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Human Papillomavirus (HPV) DNA in Penile Carcinomas in Argentina: Analysis of Primary Tumors and Lymph Nodes

María A. Picconi,[1]* Ana M. Eiján,[2] Angélica L. Distéfano,[1] Silvia Pueyo,[3] Lidia V. Alonio,[1] Susana Gorostidi,[4] Angélica R. Teyssié,[1] and Alberto Casabé[5]

[1]Departamento Virología, INEI- ANLIS, Ministerio de Salud y Acción Social de la Nación, Buenos Aires, Argentina
[2]Departamento Inmunobiología, Instituto de Oncología, Universidad de Buenos Aires, Buenos Aires, Argentina
[3]Servicio de Dermatología, Hospital Materno Infantil de San Isidro, Provincia de Buenos Aires, Argentina
[4]Departamento de Patología y, Instituto de Oncología, Universidad de Buenos Aires, Buenos Aires, Argentina
[5]Departamento de Urología, Instituto de Oncología, Universidad de Buenos Aires, Buenos Aires, Argentina

Among sexually transmitted diseases, infection by human papillomavirus (HPV) has become one of the most important. On the other hand, though epidemiological data show that some HPV types are closely associated with cervical cancer, few reports have been found with reference to penile carcinoma because of its rare occurrence. The aim of this study was to investigate the relationship between HPV infection and penile cancer in Argentina. A retrospective study was carried out on 38 white men with penile squamous-cell carcinoma. Sixty-five archival fixed biopsies taken from 34 primary penile tumors, 25 nodal metastases, 1 skin "satellite" metastasis and 5 histologically normal lymph nodes were used as specimens. HPV detection and typing were carried out by the polymerase chain reaction (PCR) using generic primers, combined with single-stranded conformational polymorphism (SSCP) analysis. HPV DNA was found in 71% patients, corresponding 81% of them to "high risk" types, with predominance of HPV 18. Both primary tumors and metastases showed concordance of HPV occurrence and type in both lesions. In 3 patients, HPV 16 was detected not only in primary tumors and metastases, but also in histologically normal lymph nodes. Our data indicate that most penile carcinomas in Argentine patients are etiologically related to HPV, especially to "high risk" genital types. The agreement in HPV detection between primary tumors and metastases suggests a potential viral role in tumor progression. HPV detection in otherwise histologically normal lymph nodes might be useful as early marker of a metastatic process. ***J. Med. Virol. 61:65–69, 2000.***

KEY WORDS: viral typing; viral oncogenesis; penile cancer; PCR-SSCP; sexually transmitted disease; metastases

INTRODUCTION

An increasing number of reports indicate that human papillomaviruses (HPVs) represent a major etiological factor in a significant portion of human cancers. They are particularly linked to the development of preneoplastic and neoplastic lesions of the cervix [zur Hausen, 1994; Bosch et al., 1995]. More than 85 different genotypes have been described; about half of them isolated from the genital mucosa. "Low-risk" HPVs, such as types 6 and 11, are often associated with benign venereal warts and low-grade cervical intraepithelial lesions (CIN), that rarely progress into cancer. Conversely, "high-risk" HPVs, such as types 16 and 18 are found mainly in high-grade CIN and cervical carcinoma [zur Hausen, 1996; Villa, 1997]. In general, HPV 16 is the predominant type throughout the world, and together with HPV 18, has been detected in 75% of human cervical cancers [Bosch et al., 1995]. Epidemiological evidence in support of this notion arose from the observation that sexual partners of men with penile carcinoma were more prone to cervical neoplasia and vice-versa [Graham et al., 1979]. Nevertheless, studies of HPV and penile carcinoma are limited because of the scarce occurrence of this malignancy. A low incidence of this neoplasm has been reported in Europe and

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*Correspondence to: Dra. María Alejandra Picconi, Departamento Virología, INEI-ANLIS "Dr. Carlos G. Malbrán," Av. Vélez Sársfield 563, (1281) Buenos Aires, Argentina.
E-mail: oncovir@sudnet.com.ar

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North America (1 per 100,000), whereas in some regions of South America, Africa and Asia, penile cancer is much more common, representing 10–20% of male malignant tumors [Malek et al., 1993]. The prevalence of penile cancer in Argentina is still unknown; there has not been any report evaluating the HPV association with this neoplasm.

HPV has been detected in tissue of penile carcinoma by using different DNA-technologies, such as Southern blot [Villa and Lopes, 1986], in situ hybridization [Iwasawa et al., 1993] and polymerase chain reaction (PCR) [Chan et al., 1994]. PCR with generic primers, combined with single-stranded conformational polymorphism (SSCP) analysis, has been applied to HPV detection and typing in cervical samples [Lizano et al., 1997; Distéfano et al., 1998].

Several studies have demonstrated the presence of HPV DNA in lymph node metastases in cervical cancer [Lancaster et al., 1986; Walboomers et al., 1987; Class et al., 1989; Fuchs et al., 1989; Burnett et al., 1992; Park et al., 1996; Kobayashi et al., 1998], but fewer reports are available on penile cancer [Wiener et al., 1992; Iwasawa et al., 1993].

In the study presented here, we have analyzed by PCR-SSCP, the presence of HPV DNA sequences in penile carcinomas, metastases and histologically normal lymph nodes.

MATERIALS AND METHODS

Study Group

Thirty-eight male Caucasian patients (ages 30–65) attending the Urology Department of the Instituto de Oncología "Angel H. Roffo," to which patients from Argentine provinces are transferred, were enrolled. Patients had clinical-histopathological diagnosis of squamous-cell carcinoma and history of phimosis. They exhibited a T2 or higher clinical stage according to UICC TNM classification criteria [Malek et al., 1993], and 23 patients showed inguinal lymph node metastases.

Sixty-five archival formaldehyde-fixed and paraffin-embedded biopsies were included. Eight-μm sections were cut by microtome; a new blade was used for each specimen to minimize block to block contamination. Samples included: A) 15 primary penile tumors (15 patients); B) 16 primary penile tumor and their lymph node metastasis (16 patients); C) 3 primary tumors, 5 inguinal node metastases and 5 histologically normal (without tumor tissue) inguinal nodes (3 patients); D) 3 inguinal node metastases (3 patients) (primary tumors were not available because they had been surgically excised in distant medical centers); and E) 1 inguinal node metastasis and 1 skin "satellite" metastasis (1 patient) (primary tumors were not available because they had been surgically excised in distant medical centers).

Informed consent was obtained for the use of these biopsies in testing.

DNA Isolation From Tissue Sections

Specimens were deparaffinized by treatment with n-octane and washed with ethanol. DNA was extracted by digestion with proteinase K and purified with phenol-chloroform extraction as described previously [Wright et al., 1990].

β-Globin Gene and HPV DNA Amplification by PCR

Isolated DNAs were tested for the β-globin gene to confirm the presence of adequate template in the samples. We have used GH20/PCO4 primers [Saiki et al., 1986] capable of amplifying a 268 bp fragment of this gene.

With regard to HPV detection, radioactive PCR reactions with GP5/GP6 generic primers that amplify a fragment of approximately 140 bp from the L1 region [Snidjers et al., 1990] were carried out. As positive controls, cloned DNA from HPV types 6, 11, 16, 18, 31 and 33 (kindly provided by H. zur Hausen, A. Lörincz and G. Orth) were included in all tests. PCR reactions were performed in 10 μl containing 100–200 ng of DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 100 μM dATP, dCTP and dTTP, 10 μm dCTP and 0.1 μl α^{32}P-dCTP 10 mCi/ml(Dupont), 10 pmol of each primer and 1 U of AmpliTaq (Perkin Elmer). Forty cycles of amplification were performed in a Perkin Elmer GeneAmp 2400 thermocycler (94°C, 30 sec; 45°C, 30 sec; 72°C, 30 sec).

HPV Typing by SSCP Analysis

Aliquots from PCR reactions were diluted 1:25 with 0.1% SDS, 10 mM EDTA; then, 2 μl of the dilution were mixed with an equal volume of denaturing solution (95% formamide, 20 mM EDTA, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and boiled for 5 min. Another aliquot from the PCR reactions was diluted 1:25 in TE buffer (10^{-2} Tris ClH pH 7.6, 10^{-3} EDTA) and then mixed with an equal volume of blue dye in glycerol. One μl of the diluted samples was loaded on nondenaturing 6% polyacrylamide gels containing 10% glycerol and run at 6 watts for 14 hr at room temperature. Gels were dried and exposed to X-OMAT-AR Kodak film, with intensifying screen at −70°C. Typing was made by comparing the migration band patterns obtained from the samples with those observed for each HPV control type.

RESULTS

Sixty-five fixed and paraffin embedded biopsies from 38 Argentine patients with penile squamous-cell carcinomas were examined to determine the presence of HPV sequences. All DNA samples amplified the β-globin gene, and were therefore considered adequate for the PCR study. Figure 1 shows an autoradiography of the HPV typing by PCR-SSCP.

The results of viral detection and typing are summa-

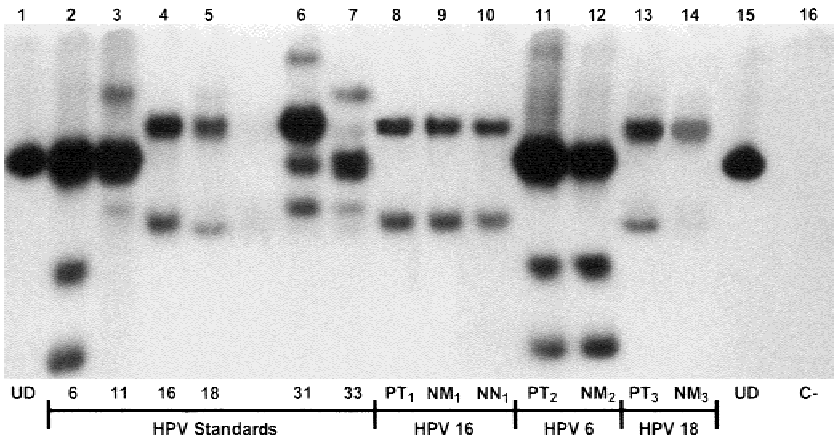

Fig. 1. PCR-SSCP analysis of HPV types in primary penile carcinoma and nodes. Genomic DNA was isolated from paraffin-embedded biopsies and subjected to PCR-SSCP analysis using GP5,6 generic primers. PCR products were denatured and electrophoresed on 6% nondenaturing polyacrylamide gels containing 10% glycerol, as described in Materials and Methods. **Lanes 1 and 15**: undenatured (UD) double stranded HPV DNA; **lanes 2–7**: cloned viral DNA corresponding to types 6, 11, 16, 18, 31, and 33; **lanes 8–14**: viral types found in primary penile tumor (PT), nodal metastases (NM) and histologically normal node (NN); the sub-index indicates the patient's number. **Lane 16**: negative control.

TABLE I. Detection of HPV DNA in Primary Penile Tumors, Metastases and Histologically Normal Lymph Nodes, Using PCR-SSCP

	HPV type detected by PCR-SSCP						
		Metastases					
		Nodal		Skin	Normal node		
Patient number	Primary tumor	1	2	3	1	2	Number of patients
1	6	6	—	—	—	—	1
2–3	16	16	—	—	—	—	2
4	16	16	16	—	16	16	1
5	16	16	—	—	16	—	1
6	16	16	16	—	16	Neg	1
7–13	18	18	—	—	—	—	7
14	UD*	UD*	—	—	—	—	1
15–19	Neg	Neg	—	—	—	—	5
20	—	16	—	16	—	—	1
21	6	—	—	—	—	—	1
22–24	16	—	—	—	—	—	3
25–28	18	—	—	—	—	—	4
29–30	UD*	—	—	—	—	—	2
31	—	16	—	—	—	—	1
32	—	18	—	—	—	—	1
33–37	Neg	—	—	—	—	—	5
38	—	Neg	—	—	—	—	1
Total							38

*UD: non determined HPV type (different than 6, 11, 16, 18, 31 or 33).

rized in Table I. HPV was detected in 27/38 (71%) of patients, 81% (22/27) of them corresponded to "high risk" types, with HPV 18 predominance. The HPV type remained undetermined by this method in 3 positive cases (11%), because the migration patterns did not correspond to any of the standard controls. Further characterization of these samples was not possible because of the limited amount of specimen.

In 19 patients with primary tumor and lymph node metastases, HPV results (positive or negative) always agreed in both lesions. Furthermore, when HPV was positive, the metastases harbored the same viral type as the primary cancer. In 3 patients from this group, HPV was detected in the primary tumor and nodal metastases, but also in the histologically normal lymph node; one of them exhibited two normal nodes, but only one was HPV positive. In the patient with nodal and skin "satellite" metastases, HPV 16 was traced in both locations.

DISCUSSION

Data on cancer incidence from most countries show that penile cancer is a rare disease. In South America, however, a high prevalence of this neoplasm has been found in Brazil and Paraguay, countries where several authors have described an association with HPV infection [Villa et al., 1986; Gregoire et al., 1995]. Our report represents the first study focusing on HPV detection in penile carcinoma and metastases in Argentina.

Despite the low viral copy number found in invasive tumors and the preservation problems related to the tissue source (fixed and paraffin embedded biopsies), we could detect one of the highest rates (71%) of HPV positivity in penile cancer ever published. When PCR had been used, the HPV DNA prevalence in this neoplasia ranged from 10% [Chan et al., 1994] to 100% [Tornesello et al., 1992]. This wide range may be due to technical and geographical differences.

By using the PCR-SSCP technique we were able to identify, in a single reaction, several ano-genital HPV types. The band patterns obtained for each type were highly specific, reproducible and similar to the ones published [Lizano et al., 1997; Distéfano et al., 1998]. Consequently, this method avoids a later restriction enzyme digestion or hybridization.

We could determine the viral type in 89% of the positive samples; however, atypical patterns were observed in 3 cases, that might correspond either to variants or to different types from the controls.

The significant prevalence of both HPV 16 and 18 in our series coincides with most reports; however we found an HPV 18 predominance. The early results from Brazil [Villa et al., 1986] indicated a high rate of HPV 18, but recently the predominance of HPV 16 has been informed [Levi et al., 1998]. The differences observed among the studies could be attributed to the HPV geographical distribution or to the lack of a representative sample.

In two patients, one with a primary tumor and the other with both tumor and metastasis, HPV 6 was detected. Although there is evidence to suggest a lack of carcinogenicity of HPV 6 and 11 (IARC Working Group, 1995], these types have previously been reported in preneoplastic and neoplastic penile lesions [Villa et al., 1986; Barraso and Jablonska, 1989; Tornesello et al., 1992, Gregoire et al., 1995]. On the other hand, it has been pointed out that up to 10% of the penile cancer lesions had developed in association with condylomata acuminata, that frequently contains HPV 6 or 11 [Malek et al., 1993]. In all reports, including ours, the detection rate of "low risk" types was considerably smaller than that of "high risk" types.

Besides the viral type, other factors should be taken into account when considering the progression of HPV induced lesions, like immuno-compromise, exposition to chemical or physical carcinogens, genetic background, etc. Phimosis, a significant risk factor in penile cancer [Maden et al., 1993; Malek et al., 1993] was consistently found in all our patients.

We have observed in this study a total accordance in HPV status in primary penile tumor and metastasis. Moreover, the agreement of viral type in both lesions, matches the data obtained on cervical carcinoma [Lancaster et al., 1986] indicating the stable persistence of integrated HPV DNA [Fuchs et al., 1989].

Another important finding from this study was the detection of HPV sequences in histologically normal lymph nodes. In 3 patients, viral DNA was observed in the primary tumor, nodal metastases and histologically normal lymph nodes. Lymphatic drainage of viral sequences, either free or cell-associated state could not be ruled out [Park et al., 1996]. In one patient with two normal nodes available, however, only one was HPV positive. In addition, we have found HPV 16 in both nodal and skin "satellite" metastases from the same patient. These findings led us to discard the possibility of a merely passive viral colonization or an undetectable hematogenic dissemination during surgery.

Therefore, HPV presence in normal lymph nodes may indicate an early metastasis, not detected by histology, as a consequence of a very low number of neoplastic observable cells. HPV nodal presence has already been described in cervical cancer [Kobayashi et al., 1998], associated with unexpected recurrences. Our results suggest a potential role of HPV in tumor progression and metastatic process.

The detection of HPV DNA in histologically normal lymph nodes arose special interest in the subject. Consequently, it could be speculated that the stable association of HPV DNA with cancer cells could be useful for the sensitive, early detection of metastases by molecular techniques as PCR. If the viral presence was indicative of metastatic cells, this fact could have prognostic significance for cancer patients. More studies are necessary to demonstrate whether HPV detection in histologically normal lymph nodes could become an early marker of a metastatic process. This study provides further evidence about the HPV etiological role, at least in this sub-set of patients with penile cancer.

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