Original Article

Association of human papillomavirus in penile cancer: A single-center analysis

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ABSTRACT

Introduction: Human papillomavirus (HPV) is a known risk factor of penile cancer (PeCa). However, studies evaluating its true association are limited. In this study, we aimed to estimate HPV prevalence and its true association with PeCa in terms of molecular biological activities.

Materials and Methods: This single-institutional prospective observational study was conducted between June 2016 and August 2019. We included 40 men with PeCa as a study group and 20 age-matched uncircumcised men who underwent circumcision for phimosis as a control group. Both the groups underwent deoxyribonucleic acid isolation for HPV subtyping followed by evaluation of relative E6/E7 messenger ribonucleic acid (mRNA) expression profile and relative telomerase activity in tissue samples. HPV-16 and -18 were categorized as high-risk, whereas HPV-6 and -11 were categorized as low-risk subtypes.

Results: The mean (±standard deviation) age of PeCa was 51 ± 15.9 years. The majority of patients had stage II disease, and the most common procedure done was partial penectomy. The overall prevalence of HPV in PeCa was 42.5% ($n = 17$) as compared to 20% ($n = 4$) in controls. Among the subtypes, the most common subtype was HPV-16 noted in 33.3% (8/24) of cases, followed by HPV-18 in 29.2% (7/24) of cases. PeCa tissues had a significantly higher relative E7 mRNA expression for HPV-18 than the control group ($P = 0.016$). The mean relative telomerase activity was significantly higher in the PeCa tissues than the control group (138.66 vs. 14.46, $P < 0.001$). A significantly higher relative telomerase activity was noted in the PeCa tissues positive for high-risk HPV subtypes than controls (141.90 vs. 14.46, $P = 0.0008$), but not between high-risk HPV-positive and HPV-negative PeCa cases (141.90 vs. 137.03, $P = 0.79$). High-risk subtypes were not associated with tumor stage ($P = 0.76$) or lymph node metastasis ($P = 0.816$).

Conclusions: HPV was associated in 42.5% of PeCa cases based on our experience from a single institution. PeCa tissues had a higher relative E7 mRNA expression for HPV-18 and relative telomerase activity as compared to controls suggesting their potential role as surrogate markers of virus-induced tumorigenesis.

INTRODUCTION

Penile cancer (PeCa) is rare with an overall incidence of 0.69/100,000 men in the United States.[1] In contrast, the incidence is up to 10% of all cancers in the developing nations of Africa, South America, and Asia.[2] It is estimated to be 3.32/100,000 men in India.[3] PeCa is associated with poor hygiene, phimosis, smoking, and balanitis xerotica obliterans; however, it has a definite causal link with human papillomavirus (HPV) infection.[1]

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The reported prevalence of HPV infection in PeCa is about 48%.[4] However, the prevalence ranged widely based on the type of lesion with 22% in verrucous carcinoma to 66% in warty and basaloid subtypes.[4] Further, based on the probability to cause malignancy, HPVs have been classified into "high-risk" and "low-risk" serotypes. HPV-16 is the most common type detected in PeCa, followed by HPV-18 and belongs to "high-risk" serotype. Type-6 and -11 are found mostly in benign lesions but also in few PeCa including nonmetastatic verrucous carcinoma and are classified as low-risk serotypes.[5]

The viral oncoproteins E6 and E7 play an important role in PeCa pathogenesis by targeting p53 and retinoblastoma tumor suppressor proteins, respectively. In cervical cancer, researchers have shown that E6 and E7 messenger ribonucleic acid (mRNA) detection was strongly correlated with the severity of cytology and histology with >90% cervical intraepithelial neoplasia Grade 3 and all cervical cancers tested positive for carcinogenic HPV E6/E7 mRNA.[6] Further, a high HPV E6/E7 mRNA expression was associated with worse overall survival in cervical cancer.[7] Thus, the detection of E6/E7 mRNA expression may achieve better specificity and positive predictive value than detection with HPV deoxyribonucleic acid (DNA) testing.[6] However, studies evaluating the role of E6/E7 mRNA expressions in PeCa are lacking.

Although HPV is directly linked to PeCa, auxiliary signature of viral activity and induced carcinogenicity such as HPV-induced cellular transformation and markers of viral transcription should be used to determine the true association of viral infection rather than a transient infection or just a contaminant. Normal cells lack telomerase and progressively lose their end at each cell cycle leading to senescence and cell death. However, cancer cells have the ability to activate telomerase which can synthesize telomeric DNA. Analyzing the telomerase activity in the tissues using genes encoding telomerase suggests carcinogenesis.[8,9] Being a rare disease, studies on the role of HPV and its clinicopathological association in PeCa are limited. Further, literature evaluating the role of HPV activity markers in PeCa are lacking. In this background, we designed this study to evaluate the role of HPV in PeCa in terms of prevalence and its clinicopathological association and comparison of E6/E7 mRNA expression profile and relative telomerase activity with age-matched controls.

MATERIALS AND METHODS

Study design

This prospective observational study was done at a single tertiary care center between June 2016 and August 2019 after institute ethical board approval (IEC/349/6/2016). Men with biopsy-proven PeCa and planned for primary penile tumor surgery in our department were included as a study group. Men with prior therapy for PeCa were excluded from the study. Preputial tissues from age-matched uncircumcised men undergoing circumcision for phimosis were taken as a control group. Patient and tumor characteristics were recorded from hospital records and it included age, history of smoking, stage, differentiation, and primary treatment underwent.

Study objectives

The primary outcome measure of the study was to evaluate the tissue HPV positivity rate in PeCa patients and controls based on DNA isolation and HPV subtyping and its clinicopathological association with respect to TNM staging. Secondary outcome measures were to compare the relative E6/E7 mRNA expression of high-risk HPV subtypes and relative telomerase activity between the two groups.

Workup of patients and surgical procedures

All men with penile tumor underwent a thorough physical examination including the assessment of primary tumor and inguinal examination. All patients had routine blood investigations including serum calcium levels. Patients then underwent penile biopsy under local anesthesia as a day care procedure to know the histopathological diagnosis. Metastatic workup included a contrast-enhanced computerized tomography (CECT) of the abdomen and pelvis to rule out pelvic and retroperitoneal lymph node metastasis. All men underwent chest X-ray, and in doubtful cases, a CECT chest was done to rule out lung metastasis. The AJCC 8th edition was used for TNM staging.[10] Men with no tumor and/or induration of proximal penis for a length of ≥2 cm from the root of penis underwent partial penectomy. A total penectomy with perineal urethrostomy was done in men with tumor involvement of proximal penis or only <2 cm length of normal penis from the root of penis. Men with ≥T2, N1, N2 underwent radical inguinal lymph node dissection as per the current international guidelines.[11]

HPV DNA isolation and typing

The tissue biopsy weighing approximately 25 mg was excised and DNA eluted using a DNeasy Qiagen kit. The purity and integrity of DNA was checked by a NanoDrop 1000 spectrophotometer and agarose gel electrophoresis. HPV typing and estimating copy number for HPV-6, -11, -16, and -18 were done using the kit purchased from Genome Diagnostics Private Limited, India. This kit contained the master mix for the real-time polymerase chain reaction (PCR) and standards for the absolute quantification of HPV subtypes in the DNA samples [Figure 1].

RNA extraction and cDNA preparation for E6/7 mRNA expression levels

The RNA was isolated from the tissue biopsy using TRIzol isolation method. The cDNA was prepared using the isolated RNA, which was used for the study of relative mRNA expression of tissue through real-time PCR.

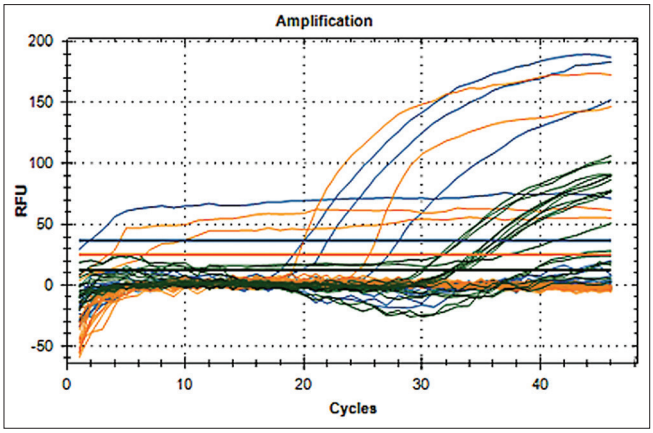

Figure 1: The graphical representation with peaks for complete graph including HPV-16 (FAM) (blue curves), HPV-18 (Rox) (orange curves, and inhibitor controls IC (Hex) (green curves) during the real-time PCR of the DNA samples of Ca penis patients using the HPV-16 and -18 reagents supplied with the kit. HPV: Human papillomavirus, PCR: Polymerase chain reaction, DNA: Deoxyribonucleic acid

Relative telomerase activity

The tissue lysate from the biopsies was used to measure the telomerase activity in the patients and controls. Telomerase activity was assayed using the TeloTAGGG Telomerase PCR enzyme-linked immunoassay (ELISA) detection kit (Roche Applied Science) according to the manufacturer's instructions. Briefly, tissue was chopped using a cryo-cutter and resuspended in cell lysis buffer without the protease inhibitor cocktail and incubated for 30 min at 4°C. Then, the samples were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was aliquoted and the total protein concentration was measured using Bradford reagent. One microgram of total cell lysate containing telomerase was used for telomerase activity measurement. The telomeric repeats (TTAGGG) to the 3'-end of the biotin-labeled synthetic P1-TS-primer were added to the cell lysate. The resulting products were immobilized via the biotin label to a streptavidin-coated microplate. Immobilized amplicons were then detected with an antibody against digoxigenin that was conjugated to horseradish peroxidase (Anti-DIG-HRP) and the sensitive peroxidase substrate TMB. Finally, the absorbance was taken at 450 nm using a microtiter plate (ELISA) reader.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation and compared with Student's *t*-test. Categorical variables were expressed as frequency (percentage) and compared with Chi-square test or Fisher's Exact test. Statistical analyses were performed using Stata®, version 14.0 software (StataCorp, College Station, Texas, USA) and GraphPad Prism software, version 3.1 (GraphPad software, San Diego, CA).

RESULTS

Table 1 describes the baseline clinicopathological characteristics. A total of 40 men with PeCa and 20 controls were included during the study period. The mean age of the PeCa patients was 51 ± 3.2 years, which was comparable with 43 ± 3.7 years of the control group. All men with PeCa had normal blood parameters including serum calcium level.

Table 1: Baseline clinicopathological characteristics

Parameters	Case (*n*=40)	Control (*n*=20)	*P*
Age in years*	51±15.9	43.1±12.2	0.06†
History of smoking	15 (37.5)	6 (30)	0.57‡
Serum calcium (mg/dL)*	9.7±0.4	8.9±0.4	0.001†
N stage			
N0	8 (20)	-	-
N1	7 (17.5)		
N2	24 (60)		
N3	1 (2.5)		
AJCC staging			
Stage 0	6 (15)	-	-
Stage I	6 (15)		
Stage II	14 (35)		
Stage III	5 (12.5)		
Stage IV	9 (22.5)		
Tumor differentiation			
Well (G1)	14 (35)	-	-
Moderate (G2)	23 (57.5)		
Poor (G3)	3 (7.5)		
Undifferentiated (G4)	-		
Primary treatment			
Circumcision	1 (2.5)	20 (100)	-
Glansectomy	1 (2.5)	-	
Partial penectomy	32 (80)	-	
Total penectomy	6 (15)	-	

All values are expressed as n (frequency), except *which is expressed as mean±SD. †Student *t*-test was used to test for the significance, ‡Chi-square or Fisher's exact test was used to test for the significance. T: Tumor, N: Nodal, AJCC: American Joint Committee on Cancer, SD: Standard deviation

Among 40 PeCa patients, 32 (80%) and 6 (15%) men underwent partial penectomy and total penectomy, respectively. A patient with tumor in the glans underwent glansectomy. However, the final histopathology showed a positive margin and subsequently underwent partial penectomy. Another patient with lesion limited to prepuce required only circumcision.

Prevalence of HPV subtypes

Among 40 tissue samples from PeCa patients, 8 (20%) were positive for HPV-16, 7 (17.5%) were positive for HPV-18, and 11 (27.5%) were positive for either HPV-16 and -18 subtypes. Similarly, 5 (12.5%) were positive for HPV-6, 4 (10%) were positive for HPV-11, and 6 (15%) were positive for either HPV-6 or -11 subtypes [Table 2].

The prevalence of HPV in our PeCa cohort was 17/40 (42.5%), with the most common serotype being HPV-16 in 33.3% (8/24), followed by HPV-18 in 29.2% (7/24) of cases. Importantly, 17.5% (7/40) of men with PeCa had multiple infections. Among them, 4/40 (10%) were positive for both HPV-16 and -18, whereas 3/40 (7.5%) were found positive for both HPV-6 and -11.

In the control group, 2/20 (10%) men were positive for any high-risk HPV types (16 or 18) and another 2/20 (10%) men were positive for any low-risk HPV types (6 or 11) [Table 2].

E6/E7 mRNA expression profile

The relative E6/E7 mRNA expression profiles for HPV-16 and -18 between PeCa cases and controls are shown in Figure 2. We noted a significantly higher expression of E7 mRNA for HPV-18 in PeCa cases as compared to the controls ($P = 0.016$). The relative E6/E7 mRNA expression for HPV-16 was higher in PeCa cases than controls; however, the difference was statistically insignificant.

Relative telomerase activity

On comparing the relative telomerase activity, PeCa cases had a significantly higher mean telomerase activity (138.66 vs. 14.46, $P < 0.001$) than controls. A higher relative telomerase activity was also noted in the PeCa tissues positive for high-risk subtypes than controls (141.90 vs. 14.46, $P < 0.0008$) [Table 3]. We also noted that the mean telomerase activity increased from 114.1 (range, 9.3–219.4), 135.5 (range, 62.1–221.1) to 176.2 (range, 98.2–236.1) in well, moderate, to poorly differentiated PeCa, respectively.

Table 2: Prevalence of human papillomavirus subtypes in the study and control groups

HPV risk groups	HPV subtypes	Cases (*n*=40), *n* (%)	Controls (*n*=20), *n* (%)	*P**
High-risk HPV types	HPV-16	8 (20)	1 (5)	0.25
	HPV-18	7 (17.5)	2 (10)	0.70
	HPV-16 or -18	11 (27.5)	2 (10)	0.19
Low-risk HPV types	HPV-6	5 (12.5)	1 (5)	0.65
	HPV-11	4 (10)	1 (5)	0.66
	HPV-6 or -11	6 (15)	2 (10)	0.70

All values are expressed in frequency (%). *Chi-square or Fisher's exact test was used to test for the significance. HPV: Human papillomavirus

Clinicopathological features

According to the TNM staging, 6, 6, 14, 2, 3, and 9 men belonged to stage 0, I, II, IIIA, IIIB, and IV, respectively. Stratified by the stages, 1/6 (16.7%), 1/6 (16.7%), 5/14 (35.7%), 2/5 (40%), and 2/9 (22.2%) were positive for either HPV-16 or -18 from stages 0, I, II, III, and IV, respectively [Table 1].

Lymph node dissection was performed in 35 patients. Among them, pathological lymph node positive was found in 14 (40%) men. In patients with pathologically lymph node-positive status, 4 (28%) men were positive for HPV-16 and 18 in the primary lesion. Similarly, in patients with pathologically lymph node-negative status, 8 (38%) men were positive for HPV-16 or 18 in the primary lesion. We noted an insignificant association between high-risk HPV positivity with different stages ($P = 0.76$) or lymph node metastasis in PeCa patients ($P = 0.816$).

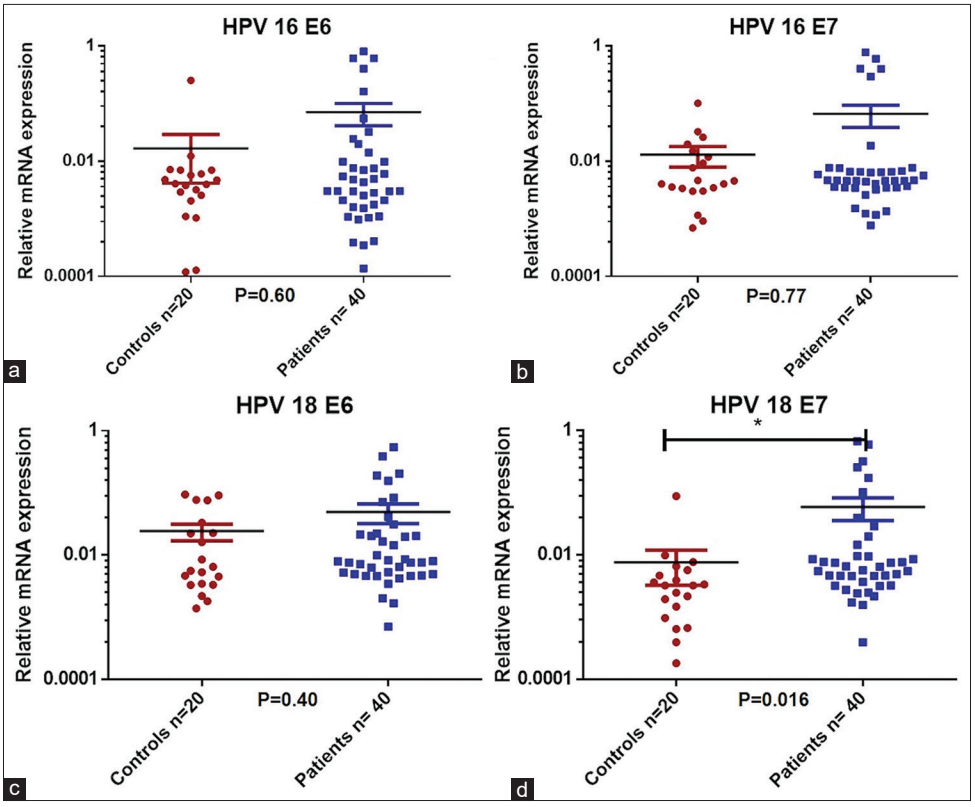

Figure 2: The graphical representation of relative E6/7 mRNA expression between PeCa and control tissues for HPV-16 (a and b) and HPV-18 (c and d). mRNA: Messenger ribonucleic acid, PeCa: Penile cancer, HPV: Human papillomavirus

Table 3: The relative telomerase activity between study and control groups

Comparison groups	Cases	Controls (*n*=20)	*P**
Cases (*n*=40) versus controls†	138.66 (9.36-236.14)	14.46 (4.37-21.77)	0.0001
High-risk HPV cases (*n*=11) versus controls‡	141.90 (9.63-221.01)	14.46 (4.37-21.77)	0.0008
High-risk HPV cases (*n*=11) versus HPV-negative cases (*n*=29)§	141.90 (9.63-221.01) versus 137.03 (9.36-236.14)		0.79

All values were expressed in mean (range). *Student *t*-test was used to test for the significance. $P < 0.05$ signifies statistical significance, †Comparison of relative telomerase activity between cases and controls, ‡Comparison of relative telomerase activity between high-risk HPV cases and controls, §Comparison of relative telomerase activity between high-risk HPV-positive and HPV-negative cases. HPV: Human papillomavirus

DISCUSSION

PeCa arises from precursor lesions caused by HPV infection, in a stepwise progression. After infection, subsequent epigenetic alterations are essential for an HPV-infected cell to turn completely malignant. The oncoprotein E6 binds and targets the tumor suppressor proteins p53 and PDZ domain proteins for proteosomal degradation, while E7 inactivates retinoblastoma tumor suppressor protein and leads to uncontrolled cellular proliferation.[12] While there is enough evidence to support that the HPV plays a major role in the carcinoma cervix and oral cancers, studies evaluating the role HPV in PeCa are scarce because of the rare occurrence of this malignancy. The reported prevalence of HPV in PeCa in the literature is varied depending on the geography, HPV subtypes evaluated, and the different techniques of DNA isolation.

The prevalence of HPV coinfection in PeCa from this single-institutional study was 42.5%. PeCa tissues had a significantly higher relative E7 mRNA expression for HPV-18 than the control group. We also found a significantly higher relative telomerase activity in the PeCa tissues and in high-risk HPV subtypes as compared to the control group.

The prevalence of HPV in PeCa was estimated to be 15%–71% depending on the detection techniques and HPV subtypes.[13] The HPV prevalence of 42.5% in our study was lower than a retrospective study from Argentina with a similar sample size.[14] Yet, our estimation was consistent with two large systematic reviews, which showed the prevalence from different countries. By estimating the data from Asia, Europe, and North and South America, Miralles-Guri *et al*.[15] and Backes *et al*.[4] reported the prevalence of HPV in PeCa to be 47.9% and 46.9%, respectively. Of note, the prevalence from our single-institutional study was lower compared with the prevalence of 55.2% to 59.3% for the Asian subpopulation mentioned in the systematic reviews.[4,15] Japan, China, Vietnam, and Thailand were the Asian countries from which the data were analyzed. Small sample size could be the possible explanation for lower prevalence in the present study. However, it would be interesting to know any geographical variances and circumcision practices exist within countries from the same continent and further studies should focus on it.

In contrast to the above, a recent study by Alemany *et al*. reported only 13.4% HPV prevalence in Asian cohort.[2] This study utilized the LiPA25 detection system for HPV detection as compared to a variety of PCR primers used for the HPV DNA detection in those systemic reviews. Although there is no literature available comparing various detection techniques of HPV DNA, the different molecular techniques used in these studies along with differential circumcision practices across countries could have resulted in low detection.[2]

In the present study, a significantly higher E7 mRNA expression for HPV-18 in PeCa cases suggests that HPV was active and involved in PeCa tumorigenesis. This signifies the direct causation and not a mere transient infection. Hence, E7 mRNA expression may serve as a surrogate marker of true association of high-risk HPV in the PeCa tissues. Although statistically insignificant, other mRNA expression profiles (HPV-16 E6/7) in our study were higher in PeCa cases than controls. Further studies with large samples may establish the exact difference. de Boer *et al*. showed a 96% HPV E6/E7 mRNA expression in stage Ib and IIa cervical cancer women.[7] Although they did not find any correlation between DNA copy number and the level of HPV E6/E7 mRNA expression, cervical cancer patients with high HPV E6/E7 mRNA expression had worse survival.[7] In context to PeCa, Alemany *et al*. studied the role of E6*I mRNA transcript in a series of invasive PeCa (n = 1010) and high-grade squamous intraepithelial lesions (n = 85) from 25 countries. In their study, mRNA assay was done for a total of 20 HPV serotypes and found that HPV E6*I mRNA detection in high-risk types was high in both penile high-grade squamous intraepithelial lesions (97.1%) and in invasive PeCa (85.1%), suggesting HPV E6*I mRNA as an additional marker of viral activity and not a mere transient infection.[2]

As a marker of immortality in carcinogenesis, telomerase activity denotes the ability of the telomerase to maintain the length of the telomere such that cancer cells will keep continuing to proliferate. Alves *et al*. showed a 85.4% telomerase activity in PeCa and the frequency of activity increased from 58.3%, 94.2%, to 100% in well, moderate, to poor differentiated PeCa, respectively.[8] In the current study, we noticed a higher relative telomerase activity in the PeCa tissues and in high-risk HPV subtypes as compared to the control group. Further, the mean telomerase activity increased from 114.1, 135.5, to 176.2 in well, moderate, to poorly differentiated PeCa, respectively.

In our study, we did not notice an increased association of high-risk HPV types with increasing TNM stages of PeCa. Furthermore, there was no significant association of lymph node metastasis with high-risk HPV-positive or HPV-negative status. Bezerra *et al.* did not find a significant association between HPV-negative and HPV-positive groups when considering lymph nodal metastasis ($P = 0.386$) and 10-year survival rate ($P = 0.83$).[16] Similarly, Wiener *et al.* found that 31% of tumors were positive for high-risk HPV types in 29 men with invasive PeCa and showed that even after adjustment for tumor stage, there was no difference between men with HPV-negative and HPV-positive status in terms of nodal metastasis.[17]

The present study is our initial attempt to evaluate the role of HPV and its true association in terms of molecular biological activities in PeCa cases. This maiden study is among the few studies in the literature in Indian subcontinent evaluating the role of E6/7 mRNA expression profiles and telomerase activity in PeCa. However, the study is not without limitations. Apart from the small sample size, we also did not evaluate the extensive panel of HPV subtypes for the estimation of HPV prevalence, follow-up data, and survival analysis. However, we primarily aimed to study the association of HPV in PeCa in terms of molecular biological activities. Yet, we believe that our data on HPV activity markers may provide some rationale in considering routine male public HPV vaccination to possibly reduce the prevalence of PeCa at least in the Indian subcontinent. Further larger sample size is needed to validate the current findings.

CONCLUSIONS

Our single-institutional study showed that PeCa was commonly related to HPV infection, with HPV-16 being the most common subtype. Further, the relative E7 mRNA expression for HPV-18 and the relative telomerase activity were higher in PeCa patients, suggesting their role as potential surrogate markers of viral activity.

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