

Presence of Human Papillomavirus DNA in Testicular Biopsies From Nonobstructive Azoospermic Men

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• **Context.**—Human papillomavirus (HPV) plays a major role in the etiology of many malignancies of diverse localization, such as uterine cervical carcinoma and its precursors. Human papillomavirus sequences have been detected throughout the male lower genitourinary tract, but the role of men as transmitters remains unclear.

Objective.—To investigate the relationship between azoospermia and the presence of HPV DNA in testicular cells.

Design.—One hundred eighty-five patients with azoospermia undergoing testicular biopsy were studied. Histologic study was done on formalin-fixed, paraffin-embedded samples from testicular biopsies, stained with hematoxylin-eosin. Molecular study to detect HPV sequences was performed on genomic DNA isolated from paraffin sections by standard protocols. Seven cases containing HPV sequences were studied after microdissection with PALM microlaser technology in order to determine the presence of

HPV DNA sequences in different cells, as well as from seminal tubules or stromal (Leydig) cells.

Results.—Human papillomavirus DNA sequences were detected in testicular biopsies of 12 patients (6.48%). Human papillomavirus type 16 was the most common genotype encountered. Among the 92 patients who underwent bilateral testicular biopsy, HPV sequences were detected in 9 patients (9.78%), all of whom showed only unilateral testicular affection, more often in the left testicle (ratio, 2:1). After microdissection, HPV DNA sequences were seen in Leydig and Sertoli cells; the presence of HPV in germinal cells could not be ruled out.

Conclusions.—Leydig cells, Sertoli cells, and probably germinal cells (cases 2, 3, and 4) harbored HPV DNA sequences. Such findings have not been previously described in testicular tissue.

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It is well established that human papillomavirus (HPV) plays a major role in the etiology of many malignancies of diverse localization. In uterine cervical carcinoma and its precursors, we have long been aware that a so-called male factor¹ exists in the sexual transmission of HPV infection. Human papillomavirus sequences have been detected throughout the male lower genitourinary tract: penis,² urethra,^{3,4} prostate,⁵ seminal vesicles, epididymis, and vas deferens,⁴ as well as seminal fluid⁶ and spermatozoa.⁷ Although asthenoazoospermia⁸ has been reported in men with genital HPV infection, the presence of HPV sequences in human testis and the probable relationship with azoospermia has not been well documented.

The purpose of this work was to investigate the presence of HPV DNA sequences in 277 surgically obtained (paraffin-embedded) testicular biopsies from 185 patients

with nonobstructive azoospermia who were studied for infertility.

MATERIALS AND METHODS

Patients

One hundred eighty-five patient candidates for intracytoplasmatic sperm injection because of severe spermatogenesis failure with complete nonobstructive azoospermia were submitted to testicular biopsies. Bilateral open testicular biopsies were surgically obtained from 92 patients; 93 underwent exclusively right-testicular biopsy. In no case was there any clinical evidence of external genital warts.

Average age of patients was 35 years (range, 25–60 years). Azoospermia was considered idiopathic (104 patients) or secondary to cryptorchidism (24), varicocele (20), orchitis (19), chemotherapy (9), Klinefelter syndrome (5), renal failure (2), ischemia (1), and thalassemia (1).

One hundred testicular samples from 50 healthy men who died in traffic accidents were studied for histologic findings associated with infertility in the general population; these were included as controls. Average age was 23 years (range, 19–30 years).

Histologic and Molecular Study

Histologic study was done on formalin-fixed, paraffin-embedded samples from testicular biopsies, stained with hematoxylin-eosin. Molecular study to detect HPV sequences was performed on genomic DNA isolated from paraffin sections by standard protocols. Several 5- μ m sections were obtained from each case; 1 was sent for histologic study, and the others were submitted to molecular study in order to detect HPV DNA. An additional sec-

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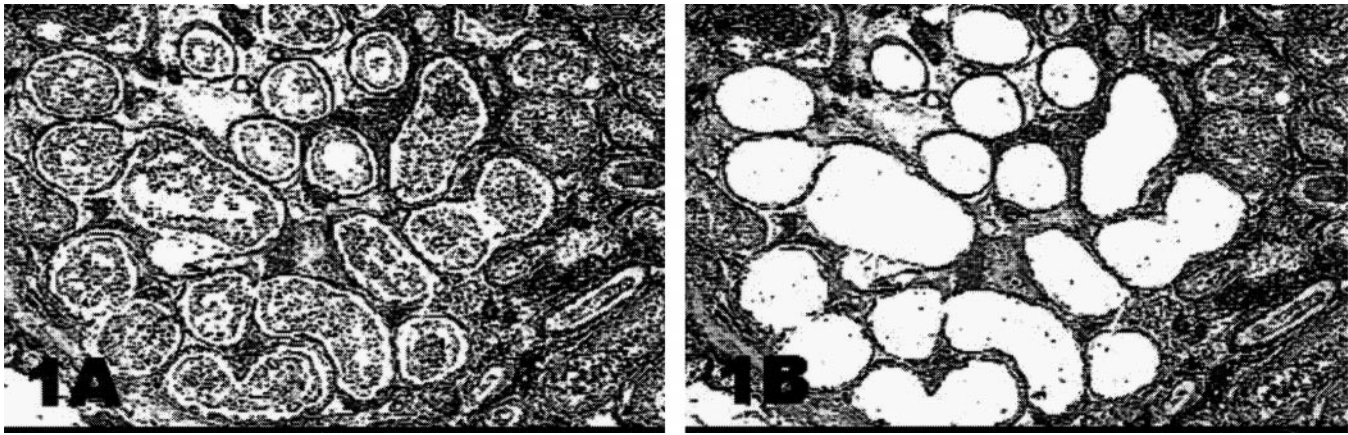


Figure 1. Microdissection of tubules. A, Selected tubules are separated from unwanted material. B, Selected tubules have been catapulted (hematoxylin-eosin, original magnification $\times 100$). Sample 9 was a positive control (high-grade cervical intraepithelial neoplasia DNA, previously confirmed for human papillomavirus [HPV] type 18 L1 gene sequence). Sample 1 was a negative control (no template DNA); sample 2 was a control on DNA extraction (extraction protocol on water). Sample 10 was a negative control (no DNA in first round of amplification). Sample 11 was a negative control (no DNA in second round of amplification). Samples 3, 4, 5, 6, 7, and 8 were DNA from testicular biopsies (positive amplification in samples 3 [case number 3], 5 [case 10], and 7 [case 7] for HPV types 16, 33, and 11, respectively, after sequencing).

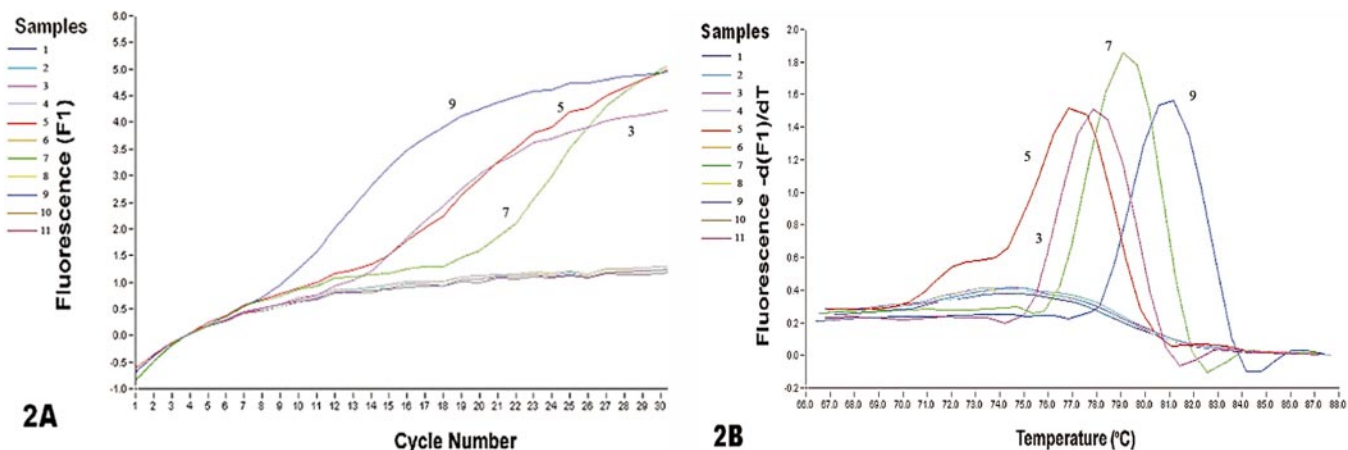


Figure 2. A, LightCycler polymerase chain reaction amplification curves (fluorescent ratio vs amplification cycle number with SYBR Green I). B, LightCycler melting curves (fluorescent ratio vs temperature with SYBR Green I). Sample 9 was a positive control (high-grade cervical intraepithelial neoplasia DNA, previously confirmed for human papillomavirus [HPV] type 18 L1 gene sequence). Sample 1 was a negative control (no template DNA); sample 2 was a control on DNA extraction (extraction protocol on water). Sample 10 was a negative control (no DNA in first round of amplification). Sample 11 was a negative control (no DNA in second round of amplification). Samples 3, 4, 5, 6, 7, and 8 were DNA from testicular biopsies (positive amplification in samples 3 [case number 3], 5 [case 10], and 7 [case 7] for HPV types 16, 33, and 11, respectively, after sequencing).

tion was studied in HPV-positive cases to confirm the findings microscopically.

Seven cases containing HPV sequences were studied after microdissection with PALM microlaser technology (PALM GmbH, Bernried, Germany) on 6- μ m paraffin sections stained with hematoxylin-eosin in order to obtain tubules (between 10–20) or Leydig cells from each of the cases. Sections (6 μ m thick) of testes were mounted on PALM membrane slides. Interstitial (Leydig) or tubular cells (Figure 1) were collected and catapulted into a tube.⁹ Laser capture microdissection parameters included a power of 90 mW, laser pulse duration of 1.2 ms, and laser spot size of 7 to 15 μ m in diameter. To avoid contamination, microdissection of tubules or Leydig cells was performed on 2 different sections of each of the positive cases. Extraction of DNA from the tubules or the interstitium (Leydig cells) and studies of the DNA were carried out separately.

Human papillomavirus DNA was detected by nested polymerase chain reaction (PCR) amplification in a fragment of the L1 gene using consensus primers MY11/09 and GP5+/GP6+.¹⁰

Adequacy of the DNA was assessed with β globin primers. In experiments made with serial dilutions from cloned DNA of HPV 16 and 18, sensitivity was 1 to 10 copies and 10 to 100 copies, respectively.

Following DNA extraction from paraffin-embedded biopsies, 100 ng DNA in a total volume of 20 μ L were used for the first-round reaction on a conventional thermal block system, which comprised 20 cycles of denaturation; 0.5 μ L of this product was used for the second-round amplification on a LightCycler Instrument (30 cycles of denaturation; Roche Diagnostics GmbH, Mannheim, Germany) and 1 cycle of denaturation for the melting curve analysis (Figure 2). SYBR Green fluorescent dye, specific for dsDNA, was included in the second-round reaction mix.¹¹ The DNAs extracted from peripheral blood lymphocytes served as negative controls. Additionally, several samples for PCR contained no DNA. Cases of cervical carcinoma previously positive for HPV were used as positive controls.

To type and confirm the HPV specificity of the PCR products, direct sequencing was carried out on the resultant amplicons.

Clinical and Histopathologic Results*							
Case No.	Age, y	Azoospermia Etiology	Main Histologic Findings	Testicle Biopsied	HPV Typing (Testicle Positive)	Laser Capture Microdissection HPV Type	
						Tubule	Interstitial (Leydig Cells)
1	34	Klinefelter	Sertoli cell only	R	18 (R)	Np†	Np†
2	33	Varicocele	Mixed‡	R	54 (R)	54	Np§
3	30	Chemotherapy	Hypospermatogenesis	R	16 (R)	16	Np§
4	35	Idiopathic	Mixed‡	Both	16 (R)	16	Np§
5	33	Idiopathic	Sertoli cell only	Both	16 (R)	Np†	Np†
6	36	Idiopathic	Sertoli cell only	Both	54 (L)	54	54
7	34	Idiopathic	Sertoli cell only	Both	11 (L)	11	11
8	31	Idiopathic	Sertoli cell only	Both	33 (L)	Np§	Np§
9	35	Chemotherapy	Sertoli cell only	Both	16 (L)	16	16
10	29	Orchitis (mumps)	Sertoli cell only	Both	33 (L)	Np†	Np†
11	33	Orchitis (mumps)	Tubular hyalinization	Both	33 (L)	Np†	Np†
12	31	Idiopathic	Sertoli cell only	Both	16 (R)	16	16

* HPV indicates human papillomavirus; R, right; Np, not performed; and L, left.

† Absence of testicular tissue.

‡ Hypospermatogenesis and Sertoli cell only.

§ Absence of or scarce Leydig cells.

Extensive precautions were taken to prevent artifactual contamination. All PCR assays were done in duplicate, and positive results were independently confirmed.

RESULTS

After microscopic study, patients' testicular biopsies were classified in accordance with the following patterns: germ cell aplasia (Sertoli cell only), 68 patients; hypospermatogenesis, 57 patients; spermatocytic arrest, 42 patients; complete tubular hyalinization, 9 patients; and mixed atrophy (spermatocytic arrest and germ cell aplasia), 9 patients. Each of the surgically obtained samples contained between 30 and 100 tubules. Similar histologic patterns were seen in both the right and left testicles of patients who underwent bilateral biopsy. A variable amount of Leydig cells was seen in the interstitium, ranging from hyperplasia to absence of Leydig cells.

Molecular study to detect HPV DNA sequences was positive in 12 cases (Table).

Histologically, HPV-positive cases showed the following patterns: germ cell aplasia (Sertoli cell only), 8 cases; mixed (hypospermatogenesis and Sertoli cell only), 2 cases; complete tubular hyalinization, 1 case; and hypospermatogenesis, 1 case. No specific histologic findings were associated with HPV infection.

One of the HPV-positive patients (case 11) showed complete tubular hyalinization with total absence of tubular cells and marked Leydig cell hyperplasia. On the other hand, no Leydig cells were seen microscopically in case 8, with tubules containing only Sertoli cells, being absolutely devoid of germinal cells.

In cases 6, 7, 9, and 12, tubules contained only Sertoli cells, and variable amounts of Leydig cells were observed in the interstitium; in these cases, following laser capture microdissection, the presence of HPV DNA sequences in Leydig cells was confirmed, and at the same time HPV DNA sequences in the tubules containing only Sertoli cells were detected. We also detected HPV DNA in microdissected tubules containing germinal cells in all maturative stages, including mature spermatids but in reduced number (cases 2, 3, and 4), corresponding histologically to hypospermatogenesis and mixed pattern. In all 7 microdissected cases, the same HPV genotype was found in intra-

luminal component (tubules) and interstitium (Leydig cells).

The frequency of HPV positivity was 6.48% (12/185 patients). Curiously, among the 92 patients who underwent bilateral testicular biopsy, we detected HPV sequences in 9 (9.78%), all of whom showed exclusively unilateral testicular affection, more often in the left testicle (ratio, 2:1). High-risk HPV types (11/12 cases) were more frequent, with HPV 16 being the most common genotype encountered (5 cases).

Control testicular biopsies showed the following patterns: germ cell aplasia (Sertoli cell only), 2 patients; hypospermatogenesis, 1 patient; spermatocytic arrest, 1 patient; and absence of histopathologic findings, 46 patients.

No HPV DNA sequences were found in the testicles from the control group.

COMMENT

Human papillomavirus infection in men is overwhelmingly subclinical, which has resulted in a potentially large number of asymptomatic carriers who serve as reservoirs and vectors for the virus. Although HPV has been studied extensively in women, data on male infection are limited. Among studies that have used PCR to detect penile HPV DNA in healthy men, results suggest that penile HPV in sexually active men is at least as prevalent as cervical HPV in among women.¹²⁻¹⁷

In an international case-control study that investigated the prevalence of HPV among the male partners of women with and without cervical cancer, investigators with the International Agency for Research on Cancer found penile HPV DNA in 3.5% to 39.0% of the control male partners and in 12.0% to 36.0% of male partners of the case patients, with prevalence varying significantly by country.^{2,17} In Spain, penile HPV infection among the male partners of control women was 3.5%.

In a similar study,¹⁸ the prevalence of penile HPV infection among uncircumcised and circumcised men in Spain was 11.7% and 2.7%, respectively, with a mean value of 7.2%, an incidence very close to our results.

We found HPV DNA sequences in 6.48% of the patients studied; 11 of 12 cases presented oncogenic HPV types,

with type 16 being the most common (5 cases), followed by type 33 (3 cases).

Recently, data from Baldwin et al¹⁹ found that nononcogenic HPV types occur more frequently in men than do oncogenic types. These data differ greatly from reports of HPV infection in women, in whom oncogenic HPV types, specifically type 16, are more common.^{20–23} In some previous studies on male HPV, the majority who tested positive for HPV had oncogenic subtypes,^{12,14,24} but in the International Agency for Research on Cancer 5-nation study, unspecified HPV types were most common.²

We had no information on HPV status of female partners in this study, but numerous data reported demonstrate differences in the characteristics of HPV infection between men and women and low correlation in HPV positivity.^{2,19}

After the initial results, 2 cases (8 and 11) were carefully reviewed in both pre- and postmolecular study slides in order to confirm the microscopic findings; thus, the presence of HPV DNA sequences in case 11, which showed complete tubular hyalinization with total absence of tubular cells and marked Leydig cell hyperplasia, indicated that probably Leydig cells harbored HPV DNA sequences (genotype 33), a fact previously described in a case of Leydig cell tumor in humans.²⁵ Likewise, no Leydig cells were seen microscopically in case 8, and the finding of HPV in this case, with tubules containing only Sertoli cells, being absolutely devoid of germinal cells, suggests that in addition to Leydig cells, Sertoli cells could also harbor HPV DNA sequences.

In order to distinguish results between tubules and interstitium, we performed laser capture microdissection, confirming the presence of HPV DNA sequences in interstitial Leydig cells, as well as in the tubules of 4 cases with germ cell aplasia (Sertoli cell only) (cases 6, 7, 9, and 12). Results were also positive in the tubules of cases 2, 3, and 4, which contained germinal cells in all maturative stages, including mature spermatids but in reduced number, corresponding histologically to hypospermatogenesis and mixed pattern. In these cases, we were not able to determine whether HPV DNA sequences were present in germinal cells, Sertoli cells, or both, because it was technically difficult to dissect them in such a way as to ensure separating the “interdigitating” cytoplasm of Sertoli cells from the germinal cells. The presence of HPV DNA sequences in tubules could explain their presence in washed spermatozoa, raising the possibility of sperm cells as a vector for HPV transmission.⁷

The overall concordance of genotypes between tubules and interstitium found in all cases in which we performed laser capture microdissection could be interpreted as contamination, a possibility that we excluded after repeated determinations with the same results.

According to our results, Leydig cells, Sertoli cells, and probably germinal cells could harbor HPV DNA sequences. Once more, these results raise the question of whether detection of HPV DNA indicates true HPV infection. The presence of HPV gene sequences should be interpreted as suggestive of the presence of HPV but should not be considered as a definitive confirmation of HPV presence. The nature of the HPV virus and the precise mechanism by which the virus infects remain unknown. Heparin/heparin sulfate²⁶ and possibly α 6 integrin²⁷ are considered among the primary attachment receptors for HPV, and they seem to be required for the initiation of productive

infection.²⁸ Heparin sulfate proteoglycans are not only involved in HPV binding but are also necessary for infection with HPV.²⁶ Heparin sulfate proteoglycans and α 6 integrin have been detected in immature rat Sertoli cells.^{29,30}

The possible relationship between testicular HPV infection and azoospermia is, in our opinion, difficult to establish. Because of this, although many HPV infections could be transient, the finding of HPV sequences in only 1 testicle of patients with bilateral testicular biopsies with similar histologic findings could suggest that HPV infection has no relationship with infertility. On the other hand, we were unable to detect HPV sequences in “normal” histopathologic testicular samples from the control group. This could be interpreted as a possible relation between absence of HPV infection and absence of histopathologic lesions, but limited by the fact that the control group was not exactly matched to the cases. The infection rate in the control group may be affected by the fact that they were much younger.

In summary, we found that the testicle could be a reservoir of HPV, a fact that highlights the importance of a “male factor” in the sexual transmission of HPV, but the role of men as transmitters remains open to debate. Although asthenoazoospermia has been reported to be higher in men with HPV infection,⁸ and although many HPV infections could be transient, our results reveal HPV sequences in only 1 testicle of patients who underwent bilateral testicular biopsy, showing identical histologic lesions. This leads us to hypothesize that HPV infection probably has a questionable relationship with nonobstructive infertility.

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