Prognostic value of p16\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\), as such p16\(^{\text{INK4a}}\) has been used as a surrogate of HPV infections.

Objective: To evaluate the prognostic factor of p16\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\). Relation between p16\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\) was positive in 8 patients (22.9%). Identification of p16\(^{\text{INK4a}}\) did not correlate with none of the histopathological factors. In this work we identified a better DFS and CSS in patients positive for p16\(^{\text{INK4a}}\) (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\(^{\text{INK4a}}\) is smaller than the one found in literature, which can suggest a less relevant part of HPV infection in the ocogenesis of penile cancer in the studied population. Identification of p16\(^{\text{INK4a}}\) did not relate with other clinicopathological factors. Tendency for a more favourable prognosis in patients with p16\(^{\text{INK4a}}\) agrees with results found in literature. The most relevant factor for prognosis is nodal staging.

Conclusions: penile cancer positive for p16\(^{\text{INK4a}}\) shows a trend for better survival, although the most relevant factor is nodal staging.

Key words: Penile cancer; HPV; p16; Prognosis.

Submitted 29 August 2019; Accepted 1 September 2019

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 socioeconomic level (12, 13) and multiple sexual partners (8).

In relation to penile cancer, HPV DNA was identified in 30-40% of cases, varies 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\(^{\text{INK4a}}\) in HPV infected cells, p16\(^{\text{INK4a}}\) expression can be used as a surrogate of active HPV infections (16). In cervical cancer and in other squamous cell carcinomas, expression of p16\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\) and mark a better cancer specific survival (CSS). However, the...
correlation between expression of p16\(^{\text{INK4a}}\) and HPV infection in penile cancer is still controversial (24).
The main aim of this paper is to evaluate the prognostic value of p16\(^{\text{INK4a}}\) expression in penile cancer. Other goals are to evaluate the epidemiological association between HPV and penile cancer in Portugal (indirect assessment by assessing expression of p16\(^{\text{INK4a}}\)) 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 reevaluated for this study.
The material was fixed in 10% formalin, embedded in paraffin and stained with haematoxylin-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\(^{\text{INK4a}}\) (mouse clone E6H4, ClINtec 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\(^{\text{INK4a}}\), the classification of Cabilla et al. (26) was adapted, and overexpression of p16\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\). 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\(^{\text{INK4a}}\) 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\(^{\text{INK4a}}\) immunoreaction**
The relation between p16\(^{\text{INK4a}}\) expression and the remaining clinicopathological results are summarized in Table 2. P16\(^{\text{INK4a}}\) immunoreaction did not correlate in a significant way (p > 0.05) with none of studied factors.

**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 ≤ 1 (p = 0.002) and N = 0 (p < 0.001).

| Table 1. Clinicopathological results. |
|-------------------------------|-----------------|
| Age (years) | N (%) |
| < 65 | 13 (37.1%) |
| > 65 | 22 (62.9%) |
| PSCC histologic subtype | |
| - Usual | 28 (80%) |
| - Noddy | 3 (8.6%) |
| - Verrucous | 2 (5.7%) |
| - Mixed Verrucous-Basaloid | 2 (5.7%) |
| Dimension (cm) | |
| < 4 | 26 (74.4%) |
| ≥ 4 | 9 (25.7%) |
| p16\(^{\text{INK4a}}\) | |
| - Positive | 8 (22.9%) |
| - Negative | 25 (72.0%) |
| 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) |
| ≥ 65                   | 6 (75.0) |
| PSCC histologic subtype|         |
| Usual                  | 6 (75.0) |
| Warthy                 | 1 (12.5) |
| Mucous                 | 0 (0.0)  |
| Mixed Warthy-Basaloid  | 1 (12.5) |
| Dimension (cm)         |         |
| < 4                    | 6 (75.0) |
| ≥ 4                    | 2 (25.0) |
| Differentiation grade  |         |
| G1                     | 4 (50.0) |
| G2                     | 3 (37.5) |
| G3                     | 1 (12.5) |
| Perineural invasion    |         |
| No                     | 7 (87.5) |
| Yes                    | 1 (12.5) |
| Lymphovascular invasion|        |
| No                     | 7 (87.5) |
| Yes                    | 1 (12.5) |
| T stage                |         |
| ≤ 1                    | 3 (37.5) |
| > 1                    | 5 (62.5) |
| Lymph node metastasis  |         |
| No                     | 8 (100.0)|
| Yes                    | 0 (0.0)  |
| Died of the disease    |         |
| No                     | 8 (100.0)|
| Yes                    | 0 (0.0)  |

only N stage presented with statically relevance (p = 0.017 and p = 0.014, respectively).

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 Cabilla 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 P16INK4a, although Olsen 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 P16INK4a (16, 18, 33). In an interesting way, positivity for P16INK4a correlates with the presence of high-risk HPV subtypes (HPV 16, HPV18 and HPV59) (18).

In this work there was no correlation between P16INK4a and histologic subtype. In the case of Usual PSCC, 6 (21.4%) were P16INK4a positive. Concerning Warty PSCC, Mixed PSCC (Basaloid + Warty) and Verrucous PSCC, the number of P16INK4a 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 P16INK4a 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 P16INK4a in > 90% of cases (16). The low number of Warty and Mixed Basaloid-Warty PSCC in our series does not allow for sustained comparisons, 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 Olsen 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 P16INK4a and other histologic characteristics. Pone et al. did not find a relation between P16INK4a and other histologic characteristics (size, clinical stage, histological grade, or lymphatic or perineural invasion), although literature indicates a relation with histologic subtype (24). Our series did not present with any Basaloid tumour, although literature indicates a relation between positive P16INK4a and this histological subtype (26). Ferrández-Pulido et al. identified a connection between positive P16INK4a and histological differentiation (P16INK4a 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 P16INK4a, as most of PEN (> 70%) are HPV+. We decided not to exclude the single patient with PEN from our work, as positivity for P16INK4a 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 P16INK4a, DSF and CSS. Nevertheless, there is a clear trend for a better outcome in patients positive for P16INK4a 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 P16INK4a positive tumours in our sample. Various works have studied the effect of HPV and P16INK4a 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 P16INK4a (119 patients, 59 P16INK4a positive, median follow-up of 30 months) (44). However, other works did not show a relation between P16INK4a and DFS. The effect of P16INK4a in CSS was studied by Sand et al. (review of 414 patients, 191 positive for P16INK4a with a HR of 0.45 (95% CI: 0.30 - 0.69) for patients positive for P16INK4a (32). The percentage of patients alive 4 or 5 years after diagnosis range from 69% to 100% for P16INK4a positive and from 51% to 77% if P16INK4a 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 P16INK4a, 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 P16INK4a had a significant better OS compared with negative P16INK4a (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 P16INK4a 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 P16INK4a and CSS of HPV positive patients, discovered that the HR of CSS P16INK4a positive patients was lower than that of HPV positive patients. This could suggest that P16INK4a 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 P16INK4a 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 P16INK4a 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 P16INK4a present with a trend for better outcome, although the most relevant factor is node stage. The probable prognosis importance of P16INK4a corroborates the indication for its determination on penile cancer.

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