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ORIGINAL PAPER

Prognostic value of p16^{INK4a} overexpression in penile cancer

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Summary ***Introduction: Penile cancer is rare, accounting for less than 1% of all male cancers in industrialized countries. It is most common in areas of high prevalence of HPV, being a third of cases attributed to the carcinogenic effect of HPV. Tumour cells infected with HPV overexpress p16^{INK4a}, as such p16^{INK4a} has been used as a surrogate of HPV infections.***
Objective: To evaluate the prognostic factor of p16^{INK4a} overexpression in penile cancer.
Methods: Retrospective analysis of patients diagnosed with penile cancer, submitted to surgery in a Portuguese Oncological Institution in the last 20 years (n = 35). Histological review of surgical pieces and immunohistochemical identification of p16^{INK4a}. Relation between p16INK4a and the following factors were studied: age, histological subtype, tumour dimensions, grade, TNM stage, perineural invasion, perivascular invasion, disease free survival (DFS) and cancer specific survival (CSS).
Results: p16^{INK4a} was positive in 8 patients (22.9%). Identification of p16^{INK4a} did not correlate with none of the histopathological factors. In this work we identified a better DFS and CSS in patients positive for p16^{INK4a} (DFS at 36 months was 100.0% vs. 66.7%; CSS at 36 months was 100.0% vs. 70.4%), although without statistical significance (p > 0.05). In multivariate analysis of histopathological factors studied, only N staging correlated with DFS and CSS (p = 0.017 and p = 0.014, respectively).
Discussion: the percentage of cases positive for p16^{INK4a} is smaller than the one found in literature, which can suggest a less relevant part of HPV infection in the oncogenesis of penile cancer in the studied population. Identification of p16^{INK4a} did not relate with other clinicopathological factors. Tendency for a more favourable prognosis in patients with p16^{INK4a} agrees with results found in literature. The most relevant factor for prognosis is nodal staging.
Conclusions: penile cancer positive for p16INK4a shows a trend for better survival, although the most relevant factor is nodal staging.

KEY WORDS: Penile cancer; HPV; p16; Prognosis.

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INTRODUCTION

In industrialized countries, penile cancer is rare, having an incidence of approximately 1/100000 in *Europe* and the *United States of America*, accounting for less than 1% of all male cancers (1-3). However, in other regions of the world, particularly *South America*, *Southeast Asia* and parts of *Africa*, the incidence of penile cancer is much greater, accounting for about 2% of all male cancers, although in some countries it can reach 10% (4, 5).
The incidence of penile cancer increases with age, reaching a peak in the six decade, although it can occur in much younger patients (1, 6).
Penile cancer is most common in areas with high prevalence of HPV, with a third of cases attributed to the carcinogenic effects of HPV (1, 7). Other risk factors identified where: phimosis (8, 9), chronic penile inflammation/ lichen sclerosus (10), psoralene and phototherapy with ultraviolet radiation A (11), smoking (8), residing in rural areas/low socioeconomical level (12, 13) and multiple sexual partners (8).
In relation to penile cancer, HPV DNA was identified in 30-40% of cases, varying in accordance to histological subtype. The histological subtypes most associated with HPV are Basaloid *Penile Squamous Cells Carcinoma* (PSCC) (76%), mixed Warty-basaloid PSCC (82%) and Warty PSCC (39%). The Usual PSCC and Papillary PSCC are not associated with HPV (14, 15). Although the classic PSCC is normally characterized as non-related to HPV, a recent metanalysis identified an association in over 30% of cases (16). The subtypes of HPV most commonly associated with penile cancer are 16 and 18 (17).
The *World Health Organization* (WHO), utilizing the hypothesis of independent pathways of carcinogenesis, categorizes PSCC regarding HPV (18, 19). The prognostic value of the association with HPV is still controversial, with recent studies showing a better outcome in HPV associated penile cancer (20-22), while others do not show significant differences (18, 23).
Various methods can be used to detect HPV in tumour cells, such as PCR amplification to detect HPV DNA. Due to the overexpression of p16^{INK4a} in HPV infected cells, p16^{INK4a} expression can be used as a surrogate of active HPV infections (16). In cervical cancer and in other squamous cell carcinomas, expression of p16^{INK4a} is used as a marker for the presence of high-risk HPV (17-19).
Progression and regression of low grade intraepithelial cervical cancer can be estimated utilizing p16^{INK4a} and mark a better *cancer specific survival* (CSS). However, the

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correlation between expression of $p16^{INK4a}$ and HPV infection in penile cancer is still controversial (24).
The main aim of this paper is to evaluate the prognostic value of $p16^{INK4}$ expression in penile cancer. Other goals are to evaluate the epidemiologic association between HPV and penile cancer in *Portugal* (indirect assessment by assessing expression of $p16^{INK4a}$) and to evaluate the association between HPV and histological subtypes of PSCC and the initial staging of the disease.

Methods

Patient selection and data collection

Retrospective analysis of all patients with the primary diagnosis of penile cancer treated in a Portuguese oncological institution, in the last 20 years. Retrospective evaluation of patient data.

Pathological and immunohistochemistry evaluation

All surgical specimens of the identified patients were revaluated for this study.
The material was fixed in 10% formol, embedded in paraffin and stained with haematoxilin-eosine, and was reviewed by a genitourinary pathologist that determined the histologic subtyping and the pathological grade using the morphologic criteria presented in the WHO classification of tumours of the penis of 2016 and tumour staging was made according the AJCC cancer staging manual of 2017 (19, 25). Immunohistochemical analysis was performed on the *BenchMark-Ultra platform* (*Ventana R*). Antigenic retrieval was performed using the *Ultraview Universe Dab Detection Kit* (*Ventana R*). Slides were then incubated with monoclonal antibody to $P16^{ink4a}$ (mouse clone E6H4, CINtec R p16 Histology, *Ventana R*).
The *Bond Polymer Refine detection system* (*Ventana*) was used for secondary antibody and visualization. Cervical squamous cell carcinoma was used as positive control, and benign skin as negative control. Cases were scored by a genitourinary pathologist. To define the expression patterns of $p16^{INK4a}$, the classification of *Cubila et al.* (26) was adapted, and overexpression of $p16^{INK4a}$ was defined as diffuse, continuous, and strong nuclear and cytoplasmic staining of the neoplastic cells. Discontinuous, focal and weak staining as well the absence of staining was interpreted as negative for $p16^{INK4a}$ overexpression.

Statistical analysis

The program SPSS 21 was used for statistical analysis. We used the Mann-Whitney test to assess the relation between clinical and pathological characteristics and $p16^{INK4a}$. Survival related to each individual factor were calculated by the Kaplen-Meyer curves. Multivariate analysis utilizing *Cox* regression was utilized for the impact of clinical and pathological factors on survival.

Results

Clinicopathological data

The total number of patients was 35, the median age was 69 (range 33-90 years) and the median tumour size was of 2.5 cm (range, 0.4-12.0). Eight patients (22.9%) presented with positive $p16^{INK4a}$ test. Relating to T staging, 1 (2.9%) presented with Tis, 13 (37.1%) T1, 11 (31.4%) T2 and 10 (28.6%) T3. The clinicopathological results are summarized in Table 1.

$P16^{INK4a}$ immunoexpression

The relation between $p16^{INK4a}$ expression and the remaining clinicopathological results are summarized in Table 2. $P16^{INK4a}$ immunoexpression did not correlate in a significant way ($p > 0.05$) with none of studied factors.

$P16^{INK4a}$ immunoexpression and prognosis

The median follow-up was 63 months (range 6-204). The Kaplan-Meyer curves of disease-free survival (DFS) and *cancer specific survival* (CSS) in relation to $P16^{INK4a}$ immunoexpression are presented in Figures 1, 2, respectively. Although a tendency to a longer survival with positive $P16^{INK4a}$ immunoexpression, this was not statically significant (DFS: $p = 0.219$; CSS: $p = 0.067$). The DFS and CSS at 3 years for patients with positive $P16^{INK4a}$ immunoexpression were 100.0% and 100.0%, respectively. The DFS and CSS at 3 years for patients with negative $P16^{INK4a}$ immunoexpression were 66.7% and 70.4%, respectively.

Other clinicopathological factors and prognosis

The disease-free survival and cancer specific survival in relation to T stage, N stage, tumour grade, perineural invasion and perivascular invasion were evaluated.
In relation to DFS, the following factors were associated with higher survival: T stage $T \leq 1$ ($p = 0.002$) and $N = 0$ ($p < 0.001$).

Table 1.
Clinicopathological results.

	N (%)
Age (years)	
· < 65	13 (37.1%)
· ≥ 65	22 (62.9%)
PSCC histologic subtype	
· Usual	28 (80%)
· Warty	3 (8.6%)
· Verrucous	2 (5.7%)
· Mixed Warty-Basaloid	2 (5.7%)
Dimension (cm)	
· < 4	26 (74.8%)
· ≥ 4	9 (25.7%)
p16INK4a	
· Positive	8 (22.9%)
· Negative	25 (78.1%)
Differentiation grade	
· G1	14 (40%)
· G2	15 (42.9%)
· G3	6 (17.1%)
T stage	
· ≤ 1	14 (40.0%)
· > 1	21 (60.0%)
Lymph node metastasis	
· No	26 (74.3%)
· Yes	9 (25.7%)
Died of the disease	
· No	26 (74.3%)
· Yes	9 (25.7%)

Table 2.
Relation between p16INK4a expression and the remaining clinicopathological results.

	N (%)		
Age (years)			
· < 65	2 (25.0%)	11 (40.7%)	0.425
· ≥ 65	6 (75.0%)	16 (59.3%)	
PSCC histologic subtype			
· Usual	6 (75%)	22 (81.5%)	0.800
· Warty	1 (12.5%)	2 (7.4%)	
· Verrucous	0 (0.0%)	2 (7.4%)	
· Mixed Warty-Basaloid	1 (12.5%)	1 (3.7%)	
Dimension (cm)			
· < 4	6 (75.0%)	20 (74.1%)	0.318
· ≥ 4	2 (25.0%)	6 (22.2%)	
Differentiation grade			
· G1	4 (50.0%)	10 (37.0%)	0.510
· G2	3 (37.5%)	12 (44.4%)	
· G3	1 (12.5%)	5 (18.5%)	
Perineural invasion			
· No	7 (87.5%)	4 (14.8%)	0.871
· Yes	1 (12.5%)	23 (85.2%)	
Lymphovascular invasion			
· No	7 (87.5%)	24 (88.9%)	0.915
· Yes	1 (12.5%)	3 (11.1%)	
T stage			
· ≤ 1	3 (37.5%)	11 (40.7%)	0.871
· > 1	5 (62.5%)	16 (59.3%)	
Lymph node metastasis			
· No	8 (100.0%)	18 (66.7%)	0.062
· Yes	0 (0.0%)	9 (33.3%)	
Died of the disease			
· No	8 (100.0%)	18 (66.7%)	0.062
· Yes	0 (0.0%)	9 (33.3%)	

Analysing CSS the following factors presented statistically significant improved survival: age < 65 years (p = 0.042), stage T ≤ 1 (p = 0.005), stage N = 0 (p < 0.001). In a multivariate Cox regression analysis with the previously described factors and HPV, the model was statistically significant in relation with DFS and CSS, although only N stage presented with statically relevance (p = 0.017 and p = 0.014, respectively).

Figure 1.
Disease free survival for p16INK4a positive and negative patients.

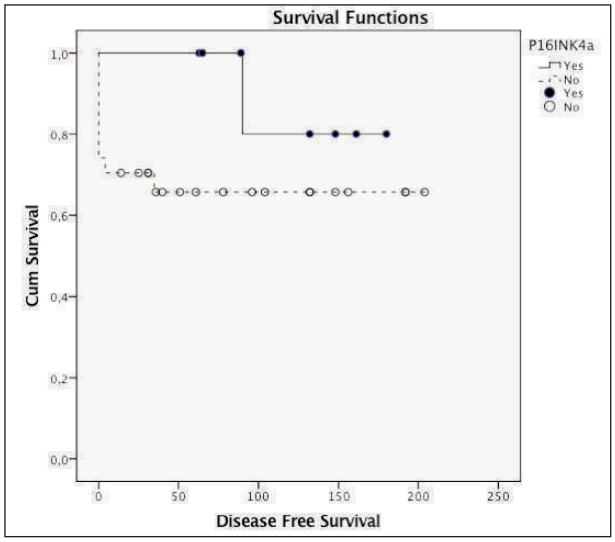
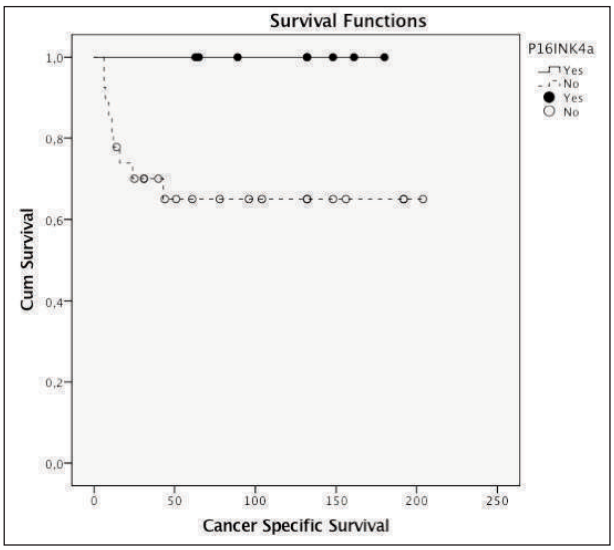


Figure 2.
Cancer specific survival for p16INK4a positive and negative patients.



Discussion

In this study, we identified 22.9% of P16INK4a positive PSCC, a value smaller than the one calculated by a recent metanalysis, which identified P16INK4a in 42.6% (95% CI; 36.2-47.0) worldwide (2995 cases) and 44.9% (95% CI; 38.4-51.1) in *Europe* (16). A Spanish study (*Barcelona*), interesting to compare due to the geographic proximity with *Portugal*, identified a P16INK4a positivity in 34.0% of 72 cases (22). In order to understand this result, it is essential to comprehend the meaning of positive P16INK4a and its correlation with the physio-pathological role of HPV infection in penile cancer. The most sensitive method for the detection of HPV in tumoral tissue is PCR amplification (27). Due to the strong correlation between active HPV and P16INK4a overexpression in neoplastic cells, it has been used as a surrogate marker for HPV (28). The incorporation of high-risk HPV (HR-HPV) in the host genome, leads to the overexpression of oncoproteins (E7 and E6). The protein E7 binds to retinoblastoma protein, leading to the increased expression of p16 (tumour suppressing protein). This overexpression can be used as a reliable marker for high-risk HPV infection (26). Sensitivity and specificity of P16INK4a expression in HR-HPV was 100% and 57%, respectively (29). This lack of specificity leads some authors to defend that identification of HPV DNA is fundamental (30). According to *Cubilla et al.*, positivity for P16INK4a in penile cancer has a strong correlation with the presence of HR-HPV (26). In the presence of a negative P16INK4a, infection with a low risk HPV genotype or absence of HPV infection can be suspected (24).

In the previously addressed metanalysis by *Olesen et al.* (16), 79.6% of HPV positive cases presented with a positive P16INK4a, while 18.5% of HPV negative cases also presented with positive P16INK4a. One of the explana-

tions for this variation and apparent incoherence may be the cut-off value used to consider a positive P16^{INK4a}, although *Olesen et al.* did not find a significant difference between different cut-offs (16). Two recent reviews identified a prevalence of HPV positive PSCC (identified by PCR amplification) of 33.1% (31) and 39.4% (32).
Nevertheless, various authors consider that a tumour can only be considered HPV positive if it presents with double positivity for HPV and P16^{INK4a} (16, 18, 33). In an interesting way, positivity for P16^{INK4a} correlates with the presence of high-risk HPV subtypes (HPV 16, HPV18 e HPV59) (18).
In this work there was no correlation between P16^{INK4a} and histologic subtype. In the case of Usual PSCC, 6 (21.4%) were P16^{INK4a} positive. Concerning Warty PSCC, Mixed PSCC (*Basaloid + Warty*) and Verrucous PSCC, the number of P16^{INK4a} positive patients was 1 (33.3%), 1 (50%) and 0 (0%) respectively. The last *World Health Organization* (WHO), divides PSCC in tumours related to HPV and non-related to HPV, as there may be prognostic importance in this division (19). Curiously, usual PSCC is identified as non-related with HPV (as are *Papillary*, *Verrucous*, *Sarcomatous* and others), although data collected from literature indicates a prevalence of HPV DNA in Usual PSCC of 32.2% and positive P16^{INK4a} of 36.9% (16). Our results and data from analysed literature, indicates that the classification Usual PSCC as independent of HPV is limited. In relation to Warty PSCC and mixed Basaloid-Warty PSCC (both classified as tumours related to HPV), literature indicates positivity for P16^{INK4a} in > 90% of cases (16). The low number of Warty and Mixed Basaloid-Warty PSCC in our series does not allow for sustained comparations, although they corroborate the limitations present on the suggest classification presented by the WHO.
In our work we did not directly study the presence of HPV, as such we cannot logically study the different HPV subtypes associated with PSCC. In developed and undeveloped countries, the predominant HRHPV associated with PSCC is HPV-16 as shown by several studies. Although uncommon in European countries, HPV-18 is the second most prevalent in PSCC in the World (22, 34). In the metanalysis conducted by *Olesen et al.*, HPV16 (68.3%), followed by HPV6 (8.1%%) and HPV18 (6.9%). were the predominant oncogenic subtypes (16).
In our study, we did not find any relation between P16^{INK4a} and other histologic characteristics. *Pone et al.* did not find a relation between P16^{INK4a} and other histologic characteristics (size, clinical stage, histological grade, or lymphatic or perineural invasion), although identified a relation with histologic subtype (24). Our series did not present with any Basaloid tumour, although literature indicates a relation between positive P16^{INK4a} and this histological subtype (26). Ferrándiz-*Pulido et al.* identified a connection between positive P16^{INK4a} and histological differentiation (P16^{INK4a} was associated with G3/4) and histological subtype (22). Some works distinguish between *penile epithelial neoplasia* (PEN) and PSCC in evaluating the importance of HPV and P16^{INK4a}, as most of PEN (> 70%) are HPV+. We decided not to exclude the single patient with PEN from our work, as positivity for P16^{INK4a} between PEN and PSCC are very similar (49.5% *vs.* 41.6%, respectively) (16). Analysing prognosis, we did not find, in this work, a significant statistical relation between P16INK4, DSF and CSS. Nevertheless, there is a clear trend for a better outcome in patients positive for P16^{INK4a} with only one patient presenting with recurrence and no case of disease related mortality. The absence of statistical significance is probably related with the low number of P16^{INK4a} positive tumours in our sample. Various works have studied the effect of HPV and P16^{INK4a} in the prognosis of PSCC. Regarding the effect HPV in DFS, *Afonso et al.* (112 patients, median follow-up of 20 months) and *Lorenzo et al.* (30 patients, median follow-up of 24 months) did not detect significant differences (35, 36). *Scheiner et al.* (72 patients) reported a better DFS at 5 years, although without statistical significance (37). Concerning the effects of HPV in CSS, in the review by *Sand et al.* (649 patients, 174 HPV+) a better CSS for patients HPV positive was noted (HR 0.61; 95% CI: 0.38-0.98) (32). Analysing the effects of HPV in Overall Survival (OS), studies did not show a significant relation (32, 38).
Tang et al. described a better DFS in patients positive for P16^{INK4a} (119 patients, 59 P16^{INK4a} positive, median follow-up of 30 months) (44). However, other works did not show a relation between P16^{INK4a} and DFS. The effect of P16^{INK4a} in CSS was studied by *Sand et al.* (review of 414 patients, 191 positive for P16^{INK4a}) with a HR of 0.45 (95% CI: 0.30- 0.69) for patients positive for P16^{INK4a} (32). The percentage of patients alive 4 or 5 years after diagnosis range from 69% to 100% for P16^{INK4a} positive and from 51% to 77% if P16^{INK4a} negative (22, 23, 29, 39-43). All the study-specific HRs are below 1 and ranging from 0.21 to 0.81, however only one (40) was statistically significant. Regarding OS, *Pone et al.* reported a better OS in patients positive for P16^{INK4a}, with a HR of 0.88 (95%CI: 0.49-1.59) (24). *Zargar-Shoshtari et al.* found that men with penile cancer positive for P16^{INK4a} had a significant better OS compared with negative P16^{INK4a} (HR = 0.33; 95% CI: 0.13-0.85) in a multivariable model adjusting for pathological nodal status, adjuvant chemotherapy and age (42). *Tang et al.* did not find a connection between P16^{INK4a} and OS (44). In this work, due to the discharge from follow-up of some patients and limitation in the quality of data collection outside our institution, we did not calculate OS.
In general, bibliography demonstrates a better survival for patients positive for HPV, as reported in other tumours related with HPV (vulvar, oropharyngeal) (32). *Sand et al.*, in the previously referred metanalysis, that analysed the HR of CSS of P16^{INK4a} and CSS of HPV positive patients, discovered that the HR of CSS P16^{INK4a} positive patients was lower than that of HPV positive patients. This could suggest that P16^{INK4a} expression may be a stronger predictor of CSS than HPV, similar to studies of neck and head cancer (32). The prognostic value of HPV is still uncertain. Some have suggested that the presence of a viral infection (HPV), might increase immune surveillance, making HPV positive cancer less aggressive than non-viral cancers (21).
In univariate analysis with other clinicopathological factors, a significant relation was found between T staging and N staging with DFS and CSS, while age > 65 years

presented with lower CSS. In multivariate analysis, only N staging correlated with survival. A work by *Wen et al.* (135 patients), reported a relation between N staging (clinical and pathologic) and CSS. In multivariate analysis, only pathologic N staging related with CSS (absence of relation between CSS and age, presence of phimoses, smoking, type of surgery, T stage or grade) (46).
Our study presented with some limitations. Our series presents a limited number of patients, with only eight P16^{INK4a} positive patients, which limits statistical results. In our work we did not evaluate the presence of HPV DNA, which can be relevant to corroborate the know connection between P16^{INK4a} status.
Lastly, due to the number present in our series, we did evaluate the influence of other factors that might influence prognosis, particularly the use of adjuvant or neoadjuvant therapies.

Conclusions

Penile cancer positive for P16^{INK4a} present with a trend for better outcome, although the most relevant factor is node stage. The probable prognosis importance of P16^{INK4a} corroborates the indication for its determination on penile cancer.

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