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Human papillomavirus (HPV) genotype distribution in penile carcinoma: Association with clinic pathological factors

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Abstract

Background

Penile carcinoma (PC) is a rare, highly mutilating disease, common in developing countries. The evolution of penile cancer includes at least two independent carcinogenic pathways, related or unrelated to HPV infection.

Objectives

To estimate the prevalence, identify HPV genotypes, and correlate with clinicopathological data on penile cancer.

Methods

A retrospective cohort study involving 183 patients with PC undergoing treatment in a referral hospital in Goiânia, Goiás, in Midwestern Brazil, from 2003 to 2015. Samples containing paraffin embedded tumor fragments were subjected to detection and genotyping by INNO-LiPA HPV. The clinicopathological variables were subjected to analysis with respect to HPV positivity and used prevalence ratio (PR), adjusted prevalence ratio (PRa) and 95% confidence interval (CI) as statistical measures.

Results

The prevalence of HPV DNA in PC was 30.6% (95% CI: 24.4 to 37.6), high-risk HPV 24.9% (95% CI: 18.9 to 31.3), and 62.5% were HPV 16. There was a statistical association between the endpoints HPV infection and HPV high risk, and the variable tumor grade II-III

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(p = 0.025) (p = 0.040), respectively. There was no statistical difference in disease specific survival at 10 years between the HPV positive and negative patients (p = 0.143), and high and low risk HPV (p = 0.325).

Conclusions

The prevalence of HPV infection was 30.6%, and 80.3% of the genotypes were identified as preventable by anti-HPV quadrivalent or nonavalent vaccine. HPV infections and high-risk HPV were not associated with penile carcinoma prognosis in this study.

Introduction

Penile carcinoma (PC) is a rare and aggressive disease with high mutilating potential. The incidence in the United States and Western Europe is estimated at 0.4%, however, in Africa, Asia and South America the incidence is about 6.0% [1–3].

Brazil is a country with a high incidence of PC, accounting for about 2% of neoplasias that affect males, its frequency is associated with the studied region and socio-economic conditions of individuals [4–7].

The origin of penile carcinoma is multifactorial, and the incidence is mainly related to poor personal hygiene, high number of sexual partners, phimosis in adulthood and infections by bacteria and viruses, such as human papillomavirus (HPV) [2, 8–11]. Phimosis is a risk factor for this carcinoma, and circumcision is considered an important factor of prevention, and countries that adopt this practice have lower prevalence of PC [8,10,11].

Human papillomavirus (HPV) is the most common cause of sexually transmitted infection (STI) [12,13] and is considered an important etiologic agent for the development of PC, however, its role is not yet fully elucidated [14,15]. The development of penile carcinoma includes at least two independent carcinogenic routes, one being related to persistent HPV infection, and the other to no associated viruses, such as inflammatory conditions (chronic balanitis, lichen sclerosus), which are favored by the presence of phimosis [16–19]. HPV are classified according to their oncogenic potential, with approximately 15 types of high oncogenic risk involved in the carcinogenic process of some tumors through the action of viral oncoproteins (E6 and E7) [20–22].

The variations in the prevalence of HPV in PC, according to the literature, are due to differences in sampling, molecular testing, and study population [23]. The overall prevalence of HPV infection in penile neoplasia has been estimated from 13.4% to 55.6% worldwide [24]. In this multicenter study, the authors present HPV positivity rates by region: Europe (32.2%; 95% CI: 27.8 to 36.9), North America (18.8%; 95% CI: 4.0 to 45.6), Latin America (36.5%; 95% CI: 32.1 to 40.9), Africa (36.8%; 95% CI: 16.3 to 61.6), Asia (13.4%; 95% CI 6.3 to 24.0) and Oceania (55.6%; 95% CI: 21.2 to 86.3) [24]. In some histological types of PC persistent HPV infection is associated with genotype 16 [19].

In Brazil, studies carried out in patients with PC found HPV prevalence ranging between 30.5% and 63.1% in the states of São Paulo and Maranhão, respectively [25,26]. In 2011, an investigation conducted in Goiânia, capital of the State of Goiás in Midwestern Brazil, HPV positivity was found in 43.3% of cases, where 50.9% were HPV16 and 25.5% were HPV-18 [27].

Squamous carcinoma (SCC) is the most common histologic type of PC, representing about 95% of cases of this neoplasm. The HPV prevalence and penile carcinoma may differ between

histologic types of squamous carcinoma [10,28,29]. Genotypic characterization of HPV in PC is important, in order to know the most frequent types. Giuliano and colleagues found that immunization with the quadrivalent vaccine resulted in 90.4% protection (95% CI: 45.8 to 98.1) against lesions related to HPV 6, 11, 16 and 18 in men [30], showing that the adoption of the HPV vaccine for men is a measure of prevention and control of this neoplasia.

The clinicopathological characteristics of penile tumors are factors that predict disease progression, the need for surgery, and death [15]. Therefore, this study aimed to estimate the prevalence and identify the HPV genotype and correlate these with clinicopathological data on penile carcinoma.

Materials and methods

Patients

This is a retrospective cohort study in patients with penile carcinoma treated in the Uro-Oncology service in a referral hospital in Goiânia, Goiás, Brazil, from January 2003 to November 2015. For this study, 225 patients received treatment during the defined period and were included in the study; of these, 42 were excluded, resulting in a total of 183 cases.

Inclusion criteria of patients in the study were: diagnosis with penile carcinoma and treatment in a referral hospital; biopsy or amputation of the penis in the institution; paraffin block with PC fragment and records located.

Cases whose paraffin blocks containing the fragment of the primary tumor were not found, and those submitted to neoadjuvant chemotherapy or penis surgery at another institution were excluded (Fig 1).

This study was approved by the Ethics Committee of the Association to Combat Cancer in Goiás (CEP / AACG) under a consolidated number CEP: 901.094.

Preparation of samples

Slides containing paraffin processed tumor tissue fragments were stained with hematoxylin and eosin and evaluated by two pathologists independently to confirm the diagnosis of PC. After the selection of the cases, the blocks were cut into slices and stored in sterile 2 mL microtubes and identified by block number.

Extraction, detection and genotyping of HPV DNA

The viral DNA extraction was performed with the following reagents: Xylol PA for removal of paraffin; Proteinase-K for cellular digestion; a commercial kit (Wizard Genomic DNA Purifications Kit—Promega) to precipitate protein: isopropanol for DNA precipitation and 70% ethanol for DNA purification.

The paraffin removal process results in loss of tissue, and therefore degradation of the DNA contained in the sample [31], so the integrity of DNA in samples for analysis were evaluated. Samples were subjected to polymerase chain reaction (PCR) using oligonucleotide primers specific for amplification of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (human housekeeping gene) (INVITROGEN) (99 bp). GAPDH negative samples were re-extracted. Each amplification used a negative control (without DNA) and positive control.

Detection and genotyping the HPV DNA was accomplished using commercial kit HPV INNO-LiPA HPV genotyping extra (Fujirebio Europe, Ghent, Belgium) that amplifies the L1 viral region (65pb) using primer SPF 10. This method uses a primer which amplifies the

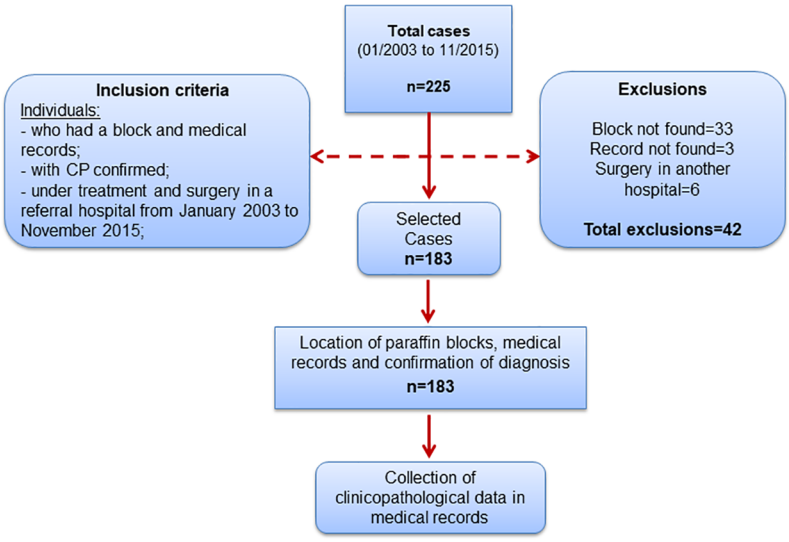

Fig 1. Flowchart of the study.

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human gene HLA-DPB1, used to monitor the quality of extraction of DNA from the sample. All reactions included negative control (without HPV-DNA) and positive control.

Genotyping was performed by reverse hybridization following amplification of the HPV L1 region, biotinylated amplicons were denatured and hybridized with specific probes fixed in parallel lines in strips. This method detected 28 genotypes, 15 genotypes of high-risk HPV, three probable high-risk, seven low risk, and three that were not classified according to risk. The tests were performed according to the manufacturer's instructions. To avoid cross-contamination between samples, specific laboratory work areas were designated for the handling of reagents and samples and for the manipulation of amplified products. Positive and negative controls were included in all DNA extractions and PCR amplification reactions. The protocols used for extraction, detection and genotyping of HPV DNA can be found in S1 Protocols.

Statistical analysis

The dependent variables were: (i) HPV infection (no or yes) and (ii) High risk HPV infection (no or yes), and the following independent variables were analyzed: (i) age, categorized as < 60 years and ≥ 60 years; (ii) phimosis (no or yes); (iii) Jackson stage (0/II or III/IV); (iv) tumor grade (I or II/III); (v) tumor invasion (superficial, deep or in situ); (vi) inguinal metastasis (no or yes); (vii) inguinal lymphadenectomy (no or yes); (viii) inguinal recurrence (no or yes); (ix) lymphovascular invasion (absent or present) and (x) death (live or dead).

The variable phimosis was not included in regression analysis due to the large number of cases missing these variables in this study [32,33].

Data were analyzed in STATA, version 14.0. Initially, descriptive analysis was performed on all variables investigated. Quantitative variables were presented as mean and standard deviation (SD) and the qualitative variables as absolute and relative frequency. Factors associated with infection with HPV were made by Poisson regression with robust variance [34,35]. Variables with p values <0.10 of bivariate analyses were included in their respective models. Age, regardless of the p value, was included in the models due to confounding potential for the control. The results of the analyses are presented as prevalence ratio (PR), adjusted prevalence ratio (PRadj) and 95% CI. In addition, the log-rank test [36] was used to compare survival of

PC patients between the following groups: (i) HPV+ versus and (ii) Low risk HPV and (ii) high-risk HPV. In all analyses, P values < 0.05 were considered statistically significant

Results

A total of 183 individuals with penile carcinomas were included in the study. Fig 1 shows the algorithm of the study.

Most patients (51.4%) were 60 years or older at the time of diagnosis of PC. Phimosis was reported by (90.1%) of the participants. Tumor grade II-III was observed in 50.2% individuals, Jackson stage III-IV in 23.6% of cases, deep tumor invasion (51.9%), inguinal metastasis (36.1%), inguinal lymphadenectomy (38.8%) and post-surgical inguinal recurrence in 8.7% of patients. Regarding the primary treatment of lesions: partial penectomy 72.7%, total penectomy in 14.2% and emasculation in 4.4% of cases. The occurrence of death due to PC happened in 18.6% of the participants, according to records (Table 1). The study database can be found in S1 Database.

The prevalence of HPV DNA was 30.6% in paraffin embedded tissue samples of PC. The high oncogenic risk genotypes were found in 24.9% of cases and low risk HPV in 3.8% (Table 2). Simple infection was found in 49 cases of HPV and multiple infection in seven cases (data not shown in table).

Among HPV DNA-positive samples, the most frequent HPV type was HPV 16 (62.5%) and HPV 18 (5.4%). HPV 6 and HPV 11 in total accounted for approximately 12.4% of penile carcinomas (Fig 2).

The clinical and pathological variables were submitted to bivariate analysis, with the outcome being the prevalence of HPV-DNA, only the variable tumor grade (II/III) remained associated after multivariate analysis (Table 3).

The same variables were analyzed with the outcome as positive for high-risk HPV, age and tumor grade [II and III] included in the multivariate analysis model, remained statistically associated with the outcome the variable tumor grade II/III (Table 4).

There was no statistical difference in survival between HPV positive and negative individuals over 10 years (long-rank test x^2: 2.14, p = 0.143) (Fig 3). Regarding individuals infected with HPV high and low risk, no statistical difference with respect to survival (long-rank test x^2: 0.97, p = 0.325) was found (Fig 4).

Discussion

To our knowledge this is the largest number of cases of PC collected with the goal of estimating the prevalence, genotypic characterization, and HPV association with clinical and pathological characteristics of this tumor in Brazil.

Penile cancer occurs more frequently in men aged 50–70 years [37]. In fact, our results showed that most individuals were 60 years of age or older. This is consistent with other studies [11,38–40]. Early diagnosis is very important in relation to both the preservation of organs and the outcome of the disease, with survival rates estimated at approximately 50% over five years [37]. However, this condition also affects young individuals, in our study 13.1% of individuals with CP were between 24 and 40 years of age (data not shown).

In this study, presence of phimosis was observed in 90.1% of PC cases, corroborating literature data [9,26,41]. The increased risk of penile cancer among men with phimosis is associated with lichen sclerosis or inadequate penile hygiene, smegma retention and therefore infection. The meta-analysis showed that childhood circumcision may have a protective effect against penile cancer [42].

Table 1. Descriptive analysis of clinical variables in patients with penile carcinoma in Goiania, Goias, Brazil.

Variables	n	%	95% CI [1]
Age (years)			
< 60	89	48.6	41.5–55.8
≥ 60	94	51.4	44.2–58.5
Phimosis (n = 151)			
No	15	9.9	6.1–15.7
Yes	136	90.1	84.3–93.9
Tumor grade (n = 182)			
I	90	49.5	43.3–56.6
II-III	92	50.5	43.4–57.7
Jackson stage (N = 174)			
0-II	133	76.4	69.6–82.1
III-IV	41	23.6	17.9–30.4
Tumor invasion (n = 181)			
Superficial	78	43.1	26.1–50.4
Deep	94	51.9	44.7–59.1
In situ	9	5.0	2.6–9.2
Inguinal metastasis			
No	117	63.9	56.8–70.5
Yes	66	36.1	39.5–43.2
Inguinal lymphadenectomy			
No	112	61.2	54.0–68.0
Yes	71	38.8	32.0–46.0
Inguinal recurrence			
No	167	91.3	86.3–94.5
Yes	16	8.7	5.4–13.7
Tumor inflammatory infiltrate (n = 137)			
Low	56	40.9	33.0–49.2
Moderate	67	48.9	40.7–57.2
Intense	14	10.2	6.2–16.4
Lymphovascular invasion (n = 164)			
Absent	137	83.5	68.1–50.6
Present	27	16.5	10.4–20.6
Primary treatment			
Partial penectomy	133	72.7	65.8–78.6
Total penectomy	26	14.2	9.9–20.0
Emasculation	8	4.4	2.2–8.4
Local Excision	16	8.7	5.4–13.7
Death			
Live	149	81.4	75.2–86.4
Dead	34	18.6	13.6–24.8

[1]95% confidence interval

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The pathogenesis of penile cancer is not well understood. A substantial percentage of penile carcinomas are associated with HPV while the remaining tumors rely on molecular mechanisms other than HPV [1,19].

Table 2. Prevalence of HPV-DNA in 183 cases of penile carcinoma in a referral hospital in Goias, Brazil.

Variables	N = 183	%	95% CI [1]
HPV+	56	30.6	24.4–37.6
HPV genotypes			
HPV high risk	45	24.9	18.9–31.3
HPV low risk	7	3.8	1.9–7.7
Undetermined*	4	2.2	0.9–5.5

[1]95% confidence interval

*four HPV positive samples did not have a genotype identified by LiPA, being called X

https://doi.org/10.1371/journal.pone.0199557.t002

The prevalence of HPV-DNA in penile carcinoma in this study was 30.6% (95% CI: 24.4–37.6). In a multicenter study conducted in 25 countries, the prevalence of HPV in PC was 33.1% (95% CI: 30.2–36.1) [24]. However, other studies conducted in the same population found a similarity in HPV positivity [24,40,43,44]. Other studies estimated higher rates of HPV positivity, being 46.9% (95% CI: 44.4–49.6) [45]; 47.8% (95% CI: 45.0–50.6) [23], 60.7% (95% CI: 51.9–69.0) [46] in paraffin-packed PC samples.

In Brazil, the prevalence of HPV in penile carcinoma ranges from 30.5 to 63.1% in paraffin-embedded samples [25–27,46,47,48]. This difference observed in HPV-DNA positivity may be related to the methodology of the studies, the selection of samples (paraffin or cryopreserved), incidence of HPV in the geographical regions, methods of viral detection, and population studied [14,16,24,43,49].

In this study, the prevalence of HPV and HPV types was estimated in 183 cases of PC, however, the research does not allow for inferences regarding viral activity, whether this viral infection is transcriptionally active or not. Other active infection markers would need to be investigated, for example, to evaluate expression of the $p16^{INK4a}$ protein and detection of HPV E6*I mRNA [19,24]. However, this study is relevant from an epidemiological point of view and by virtue of the large number of PC cases included in the research.

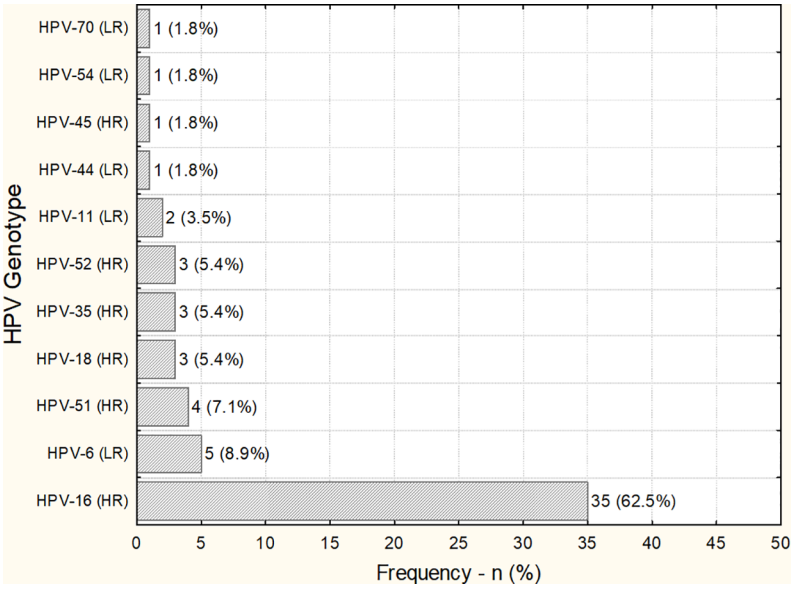

Fig 2. HPV genotype distribution in a population with PC. HR: High risk; LR: Low risk.

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Table 3. Bivariate and multivariate analysis of factors associated with HPV infection.

Variables	Total (N = 183)	HPV+ (%)	PR (95% IC)	P[1]	Adjusted PR (95% IC)	p[1]
Age (years)						
< 60	89	28 (31.5)	1.00		1.00	
≥ 60	94	28 (29.8)	0.94 (0.61–1.46)	0.807	0.96 (0.61–1.47)	0.870
Phimosis*						
No	15	8 (53.3)	1.00			
Yes	136	37 (27.2)	0.51 (0.23–1.09)	0.084		
Jackson stage						
0-II	133	40 (30.1)	1.00			
III-IV	41	13 (31.7)	1.05 (0.62–1.77)	0.842		
Tumor grade						
I	90	20 (22.2)	1.00		1.00	
II-III	92	35 (38.0)	1.71 (1.07–2.73)	0.024	1.70 (1.06–2.72)	**0.025**
Tumor invasion						
Superficial	78	24 (30.8)	1.00			
Deep	94	29 (30.9)	1.00 (0.63–1.57)	0.991		
In situ	9	2 (22.2)	0.72 (0.20–2.57)	0.616		
Inguinal metastasis						
No	117	33 (28.2)	1.00			
Yes	66	23 (34.8)	1.23 (0.79–1.91)	0.346		
Inguinal lymphadenectomy						
No	112	32 (28.6)	1.00			
Yes	71	24 (33.8)	1.18 (0.76–1.83)	0.453		
Inguinal recurrence						
No	167	52 (31.1)	1.00			
Yes	16	4 (25.0)	0.80 (0.33–1.93)	0.625		
Tumor inflammatory infiltrate						
Low	56	13 (23.2)	1.00			
Moderate	67	19 (28.4)	1.22 (0.66–2.25)	0.521		
Intense	14	3 (21.4)	0.92 (0.30–2.81)	0.888		
Lymphovascular invasion						
Absent	137	40 (29.2)	1.00			
Present	27	9 (33.3)	1.41 (0.62–2.07)	0.663		
Death						
Live	149	51 (32.2)	1.00			
Dead	34	8 (23.5)	0.73 (0.28–1.40)	0.344		

Abbreviations: PR: prevalence ratio; 95% CI: 95% confidence interval

[1]Wald chi-square test

* Variable excluded from the multiple regression model due to the large amount of missing data.

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The prevalence of high-risk oncogenic HPV genotypes in this study was 24.9% (45/183) and HPV-16 was identified in 62.5% of DNA-HPV positive samples, corroborating national and international data [15,18,24,26,47,50]. In a multicenter study, HPV 16 and 18 were detected in 70% of PC cases [24]. A similar finding was observed in this investigation, where 67.8% of the cases were infected with HPV 16 and 18.

HPV 6 and 11 are responsible for the development of 90% of genital warts [45] and have been identified in 12.5% of PC cases, similar to that found in other studies [26,48]. In our

Table 4. Bivariate and multivariate analysis of factors associated with infection by high-risk HPV.

Variables	Total (N = 179)	HPV High Risk Pos	PR (95% IC)	p[1]	Adjusted PR (95% IC)	p[1]
Age (years)						
< 60	87	21 (24.1)	1.00		1.00	
≥ 60	92	24 (26.1)	1.08 (0.64–1.79)	0.765	1.09 (0.66–1.81)	0.730
Phimosis *						
No	14	5 (35.7)	1,00			
Yes	134	25 (18.7)	0.52 (0.19–1.36)	0.185		
Jackson stage						
0-II	130	32 (24.6)	1.00			
III-IV	40	10 (25.0)	1.01 (0.54–1.88)	0.961		
Tumor grade						
I	89	16 (18.0)	1.00		1.00	
II-III	89	28 (31.5)	1.75 (1.01–3.00)	0.043	1.76 (1.02–3.02)	**0.040**
Tumor invasion						
Superficial	76	20 (26.3)	1.00			
Deep	92	23 (25.0)	0.95 (0.56–1.59)	0.846		
In situ	9	1 (11.1)	0.42 (0.06–2.79)	0.372		
Inguinal metastasis						
No	115	27 (23.5)	1.00			
Yes	64	18 (28.1)	1.19 (0.71–2.00)	0.491		
Inguinal lymphadenectomy						
No	110	26 (23.6)	1.00			
Yes	69	19 (27.5)	1.16 (0.69–1.94)	0.558		
Inguinal recurrence						
No	163	42 (25.8)	1.00			
Yes	17	4 (23.5)	0.72 (0.25–2.09)	0.555		
Tumor inflammatory infiltrate						
Low	56	10 (17.9)	1.00			
Moderate	65	15 (23.1)	1.29 (0.62–2.65)	0.484		
Intense	14	3 (21.4)	1.20 (0.37–3.80)	0.757		
Lymphovascular invasion						
Absent	135	31 (23.0)	1.00			
Present	26	8 (30.8)	1.33 (0.69–2.58)	0.382		
Death						
Live	145	38 (26.2)	1.00			
Dead	34	7 (20.6)	0.78 (0.38–1.60)	0.509		

Abbreviations: PR: prevalence ratio; 95% CI: 95% confidence interval

[1]Wald chi-square test

* Variable excluded from the multiple regression model due to the large amount of missing data.

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series, HPV vaccine types were detected in 80.3% CP cases. This study emphasizes the need for vaccine-related immunity in males to ensure a reduction in the overall burden of infection and diseases caused by HPV [30].

The role of HPV as a prognostic factor in penile cancer remains unclear [51]. It is uncertain whether cancers involving HPV infection have better survival profiles than cancers without HPV infection (15,40). In this series of cases, we observed no association between HPV and

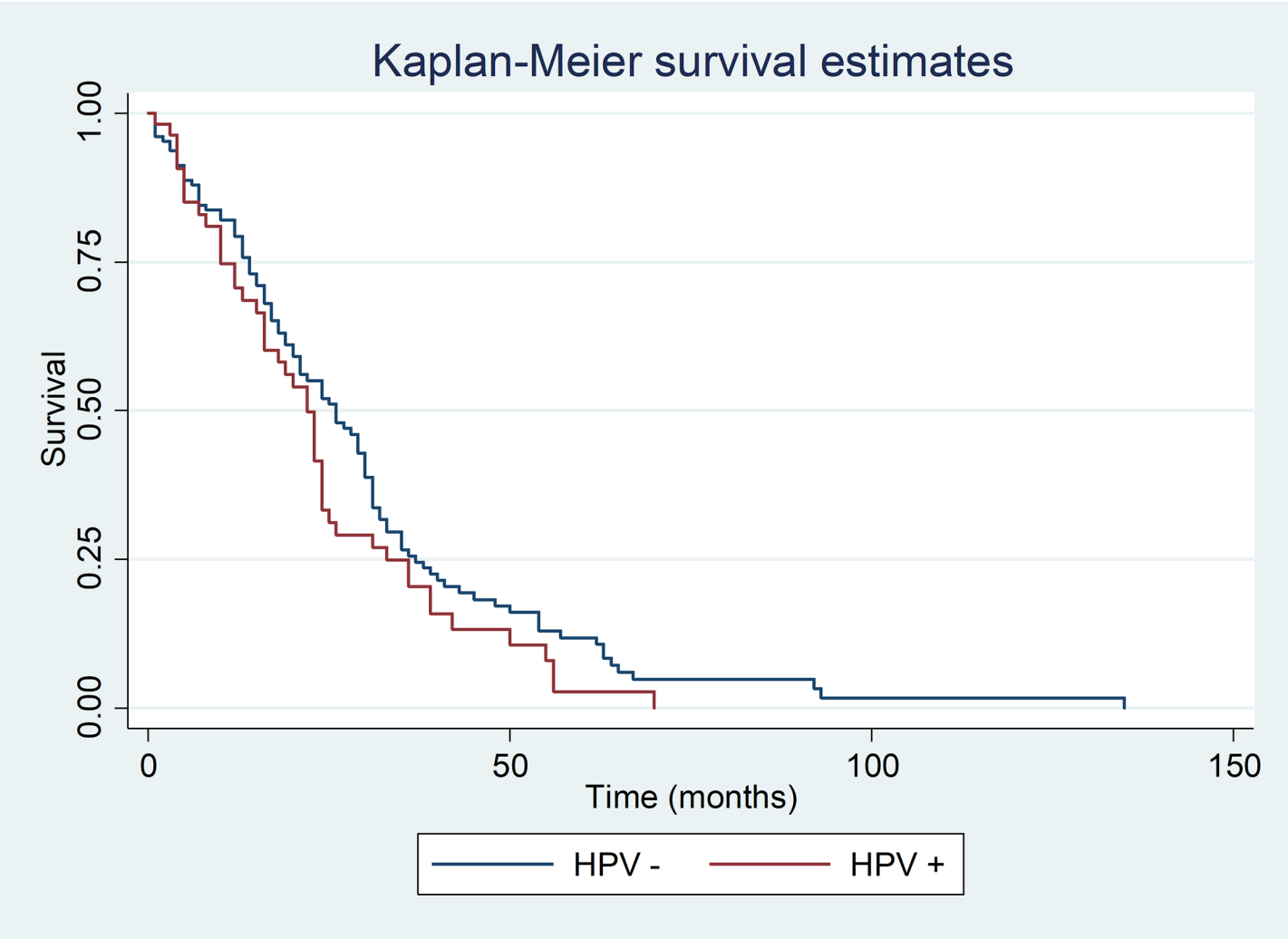

Fig 3. Curve of survival of patients positive and negative for HPV.

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high-risk HPV negative and positive patients when considering lymph node metastasis, this being one of the primary factors related to patient survival.

In this study, infection by HPV and high-risk HPV was associated with tumor grade II / III ($p = 0.025$ / $p = 0.040$) in multivariate analysis, as well as in other studies [15,46,52]. This pathological variable indicates a worse prognosis of lesions [25], as the development of lymph node metastases increases according to the degree of cell indifferentiation [1].

A recent Netherlands study reported that High-risk HPV positive tumors appear to providence a significant survival benefit over High-risk HPV negative tumors on multivariable analsysis (hazard ratio [RH], 0.2; $p = 0.034$) [15]. However, these results differ from those observed in this study because there was no association between HPV (long-rank test x^2: 2.14, $p = 0.143$) and high-risk and low-risk HPV (long-rank test x^2: 0.97; $p = 0.325$) and disease-specific survival rate at 10 years. It is possible that these conflicting results may be due to different study designs, sample sizes and sampling methods for DNA-HPV (19,25).

This study has some limitations, by its own design. Because it was a retrospective investigation, there was no possibility of retrieving clinical and histopathological data not recorded in

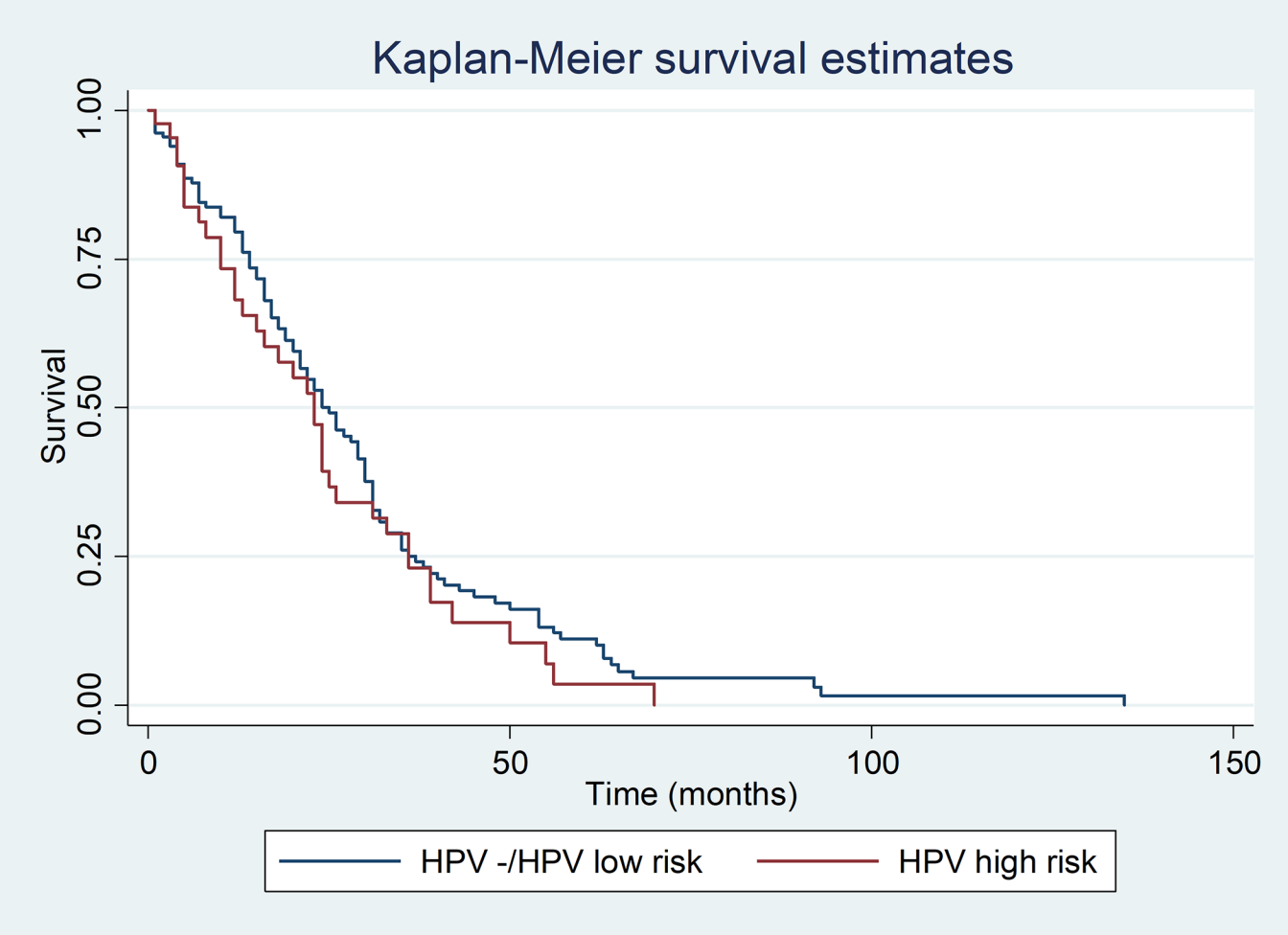

Fig 4. Survival curve of the patients in relation to HPV positivity and negativity low and high risk.

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medical records. In addition, it was not possible to identify the histological subtypes of the cases of penile carcinoma and to correlate them with the prevalence of HPV, since the majority of anatomopathological reports of the tumors did not include the histological subtype. The study also did not investigate the transcriptional activity of HPV in PC, however, the study design was to estimate the prevalence of HPV and to correlate positivity with clinicopathological factors of PC cases.

Conclusion

The results of the study showed that 80.3% of the types of HPV identified (16, 18, 6 and 11) in individuals with PC are immunopreventable types using the quadrivalent or nonavalent anti-HPV vaccine. This information emphasizes the importance of HPV vaccination in males, especially in developing countries, with a high incidence of penile carcinoma.

Supporting information

S1 Database. Study database.
(XLSX)

S1 Protocols. Protocols used for extraction, detection and genotyping of HPV DNA.
(PDF)

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