

## PREVALENCE OF HUMAN PAPILLOMAVIRUS IN PENILE CARCINOMA

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### ABSTRACT

**Introduction:** Squamous cell carcinoma accounts for 95% of penile malignant tumors, and its association to human papillomavirus (HPV) has been studied by several authors. Our study describes the prevalence of HPV DNA in penile squamous cell carcinomas in Brasília, Brazil.

**Materials and Methods:** Human papillomavirus DNA was amplified by polymerase chain reaction (PCR) using GP5+/GP6+ and pU-1M/pU-2R primers, after its extraction from paraffin embedded material from 59 patients with penile carcinoma. HPV isolates were typed by restriction enzymes digestion and automated sequencing.

**Results:** Good quality DNA was obtained from 37 out of the 59 samples previously selected. Prevalence of HPV DNA ranged from 13.5% (5/37) to 64.8% (19/24) when we considered the amplification with pU-1M/pU-2R or GP5+/GP6+ primers, respectively. HPV-16 was the only type detected.

**Conclusions:** Our data indicate that GP5+/GP6+ PCR allow a good rate of HPV DNA detection and typing in formaldehyde-fixed and paraffin-embedded tissues. The HPV DNA prevalence can be considered high when compared to data from other South America studies. The high frequency of HPV-16 and the low occurrence of HPV-18 are in agreement with our results from a female population research in the same region of Brazil. Further investigation should be conducted in order to evaluate the frequency of HPV types associated to other penile epithelial disorders and different kinds of neoplasias, as well as in asymptomatic subjects in the central region of Brazil.

**Key words:** penis; tumors; carcinoma; HPV; RT-PCR  
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### INTRODUCTION

Penile cancer is a serious worldwide health problem, despite its scarce occurrence. The higher rates of the disease are found in developing countries. In Brazil, the relative frequency of this tumor among all male neoplasias has been reported as 2.1% (1). By far, the most common histologic type is the squamous cell carcinoma (SCC), which accounts for more than 95% of the cases (2). Its etiology appears

to be multi-factorial, with a history of smoking, phimosis and poor hygiene commonly associated (3). Similarly to cervical SCC, penile SCC has been related to human papillomavirus (HPV) (4).

Human papillomavirus is a member of the Papillomaviridae family. The nonenveloped, icosahedral capsid carries a single molecule of circular double-stranded DNA, ranging from 6800 to 8400 base pairs (bp) (5). More than 40 different genotypes have already been described in the mucous epithelia

of the urogenital surface and aero-respiratory tract. The mucosotropic genotypes are classified in two distinct groups: "low-risk" group, often associated with benign warts and low-grade intraepithelial lesions, and "high-risk" group, associated with invasive carcinomas (6,7). The virus targets the basal cell layer of the epidermis, where a latent infection may be established. Migration of cells to the skin surface during differentiation allows the progression to active infection (8).

The studies performed so far have indicated that HPV has an etiological role in human malignant neoplasias. As early as 1976, Meisels & Fortin (9) reported findings of HPV infection in patients with cervical cancer. The frequent association of HPV-16, -18, -31, and -33 with malignant change is now generally accepted. Other types such as -35, -39, -41 to -44, -51, -52, and -56 have also been implicated in cervical, vulvar, or penile cancer. The more common types 6 and 11 are considered to carry a low risk of malignant progression. However, instances of cancer associated with virtually every HPV type have been reported (7). These evidences include: viral DNA is found in close to 90 % of anogenital and cervical tumors (6); most of the virus-positive tumors contain integrated viral DNA (7); and the E6 and E7 viral proteins of high-risk types are able to immortalize human's foreskin and cervical keratinocytes (10).

This transversal study is the first report on the prevalence of HPV DNA detected by molecular methods using 2 different sets of primers. We have used paraffin-embedded tissue fragments from penile squamous cell carcinomas, from patients originally from Brasilia, central region of Brazil. Moreover, it also describes the frequency of HPV types detected in the samples.

## MATERIALS AND METHODS

### Samples

Fifty-nine formaldehyde-fixed and paraffin-embedded tissue fragments from patients attended at Brasília public hospital from 1994 to 1999 were included in the study. The material, obtained from males with penile invasive squamous cell carcinoma, was

analyzed in order to detect HPV DNA sequences. Subjects were married, 27 to 82 years old (mean age of 54 and median of 60), mainly agriculture workers, with history of penile lesions varying from 2 months to 5 years.

The inclusion criteria was the histopathologic diagnosis of squamous cell carcinoma. Seventy per cent were histologically classified as well-differentiated squamous cell carcinoma, whereas 20% were moderately and 10% poorly-differentiated. Socio-demographic, clinical and epidemiological data from subjects were recovered from hospital files, whenever available.

### DNA Isolation from Tissue Sections

Each paraffin block was cut into 15- $\mu$ m sections using a new blade for each specimen to minimize block-to-block contamination. Tissue sections were deparaffinized by treatment with xylene and washed with ethanol. The proteins were digested by proteinase K [200  $\mu$ g/ml], overnight, at 55°C, and the extracted DNA purified by resin from the Nucleon HT kit (Amershan Pharmacia Int.).

### PCR Amplification

To evaluate the quality of the DNA isolation procedure, samples were submitted to polymerase chain reaction (PCR) (11) of the  $\beta$ -globin human gene sequence, using a pair of primers named pCO3/pCO4. DNA amplification for HPV detection was performed in two distinct genomic regions: E6-E7, using primers pU1M/pU2R, and L1, using primers GP5+/GP6+. The oligonucleotides sequences used as PCR primers are shown in Table-1 (11-13).

PCR reactions were performed in a final volume of 50  $\mu$ l, containing 10  $\mu$ l of extracted DNA, 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 250  $\mu$ M dATP, dCTP, dTTP and dGTP, 2.4 pmol of each primer and 2.5 U of Taq DNA polymerase. Forty cycles of amplification were conducted in an MJ Research PTC-100 thermocycler (first cycle: 95°C-5 min, 40°C-3 min, 72°C-3 min; second to 39<sup>th</sup> cycle: 95°C-1 min, 40°C-3 min, 72°C-3 min; 40<sup>th</sup> cycle: 95°C-1 min, 40°C-3 min, 72°C-3 min). PCR products were analyzed by electrophoresis on 1.5% agarose gels followed by 10 mg/ml ethidium bromide staining.

**Table 1 - Sequences of the oligonucleotides used as PCR primers and size of amplified DNA region.**

Gene	Primers	Sequences	Size (bp)
HPV E6-E7	pU-1M pU-2R	5'-TGTCAAAAACCGTTGTGTCC-3' 5'-GAGCTGTGCGCTTAATTGCTC-3'	230 to 270
HPV L1	GP5+ GP6	5'-TTTGTTACTGTGGTAGATACTAC-3' 5'-GAAAATAAACTGTAAATCATATTC-3'	150
Human $\beta$ -globin	PCO3 PCO4	5'-ACACAACCTGTGTTCACTAGC-3' 5'-CAACTTCATCCACGTTCCACC-3'	110

**HPV Typing by RFLP Analysis**

Samples with positive HPV DNA were typed by restriction fragment length polymorphism (RFLP). The products generated by pU-1M/pU-2R primers were digested by the following enzymes: Acc I, Ava II, Bgl II and Rsa I (3-4 U) (12). For GP5+/GP6+ (20) PCR products, we used Rsa I (5 U) digestion (14). The pattern of length polymorphism for each sample was analyzed by electrophoresis on 8% polyacrylamide gels, followed by 10 mg/ml ethidium bromide staining.

**Automated Sequence and BLAST Analysis**

Automated sequencing followed by Basic Local Alignment Search Tool (BLAST) (15) was performed, using the amplified 150 bp fragments with GP5+/ GP6+ primers of five HPV DNA samples to confirm enzyme digestion typing.

**RESULTS**

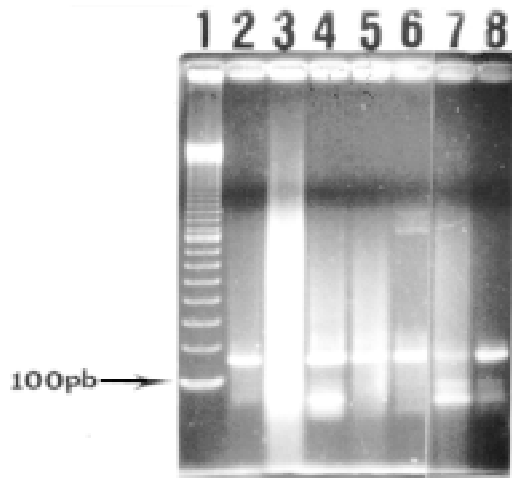
Twenty-two (37.3%) samples proved no significant PCR amplification of the gene fragments tested, either  $\beta$ -globin gene or HPV DNA fragment, and were excluded of our series. In the 37 samples suitable for PCR amplification, 24 were positive for HPV DNA after amplification with GP5+/ GP6+ primers (Figure-1). The presence of HPV DNA was also detected in 5 out of 37 samples when pU1M/pU2R primers were used for amplification (Figure-

2). The results of HPV DNA detection are summarized in Table-2. The overall prevalence of HPV DNA ranged from 13.5% to 64.9%, when we considered the results of PCR by pU-1M/pU-2R and GP5+/GP6+, respectively.

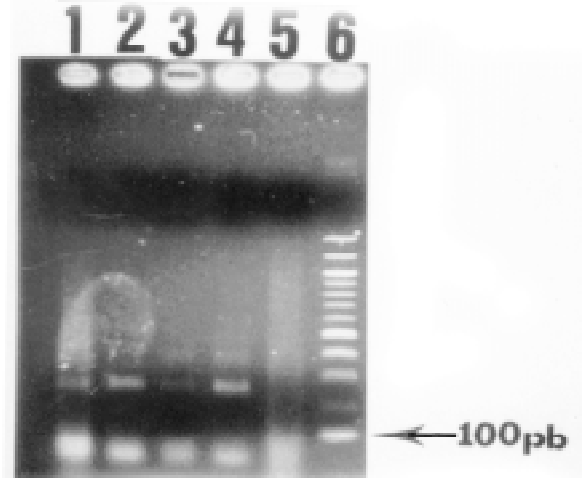
HPV-16 was found in 22 of the 24 isolates by digestion with Rsa I. Two samples could not be typed because there was not enough DNA to repeat the amplification procedure. The five isolates amplified by pU-1M/pU2R were also typed as HPV-16. Therefore, the prevalence of HPV-16 in the HPV DNA positive samples submitted to typing was 100%. In order to check the HPV-16 typing using Rsa I analysis, five positive samples, different from those amplified by pU-1M/pU2R, were automatically sequenced and analyzed by BLAST, confirming the presence of HPV-16.

**Table 2 - Matched and unmatched results when the 2 sets of primers were compared.**

		HPV DNA detection by GP5+/GP6+	
		Positive	Negative
HPV DNA detection by pU1M/pU2R	Positive	5	0
	Negative	19	13



**Figure 1** - Agarose gel electrophoresis of PCR products using GP5+/GP6+ primers, Lane 1: molecular weight marker (100 pb DNA ladder, Gibco) Lanes 2, 4-8: HPV DNA positive samples, showing 150 pb DNA fragments. Lane 3: DNA negative sample. Ethidium bromide staining.



**Figure 2** - Agarose gel electrophoresis of PCR products using pU-1M/pU-2R primers. Lane 1-4: HPV DNA positive samples, showing 238 pb DNA fragments. Lane 5: DNA negative sample. Lane 6: molecular weight marker (100 pb DNA ladder, Gibco). Ethidium bromide staining.

## DISCUSSION

Data on cancer incidence show that penile carcinoma is a rare disease in most developed countries. A high prevalence of this tumor in Brazil associated to HPV infection has been previously reported (16). Our study is the first report on HPV detection in penile squamous cell carcinoma in the city of Brasília, located in the central region of Brazil. The prevalence of HPV DNA detected in this sampling can be considered high (64.9%) when compared to data from other regions of Brazil (30%) (2) and Argentina (71%) (17).

As previously discussed by other authors, HPV prevalence associated to penile carcinoma can vary significantly in distinct geographical areas (17). As our results indicate, one shall not use the information on HPV prevalence from other cities of Brazil (1,16) to infer the prevalence in one specific region. Variations in HPV prevalence, as well as in the frequency of HPV types, have implications in: vaccine testing; choice of diagnostic methods, and epidemiological studies involving disease control (8).

Despite the preservation problems usually related to paraffin-embedded tissue fragments (17,18),

by using the nucleon HT kit, we were able to obtain a good quality DNA in 62.7 % (37/59) of the tested samples. The DNA extraction procedure adopted was the one that presented the highest percentage of recovery, although four other different methods had been tested. The percentage of recovery of archival samples obtained can be considered acceptable, since there is always some percentage of loss for different reasons (1,2).

The discrepant prevalence of HPV DNA accounted for pU-1M/pU-2R and GP5+/GP6+ PCRs products may be due to the technical limitations. Partial degradation of DNA could possibly have affected the amplification sensibility of pU-1M/pU-2R primers, determining the prevalence rate 4.8 lower than the rate defined by GP5+/GP6+ amplification. As the pair of primers for E6/E7 genes (pU1M/2R) amplifies a longer DNA fragment if compared to GP5+/GP6+, it would be expected that degraded DNA samples presented a higher efficiency of amplification for lower fragments.

The use of Rsa I digestion for HPV typing with GP5+/GP6+ amplified products was first reported in 1990 (14). Recently, we have analyzed all HPV DNA sequences available at the GenBank. We noticed that, considering the HPV variation that oc-

curs in Brasília (19), the only other HPV type that might be misinterpreted as HPV-16 is the HPV-35, when (1,16) Rsa I digestion is used as a typing method. This latter HPV type can be detected in association with high-risk lesions, but not in invasive carcinomas (8).

The significant prevalence of HPV-16, a "high-risk" type (6), is compatible with the histopathologic diagnosis of squamous cell carcinoma, also referred by other authors (20), although other reports from Brazil (1,16) and Argentine (17) have found a predominance of HPV-18. The high prevalence of HPV-18 associated to cancer is not detected in the central area of Brazil, neither in the male nor in the female populations studied so far (19).

As only HPV-16 was detected in all analyzed samples, no correlation could be established considering prognostic factors, association to clinical or epidemiological data and HPV types.

We consider that other studies should be conducted in order to evaluate the frequency of HPV types associated to other penile epithelial disorders and different kinds of neoplasias, as well as in asymptomatic subjects in the central region of Brazil.

## CONCLUSIONS

GP5+/GP6+ PCR allow an acceptable rate of HPV DNA detection and can be used for formaldehyde-fixed and paraffin-embedded tissues.

HPV prevalence associated to penile carcinoma seems to be higher in Brasília than in other regions of the South of Brazil, although compatible to what was found in Argentina.

HPV-16 was the only type detected in penile cancer in this target population. Further investigation should be conducted in order to define the distribution of other penile epithelial disorders and different kinds of neoplasias, as well as in asymptomatic subjects.

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