ASSESSMENT OF LYMPH VESSEL DENSITY (LV AND LYMPHO-VASCULAR INVASION (LVI) IN SQUAMOUS CELL CARCINOMA (SCC) OF THI PENIS USING D2-40 AND P53 IMMUNOSTAININ AND CORRELATION OF THESE MARKERS WIT LYMPH NODE METASTASIS.

A DISSERTATION SUBMITTED IN PART FULFILMENT OF THE REQUIREMENTS FOR THE M.D. DEGREE BRANCH III (PATHOLOGY) EXAMINATION OF THE TAMIL NADU DR. M.G.R.MEDICAL UNIVERSITY CHENNAI TO BE HELD IN APRIL 2016.

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DECLARATION

This is to declare that the dissertation entitled "**ASSESSMENT OF LYMPH VESSEL DENSITY (LVD) AND LYMPHO-VASCULAR INVASION (LVI) IN SQUAMOUS CELL CARCINOMA (SCC) OF THE PENIS USING D2-40 AND P53 IMMUNOSTAINING AND CORRELATION OF THESE MARKERS WITH LYMPH NODE METASTASIS**" is the original work done by me, in part fulfilment of rules and regulations for the M.D. Branch III (Pathology) Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, to be held in April 2016. I have independently reviewed the literature, standardized the data collection methodology and carried out the evaluation towards completion of the dissertation.

Dr. Miriam M Bidari,

Postgraduate registrar,

Department of Pathology,

Christian Medical College,

Vellore- 632004.

CERTIFICATE

This is to certify that the dissertation entitled **"ASSESSMENT OF LYMPH VESSEL DENSITY (LVD) AND LYMPHO-VASCULAR INVASION (LVI) IN SQUAMOUS CELL CARCINOMA (SCC) OF THE PENIS USING D2-40 AND P53 IMMUNOSTAINING AND CORRELATION OF THESE MARKERS WITH LYMPH NODE METASTASIS"** is a bonafide work done by Dr. Miriam M Bidari under my guidance, in part fulfillment of the requirement for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai, to be held in April 2016. The candidate has independently reviewed the literature, standardized the methodology and carried out the evaluation towards completion of the thesis.

Dr. Ramani Manoj Kumar MBBS, MD,

Associate Professor,

Department of Pathology,

Christian Medical College,

Vellore

CERTIFICATE

This is to certify that this dissertation titled "**ASSESSMENT OF LYMPH VESSEL DENSITY (LVD) AND LYMPHO-VASCULAR INVASION (LVI) IN SQUAMOUS CELL CARCINOMA (SCC) OF THE PENIS USING D2-40 AND P53 IMMUNOSTAINING AND CORRELATION OF THESE MARKERS WITH LYMPH NODE METASTASIS**" is a bonafide work done by Dr.Miriam M Bidari, in part fulfilment of rules and regulations for the M.D. Branch III (Pathology) Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, to be held in April 2016.

Dr. Banumathi Ramakrishna, MBBS, MD, MAMS,

Professor & Head,

Department of Pathology,

Christian Medical College,

Dr Alfred Job Daniel, D Ortho, MS (Ortho), DNB (Ortho),

Principal,

Christian Medical College,

Vellore.

TITLE OF THE ABSTRACT: "Assessment of lymph vessel density (LVD) and lympho-vascular invasion (LVI) in squamous cell carcinoma (SCC) of the penis using D2-40 and p53 immunostaining and correlation of these markers with lymph node metastasis"

DEPARTMENT: GENERAL PATHOLOGY

NAME OF THE CANDIDATE: DR MIRIAM BIDARI.

DEGREE AND SUBJECT: MD PATHOLOGY

NAME OF THE GUIDE: Dr. Ramani Manoj Kumar MBBS, MD,

OBJECTIVES:

1. To detect early metastasis in amputed specimens of penis, by using two immuno markers, D2-40 and p53 and correlation of these with the histopathological variables and clinical stage
2. Also to compare which marker is the better predictor for metastasis.

METHODS: Study was retrospective of total 49 cases of partial /total amputation with lymphadenectomy of penile cancers diagnosed between January 2006 to september2014 in the Department of General pathology; Christian medical college and hospital were

retrieved. These 49 cases were divided into metastatic and non metastatic groups on H&E sections in which 16/49 had metastasis. Two immunomarkers D2-40 and p53 were done on the amputed specimens to predict early metastasis. The p53 tumor density was calculated in 100 cells with 20% cut off and was graded as positive when more than 20% of the tumor cells with positive nuclear staining. For D2-40, LVD was calculated in the peritumoral and normal area by taking 10 % cutoff of positive staining of lymphatic cells within the lymphatics. All the pathological and clinical variables were calculated by Chi square test. In our study only the histopathological stage of the tumor statistically correlated with the p53 tumor marker. D2-40 did not show any correlation statistically with any pathological or clinical variable..

RESULTS:

Total 49 cases of partial amputed specimens had conventional SCCs and most common grade was grade I and common site of presentation was glans with mean depth of 0.9mm. Total 16 cases had lymph node metastasis, 17 /49 had LVI. Most common histopathological stage was T2 and most of our patients were clinically N0 status. Most common node involved was right superficial inguinal lymph node. 13 /49 cases with increased p53 tumor density and 11/49 showed increased LVD by D2-40 in 16 metastatic nodes and 23 in 33 nonmetastatic nodes. P value of 0.05 was considerd significant in our study. P53 had a sensitivity of (81.3%) and specificity of (54.4%) with positive predictive value of (36.1%)(CI20.8%-53.8%) and negative predictive value of (76.9%) (CI 46.2%-95.0%). D2-40 had (68.8%) sensitivity and (42.4%) specificity with positive predictive value of (36.7%)(CI 19.9%-56.1%) and negative predictive value of (73.7%)(CI 48.8%-

90.9%). Both the markers did not correlate statistically with histopathological and clinical variables like phimosis, BXO, clinical node status, tumor type, tumor grade, depth of invasion, LVI. Both had low positive predictive value. Therefore in our study p53 and D2-40 cannot predict the early lymph node metastasis because of low positive predictive value, even though they are sensitive.

To conclude, both the markers had low specificity and low positive predictive value, which suggest that they are not able to predict lymph node metastasis even though they are sensitive and therefore they are not helpful to decide for prophylactic lymphadenectomy. D2-40 can be used as a good adjunct along with H& E sections on the initial amputated specimens to detect the definite LVI which needs to be confirmed by D2-40 staining in difficult and doubtful situations, on routine histology.

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Key words: SCC, Lymphatic vessel density (LVD), Lymphovascular invasion (LVI), D2-40 and p53.

INTRODUCTION

Squamous cell carcinoma of the penis is an uncommon tumor in the affluent developed countries (age standardized incidence rate 0.3-1/100,000 according to WHO). However, in developing countries like India, it is very common and the incidence is similar to that of cervical cancer. It accounts for about 0.4%-0.6% of all tumors in males in USA and Europe, and is about 20% of all malignancies in Asian countries. This depicts that environmental factors play a major role. According to the Western literature, the mean age at presentation is 60 years; however, in areas of high incidence, penile carcinoma presents at an earlier age. In India, the age of presentation can be as low as 35 years[1-2]. Penile cancers with involvement of lymph nodes have a poor prognosis. Indeed the prognosis of carcinoma of penis depends upon an accurate diagnosis of the lymph node status.[3]. Involvement of the lymph node is the most important predictor of outcome for survival in penile carcinoma. However clinical estimation of the lymph node status in these patients is still a major problem, since 20-25% of clinically node negative patients can have occult nodal metastasis[3-4]. On the other hand, traditional elective lymphadenectomy accounts for overtreatment in up to 80% of patients and is associated with high morbidity (30-90%) [4-5].

Many groups have tried to recognize prognostic factors at the earliest to overcome the surgical morbidities such as life threatening septicemia or even death in few cases.

Some researchers have suggested that the lymph vessels have a passive role in lymphatic spread as the tumor cells permeate the already existing dilated, functional lymphatics of the peritumoral tissue.

Dissemination into the adjacent lymph nodes is the first step in the process of metastasis of numerous common malignancies like carcinoma of the breast, head and neck, bladder etc. This mechanism of spread is responsible for the outcome of the disease. Sentinel nodes are the first to be affected and biopsy of these sentinel lymph nodes is done as diagnostic measure to locate this affected node and be removed in cases of metastasis. The advancement in the field of molecular technology has made it possible to recognize and distinguish many malignant diseases using tumor suppressor genes and oncogenes. Therefore prediction of lymph node metastases by using immunomarkers could reduce the incidence of unnecessary lymphadenectomy. According to Minardi et al, palpable inguinal lymph nodes show positivity for D2-40 in only 50- 60% of patients, whereas occult metastasis was detected in 20% of patients with nonpalpable lymph nodes.[7].

By using p53 Martin et al and Zhu et al showed that there is increased risk of lymph node metastasis with increased p53 expression in the tumor and these patients have poor outcome.[5, 8, 9]. Thus, here we attempt to predict early metastasis in these tumors, so that major surgery can be avoided and thereby prevent morbidity in a subset of patients.

Aims and Objective

- To determine the lymph vessel density (LVD) in penile squamous cell carcinomas by using immunohistochemical marker D2-40.

- To determine lymphovascular invasion (LVI) by tumor cells in these carcinomas.

- To determine whether there is a relationship between normal and peritumoral LVD and the presence of lymph node metastasis in ilioinguinal/ inguinal block dissection specimens of these patients.

- To determine whether nuclear positivity for p53 correlates with lymph node metastasis.

REVIEW OF LITERATURE

Definition: Squamous cell carcinoma of penis is a tumor arising from the squamous epithelium of the prepuce and the glans. The histology is similar to squamous cell carcinoma of female genital tract (cervix, vagina and vulva), anus, and the oropharynx.,

SCC is referred to as "**epidermoid carcinoma**" and "**squamous cell epithelioma**", by the clinicians, though the use of these terms is no longer in vogue in pathology.

Epidemiology: In western countries, squamous cell carcinoma of the penis accounts for 1.00 per 100,000 males.. This incidence is also affected by race and ethnicity [1,2], with highest incidence found in white Hispanics (1.01 per 100,000) and white non Hispanics(0.51 per 100,000). In contrast, in India incidence of penile cancer is much higher and represents 10-20% of all malignancies in men, with age adjusted incidence of 0.7-3 per 100,000 people. In places like Uganda the incidence is much higher where it is the most common malignancy. Incidence of penile carcinoma in India is almost equal to the incidence of cervical cancer .

This indicates that environmental factors play an important role in the causation of the disease. According to the Western literature, the mean age at presentation is 60 years; however, in areas of high incidence, penile carcinoma presents at an earlier age. In India, the age of presentation can be as low as 35 years[3].

Important risk factors include cultural habits, social factors, hygienic and religious practices like circumcision. Therefore penile cancers are uncommon in communities like Jews, Muslims, and Ibos of Nigeria that practice circumcision in new born or before puberty. The risk of carcinoma penis is reduced by 3-5 times by early circumcision. However circumcision in adult hood does not protect against penile cancer**.**

Risk Factors: [4, 5]

Case control studies recognize strong risk factors with odds ratio of more than 10, and include:

Chronic inflammatory conditions such as balanitis xerotica obliterans .

Phimosis

Treatment with PUVA

Multiple sexual partners,

Cigarette smoking and

Human papillomavirus (HPV) infection with high risk strains such as HPV 16 &18.

.

Dysplastic epithelium shows infection with HPV in 70-100% of cases, where as in invasive penile carcinoma it accounts for only 40-50 %.

These factors have been confirmed by case control population based study [6]. In 80% of the tumor samples, HPV DNA was isolated, out of which 69% were positive for Human papilloma virus 16(HPV16).

This proposes that oncogenesis is initiated by this virus by interaction with oncogenes and tumor suppressor genes, such as p53 protein[7]. Being sexually transmissible, the incidence increases with increased sexual activity and with earlier age of onset of sexual activity. However the prognosis of the patient is not affected by the presence of high risk HPV DNA in penile cancer. Few of the studies have proved that there is no difference between HPV DNA-positive and negative patients for metastasis in lymph node over a period of 10 years[8].

HPV Types and Oncogenic Potential

Classification	HPV Types
High risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Probably oncogenic	26, 53, 66, 68, 73, 82
Low risk	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81

{Documented from Muñoz et al}

Classification

SCC of the penis has been classified according to

Histological appearance of the tumors

Patterns of growth

Relationship to HPV infection.

i) **Histological classification**: The WHO has classified penile SCC according to the histological variants[3]

Squamous cell carcinoma, (SCC), conventional, and

Variants of SCC

- Condylomatous (Warty) carcinoma
- Basaloid
- Verrucous carcinoma
- Papillary carcinoma, (NOS) not otherwise specified
- Mixed carcinoma
- Sarcomatoid(spindle cell)
- Adenosquamous

Mills et al have described the following additional histological variants[9]

- Pseudohyperplastic SCC
- Carcinoma cuniculatum
- Acantholytic SCC
- Clear-cell SCC

The majority of SCC (approximately 60-70%) show conventional non verruciform keratinizing SCC.

Characterization of Penile SCC Histologic Types that have Higher Distributions

Carcinoma Subtype	Distribution (%)	Main Features	Metastatic Rate (%)	Prognosis
Usual	48–65	Most common subtype, morphologically similar to SCC from other sites	28–39	Intermediate
Basaloid	4–10	HPV-related aggressive tumor composed of deeply invasive tumor nests of basaloid cells	50–100	Ominous
Verrucous	3–8	Low-grade verruciform tumor, usually invading only superficial anatomic levels	Null	Excellent
Warty	7–10	HPV-related verruciform tumor similar to warty carcinomas from other sites	17–18	Good
Papillary	5–15	Low-grade verruciform tumor frequently invading superficial erectile tissues	12	Good

Documented from Chaux et al

ii.Classification of penile carcinomas according to patterns of growth

1) Superficial spreading pattern-Horizontal growth, superficial invasion

2) Vertical growth pattern – Deep invasion

3) Verrucous

4) Multicentre –Combination of the above

Histological growth patterns have a direct correlation with prognosis according to WHO, Cubilla and Mills[3, 9, 10].

iii. Classification of penile carcinomas according to relationship with HPV infection

Cubilla et al categorized penile SCCs according to the association with HPV infection [10]

Related to HPV infection

1) Warty carcinoma

2) Warty/basaloid carcinoma

3) Giant condyloma.

4) Basaloid carcinoma - an aggressive variant

Unrelated to HPV infection

1) Verrucous carcinoma

2) Hybrid verrucous / SCC

3) Papillary carcinoma

This confirms that hr HPV DNA is strongly associated with the basaloid/warty subtypes and is only weakly associated with keratinizing/other SCCs. There is only very weak or almost nil association with verrucous carcinoma of the penis.

Grading:

Histological grading is based on Modified Broder's grading of skin SCCs [11]:

Grade 1 (G1) well differentiated with well formed keratin pearls

Grade 2 (G2) moderately differentiated with individual cell keratinisation

Grade 3 (G3) poorly differentiated tumor with lack of keratin formation.

Cubilla et al[10] have categorized penile SCCs into three grades based on growth pattern and / or histological appearance:

1) High-grade – Basaloid carcinoma with vertical growth pattern.

2) Intermediate-grade – SCC with superficial spreading growth pattern.

3) Low-grade–Verrucous carcinoma

Squamous cell carcinoma (SCC) of conventional type:

Keratinizing SCC similar to other sites, vulva, anal and esophagus.

Gross: Irregular granular polypoidal mass, sometimes ulcerated with solid cut surface .Superficial and deep infiltration into different anatomic layers of penis is usually seen.

Microscopically: This is an infiltrating keratinizing SCC that is graded based on Modified Broder's three tier system as mentioned above.

According to WHO, Cubilla and Mills[3, 9, 10] histopthological variants broadly studied are:

Basaloid carcinoma: Is an aggressive, high grade and deeply invasive penile neoplasm usually associated with HPV. It accounts for 5-10% of all penile cancers.

More than half of patients show enlarged lymph nodes as a result of metastasis, at the time of presentation. Glans is the commonest location.

Grossly, this is a flat ulcerated irregular mass with a solid tan cut surface that usually replaces corpus spongiosum and corpus cavernosa.. Microscopically, tumor has solid nests formed by monotonous small tumor cells often with central comedonecrosis, surrounded by peripheral clefts.

Verruciform carcinomas.

These are exophytic tumors with a verruciform gross appearance and are low- to intermediate-grade malignancy which involves glans or fore skin.

Grossly they present as grey white exophytic mass. Microscopically, they are well differentiatiated papillary neoplasms with marked acanthosis and hyperkeratosis with subtle fibrovascular cores. Koilocytic changes are absent. Tumors have broad based, pushing border. It is HPV unrelated. These are slow growing tumors which recur locally but metastasis is rare.

Warty (condylomatous) carcinoma: Consists of 20% of verucciform neoplasms.

Grossly, these are cauliflower like, tan white lesion that involves the glans, coronal sulcus or foreskin. Tumor can reach up to a maximum size of 5. cm.

Microscopically, it is hyper-keratotic with papillary arborizing growth pattern. The papillae have thin fibrovascular cores. The tumour cells show low to intermediate

grade morphology with conspicuous koilocytotic atypia. Nuclei are large, hyperchromatic and wrinkled with binucleation.. These tumours are HPV unrelated. Therefore regional lymph node involvement is not generally seen.

Papillary carcinoma, not otherwise specified (NOS):

Grossly they are exophytic, grey-white firm tumors. Microscopically, they are well differentiated, with hyperkeratosis and complex papillae containing thin fibrovascular cores. The tumor has an infiltrative pattern.

Sarcomatoid carcinomas (spindle cell): Form about 4% of penile carcinomas, show high grade morphology and are composed of predominantly spindle cells.

Grossly they are large; polypoidal with ulcerated surface commonly located in the glans and are deeply infiltrative.

Microscopically, bulk of the tumor is composed of atypical spindle cells with interlacing fascicles with areas reminiscent of fibrosarcoma or leiomyosarcoma frequently mixed with pleomorphic giant cells simulating malignant fibrous histiocytoma. Areas of myxoid matrix may be prominent. Pseudovascular pattern mimics angiosarcoma .. Mitotic activity is brisk and areas of necrosis are quite common. Foci of heterologous differentiation such as bone and cartilage (osteosarcomatous and chondrosarcomatous components) may be obvious in a few cases.

Mixed carcinomas: Mixture of various types mentioned above, with moderate to high grade SCC.

Adenosquamous carcinoma:

Tumors showing both glandular and squamous differentiation in the glans and coronal sulcus. These tumors are deeply invasive.

Staging:

American Joint Committee on Cancer: (T-Tumor, N-Node, M-Metastasis) [12]

(pT) primary tumor

pTX primary tumor cannot be assessed

pT0 no evidence of primary tumor

pTis carcinoma in situ

pTa noninvasive verrucous carcinoma (broad pushing invasion allowed, destructive is not)

pT1a -Tumor invades sub epithelial connective tissue without lymphovascular invasion or is not poorly differentiated. .

pT1b - Tumor invades sub epithelial connective tissue and either has lymphovascular invasion or is poorly differentiated.

pT2 - Tumor invades corpus spongiosum or corpus cavernosum

pT3 - Tumor invades urethra or prostate.

pT4 -Tumor invades other adjacent structures

(N)- Regional Lymph Nodes

Nx -Regional lymph nodes cannot be assessed:

N0- No regional lymph node metastasis.

N1- Metastasis in a single superficial inguinal lymph node.

N2- Metastasis in multiple or bilateral superficial inguinal lymph nodes.

N3- Metastasis in deep inguinal or pelvic lymph node(s), unilateral or bilateral

M -Distant Metastasis

Mx Distant metastasis cannot be assessed.

M0- no distant metastasis

M1- distant metastasis

European Association of Urology guidelines classify patients into 3 risk groups[3, 5]

Low: pTis, pTa G1-G2, pT1 G1

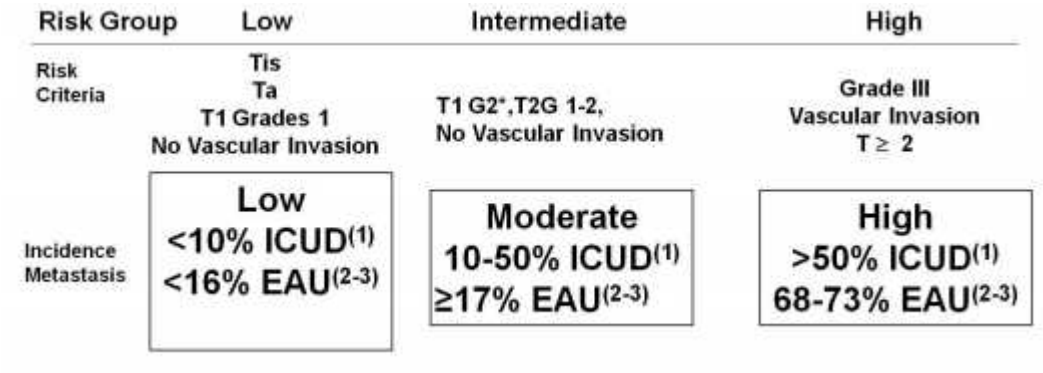
Intermediate: pT1 G2

High: pT2/T3G2/ G3

The risk of inguinal metastasis as per the above classification is 4% for low risk, 34.8% for intermediate risk factors,and 45.8% for high risk patients[13].

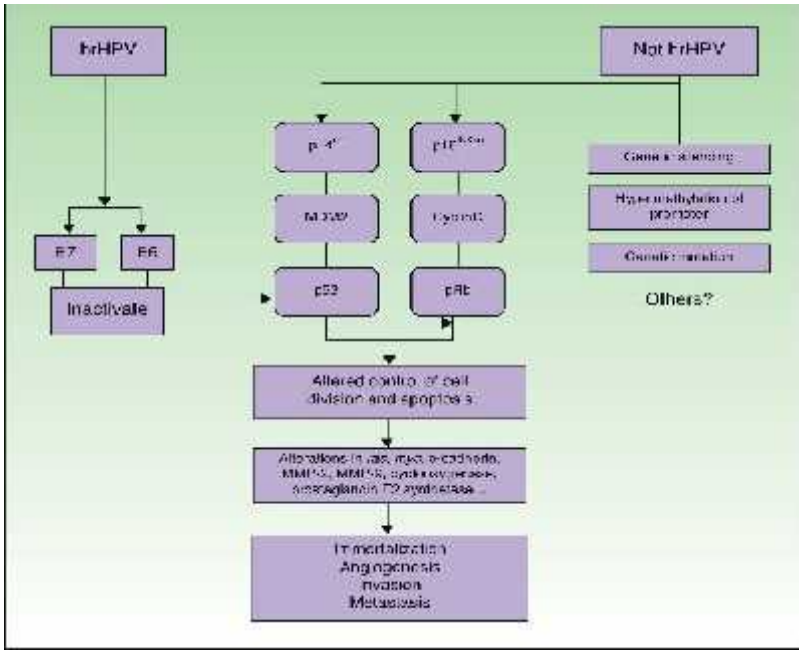
Novara et al[14] in their study, however showed that the above risk stratification did not have a high prognostic precision. A new classification was proposed by Ornalles et al[15] who divided these patients into 3 different risk groups depending upon the histological grade of the tumor and pathologic stage. Their patients were accordingly categorized as low- T1G1 and T1G2, intermediate -T2G1, T2G2, T3G1 and T3G2, and high- T1-3G3 and T4G1-3.

They found significant differences in the cancer specific survival for 10 years between the low and intermediate risk patients and the intermediate and high risk patients.



Penile carcinoma prognostic factors for inguinal lymph node metastasis

Pathogenesis: The molecular pathways which are involved in the etiology and pathogenesis of penile cancers are of different mechanisms. 42% of the carcinoma of penis are caused by hrHPV infection while the remaining is caused by HPV-independent molecular mechanisms.[16]



Molecular events in carcinogenesis in penile cancers related to HPV and Non HPV infection [16]

The initiating event is infection by hrHPV and its persistence within the squamous epithelium, followed by a sequence of epigenetic modifications that procure into causing malignant transformation of the infected cell. Oncoproteins E6 and E7 are expressed by hrHPV virus, which causes inactivation of the tumor suppressor gene products p53 and pRb. E6 and E7 play a major role in maintaining and inducing the transformed phenotype of the infected cell.

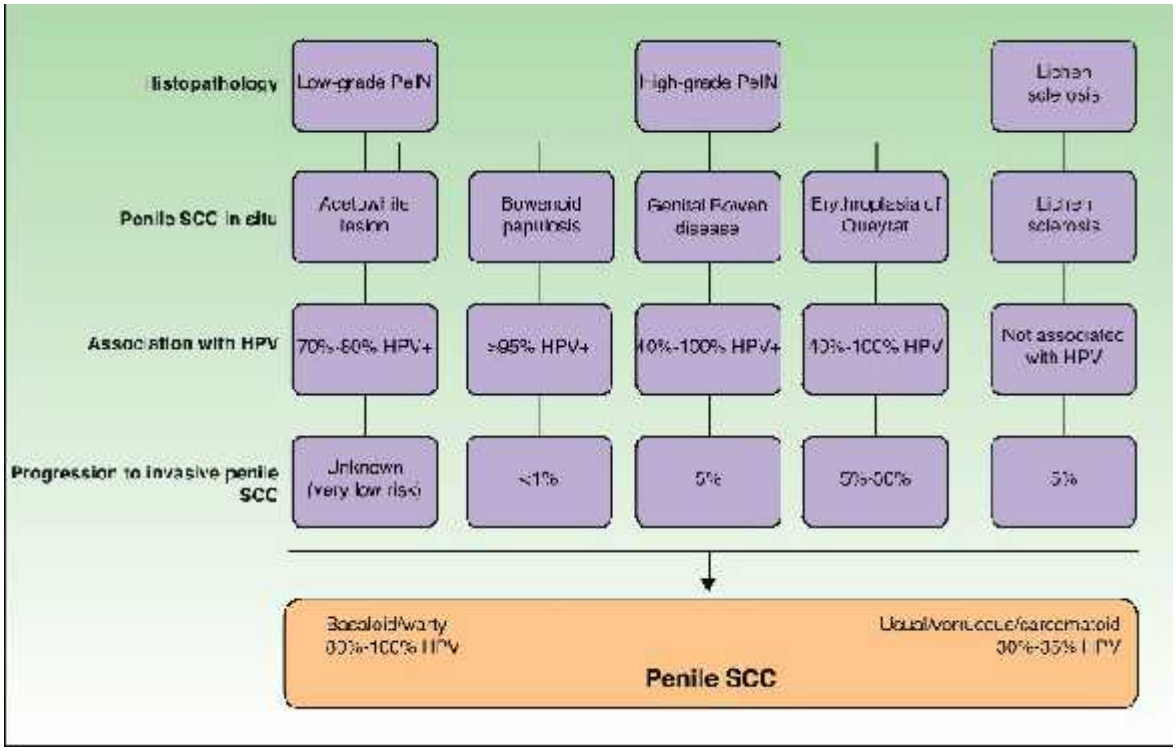
P14arf/MDM2/p53 and p16^{INK4a}/cyclinD/Rb pathways are altered by hrHPV types and ultimately cause cell division and apoptosis. The pathways involved in HPV-associated carcinoma of penis are the same as those that

are altered in penile carcinomas caused by Non HPV types with the involvement of the following mechanisms:

Silencing of tumor suppressor genes,

Hypermethylation of promoter genes, and

Overexpression of oncogenes.[16-20]



Clinico histopathological correlation in carcinoma penis PeIN indicates penile intraepithelial neoplasia

Mode of spread:

The mode of spread in penile carcinomas is predominantly lymphogenic. Traditionally, the regional lymph nodes of the penis are divided into the superficial and the deep groups which are situated in the inguinal region. In carcinoma of penis,Cloquet's or Rosenmuller's node, is the first draining lymph node, located in the inguinal region and demarcates the transition between pelvic groups and inguinal groups.

The second group of regional nodes is located in the pelvic region groups of lymph nodes. EAU guidelines suggest that modified bilateral lymphadenectomy should be done for all cases with pT1G2 and in aggressive types like high histologic grade, sarcomatoid carcinoma or increase in average depth of invasion with clinically unaffected nodes. Radical inguinal lymphadenectomy should be done for patients with metastatic nodes. Pelvic inguinal lymphadenectomy should be performed, if more than two inguinal nodes are histologically involved. In a case of penile carcinoma, with inguinal metastases clinically, inguinal lymph node dissection is to be done along with the excision of the primary lesion. However, clinically if the nodes are nonpalpable (cN0), the management of the patient is controversial since a routine prophylactic treatment of inguinal lymph node dissection leads to over-treatment in a vast majority of patients because the incidence of occult lymph node metastases is only around 20%[21-22].

According to the study by Naumann et al [22] conventional H&E examination of nonsentinel lymph node biopsy fails to detect lymphatic spread in penile cancer. Therefore step section method at 3 levels helps to detect early metastatic disease, but this procedure is time consuming.. So if early lymph node can be predicted using D2-40 or p53, unnecessary lymphadenectomy can be prevented.

Reference	Patients, n	Ta, T1	T2	T3	T4	G1	G2	G3
Ornellas et al [6]	350	18	46	64	50	–	–	–
Hungerhuber et al [78]	102	14		52		29	46	82
Narayana et al [79]	117	10		56		–	–	–
Solsona et al [80]	66	4	64			19	65	85
Lopes et al [17]	145	50	55	53	29	47.5	64	67
Ficarra et al [9]	175	11	20		64	9	29	
Naumann et al [10]	20	50	–	–	–	–	50	–

Figure: 1 Frequency of lymph node metastases in % in grade and stage {Documented from EAU 20014}

Prognostic factors:

Lymph node metastasis in carcinoma of penis has poor prognosis. Diagnosis of lymph node metastasis at the earliest is the gold standard method to improve the tumor free survival of the patient. Hence the therapeutic adequacy and the prognosis in penile cancer depend upon an accurate diagnosis of the presence or absence of lymph node metastasis.

Circumcision done in childhood offers maximum protection against the disease. However circumcision in the adult does not afford any protection to development of carcinoma. The most important environmental predisposing factors are poor hygiene of the penile area, cigarette smoking and phimosis which play role in carcinoma of penis. The disease at the earliest can be treated by conventional resections, or in some patients by organ preserving techniques (Mohs micrographic surgery), and laser and radiation therapy..

Partial or total penectomy is required for more advanced primary disease. Prophylactic / therapeutic lymph node dissection is done for metastatic inguinal nodes and depending on the disease status, unilateral or bilateral inguinal or ilioinguinal lymphadenectomy might be needed. (Please see the flow chart below). They are actually the late manifestations of disease progression .The mechanisms involved are angiogenesis and invasion. These factors are predictive for early lymph node metastasis [24-26]

The prognosis depends upon the [5]

Tumor size,

Location (Glans/foreskin/sulcus, corpus spongiosum or cavernosum)

Histological subtype

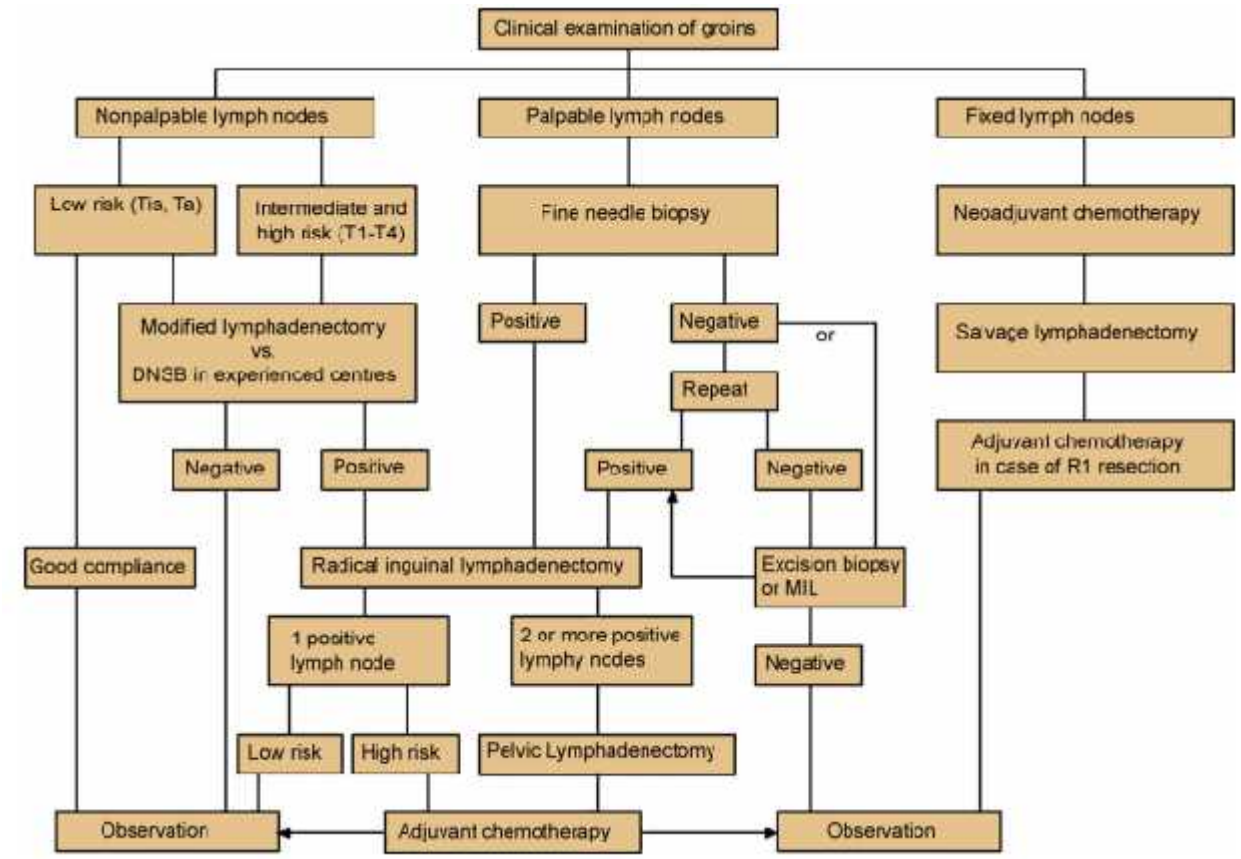
Histological grade

Growth pattern

Depth

Lymphatic invasion

Vascular invasion



Flow chart for the approach to the management of ca penis [5]

Tumors involving exclusively the foreskin carry a better prognosis than those of the glans penis as the former are generally low-grade and superficially invasive. There is a correlation between pattern of tumor and the presence of regional metastasis and survival. The verruciform tumors have negligible incidence of metastasis. The incidence of metastasis of superficial spreading carcinomas is 10% where as it is 67% in patients who have tumors with vertical growth pattern.

Excluding verruciform neoplasms (which tend to be large but well differentiated), there is a correlation between larger tumor size and metastasis[9.] There is also a correlation between histological type and regional metastasis and its outcome. Metastasis and mortality in verrucous, papillary, and warty (condylomatous) carcinomas are rare. Basaloid and sarcomatoid carcinoma show a high rate of metastasis and mortality because as they are deeply invasive with their vertical growth pattern and therefore with early lymph node metastasis. The prognostic role of HPV in patients with penile cancer is not yet well established. A large series found that high-risk HPV-related cancers were associated with an overall better survival. The histological grade correlates with prognosis.

A major study found an incidence of regional metastasis in 24%, 46%, and 82% of patients with well-, moderately, and poorly differentiated tumors, respectively. Few studies have found that any proportion of grade 3 adversely affects prognosis. There are studies which show that there is relation between depth of tumor infiltration and the presence of inguinal metastasis in penile carcinomas[5.]

Measurement of depth of tumor invasion is from the basement membrane of adjacent squamous epithelium to the deepest point of infiltration. In cases of large, destructive, and bulky exophytic tumors, it is measured from the tumor surface to the deepest point of invasion.

This measurement excludes the keratin layer. Superficial neoplasms, especially those infiltrating only lamina propria (<5 mm), have minimal to almost no risk of regional spread. The tumors with more than 5 mm deep are at a higher risk for invasion and in such cases corpora spongiosa and corpora cavernosa are usually involved.

Therefore three major important histopathologic factors related to LN metastasis are histological grade, depth of invasion, and vascular invasion. Furthermore the histological grade and depth of invasion together is thought to strongly predict metastasis and outcome of survival. But the most important amongst all of them is nodal metastasis in carcinoma of penis.

Clinically nonmetastatic groups have better outcome of survival with probabilities between 75% and 93%, and those with pathologically confirmed negative nodes have 5-year cancer-specific survival probabilities ranging from 85% to 100%. Those cases that have multiple lymph node metastasis have poor survival outcome than those who have single lymph node metastasis. Patients with clinical N0 stage develop recurrence later in life due to high histological grade and deeper invasion. Their survival outcome is reduced due to these factors.

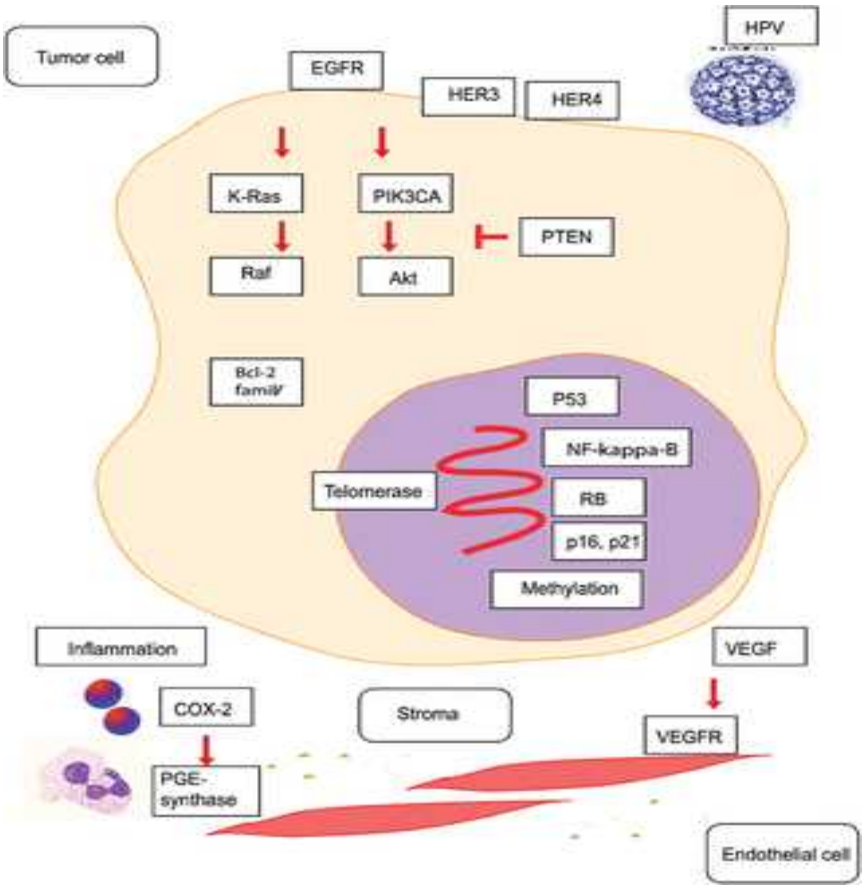
Recently, Alkatout et al[42] have shown that deep tumor infiltration is a predictor of pN+ disease with penile carcinoma; therefore there was 5 fold increased risk of having lymph node metastases with an infiltrating pattern than in those with pushing pattern of infiltration.

Therefore the studies show that an infiltrating pattern of invasion is also one of the independent predictors of recurrence in many other epithelial tumors like oral cavity and cervical squamous cell carcinoma[43], The tumour–host tissue reaction depicts the pattern of invasion, defined as infiltrating in the form of clusters of small, solid strands of cell tumours that broadly infiltrate the stroma and pushing infiltration in which tumour cells invade with well-defined tumour host interface[44] signifying the characteristics of infiltrative tumours,. Previous studies have shown that 12%-24% of patients have occult micrometastases in clinical N0 patients.[45]

Therefore prophylactic bilateral inguinal lymph node dissection can considerably improve the prognosis of patients with occult inguinal metastasis.

Conversely this type of surgery has resulted in major complications in 24%-87% of patients. 3% of them died as adverse effect of the surgery.. The other common complications are lymphedema, lymphocoele, wound infection and wound separation, skin edge necrosis and skin flap necrosis[46, 47]. There is overtreatment in 75%-90% of patients who undergo prophylactic, inguinal lymphadenectomy in whom micrometastases were not present.

Therefore the histopathological factors like depth, grade and lymphovascular invasion of the tumor and the molecular features of the primary tumor might help to predict regional lymph node involvement.



Pathway for p53 tumorigenesis

Molecular Markers in Penile Carcinomas:

Recent studies suggest that in many cancers poor prognosis is related to lymph angiogenesis with formation of new lymphatic channels. There are many recent markers for early detection of lymph node metastasis which will enhance the 5 year survival of these patients, these are p53, Ecadherin, MMP-2, MMP-9, Ki67 and recently studied marker is D2-40.

Though there are many articles published regarding these markers, only very few studies have come out regarding the use of D2-40 in penile SCC. There are several studies to predict the prognostic outcome of histopthological factors which define those patients at increased risk of nodal metastasis and who undergo prophylactic lymphadenectomy. These are grade, tumour stage and the presence and absence of lymphovascular invasion. [13, 26-28]. Some studies showed that 82% of patients had unnecessary prophylactic lymphadenectomy when only tumor stage and grade were taken into consideration [29]. Hence it appears that more distinct predictive factors are needed for identifying patients at high risk for lymph node metastasis. As we know lymph node metastasis occurs due to a sequence of events such as mutations in gene encoding their products. Hence early detection of these encoding proteins would allow identifying cancers that are at high risk for metastasis and thus would help to develop targeted therapy.

It was also noted that certain immunohistochemical marker would help to detect occult metastasis at the earliest. In this aspect Lopes et al[25] studied the prognostic implications of p53 in patients with carcinoma of penis.

As we know p53 is a protein which is responsible for repair of DNA damage at G1 phase of the cell cycle. Therefore the mutation of p53 leads to failure to repair the DNA damage, which leads to a mutant p53 which has a longer shelf life.

This p53 gets collected in the cell and can be highlighted by tumor immunostains which shows nuclear overexpression of mutated p53. This mechanism is HPV independent pathway of carcinogenesis. Another mechanism of HPV dependent mechanism is inactivation of p53 gene. Therefore over expression of p53 is indicative of carcinogenesis and serves as a useful marker [5]. There is 4.8 -fold increased risk for metastasis with increased p53 positive tumor cells than with patients with negative p53. There have been studies where it is shown that overexpression of p53 by the tumor cells is directly proportional to lymphatic invasion leading to lymph node metastasis. On multivariate study the lymphatic invasion by neoplastic cells and p53 positivity for tumor cells were independent prognostic factors for lymph node metastasis[25,27]. There was p53 nuclear staining in tumor cells and any tumor with more than 20% for tumor cells showing p53 staining was considered 'positive'.

Many studies have used different cut off for p53 positive tumor cells. One of these is Martins et al [27] which used 10% cut-off, which showed that increased nuclear positivity is a predictor of lymph node metastases and the outcome of survival for the patients. Few other studies have taken 5% as cut off [50].

Same marker was used at various other sites such as, Cabelguenne et al, whose major study was in head and neck tumors, and Maeda et al, who analysed in gastric carcinoma and also Unal et al observed that tumor cells showed over expression for p53 in cases of carcinoma tongue with positive p53.

D2-40 is recently studied in SCC of esophagus which showed increased LVD in intratumoral area than peritumoral area with increased expression in cancer cells and tumor stromal cells. But only the cases with increased LVD in intratumoral area correlated with lymph node metastasis[53.]

Use of D2-40 in SCC of penis has been done by only one group, Minardi et al whereas it has been studied extensively by other people, in different sites of cancers..[28]

Recent updates show that angiogenesis and lymph angiogenesis can cause the disease spread via lymphatics and blood vessels. The D2-40 which is a monoclonal antibody detects an antigen with a fixation resistant epitope. The podoplanin(D2-40) antigen is expressed in lymphatic endothelial cells specifically and therefore is helpful in lymphatic vessel identification and estimation of their density. D2-40 stains the cytoplasm of lymphatic endothelial cells. D2-40 staining is estimated in the primary tumor as part of the initial examination alongside H&E stain.

It is not estimated in the lymph node specimen. The density of D2-40 positive lymphatic vessels were estimated at different areas, i.e peritumoral, intratumoral,and normal tissue respectively from diagnosed cases of carcinoma penis in partially/totally amputated specimen.

This marker is also expressed in squamous cell carcinomas at different sites like lung, , breast, cervix, prostate, esophageal, head and neck cancers & melanoma . It is a new marker, not studied much in penile cancers. It is involved in infiltration as well as

increased cell migration [33] .Hence D2-40 is used as a prognostic marker to evaluate LVD and LVI in tumors like melanoma, carcinomas of breast ,endometrium, uterine cervix, stomach, and bladder [33-41]. All these studies showed that there is increased density of lymphatic channels in the peripheral zone of tumor which was highlighted by D2-40. Minardi et al showed that D2-40 is sensitive (89%) in predicting lymph node metastasis by detecting the lymphatic invasion in contrast to H&E staining which is sensitive by 41% only. Compared to H&E staining, D2-40 was found to be more accurate in predicting lymph node metastasis.[37].

Same group showed that intratumoral tissue had collapsed and compressed lymphatics and high positivity for epithelial cells for D2-40., while the peritumoral compartment showed high lymphatic density than in intratumoral tissue, decreased expression of D2-40 by the epithelial cells in this region, while in normal tissue D2-40 was weakly positive for lymphatic vessels as well the epithelium.

The basal layer of the epithelium was mainly stained. LVD was calculated by Minardi et al by choosing 10 hot spot fields at 10x. LVD was calculated in peritumoral, intratumoral and normal tissue at magnification x200.

Overall, they found a greater LVD in peritumoral tissue as compared to intratumoral tissue, which is similar to the study carried by other groups for clear cell renal cell carcinoma, gastric and prostatic adenocarcinoma, endometrial carcinoma, lung carcinoma and transitional cell carcinoma of the urinary bladder [32,-40].

An interesting significant finding of their study was that intratumoral LVD was lower than peritumoral LVD in cases with lymph node metastases. This finding suggests that the LVD within the tumor might not be as significant as LVD in the peritumoral tissue in predicting the likelihood of LN metastases.

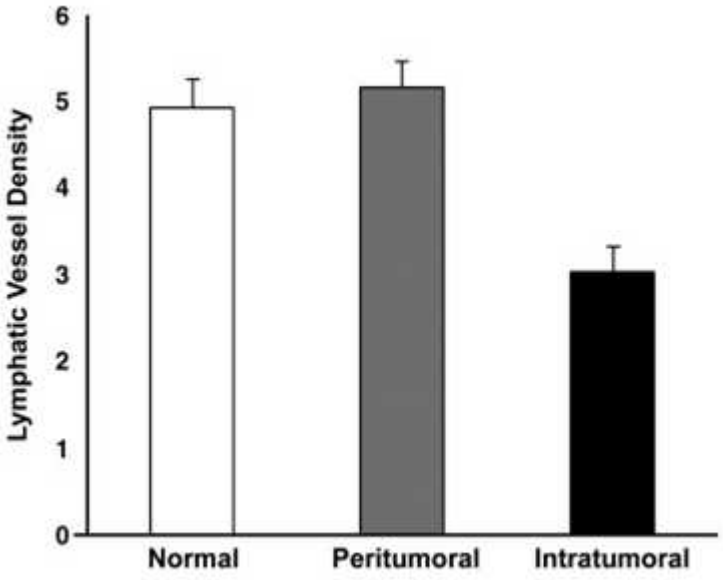


Figure: 2 Relationship of LVD with each compartment

Their findings correlated with another group who found that intratumoral LVD was inversely proportional to lymph node metastasis and peritumoral LVD directly correlated with lymph node involvement in non–small cell lung carcinoma. It was also shown that, LVD did not correlate with intratumoral vascular endothelial growth factor expression (VEGFE) [40].

In our study we plan to evaluate staining of D2-40 with average number of LVD in 10 hot spot fields at magnification x200. Cell positivity will be evaluated in the intratumoral, normal, and peritumoral tissue. The peritumoral tissue is the squamous epithelium immediately around the invasive tumor, which shows dysplastic changes which are mild to severe.

The area beyond this tissue is normal epithelial tissue, without any dysplasia or atypia. The immunoreactivity is considered negative if it stains less than 10% of endothelial cells and positive if it stains greater than 10% of cells. Occult metastasis, detected by immunomarkers, are taken as a causative factor for recurrence in node negative patients with cancer. 20% of clinically node (cN0) - negative patients will have occult metastasis. SCC with occult metastasis has greater risk of recurrence. There was also a study done by Niakosari et al where retrospective melanoma cases with routine hematoxylin-eosin staining were compared with D2-40 histochemical marker for confirmation of lymphatic invasion. Immunostaining with D2-40 increases the incidence of detection of lymphatic invasion relative to conventional hematoxylin-eosin staining in melanoma. In both frozen as well as formalin fixed tissues, D2-40 is a highly specific marker of lymphatic endothelium [31].

Study conducted by Yamauchi et al with combination of H&E and D240 in breast carcinomas confirmed that the latter helps the pathologist to find tumor nests suspicious of being emboli in H &E[48]. Recently Minardi investigated the role of CD44 immunoexpression within tumoural and non-tumoural tissue in order to predict its role in early metastasis and further stratify the need of lymph node dissection. CD44 is a cluster differentiation cell surface receptor for hyaluronic acid, and has been implicated in tumourigenesis and metastasis[49].

Recently, a study was carried by Wang et al of D2-40 LVD in esophageal SCC. They estimated LVD in three different compartments i.e peritumoral, tumoral and normal tissue as well as looked for D2-40 expression in tumor epithelial cells and stromal cell reactivity as a predictor of lymph node metastasis. These were correlated with clinico pathological variables and survival outcome of the patients were studied. They found that there was high intratumoral LVD, and not high peripheral LVD and increased expression of D2-40 staining of tumor cells and not in tumor stromal cells were responsible for lymph node metastasis. This increased intratumoral LVD was inversely related to overall survival of the patient.[53] This study was in contradiction to all other studies.

Materials and Methods:

All the procedures implicated in the study were approved by the Institutional Review Board of Christian Medical College, Vellore. Resection specimens for squamous cell carcinoma of the penis received in the department of General Pathology, Christian Medical College from. January 2006 to September 2014 were utilized for the study. The study was retrospective and archival stained and mounted slides and formalin fixed paraffin embedded tissue blocks were retrieved. Clinical details were obtained from clinical work station and the initial pathological diagnosis was reviewed from Pathology work station using PACS (Picture archival and communication System). The samples had been fixed overnight in 10% buffered formalin and they were embedded in paraffin wax using conventional methods. Haematoxylin and eosin stained slides of all cases of carcinoma of penis were reviewed. 3-4 micron sections were taken from the paraffin embedded blocks for performing immunohistochemistry (D240 and p53) by using the standard DAKO protocols. In the current investigation, we initially reviewed 57 diagnosed cases of SCC of penis that underwent partial or total amputation with lymphadenectomy from a period of 81/2 years (January 2006 to September 2014), out of which 8 cases were excluded as paraffin blocks were not available or adequate for immunohistochemical study.

The remaining 49 cases were divided into those with metastatic nodes and those with no nodal metastasis.. Accordingly there were 16 cases that had pathological lymph node metastasis and 33 cases that did not have metastasis. All available clinical parameters and pathological variables were included in the study so as to compare their relation with these prognostic markers. Paraffin blocks with complete squamous epithelium which included peritumoral, tumoral and normal tissue was a must criteria for D2-40 study.

Cases that were excluded were "slide review only" cases, amputation specimens referred from other hospitals without lymph node status or clinical details, patients who underwent wedge biopsy only or amputation in our center but without lymph node dissection, and blocks without complete epithelium.

Following variables were studied:

- Age of the patient.
- Presence of phimosis.
- Balanitis xerotica obliterans
- Circumcision.
- Clinical stage.
- Gross tumor size
- Depth of invasion.
- Histological type
- Histological grade

- Infiltration of corpus cavernous/spongiosum
- Urethral infiltration
- Lympho vascular invasion on H&E(LVI)
- Density of p53 in the tumor.
- Lymphatic density assessment by D2-40 in peritumoral/normal area..
- Pathological involvement of superficial and deep inguinal and pelvic lymph nodes.

Histological grade was classified as per the Broder's system as G1 (well), G2 (Moderate) and G3 (poorly) differentiated. Lymphatic emboli were defined as the presence of tumor emboli within the endothelium-lined lymphatic spaces highlighted by D2-40.

Quantification of Lymphatic vessel density by D2-40:

Using D2-40 the density of the lymphatics in the peritumoral and normal area was evaluated. The extent and the intensity of staining were evaluated in peritumoral and normal area. The peritumoral compartment is that covered by squamous epithelium immediately around the invasive tumor, which shows dysplastic changes of various grades. The area beyond this tissue is the normal tissue without any epithelial dysplasia.

The sections were first scanned at 4x to identify hot spot fields with the maximum density of staining for D2-40 positive endothelial cells. The "Hotspot" region was then taken as the field for counting. The area with the greatest number of discrete lymph vessel staining was counted. The counting was done at magnification 200x and the counts were expressed as highest number of lymphatics in 10 hot spot fields. Mean of number of lymphatics in peritumoral and normal area were calculated for each case. Lymphatics of normal area were used as control for comparison of lymphatics in peritumoral area. The immunoreactivity is considered negative if only less than 10% of endothelial cells were positive and it is considered positive if greater than 10% cells stained with D2-40 cells. The basis of this cut off value was the study by Minardi et al. Clusters of D2-40 positive cells, irrespective of presence or absence of a lumen was also counted as one lymphatic channel in the peritumoral area.

We found that most intratumoral lymphatics were malfunctional, small with collapsed lumen, in contrast with the widely open lymphatics in peritumoral regions[51]. This also helped us to detect tumor emboli within small lymphatics that was missed in the original histological section.

Quantification of Tumor density for p53:

p53 density was calculated in 100 tumor cells under low power field (10x) and expressed in per cent. p53 was graded as positive when at least 20% of the tumor cells showed nuclear staining.

Immunohistochemistry Method:

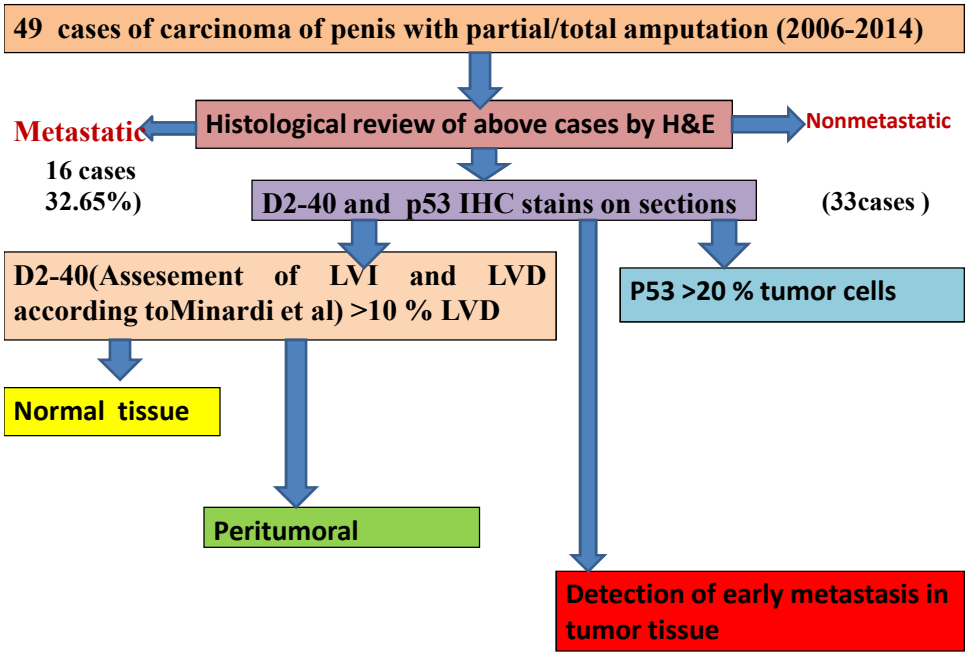
D2-40 and p53 immunostains was carried out using automation by Ventanna Bench Mark XT autostainer, using DAKO reagents and the same guidelines were used for both markers:

Paraffin embedded tissue sections were cut at 4µ thickness and floated in poly L-Lysine coated slides and incubated overnight at 37˚C. These slides were then treated with 4% milk solution for 10 minutes to eliminate the hydrophobic effect and give positive charge to the slides. Then the slide labels were bar coded and the labeled slides were loaded in Ventanna Benchmark XT autostainer (a fully automated immunostainer). Individual protocols have been designed in the software attached to the machine for each marker. Specific protocols were selected according to the marker. A standard protocol was used for most of the markers with minimal variation in certain cases..

The steps included in this protocol were as follows:

- Deparaffinization followed by liquid cover slip application.
- Heat induced antigen retrieval by treating with standard CC1 solution (pH patent with the company) for one hour at 90˚C..
- Primary antibody was added and incubated for 40 minutes at 37˚C.
- Secondary antibody (Multimer) was added and incubated for 8 minutes.
- Slides were counterstained with Haematoxylin and incubated for 8 minutes.
- Incubation with the bluing reagent for 4 minutes.
- Slides were brought to 80% alcohol (2 changes) to remove the liquid cover slip.
- Dried and mounted in DPX.
- Controls: colonic adenocarcinoma for p53 and skin for D2-40.

Figure: 3 Algorithm of assessment of D2-40 and p53 in Carcinoma of penis



Statistical Analysis

- Data was analysed using a statistical software STATA version 13.1.
- Descriptive statistics for continuous data were expressed as mean with S.D or median.
- Categorical data was expressed as frequencies and percentages.
- Sensitivity and specificity for D2-40 in carcinoma penis were obtained from ROC curves.
- Association between D2-40 and p53 with clinical stage, histological grade and lymphovascular invasion was calculated using chi square test.
- P value of <0.05 was considered significant.
- The following formula was used to calculate the sample size.

  N=4xpxq/d2

  P=sensitivity/specificity.

  Q=1-P d=precision-10%

Results

A total of 49 SCCs of penis with lymphadenectomy were included in our study from January 2006 to September 2014 from the department of General pathology, Christian Medical College, Vellore.

Mean age of presentation in the study was 53 with a range of 35-81 years. (Figure 1)

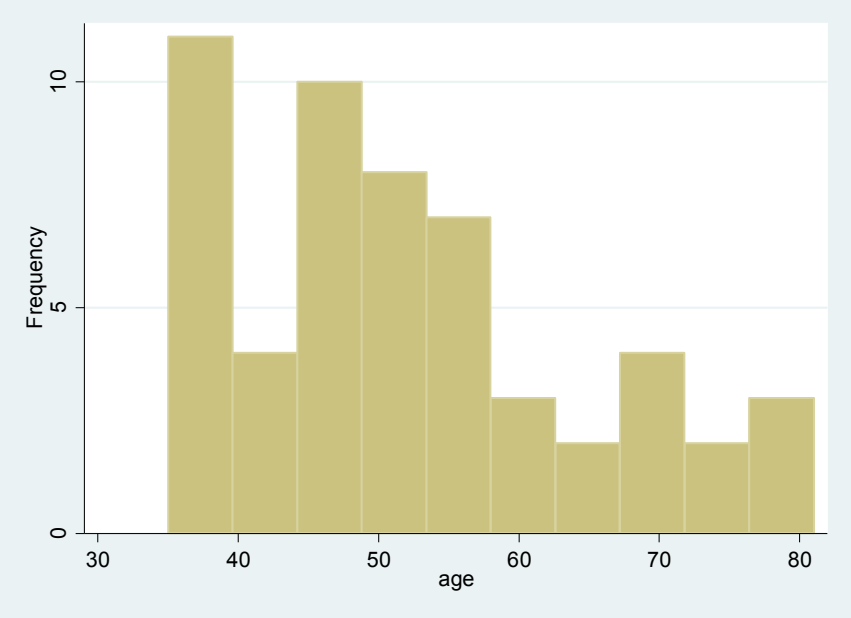


Figure1: Age distribution of study cohort.

3(6.12%) patients had balanitis xerotica obliterans, 10(20.41%) had phimosis, 3(6.12%) patients had undergone circumcision. Majority (43/49) of our patients underwent partial amputation while only 6 patients had total amputation.

The most common site of tumor was the glans (44/54-89.80%) and majority of them presented with ulcerating lesion. The remaining tumors were located in the prepuce 3(6.12%) and coronal sulcus 2 (4.08 %.)

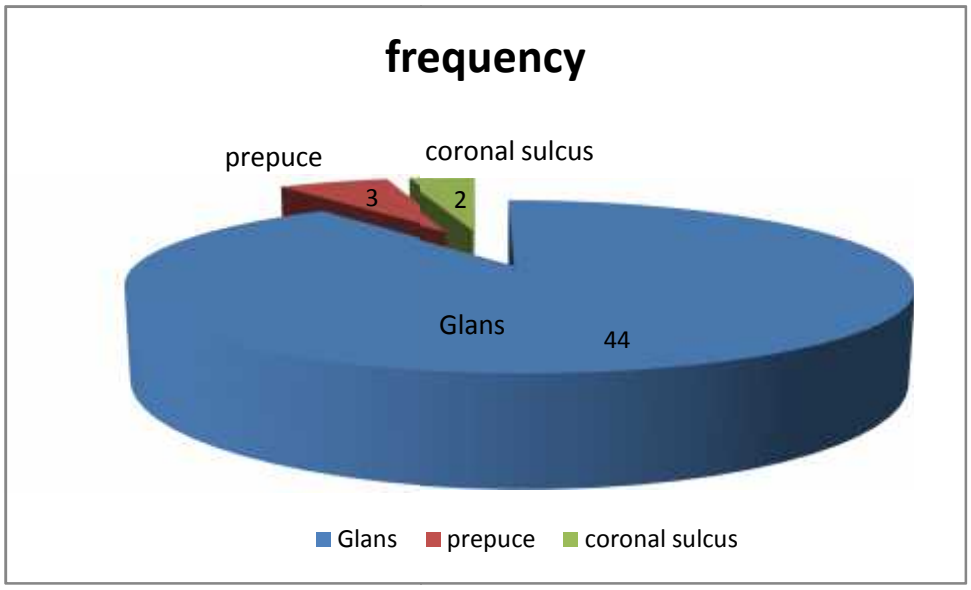


Figure 2: Distribution of tumour site in study cohort

Common histological type of the tumour was conventional squamous cell carcinoma.

Most of the cases 30 (61.22%) were of grade 1 histology, 15(30.61%) of grade 2 and 4(8.16%) were of grade 3 histology.

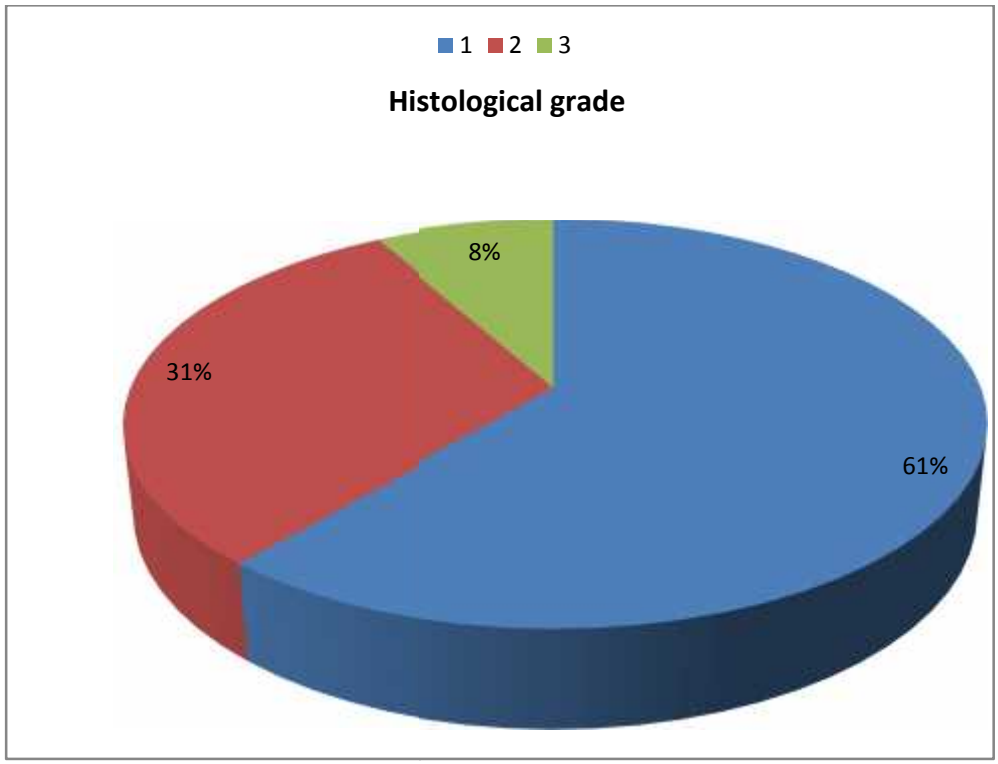


Fig 3: Distribution of histological grade

17/49(34.69) cases showed lymphovascular invasion.

35(71.43%) showed invasion into corpus cavernosum and/or spongiosum.

Mean depth of invasion was 0.9mm, maximum depth of 3cm and minimum depth of 0.1mm.

Pathologically nodal metastasis was found in 16 of the total 49 cases (32.65%). Of these 13/49(26.53%) cases showed metastasis in the right superficial inguinal lymph node groups, 9/49(18.37%) in left superficial inguinal lymph node group, 4/49(8.16%) in the right deep inguinal lymph nodes, 2/49(4.08%) in the left deep inguinal lymph nodes, and 3 (6.12%) right pelvic lymph node.

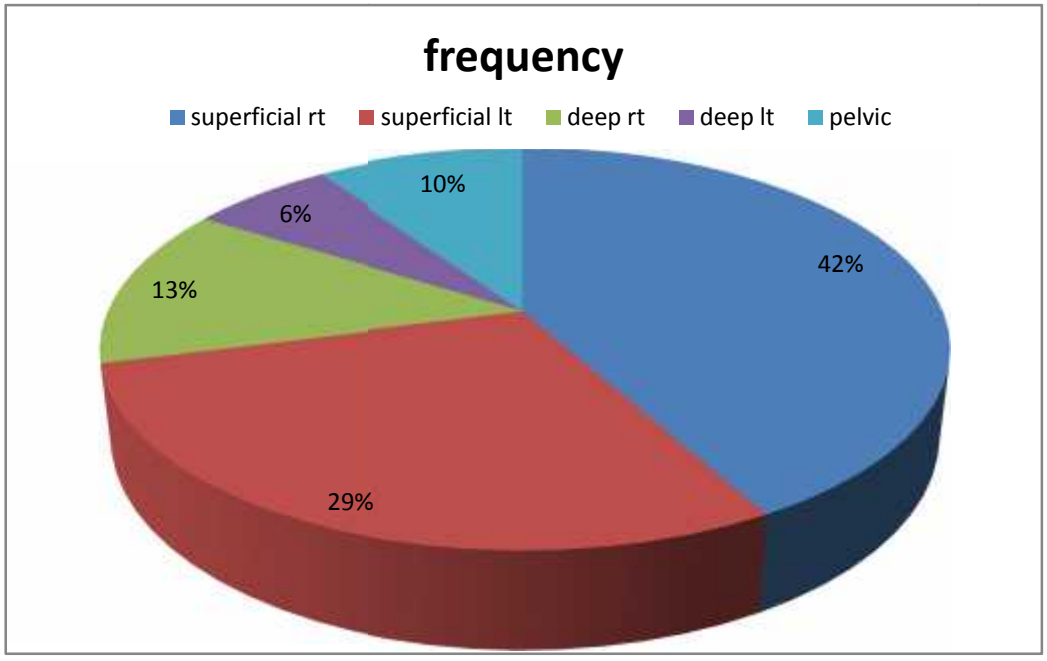


Figure 4: Distribution of pathological node positive status

Clinical node status was N0 in 32/49(65.30%), N1 in 6/49(12.24%) cases, N2 in 1/49(2.04%), N3 in 3/49 (6.12%) and Nx 7(14.29%)

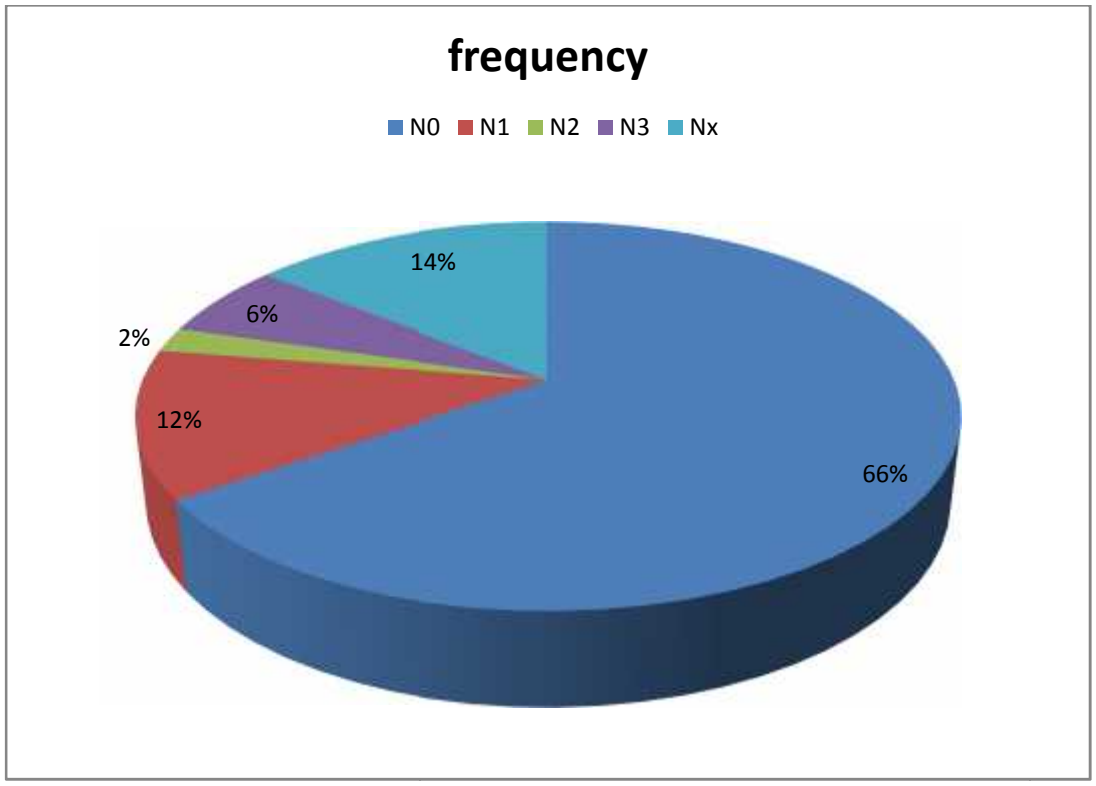


Fig 5: Distribution of clinical node

Commonest pathological stage noted was pT2 23/49(46.94%), followed by T1 in 14/49(28.57) & pT3 in 12/49(24.49)

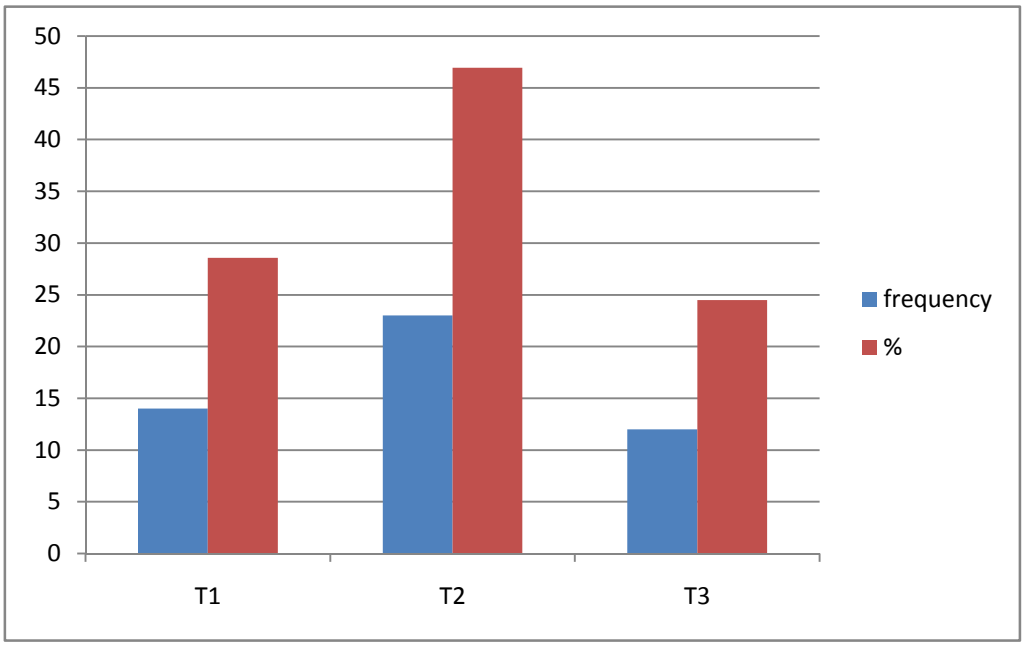


Fig 6: Distribution of T stage

Table: 1 for p53 cases above and below the cut off

P53cut off	Frequency	%
<20	13	26.53
>20	36	73.47
Total	49	100.00

Assessment of p53 density showed that 36 of the total 49 cases had a high (> 20 %) p53 density while the remaining cases had less than 20 % tumor density in the tumor cells (73.47%).

Table: 2 Depicts Lymph node status in relation with p53

	P53 positive	P53 negative	Total lymph nodes
Metastatic	13	3	16
Nonmetastatic	23	10	33
Total cases	36	13	49

The above table shows that of the 36 cases that had high p53 density, only 13 cases showed nodal metastasis and 23case did not show nodal metastasis.

This result shows that p53has a sensitivity of s 81.3% sensitive and specificity of 30.3%, thus giving a positive predictive value of 36.1% (CI 20.8%-53.8%) and negative predictive value of 76.9% (CI 46.2%-95.0%). Overall p53 does not show statistical significant correlation with nodal metastasis.

The following variables do not show t statistically significant correlation with p53 and are therefore independent factors for prognosis.

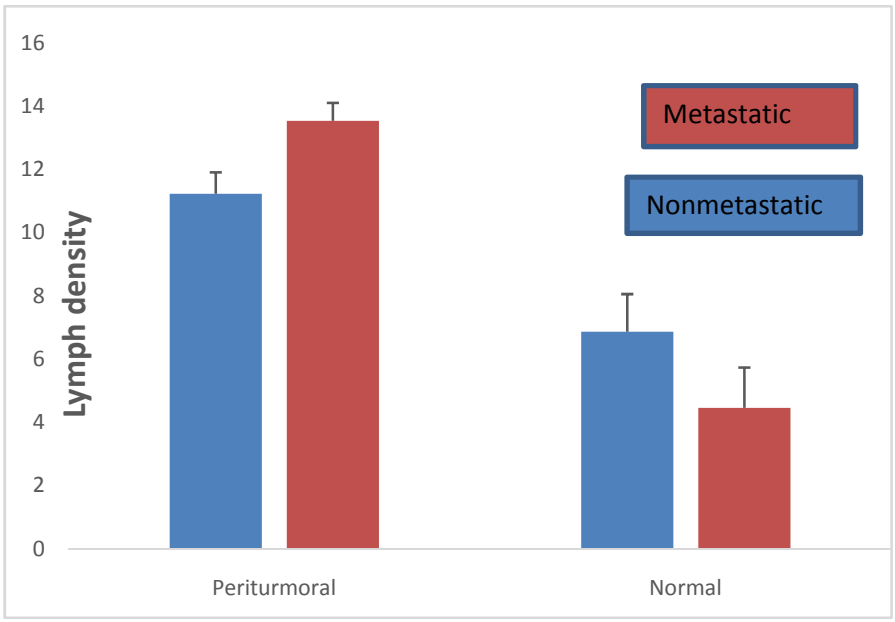
Age (p0.4), BXO (p 0.7), phimosis (p 0.7), circumscision (p 0.7), Node status (p0.4), histological grade (p 0.3), depth of invasion (p 0.9), pathological lymph node status (p0.3) and lymph vascular invasion (p0.7).

It is seen that the only statistically significant correlation with p53 tumor density was the T stage with value of 0.04. .Hence involvement of corpus is statistically significant with

(p 0.01).

All 49 cases had tumor free skin, corporal and urethral margins (100%)

On immunohistochemistry 36 patients had increased p53 density (>20 cut off) in the amputated specimens, of which 23 cases had negative nodes. Only 13 cases showed nodal metastasis and therefore in these cases p53 was early predictor of metastasis.



: **Figure: 7 Comparison of lymphatic density in normal and peritumoral tissue in metastatic and Nonmetastatic groups in carcinoma penis**

Above figure shows that there is increased LVD in the peritumoral area compared to normal area in metastatic groups. In nonmetastatic groups this was not increased.

Table: 3 D2-40 positve and Negative cases in metastatic and nonmetastatic nodes.

Lymph node	p53		Total
Positive	11	5	16
Negative	19	14	33
Total	30	19	49

Above table depicts that 11/16 positive node cases showed increased LVD in the initial amputed specimens. 19/33 negative lymph node cases s also showed increased LVD. D2-40 showed sensitivity of 68.75% and specificity of 42.42% at 1.87 cut point with positive predictive value of only 36.7% (CI 19.9%-56.1%) and negative predictive value of 73.7% (CI 48.8%-90.9%). Therefore it suggests that D2-40 is sensitive but it is not specific to predict early metastasis.

ROC for D2-40 is 0.48 with confidence interval of (0.3158-0.6576) only, which suggests that D2-40 is not able to discriminate between the patients case who might go on to have nodal metastasis and those who may not.. Therefore it is not able to predict early metastasis as it is not specific marker even though it is only sensitive.

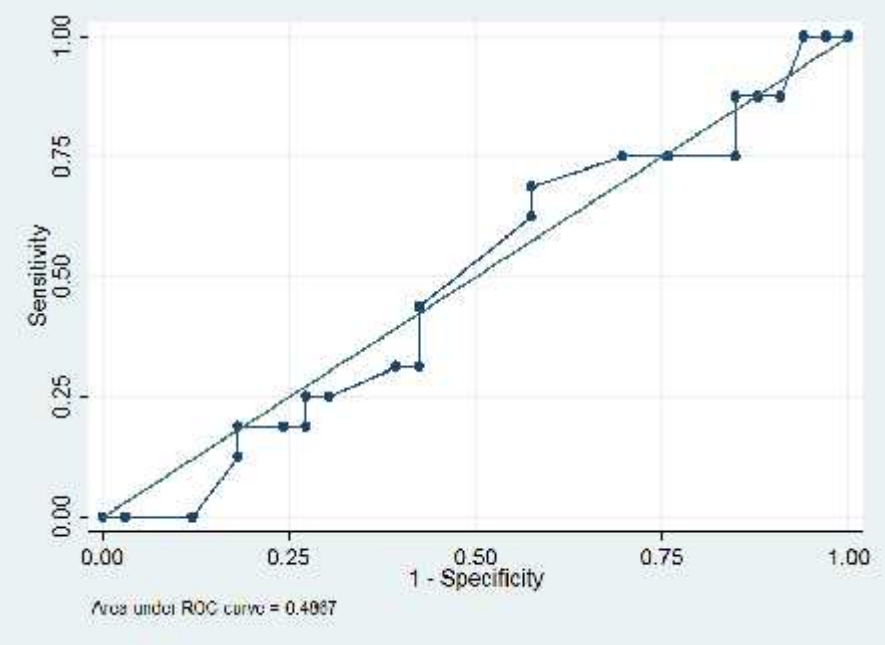


Fig8: ROC for D2-40

Comparison with other variables:

D2-40 is statistically not significant with any variables as follows:

Phimosis (p 0.5), circumcision (p0.3), T stage (p 0.06), N stage (p 0.7), histological grade (p 0.3), BXO (p 0.8), LVI (p 0.3), histopthological lymph node status (p 0.4)

ILLUSTRATIONS

Fig:9 H&E Conventional SCC {10X}

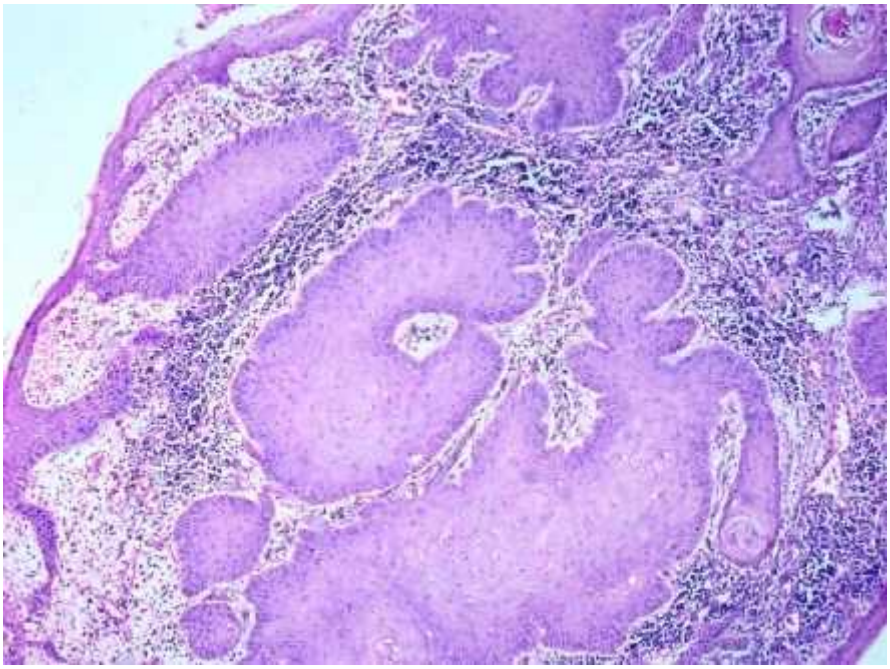


Fig: 10 SCC with peritumoral and normal area {10X}

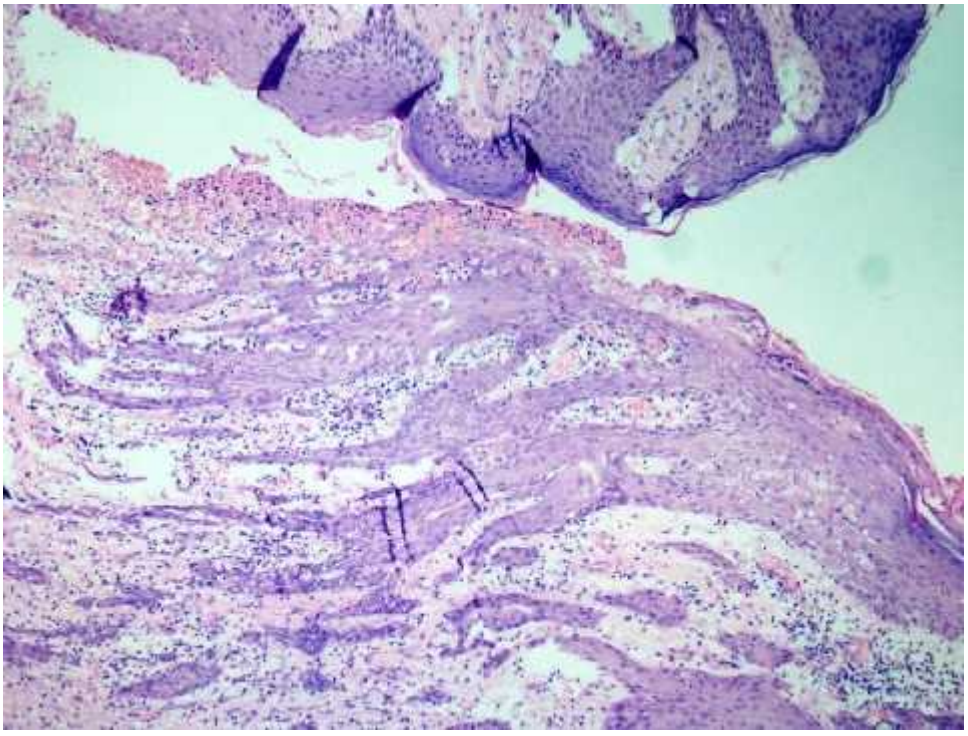


Figure: 11 D2-40 Peritumoral & Tumoral area {10X}

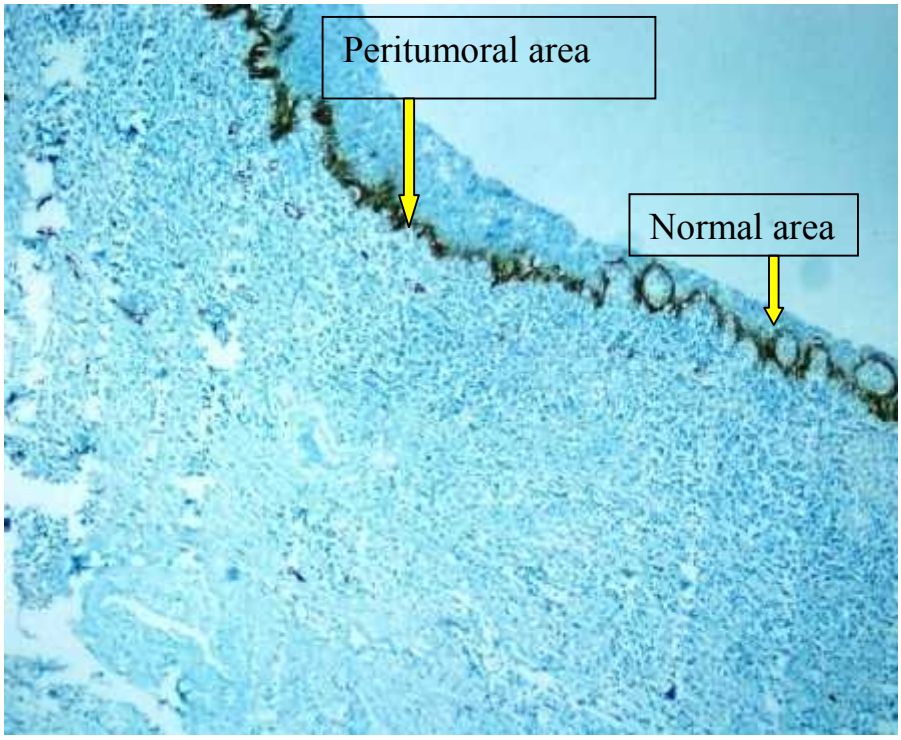


Fig 12 D2-40 Peritumoral area with increased LVD {10X}

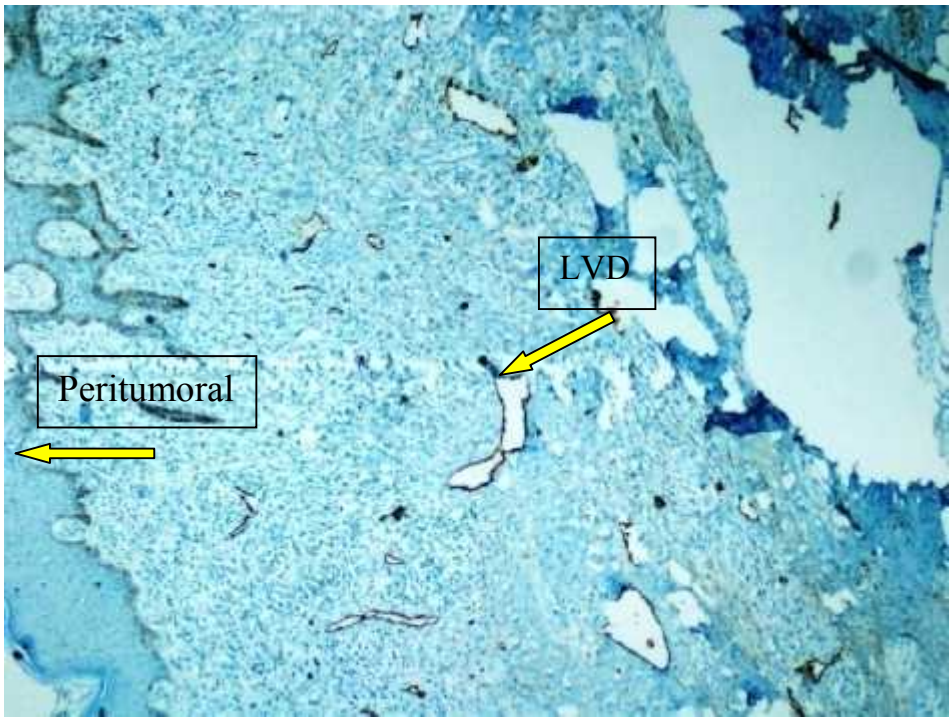


Fig: 13 Peritumoral with increased LVD and LVI {10X}

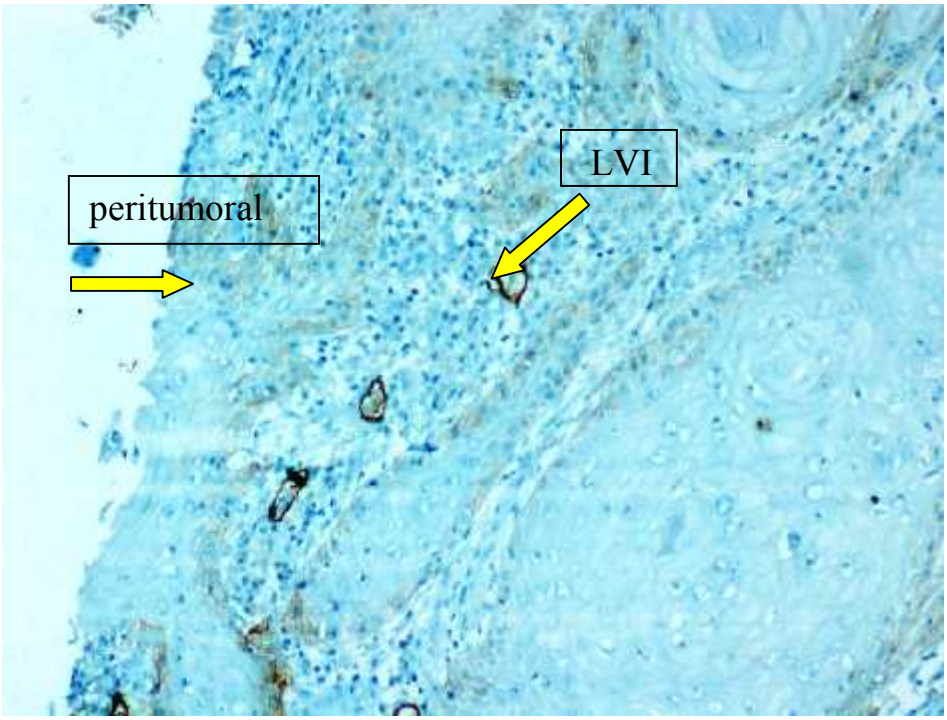


Fig 14 LVI {20X}

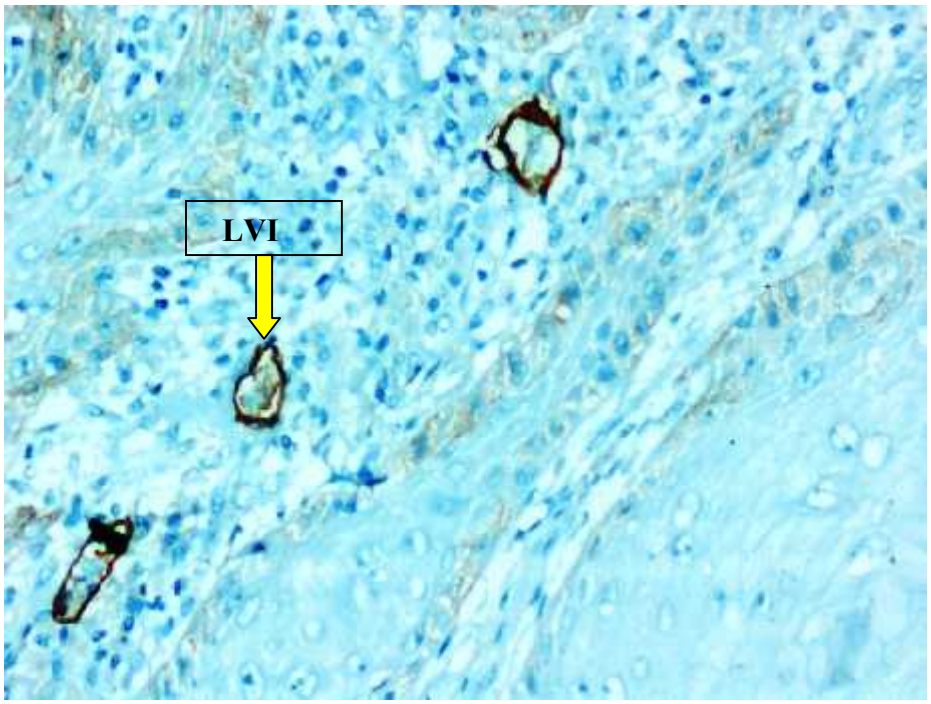


Fig 14 D2-40 in normal area {10X}

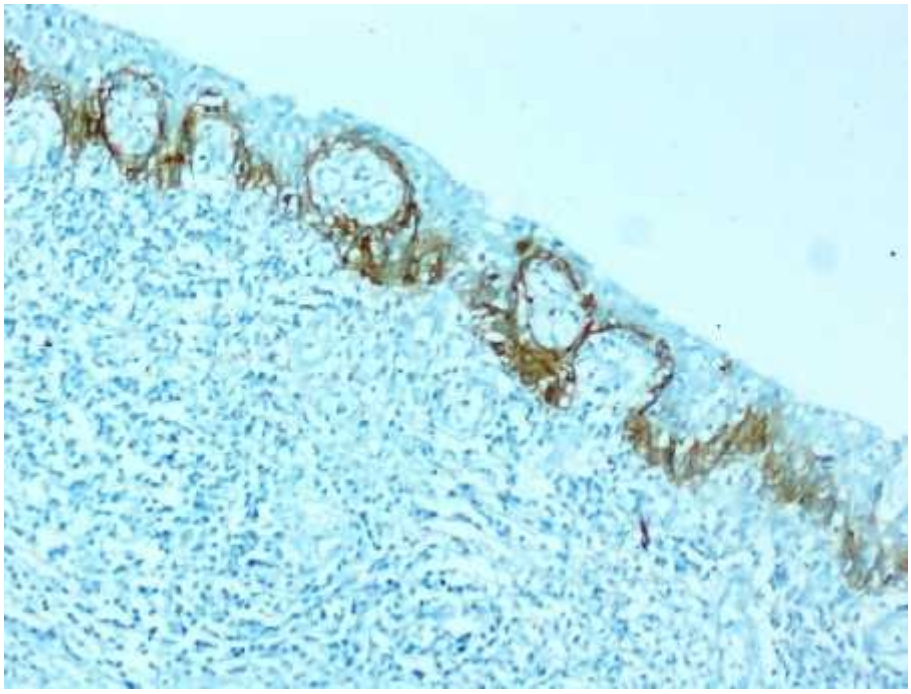


Figure: 15 D2-40 Tumoral area with collapsed lymphatic {20X}

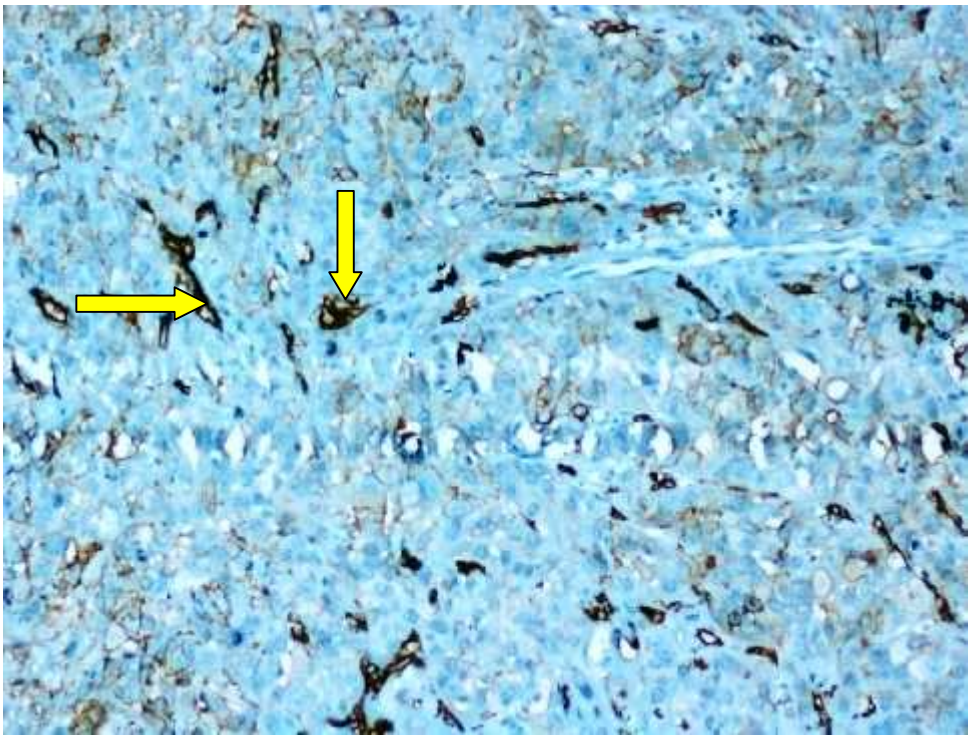


Fig: 16 D2-40 Increased LVD one showing tumor emboli {20X}

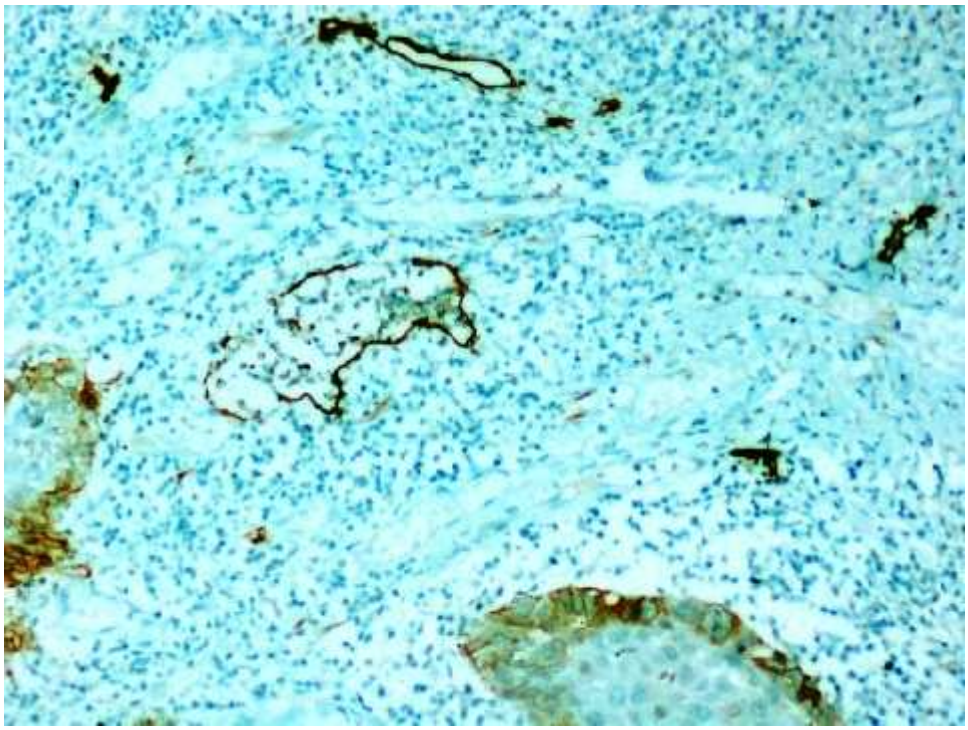


Figure: 16 D2-40 highlighting lymphatic vessel with tumor emboli {20X}

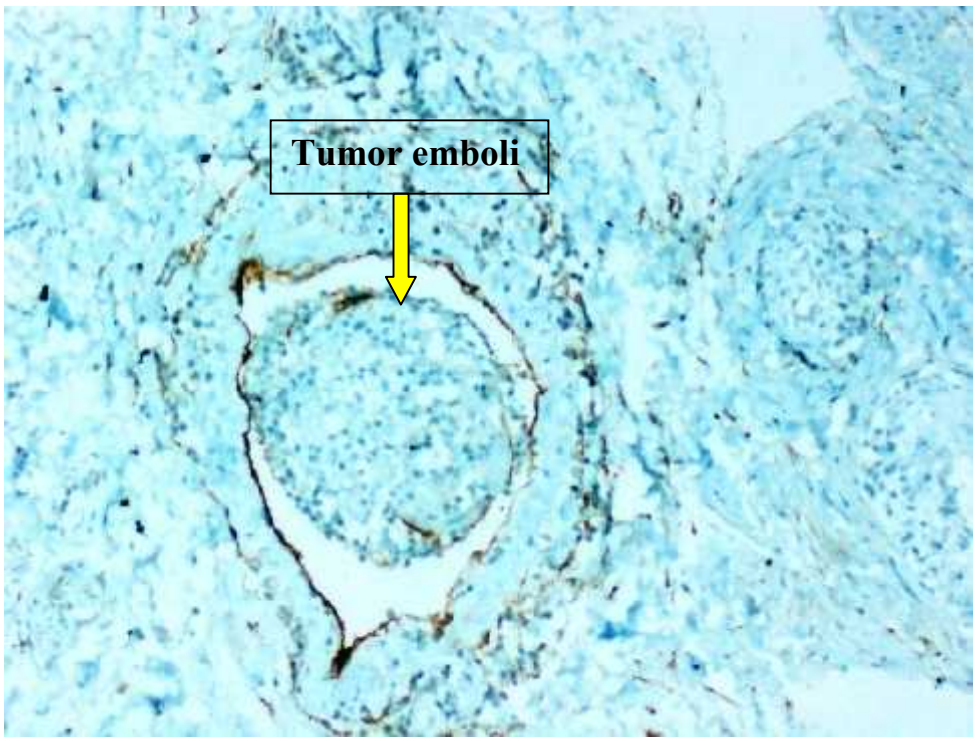


Figure:17 Increased p53 tumor density (4X)

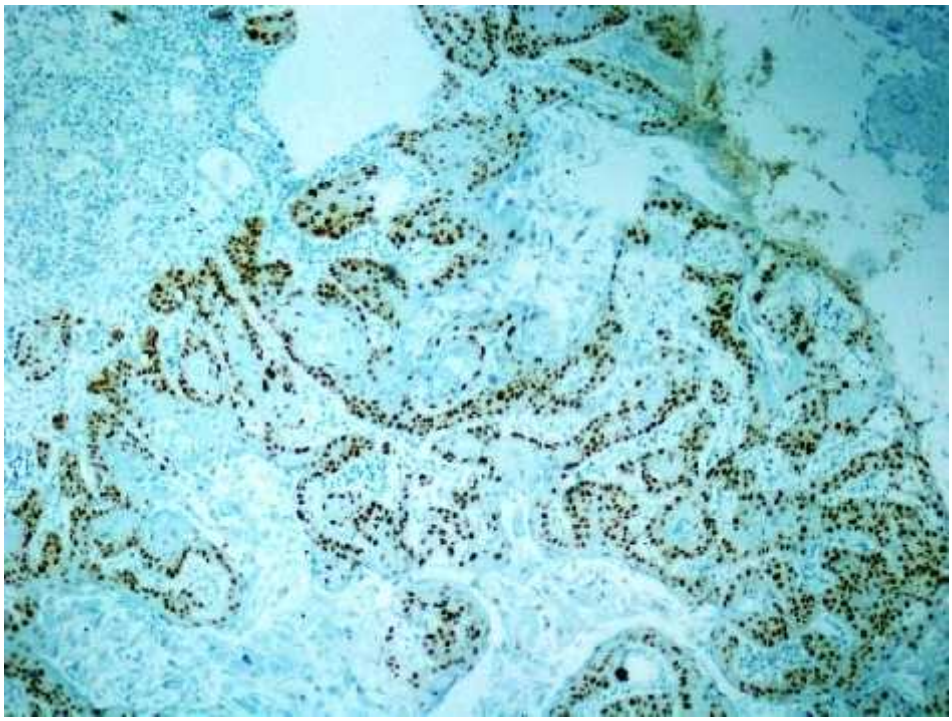


Figure:18 p53 high tumor density (20X)

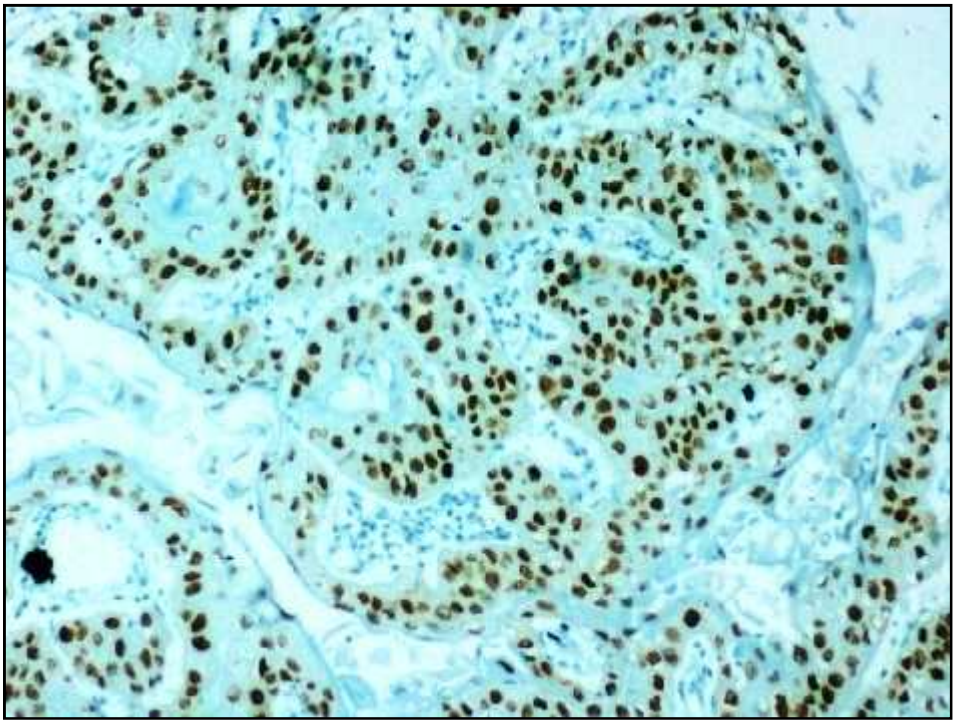
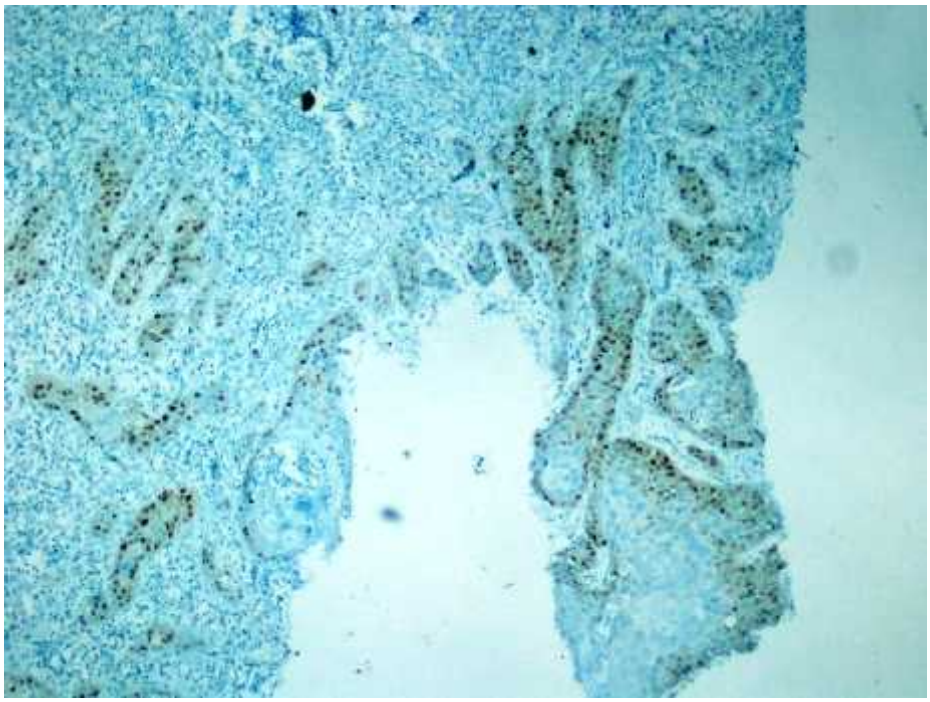


Figure: 19 Decreased tumor density <20% p53 (10X



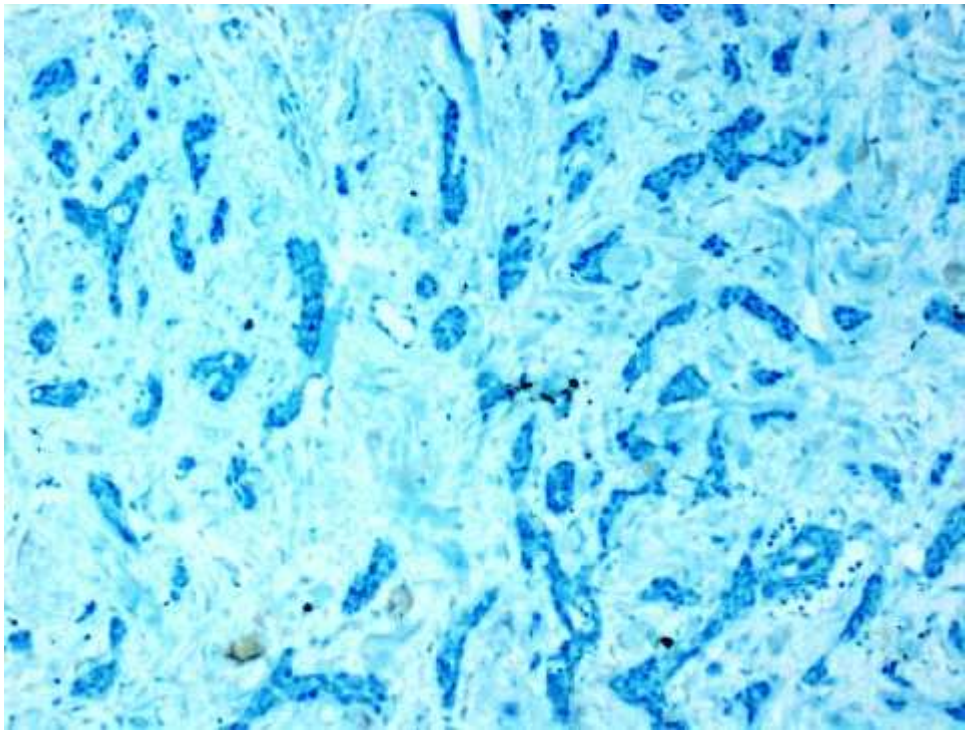


Fig: 20 Absent p53 in well differentiated SCC {10}

Discussion

The most important prognostic factor in squamous cell carcinoma of penis is lymph node metastasis. European Association of Urology guidelines stratify patients into 3 risk groups: low (Tis, pTaG1-G2, pT1G1), intermediate (pT1 G2), and high (pT2/T3 G2/ G3)[5]. The therapy of choice is node dissection for patients with clinically palpable inguinal lymph nodes and for those with unfavorable histopthological characteristics such as basaloid, sarcomatoid patterns of growth and increased depth of invasion. In the current study we have tried to analyse the role of D2-40 and p53 as predictive factors for penile carcinoma to better define the strategies for treatment of penile cancer. We evaluated the association of D2-40 and p53 with the clinical and histopthological variables related to potential lymph node metastasis .This is also a comparative study to see which would be a better predictor for early lymph node metastasis between D2-40 and p53. In this study we also discovered that D2-40 could be useful in detecting lymphatic invasion that is generally difficult on routine histological sections.

The expression of p53 has been shown to have prognostic importance in head and neck SCC by Lopes et al. Recently D2-40 is also being used in the study of predictor of lymph node metastasis in tumors of bladder, breast, Gastro intestinal tract[29-35] etc.

Our study is based on a recent original article by Minardi et al and Lopes, for D2-40 and p53 expression in predicting early lymph node metastasis respectively. To date, there has not been any reported study regarding the usefulness of the markers in penile carcinoma from India. Being a referral centre many cases of penile carcinoma are diagnosed every year in our hospital. Hence our proposal to study the response of the two immune markers in penile SCC was put forward to our IRB. The study was carried out on 49 patients and the aim was to assess the correlation of the above markers with age, clinical stage of the disease, histological type, histological grade, depth of invasion, common nodes involved, and association of precancerous lesions like phimosis and BXO and status of margins. This was done by calculating the lymphatic density and tumor density by D2-40 and p53 respectively. They were evaluated by using the cut off value for each as mentioned by Minardi et al and Lopes et al respectively. The sensitivity and specificity, positive predictive value, and P value for each marker were calculated. The mean age of presentation in this population of study was 53 with a range of (35 -81) in total of 49 cases and this is similar to the study by WHO.

10(20.41%) cases presented with phimosis and 3 (6.12%) with BXO, which was confirmed on histopthological slides during the initial diagnosis. These variables did not have statically significant correlation with the above tumor markers. Most of the patients had undergone partial amputation, only six underwent total amputation.

Similar sampling was seen in other studies[25-28,] Of these 49 patients, histopthological evaluation showed 16cases with lymph node metastasis, and the most common lymph node group involved was right superficial 13/49(26.53%) group of lymph nodes. Clinically however the commonest presentation was N0 stage that formed 29 (59.18%) and these findings are similar to the study by Lopes et al.

Our analysis showed that majority of our patients (23/49) presented in pathological stage pT2 followed by pT1 and this was similar to studies by Minardi et al and Lopes. group[24, 25, 28.] However some other authors have noted pT3 and pT4 as the common stage[25.]. . The commonest site of involvement was glans penis (44/49), which was similar to findings in other studies[25-28]. The predominant histological type in our study was conventional squamous cell carcinoma which was also similar to other studies [25-27].

Most common histological grade of the tumor was well differentiated SCC followed by moderately differentiated and few cases (5%) were poorly differentiated which is parallel to Lopes et study. A recent study states that higher grade and more aggressive tumors are prone to disseminate, early even without lymph angiogenesis, while the more differentiated tumors need denser lymphatic network in the form of increased LVD for their metastatic spread[51]

All 49 cases had tumor free skin, soft tissue and urethral resection margins. Lymphovascular invasion was seen in all the 16 cases that histologically had metastatic lymph nodes. It was stated by Lopes et al that there is strong association of this variable with increased chances of metastasis[25].

In our study 11/16 node positive cases and 19/33 node negative cases showed increased LVD in the peritumoral region, using D2-40. In all the above case there was increased LVD in peritumoral area with decreased expression in the epithelial cells seen in peritumoral compartment and decreased LVD in intratumoral compartment with increased epithelial cell staining while the normal tissue showed weak expression of D2-40 both in lymphatic vessels and epithelium . Similar observation was found in the study done by Minardi et al in carcinoma penis and Cheng et al in carcinoma prostate[38]

The intratumoral lymph vessels were collapsed and were therefore malfunctioning, with reduced D2-40 staining. It was also noted that in this area the tumor cells also showed D2-40 staining. But converse was seen in a study by Faoro et al in non small cell carcinoma of lung where there was increased LVD in intratumoral as well as peritumoral tissue than in the uninvolved adjacent lung parenchyma but their study did not show significant association with lymph node stage [52] .

Study of D2-40 was also carried in esophageal SCC recently where there was high intratumoral LVD than peritumoral LVD with increased expression in cancer cells and not in stromal cells were associated with lymph node metastasis, recurrence and overall survival of the patients[53] .

D2-40 showed sensitivity of 68.75 and specificity of 42.42% at 1.87 cut point with positive predictive value of only 36.7% and negative predictive value of 73.7%. Therefore it suggests that D2-40 is sensitive but it is not specific to predict early metastasis, with positive predictive value of only 36.7%.

ROC for D2-40 is 0.48 with confidence interval of (0.3158-0.6576) only, which suggests that D2-40 is not able to discriminate between the patients who might go to nodal metastasis and those who may not are not. Therefore it is not able to predict early metastasis as it is not specific marker even though it is sensitive.

The increased LVD in peritumoral compartment was found to predict lymph node metastasis in Minardi's study however the present study did not show statistical significance. This did not correlate with the histological grade or clinical stage either.. D2-40 is useful to confirm lymph node metastasis[3].

D2-40 was not statistically significant in correlating with other histopthological or clinical variables such as , phimosis (p0.5), circumcision (p0.3), T stage (p0.06), N stage (p0.7), histological grade (p0.3), BXO (p0.8). LVI (p0.3), or histopthological lymp node status (p0.4).

Histopthological stage was only parameter which was statistically significant in p53 study. Evaluation of p53 in our study showed that there were 36 cases with more than 20% p53 tumor density and 13 cases with less than 20% p53 density. Out of 16 positive lymph node cases, 13 cases showed high density. However 23/33 negative lymph nodes also showed increased tumor density for lymph node metastasis.. Present study showed that increased p53 tumor density had increased LVI with increased nodal metastasis. . Analysis showed that only the histological stage statistically correlated with p53 which is directly proportional to the lymph node metastasis. This was similar to the study done by Martins et al and Zhu et al. In our study majority were at T2 stage, and this stage the marker was sensitive but yet was not a predictor of early metastasis as the statistical positive predictive value was low. This marker statistically did not correlate with other multivariate variables. Relationship of each marker with other variables calculated by Chi square test showed that, age (p0.4), BXO (p 0.7), phimosis(p 0.7), circumscision (p 0.7) , Node status (p 0.4), histological grade (p 0.3), depth of invasion (p 0.9), pathological lymph node status (p 0.3) and lymph vascular invasion (p 0.7) did not statistically correlate with p53.

Therefore they are independent marker of prognosis. Only T stage with pvalue of 0.04 is statistically correlated with p53 .Hence involvement of corpus is statistically significant with (p 0.01). This marker has sensitivity of 81.3% and specificity of 30.3%. In our study p53 was more sensitive than D2-40.

Conclusion:

- Total 49 cases of partial total/ amputation of penis were studied.
- The sensitivity of p53 to detect early metastasis in SCC of penis was 81.3% and specificity was 30.3%.
- The sensitivity of D2-40 to detect early metastasis in SCC of penis was 68.8% and specificity was 42.4%.
- Therefore p53 is very sensitive to detect even mild increased tumor density in SCC of penis at 20 % cut off.
- p53 is more statistically sensitive in comparison with D2-40.
- D2-40 is sensitive in detecting increased LVD in conventional well differentiated SCC in our study, as most of our cases constituted well differentiated SCC.
- The increased density was more in the peritumoral area than in the normal area.
- The intratumoral lymphatics in our study were all collapsed and malfunctioned and were difficult to assess. Therefore they were not assessed.
- D2-40 highlighted the tumor emboli.
- Adjacent blood vessels were not stained by D2-40.
- Among the different clinicopathological variables assessed, p53 correlated only with the pathological stage of the disease.

- D2-40 was independent marker, it did not correalate with any of the clinicopathological variables included in our study.
- The positive predictive value for p53 to predict metastasis was only 36.1 %(CI 20.8% -53.8%) and negative predictive was 76.9% (CI 46.2%-95.0%) and for D2-40 positive predictive value was 36.7% (CI 19.95-56.1%) and negative predictive was (48.8%-90.9%).

To conclude, this is the only study done in India by comparing the two markers to detect early metastasis in partial /total amputed specimens in SCC of penis. p53 and D2-40 are extensively studied in different carcinomas at different sites. Both the markers had low specificity and low positive predictive value, which suggest that they are not able to predict lymph node metastasis even though they are sensitive and therefore they are not helpful to decide for prophylactic lymphadenectomy. Specificity could have been achieved, if the sample size was large and if the cut off for p53 was kept at 5%. D2-40 can be used as a good adjunct along with H& E sections on the initial amputated specimens to detect the definite LVI which needs to be confirmed by D2-40 staining in difficult and doubtful situations, on routine histology.

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ABBREVATIONS:

SCC: Squamous cell carcinoma.

LVD: Lymph vessel density.

LVI: Lympho vascular invasion

VEGF: Vascular endothelial growth factor

BXO: Balanitis xerotica obliterans

NOS: Not otherwise specified.

hr HPV: High risk Human Papilloma Virus

ANNEXURE

Squamous cell carcinoma of Penis: D2-40 and p53 immunostaining pattern.

Proforma sheet:

Name: Age: Hosp No. Biopsy

Clinical features Clinical Stage:

Clinical stage:

Gross:

Type of specimen: Total/Partial amputation

Tumor size:

Depth of invasion

No. of lymph nodes sampled

Microscopy:

Histologic type: Histologic grade: Well/Moderately/Poor

Tumor thickness/Depth-----mm

Vascular invasion:Present/Absent

	No of lymph nodes sampled		No. of lymph nodes positive
Superficial	Right		
	Left		
Deep	Right		
	Left		

IHC:

D2-40 cutt off 10%

- Peritumoral- LVD
- (Normal tissue- LVD (in%)

P53: Present/Absent Tumor density (in %) <20% / >20%