

A Multifaceted Study of Human Papillomavirus and Prostate Carcinoma

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BACKGROUND. The presence of human papillomavirus (HPV) in the prostate and its role in prostate carcinoma are in dispute. To address these issues, two laboratories with extensive HPV experience were selected to test specimens from two populations at different risk for prostate carcinoma, using three different polymerase chain reaction (PCR) assays and two serologic assays for HPV.

METHODS. The cases were comprised of 51 African-American (men at high risk for prostate carcinoma) and 15 Italian (men at intermediate risk for prostate carcinoma) men with prostate carcinoma. Controls were 108 African-American men and 40 Italian men with histologically proven benign prostatic hyperplasia (BPH). Prostate tissue was obtained from each patient at surgery and immediately frozen in liquid nitrogen. The PCR primer sets included two (MY09/MY11 and GP5+/GP6+) that amplify different regions of L1 and a third (WD66,67,154/WD72,76) targeted to E6. Sensitivity in the 2 L1 PCR assays was shown to be 1 HPV DNA genome per 100 cells. Serum antibodies to HPV-16 and HPV-11 virus-like particles (VLPs) were detected using enzyme-linked immunosorbent assays.

RESULTS. All available prostate carcinoma tissue specimens (n = 63) and BPH specimens from selected controls (n = 61) were tested by PCR. Human β -globin DNA could be amplified from all specimens except three carcinomas, but no HPV DNA was detected in any case or control specimens by MY09/MY11 or E6 PCR. Microdissection of 27 carcinoma specimens was conducted to minimize nontumor DNA, but results remained negative by MY09/MY11 and GP5+/GP6+ PCR. In addition, serum specimens in cases (n = 63) and controls (n = 144) showed no differences in their responses against HPV-16 ($P = 0.54$) or HPV-11 VLPs ($P = 0.64$).

CONCLUSIONS. The findings suggest that HPV is not associated with prostate carcinoma, and that HPV DNA is not at all common in the prostate glands of older men. *Cancer* 1998;82:1118–25. © 1998 American Cancer Society.

KEYWORDS: human papillomavirus, prostate carcinoma, benign prostatic hyperplasia, polymerase chain reaction, serology.

Human papillomavirus (HPV), a sexually transmitted DNA virus linked to the development of cervical carcinoma and other anogenital tumors, was detected in prostate tissues in several recent investigations.^{1–13} Prostate carcinoma (PCa) is the most common malignancy in American men.¹⁴ Identification of an infectious agent etiologically linked to PCa would be an important discovery. It also is important to know whether the prostate is a reservoir for HPV infection, and therefore might play a role in the transmission of this virus to sexual partners.

Hypotheses that environmental factors could play a role in PCa tumorigenesis have been prompted by increasing PCa incidence in some countries and the heterogeneous racial, religious, and regional distributions of cases.^{15–22} Furthermore, several reports, but not all,

have suggested that PCa specifically might be associated with sexually-related exposures.²³⁻²⁷ Indeed, a number of laboratory studies in the past reported evidence of viruses in prostate tissue, especially herpes viruses.²⁸⁻³² However, the latter observations have not yet been confirmed using current sensitive DNA detection methods.³³

In contrast, HPV DNA in the prostate has been demonstrated by polymerase chain reaction (PCR) and other methods in recent studies (Table 1).¹³ Several of these investigations showed a clear cancer association with HPV, but others reported that HPV was equally prevalent in benign prostate hypertrophy (BPH) and even in normal prostate tissue. Additional studies did not detect HPV in any prostate tissues.³³⁻³⁵ Complicating matters further, most investigations in the prostate have been small, and have used varied clinical and laboratory approaches. However, the detection of HPV in these studies cannot be ascribed easily to particular methods of specimen collection, preservation, DNA testing, or patient populations.

To assess whether HPV is associated with PCa, or at least can be detected in prostate specimens, we selected two laboratories with extensive HPV experience to test specimens obtained from two distinct populations at different risk of PCa using a multifaceted laboratory approach.³⁶⁻³⁸ Specifically, to minimize the chance of false-negative results due to the selection of laboratory methods, Laboratory (Lab) 1 used three different sensitive HPV DNA PCR assays targeted to different conserved regions of the viral genome, and as a novel step, Lab 2 tested patients' sera for antibodies (immunoglobulin G) to HPV capsid proteins, humoral immune responses that are associated highly with HPV-related cervical, anal, and vulvar carcinoma.³⁹⁻⁴¹ Finally, to minimize the possibility of missing an association due to patient characteristics, we tested specimens from both African-American men, who have the highest PCa incidence in the world, and Italian men, an intermediate risk group.²²

METHODS

Subjects

Between 1991 and 1994, 51 African-American men with PCa and a comparison group of 108 patients with BPH (controls) were enrolled sequentially as they presented at Howard University Hospital, Washington, DC. During the same period, 15 Italian men with PCa and 104 controls with BPH similarly were enrolled as they presented at Malpighi Hospital, Bologna, Italy. All subjects enrolled at Howard University were included in the current investigation. In contrast, in Italy subjects with BPH were frequency-age matched to PCa cases, using 5-year age groups and a 3:1 ratio. Not all

cases had 3 appropriate controls available, however, and 40 individuals were selected as controls for the current analysis.

Clinical Methods

All patients underwent surgery for resection or had biopsy of the prostate. In the surgery suite, each prostate tissue specimen was sliced longitudinally in two halves; one half of each specimen was placed immediately in vapor-phase liquid nitrogen for DNA studies and the other half was placed in formaldehyde for histology. Although the two halves of each tissue specimen were contiguous, it remained possible that tissue heterogeneity could lead some specimens to contain few abnormal cells. Therefore, in a random subset of PCa specimens the tissues were microdissected to separate tumor cells. In brief, tumor specimens were ethanol fixed, paraffin embedded, and sectioned, generating a first and last section for hematoxylin and eosin staining for determining the location of cancer cells, and four 50- μ thick sections from each tumor specimen then were dissected under magnification using a small blade.

At Howard University, histologic tumor grade was assessed by Gleason score, whereas Broder grade was used in Italy. A single classification scheme was not imposed because these data were used only for descriptive purposes and had no bearing on the findings. All patients had blood samples drawn at entry into the study, separated within 2 hours into serum, plasma, and buffy coat, and immediately frozen. Blood specimens and frozen tissue sections were maintained at or below -80°C until testing.

Laboratory Methods

HPV DNA Testing

All samples were processed by Lab 1 in a BioSafety Cabinet in a laboratory physically separate from where the PCR amplification was performed. Frozen prostate tissue from all carcinoma cases ($n = 63$; 1 Howard University and 2 Malpighi Hospital cases had no testable specimens available) were tested for the presence of HPV DNA. A similar number of specimens from controls (i.e., patients with BPH), individually matched to cases by age (within 5 years) and hospital, were identified and also sent for HPV DNA testing (61 selected controls had available specimens).

To extract DNA, frozen prostate tissue specimens approximately 0.1 cm \times 0.2 cm in dimension were thawed, digested overnight in 500 μL of buffer (10 mM ethylenediamine tetraacetic acid, 100 mM Tris-CL, and 2% laureth 12 [pH 8.5]) containing 400 $\mu\text{g}/\text{mL}$ of proteinase K at 55°C , and inactivated the next day by heating specimens to 95°C for 10 minutes. The

TABLE 1
Summary of Studies Investigating HPV in the Prostate

Reference	HPV detection	Collect (storage)	Subjects			HPV types			
			No.	Type	HPV	6	16	18	X
McNicol and Dodd ¹ (1990)	Southern blot for HPV-16, and -18	Mostly TURP, also SPP (frozen)	4	PCa cases	75%				
			12	BPH controls	33%				
McNicol and Dodd ² (1990)	E6 PCR for HPV-16, -18	Mostly TURP, also SPP and autopsy (frozen)	4	PCa cases	100%		4	3	
			15	BPH controls	93%		14		
			5	normal autopsies	20%		1		
Masood et al. ³⁴ (1991)	ISH for HPV-6, 11, 16, 18, 31, 33, and 35	Biopsies/TURP (paraffin)	20	PCa cases	None				
			20	BPH controls	None				
McNicol and Dodd ³ (1991)	E6 PCR for HPV-16, 18	TURP/SPP (frozen)	27	PCa cases	52%		14	1	
			56	BPH controls	63%		34	3	
Anwar et al. ⁴ (1992)	E6 PCR for HPV-16, 18, and 33	TURP/SPP and autopsy (paraffin)	68	PCa cases	41%		11	17	5
			10	BPH controls	None				
			10	normal autopsies	None				
Rotola et al. ⁵ (1992)	E6 PCR for HPV-6/11, and 16	Collection NS (frozen)	8	PCa cases	NS	4	6		
			17	BUK lesions	NS	14	11		
			62		NS	18	27		
Effert et al. ³⁵ (1992)	"Differential" E6 PCR for HPV-16, and 18	Collection NS (frozen)	30	PCa cases	None		2	2	
			8	Cervical carcinoma	38%				
Serfling et al. ³³ (1992)	L1 Consensus Primer PCR	Collection NS (frozen)	30	PCa and BPH subjects	None				
Ibrahim et al. ⁶ (1992)	L1 PCR and ISH	Biopsies/TURP/SPP (paraffin and frozen)	40	PCa cases	15%		6		
			12	BPH controls	None		2		
			17	Normal	12%				
Sarkar et al. ⁷ (1993)	E6/E7 PCR for HPV-6/11, 16, and 18; also Southern blot	Surgical not TURP (paraffin- microdissection)	23	PCa and PIN cases	13%		3		
Dodd et al. ⁸ (1993)	Reverse transcription PCR for E6/E7 mRNA of HPV-16	Collection NS (frozen)	7	PCa cases	43%		3		
			10	BPH controls	50%		5		
Tu et al. ⁹ (1994)	L1 consensus primer PCR	Surgical not TURP (tumors-paraffin metastases-frozen)	43	PCa cases	2%		1		
			17	metastases	6			1	
			1	normal	None				
Moyret-Lalle et al. ¹⁰ (1995)	E6 PCR for HPV-16, and - 18	Collection NS (frozen)	17	PCa cases	53%		9		
			22	BPH controls	32%		7		
Wideroff et al. ¹¹ (1996)	L1 consensus primer PCR, and E6 PCR for HPV-6, 11, 16, 18, 31, 33, and 45	TURP and SPP (paraffin)	56	PCa cases	L1 13% E6 0%				
			42	BPH controls	L1 10% E6 0%				
Suzuki et al. ¹² (1996)	L1 consensus primer PCR	Surgery or autopsy	51	PCa cases	16%				

HPV: human papillomavirus; TURP: transurethral resection of the prostate; SPP: suprapubic resection of the prostate; PCa: prostate carcinoma; BPH: benign prostate hypertrophy; PCR: polymerase chain reaction; ISH: in situ hybridization; NS: not specified; BUK: bladder, ureters, kidneys, and urethra; PIN: prostatic intraepithelial neoplasia.

adequacy of the DNA in each specimen for PCR amplification was determined by detection of a 268-base pair (bp) fragment of the β -globin gene after amplification using the GH20/PC04 primer set. To detect HPV DNA, three different PCR-based assays were used, as follows: 1) MY09/MY11, which amplifies a highly conserved 450-bp region from the L1 open reading frame (ORF) of most mucosotropic HPV types (amplicons were detected by Southern blot hybridization with a radiolabeled "generic probe" as described)^{36,37}; 2) GP5+/GP6+, which amplifies a 150-bp region within the L1 ORF of most mucosotropic HPV types (amplicons were detected using the same "generic probe" described for the MY09/MY11 PCR)^{42,43}; and 3) WD66,67,154/WD72,76, which amplifies a 240-bp region in the E6 ORF from the HPV types most commonly associated with cervical carcinoma (amplicons were detected by Southern blot hybridization with radiolabeled whole genome probes of HPV-11, 16, 18, 51, and 61, as described).^{44,45}

For MY and E6 PCR, assays were run as follows. To 10 μ L of the digested specimens 90 μ L of PCR buffer was added, yielding a final concentration of 10 mM Tris-HCl, 50 mM KCl, 4.0 mM MgCl₂, 200 mM of each dNTP, and 2.5 units of Taq polymerase, as well as primers. The concentration of primers in MY PCR was 50 pmol each (in E6 PCR) and was 10 pmol WD72, 40 pmol WD76, 10 pmol WD66, 40 pmol WD67, and 10 pmol WD154 as described.⁴⁴ Thirty-five cycles of amplification were conducted (20 seconds at 94 °C for denaturing, 30 seconds at 55 °C for annealing, and 30 seconds at 72 °C for extension, with a final extension of 5 minutes) using a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Oak Brook, IL). GP5+/GP6+ PCR was conducted as previously described.^{36,42} The products of amplifications were denatured, 10 μ L of each product was drawn onto nylon membranes, immobilized by heating at 80 °C for 1 hour under vacuum, and hybridized with the described radiolabeled probes.

To determine whether HPV DNA could be detected in specimens in which tumor cell DNA was predominant, paraffin embedded carcinoma specimens were microdissected in a random subset of cases ($n = 27$), as described earlier. These microdissected samples were pretreated with 1 mL of octane for 5 minutes to remove paraffin. The octane then was removed and the pellets were washed twice using 500 μ L of ethanol and dried. DNA testing was performed using MY09/MY11 PCR as well as the GP5+/GP6+ system, because this system amplifies a small HPV DNA fragment and is preferable in samples extracted from paraffin.⁴⁶

Positive and negative controls were run with each amplification. Negative controls included a water specimen placed every 20th sample during processing,

and a cellular DNA positive/HPV DNA negative specimen (cell line Huh7) tested in every amplification. All negative controls tested negative in all experiments. HPV DNA positive controls were comprised of a 200-cell aliquot of the SiHa cell line that contains 1-2 copies per cell of integrated HPV-16 and a previous sample known to contain HPV-31.⁴⁷ Each amplification had these two positive controls, and they tested positive in all experiments. The sensitivity of the MY09/MY11 and GP5+/GP6+ PCR systems was evaluated further by serially diluting SiHa cells. MY09/MY11 and GP5+/GP6+ were able to detect HPV-16 DNA in 20–50 and in 10–20 SiHa cells, respectively (data not shown). This suggests that the PCR assays had sufficient sensitivity to detect 1 HPV DNA copy per 100 prostate cells; because each test sample contained 50–100 ng of cellular DNA, the equivalent of approximately 10^4 cells.

Serology

In Lab 2, HPV-16 and HPV-11 virus-like particles (VLPs) were prepared from sf9 insect cells infected with a recombinant baculovirus expressing the L1 and L2 viral capsid proteins purified by the method of Kirnbauer et al.⁴⁸ The purified VLP preparations then were used to coat the wells of a 96-well polystyrene microtiter plate (Corning-Costar, Cambridge, MA) at a total protein concentration of 10 μ g/mL and a volume of 50 μ L in phosphate-buffered saline (PBS) [pH 7.4] and held overnight at 4 °C. Control wells were coated with PBS. After washing the plate 5 times with PBS-0.05% Tween 20 (PBS-T), 50 μ L of test serum diluted 1:10 in PBS-0.5% nonfat milk was added to each of 2 antigen and control wells. The plate was incubated for 2 hours at 37 °C, then washed 5 times with PBS-T. VLP-reactive antibodies were detected with horseradish peroxidase-conjugated recombinant Protein G (Zymed Laboratories, San Francisco, CA) diluted 1:20,000 in PBS-T after 30 minutes incubation with the conjugate, followed by 30 minutes incubation with freshly prepared ABTS and hydrogen peroxide solution (Kirkegaard and Perry, Gaithersburg, MD). Optic density (OD) was read in a dual wavelength microtiter plate reader (Molecular Devices Corp., Menlo Park, CA) at 405 nanometers (nm) with a reference wavelength of 490 nm. Seropositivity in the HPV enzyme-linked immunosorbent assays (ELISA) were determined using previously determined OD cutoff values. Human serum samples previously found to be reactive in the HPV-16 and HPV-11 ELISAs and a mouse monoclonal antibody directed against conformational epitopes on intact HPV-16 virions (gift of Shin-je Ghim, Georgetown University School of Medicine, Washington, DC) were used as positive controls. The negative control was PBS-0.5% nonfat dry milk, the diluent for serum samples.

TABLE 2
Characteristics of Patients

	Howard University (U.S.)	Malpighi Hospital (Italy)
BPH	N = 108	N = 40
Age (yrs) mean (median)	67 (69)	67 (66)
Race		
African-American	100%	0%
White	0%	100%
Surgery ^a		
TURP	83%	82%
Surgical ^b	17%	18%
Carcinoma	N = 51	N = 15
Age (yrs) mean (median)	72 (73)	67 (64)
Race		
African-American	100%	0%
White	0%	100%
Surgery		
TURP	80%	75%
Surgical ^b	20%	25%
Grade ^c		
Low (Gleason 1-3 or Broder 1)	20%	0%
Moderate (Gleason 4-6 or Broder 2)	42%	20%
High (Gleason 7+ or Broder 3+)	38%	80%

BPH: benign prostate hypertrophy; TURP: transurethral resection of the prostate.

^a Data regarding surgical procedures was not available from one Italian and four African-American controls, and seven African-American cases.

^b Open resection or biopsy.

^c Tumor grade was not available from six African-American cases. Tumor grade was determined by Gleason score in the U.S. and by Broder's grade in Italy.

Statistical Methods

In exploratory data analysis, categoric data were summarized in contingency tables and analyzed by the chi-square or Fisher's exact tests. Continuous data (e.g., antibody responses and age) first were examined for normality, and appropriate transformations were made of the data prior to any statistical tests. Antibody responses were determined by subtracting background mean ELISA values (i.e., the OD of each serum sample in the wells without viral antigen) from the mean OD values of each sample in wells with viral antigen. To normalize their distribution, antibody responses were log-transformed, and the log-transformed values were compared between cases and controls using the student's *t* test. These analyses then were stratified to control for race and nationality.

RESULTS

Subjects

Patient characteristics are shown in Table 2. Consistent with the study design, all patients enrolled

through Howard University were African-American and all patients enrolled through Malpighi Hospital were white and Italian. The age distributions were fairly similar in all patient groups. Indeed, Italian cases and controls had the same mean age ($P = 0.56$), consistent with the use of frequency age-matching. However, among African-Americans, the difference in mean age between cases and controls (5 years) was statistically significant ($P < 0.01$). Transurethral resection of the prostate was the most common method through which prostate tissue samples were obtained in cases and controls in both national-racial groups.

HPV DNA

PCR was conducted on frozen prostate tissue from 63 PCa cases (49 African-Americans and 14 Italians), and on specimens from 61 age-matched and race/nationality-matched BPH controls (48 African-Americans and 13 Italians). Only three patients' specimens were β -globin negative (three African-American PCa cases). However, no HPV DNA was detected in any frozen prostate tissue specimens using MY09/MY11 or E6 PCR. Likewise, all 27 tumor DNA-enriched specimens obtained after microdissection tested positive for β -globin amplification, but none were positive for HPV DNA using either MY09/MY11 or GP5+/GP6+ PCR (Table 3).

HPV Serology

Figure 1 shows serologic results for cases ($n = 63$) and controls ($n = 144$) in the HPV-16 VLP ELISA (specimens were not available from 2 cases and 4 controls among African-Americans, and 1 Italian case). The distribution of these OD values in the assay essentially were identical regardless of case-control status. That is, cases and controls had similar geometric mean ($P = 0.54$) and median values, as well as similar interquartile ranges and a similar frequency of zero values. Based on the predetermined serum cutoff value, HPV-16 seroprevalence was similar ($P = 0.44$) in cases (1.6%) and controls (4.9%). Likewise, HPV-11 VLP antibody responses were not related to case-control status. Cases and controls again had similar geometric mean ($P = 0.64$) and median values, similar interquartile ranges, a similar frequency of zero values, and similar seroprevalence (data not shown). There was no correlation of antibody responses with age, and stratification of the analyses by race/nationality had no effect on the findings.

DISCUSSION

We tested PCa cases and patients with BPH for evidence of HPV infection to assess whether HPV might play a role in the development of PCa and whether

TABLE 3
Human Papillomavirus DNA Results

	No.	β -globin	MY09/M11 ^a	GP5+/GP6+ ^a	E6 ^a
BPH specimens	61	100%	0%	ND	0%
Carcinoma specimens	63	95%	0%	ND	0%
Microdissected tumor cells	27	100%	0%	0%	ND

BPH: benign prostate hypertrophy; ND: not done.

^a Percent positive.

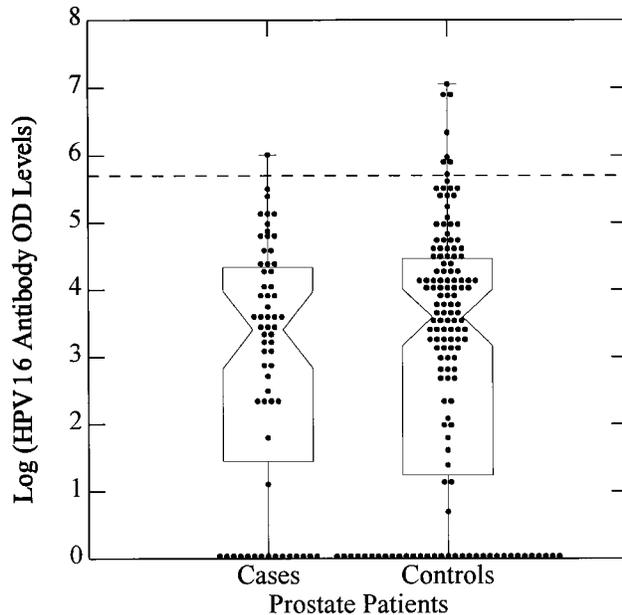


FIGURE 1. Immunoglobulin responses to HPV-16 virus-like particles (VLPs) in prostate carcinoma cases and controls. The values shown are optic density (OD) results in a serum enzyme-linked immunosorbent assay test. VLPs based on the L1 region of HPV-16 were used as antigen. Boxplots are interpreted as follows: the waist is the median, diagonal lines indicate 95% confidence intervals about the median, lower and upper horizontal lines indicate 25th and 75th percentiles of the distribution, and upper and lower bars indicate the range of the data excluding outliers. The dotted horizontal line across the figure shows the serum cutoff value in the assay, an OD of 300 (log = 5.7).

HPV infects the prostate. Recent studies of HPV in the prostate have reported conflicting findings and used varied methods.^{1-13,33-35} Therefore, to address these issues, in this study we used several complementary laboratory approaches, including three different HPV PCR tests and two HPV antibody assays, in two separate populations at different risk of PCa. However, not a single prostate tissue specimen was positive for HPV DNA in any of the PCR assays. Also, there was no

evidence of an association between antibody responses to HPV and PCa. Thus, our data strongly suggest that HPV is not associated with PCa, and in addition, that HPV DNA is not at all common in the prostates of older men.

Consistent with these negative results, the relation between PCa and sexual behavior has not been reported consistently in case-control studies.^{26,27,49} Likewise, populations at low risk of sexually transmitted diseases, such as Mormons in Utah,¹⁸ have a high PCa incidence, and PCa risk is not increased among older (e.g., age \geq 60 years), never married men, a group that has been used as a population surrogate for prevalent homosexuality and other high risk sexual behaviors in examining the relation between acquired immunodeficiency syndrome and other malignancies.⁵⁰

For many additional reasons the failure to detect HPV DNA in prostate tissue specimens in this study is unlikely to be due to laboratory methods. First, the DNA were in good condition for testing by PCR. That is, tissue specimens initially were snap frozen (which is considered optimal for preservation of DNA); essentially all DNA samples tested positive for human β -globin (a sensitive but nonspecific marker of the adequacy of DNA for PCR); and tumor specimens microdissected to minimize nontumor DNA still were β -globin positive and HPV negative. Second, we used three different PCR assays designed to amplify distinct highly conserved regions of the HPV genome. The MY09/MY11 PCR assay we used, which amplifies a region of the L1 ORF, most likely is the most widely employed HPV PCR method in the U.S. We also used GP5+/GP6+ PCR, which amplifies a smaller region of L1, in case the HPV genome was fragmented, and E6 PCR, in case (unlike in other anogenital tissue) the L1 open reading frame was lost in HPV infection of the prostate. The laboratory that conducted these tests has conducted numerous studies of HPV DNA in anogenital tumors, and in this investigation the laboratory demonstrated that the MY09/MY11 and the GP5+/GP6+ PCR assays they employed could detect 1 HPV

DNA copy per 100 prostate cells. In summary, if HPV DNA was present clonally in prostate carcinoma specimens we should have been able to detect it.

Subjects also were tested for antibodies to HPV. The HPV-16 VLP ELISA used strongly discriminates women with cervical neoplasia and men with anal carcinoma from controls.^{38,39,40} Indeed, HPV-16 is the virus responsible for most cervical and anal carcinomas,⁵¹ and most positive studies of the relation between HPV and PCa specifically implicated HPV-16.¹⁻¹³ However, there was no association at all between HPV-16 antibody responses and the presence of PCa. Similarly, there was no relation between antibody to HPV-11 VLPs and PCa. Thus, the serologic data are consistent with the negative PCR results and suggest that HPV plays no role in the development of PCa.

Finally, patient characteristics are unlikely to explain the negative results in this investigation. We studied African-Americans, men with the highest PCa rates in the world,²² and who as a group have a relatively high number of lifetime sex partners, initiate intercourse at a young age, and experience a high incidence of sexually transmitted diseases,²⁴ and we studied Italians, men who have an intermediate risk of PCa relative to other races and nationalities.²²

The data from this study strongly suggest that HPV does not have a role in the development of PCa. Moreover, the negative PCR data indicate that HPV DNA, and, by extension, HPV infection, is not at all common in the prostate glands of older men, such as those tested. Future research should focus on determining whether PCa indeed is linked to high risk sexual behavior by using biologic (e.g., serologic) markers of lifetime exposure to sexually transmitted infections, which are invulnerable to recall bias.

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