Expression of PD-L1, CD 8, P16, and HPV in Penile carcinoma and their correlation with clinicopathological parameters

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सेवा में,

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Subject: Submission of M.D thesis.

आदरणीय महोदय,

This is to submit that the M.D thesis by the Academic Junior Resident (July 2020 Batch) of our Department ~~have~~ has been duly completed and signed and is ready for submission. Please accept the same.

Details of her thesis are attached herein:

Name of candidate	Thesis topic
Dr. Deepsikha Bhanja	EXPRESSION OF PD-L1, CD 8, P16, AND HPV IN PENILE CARCINOMA AND THEIR CORRELATION WITH CLINIC-PATHOLOGICAL PARAMETERS.

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डॉ. पूनम ऐलेन्स
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DECLARATION

I hereby declare that the thesis titled **"Expression of PD-L1, CD 8, P16, and HPV in Penile carcinoma and their correlation with clinico-pathological parameters"** embodies the original work carried out by the undersigned in All India Institute of Medical Sciences, Jodhpur.

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CERTIFICATE

This is to certify that the thesis titled **"Expression of PD-L1, CD 8, P16, and HPV in Penile carcinoma and their correlation with clinico-pathological parameters"** is the bonafide work of **Dr. Deepsikha Bhanja** carried out under our guidance and supervision, in the Department of Pathology and Lab medicine, All India Institute of Medical Sciences, Jodhpur.

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"Arise, awake and stop not till the goal is reached." – Swami Vivekananda

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SYNOPSIS

Penile Carcinoma is predominantly found in developing countries. Human Papilloma Virus(HPV) infection and p16,a surrogate marker of HPV, PD-L1, an important molecule responsible for immune evasion and CD8 infiltration has been used to evaluate tumour activity and prognosis. As the studies are lacking in developing countries, so we planned this study to examine any possible ethnic variation of tumour microenvironment and to guide future personalized immunotherapy.

This ambispective observational study included 42 patients small biopsies, partial and total penectomy specimens of diagnosed cases of penile carcinoma. After reviewing H&E slides, IHC markers of PD-L1, CD8, HPV, and p16 were applied to the representative slide and correlated with assessed clinicopathological parameters. (61.9%) of all patients were of 65 years or more in age and 71.4% of all patients were addicted to smoking tobacco. Commonest (61.9%) surgery undergone by patients was a partial penectomy, followed by a small biopsy (26.2%). Glans was the commonest site involved in more than half (52%) of patients, followed by shaft of penis (11.9%) and around one fourth (28.6%) had multifocal involvement. One third of all tumors were well differentiated and 64.3% were moderately differentiated. Evaluation of pathological staging revealed majority of patients to be in (38%) pT2-pT4. Median size of tumors (excluding cases of small biopsy) was 26.25 cubic centimeter with a interquartile range of 6.07 cm^3 and 58.3 cm^3. Mean depth of invasion was 2.66 cm with SD of 1.41cm. Regarding IHC findings, PD-L1, HPV and p16 were positive in 54.8%, 31% and 26.2% cases respectively. Mean CD8 score in IHC study was 39.17 with a SD of 20.78%.

This study underscores the need for evaluation of PDL-1 and its correlation with tumour infiltrating lymphocytes by CD8. The tumour histology correlating well with presence of HPV by HPV and p16 immunohistochemistry indicating p16 as the surrogate marker for HPV.

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ARB	Antigen Retrieval Buffer
CAP	College of American pathologists
CD	Cluster of Differentiation
CDK	Cyclin Dependent Kinase
CI	Confidence Interval
CSS	Cancer Specific Survival
DAB	Diaminobenzidine
DC	Dendritic Cells
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FOX	Fork head box protein
GLOBOCAN	Global Cancer Observatory
H&E	Haematoxylin and eosin
HPV	Human papillomavirus
HCl	Hydrochloric acid
HR	High Risk
LR	Low Risk
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IQR	Inter Quartile Range
IRF9	Interferon regulatory factor 9
ISH	In situ hybridization
LNM	Lymph Node Metastasis
LVI	Lymphovascular Invasion
MHC	Major Histocompatibility Complex
NK	Natural Killer
NOS	Not Otherwise Specified
PC	Penile Carcinoma
PCR	Polymerase Chain Reaction
PD 1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1

PeIN	Penile Carcinoma In Situ
PeSCC	Penile Squamous Cell Carcinoma
PLL	Poly-L-Lysine Solution
PNI	Peri neural Invasion
PUVA	Psoralen and Ultra Violet A
Rb	Retinoblastoma
SCC	Squamous Cell Carcinoma
SD	Standard Deviation
TCR	T Cell Receptor
TIL	Tumour Infiltrating Lymphocytes
TRIS	Tris(hydroxymethyl)aminomethane
V/V	Volume/Volume
WHO	World Health Organization

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INTRODUCTION

Penile carcinoma is a rare disease and most commonly found in developing countries(1). Indeed, the rates of penile carcinomas have been reported to represent up to 10% of men's malignant disease(2). Although penile carcinomas can present in younger males, it generally remains a disease of the elderly, with a mean age of diagnosis is around 60 years(3). It has been well established that penile squamous cell carcinoma (SCC) can arise from chronic inflammatory conditions and infections with the human papillomavirus (HPV)(1). In 2016, WHO and the International Society of Urological Pathology stratified the histological classification of penile SCC as Usual HPV related vs. Non-HPV-related neoplasms(4).

In urban India, the age-adjusted incidence of penile carcinoma ranges from 0.7-2.3 cases /100,000 men, accounting for more than 6% of all malignancies(5). Fu et al suggested, an estimated age- standardized incidence of penile cancer worldwide to be 0.80 per 100,000 person-years in 2018, and the incidence predicted to increase by more than 56% by 2040, as per the global cancer registries (GLOBOCAN) cancer tomorrow prediction tool(68). Incidence varies from 0.7-2.3 cases per 100,000 men in urban India and 3 cases per 100,000 men in rural India(23).

Approximately 60-100% of penile intraepithelial lesions are HPV DNA positive(6). HPV DNA is detectable in about 50% of all penile cancer in India(7). The two most important risk factors for penile cancer are HPV infection and phimosis, and the risk increases with the number of sexual partners, a history of genital warts, and concomitant sexually transmitted disease. HIV infection, poor hygiene, smegma, balanitis, phimosis, paraphimosis, lichen sclerosis, immunosuppression, and PUVA treatment(1). The estimated overall prevalence of HPV in penile cancer is 42% to 48%, with the most commonly involved HPV subtypes being HPV 16

and 18 (8). HPV DNA has been found in approximately 90% of dysplasia cases and 100% condylomata of the penis(9). More than 95% of penile squamous cells originate from the glans, preputium, or sulcus coronaries(3). Among HPV, basaloid type is most common in America(10).

From the diagnostic view, most of the penile lesions can be classified using H&E stain and IHC. The molecular analysis may help in challenging cases. Patients with HPV positive neoplasms have a prognosis better than HPV negative neoplasms, although this fact is not clear in case of penile cancer(11,12). So, the identification of the virus in the tumour tissues becomes essential factor.

HPV is a common cause of penile SCC and can be diagnosed by tumour histology and confirmed by over expression of p16 on IHC. It is recommended that immunohistochemical staining for p16 be utilized as a surrogate indicator of HPV. The p16 immunohistochemistry can be used as an indicator of HPV and a prognostic marker of squamous cell carcinomas at various sites(13,14).

Programmed death-ligand 1(PD-L1) is a co-inhibitory molecule that impairs the T-cell response by downregulating T-cell proliferation and cytokine production(15). Tumour cells often upregulate PD-L1 and thereby evade the host immune system(16). Notably, PD-L1 expression has been seen in squamous cell carcinoma of the head and neck, and skin(17). In response to malignancy, the host immune system engages various immune cell types and cell signalling pathways. Tumour infiltrating lymphocytes (TILs) are immune cells recruited as a defence mechanism against tumour cells. Increased TILs have been frequently correlated with a favourable prognosis in melanoma and solid tumours like ovarian and colorectal cancers(18,19). Effector CD8 (killer)T-cells primarily responsible for most anti-tumour activity(20). CD8 (killer)T-cells density in tumour tissue has been correlated with anti-tumour immunity and inversely correlated with disease severity(21).

Although penile carcinoma is more common in developing countries, data from India is scarce. So, in this study we evaluated incidence of PDL1, p16, HPV and density of CD8 in histopathologically confirmed tissues of penile carcinoma and correlated them with clinicopathological parameters.

REVIEW OF LITERATURE

Penile cancer represents 20-30% of all cancers diagnosed in men who live in Asia, Africa, and South America(3). In urban India, the age-adjusted incidence of penile cancer ranges from 0.7-2.3 cases per 100,000 men. In rural India the rate of penile cancer is 3 cases per 100,000 men(22,23).

Risk Factors

Uncircumcised men develop penile carcinoma more frequently than those who have had early circumcision(24). The risk for penile cancer is 3.2 times greater among men who were never circumcised(25). Male circumcision is associated with a reduced risk of penile HPV infection(26). The incidence of SCC arising in the setting of long-standing lichen sclerosus constitutes significant case burden(28).

A significant association of lichen sclerosus with special (usually HPV- unrelated) variants of SCC such as usual, pseudohyperplastic, verrucous, and papillary SCCs has been demonstrated in the WHO 2016 fourth edition(1).Occasionally, hyperplasia of basal cells may be noted, especially in association with the basaloid variant of SCC(10).

Penile SCCs as classified by WHO classification of urinary male genital tumours, 2022 (29):-

Precursor lesions (lesions are not graded; all are considered high-grade) Penile intraepithelial neoplasia, HPV-associated

Common patterns: basaloid (undifferentiated) and warty (condylomatous, Bowenoid)
Other (less frequent) patterns: pagetoid and clear cell
Differentiated penile intraepithelial neoplasia, HPV-independent
Invasive carcinoma
HPV-associated squamous cell carcinoma
Subtypes:
Basaloid
Warty
Clear cell Lymphoepithelioma-like Mixed
HPV-independent squamous cell carcinoma
Subtypes:
Squamous cell carcinoma, usual type (includes pseudohyperplastic and pseudoglandular)
Verrucous carcinoma (includes carcinoma cuniculatum)
Papillary
Sarcomatoid carcinoma
Mixed
Squamous cell carcinoma NOS (invasive keratinizing carcinoma without special features, for which evaluation of p16 is not available)
Adenosquamous carcinoma
Mucoepidermoid carcinoma

PATHOLOGIC STAGE CLASSIFICATION (pTNM, AJCC 8th Edition)(30)

pT Category

___ pT not assigned (cannot be determined based on available pathological information)

___ pT0: No evidence of primary tumor

___ pTis: Carcinoma *in situ* (Penile intraepithelial neoplasia [PeIN])

___ pTa: Noninvasive localized squamous cell carcinoma

pT1: (Glans) Tumor invades lamina propria; (Foreskin) Tumor invades dermis, lamina propria, or dartos fascia; (Shaft) Tumor invades connective tissue between epidermis and corpora regardless of location; All sites with or without lymphovascular invasion or perineural invasion and is or is not high grade

___ pT1a: Tumor is without lymphovascular invasion or perineural invasion and is not high grade (i.e., grade 3 or sarcomatoid)

___ pT1b: Tumor exhibits lymphovascular invasion and / or perineural invasion or is high grade (i.e., grade 3 or sarcomatoid)

___ pT1 (subcategory cannot be determined)

___ pT2: Tumor invades into corpus spongiosum (either glans or ventral shaft) with or without urethral invasion

___ pT3: Tumor invades into corpora cavernosum (including tunica albuginea) with or without urethral invasion

___ pT4: Tumor invades into adjacent structures (i.e., scrotum, prostate, pubic bone)

pN Category

___ pN not assigned (no nodes submitted or found)

___ pN not assigned (cannot be determined based on available pathological information)

___ pN0: No lymph node metastasis

___ pN1: less than or equal to 2 unilateral inguinal metastases, no extranodal extension

___ pN2: greater than or equal to 3 unilateral inguinal metastases or bilateral metastases, no ENE

___ pN3: Extranodal extension of lymph node metastases or pelvic lymph node metastases

pM Category (required only if confirmed pathologically)

___ Not applicable - pM cannot be determined from the submitted specimen(s)

[#] *Including lymph node metastasis outside the true pelvis, lung, liver, cutaneous nodules distant from the primary site, and bone.*

___ pM1: Distant metastasis present

Role of Human Papillomavirus (HPV)

Human papillomavirus (HPV) infection and its neoplastic implications dominate cervical epithelial pathology and HPV infection has generally been thought to be required for the development of cervical cancer(31). Penile carcinoma has a multifactorial etiology, the most common risk factors being human papillomavirus (HPV) infection, phimosis and poor hygiene, as well as lack of circumcision, lichen sclerosis and inflammatory conditions (balanitis xerotica obliterans), premalignant lesions (Bowen's disease, erythroplasia of Queyrat), compromised immune system, obesity, smoking, UVA phototherapy, increasing number of sexual partners and socioeconomic status(8).

HPV infection has been linked to penile carcinoma, the exact mechanism involved in its pathogenesis not being fully elucidated. HPV has been linked with other malignancies including cervical cancer, anal cancer and oropharyngeal cancer. More than 20% of patients with penile cancer have been tested positive for HPV infection, HPV prevalence depending on the method of sampling, processing methods and the anatomic sites or specimens sampled. The prevalence of HPV seems to be much higher in uncircumcised men compared to circumcised patients(32). HPV DNA is detected in up to 90% of cervical tumour cells and ~68% of tonsillar tumour cells(33,34).

It can lead to a variety of disease processes, including genital warts, dysplastic lesions and invasive malignancies of the anus, penis, vulva, vagina, cervix and oropharyngeal cancers(35). HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are carcinogenic in the uterine cervix, according to the International Agency for Research on Cancer(34). HPV is a 55-nm icosahedral, nonenveloped, 8000-base-pair, double-stranded DNA virus(36). An early (E) gene area, a late (L) gene region and a noncoding section with regulatory elements make up the HPV genome(37). Early in the development cycle, the E1, E2, E5, E6 and E7 proteins are expressed and are needed for viral replication and cellular

transformation. Malignant transformation is caused by the E6 and E7 proteins, which target the human cell cycle regulators p53 and Rb (retinoblastoma protein) degradation(36,38). HPV targets basal keratinocytes after microtrauma resulting in exposure of these cells to the virus and the virus completes the replication cycle in these cells(39). CD4+ T cell regulation is particularly important in controlling HPV infections. The viral oncogenes E6 and E7 interfere significantly with apoptosis and cell cycle control in transforming HPV infection(38). It is known that the E6 protein produced by high-risk HPV types 16 and 18 can combine with the p53 protein and cause the same functional consequence as a p53 gene mutation. The E7 protein of HPV-16 is also shown to bind to the Rb protein encoded by the retinoblastoma gene (Rb1). The interaction between E7 and Rb1 is responsible for the significant elevation of p16 protein expression in high-risk human papillomavirus (HR-HPV) infected lesions. The absence of block-type p16 immunopositivity in lesions infected with low-risk human papillomavirus (LR-HPV) types is explained by the fact that LR-HPV E7 proteins do not trigger p16 overexpression(40). The p53 and Rb proteins participate in the activity at the G1-S cell cycle checkpoint that normally causes cells with DNA damage to undergo either cellular arrest at G1 or apoptosis(41). The cellular tumour suppressor protein p16INK4a (p16) has been identified as a biomarker for transforming HPV infections(42). Affected cells overexpress p16 to compensate for the irregular cell cycle activation; however, because E2F is produced via E7 rather than CDK4/6, p16 expression does not affect cell cycle activation. Literature shows that high T-cell response to E2 protein is linked to a lack of cervical disease development in women with HPV type 16 infection(42). HPV-independent cervical carcinomas are often more aggressive than HPV-associated carcinomas in other anatomical locations such as the oropharynx and the vulva, a feature that is becoming clinically important(43). Persistent HPV infection is the most significant risk factor for cervical cancer. Controlling the development of HPV infection is enhanced by a cell mediated immune response. The introduction of human papillomavirus (HPV) DNA testing into clinical practice raised hopes for improved primary screening, triage and post-treatment monitoring. The discovery of HPV as an etiological factor in HPV related cancers opens up the possibility of controlling these cancers by vaccines and other targeted therapies(6,32).

Programmed death -1/ Programmed death ligand- 1 (PD-1/PD-L1) The T cell-based immune system has been developed to recognize and eliminate abnormal cells, such as

pathogen-infected cells and cancer cells(16). The binding of the T cell receptor (TCR) on T cells to peptide-major histocompatibility complexes (MHC) on target cells results in the detection of such aberrant cells. These checkpoint pathways play an important role in preventing tissue damage and maintaining self-tolerance by controlling the amount and functional activity of antigen-specific T lymphocytes. Among all immune checkpoints, the PD-L1/PD-1 pathway has stood out because of its proven value as a therapeutic target in a large number of malignancies. regulated by several inflammatory cytokines and PD-1/PD-L1 binding can trigger active T-cell death and interleukin-10 (IL-10) expression as a negative feedback mechanism(16). Anti-PD-1 checkpoint inhibitor immunotherapy has enhanced tumour response and survival. Pembrolizumab was demonstrated in phase Ib KEYNOTE-012 and single-arm phase II KEYNOTE-055 studies to have an 18% response rate and a median overall survival of 6 to 8 months in treated, recurrent and metastatic patients. Programmed cell death protein 1 (PD-1) PD-1, also known as CD279, was identified in 1992 in IL-3-deprived LyD9 (murine hematopoietic progenitor) and 2B4-11 (murine T-cell hybridoma) cell lines.(17) PD-1 is a 55kDa transmembrane protein of 288 amino acids that includes an extracellular N-terminal domain (IgV-Like), a membrane-permeating region and a cytoplasmic tail with two tyrosine bases at the N and C end(15). PD-1 is an inhibitor of both adaptive and innate immune responses and is found on activated T, NK and B lymphocytes, macrophages, dendritic cells (DCs) and monocytes(44). It is overexpressed in tumor-specific T cells. Transcription factors such as a nuclear factor of activated T cells, NOTCH, Fork head box protein (FOX) O1and interferon (IFN) regulatory factor 9 (IRF9) may be involved in PD-1 transcription(45). PD-1 is produced in exhausted T cells (CD8) during persistent infections and the FOXO1 transcription factor attaches to the PD-1 promoter to boost its expression. Leakage from cancer cells increases the expression of the c-FOS component, which increases the expression of PD-1. So PD-1 plays two opposing roles, as it can be both beneficial as well as harmful(15).

The role played by HPV in carcinogenesis of the penis appears to be similar to cervical cancer. HPV encodes the E6 and E7 oncogenes which are required for malignant transformation and maintenance of host cells. The viral oncoproteins (E6 and E7) may compromise the regulation

of the host cell cycle and lead to an uncontrolled proliferation(38). P16 is a tumor suppressor gene and its protein is physiologically expressed in normal tissues(46). The inactivation of the retinoblastoma gene (pRb) by HPV E7 results in overexpression of p16INK4a due to the lack of negative feedback loop between pRb and p16 protein. The overexpression of p16INK4a in tumor cells has been shown to correlate with high-risk HPV DNA detection in PC(47).

Penile Carcinoma arises from precursor lesions caused by HPV infection, in a stepwise progression(8). After infection, subsequent epigenetic alterations are essential for an HPV-infected cell to turn completely malignant. The oncoprotein E6 binds and targets the tumour suppressor proteins p53 and PDZ domain proteins for proteasomal degradation, while E7 inactivates the retinoblastoma tumour suppressor protein and leads to uncontrolled cellular proliferation(40). While there is enough evidence to support that HPV plays a major role in carcinoma cervix and oral cancers, studies evaluating the role of HPV in Penile Carcinoma are scarce because of the rare occurrence of this malignancy. The reported prevalence of HPV in Penile Carcinoma in the literature is varied depending on the geography, HPV subtypes evaluated, and the different techniques of DNA isolation(6).

A focus was drawn to the tumour-associated immune cell response in recent years(48). Attempts on measuring this answer have been made in different tumour entities to generate information on the patients' outcome. From a previous study, it is known that squamous cell carcinomas of the penis related to infection by HPV are associated with a different amount and composition of tumour infiltrating lymphocytes than non-HPV related squamous cell carcinomas of the penis(49). Additionally, studies attempting to gain knowledge on the amount and prognostic impact of immune cells were conducted(50). In general, from an immunological point of view, tumours are separated into subgroups with low immune cell infiltrate, medium amount of immune cell infiltrate and high immune cell infiltrate(49). Tumour-infiltrating lymphocytes (TILs) are an important component of and are closely related to the antitumor immune response and prognosis in penile carcinoma. There were higher numbers of $CD8^+$ cytotoxic T-lymphocytes (CTLs) and $FOXP3^+$ Tregs in HPV^+ peSCC than in HPV^- tumours, which indicated a stronger cytotoxic immune response and immune escape in HPV^+ penile cancer(51).

The PD-1/PD-L1 immune checkpoint pathway is one of the major targets of a new generation of immunotherapeutics(16). PD-1, a co-inhibitory receptor presents on a subset of CD8-positive cytotoxic T-cells, interacts with its ligand PD-L1 on tumour cell membranes which results in suppression of T-cell activation and proliferation, thereby, dampening of the host anti-tumour immune response. Therefore, it inhibits the PD-1/PD-L1 interaction. Hence it should augment tumour cell killing by cytotoxic T-cells. Indeed, immunotherapeutic approaches targeting PD-1 or PD- L1 have been used to enhance anti-tumour activity in preclinical models, and anti-PD-1 and anti-PD-L1 immunotherapeutics have yielded promising results in this area(15). So in present study, we have applied IHC PDL1 to see the presence of it in tumours and TILs and CD8 to check the immune response against the tumour. We have applied IHC HPV and its surrogate marker p16 to check HPV-associated penile carcinomas.

The CAP(2017) protocol recommends the use of the TNM staging system of the American Joint Committee on Cancer (AJCC) for carcinoma of the penis(52). By AJCC convention, the designation T refers to a primary tumour that has not been previously treated. The symbol p refers to the pathologic classification of the TNM, as opposed to the clinical classification, and is based on gross and microscopic examination. pT entails a resection of the primary tumour or a biopsy adequate to evaluate the highest pT category, pN entails removal of nodes adequate to validate lymph node metastasis, and pM implies microscopic examination of distant lesion. Pathologic staging is usually performed after surgical resection of the primary tumour(30).

P16 is a tumour suppressor gene and its protein is physiologically expressed in normal tissues. The inactivation of the retinoblastoma gene (pRb) by HPV E7 results in overexpression of p16 due to the lack of negative feedback loop between pRb and p16 protein. The overexpression of p16 in tumour cells has been shown to correlate with high-risk HPV DNA detection in PC(14).

Cubilla et al in 2001 evaluated the prevalence of HPV DNA in different histological subtypes of penile carcinoma, dysplasia, and condyloma using a novel, sensitive SPF10 HPV polymerase chain reaction assay and a novel genotyping line probe assay, allowing simultaneous identification of 25 different HPV types. They found keratinizing squamous cell carcinoma and verrucous carcinoma were positive for HPV DNA in only 34.9 and 33.3% of cases, respectively, HPV DNA was detected in 80% of basaloid and 100% of warty tumour

subtypes. HPV was preferentially associated with warty, basaloid, and high- grade tumours and not with typical SCC, papillary, or verrucous carcinomas. The overall prevalence of HPV DNA in penile carcinoma (42%) is lower than that in cervical carcinoma (∼100%) and similar to vulvar carcinoma (∼50%)(9).

Lont et al in 2006 investigated in a retrospective study of 171 patients, the prevalence of high-risk HPV in a large series of penile squamous-cell carcinomas (SCCs) and to determine the relationship between HPV and survival. Formalin-fixed, paraffin-embedded tumour specimens of 171 patients with penile carcinoma were tested for high-risk HPV DNA presence by GP5+/6+-PCR. The clinical course of the patients and the histopathological characteristics of the primary tumours were reviewed. High-risk HPV DNA was detected in 29% of the tumours, with HPV 16 being the predominant type, accounting for 76% of high-risk HPV containing SCCs. Disease-specific 5-year survival in the high-risk HPV-negative group and high-risk HPV-positive group was 78% and 93%, respectively (log rank test $p = 0.03$). In multivariate analysis, the HPV status was an independent predictor for disease-specific mortality ($p = 0.01$) with a hazard ratio of 0.14 (95% CI: 0.03–0.63). Results indicated that the presence of high-risk HPV (29%) confers a survival advantage in patients with penile carcinoma(65).

Chaux et al in 2009 College of American pathologists (CAP) in 2017 recommend a method to grade penile SCCs as follows: Grade 1 is an extremely well-differentiated carcinoma, with a minimal deviation from the morphology of normal/hyperplastic squamous epithelium. Grade 2 tumours show a more disorganized growth as compared to grade 1 lesions, higher nuclear-to cytoplasmic ratio, evident mitoses, and, although present, less prominent keratinization. Grade 3 are tumours showing any proportion of anaplastic cells, identified as solid sheets or irregular small aggregates, cords or nests of cells with little or no keratinization, high nuclear-to-cytoplasmic ratio, thick nuclear membranes, nuclear pleomorphism, clumped chromatin, prominent nucleoli, and numerous mitoses(54).

Kamran Zargar-Shoshtari et al(2016) studied in a group of 57 patients treated for invasive PC. They did tissue microarrays of 57 cases of invasive penile squamous cell carcinomas were immunohistochemically stained for p16 and p53. HPV in situ hybridization (ISH) for high-risk subtypes was also performed. p16 and HPV ISH were positive in 23 (40%) and 24 (42%) of the cohort, respectively. The proportion of warty, basaloid, or mixed warty basaloid tumour subtypes were significantly greater in the p16-positive patients (48% vs. 3%; P < .01). p53 expression was negative in 31 (54%) cases. Only in p16-negative patients, positive p53 status was associated with pN+ disease (odds ratio, 4.4 [95% confidence interval (CI), 1.04-18.6]). In Kaplan-Meier analysis, the unadjusted estimated OS was insignificantly longer in p16-positive patients (median OS, 75 vs. 27 months; P = .27) and median CSS was not reached (P = .16). In a multivariable Cox proportional hazard model, when controlling for pathological nodal status and adjuvant chemotherapy, p16 status was a significant predictor for improved CSS (hazard ratio, 0.36 [95% CI, 0.13-0.99]). The worst CSS was seen in pN+ patients with double negative p16 and p53 expression (8 vs. 34 months; P = .01). In this cohort, p53 and p16 status showed clinical utility in predicting nodal disease as well as survival(63).

Alemany et al.in 2016 studied the role of E6 mRNA transcript in a series of invasive PC (*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 mRNA detection in high-risk types was high in both penile high-grade squamous intraepithelial lesions (97.1%) and in invasive PC (85.1%), suggesting HPV E6 mRNA as an additional marker of viral activity and not a mere transient infection(58).

Ottenhof et al in the year 2018 aim to identify immunological prognosticators for lymph node metastases (LNM) and disease-specific survival (DSS) in penile SCC. For this retrospective observational cohort study was done in 213 penile SCC patients in the Netherlands Cancer Institute. They observed HPV-negative patients with penile cancer were more likely to have PD-L1–immunoreactive tumour cells. HPV plays a role in carcinogenesis of the penis similar to pathogenesis of cervical cancer. HPV encodes the E6 and E7 oncogenes which are required for malignant transformation and maintenance of host cells. The viral oncoproteins (E6 and E7) may compromise the regulation of the host cell cycle and lead to an uncontrolled

proliferation. they observed a significant difference only in differentiation grade ($p<0.01$). They studied on 213 cases, hrHPV was positive in 52 cases and are negative for hrHPV in 152 cases. The studied the higher number of diffusely PD-L1 positive tumours in the hrHPV negative group of their cohort, however, it matches the hypothesis that a more mutated tumour type will have higher T-cell inhibition properties, partially having poorer survival(53).

Olesen et al(2019) did a meta-analysis study of 52 studies showed a pooled prevalence of 50.8% (44.8–56.7) of HPV infection in PC with a rate of 68.3% (58.9–77.1) of HPV16. A large proportion of penile cancers and penile intraepithelial neoplasia are associated with infection with HPV DNA (predominantly HPV16), emphasising the possible benefits of HPV vaccination in men and boys(55).

Singh et al. 2019 did a study Human papillomavirus-associated carcinoma penis, a comparative study for histopathological correlation and its outcome. The total number of HPV-positive cases was 103, so the incidence of HPV in carcinoma penis was 45.5%. The incidence of HPV type 16 was 90.3%, and the incidence of HPV type 18 was 41.7%. Large proportion of penile carcinomas are associated with HPV in India and are predominantly warty basaloid type and is strongly associated with p16 immunostaining(7).

Eich et al. in 2019 studied the morphology, outcomes of p16, HPV in squamous cell carcinoma of the penis in 102 patients. 46% of the tumours were HPV- related subtypes, while 52% were p16 positive. Tumour histology correlated well with p16 positivity ($p<0.01$), and p16 IHC predicted HPV in 25/26 cases. HPV is a common cause of penile SCC and is diagnosed by tumour histology and confirmed by the overexpression of p16 on IHC(57).

Sharma et al studied in 2022 that PC was commonly related to HPV infection, with HPV-16 being the most common subtype. They found 22% of verrucous carcinoma to 66% in warty and basaloid subtypes. 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 Penile Carcinoma, followed by HPV-18, and belongs to the “high-risk” serotype. Type-6

and -11 are found mostly in benign lesions but also in a few Penile carcinomas including nonmetastatic verrucous carcinoma and are classified as low-risk serotypes(56).

Joshi et al in august 2022 did a systematic review and metanalysis in which their results have also demonstrated the prognostic value of immune cells such as tumour-associated macrophages, immune markers such as programmed death ligand-1, and HPV-status in penile cancer. Immune-based therapies including immune-checkpoint blockade, adoptive T cell therapies, and HPV-targeting therapeutic vaccines are each promising candidate therapies, although these treatments are largely unexplored in penile cancer; however, they are currently being evaluated prospectively(64).

Portillo et al. in 2020 showed the recommendations on using immunohistochemical and molecular biomarkers in penile cancer. 21/53 (40%) of penile SCCs, including those with advanced disease, were positive for tumoral PD-L1 expression. The study depicts a representative PD-L1 staining pattern. PD-L1 was expressed by a significant proportion of advanced penile SCC. 44% (15/34) of stage pT2 and 38% (6/16) of tumors with lymph node metastasis were positive for PD-L1. Expression of PD-L1 in stromal immune cells was identified in 26% (14/53) of cases. PD-L1 positivity did not correlate with tumor recurrence or progression in their study(67).

Cocks et al. in 2016 studied the immune checkpoint status in penile squamous carcinoma. 21/53 (40%) of penile SCCs, including those with advanced disease, were positive for tumoral PD-L1 expression. The study depicts a representative PD-L1 staining pattern. PD-L1 was expressed by a significant proportion of advanced penile SCC. 44% (15/34) of stage pT2 and 38% (6/16) of tumors with lymph node metastasis were positive for PD-L1. Expression of PD-L1 in stromal immune cells was identified in 26% (14/53) of cases. PD-L1 positivity did not correlate with tumor recurrence or progression. suggested that in response to malignancy, the host immune system engages a variety of immune cell types and cell signalling pathways. Tumour infiltrating lymphocytes (TILs) are immune cells recruited as a defence mechanism against tumour cells. Increased TILs have been frequently correlated with a favourable prognosis in melanoma and solid tumours including ovarian and colorectal cancers. Effector

CD8 (Killer) T-cells are primarily responsible for most anti-tumour activity while a population of CD4 cells that express Forkhead Box P3 (FOXP3) transcription factor (also known as regulatory T cells or TRegs) are involved in suppressing antitumor immune responses. They have noted that CD8 expression was high in both tumour and stromal immune cells (42% and 47% respectively), as was FOXP3 expression (49% and 51% respectively). The ratio of CD8:FOXP3 was <1 in stromal immune cells and increased in tumour immune cells(59).

Deng et al (2016) studied expression pattern of PD-L1 in PeSCC tumour cells and TILs as well as their association with common clinicopathological features and CSS. The expression of PD-L1 in TILs was significantly correlated with nodal status, G grade (p D 0.012), extent of TILs (p D 0.002) and CD8 positive TILs (p =0.001). PD-L1 was positively correlated with interferon -gamma and CD8 gene expression. 36 (69.2%) patients with PD-L1-negative tumours presented with positive TILs, no significant association was observed between TILs and CSS in this subpopulation of patients. The expression of PD-L1 in tumour cells was correlated with the extent of TILs and $CD8^+$ TILs. They hypothesized that PD-L1 expression in tumour cells can be constitutive and/or induced by TILs in PeSCC. To confirm this hypothesis, they also evaluated the baseline levels of PD-L1 in three primary PeSCC cell lines and in the normal human keratinocyte cell lines.

Mager's et al 2019 studied on genitourinary malignancies includes identification of novel groups of "high risk" patients undergone biopsy after abnormal screening. Immunohistochemistry was important methodology that identified specific molecular subtypes for diagnosis, prognosis and prediction.

Muller et al in the year 2022 did a cohort of 60 patients having well-defined penile invasive carcinomas the average age was between range 41–85 years. Thirty three patients were within the age group of 65 forming 55 % of total population. Twenty seven patients were above the age group of 65 forming 45% of total population. They have classified tumour staging into T1a, T1b, T2, T3,T4 and TX. 23 patients were categorized as T1a, 7 patients were categorized as T1b, 23 patients were categorized as pT2. 3 Patients were categorized into pT3 and 2 patients were categorized as pT4. T2–3 penile cancers are heterogeneous, and a modified

clinicopathological staging system that incorporates lymphovascular embolization may better predict the prognosis of patients with penile cancer than does the 8th AJCC-TNM staging system.(60)

Udager et al in 2018 used an anti-PD-L1 primary antibody (clone 5H1), immunohistochemistry was performed on whole tumour sections from thirty-seven patients with penile SqCC treated at their institution between 2005 and 2013. PD-L1 positive tumours were defined as those with membranous staining in ≥5% of tumour cells. Association between PD-L1 expression and clinicopathologic parameters was examined using Fisher's exact test. Correlation between PD-L1 expression in primary tumours and matched metastases was assessed using the Spearman rank correlation coefficient (ρ). They did the study on twenty-three (62.2%) of thirty-seven primary tumours were positive for PD-L1 expression, and there was strong positive correlation of PD-L1 expression in primary and metastatic samples (ρ = 0.72; $0.032 < P < 0.036$). Primary tumour PD-L1 expression was significantly associated with usual type histology ($P = 0.040$) and regional lymph node metastasis ($P = 0.024$), as well as decreased cancer-specific survival ($P = 0.011$)(61).

Bacco et al(2019)studied that out of 35, 18 (51.4%) were PD-L1 +. PD-L1+ were associated with larger tumours, (p=0.027). There was an association between PD-L1+ and p16 expression (p=0.002). PD-L1+ was more frequent in grade II and III than grade I (77.8% vs. 22.2%) and was expressed in all patients with grade III. PD-L1+ was predominant in lesions affecting glans (94.4%) and urethra (72.2%). Considering tumour grade, were grade I in 22.2%, grade II in 61.1% and grade III in 16.6%. There was statistical correlation between PD-L1+ and p16 expression *(p=0.002)*. Patients with PD-L1+ had a trend to present high-grade tumours, grade II and III in 77.8% of the lesions vs. 22.2% grade I. PD-L1 was expressed in all patients with grade III PSCC(62).

Ahmed et al in 2020 reported that the majority of CD-8+ Tumour-infiltrating lymphocyte in HPV- associated head and neck SCC express PD-L1, suggesting the benefits of using immune checkpoint blockades in these patients(66)

AIMS AND OBJECTIVES

AIM

To study the expression of PDL1, CD 8, HPV and p16 in penile carcinoma and their correlation with clinicopathological parameters

OBJECTIVES

1. To study the immunohistochemical expression of PDL1, CD 8, HPV, and p16 in penile carcinoma.
2. To study the clinicopathological parameters of patients with penile carcinoma.

MATERIAL AND METHODS

The present study was an ambispective observational study. This study included small biopsies, partial and total penectomy specimens of diagnosed cases of penile carcinoma received in the Department of Pathology at AIIMS Jodhpur from January 2017 to July 2022.(AIIMS/IEC/2021/3384). Samples from departmental archives were also retrieved and analysed in the study. The clinical parameters were retrieved from in-patient and out-patient data from the clinical departments.

After reviewing all the H&E slides, IHC markers PD-L1, CD8, HPV, and p16 were performed on the representative slide. Their expression was noted and correlated with assessed clinicopathological parameters. The histopathological typing and grading were done according to WHO 2022 classification of tumours of the genitourinary system, 5th Ed.

INCLUSION CRITERIA:

- All diagnosed cases of penile carcinoma.

EXCLUSION CRITERIA:

- Non-neoplastic lesions of the penis.
- Secondary metastatic deposits to penis
- Patient not willing to participate in the study

SAMPLE PROCESSING, STAINING AND IMMUNOHISTOCHEMISTRY

- After approval from the Institutional ethics committee, the study was started.
- Informed consent was obtained from the patients.
- Paraffin blocks were prepared using routine histopathological techniques. Thin sections (4-5 μm) were stained with routine Haematoxylin and Eosin (H&E).
  Light microscopy results were recorded and histopathological grading was given.
  The appropriate representative block was subjected to immunohistochemistry (IHC).

Grossing of partial and total penectomy specimen

Penile carcinomas: Measurement of the specimen was taken and the specimen was sectioned and kept for fixation in 10% formalin overnight. The following day, the dimension of the tumour, location and appearance were noted.

The representative sections were taken, and the tissue was processed as follows:

1. Dehydration was carried out by passing the sections through a series of ascending grades of ethyl alcohol, from 50%, 70%, 95% to absolute alcohol.
2. The clearing was done by passing the tissue through two changes of xylene.
3. Impregnation was done in molten paraffin wax which had a melting point of 54 – 62℃.
4. Embedding: Embedding station (Leica EG 1150 H) was used through which a small amount of liquid paraffin was layered into aluminium molds. Properly oriented tissues were placed inside the molds, which were then filled with liquid paraffin 60 – 62℃ and allowed to cool and harden.
   The lower portion of the cassette with an identification number was used as the final block.
5. Microtomy: Microtome (Leica-RM2255) was used and thin ribbons (4-5 μm) were cut and floated in warm water (~56℃) for expansion of the curled sections. These sections were then collected on frosted glass slides and kept for drying.

2) STAINING OF SECTIONS: (For H and E Stain)

1. Deparaffinization – The glass slides containing the tissue sections were kept over the hot plate at 60 ℃ for 10 minutes, followed by two changes in xylene (Xylene I & Xylene II), 10 minutes each.
2. Hydration – Through graded alcohol (100%, 95%, 70%, 50%) to water, 10 minutes each.
3. Haematoxylin – The sections were kept in Harris's Haematoxylin for 5 minutes.
4. Washing – The sections were washed well in water for 2 minutes.
5. Differentiation – Done in 1% acid alcohol (1% HCl in 70% alcohol) for 10 seconds.
6. Washing – Done under running tap water (usually for 15 – 20 minutes) until the sections 'blue'.
7. Eosin – Stained in 1% Eosin Y for 10 seconds.
8. Washing – Done in running tap water for 2 minutes.
9. Dehydration – Through graded alcohol (50%, 70%, 95%, 100%), 10 minutes each.
10. Clearing –Through xylene (Xylene II & Xylene I), 2 minutes each.
11. Mounting – The sections were mounted in DPX with a cover slip.

3) IMMUNOHISTOCHEMISTRY ANTIBODIES USED:

• **Primary antibody:**

Primary antibody	Clone	Make	Concentrated/ Ready to use
1. **PD-L1**	CAL 10, 6 mL	BioCare, USA	Ready to use (Prediluted)
2. **HPV monoclonal antibody (Recombinant major capsid of p16)**	Clone BSB-66, 7 mL	BioSB	Ready to use
3. **Tinto p16 antibody**	(RM 267),	BioSB	Ready to use
4. **CD 8**	[IHC542]	Genome GeneAb™	Ready to use

• **Secondary Antibody:** Bond Polymer Refine Detection, Leica

- Peroxide block, 3-4%(v/v)
- Post Primary, Rabbit anti mouse IgG in 10% (v/v) animal serum in tris-buffered saline
- Polymer, Anti-rabbit Poly-HRP-IgG containing 10% (v/v) animal serum in tris-buffered saline
- DAB Part 1, in stabilizer solution
- DAB Part B ≤0.1% (V/V) Hydrogen peroxide in stabilizer solution
- DAB Part B ≤0.1% (V/V) Hydrogen peroxide in stabilizer solution
- Haematoxylin, 0.1%

PREPARATION OF REAGENTS:

A**. Preparation of Buffer–Two types of buffers was used.**

1. Wash Buffer

2. Antigen Retrieval Buffer (ARB)

Wash buffer preparation: 6 gm powdered TRIS buffer salt was dissolved into 1 litre of distilled water and pH was set at 7.4.

ARB preparation: 6.05 gm TRIS salt and 0.744 gm EDTA salt were dissolved in 1 litre of distilled water, pH was set at 9.0.

Note:-

• To increase the pH, NaOH solution was added drop by drop and pH was titrated.

• To decrease the pH, HCl was added drop by drop and pH was titrated.

B. Preparation of Poly-L-Lysine Solution (PLL Solution):

1 ml of PLL was diluted with 9 ml of distilled water (1 in 10 dilutions).

C. Slide Coating Procedure:

- Step 1: Diluted PLL solution was taken in a clean container/Coplin jar
- Step 2: Both sides of glass slides were cleaned with tissue paper
- Step 3: The clean slides were immersed in a PLL solution for 5 minutes
- Step 4: After 5 minutes, the coated slides were removed and kept overnight for air dry. The coated slides were kept at room temperature. Tissue sections of 4 µ thickness were obtained on the PLL coated slides. Baking: The slides were kept at 60℃ for 1 hour and then cooled to room temperature.

IHC STAINING PROCEDURE

Step 1: Deparaffinization – The slides were kept in Xylene I (10 minutes), followed by Xylene II (10 minutes).

Step 2: Rehydration – The slides were kept in 100%, 70% and 50% alcohol for 5 minutes each followed by running tap water for 5 minutes.

Step 3: Antigen retrieval – by pressure cooker method(38). 200 ml of clean tap water was taken in the empty pressure cooker and heated up to the steam formation. The slides were placed in a rack. 300 ml of ARB was put in the container and the rack with slides was placed inside the container. Then the container, containing the rack with slides, was placed inside the pressure cooker and lid was closed. After two whistles the

pressure was released by lifting the air vent and allowed to cool till it reached the room temperature.

Step 4: Wash – Slides were washed in Wash Buffer (pH7.4) thrice at a 1-minute interval.

Step 5: Peroxide blocking – Blocking reagent was added to the sections and incubated for 10 minutes in Humidity chamber at room temperature. This step prevents unwanted, non-specific background staining.

Step 6: The peroxide was decanted and not washed with buffer.

Step 7: Primary antibody – PD-L1 to the sections and incubated in Humidity chamber for one hour.

Step 8: Wash – After that slides were washed in Wash Buffer (pH 7.4) thrice at a 1-minute interval.

Step 9: Amplifier – Amplifier was added over the sections and incubated for 30 minutes in Humidity chamber at room temperature.

Step 10: Wash – The slides were washed in Wash Buffer (pH 7.4) thrice at a 1-minute interval.

Step 11: HRP label – The HRP was added and incubated for 30 minutes in Humidity chamber at room temperature.

Step 12: Wash – The slides were washed in Wash Buffer (pH 7.4) thrice at 1-minute interval.

Step 13: DAB – The DAB chromogen was applied to the sections and incubated in Humidity chamber for 10 minutes, avoiding light exposure as much as possible.

Step 14: Wash – The sections were washed in distilled water twice at 1-minute interval.

Step 15: Counter stain – Slides were counterstained using Harris Hematoxylin for 2-3 minutes.

Step 16: Wash – The slides were washed in running tap water for 5 minutes.

Step 17: Dehydration – was done in graded alcohol (50%, 70%, 95%, 100%), 1 minute each.

Step 18: Mounting – Slides are air dried, mounted with DPX and examined under the microscope.

STAINING FOR PD-L1:

IHC was performed using commercially available ready to use monoclonal antibody for PD-L1. With each batch, appropriate controls were also run. Section from the endometrial biopsy with normal histology was taken as a positive external control for all the five antibodies.

INTERPRETATION OF IMMUNOHISTOCHEMICAL STAINS:

Staining of PD-L1

PD-L1: Circumferential membranous staining and cytoplasmic staining

Combined Proportion score(CPS) - The CPS was defined as the total number of tumour cells and immune cells (including lymphocytes and macrophages) stained with PD-L1 divided by the number of all viable tumour cells, then multiplied by 100

CPS (%) = Number of PD-L1 staining cells (tumour cells, lymphocytes, macrophages)/Total number of viable tumour cells × 100

Expression of PD-L1 in the tumour was quantified manually and classified as positive when staining (PD-L1: membranous) was present in ≥1% of tumour cells.

Staining extent was further characterized in the following subcategories:

1–5%, 6–10%, 11–25%, 26–50%and >50%. The 1% threshold for positivity was selected based on data demonstrating immune response to PD-L1 inhibition. Immune microenvironment staining was scored positive, when ≥1% of peritumoral and intertumoral immune cells showed reactivity. It was subdivided as 1–10%, 11–25%, 26–50% and >50%.(68)

Tumour infiltrating lymphocytes (TILs):

Staining in the peritumoral immune compartment was considered positive if membranous or cytoplasmic staining was seen in lymphomononuclear cells in

association with the tumour. Number of CD8+ lymphocytes in the highest density area (hot spot) per HPF (X40) both in tumour and in the stroma.(80)

Staining of CD 8

Present study calculated the % immune cell infiltrate inside tumour or peritumoral areas.

Interpretation of IHC

Positive: Any membranous staining in tumour infiltrating cells.

Negative: Complete absence of membranous staining within the tumour cells with concurrent internal control positive.

Staining of HPV

Interpretation of IHC

Positive: Any nuclear staining within the tumour cells.

Negative: Complete absence of nuclear staining within the tumour cells.

Staining of p16

Interpretation of IHC

Positive: Block positivity (both nuclear and cytoplasmic staining) within the tumour cells

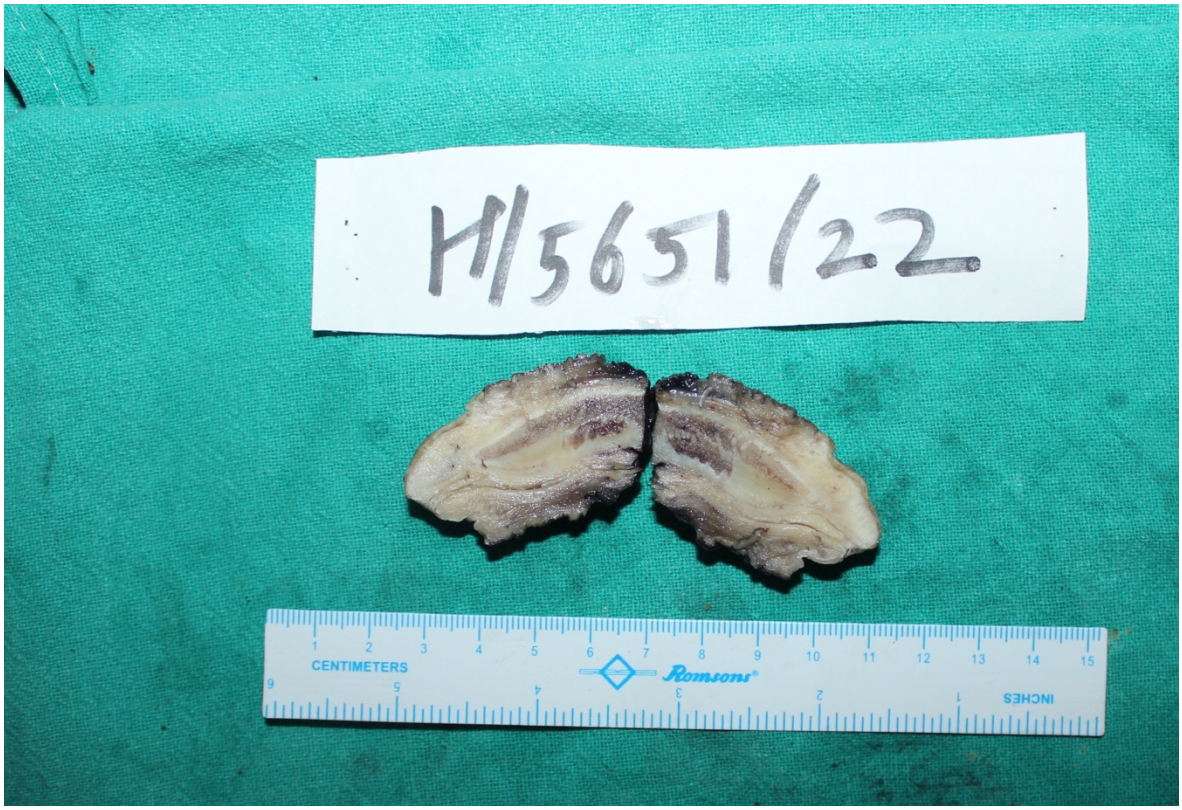
Negative: Complete absence of nuclear staining within the tumour cells. Only nuclear or only cytoplasmic staining has not been considered.

STATISTICAL ANALYSIS

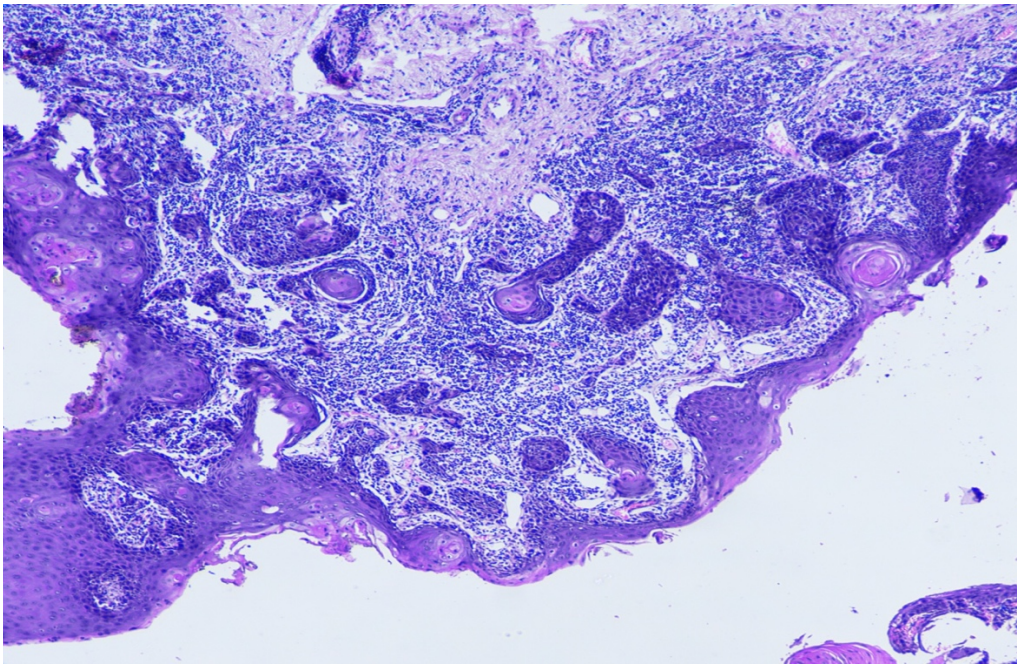
Sample Size calculation

Present study was time-bound, wherein all patients of penile carcinoma were analysed from January 2017 to June 2022. However; considering COVID-19 situation a total of 42 cases of penile carcinoma were included in the study.

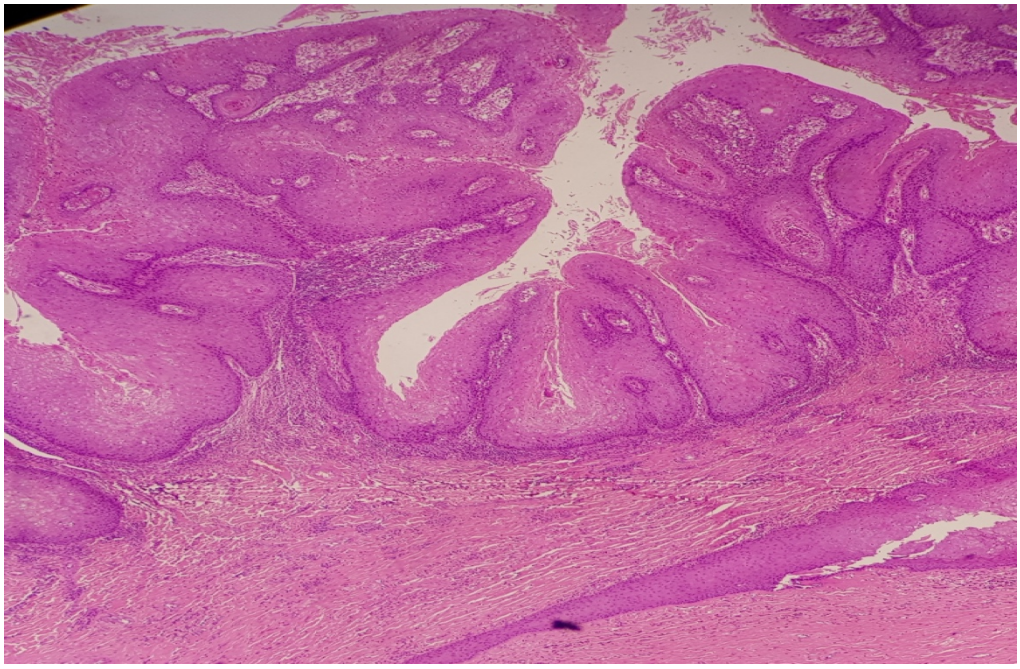
Since the study was time-bound, hence sample size calculation was not done.



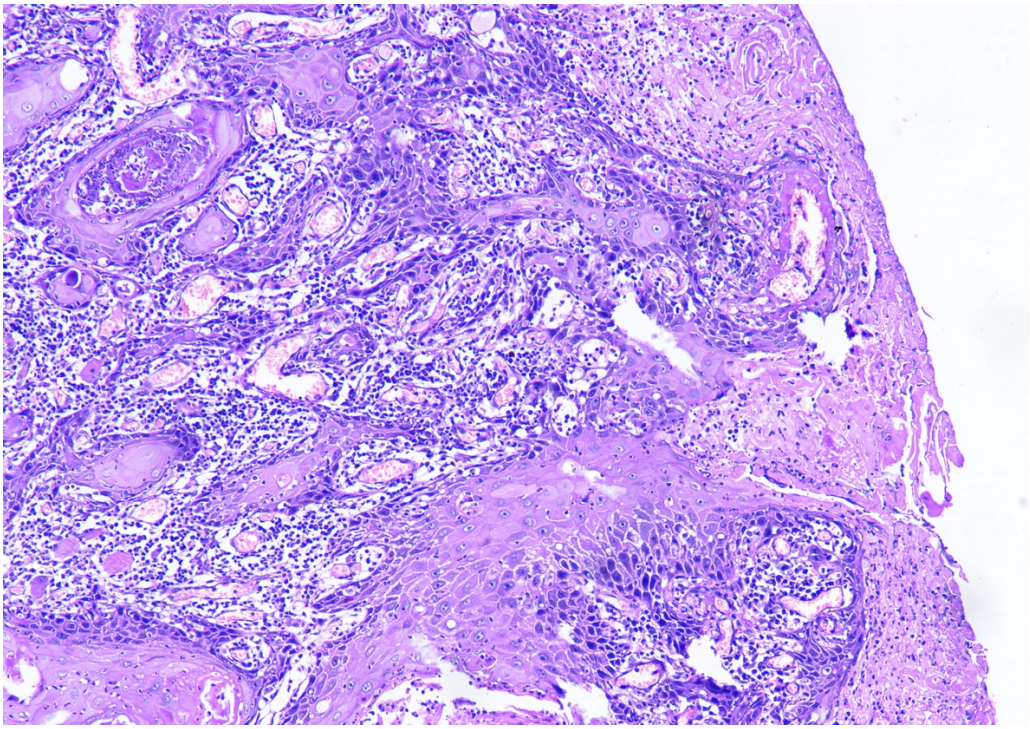
Picture 1) Gross image of partial penectomy 2) Cut surface of specimen



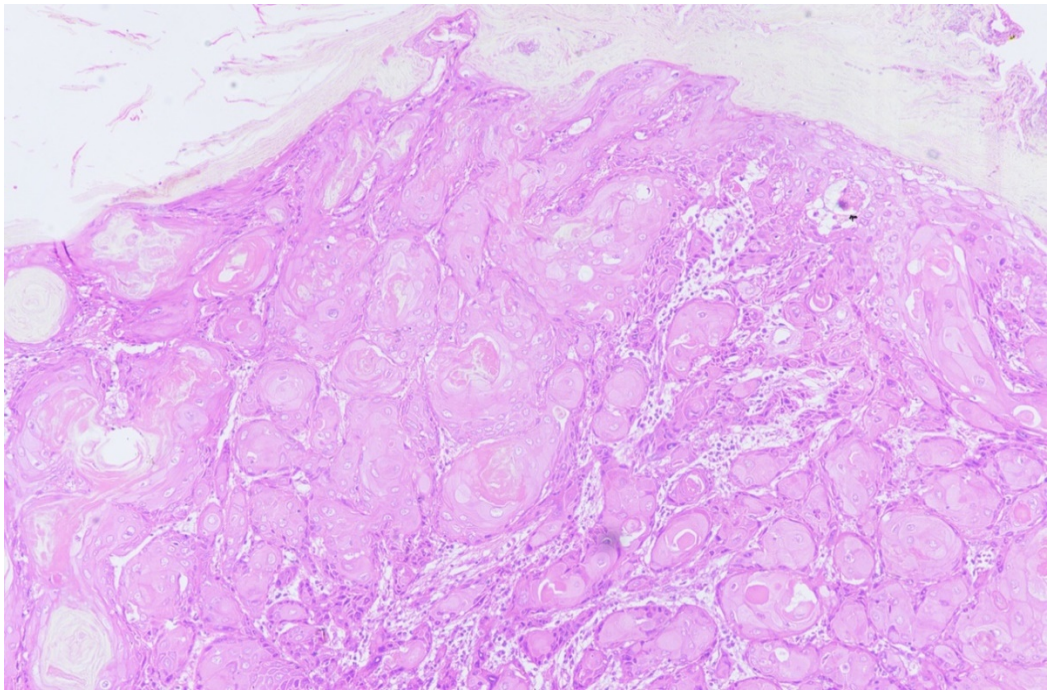
Photomicrograph 3: Squamous cell carcinoma arising from overlying epithelium (H and E,10X)



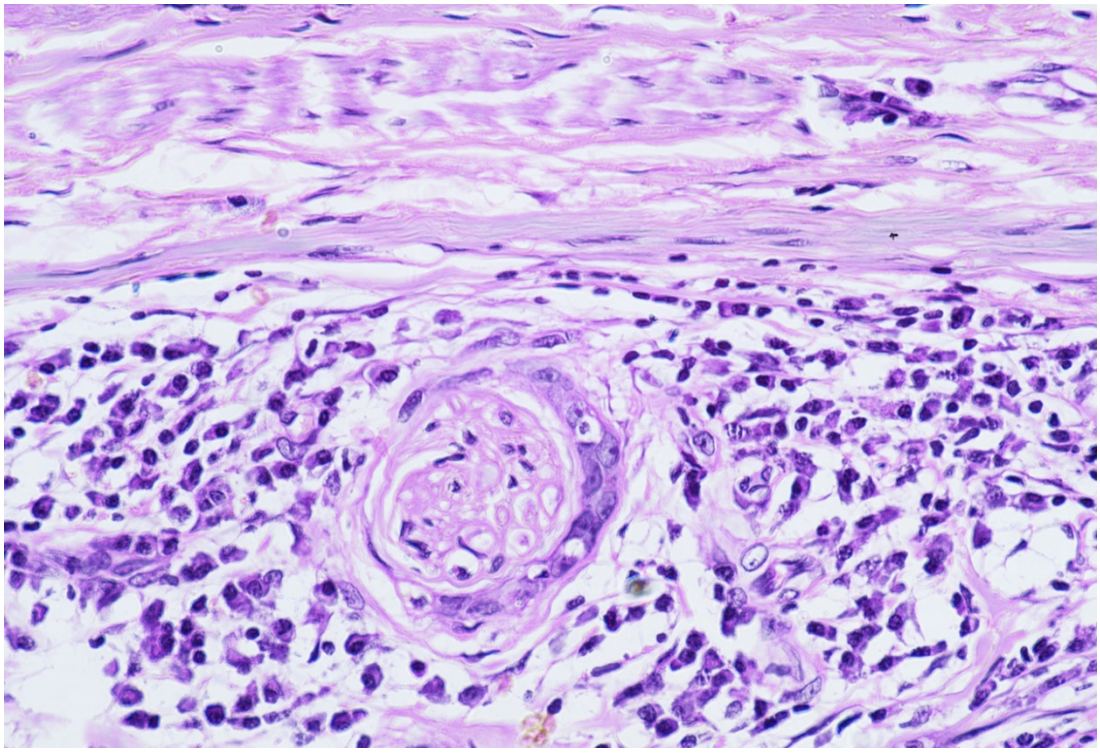
Photomicrograph 4: Verrucous carcinoma (H and E,4X)



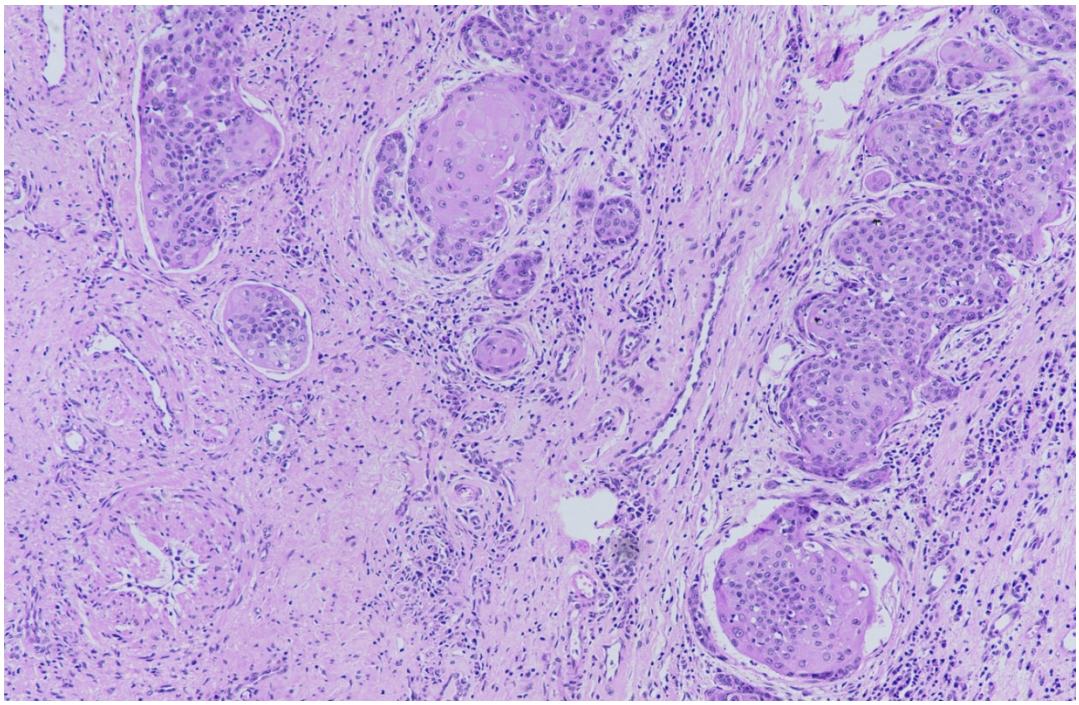
Photomicrograph 5: Well differentiated squamous cell carcinoma (H and E,10X)



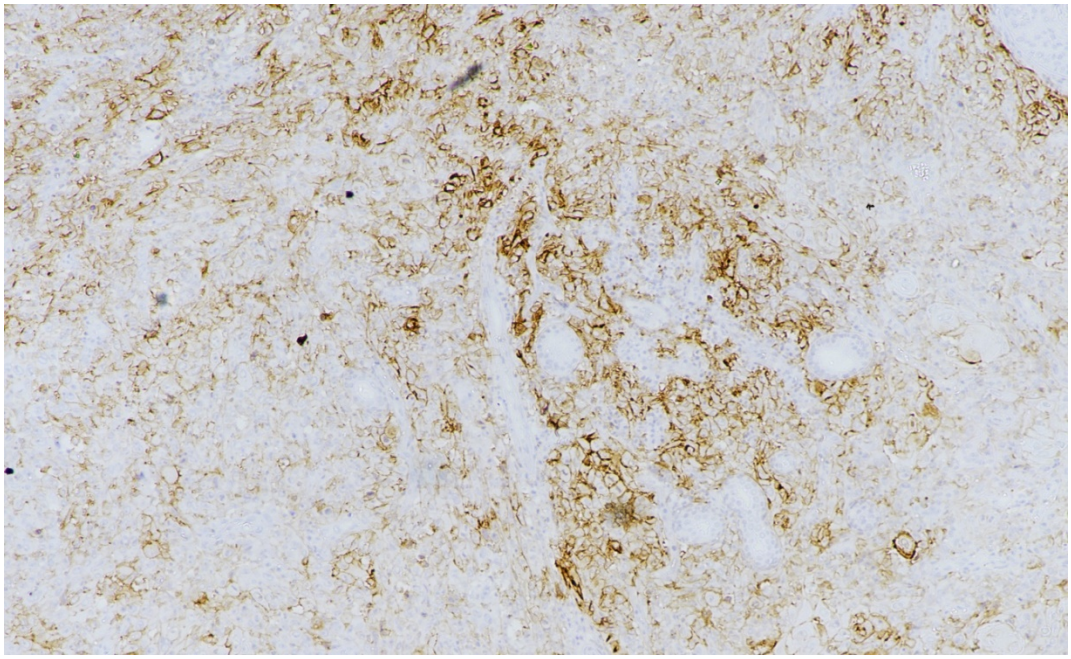
Photomicrograph 6: Moderately differentiated squamous cell carcinoma(H and E, 10 X)



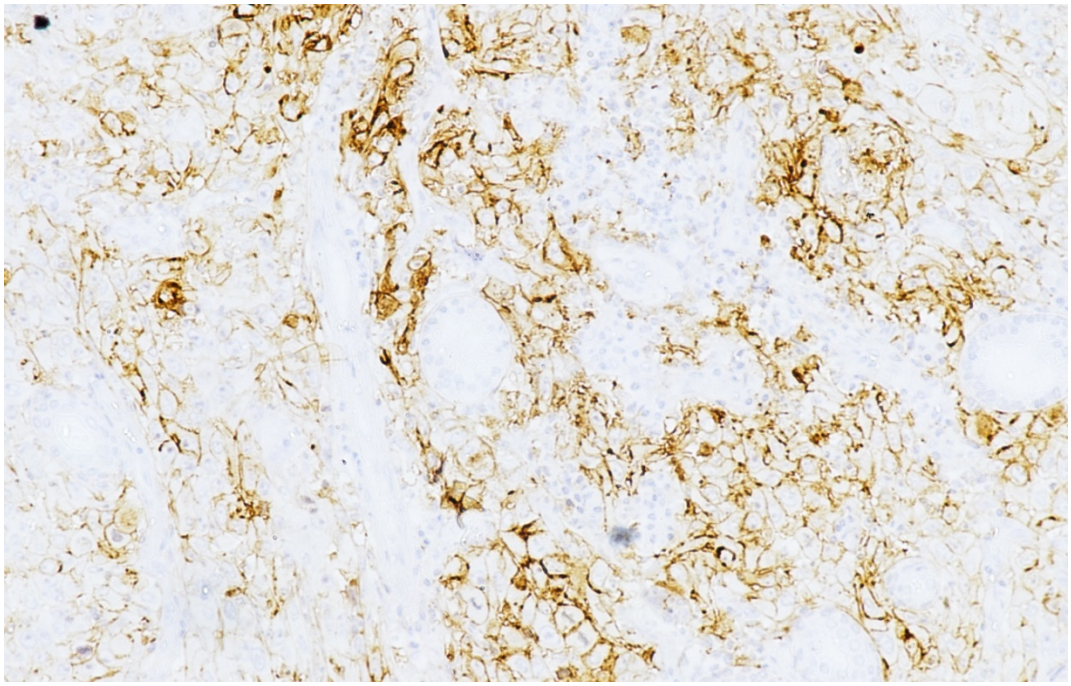
Photomicrograph 7: H and E section show PNI of tumour cells (40X)



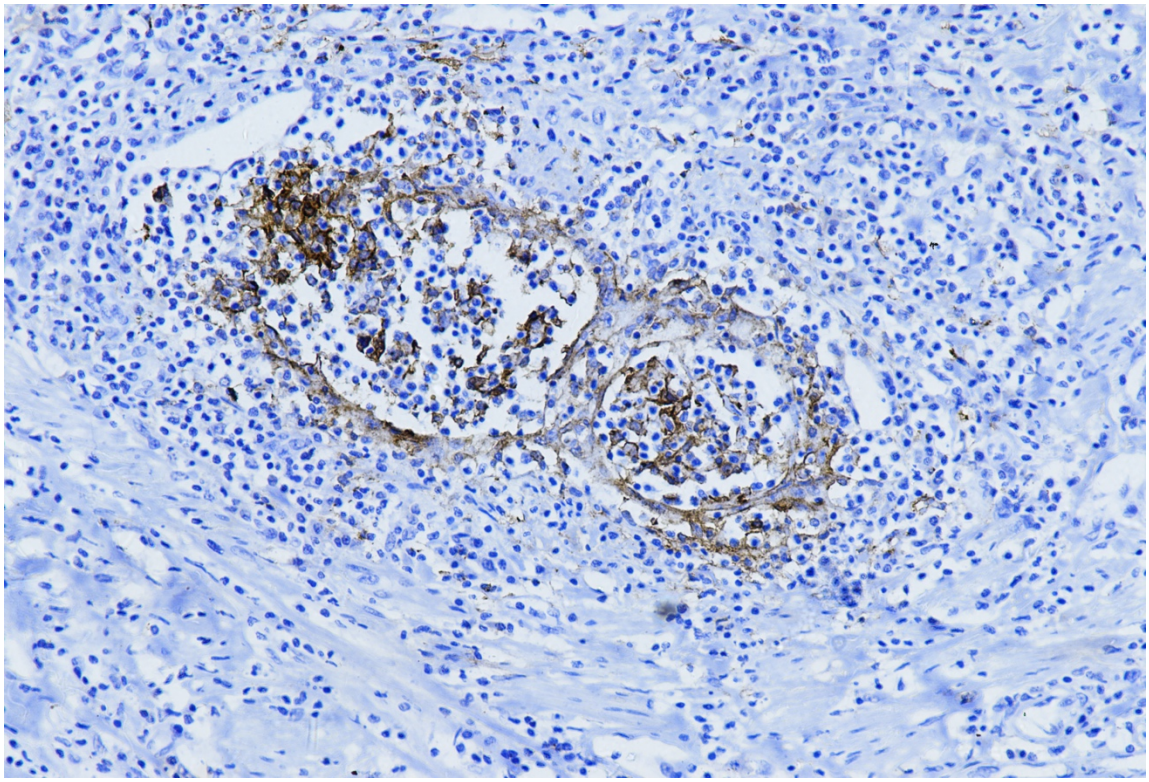
Photomicrograph 8: Case of moderately differentiated PeSCC showing multiple LVI 10X



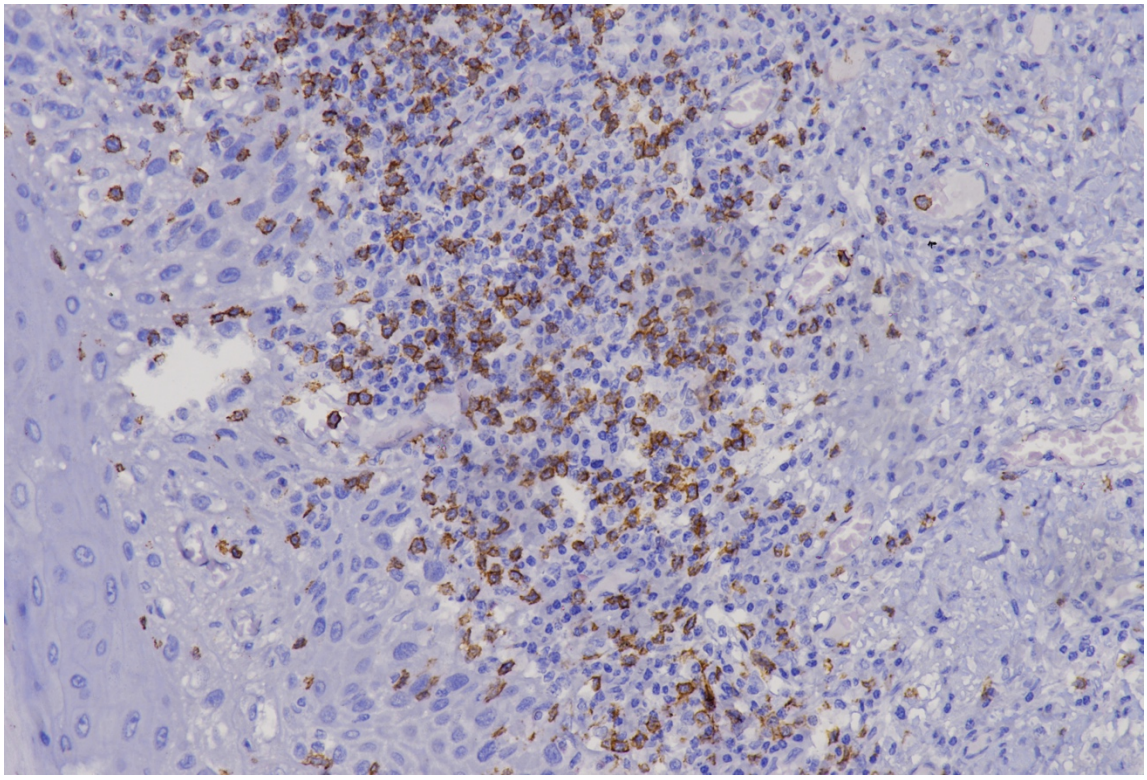
Photomicrograph 9: A case of moderately differentiated squamous cell carcinoma of penis showing positive membranous expression of PD-L1(10X)



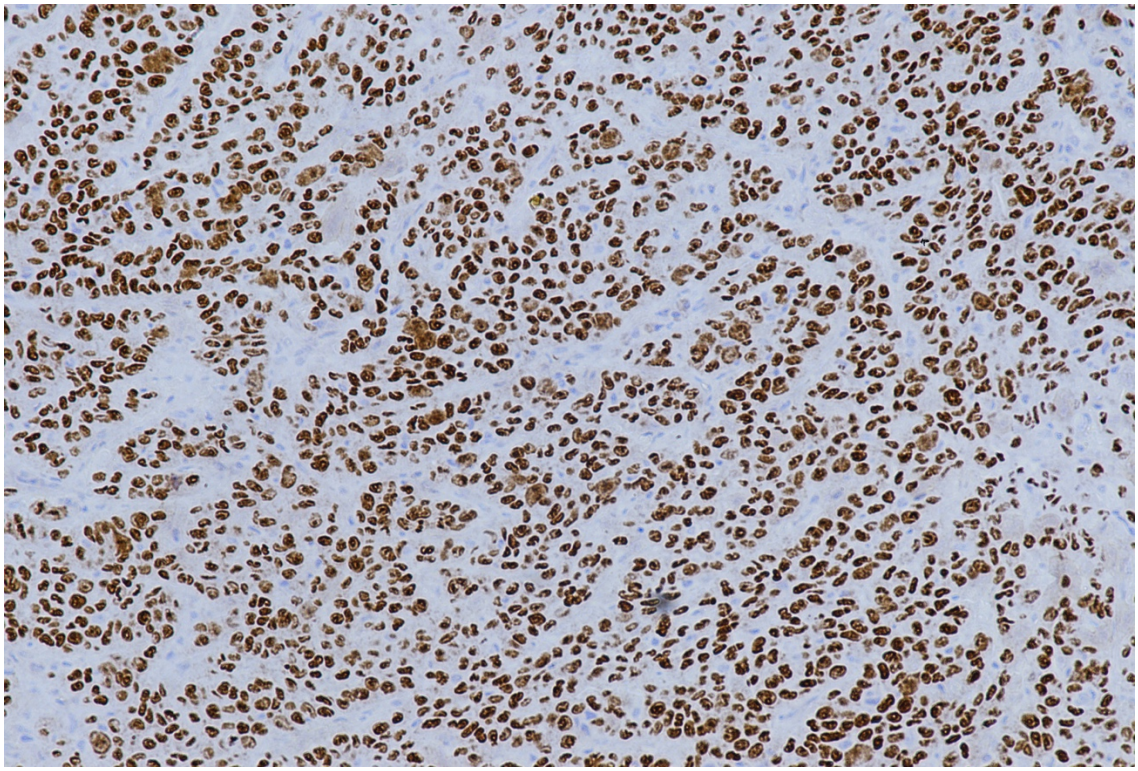
Photomicrograph 10: PD-L1 expression in tumour cells (40X)



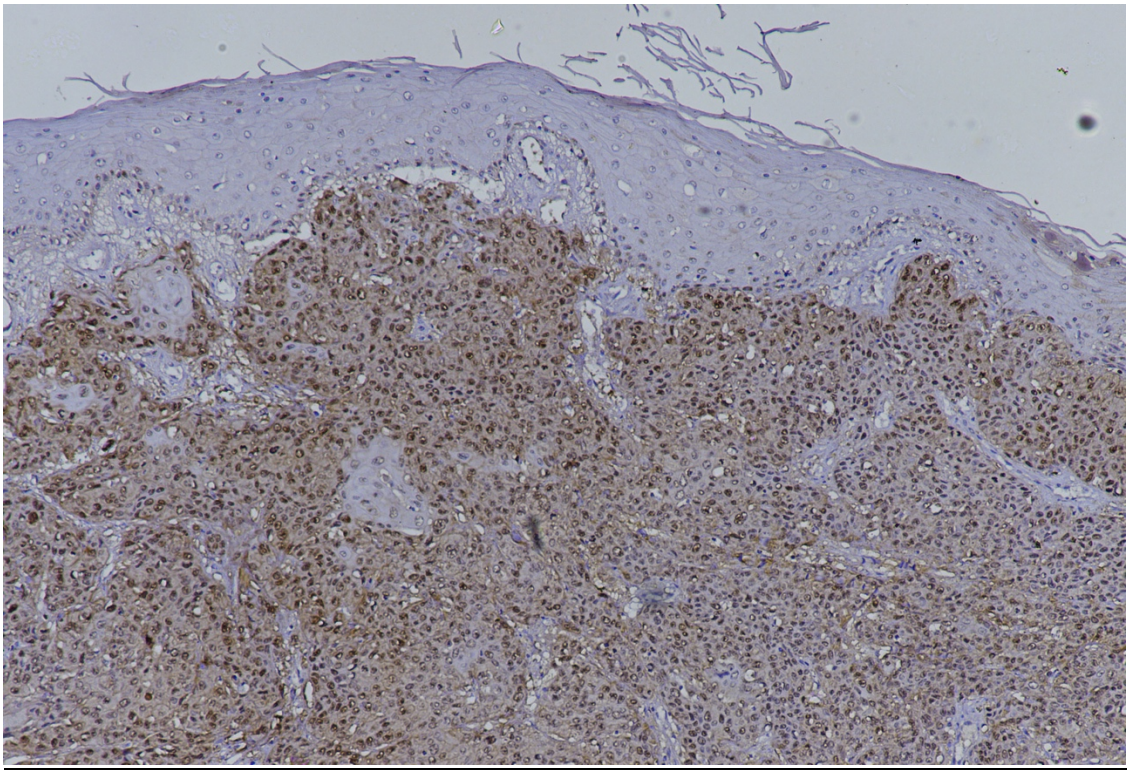
Photomicrograph 11: PD-L1 expression in TILs (40X)



Photomicrograph 12: CD 8 expression in lymphocytes (10X)



Photomicrograph 13: Nuclear expression of HPV in tumour cells(10X)



Photomicrograph 14: Block positivity of p16 in tumour cells(10 X)

RESULTS

This study was a prospective and retrospective study done from January 2017 to 31st December 2022 in the Department of Pathology and Lab Medicine in All India Institute of Medical Sciences, Jodhpur, Rajasthan, India. This study evaluated incidence of PDL1, p16, HPV and density of CD8 in histopathologically confirmed tissues of penile carcinoma and correlated them with clinicopathological parameters. A total of (n= 42) patients were selected from departmental archive and system records after considering exclusion and inclusion criteria.

PARAMETERS		NUMBER (%) OR MEAN(SD) OR MEDIAN(IQR)
Age	≤65years >65years	26(61.9) 16(38.1)
Smoking	Yes No	30(71.4) 12(28.6)
Type of surgery	Small biopsy Partial penectomy Total penectomy	11(26.2) 26(61.9) 5(11.9)
Site	Glans Prepuce Shaft Glans and prepuce Glans and shaft	22(52.3) 3(7.2) 5(11.9) 6(14.3) 6(14.3)
Focality	Unifocal Multifocal	30(71.4) 12(28.6)
Differentiation	Well Moderate Poor	14(33.3) 27(64.3) 1(2.4)
Pathologic stage	Not staged pT1 pT2-4	15(35.7) 11(26.2) 16(38.1)
Tumour size (CC)[1]	----------	26.25(6.07,58.3)
Depth of invasion(cm)[1]	----------	2.66(1.41)
Histological type	Usual Warty Basaloid Verrucous	36(85.7) 5(11.9) 1(2.39) 1(2.39)
LVI	Yes No	7(16.7) 35(83.3)
PNI	Yes No	11(26.2) 31(73.8)
PDL1 status	Positive Negative	19(45.2) 23(54.8)
HPV status	Positive Negative	13(31.0) 29(69.0)
p16 expression	Positive Negative	11(26.2) 31(73.8)
CD8 Density score	----------	39.17(20.775)

TABLE 1-Baseline clinicopathological data(N=42)

1-Small biopsies have been excluded(N=31)

Figure 1- Pie chart showing age distribution(N=42)

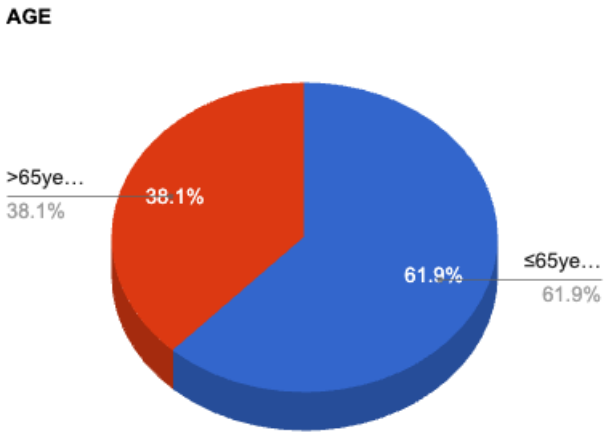


Figure 1 shows distribution of patients according to age.

61.9% patients were in the age group of 65 years or more. 38.1 % of patients were of the age below 65 years.

Figure 2- Age distribution chart(N=42)

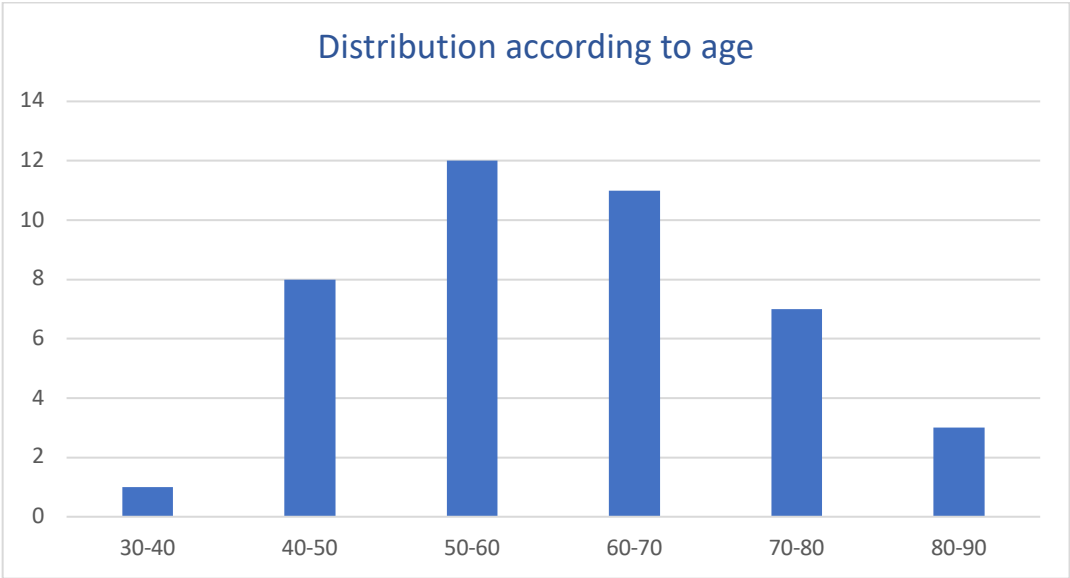
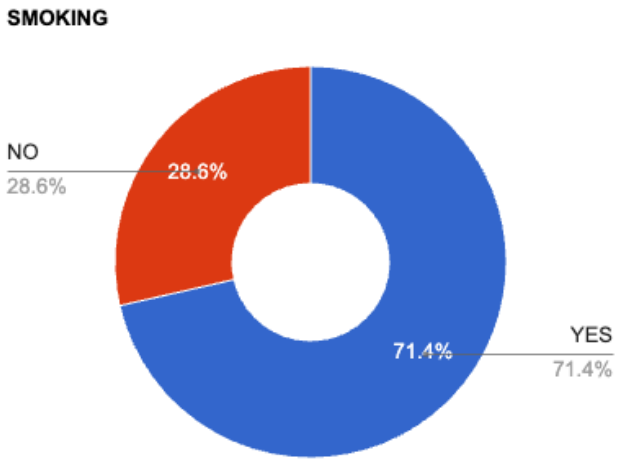
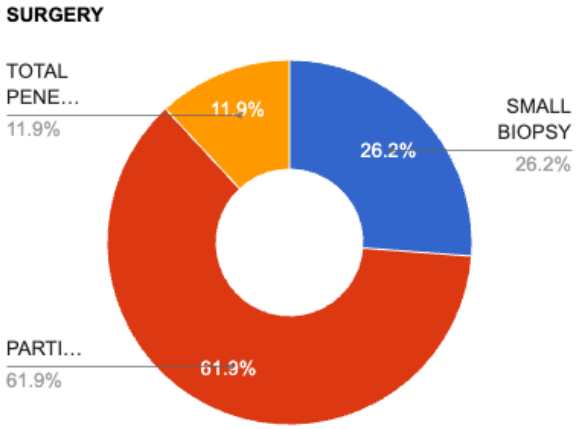


Figure 3- Pie chart showing distribution of patients smoking status (N=42)



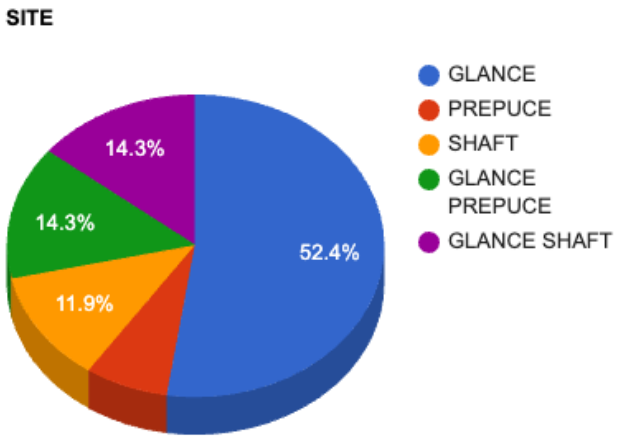
71.4% patients were addicted to smoking. 28.6% patients are non-smoker.

Figure 4- Pie chart showing type of surgery (N=42)



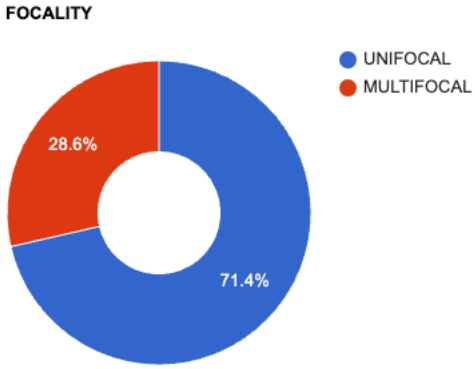
61.3% patients had undergone partial penectomy and 11.9 % patients had total penectomy. 26.2% cases of small biopsy.

Figure 5- Pie chart showing site of involvement (N=42)



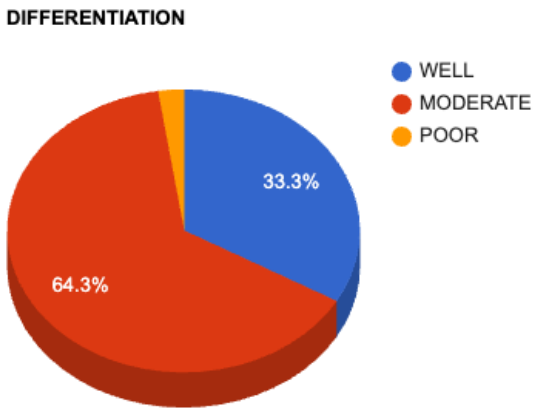
Glans was the commonest site of involvement in 52.4% patients, followed by shaft (11.9%). Six cases (14.3%) had multifocal involvement, involving Glans and prepuce. Another six cases (14.3%) had multifocal involvement, involving Glans and shaft.

Figure 6- Pie chart showing focality of tumour (N=42)



Glans was the commonest site of involvement in 28.6% multifocal cases. Nearly three fourth (71.4%) cases had unifocal involvement.

Figure 7- Pie chart showing differentiation (N=42)



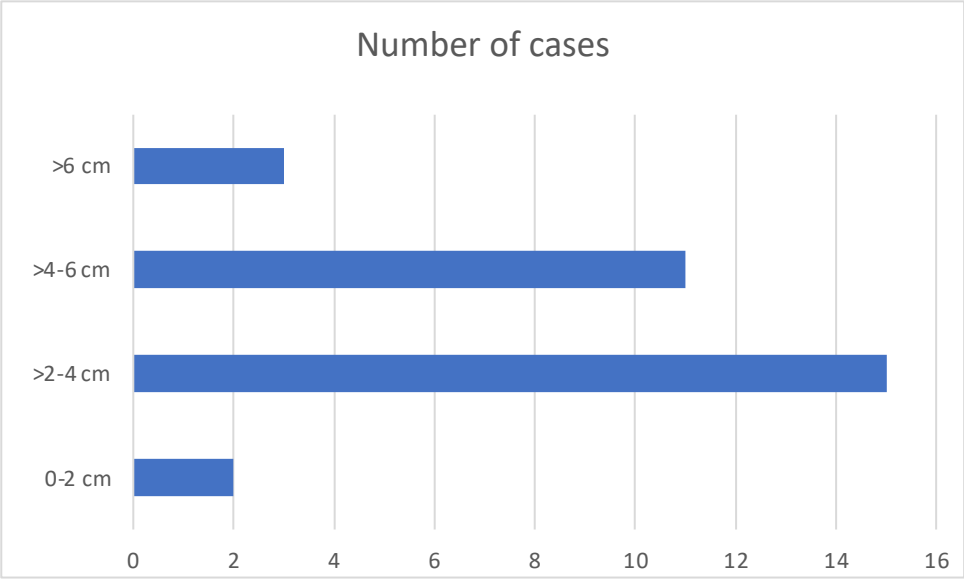
Most of the cases (64.3%) were moderately differentiated. One third (33.3%) case were well differentiated. Only one case was poorly differentiated.

Table 2, shows size distribution of tumour

(According to maximum dimension of tumour)

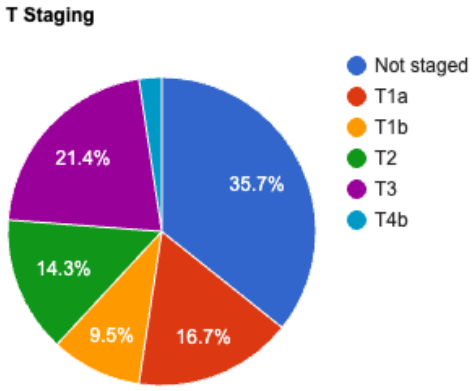
Size	Number of cases
0-2 cm	2
>2-4 cm	15
>4-6 cm	11
>6 cm	3

Fig 8, shows distribution of patients according to maximum dimension of tumour.



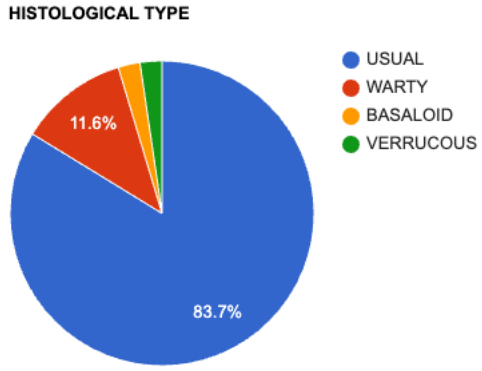
Small biopsies have been excluded.

Figure 9- Pie chart showing detailed T stage distribution(N=42)



pT3 was most frequent among the patients who were classified according to T stage. 16.7% cases were classified as pT1a, 9.5% as pT1b, 14.3% were pT2 and rest were pT4. While majority of patients could not be staged because of small biopsy or unavailability of data.

Figure 10- Pie chart showing distribution of histological type (N=42)



While majority (83.7%) of patients were of usual type. 11.6% were warty type and 2.39% were verrucous and basaloid type.

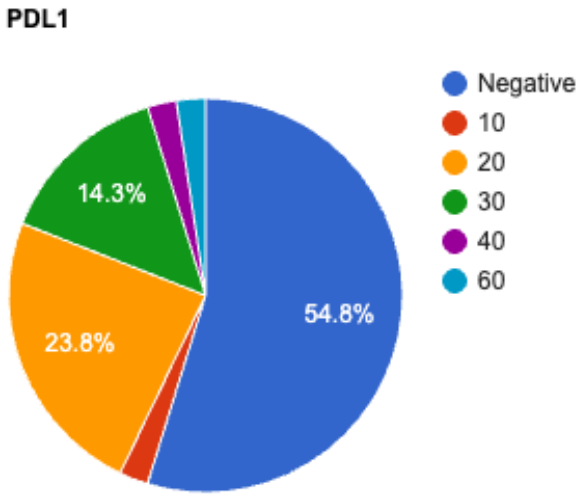
TABLE 3 -Association of PDL1 status with clinicopathological parameters (N=42)

(Pearson's Chi-Squared Test)

Parameters		PDL1 Status/ Number (%)		P value
		Positive	Negative	
Age	≤65years >65years	11(42.3) 8(50)	15(57.7) 8(50)	0.627
Smoking	Yes No	12(40) 7(58.3)	18(60) 5(41.7)	0.281
Type of surgery	Small biopsy Partial penectomy Total penectomy	4(36.4) 12(46.2) 3(60)	7(63.6) 14(53.8) 2(40)	0.671
Differentiation	Well Moderate Poor	10(71.4) 8(29.6) 1(100)	4(28.6) 19(70.4) 0(0.0)	**0.021**
Pathologic stage	Not staged pT1 pT2-4	5(33.3) 6(54.5) 8(50)	10(66.7) 5(45.5) 8(50)	0.499
Site	Glans Prepuce Shaft Glans and prepuce Glans and shaft	10(45.5) 0(0.0) 3(60.0) 4(66.7) 2(33.3)	12(54.5) 3(100) 2(40.0) 2(33.3) 4(66.7)	0.641
LVI	Yes No	2(28.6) 17(48.6)	5(71.4) 18(51.4)	0.332
PNI	Yes No	3(27.3) 16(51.6)	8(72.7) 15(48.4)	0.163
HPV	Positive Negative	6(46.1) 13(44.9)	7(53.9) 16(55.1)	0.936
P16	Positive Negative	5(45.5) 14(45.2)	6(54.5) 17(54.8)	0.987

In present study, 11 out of 42 cases (42.3%) were of age < 65 years expressing PD-L1. 8 cases out of 42 (50%) are of age > 50 years expressed PD-L1. In present study, observation of PD-L1 expression between smokers and non-smokers was done. 12 smokers (40%) out of 30 smokers show expression of PD-L1. 7 cases (58.3%) are non-smokers but show expression of PD-L1. No statistical association between PD-L1 and smoking (p = 0.281 was found. In present study, 10 cases (71.4%) out of 42 cases show well differentiated form of PC and PD-L1 positivity. 8/42 cases (29.6%) are positive for PD-L1 and show histological grade (G2) as well as PD-L1 positivity. 1 case is poorly differentiated and showed expression of PD-L1. Significant association was noted between PD-L1 and histological grade. **(p=<u>0.021</u>).**

Figure 10- Pie chart showing PD-L 1 CPS score distribution(N=42)



While majority (46.2 of patients were PDL1 positive, 20 was most frequent (23.8%) score among the patients who were classified according to PDL1 total score. 14.3% had a score of 30.

TABLE 4- Association of HPV status with clinicopathological parameters(N=42)

(Pearson's Chi-Squared Test)

Parameters		HPV Status/ Number (%)		P value
		Positive	Negative	
Age	≤65years >65years	8(30.8) 5(31.2)	18(69.2) 11(68.8)	0.974
Smoking	Yes No	6(20) 7(58.3)	24(80) 5(41.7)	**0.015**
Type of surgery	Small biopsy Partial penectomy Total penectomy	5(45.5) 8(30.8) 0(0.0)	6(54.5) 18(69.2) 5(100)	0.190
Differentiation	Well Moderate Poor	5(35.7) 8(29.6) 0(0.0)	9(64.3) 19(70.4) 1(100)	0.734
Pathologic stage	Not staged pT1 pT2-4	6(40) 4(36.4) 3(18.8)	9(60) 7(63.6) 13(81.3)	0.399
Site	Glans Prepuce Shaft Glans and Prepuce Glans and shaft	7(31.8) 3(100) 0(0.0) 0(0.0) 3(50)	15(68.2) 0(0.0) 5(100) 6(100) 3(50)	0.215
Histological type	Usual Warty Verrucous Basaloid	11(31.4) 2(40.0) 0(0.0) 0(0.0)	24(68.6) 3(60.0) 1(100.0) 1(100.0)	0.730
LVI	Yes No	2(28.6) 11(31.4)	5(71.4) 24(68.6)	0.881
PNI	Yes No	4(36.4) 9(29.0)	7(63.6) 22(71.0)	0.651
P16	Positive Negative	6(45.5) 7(22.6)	5(45.5) 24(77.4)	**0.049**

Most smokers are HPV negative(p=0.015). Most HPV negative tumors were also p16 negative(p=0.049). Majority of HPV negative cases had more radical surgery, like 69.2% partial penectomy and all cases of total penectomy. Moreover, 81.3% of advanced(pT2-4) case were HPV negative. However, these differences were not statistically significant.

TABLE 5- Association of p16 status with clinicopathological parameters(N=42) (Pearson's Chi-Squared Test)

Parameters		P 16 Status/ Number (%)		P value
		Positive	Negative	
Age	≤65years >65years	6(23.1) 5(31.3)	20(76.9) 11(68.8)	0.559
Smoking	Yes No	7(23.3) 3(33.3)	23(76.7) 8(66.7)	0.505
Type of surgery	Small biopsy Partial penectomy Total penectomy	5(45.5) 5(19.2) 1(20.0)	6(54.5) 21(80.8) 4(80.0)	0.239
Site	Glans Prepuce Shaft Glans and prepuce Glans and shaft	6(27.3) 1(33.3) 3(60) 0(0.0) 1(17)	16(72.7) 2(66.7) 2(40) 6(100) 5(83)	0.458
Differentiation	Well Moderate Poor	2(14.3) 9(33.3) 0(0.0)	12(85.7) 18(66.7) 1(100.0)	0.351
Pathologic stage	Not staged pT1 pT2-4	4(40.0) 2(18.2) 3(18.8)	9(60.0) 9(81.8) 13(81.3)	0.316
LVI	Yes No	2(28.6) 9(25.7)	5(71.4) 26(74.3)	0.875
PNI	Yes No	3(27.3) 8(25.8)	(72.7) 23(74.2)	0.924

Table 5 describes association of p16 status with clinicopathological parameters.

Majority of p16 negative cases had more radical surgery, like 80% partial penectomy and total penectomy. Moreover, 80% of advanced(pT2-4) case were p16 negative.

TABLE 6- Association of CD8 status with clinicopathological parameters(N=42) (Kruskal Wallis Test and Man Whitney U Test)

Parameters		CD8 Density Median (IQR)	P value
Age	≤65years >65years	30(30,50) 45(13,60)	0.979[1]
Smoking	Yes No	20(30,60) 40(20,60)	0.690[1]
Type of surgery	Small biopsy Partial penectomy Total penectomy	40(20,60) 30(30,50) 30(30,30)	0.661[2]
Site	Glans Prepuce Shaft Glans and prepuce Glans and shaft	35(20,60) 60(10,80) 60(35,65) 35(18.75,52.5) 30(17.5,32.5)	<0.001[2]
Differentiation	Well Moderate Poor	30(30,40) 40(20,60) 60(60,60)	0.330[2]
Pathologic stage	Not staged pT1 pT2-4	30(20,60) 40 (30,50) 30 (20,55)	0.947[2]
LVI	Yes No	50(30,60) 30 (20,60)	0.274[2]
PNI	Yes No	50(20,60) 30 (20,60)	0.478[2]

1- Man Whitney U Test

2- Kruskal Wallis Test

Tissues from prepuce and shaft had higher CD8 score compared to other sites($p<0.001$). Poorly differentiated cases had higher (median-60) CD8 scoring. No other parameters revealed any statistically significant association.

TABLE 7- Inter-Correlation of CD8, P16, PDL and HPV (N=42)

(Spearman's rho Test)

Parameters			**PD-L1**	**CD8**	**p16**	**HPV**
Parameters	PD-L1	Correlation Coefficient	1.000	-0.054	-0.022	0.047
		P Value	.	0.735	0.889	0.768
	CD8	Correlation Coefficient	-0.054	1.000	0.201	0.213
		P Value	0.735	.	0.201	0.176
	p16	Correlation Coefficient	-0.022	0.201	1.000	0.304
		P Value	0.889	0.201	.	**0.049**
	HPV	Correlation Coefficient	0.047	0.213	0.304	1.000
		P Value	0.768	0.176	0.050	.

PDL1 was negatively correlated with CD8 and p16 and positively correlated with HPV. CD8 was positively correlated with p16 and HPV. There was significant positive correlation between HPV and p16(p=0.049). No other parameters revealed any statistically significant correlation.

TABLE 8- Correlation of tumour size and depth of invasion with CD8, P16, PDL and HPV (N=31)

(Spearman's rho Test) (Small biopsies have been excluded)

Parameters	**PDL1**		**CD8**		**P16**		**HPV**	
	Correlation Coefficient	P Value	Correl-ation Coeffi-cient	P Value	Correl-ation Coeffi-cient	P Value	Correl-ation Coeffi-cient	P Value
Tumour size	-0.229	0.216	-0.170	0.360	0.164	0.376	0.061	0.741
Depth of Invasion	-0.093	0.616	-0.140	0.452	0.100	0.589	0.016	0.929

Table 8 describes correlation of HPV, p16, PDL1 and CD8 status with tumour size and depth of invasion. Both tumour size and DOI were negatively correlated with PDL1 and CD8, positively correlated with p16 and HPV. However, parameters were not statistically significant.

DISCUSSION

This was an ambispective hospital-based observational study conducted in the Department of Pathology and Lab Medicine at All India Institute of Medical Sciences, Jodhpur from January 2017 to July 2022 in patients diagnosed with PeSCC. Due to the COVID-19 situation, we received a total of 42 diagnosed cases of penile invasive squamous cell carcinoma only.

The clinico-epidemiologic profile and patient characteristics are assessed in present study and examined histopathologically, tumour sections were reviewed and an appropriate section was chosen for IHC. Expression of PD-L1, CD8, p16, and HPV status were assessed in tumour cells on IHC. Correlation and association between the clinico-pathological parameters and IHC markers were done.

Clinico-pathologic parameters

Penile cancer is an unusual malignancy with higher incidence in developing countries like India, as compared to the western world(23).

The association of penile cancer has been demonstrated with factors like poor hygiene, phimosis,

smoking, and balanitis xerotica obliterans; however, it has a definite causal link with HPV infection. HPV is a known risk factor for penile cancer. However, studies evaluating its true association are limited(1).

The overall reported prevalence of HPV infection in PC is about 48%. However, the prevalence ranged widely based on the type of histological variant. Penile Carcinoma arises from precursor lesions caused by HPV infection, in a step wise progression. The reported prevalence of HPV in Penile Carcinoma in the literature is varied depending on the geography, HPV subtypes evaluated, and the different techniques of DNA isolation(58).

The PD-1/PD-L1 immune checkpoint pathway is one of the major targets of a new generation of immunotherapeutics. So, in present study, we have applied IHC PDL1 to evaluate its presence in tumours and TILs and CD8 to check the immune response against the tumour. We haveperformed immunohistochemistry HPV and its surrogate marker p16 to check HPV-associated penile carcinomas(62).

Age

Present study showed 26 cases (69.1%) in ≤ 65 years age group and 16 cases (38.1%) in 65 years age group. The mean age of the cases affected by Penile Carcinoma was 59.69 years with a standard deviation of 12.94 years. Present study results are in concordance with that of Cocks' et al which reported a mean age of 65 years in 53 cases of penile carcinoma and with study done by Muller et al which analysed a cohort of 60 cases of penile carcinomas where in the average age was between 41–85 years (59, 69). Similar study was done by Martin et al in 2020(47) in which mean age of patients at the time of diagnosis were 57.4 years ranging from 20 to 90 years. In present study the mean age is 59 years, indicating that advanced penile squamous cell carcinomas affect higher age group.

Smoking

In present study population, 30 cases (71.4%) were tobacco smokers. Smoking has proved to be a well-known risk factor in multiple studies. Daling et al demonstrated that cigarette smoking was associated with a 4.5-fold risk of invasive penile cancer(70). Harish et al in 1995 did a study on Indian population describing the role of tobacco use as risk factor in early invasive penile carcinoma. They found 229 of 503 patients (45%) with a history of smoking(71). Present study showed higher prevalence of smoking with respect to penile carcinomas as noted by western and Indian studies.

Site and focality

Present study highlighted glans as the commonest site involved by tumour in more than half (52%) of patients, followed by shaft of penis (11.9%) and around one fourth of the patients (28.6%) had multifocal involvement. Similar study done by Lorga et al showed, that glans as the most common site of penile carcinoma, accounting for up to 48% of cases, followed by the prepuce (21%), glans and prepuce (9%), coronal sulcus (6%) and uncommonly the penile shaft 2%)(8). This is in concordance with review of penile SCC in the U.S. data which showed 34.5% of patients had the primary lesion on glans, 13.2% on prepuce and 5.3% on the shaft(72).

Tumour grade

In present study, 14 cases of penile SCC were well differentiated constituting 33.3 % of total cases. 27 cases showed moderate differentiation comprising of 64.3% of total cases.1 case showed poorly differentiated histology comprising of 2.4 % of total cases. Histological grade has been consistently reported as an influential predictive factor of groin metastasis and dissemination of penile cancer in literature. Chaux et al did a study on penile carcinoma wherein 4 (12.1%) cases were classified into grade G1, 19 cases (47%) were graded as G2 and 10 (30.3%) cases were graded as G3(54). Bacco et al studied 35 patients, out of which 13 (37%) were classified as Grade 1, 19 (54.2) were classified as Grade 2 and 3(8.5%) were classified as Grade 3(54,62). Present study showed moderately differentiated carcinomas (G2) (64.3%) as the most common histological grade which is in concordance to other studies as cited above.

Pathological stage

The CAP (2017) protocol recommends the use of the TNM staging system of the American Joint Committee on Cancer (AJCC) for carcinoma of the penis (30). Present study categorized 27 cases for pathological stage. 21.4% cases (9/42) were categorized as pT3, 16.7% (7/42) cases were classified as pT1a, 9.5% (4/42) as pT1b, 14.3% (6/42) were pT2 and (1/42) was pT4. Similar study done by Muller et al in a cohort of 60 patients, showed 23 cases as T1a, 7 cases were categorized as T1b, 23 cases were categorized as pT2, 3 cases were categorized into pT3 and 2 patients were categorized as pT4(69). Pathologic staging is usually performed after surgical resection of the primary tumour. In present study, a total of 15(35.7%) cases were not staged. This was because either tissue was from a small biopsy (excluded according to CAP protocol) or unavailability of data.

Lymphovascular invasion (LVI) and perineural invasion (PNI)

In present study, 7 cases out of 42 (16.7%) showed the presence of LVI and 35 cases (83.3%) out of 42 did not show LVI. Study by Frankhauser et al(23) in 2022 showed 554 men with T1G2 penile cancer, pooled from 6 European institutions. ILN metastases were observed in 46/554 men (8.3 %). This is also in concordance with study done by Chengbio chu et al(51) which analysed 158 cases, out of which 20 (11.6%) cases showed presence of LVI.

In present study 11 cases, out of 42(26.2 %) were showing perineural invasion (PNI). Study by Chengbio chu et al showed 27 cases (15.7%) with nerve invasion. Meta-analysis by Zhou et al(72) in 2018 demonstrated that out of total 1001 PC patients, 298 patients (29.7 %) presented with PNI. Study done by Elsa et al in 2008 showed, perineural invasion in 48 of 134 cases (36%), and groin metastasis was found in 33 cases (69%) of these cases showing perineural invasion(73). The range of LVI and PNI in cited literature and in present study shows concordance. In present study total number of small biopsies were 31. None of didn't show presence of LVI or PNI. In view of a smaller number of cases, the statistical association of LVI and PNI in present study is not significant.

Expression of PDL1

In present study out of 42 cases, positive expression of PDL1 was noted in 19(45.2%) cases. Expression of PD-L1 in stromal immune cells exclusively was identified in 4 cases (17%) whereas expression of PD-L1 in tumour cells and TILs was in 19 cases (83 %) cases. This is in harmony with study done by Cock's et al in which PD-L1 was expressed in 21/53 (40%) of penile SCCs(59). In their study, expression of PD-L1 in stromal immune cells was identified in 26% (14/53) of cases and 39 cases out of 53 showed tumour positivity for PD-L1. Similar study done by Bacco et al show expression of PD-L1 in 35 patients, 18 cases (51.4%) expressed positivity for PD-L1. Similarly, Davidsson et al hypothesized in 2018 in a well-defined penile SqCC cohort of 222 patients was evaluated for PD-L1 expression on tumour cells and TIICs. (74)32.1% of the tumours and 64.2% of the TII cells expressed PD-L1 in their study. Present study revealed a slightly average percentage(45.2%) of tumour PD- (compared to other reports on penile cancer (range 40–62%). Results from other PC studies investigating PD-L1 expression on immune cells have been more variable, ranging from 26% to nearly 80%; our

findings for PD-L1 (positivity was both in tumour and tumour infiltrating cells) are within this range(17,29).

In present study, CPS(Combined positive score) was assigned to each positive case in which 14.3 % cases had CPS of 30. 23.8 % had CPS of 20. Majority cases (10 out of 42 cases)were assigned CPS score of 20, followed by CPS score of 30 observed in 14.3% cases (6/42). This is in concordance with Montella et al which analysed 72 PC cases and found 57 cases (79%) to be positive for PD-L1. Among these, 18 cases (25%) had CPS score between 1 to <20 and 39 cases (54%) had CPS score of >20 (75).

In present study, 11 out of 42 cases (42.3%) were of age < 65 years that expressed PD-L1. 8 cases out of 42 (50%) were of age > 50 years that expressed PD-L1. However, no statistical significance or association was not obtained (p= 0.627) probably due to lesser number of cases. In present study, expression of PD-L1 between smokers and non-smokers was assessed. 12 smokers (40%) out of 30 showed expression of PD-L1. 7 cases (58.3%), non-smokers showed expression of PD-L1. No statistical association between PD-L1 and smoking (p = 0.281) was found. In present study, 10 cases (71.4%) out of 42 showed well differentiated PC and PD-L1 positivity. 8/42 cases (29.6%) were positive for PD-L1 and showed histological grade (G2) as well as PD-L1 positivity. 1 case showed poorly differentiated PC and showed positive expression of PD-L1. Significant association was noted between PD-L1 and histological grade. **(p=<u>0.021</u>).**

In present study, 22 cases showed presence of tumour in glans, out of which 10 cases (45.5%) were positive for PD-L1. 3 cases out of 5 (60.0%) showed presence of tumour in shaft and positivity of PD-L1. 4 cases out 6 cases (66.7%) showed presence of tumour in glans and prepuce and PD-L1 positivity. 3 cases out 42(60.0%) had presence of tumour in prepuce, but didn’t show expression for PD-L1.

Total 7 cases out 42 cases showed presence of LVI as mentioned above (Table 1). 2 (28.6%) show presence of LVI as well immune reactivity for PD-L1 and 17 cases (48.6%) were immune reactive for PD-L1 but did not show any presence of LVI. 3 cases out of 11 (27.3%) showed PD-L1 expression and presence of PNI. The correlation coefficient of PD-L1 and tumour is -0.229 with a p value (p=0.216). The correlation coefficient of PD-L1 and depth of Invasion is (-0.093) with (p=0.616).

So, in present study PD-L1 is associated with histological grade. Differentiation status significantly improves with PDL1 positivity(p=0.021). Similar study by Davidsson et al(74) in 2019 showed PD-L1 positivity in tumour cells to be associated with higher tumour grade and more advanced stage. They noted 48.48% cases of grade 2 (moderately differentiated tumour) which expressed PD-L1.

Other clinicopathological parameters revealed no statistically significant association, due to a smaller number of cases. No association of PD-L1 was noted with smoking, type of surgery, pathological stage, site, LVI, PNI, HPV status and p16. In study done by Cocks et al(59) PD-L1 expression did not correlate with patient age, tumour location, histologic subtype, tumour stage, anatomic depth of invasion or tumour grade.

Expression of HPV and p16

p16 positivity has a high correlation with HPV 16 DNA detection, commonly used nowadays as a surrogate marker for HPV-driven cancers(57). In present study, We noted the expression of IHC HPV and p16 in confirmed cases to see the association of penile squamous cell carcinoma and HPV. In present study, 31% were HPV positive cases and 26.2% were p16 positive cases. An analysis done by Martin et al on 52 cases showed a pooled prevalence of 50.8% (44.8–56.7) of HPV infection in PC with a rate of 68.3% (58.9–77.1) of HPV16 (14). Present study is in concordance with study done by Eich et al which did morphological study and correlated with IHC. 46% of tumours displayed an HPV-related subtype, while p16 was positive in 52% of all cases. Therefore, mean range of expression of HPV was around (4-60%) in that study(57). 8 patients out of 26 (30.8%) were in the age group of <65 years and were positive for expression of HPV. 5 patients (31.2%) out of 16 were above the age of 65 years and expressed HPV. 18 cases out of 26(69.2%) were within the age group of <65 years and did not express HPV. 11 patients (68.8%) out of 16 were in the age group of >65 years and did not express HPV. 6 patients (20%) out of 30 were smokers and gave nuclear positivity for HPV.7 cases (58.3%) out of 12 were non-smokers and expressed HPV. In present study, a significant association between smoking and HPV are noted (p= 0.015).

11 cases in present study were small biopsies, 5 (45.5%) of which expressed HPV whereas 6 biopsies were negative for HPV. HPV is expressed in 5 cases (35.7%) out of 13 cases expressed HPV and are grade 1(well differentiated tumors). 8 patients (29.6%) out of 27 cases expressed

HPV. None expressed HPV, which showed features of poorly differentiated. This finding suggests that well differentiated tumors have tendency to show HPV positivity. However, due to smaller number of cases no statistical significance was seen. The findings of present study were in concordance with study done by martin et al in which 70% (23/33) of the cases showed well and moderately differentiated tumours (Grade I/II) and were not associated with HPV infection (adjusted p-value = 0.006).

7 cases (31.8%) out of 22 showed presence of tumour in glans with HPV positivity. There is no significant association between site and HPV positivity (p=0.215). 4 cases (36.4%) out of 11 were pT1 and expressed HPV. 3 cases (18.8%) out of 16 fell in pT2-pT4 stage and expressed HPV. 24 cases (68.6%) out of 35 were HPV positive and show histological. 3 cases (60.0%) out of 5 cases are warty type and expressing HPV.1 case (100.0%) of basaloid type and 1 case (100.0%) of verrucous variant expressing HPV. Similar study done by Rubin et al(9) in 2001 have found that only a third of penile cancers are related to HPV and that HPV is preferentially associated with warty, basaloid, and high- grade tumours and not with typical SCC, papillary, or verrucous carcinomas

2 cases (28.6 %) out of 7 showed presence of LVI and expression of HPV. 11 cases (31.4%) out of 35 did not show presence of LVI but showed expression of HPV. While 4 cases (36.4%) showed presence of PNI and expression of HPV.

In present study, 6 cases (45.5%) out of 11 were positive for both p16 and HPV. 24 cases (77.4%) were negative for p16 and HPV. 7 cases (22.6%) out of 11 showed expression of HPV but were negative for p16. A significant association of p16 and HPV were noted in present study (p=**0.049**). Study done by Eich et al showed concordance with the present study in which tumour histology correlated well with p16 positivity (p<0.001) and p16 IHC accurately predicted the presence of HPV in 25/26 (96%) cases(57).

In present study, 26.2% cases (11/42) showed expression of p16. Number of patients showing expression of p16 in patients are 6 cases (23.1%) out of 42. 6/26 cases (23.1%) show positivity for p16 and were in the age group of <65 years. (5/'[42) (31.3%) showed positive expression of p16, and were within the age group of >65 years. 7 cases (23.3%) were smokers and showed block positivity for p16. While 3/11(33.3%) cases are non-smokers but show p16 positivity(p=0.505). Similar study was done by Martin et al which showed p16 overexpression in 12 cases of which 8 cases were smokers and 4 were non-smokers (14).

However, no association was noted between p16 and smoking. 2 cases (14.3%) out of 14 showed well differentiated PC and p16 positivity. 9 cases (33.3%) out of 27 showed presence of moderately differentiated PC. 1 case was poorly differentiated but did not express p16. In current study no association between histological grade and p16 was noted. Also, no association between type of surgery and p16 was noted (p=0.351). 2 cases (28.6%) out 7 showed presence of LVI and positive expression of p16, while 9 cases (25.7%) out of 35 showed presence of LVI but expression of p16 was negative. 3 cases (27.3%) out of 11 showed presence of PNI and expression of p16.

Relation of PD-L1, HPV and p16

The present study showed an inverse relationship between PD-L1 and p16 (Correlation coefficient (-.022). 13 cases (68.4%) were negative for PDL1 and HPV and 9 were positive for PD-L1 and HPV. HPV-negative PC cases more likely showed PD- L1–immunoreactive tumour cells. This finding showed concordance with study done by Ottenhof et al(53), which studied the higher number of diffusely PD-L1 positive tumours in the hrHPV negative group of their cohort, however, it matches the hypothesis that a more mutated tumour type will have higher T-cell inhibition, partially having poorer survival. However, no significant correlation was noted between PD-L1, p16, HPV, site of tumour, LVI, PNI, tumour differentiation, pathological stage and type of surgery.

Expression of CD 8

In the present study, mean CD8 density score in all of the cases was 39.17 with a SD of 20.775. In the age group of ≤65years median CD8 density was 30(30,50), on the other hand in >65 years age group CD8 density was more with a median of 45(13,60). However, this difference was not statistically significant. (p=0.979). In a recent study by Hladek et al (2022)(76), they could not demonstrate any variability of CD8 density according to difference in age group. In present study, CD8 density was less in smokers with a median of 20(30,60), compared to non-smokers with a median of 40(20,60). Despite this difference, statistical significance was not obtained (p=0.661). Present study could not find any reference depicting the effect of smoking on CD8 density in penile carcinoma cases. Smoking has been proven to have a distinct immunosuppressor effect(77). Therefore, this may explain, reduced density of CD8 cells in PC tissues in case of smokers. In present study, we analyzed CD8 density according to type of

surgery cases of small biopsy [median 40(20,60)] was marginally higher compared to partial penectomy [median 30(30,50)] and total penectomy [30(30,30)] cases. This difference was not statistically significant($p=0.661$). However, present study depicted significantly differential density of CD8 cells in different site of tumour origin. Tumours from prepuce and shaft had high CD8 density with median of 60(10,80) and 60(35,65) respectively. On the other hand, in tumours from glans median density of CD8 was [35(20,60)] and multifocal cases [35(18.75,52.5), 30(17.5,32.5)] had lower density. This difference was statistically highly significant($p<0.001$). An extensive literature search revealed no reference that examined the above-mentioned parameter. Present study may be first to report a highly differential CD8 density according to variability in site of origin in case of penile carcinoma. In the present study, well differentiated tumours had a CD8 density of 30(30,40), moderately differentiated had CD8 density of 40(20,60) and poorly differentiated had CD8 density of 60(60,60). However, this difference was not statistically significant. Hladek et al (2022)(78), in a study of 55 therapy naïve penile carcinoma cases did not find any significant difference in CD8 density according to grading of the tumour. Cocks et al (2016) studied CD8 density separately in tumour lymphocytes and stromal lymphocytes. They also reported no significant correlation with grading. Present study could not demonstrate any significant association of T stage with differential CD8 density. pT1 tumours had a median density of 40 (30,50). Advanced cases (pT2-4) had a median density of 30 (20,55). Cocks et (2016)(59) and Hladek et al (2022)(78) also did not find any correlation of tumour stage with CD density. However, Hladek et al (2022)(78) showed a significant negative correlation of CD3 score with tumour staging($p=0.03$). CD8 score, in the present study, LVI and PNI positive cases had more CD8 density [50(30,60), 50(20,60)] compared to negative cases [30 (20,60), 30 (20,60)]. Although, this was not statistically significant. Above mentioned studies by Cocks et (2016) and Hladek et al (2022) also reported that CD8 scores did not have any correlation with LVI and PNI.(59)

Correlation PD-L1 and CD8

In literature it has been well demonstrated that the PD-1/PD-L1 axis plays a crucial role in tumour immune evasion(62). PD-L1 can be found on tumour cells or infiltrating immune cells. So present study tried to correlate PD-L1 and tumour infiltrating lymphocytes. Tumour immune cells are correlating with tumour site, Glans is the most common site for penile

carcinoma. It has been observed that more CD-8 infiltrating immune cells are present in case where glans is the common site for tumour **(p=<0.001).** Tumour infiltrating lymphocytes had a negative correlation with PD-L1 (Coefficient -0.054). No other statistically significant correlation or association was noted. Study done by Deng et al demonstrated(79) expression pattern of PD-L1 in PeSCC tumour cells and TILs as well as their association with common clinicopathological features and CSS. The expression of PD-L1 in TILs was significantly correlated with nodal status, grade (p = 0.012), extent of TILs (p D 0.002) and CD8 positive and TILs (p =0.001). The present study did not get any statistical association of PD-L1 and CD8.

SUMMARY

This study was an ambispective study evaluating incidence of PDL1, p16, HPV and density of CD8 in histopathologically confirmed tissues of penile carcinoma and correlated them with clinicopathological parameters. The salient findings are summarized below:

- Majority (61.9%) of all patients were of 65 years or more in age.
- 71.4% of all patients were addicted to smoking tobacco.
- Commonest (61.9%) surgery undergone by patients was a partial penectomy, followed by a small biopsy (26.2%). Few (11.9%) patients had undergone total penectomy.
- Glans was the commonest site involved in more than half (52%) of patients, followed by shaft of penis (11.9%). Only 7.2% had involvement in prepuce and around one fourth (28.6%) had multifocal involvement.
- One third (33.3%) of all tumors were well differentiated and 64.3% were moderately differentiated.
- Evaluation of pathological staging revealed majority of patients to be in(38%) pT2-pT4, while 35.5% patients could not be staged based on available record. 26.2% cases were in pT1 stage.
- Median size of tumors (excluding cases of small biopsy) was 26.25 cubic centimeter with a interquartile range of 6.07 cm^3 and 58.3 cm^3.
- Mean depth of invasion (excluding cases of small biopsy) was 2.66 cm with SD of 1.41cm.
- Most of the cases were negative for lymphovascular (83.3%) and peri neural (73.8%) invasion.
- IHC of PDL1, HPV and p16 were positive in 54.8%, 31% and 26.2% cases respectively.
- Mean CD8 score in IHC study was 39.17 with a SD of 20.78%.
- Differentiation status significantly improves with PDL1 positivity(p=0.021). Most of the LVI (71.4%) and PNI (72.7%) positive cases were, PDL1 negative. However, this difference was not statistically significant.
- Most smokers are HPV negative(p=0.015). Most HPV negative tumors were also p16 negative(p=0.049). Majority of HPV negative cases had more radical surgery, like 69.2% partial penectomy and all cases of total penectomy. Moreover, 81.3% of

advanced(pT2-4) case were HPV negative. However, these differences were not statistically significant.

- Majority of p16 negative cases had more radical surgery, like 80% partial penectomy and total penectomy. Moreover, 80% of advanced(pT2-4) case were p16 negative. However, no parameters revealed any statistically significant association.
- Tissues from prepuce and shaft had higher CD8 score compared to other sites($p<0.001$).
- PDL1 was negatively correlated with CD8 and p16 and positively correlated with HPV. CD8 was positively correlated with p16 and HPV. However, these correlations were not statistically significant. There was significant positive correlation between HPV and p16($p=0.049$).
- Both tumor size and DOI were negatively correlated with PDL1 and CD8, positively correlated with p16 and HPV. However, none of the parameters revealed any statistically significant correlation.

CONCLUSION

- In conclusion, Majority (61.9%) of all patients were of 65 years or more in age and 71.4% of all patients were addicted to smoking tobacco. Glans was the commonest site involved in more than half (52%) of patients, followed by shaft of penis (11.9%). One third (33.3%) of all tumors were well differentiated and 64.3% were moderately differentiated. Evaluation of pathological staging revealed majority of patients to be in (38%) pT2-pT4, while 35.5% patients could not be staged based on available record. Most of the cases were negative for LVI (83.3%) and peri neural (73.8%) invasion. IHC of PDL1, HPV and p16 were positive in 54.8%, 31% and 26.2% cases respectively. Mean CD8 score in IHC study was 39.17 with a SD of 20.78%. Differentiation status significantly improved with PDL1 positivity($p=0.021$). Most smokers were HPV negative($p=0.015$). Most HPV negative tumors were also p16 negative($p=0.049$). Tissues from prepuce and shaft had higher CD8 score compared to other sites($p<0.001$). Other clinicopathological association and correlations were not significant.

Limitations of study

1.Number of cases were less (N=42)due to COVID-19 situation

2. Subtyping of HPV into high risk type and low risk could not be done due to limited budget constraint.

3. Survival rate could not be assessed because of no follow up of patients due to COVID -19 situation.

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Annexure 1

Ethical Clearance Certificate

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर

All India Institute of Medical Sciences, Jodhpur

संस्थागत नैतिकता समिति

Institutional Ethics Committee

No. AIIMS/IEC/2021/ 3549 **Date: 12/03/2021**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2021/3384**

Project title: **"Expression of PDL1, CD8, P16 and HPV in penile carcinomas and their correlation with clinicopathogical parameters"**

Nature of Project:	**Research Project Submitted for Expedited Review**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr. Deepsikha Bhanja**
Guide:	**Dr. Jyotsna Naresh Bharti**
Co-Guide:	**Dr. Poonam Abhay Elhence, Dr. Meenakshi Rao, Dr. Deepak Vedant, Dr. Gautam R Choudhary & Dr. Mahendra Singh**

Institutional Ethics Committee after thorough consideration accorded its approval on above project.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

Please Note that this approval will be rectified whenever it is possible to hold a meeting in person of the Institutional Ethics Committee. It is possible that the PI may be asked to give more clarifications or the Institutional Ethics Committee may withhold the project. The Institutional Ethics Committee is adopting this procedure due to COVID-19 (Corona Virus) situation.

If the Institutional Ethics Committee does not get back to you, this means your project has been cleared by the IEC.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member Secretary

Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
अनुसंधान अनुभाग
Research Section

No.: AIIMS/RES/2021/6486 Dated: 11/09/21

To
Dr. Jyotsna Naresh Bharti
Associate Professor,
Department of Pathology,
AIIMS, Jodhpur

Subject: Transfer of principle guide responsibility of ongoing MD thesis of Dr. Deepsikha Bhanja, July 2020 Batch.

Dear Dr. Jyotsna,

This is in reference to your letter no. AIIMS/JDP/PATH/5196/2021 dated 31/08/2021. I am directed to inform you that Dean (Research) has accorded his permission to appoint Dr. Deepak Vedant as a Guide for MD Thesis of Dr. Deepsikha Bhanja titled "Expression of PDL-1, CD8, P16, and HPV in penile carcinoma and their correlation with clinicopathological parameters", if he is eligible for guideship as per the institute guidelines.

Dr. Jaykaran Charan
Sub Dean (Research)
Sub Dean (Research)
All India Institute of Medical Sciences
Jodhpur (Raj.) 342005 India

Copy to:
1. Dr. Deepak Vedant (Guide), Assistant Professor, Dept. of Pathology, AIIMS, Jodhpur.
2. Dr. Poonam Elhence, Professor & Head, Department of Pathology, AIIMS, Jodhpur.
3. Dr. Deepsikha Bhanja, PG Student.
4. Member Secretory, IEC
5. Dean (Academics)

14/09/2021

Annexure 2

Informed consent form(English)

All India Institute of Medical Sciences
Jodhpur, Rajasthan
Informed consent form

Title of the project: Expression of PDL1, CD 8, HPV high-risk type, and p16 in penile carcinoma its correlation with clinicopathological parameters.

Name of the Principal Investigator: **Dr. Deepsikha Bhanja Tel. No. 9679849059**

Patient/Volunteer Identification No. : _____

I, _____ S/o or D/o _____
R/o

_____
give my full, free, voluntary consent to be a part of the study "_____
_____", the procedure and nature of which has been explained to me in my own language to my full satisfaction. I confirm that I have had the opportunity to ask questions.

I understand that my participation is voluntary and is aware of my right to opt-out of the study at any time without giving any reason.

I understand that the information collected about me and any of my medical records may be looked at by responsible individual from _____ (Company Name) or from regulatory authorities. I give permission for these individuals to have access to my records.

Date: _____ _____

Place: _____ Signature/Left thumb impression

This to certify that the above consent has been obtained in my presence.

Date: _____ _____

Place: _____ Signature of Principal Investigator

Witness 1

_____

Signature

Name: _____

Address: _____

2. Witness 2

_____

Signature

Name: _____

Address: _____

Annexure 3

Informed consent form(Hindi)

All India Institute of Medical Sciences

Jodhpur, Rajasthan

Informed consent form (Hindi)

थीसिस/निबंध का शीर्षक: पेनाइल कार्सिनोमा में पीडीएल 1, सीडी 8, पी 16 और एचपीवी हाई-रिस्कप्रकार का अभिव्यक्ति, और क्लिनिको-पैथोलॉजिकल मापदंडों के साथ इसका संबंध।

पीजी छात्र का नाम: डॉ. दीपसिखा भंज। **टेलन**: 9679849059

रोगी / स्वयं सेवक पहचानसंख्या: _____

मैं, _____पुत्र/ पुत्री_____

पता_____

अध्ययन "_____" का एक भाग बनने के लिए मेरी पूर्ण, स्वतंत्र, स्वैच्छिक सहमति दें, जिसकी प्रक्रिया और प्रकृति मुझे अपनी पूरी संतुष्टि के लिए अपनी भाषा में समझाई गई है। मैं पुष्टि करता हूं कि मुझे प्रश्न पूछने का अवसर मिला है।

मैं समझता हूं कि मेरी भागीदारी स्वैच्छिक है और मुझे किसी भी कारण दिए बिना किसी भी समय अध्ययन से बाहर निकलने के मेरे अधिकार की जानकारी है।

मैं समझता हूं कि मेरे और मेरे मेडिकल रिकॉर्ड के बारे में एक त्रितकी गई जानकारी को _____ (कंपनी नाम) या विनियामक प्राधिकरणों से जिम्मेदार व्यक्ति द्वारा देखा जा सकता है। मैं इन व्यक्तियों को अपने अभिलेखों तक पहुंच के लिए अनुमति देता हूं I

तारीख: _____

जगह: _____ हस्ताक्षर / बाएं अंगूठे का छाप_____

यह प्रमाणित करने के लिए कि मेरी उपस्थिति में उपरोक्त सहमति प्राप्त की गई है I

तारीख: _____

जगह: _____ पीजी छात्र के हस्ताक्षर_____

Annexure 4

Patient Information Sheet(English)

PATIENT INFORMATION SHEET

1. Risks to the patients: No interventions or life-threatening procedures will be done.
2. Confidentiality: Your participation will be kept confidential. Your medical records will be treated with confidentiality and revealed only to doctors/ scientists involved in this study. This study's results may be published in a scientific journal, but you will not be identified by name.
3. Provision of free treatment for research-related injury is not applicable.
4. Compensation of subjects for disability or death resulting from such injury: Not Applicable
5. Freedom of the individual to participate and to withdraw from the research at any time without penalty or loss of benefits to which the subject would otherwise be entitled.
6. You have complete freedom to participate and to withdraw from the research at any time without penalty or loss of benefits to which you would otherwise be entitled.
7. Your participation in the study is optional and voluntary.
8. The copy of the results of the investigations performed will be provided to you for your record.
9. You can withdraw from the project at any time, which will not affect your subsequent medical treatment or relationship with the treating physician.
10. Any additional expense for the project, other than your regular expenses, will not be charged to you.

Annexure 5

Patient Information Sheet(Hindi)

रोगी सूचना पत्रक

1. रोगियों के लिए जोखिम कोई हस्तक्षेप या जीवन धम की प्रक्रिया नहीं की जाएगी।

2.गोपनीयता आपकी भागीदारी को गोपनीय रखा जाएगा। आपके मेडिकल रिकॉर्ड को गोपनीयता के साथ इलाज किया जाएगा और केवल इस अध्ययन में शामिल डॉक्टरों / वैज्ञानिकों को पता चलेगा। इस अध्ययन के परिणाम एक वैज्ञानिक पत्रिका में प्रकाशित हो सकते हैं, लेकिन आपको नाम से पहचान। नहीं जाएगा।

3. अनुसंधान संबंधी चोट के लिए नि: शुल्क उपचार की व्यवस्था लागू नहीं।

4. ऐसी चोट से उत्पन्न विकलांगता या मृत्यु के लिए विषयों का मुआवजा लागू नहीं है।

5.किसी भी समय दंडया लाभों के नुकसान के बिना किसी भी समय भाग लेने के लिए व्यक्ति को स्वतंत्रता लेने औरअनुसंधान सेवा पस लेने के लिए स्वतंत्रता, जिसके तहत विषय अन्यथा हकदार होगा

6.आपकोजुर्माना या लाभ के नुकसान के बिना किसी भी समय भाग लेने और अनुसंधान सेवा पसलेने की पूरीआजादी है, जिस पर आप अन्यथा हकदार होंगे।

7. अध्ययन में आपकी भागीदारी वैकल्पिक और स्वैच्छिक है।

8. प्रदर्शन की जांच की परिणामों की प्रति आपके रिकॉर्ड के लिए आपको उपलब्ध कराई जाएगी।

9. आप किसी भी समय परियोजना सेवा पसले सकते हैं, और यह आपके बाद के चिकित्सा उपचारया उपचार चिकित्सक के साथ संबंध को प्रभावित नहीं करेगा।

10. परियोजना के लिए कोई भी अतिरिक्त व्यय, आपके नियमित खर्चों के अलावा, आपसे शुल्क नहीं लिया जाएगा।

Annexure 6

All India Institute of Medical Sciences (AIIMS), Jodhpur

Department of Pathology

PROFORMA

Date: **I.D.:**

Name:

Age/ Sex:

Address:

Relevant clinical History:

a. Smoking
b. Bleeding from or under the foreskin
c. Any growth in the penis
d. Multiple sexual partners
e. Chemotherapy/Radiotherapy

Gross/ Histopathology Features [CAP Protocol 2017]

- Presence of Foreskin
- Tumor site/size
- Growth pattern
- Tumor Deep Borders
- Unifocal / Multifocal
- Tumor Thickness/Depth of invasion
- Typing / Differentiation/PeIN
- Tumour in surgical margin / Extension
- Pathological tumour staging [AJCC 8th Ed]
- Invasion- LVI/PNI/LN Metastasis
- Ancillary Study: Immuno-scoring/ Immunoexpression

PDL-1	Cytoplasmic or Membranous staining	>5% of both in tumor and in stroma/HPF
CD 8	Membranous	Number of CD8+ lymphocytes in the highest density area (hot spot) per HPF (X40) both in tumor and in the stroma.
p16	Complete Cytoplasmic and Nuclear staining	All tumor cells.
HPV	Nuclear staining	All tumor cells.

Clinical details:

Presentation

Imaging if any

Biopsy report

Surgery performed

Final histopathology report

Perioperative outcome

Follow up

Annexure 7
Master Chart

H-number	Age	Address	Smoking	Bleeding from	Any growth in	Multiple sexu	Chemotherap	Type of surg	Tumor size	Localisation	Growth patte	DOI	Unifocal/Multi	Histological	Differentiation	Tumor in surg	T stage	LVI	PNI	PDL1	CD8	p16	HPV	pdl1tls	Combine positive	Immune cell infiltrate	
H/2980/17		46 Mallya ka bas, Jo	Yes	NA	Yes	NA	Yes	Partial penectomy	6x4x4 cms	ulceroproliferative	growth involving	4 cms	Unifocal	usual	Moderately differe	Free of tumour	pT1b	Yes	Yes	0	40	0	1		0	2	
H/3569/17		59 Fateh Garh, Jalor	Yes	NA	Yes	NA	Yes	Small biopsy	1x1x0.8	Body of penis	Ulcero-proliferati	NA	MULTIFOCAL	usual	Moderately differe	Free	pT1c	NO	No	0	60	1	0		0	3	
H/3385/17		31 Jalore	yes	No	Yes	NA	Yes	Partial penectomy	4.5x3x2 cm	shaft	Fungative	2 cm	Unifocal	usual	Well-differentiated	Free	pT1a	NO	No	40	40	0	0		40	2	
H/8916/18		50 Barmer	No	Yes	Yes	NA	Yes	Partial penectomy	4.5x3.5x3.7cm,	arising from prepu	Ulcero-proliferati	3.7cm	Unifocal	usual	Moderately differe	Free	pT1b	NO	Yes	0	80	0	1	positive	0	3	
H/8975/18		82 Soorsagar, , Jodh	Yes	NA	Yes	NA	NM	Partial penectomy	4.5x4.5x3.5cm	Tip and shaft	Ulcero-proliferati	3.5 cm	Multifocal	usual	Well-differentiated	Free	pT1b	NO	No	20	10	1	1		20	1	
H/5975.2019		53 Nagaur, Ladnu	No	No	Yes	NA	NM	Partial penectomy	3x3x1.2 cms	Glans penis	Ulcero-proliferati	1.2 cm	Unifocal	usual	Moderately differe	free	pT3	NO	Yes	0	50	0	1	positive10	0	2	
H/1375/2019		50 Jalore, Aksli	Yes	Yes	Yes	NA	NM	Partial penectomy	2.5x3x3.5cm	Glans penis	Fungative	3.5 cm	Multifocal	usual	Moderately differe	free	pT2	NO	Yes	0	50	0	0		0	2	
H/3380 /2019		47 Rabariyo Ka Ba	No	No	Yes	No	NM	Partial penectomy	4x3.5x3.5cm	Glans penis	Ulcero-proliferati	3.5 cm	Unifocal	usual	Well-differentiated	Free	pTx	NO	No	30	20	0	0		30	1	
H/2301/18		88 Mandore	Yes	No	Yes	NA	NM	Partial penectomy	4x2x1 cm	Glans Penis	Ulcerative	1 cm	Unifocal	usual	Moderately differe	NM	pT1b	NO	No	0	60	0	0		0	3	
H/4930/17		73 Jodhpur	No	Yes	Yes	NA	NM	Small biopsy	0.5x0.5x0.2cm	prepuce	Ulcero-proliferati	NA	Unifocal	usual	Moderately differe	NM	pTx	NO	No	0	10	1	1		0	3	
RH/2682/2018:		76 Chandpol, , Jodhj	Yes	No	no	Yes	NM	Partial penectomy	4x3.5x2cm	Glans Penis	Ulcerative	2 cm	Unifocal	usual	Moderately differe	NM	pTx	NO	No	0	10	0	0		0	1	
RH/561/19		83 Jodhpur,,Dev Na	yes	NA	Yes	NA	NM	Small biopsy	1.8x1.2x0.5cm ar	Glance	Ulcero-proliferati	NA	Unifocal	usual	Moderately differe	NM	pTx	NO	No	20	80	1	1		20	3	
H/6297/19		75 Rajiv Nagar ,Jod	yes	Yes	Yes	No	NM	Small biopsy	4x3.5x2cm	Glance	Verruciform	NA	Unifocal	usual	Well-differentiated	Free	pT1a	NO	No	20	40	0	0		20	2	
H/3109.2019		64 Khudiyala	Yes	Yes	Yes	No	No	Partial penectomy	4.8x3x3.5cm	Distal penis	Ulcero-proliferati	3.5 cm	Unifocal	usual	Well-differentiated	free	pT3	NO	No	30	30	0	1		30	1	
H/0118.03		46 Jhunjhunu	yes	No	Yes	Yes	NM	Partial penectomy	1.9x2x2.8cm	glans, prepuce	Fungative	2.8 cm	MULTIFOCAL	usual	Moderately differe	free	pT3	NO	No	20	20	0	0		20	1	
H/1624/17		64 Masuriya	yes	NA	yes	No	NM	Small biopsy	1.8x1.2x0.5cm and	1.2x0.8x0.4cm	Ulcerative	NA	Unifocal	Warty	Moderately differe	NM	pTx	Yes	Yes	0	20	1	0		0	1	
H/4360/17		54 Tinwari,,Jodhpur	Yes	No	Yes	No	NM	Partial penectomy	5.5x4x2.5cm	Glance	ulcerating and infi	2.5cm	Unifocal	usual	Moderately differe	Free	pTx	NO	Yes	0	30	1	0		0	1	
H/162/4/17		73 Nagaur	yes	Yes	Yes	NA	Yes	Small biopsy	1.8x1.2x0.5cm	Glans	ulcerating and infi	NA	Unifocal	usual	Moderately differe	NM	pTx	Yes	Yes	0	60	0	0		0	3	
H/74/18		42 PALI	yes	NA	Yes	NA	NM	Partial penectomy	4X2.8X0 CM	SHAFT	Ulcerative	3 cm	Unifocal	usual	Moderately differe	FREE	pT2	NO	No	0	70	1	0	positive5%	0	3	
H/2685/18		66 PALI	yes	NA	Yes	NA	Yes	Partial penectomy	3X1.5X1.2	PREPUCE	ulcerating and infi	1.2cm	Unifocal	Warty	Moderately differe	FREE	pTx	NO	No	0	60	0	1		0	3	
H/752/19		52 JHALOR	yes	NO	yes	NA	NM	Partial penectomy	6X5X4.5	DISTAL GLANCE	Ulcerative	4.5cm	Unifocal	usual	Moderately differe	FREE	pT3	NO	No	0	20	0	0		0	1	
H/137/20		50 NAGORE	no	NA	Yes	NA	NM	Total penectomy	2X1.5X1.7	GLANCE	ULCERATED INFILTRATIVE		Unifocal	usual	Well-differentiated	FREE	pT2	Yes	No	30	30	0	0		30	1	
H/853/21		66 PALI	No	NA	Yes	NA	Yes	Partial penectomy	4X4.3X0.5	GLANCE AND FC	PUSHING AND IN	5MM	MULTIFOCAL	usual	Moderately differe	FREE	pT1a	NO	No	20	20	0	0		20	1	
H/659/21		55 JODHPUR	yes	NA	yes	NA	NM	Partial penectomy	1.7X1.7X0.3	GLANCE	FLAT	2MM	Unifocal	usual	Well-differentiated	FREE	pT1a	NO	No	30	30	0	0		30	1	
H/674/21		56 jodhpur	yes	Yes	Yes	NA	NM	Small biopsy	0.3x0.2 cm	GLANCE	KERATINIZING	NA	Unifocal	usual	Moderately differe	NM	pTx	NO	No	0	10	0	1		0	1	
H/264/1/21		45 JHALOR	yes	Yes	Yes	NA	NM	Total penectomy	7X5.3X4.2	GLANCE AND SHAFT		4.2 cm	MULTIFOCAL	usual	Well-differentiated	FREE	pT3	NO	No	0	30	0	0		0	1	
H/465/5/19		64 Masuriya	no	NO	yes	no	yes	small biopsy	4x2x1.3	Glance	Fungative	NA	Unifocal	usual	Moderately differe	NM	pTx	NO	No	0	40	0	0		0	2	
H/305/5/21		57 Jodhpur	no	NO	no	yes	no	Small biopsy	3x1.8x1.5	Glance	fungative	NA	Unifocal	Warty	Well-differentiated	(G1)	pTx	NO	No	30	60	0	1		30	3	
H/5765/19		72 jodhpur	yes	NA	yes	no	yes	Partial penectomy	3.5x3x2.5 cm	Glance	ulcerative	2.5cm	Unifocal	Usual	Moderately differe	free	pT2	NO	Yes	20	10	0	0		20	1	
H/2185/17		64 Masuriya	yes	no	yes	no	no	partial penectomy	2.3x2.2x1.2	GLANCE AND FC	Fungative	5mm	MULTIFOCAL	Usual	Moderately differe	NM	pT2	Yes	No	0	50	0	0		0	2	
H/93/21		51 JALORE	yes	NO	Yes	NA	Yes	Partial penectomy	2.3X1.9X1	GLANCE	ulcerating and infi	1 cm	Unifocal	Usual	Well-differentiated	FREE	pT2	NO	No	20	30	0	0		20	1	
H/742/21		71 NAGORE	yes	Yes	Yes	NA	Yes	Partial penectomy	3.2X1.2X1.3	GLANCE AND FC	INFILTRATIVE	1.3cm	MULTIFOCAL	basaloid	Poorly differentiati	NM	pT3	Yes	Yes	30	60	0	0		30	3	
H/511/0/21		40 JODHPUR	yes	NA	Yes	NA	NM	Total penectomy	7X5.3X4.5	Glans	Ulcerative	4.5 cm	Unifocal	Warty	Moderately differe	VASCULAR +	pTx	NO	No	20	30	0	0		20	1	
H/429/1/21		66 JODHPUR	yes	NO	Yes	NA	NM	Partial penectomy	2.9X1.9X0.5	GLANCE AND PE	ulcerating and infi	4MM	MULTIFOCAL	Verrucous	Moderately differe	FREE	pT1a	NO	No	0	30	0	0		0	1	
H/264/1/21		45 JHALOR	yes	YES	Yes	NA	NM	Total penectomy	7X5.3X4.2	GLANCE AND SH	ulcerating and infi	4.2 cm	MULTIFOCAL	Usual	Well-differentiated	FREE	pT3	NO	No	0	20	0	0		0	1	
H/853/21		66 PALI	yes	NA	Yes	NA	Yes	Partial penectomy	4X4.3X0.5	GLANCE AND FC	PUSHING AND IN	5MM	MULTIFOCAL	Usual	Moderately differe	FREE	pT1a	NO	No	0	50	0	0	positive	0	2	
H/546/21		72 Jodhpur	yes	yes	yes	no	no	Total penectomy	6x5x5cm	Scrotum	ulcerating and infi	5mm	Unifocal	Usual	Moderately differe	NM	pT4b	NO	No	20	60	1	0		20	3	
H/582/4/21		56 Jodhpur	yes	No	Yes	No	No	Small biopsy	0.6x0.3x0.2	GLANCE	ulcero proliferativ	NA	Unifocal	Warty	Well-differentiated	(G1)	pTx	No	No	0	30	0	0		0	1	
H/441/4/21		66 Jalore	No	No	Yes	No	No	Partial Penectomy	3.2x3x2.5	GLANCE	ulcerorinfiltrative	2.5cm	Unifocal	Usual	Moderately differe	NM	pT3	Yes	Yes	0	60	1	1		0	3	
H/693/4/22		41 Jalore	No	No	Yes	No	No	Small biopsy	1.1x0.5x0.3	GLANCE	Fungative	NA	Unifocal	Usual	Moderately differe	NM	pTx	Yes	No	20	80	1	1		20	1	
H/666/5/22		68 Barmer	no	yes	no	no	no	Partial penectomy	3x2.7x4.8 cm	Prepuce, Glance	ulcero proliferativ	8 MM	MULTIFOCAL	Usual	Moderately differe	FREE	pT3	No	Yes	10	15	0	0		10	1	
H/693/4/22		62 Jalore	no	no	yes	NA	NM	Partial penectomy	3.1x2.8x4 cm	Prepuce	Ulcerative	4 cms	Unifocal	Usual	Well-differentiated	NM	pT1a	No	No	60	40						