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ORIGINAL ARTICLE

Prevalence of human papillomavirus and implication on survival in Chinese penile cancer

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Abstract

We assessed the prevalence of HPV DNA in a large series of Chinese penile cancer and examine its association with the histological subtype, $p16^{INK4a}$ expression, and prognosis. We pathologically categorized 226 invasive penile squamous cell carcinomas and assessed HPV genotyping by real-time PCR and $p16^{INK4a}$ immunohistochemistry. The results were correlated with histopathological and clinical parameters and disease-specific survival (DSS). HPV DNA was detected in 32.7% (74/226) of penile cancer cases. The most frequent genotype was HPV 16 (64/74, 86.5%), followed by HPV 18 (6/74, 8.1%). Fifty-nine (26.1%) cases were positive for the $p16^{INK4a}$ expression, and $p16^{INK4a}$ expression had a sensitivity of 56.8% (95% CI, 45.2–68.3%) and a specificity of 88.8% (95% CI, 83.8–93.9%) for defining HPV status. HPV DNA ($P = 0.019$), $p16^{INK4a}$ ($P = 0.038$), age ($P = 0.018$), grade of differentiation ($P = 0.001$), lymph nodes ($P < 0.001$), T stage ($P < 0.001$), M stage ($P < 0.001$), and lymphovascular invasion (LVI, $P = 0.001$) were prognostic factors for DSS. HPV-positivity (HR 0.334; 95% CI, 0.158–0.705, $P = 0.004$) was still a significant prognostic factor for DSS in the multivariate Cox regression model. HPV DNA was observed in one third of Chinese penile carcinoma cases. The p16INK4a expression can indicate high-risk human papillomavirus (HR-HPV). HPV-positive penile tumors confer a survival benefit over HPV-negative tumors.

Keywords Penile cancer · HPV · p16INK4a · Prevalence · Survival

Introduction

Penile cancer (PC) is a rare and highly disabling disease with an annual increase of 26,300 new cases worldwide [15]. The overall incidence of PC was $0.6/10^5$ and mortality was $0.18/10^5$ in Chinese male in 2011 reported by the National Central Cancer Registry (NCCR) of China [26]. According to previous research [11, 17], the prevalence of penile cancer has been found to be related to a variety of factors, such as HPV, phimosis, poor sanitation, smoking, multiple sexual partners, genital warts, or other sexually transmitted diseases. Human papillomavirus (HPV) infection has been identified of playing an important role in the development of penile cancer [22]. Two major causative pathways that occur in the carcinogenesis of penile cancer have been described. One has been its association with HPV infection and the other with inflammation, phimosis or sclerosing moss, and lichen planus [5]. In 2016, a new WHO classification for penile cancer was released in which penile squamous cell carcinomas are now classified as HPV- or non-HPV-related [23, 24].

The overall prevalence of HPV DNA in penile carcinoma ranges between 11.6 and 100% but often has a large between-study heterogeneity [24]. Most of the previous researches have indicated that HPV16 was the most common HPV type among HPV DNA-positive penile cancers, followed by HPV6 and HPV18 [1, 24]. Moreover, in penile invasive cancers, the

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HPV infection rate varied with geographical position, race, and ethnicity [1]. The assessment of HPV status was already a strong recommendation in the EAU guidelines for the pathological evaluation of penile carcinoma specimens [18].

The $p16^{INK4a}$ expression is often used as a surrogate marker for the presence of high-risk human papillomavirus (HR-HPV) in cervical cancer and other organs, such as the head and neck carcinoma [6, 28]. Also, it has been shown that the $p16^{INK4a}$ expression has a strong connection to the presence of HR-HPV in penile cancer [13, 32]. In addition to HPV DNA, $p16^{INK4a}$ has shown prognostication potential in penile cancer. However, the prognostic value of HPV status and P16 status in penile cancer is still inconclusive. Some studies have shown that the positivity of HPV and the P16 protein was a good predictor of prognosis [1, 24], while others had contradicting results [4, 12, 20, 32]. In a recent review, HPV- or p16-positive penile squamous cell carcinomas (SCC) demonstrated significantly better clinical outcomes (HR_{HPV} 0.61; 95% CI, 0.38–0.98; HR_{p16} 0.45; 95% CI, 0.30–0.69) compared with HPV-negative ones [30]. In China, Jianpo Zhai et al. (2013) has shown that the HPV DNA prevalence in Chinese patients was relatively high, at 25.9% (7/28), but its presence did not provide survival advantage [33]. However, due to the limited number of specimens used in such study, more researches are needed to elucidate their correlation in Chinese patients.

Hence, the aim of the current study is to analyze the prevalence of HPV-DNA and expression of $p16^{INK4a}$ in a large series of Chinese PC tissue samples and correlate these results with clinicopathological features and the patients' survival. In addition, we also aimed to evaluate the association between the histological subtypes of the 2016 WHO penile cancer classification to that of the prevalence of HPV DNA and expression of $p16^{INK4a}$.

Materials and methods

Patients

The cohort comprised of 226 patients who were treated for invasive PC from 1999 to 2013 at the Sun Yat-sen University Cancer Center (SYSUCC) (Guangzhou, China) and had tissue available for this study. The formalin-fixed paraffin-embedded (FFPE) tissue blocks of the investigated patients were preserved in the pathological archives of SYSUCC and consisted of 186 primary tumors and 40 lymph node metastatic specimens. Their respective clinical and pathological data were retrieved from our electronic medical records and tumor registry.

Pathologic evaluation

The following pathological variables were investigated by two pathologists (Chu CB, Lu JL): tumor histology, grade, pathological T and N stage, presence of lymphovascular and perineural invasion, and necrosis. The tumor histology subtypes were classified as HPV- or non-HPV-related carcinoma in whole tissue sections using the morphological criteria presented in the pathology of penile cancer [23]. The grading of the examined tumors followed the three-tiered International Society of Urological Pathology/World Health Organization system [8]. Pathological parameters were evaluated according to the Eighth Edition of the Tumor-Node-Metastasis (TNM) Staging Classification for Penile Cancer [25].

HPV DNA detection and typing

The detection of HPV on the DNA extracts from FFPE tissues was performed using a Hybribio Assay (HybriMax, Chaozhou Hybribio Limited Corp., Chaozhou, China) that detects the 23 HPV types using real-time polymerase chain reaction (PCR). The kit could identify 13 HR-HPVs (subtype, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), 5 low-risk HPVs (LR-HPVs) (subtype, 6, 11, 42, 43, and 44), and other HPV types commonly found in Chinese populations (subtype, 53, 66, 73, 82 and 81/CP8304). DNA was extracted from FFPE tissues using QIAamp® DNA FFPE Tissue (Q) (Cat. no. 56404, Qiagen, Hilden, Germany), and real-time PCR was performed with 2 ul of DNA buffer, 17.5 ul of Master mix (containing probes and primers), and 0.5 ul of Taq DNA polymerase. The PCR conditions were 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min, with data collection at each cycle during the 60 °C phase on a LightCycler 480 (Roche Diagnostics and Rotor-Gene Q, Qiagen).

Immunohistochemistry for $p16^{INK4a}$ expression

Immunohistochemistry was performed to determine the expression of $p16^{INK4a}$ according to the manufacturer's protocol using a mouse monoclonal primary antibody $p16^{INK4a}$ (Clone 6H12, IgG2b/Newcastle) at a dilution of 1:100. The expression of $P16^{INK4a}$ was classified as four patterns: 0, no stain; 1, weak and individual; 2, moderate with small clusters; and 3, strong and diffuse (Fig. 1). Only pattern 3 was represented positive for $p16^{INK4a}$ expression [20].

Follow-up and statistical methods

All patients were followed for every 3 months until the second year after surgery, every 6 months in the 3rd and 4th years, and then on a yearly thereafter. Follow-up data were recorded until March 2019 and comprised of information concerning the patients' disease status and disease-specific mortality.

Data were tabulated using Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and analyzed with SPSS V22 (IBM Corp., Armonk, NY, USA). Categorical

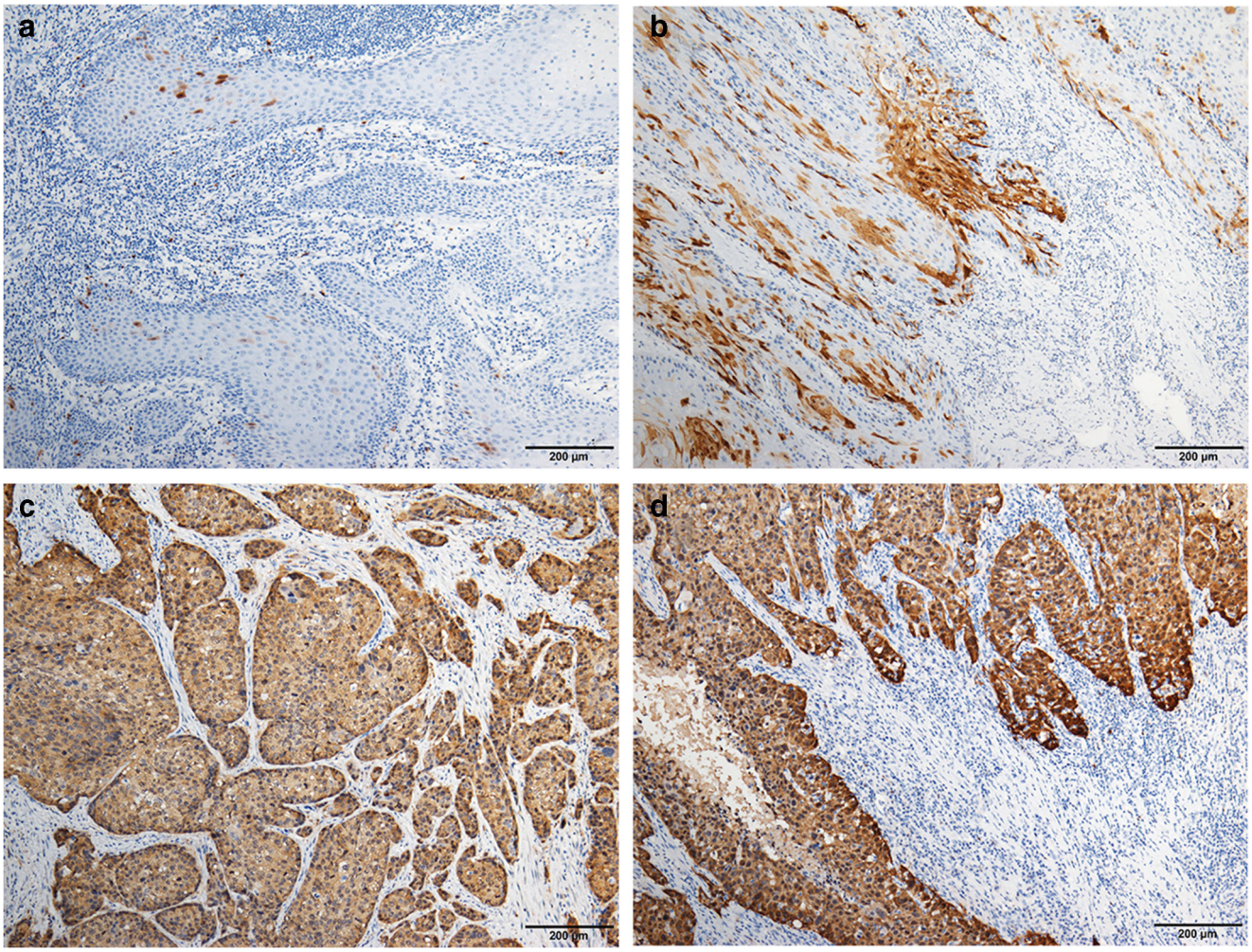

Fig. 1 Patterns of p16 expression in penile carcinomas. **a** Pattern 1, weak and individual. **b** Pattern 2, moderate with small clusters. **c** Pattern 3 in primary tumors, strong and diffuse. **d** Pattern 3 in lymph node metastatic specimens

variables were estimated with the chi-square test or Fisher-exact chi-square test and continuous variables with the Mann-Whitney *U* test. Survival analysis was estimated by the Kaplan-Meier method and compared by the log-rank test. The Cox regression proportional hazard model was used for multivariate analysis, predicting disease-specific survival (DSS). The statistical significance threshold used was $P \leq 0.05$.

Results

Clinicopathological data

The patients' median age at the time of surgery was 52 (ranging from 24 to 86) years, and the median follow-up time was 57 (2–209) months. There was no difference in the ages between HPV-positive and HPV-negative patients (Mann-Whitney *U* test, $P = 0.833$). Of the patients' specimens, 82.3% (186/226) were primary lesion, and the rest (17.7%, 40/226) were focal lymph nodes. The most common histological subtype was usual SCC (80.4%, 144/179), and 89.4% (160/179) were found to be non-HPV-related histological subtypes. In some cases, the clinicopathological data could not be evaluated since they only received primary cancer focus resection or lymph node resection in our hospital.

HPV genotyping

The presence of HPV DNA was detected in 74 (32.7%) of 226 samples, 80.1% (60/74) of which corresponded to only 1 HPV genotype infections. Of these, the most frequent were HPV 16 (64/74, 86.5%), followed by HPV 18 (6/74, 8.1%). Seventy-one cases (95.9%) were infected with HR-HPV, and 3 cases (4.1%) were infected with low-risk HPV (LR-HPV) (HPV6, HPV43, and HPV73 (1 type for each case)) (Fig. 2). The HR-HPV subtypes were observed either alone or together with other subtypes. Overall, 18.9% (14/74) of the investigated penile cancer cases contained HPV DNA from multiple types of HPV.

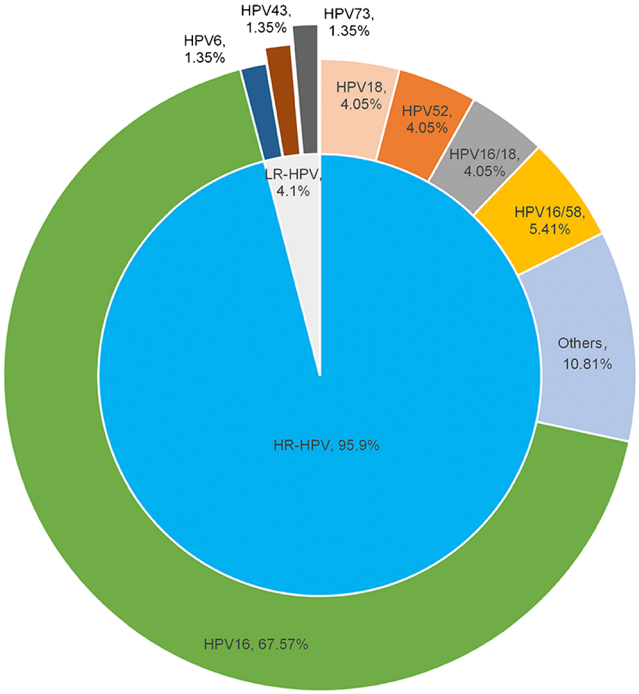

Fig. 2 Distribution of HPV genotype ($n = 74$)

P16^{INK4a} protein and HPV

Distribution of the p16^{INK4a} expression patterns and the entire HPV status are shown in Fig. 3. Fifty-nine (26.1%) were positive for the p16^{INK4a} expression, and 167 (73.9%) were negative. P16^{INK4a} immunopositivity, considering the observed staining pattern 3, was significantly associated with HR-HPV infection ($P < 0.001$). p16^{INK4a} had a sensitivity of 57.7% (95% CI, 45.5–69.2%) and a specificity of 88.4% (95% CI, 82.0–92.8%) for defining HR-HPV status. LR-HPV infection was found in 3 cases. Among them, only 1 case, who was usual SCC with HPV-43, had p16^{INK4a} over-expression (pattern 3).p16^{INK4a} was negative in the remaining 2 LR-HPV-positive tumors.

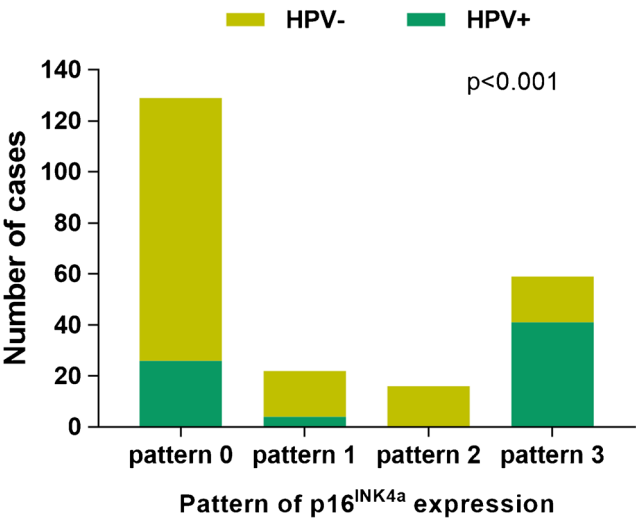

Fig. 3 Distribution of the p16INK4a expression patterns stratified by HR-HPV status. HR-HPV, high-risk human papillomavirus

Association of HPV and p16^{INK4a} protein with histopathological and clinical parameters

The clinicopathological features of the patients stratified by tumor HPV DNA status and P16^{INK4a} protein are presented in Table 1. Tumor grade was associated with HPV presence ($P = 0.029$) and p16^{INK4a} immunopositivity ($P = 0.021$). HPV-positive or p16^{INK4a} immunopositivity patients tended to have higher tumor grade than HPV-negative or P16^{INK4a}-negative patients. Pure and mixed basaloid carcinomas were observed in 18.75% (3/16 cases) of all grade 3 tumors. When basaloid carcinomas were excluded from the analyses, no significant differences were observed between tumor grade and HPV ($P = 0.056$) or p16^{INK4a} ($P = 0.154$). The histological subtypes were correlated with P16^{INK4a} ($P = 0.007$), where 62.5% of the tumor in HPV-related histologic subtypes were p16^{INK4a} over-expression compared with 26.9% in non-HPV-related histologic subtypes. However, the difference for histologic subtypes in association with HPV positivity did not reach statistical significance ($P = 0.082$). In addition, age and other histological characteristics were not significantly associated with HPV or p16^{INK4a}.

Table 2 depicts the histological subtypes, HPV status, and P16^{INK4a} expression within these subtypes. The most common subtype was the usual carcinomas (80.4%), followed by warty carcinomas (7.8%), papillary not otherwise specified (5.0%), verrucous (3.4%), basaloid carcinoma (1.7%), and sarcomatous carcinomas (1.1%). Considering the histologic diagnosis in penile cancer, HPV and P16^{INK4a} prevalence varied by the histologic subtypes with the highest prevalence in basaloid and warty carcinomas; 100% of pure or mixed basaloid carcinomas were p16^{INK4a} over-expression. LR-HPV infection was found in 3 cases, of which 2 corresponded to usual SCC and the remaining 1 to warty carcinomas (all was of grade 1). From the morphologic point of view, these LR-HPV tumors were undistinguishable from HR-HPV-positive and HPV-negative tumors with similar histopathologic classification. We were unable to distinguish these LR-HPV carcinomas from HR-HPV-positive and HPV-negative carcinomas by histomorphology.

Survival analysis

The hazard ratios for disease-specific survival (DSS) to HPV DNA positivity, p16^{INK4a} over-expression, and clinicopathological features, using Cox proportional hazard regression model, are shown in Table 3. Age ($P = 0.018$), grade of differentiation ($P = 0.001$), lymph nodes ($P < 0.001$), T stage ($P < 0.001$), M stage ($P < 0.001$), and lymphovascular

Table 1 Clinicopathological features stratified by tumor HPV DNA status and $P16^{INK4a}$ protein

Variable		HPV DNA			$p16^{INK4a}$ over-expression		
	n	Positive	Negative	*P* value	Positive	Negative	*P* value
Age				0.833[a]			0.719[a]
Median (range)	226	51 (24–86)	53 (26–82)		51 (25–86)	53 (24–82)	
Tumor grade				**0.029**			**0.021**
I	110	33 (30.0%)	77 (70.0%)		26 (23.6%)	82 (76.4%)	
II	54	22 (40.7%)	32 (59.3%)		18 (33.3%)	36 (66.7%)	
III	16	10 (62.5%)	6 (37.5%)		9 (56.3%)	7 (43.8%)	
T stage				0.525			0.338
I–II	174	56 (32.2%)	118 (67.8%)		46 (25.7%)	133 (74.3%)	
III–IV	26	10 (38.5%)	16 (61.5%)		9 (34.6%)	17 (65.4%)	
LN metastasis				0.930			0.093
Positive	117	38 (32.5%)	79 (67.5%)		25 (21.4%)	92 (78.6%)	
Negative	109	36 (33.0%)	73 (67.0%)		34 (31.2%)	75 (68.8%)	
M stage				0.302[b]			0.292[b]
Yes	10	5 (50.0%)	5 (50.0%)		4 (40.0%)	6 (60.0%)	
No	216	69 (31.9%)	147 (68.1%)		55 (25.5%)	161 (74.5%)	
LVI				0.113			0.718
Yes	21	11 (52.4%)	10 (47.6%)		7 (33.3%)	14 (66.7%)	
No	156	54 (34.6%)	102 (65.4%)		46 (29.5%)	110 (70.5%)	
Perineural invasion				0.406			0.621
Yes	27	8 (29.6%)	19 (70.4%)		7 (25.9%)	20 (74.1%)	
No	150	57 (38.0%)	93 (62.0%)		46 (30.7%)	104 (69.3%)	
Necrosis				0.270			0.169
Yes	21	10 (47.6%)	11 (52.4%)		9 (42.9%)	12 (57.1%)	
No	156	55 (35.3%)	101 (64.7%)		44 (28.2%)	112 (71.8%)	
Histological subtypes				0.082			**0.007[b]**
HPV-related	16	9 (56.3%)	7 (43.7%)		10 (62.5%)	6 (37.5%)	
Non-HPV-related	163	56 (34.4%)	107 (65.6%)		43 (26.9%)	120 (73.6%)	

[a] Mann-Whitney *U* test

[b] Fisher-exact test

Bold value indicates a significant difference

LN, lymph nodes; LVI, lymphovascular invasion

invasion (LVI, $P = 0.001$) were prognostic for DSS. Regarding DSS for HPV and $p16^{INK4a}$ status, it was observed that HPV-positive and $p16^{INK4a}$ over-expression patients had a higher DSS (Fig. 4) (log-rank $P = 0.019$ and 0.038,

Table 2 Association of HPV DNA positivity and $p16^{INK4a}$ protein with clinicopathological features in patients with penile SCC

Histological subtypes (WHO 2016)		*n*	%	HPV (%)	$P16^{INK4a}$ (%)
Non-HPV-related	Usual	144	80.4	54 (37.5)	42 (29.2)
	Papillary	9	5.0	2 (22.2)	2 (22.2)
	Verrucous	6	3.4	1 (16.7)	1 (16.7)
	Pseudoglandular	1	0.6	0 (0)	0 (0)
HPV-related	Warty	14	7.8	6 (42.9)	5 (35.7)
	Basaloid	3	1.7	2 (66.7)	3 (100)
Others	Sarcomatous	2	1.1	0 (0)	0 (0)

SCC, squamous cell carcinoma

Table 3 Univariate and multivariate analyses of clinicopathological and HPV status for cancer-specific survival ($n = 226$)

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age	1.860 (1.110–3.117)	**0.018**	1.791 (0.879–3.648)	0.108
Tumor grade	2.004 (1.348–2.978)	**0.001**	1.015 (0.594–1.735)	0.956
T stage	2.813 (1.498–5.281)	**0.001**	1.600 (0.743–3.448)	0.230
LN metastasis	40.282 (9.843–164.854)	**0.000**	38.075 (8.678–167.044)	**0.000**
M stage	8.868 (4.322–18.194)	**0.000**	5.186 (1.876–14.334)	**0.002**
LVI	3.236 (1.623–6.453)	**0.001**	1.166 (0.514–2.643)	0.898
Perineural invasion	2.011 (0.986–4.100)	0.055		
Necrosis	1.979 (0.916–4.278)	0.082		
Histological subtypes (wart/bas)	0.207 (0.029–1.506)	0.120		
HPV status	0.483 (0.263–0.888)	**0.019**	0.388 (0.183–0.824)[a]	**0.014**[a]
P16	0.489 (0.249-0.961)	**0.038**	0.560 (0.231–1.356)[b]	0.199[b]

HR, hazard ratio; CI, confidence interval; LN, lymph nodes; LVI, lymphovascular invasion

[a] multivariate analysis excluding P16

[b] multivariate analyses excluding HPV status

Bold value indicates a significant difference

respectively). In a Cox proportional hazard model, HPV-positive (HR 0.334; 95% CI, 0.158–0.705, $P = 0.004$) was still a significant prognostic factor for DSS, after adjustment for age, grade of differentiation, lymph node status, T stage group, M stage, and LVI (Table 3).

Discussion

This study is the largest, to our knowledge, to assess the HPV prevalence, type distribution, $P16^{INK4a}$ expression, and their association with clinical and pathological parameters of PC in China. The HPV DNA prevalence reported in this study for penile carcinoma (32.7%) was consistent with previous observations in a multicenter study conducted in 25 countries [1]. However, it was lower than that found in systematic reviews by Backes et al. (50%) [3], Tina Bech Olesen et al. (50.8%) [24], and one from the USA in 2014 (63%) [31]. The PC cases of this study had a higher rate of HPV positivity, in sharp contrast to what was found in Asia (13.4%) by Alemany L et al. [1]. In a recent Japanese study, 41% of penile cancer were HPV-positive while only included 34 PC samples [29]. These results indicate a significant geographical difference in HPV infection prevalence.

HPV-related penile tumors had a higher HPV DNA prevalence than non-HPV-related penile tumors. In the present study, we found that cases with warty-basaloid morphologic features were strongly related to HPV DNA positivity and

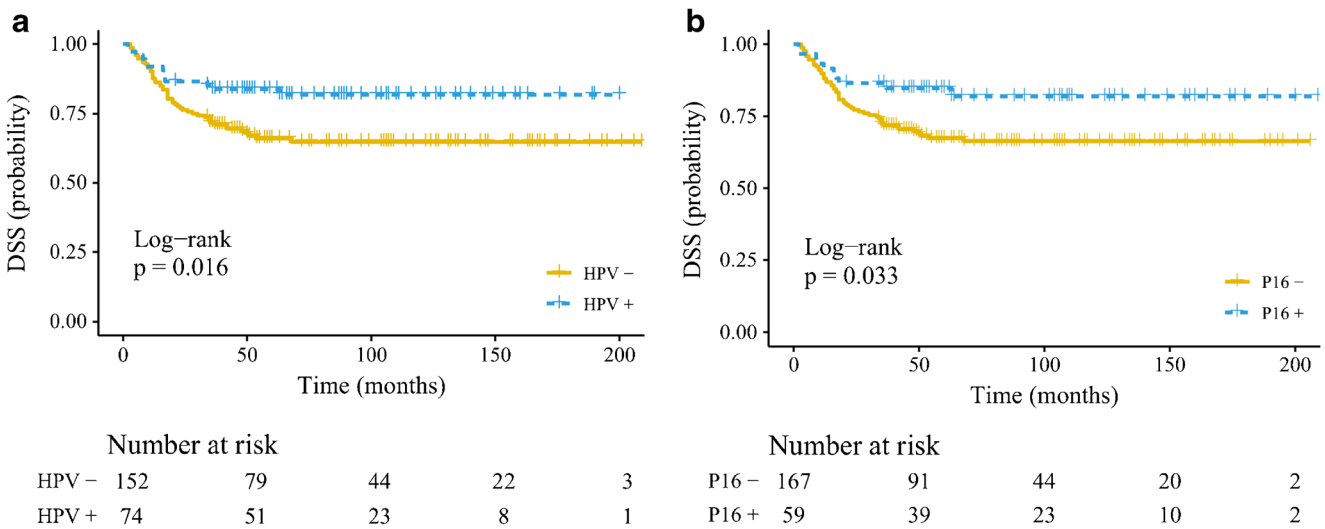

Fig. 4 Survival curve of patients positive and negative for HPV (**a**) and P16 (**b**)

$p16^{INK4a}$ over-expression, which is largely consistent to that previously established in other studies [7, 9, 14, 24]. Furthermore, the HPV DNA prevalence in verrucous carcinoma was similar to the previous analysis [3, 22]. It is worth noting that the usual type of SCC, which is classified as non-HPV-related according to the latest WHO classification, had a high HPV DNA prevalence of 37.5%. In consequence, it was unreasonable to assess HPV status simply by classifying penile tumors as HPV- or non-HPV-related.

Furthermore, significantly fewer well-differentiated tumors were found among the HPV-positive tumors, which was in accordance with some previous studies [13, 16]. Nevertheless, no significant differences between tumor grade and HPV ($P = 0.056$) or $p16^{INK4a}$ ($P = 0.154$) were found when we excluded tumors with basaloid and warty features from the analyses.

The findings of this study validated HPV16 as the most common oncogenic HPV genotype, which accounted for more than 70.4% of the single HR-HPV infections in penile cancer. HPV subtypes 6, 11, 16, 18, 31, 33, 45, 52, and 58 together accounted for approximately 90.5% of HPV DNA-positive penile cancers. According to our data, the predominance of HPV16 in penile cancer is underlined, which highlights the potential preventive effect of the available new 9-valent HPV vaccines.

The $p16^{INK4a}$ expression was related to the presence of HR-HPV in penile carcinoma samples, and LR-HPV cases had a lower $p16^{INK4a}$ upregulation than HR-HPV cases. Besides, the $p16^{INK4a}$ protein was related to tumor subtypes other than other histological prognostic parameters. Our data evinces that $p16^{INK4a}$ expression can be used as a significant marker of HR-HPV infection [10].

We examined the prognostic significance of HPV and $p16^{INK4a}$ status on survival in men diagnosed with penile cancer. We found that men with HPV DNA or P16-positive penile cancer had a significantly better DSS compared with those without HPV- or P16-negative penile cancer.

Our results support the hypothesis that HPV-positive and HPV-negative penile cancers differ in relation to survival outcome, which has also been found in other HPV-associated cancers including vulvar and oropharyngeal SCC [2, 27]. However, the explanation for the prognostic significance of HPV status is still unclear. It has been suggested that the presence of viral infection in HPV-associated cancers might strengthen immune surveillance, which consequently makes the HPV-positive cancers less aggressive compared with HPV-negative cancers [2, 19]. In vulvar precancerous lesions, HPV-negative lesions progress to invasive carcinoma more quickly compared with HPV-positive lesions [21], which indicated that HPV-related precancerous lesions generally develop through a slower invasive pathway and have a better prognosis in all stages of the cancerization. Furthermore, for head and neck cancers, it has been revealed that HPV-positive cancers could have a lower degree of gross genetic alterations or that the HPV status of the tumor may determine the molecular structure of the tumor, which could potentially affect the response to therapy [2].

The limitations of this study should be pointed out. First, there are inherent biases and potential errors associated with retrospective and single-center study, and a 5-year follow-up period was not achieved in all cases. Second, we used a mixed set of specimens, containing primary tumors as well as lymph node metastases. However, the histologic subtypes of lymph node metastases could not be assessed. Perhaps, this could in part explain the disappointing fact that the presence of HPV DNA did not correlate well with histologic subtypes of squamous cancer. Third, by using FFPE material, it may be that the proportion of HPV positivity was underestimated.

Conclusion

We observed that the HPV DNA prevalence was 36.4% in penile cancer and P16-positive in 26.1% of those cases. $p16^{INK4a}$ expression was related to HR-HPV DNA and is an important marker of HR-HPV infection. Besides, this study suggests that men with HPV DNA-positive or p16 penile cancer have a significantly better DSS compared with those with HPV/p16-negative.

Author's contributions Conceptualization: Chengbiao Chu, Kai Yao, Yun Cao; Methodology: Chengbiao Chu, Keming Chen, Xingliang Tan, Jiangli Lu, Yuanzhong Yang, YiJun Zhang; Formal analysis and investigation: Chengbiao Chu, Keming Chen; Writing, original draft preparation: Chengbiao Chu; Writing, review and editing: Chengbiao Chu, Kai Yao, Yun Cao; Funding acquisition: Kai Yao, Yun Cao; Resources: Chengbiao Chu, Xingliang Tan, Kai Yao, Yun Cao; all authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study protocol was approved by Independent Ethics Committee of Sun Yat-sen University Cancer Center.

Data availability The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn), with the approval RDD number as RDDB2019000656.

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