissues obtained from the cecal and ascending colon regions is as common as in the tissues obtained from rectosigmoid locations suggests that this infection might not be a result of direct spread from anogenital sites.

E2 detection was used in this study as a means to evaluate HPV DNA integration, because the viral E2 gene is often disrupted during HPV DNA integration. Although the assay can only detect integrated viral DNA in the absence of episomal HPV DNA, only one-third of the HPV-positive samples had
intact E2. Even though the overall numbers are low, the viral DNA seemed to have integrated relatively frequently in the tumor tissues relative to the tumor-adjacent tissues.

In summary, colorectal HPV16 infection seems to be common in colorectal cancer tissues and adjacent nontumor tissues, suggesting that HPV might play a role in the pathogenesis of colorectal cancer. However, where and how HPVVs come to colorectal tissues remains to be investigated.

References


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