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ORIGINAL ARTICLE

Nuclear loss and cytoplasmic expression of androgen receptor in penile carcinomas: role as a driver event and as a prognosis factor

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Abstract

Androgen receptor (AR) is a member of the steroid and nuclear family receptor that acts as transcription factor. AR signaling plays pivotal role in the development and progression of prostate cancer. However, the role of AR in penile cancer (PeCa) is poorly explored. Our previous molecular studies unveiled frequent *AR* mRNA loss in PeCa, which was further predicted as a major driver alteration in this neoplasm. Herein, we assessed the AR protein expression in 59 usual PeCa tissues and 42 surrounding normal tissues (SNT) by immunohistochemistry using a tissue microarray. In a paired analysis, we found a total absence of nuclear AR expression in PeCa while 95.2% of SNT samples presented strong nuclear AR expression ($P < 0.001$). Interestingly, 17 of 42 PeCa presented weak or moderate cytoplasmic AR staining, contrasting with 5 of 42 SNT ($P = 0.008$). Increased levels of AR cytoplasmic expression were related with poor prognosis features including advanced clinical staging ($P = 0.044$), compromised surgical margins ($P = 0.005$), and pathological inguinal node status ($P = 0.047$). Furthermore, AR cytoplasmic expression was also related with shorter overall survival ($P = 0.032$). In conclusion, the frequent loss of nuclear AR protein levels suggests a potential function in PeCa development. Based on this result, the androgen deprivation therapy is not indicated for PeCa patients. In addition, the AR cytoplasmic expression found in a significant number of cases (40.5%) showed prognostic value and pathways activated by the non-genomic AR signaling may represent a promising therapeutic strategy.

Keywords Penile carcinoma · Androgen receptor · Cytoplasmic AR expression · Immunohistochemistry

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Introduction

The molecular action of androgens is mediated by the androgen receptor (AR), an important steroid hormone receptor that plays a critical role in male sexual differentiation, development, and maintenance of male characteristics [1, 2]. The human AR is a soluble protein (110 kD composed by 919 amino acids) encoded by the *AR* gene mapped on Xq11-12 [3, 4]. In the canonical pathway, AR is activated in the cytoplasm through binding to androgenic hormones, as testosterone or dihydrotestosterone (DHT), leading to AR homodimerization and translocation into the nucleus [5].

In the nucleus, AR acts as a transcription factor, binding to specific DNA regions named AREs (androgen response elements), which are found in a large number of target genes in different cell types [5, 6]. In the cytoplasm, AR function is often referred as non-genomic signaling. While still in the cytoplasm, AR can bind to several proteins and activates SRC, RAS, MAPK, AKT, EGFR, and PI3K among others

[7, 8]. Non-genomic AR signaling promotes tumor cell survival, proliferation, migration, invasion, and metastasis [9].

In prostate cancer, AR overexpression has been found in epithelial cells during the development and progression of the disease [10, 11]. Androgen deprivation therapy to intercept AR signaling pathway is the standard treatment used for advanced prostate cancer patients [11]. However, despite an initial favorable response, almost every patient progress to a more aggressive, castrate-resistant phenotype. In other urological tumors, as in penile cancer (PeCa), the role of AR in the development and progression of the disease and its potential as a therapeutic target is unknown.

Penile cancer is a rare disease in developed countries; however, it is more common in poor regions [12]. The human papillomavirus (HPV) infection has been described in 22.2 to 63% of penile carcinomas [13–19], being the HPV16 the predominant type. The large range of the HPV positivity among different studies may be due to sampling methods, HPV detection procedures, histopathological subtypes, and population cohorts [20].

Molecular alterations in PeCa have been described only recently by our group and others [19, 21–30]. Interestingly, contrary to the findings described in breast and prostate cancers, *AR* mRNA is down-expressed in PeCa [19]. Moreover, *AR* alteration was pointed out as one of the main putative drivers in PeCa [28]. The molecular mechanism leading to AR down-expression in PeCa is poorly clarified. Nonetheless, X chromosome loss [31], overexpression of microRNAs targeting the *AR* gene [19, 28], and hypermethylation of the *AR* gene promoter [23] have been described as mechanisms leading to *AR* downregulation.

In this study, the expression of the AR protein was investigated in a cohort of penile carcinomas. To our knowledge, this is the first study reporting the protein AR expression levels in PeCa. Total loss of AR protein expression in nuclei of PeCa cells corroborates our previous results using mRNA expression analysis. In addition, we found that a weak/moderate AR cytoplasmic expression in tumor cells was significantly associated with poor prognosis.

Material and methods

Patients

Fifty-nine usual penile carcinomas (PeCa) and surrounding normal tissues (SNT) from 42 matched samples were obtained from patients who underwent tumor resection at Barretos Cancer Hospital, Barretos, SP, Brazil. All tumor samples were confirmed histologically as usual penile squamous cell carcinomas (SCC) or normal (non-neoplastic) tissues. Written informed consent was obtained from all patients. The Institutional Human Research Ethics Committee approved the study (Protocol 363-2010). The clinicopathological data are summarized in Table 1. In average, the follow-up was 43.1 months. Almost half of PeCa cases (49.2%) presented histological grade II and 30 cases (50.8) presented lymph node metastasis. Perineural invasion and surgical positive surgical margins were observed in 12 (21.3%) and 10 cases (18%), respectively. The p16 protein expression, which is a surrogate indicator of HPV infection [16], was also investigated. In 21 of 59 cases (with fresh frozen tumor samples available), the HPV status was also evaluated using the LINEAR ARRAY HPV Genotyping Test kit (ROCHE, Pleasanton, CA, USA).

AR protein expression analysis

Formalin-fixed paraffin embedded (FFPE) blocks containing representative samples of PeCa and SNT were histopathologically revised using hematoxylin and eosin (HE) stained slides. Three representative tissue cores (1 mm) from each sample (PeCa and SNT) were punched and arrayed on a recipient paraffin block (TMA arrayer MTA1, Estigen, Tartu, Estonia). The punches were taken from random regions of the samples. The sections were mounted on conventional slides with 3-aminopropyltriethoxysilane (Sigma-Aldrich Co.; St Louis, MO). The tissue microarray (TMA) also contained non-penile cancer tissues samples as controls of the reaction.

Immunohistochemical reaction (IHC) was carried out using Envision + Dual Link kit (Dako, Agilent, Santa Clara, USA). Heat-induced retrieval of antigen epitopes was performed in Dako Target Retrieval solution pH 9 using Dako PT LINK. AR monoclonal antibody (Clone AR441, Dako, USA) diluted at 1:50 was applied for 30 min of incubation. Negative (primary antibody replaced by phosphate-buffered saline (PBS) and positive controls (FFPE tissue section from AR-positive cases) were included in the analysis.

Nuclear AR staining was defined as positive (1) or negative (0), while AR cytoplasmic expression was scored semi-quantitatively. The cytoplasmic score used was the sum of the percentage of AR-positive cells (0: negative; 1: less than 25% of positive cells; 2: 26 to 50% of positive cells; and 3: more than 50% of positive cells) and the staining intensity (0: negative; 1: weak; 2: moderate; and 3: strong). Samples with scores 0–2 were considered negative (0) and scores 3–6 were considered positive (1). The analysis was carried out by two observers and the samples were scored blinded with respect to clinical patient data. In case of discrepant findings, a consensus score was used.

Statistical analysis

The IHC results of paired tumors and SNT were compared using McNemar's test. Clinical and histopathological parameters were associated with the AR cytoplasmic expression by Fisher's exact test, and the area under the receiver operating

Table 1 Clinical and histopathological features of 59 usual penile carcinomas

Clinical and histopathological features	Number
Average age (range)	60.5 years (25–92)
Follow-up (range)	43.1 months (0.3–120)
Histological grade	
I	20 (32.8%)
II	30 (49.2%)
III	9 (16.4%)
T (tumor size) stage	
T1–T2	37 (62.7%)
T3–T4	18 (30.5%)
ND	4 (6.8%)
Lymph nodes involvement	
N0	26 (44.1%)
N+	30 (50.8%)
ND	3 (5.1%)
Distant metastasis	
Yes	2 (3.4%)
No	49 (83.1%)
ND	8 (13.5%)
Perineural invasion	
Presence	12 (21.3%)
Absence	39 (65.6%)
ND	8 (13.1%)
Vascular invasion	
Presence	12 (19.7%)
Absence	37 (63.9%)
ND	10 (16.4%)
Surgical margins	
Positive	10 (18.0%)
Negative	47 (78.7%)
ND	2 (3.3%)
Type of surgery	
Partial penectomy	50 (84.7%)
Total penectomy	7 (11.9%)
Glandectomy	2 (3.4%)
Recurrence	
Yes	14 (24.6%)
No	37 (62.3%)
ND	8 (13.1%)
Death by disease	
Yes	24 (41.0%)
No	34 (57.4%)
Loss of follow-up	1 (1.6%)

SCC squamous cell carcinoma, *ND* not determined

characteristics curve (AUC) was estimated in relation to the pathological inguinal lymph node status. Overall and disease-free survival analysis were performed by Kaplan-Meier and log rank test (date of surgery as onset). Multivariate analysis was performed using Cox model with proportional risks to test independency of the variables on survival. The statistical analysis was performed using SPSS (v21.0; SPSS) adopting a two-tailed test P value < 0.05 as significant.

Results

PeCa presents negative nuclear AR expression and weak/moderate cytoplasmic AR expression

Nuclear (0 or 1 score) and cytoplasmic (0 or 1 score) AR protein expression levels were compared in 42 paired PeCa and SNT samples. Nuclear AR expression was found in 40 SNT samples (95.2%), while no nuclear expression was observed in all PeCa samples. AR nuclear immunostaining in PeCa presented reduced levels compared to paired SNT samples (P value = 2.5×10^{-10}) (Table 2). Cytoplasmic AR expression was detected in 40.5% (17/42) of PeCa samples and in five SNT samples (11.9%) ($P = 0.008$). The paired analysis demonstrated that PeCa samples presented negative nuclear immunostaining compared to SNT. However, when the cytoplasm compartment was evaluated, PeCa samples presented higher number of cases with weak/moderate AR expression (Table 2 and Fig. 1). The analysis of the remaining 17 PeCa unpaired samples confirmed these findings. Absence of nuclear AR expression was detected in all cases, and five PeCa samples (29.4%) presented weak/moderated cytoplasmic immunostaining.

Table 2 Percentage of cells with AR-positive nuclear or cytoplasm IHC score in 59 usual PeCa and 42 SNT samples

AR-positive IHC score			
PeCa samples	Number (%)	SNT samples	Number (%)
Paired cases	42	Paired cases	42
Nucleus*	0 (0)	Nucleus	35 (83.3)
Cytoplasm#	17 (40.5)	Nuclei and cytoplasm	5 (11.9)
Negative	25 (59.5)	Negative	2 (4.8)
Unpaired cases	17		
Nucleus	0 (0)		
Cytoplasm	5 (29.4)		
Negative	12 (70.6)		

AR androgen receptor, *SNT* surrounding normal tissues

40/42 SNT samples (95.2%) presented AR expression

* PeCa nuclear AR expression versus SNT nuclear AR expression—McNemar's test P value = 2.5×10^{-10}

PeCa cytoplasmic AR expression versus SNT cytoplasmic AR expression—McNemar's test P value = 0.008

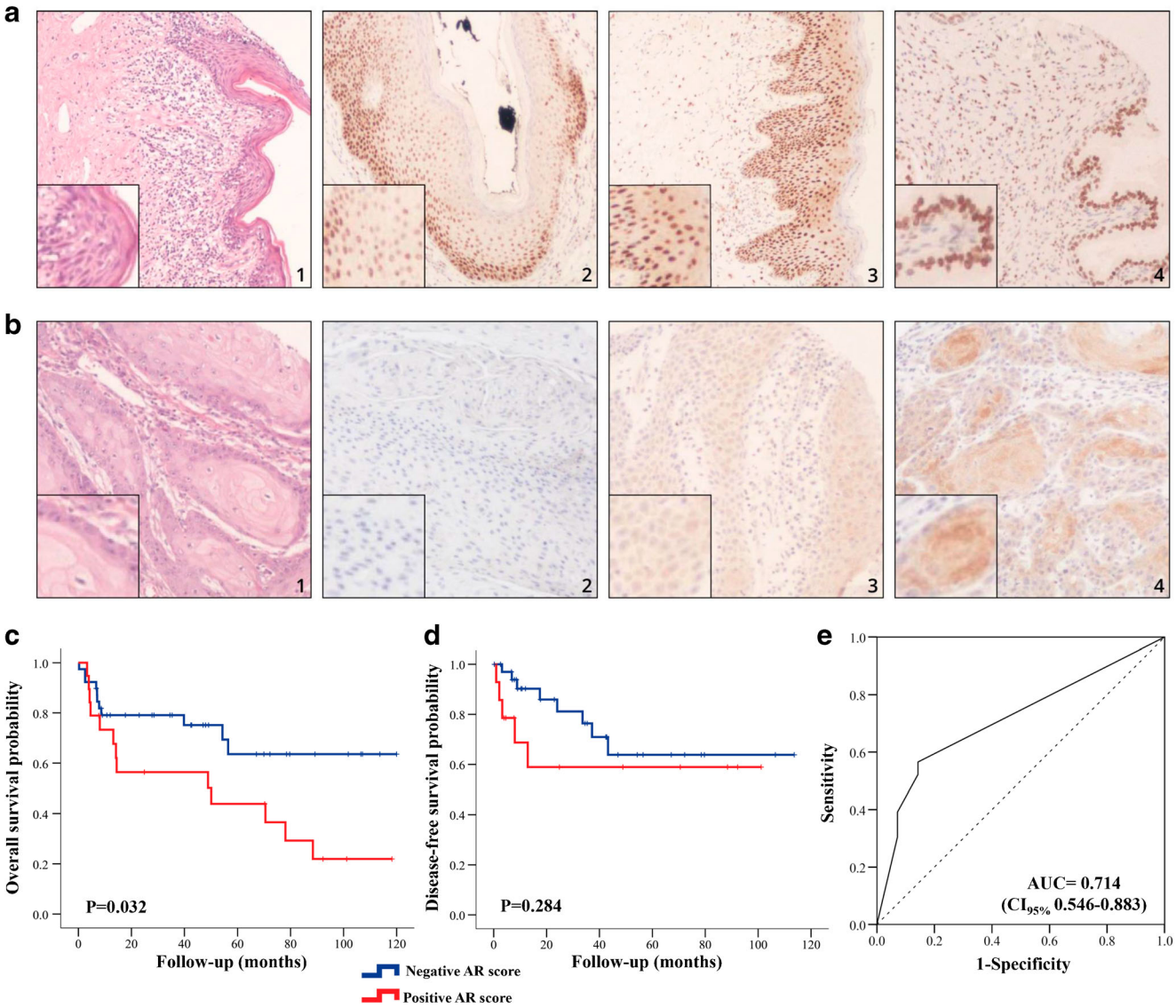

Fig. 1 AR protein expression evaluated by immunohistochemistry in surrounding normal tissues (SNT) (**a**) and in penile carcinomas (PeCa) samples (**b**). SNT (*a1*) and PeCa (*b1*) staining with hematoxylin-eosin. Nuclear AR positivity (*a2*) and both nuclear and cytoplasmic AR expression (*a3*). Human prostatic adenocarcinoma was a positive control (*a4*). PeCa tissues with no AR expression (*b2*), weak (*b3*), and moderate (*b4*) cytoplasmic expression (× 100 magnification). PeCa patients with cytoplasmic AR positivity showed significant shorter overall survival (**c**) but no differences were found in the disease-free survival (**d**). The ROC curve revealed that cytoplasmic AR scores (semi-quantitative scores based on percentage of positive cells and staining intensity ranging from 0 to 6) showed a potential power in predicting inguinal lymph node metastasis (**e**). SNT, surrounding normal tissues; Blue line, negative AR cytoplasmic expression; Red line, weak or moderate AR cytoplasmic expression; AUC, area under the ROC curve; CI95%, confidential interval of 95%

HPV status was not associated with AR expression levels

The HPV status was evaluated by p16 expression and genotyping. Among 59 PeCa, 18 cases were positive for p16 staining (30.5%) and no significant association was found between p16 and AR expression levels ($P = 0.766$, Table 3). By genotyping analysis, seven of 21 PeCa cases (33.3%) presented high-risk HPV (five cases HPV16+ and two HPV33+, Table 3). No association was detected between HPV status and AR expression levels ($P = 0.638$).

Relation of cytoplasmic AR expression with survival

The cytoplasmic AR scores (negative versus positive) were compared with clinical features using the 59 PeCa samples (Fisher's exact test). Advanced clinical stage ($P = 0.037$), compromised surgical margins ($P = 0.008$), clinical inguinal

Table 3 Androgen receptor cytoplasmic IHC score versus prognostic clinical features

Variables	AR score		*P* (Fisher's exact test)
	Negative (%)	Positive (%)	
p16 immunostaining (*N* = 59)			
No	27 (65.9)	14 (34.1)	0.766
Yes	13 (72.2)	5 (27.8)	
HPV genotyping (*N* = 21)			
No	10 (71.4)	4 (28.6)	0.638
Yes	4 (57.1)	3 (42.9)	
Grade			
I/II	35 (70)	15 (30)	0.450
III	5 (55.6)	4 (44.4)	
Tumor size			
T1/T2	25 (67.6)	12 (32.4)	1.000
T3/T4	12 (66.7)	6 (33.3)	
Clinical lymph node status			
cN0	21 (80.8)	5 (19.2)	*0.047*
cN1	16 (53.3)	14 (46.7)	
Pathological lymph node status*			
pN0	12 (85.7)	2 (14.3)	*0.035*
pN1	11 (47.8)	12 (52.2)	
Vascular invasion			
No	27 (73)	10 (27)	0.080
Yes	5 (41.7)	7 (58.3)	
Perineural invasion			
No	25 (64.1)	14 (35.9)	0.728
Yes	9 (75)	3 (25)	
Surgical margins			
Negative	36 (76.6)	11 (23.4)	*0.008*
Positive	3 (30)	7 (70)	
Distant metastasis			
No	31 (63.3)	18 (36.7)	0.551
Yes	1 (50)	1 (50)	
Clinical stage			
I/II	25 (78.1)	7 (21.9)	*0.037*
III/IV	10 (47.6)	11 (52.4)	

* Patients submitted to inguinal lymph node dissection

node status ($P = 0.047$), and pathological lymph node status ($P = 0.047$) were associated with cytoplasmic AR expression (Table 3). In addition, a lower overall survival was associated with cytoplasmic AR expression ($P = 0.032$) (Fig. 1C). The multivariate analysis revealed no significance ($P = 0.193$, data not shown) probably due to the small number of cases and events (24 of 59 patients died from the disease). AR cytoplasmic score presented a higher potential to predict lymph node metastasis (AUC = 0.714) than other clinicopathological features related to tumor aggressiveness, as vascular invasion (AUC = 0.705), perineural invasion (AUC = 0.639), surgical margins (AUC = 0.598), tumor grade (AUC = 0.512), and tumor dimension (AUC = 0.506).

Discussion

Androgens and its receptor are necessary for the normal development and maintenance of male genital tract organs, including the penis. AR expression in penile tissues suggests its role in the growth, differentiation, and maintenance of the epithelial cells [32].

Androgen receptor has been described as a major oncogenic driver in prostate cancer [9]. In penile tumors, the *AR* gene was one of the top 10 putative drivers involved in genomic loss and regulated by oncomiRs, which was a plausible explanation for the mRNA lower expression levels [28]. In parallel, we showed that *AR* is at least 30 folds down-expressed in PeCa compared to normal tissues giving additional support for the involvement of this gene in penile carcinogenesis [19]. Moreover, different studies showed *AR* genetic and epigenetic alterations. Using a panel of 236 cancer-related genes, Ali et al. [21] reported two *AR* mutations in 20 PeCa cases. A high-density genome-wide methylation arrays performed in 38 PeCa and 11 normal tissues revealed *AR* hypermethylation in penile tumors [23]. Mutations have been described to promote AR loss of function [10].

In the present study, none of the tumor samples presented nuclear AR expression, while almost every surrounding normal tissue presented nuclear AR expression. These findings suggest that during the process of dedifferentiation, AR nuclear expression is lost in penile cancer cells. Only two SNT samples (from patients with high-grade and advanced tumor stages, T4N3 and T2N3) presented absence of nuclear AR expression. Furthermore, five SNT cases presented both nuclear and cytoplasmic expression. Interestingly, the histopathological revision showed areas of penile intraepithelial neoplasia (PIN) in three of these five SNT samples.

Cytoplasmic AR positivity was observed in 22/59 PeCa samples (37.3%), suggesting an accumulation of AR in the cytoplasm during the tumorigenesis process. The accumulation of AR in the cytoplasm may be attributed to the presence of *AR* variants (AR-Vs) that do not translocate into the nucleus and may have exclusively cytoplasmic functions [33, 34]. Alternatively, AR mutations in the nuclear localization signal (NLS) reduces the binding affinity to importin-α (importin-α–importin-β complex transport AR to the nucleus), which results in import defects of AR into the nucleus [35–37].

The cytoplasmic expression of AR was correlated with poor prognosis features, including advanced clinical staging,

compromised margins, and inguinal metastasis. Analysis of AR protein expression by immunohistochemistry presented higher capability to predict lymph node metastasis compared with other clinicopathological features. By establishing a score equal or higher than three as a threshold, we were able to identify more than half of the patients presenting confirmed lymph node metastasis (52% sensitivity) with high specificity (86%). Markers that can be readily evaluated by IHC would be of great value to better select patients to inguinal node resection and adjuvant treatment. Although other markers have already been proposed [38, 39], none is currently used in the clinical practice.

We also observed that patients who presented AR cytoplasmic staining showed a lower overall survival ($P = 0.032$). Expression of AR splice variants, mainly the splice variant 7 (AR-V7), has been associated with poor response to androgen receptor inhibitors therapy in castration-resistant prostate cancer [40] and shorter survival compared with patients with normal levels of AR-Vs [41]. Although some AR-Vs are able to translocate into the nucleus, Guo et al. [42] found immunofluorescence staining of AR-V7 in both nucleus and cytoplasm. In our study, the antibody used for AR expression analysis corresponds to the amino acids 299-315 (N-terminal domain) in the sequence of the human AR (UniProt P10275). This monoclonal antibody allows the detection of several AR-Vs, but is not specific for any particular AR isoform.

In addition to the AR-V and potential mutations that may be a cause of AR cytoplasmic accumulation, AR also has a non-genomic signaling function that occurs mainly in the cytoplasm [7]. AR is able to activate kinase-signaling cascades, for example SRC, PI3K, and MAPK signaling pathways [9]. Considering that SRC, PI3K, and MAPK signaling inhibitors are clinically available, pharmacological inhibition of these AR non-genomic signaling pathways could offer additional therapies for PeCa patients with cytoplasmic AR expression. Interestingly, our previous reports in PeCa tissues [19, 26] and cell cultures [43] revealed the dysregulation of PI3K and MAPK signaling pathways.

In addition to the clinicopathological and morphological characterization, the penile SCCs were recently classified as HPV-related and non-HPV-related (2016 World Health Organization; [44]). Specific PeCa histological subtypes (basaloid and warty) are consistently associated with HPV positivity; while more keratinized subtypes, such as usual and verrucous SCCs, present lower HPV infection rates [45]. Although usual SSCs are frequently HPV negative, a subset of cases has been described as HPV positive. Recently, Alemany et al. [18] evaluated 1010 penile cancers from 25 countries and detected HPV DNA in 33.1% of PeCa, the proportion of HPV infection in non-warty-basaloid SCC was 14.7%. Miralles-Guri et al. [14] described HPV positivity in 76% of basaloid, 43.5% of keratinizing, and 24.5% of verrucous SCC. In agreement, several other studies have reported HVP positivity in keratinizing tumors, including the usual subtype [19, 46, 47].

Immunostaining for p16 protein is a practical alternative for HPV testing based on the high correlation between HPV detection and p16 overexpression [48]. The combined p16 immunostaining and HPV DNA PCR testing represent an attractive strategy for the reliable diagnosis of HPV-induced cancer (reviewed by Prigge et al. [49]). Using both procedures, we detected HPV positivity in 30.5% (p16 expression) and 31.3% (HPV genotyping) of cases. Our concordance rate among p16 immunostaining and viral DNA was 68%. According to Cubilla et al. [50], p16 overexpression presented 67% of sensitivity and 91% of specificity to define the HPV status. Considering only the IHC results, 18 of 59 cases were positive for p16 staining (30.5%) and no significant association was found between p16 and AR expression levels ($P = 0.766$). Although only usual PeCa were included in our set of cases, the major limitation of our study is the small sample size ($N = 59$). Furthermore, the presence of fresh tumor tissues was a limiting factor to investigate the presence of viral DNA ($N = 21$).

In conclusion, we found loss of AR expression in the nucleus of PeCa cells when compared to normal tissues. Cytoplasmic AR immunostaining was observed in a significant number of these cases and was related with poor prognosis and shorter overall survival. The presence of non-genomic AR signaling in the cytoplasm suggests a potential option for target therapy in PeCa. Although *AR* revealed to be an oncogenic driver in penile tumors, the mechanisms by which AR is retained in the cytoplasm of PeCa require further investigation.

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Compliance with ethical standards

The Institutional Human Research Ethics Committee (Barretos Cancer Hospital) approved the study (Protocol 363-2010).

Informed consent Written informed consent was obtained from all patients.

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

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