

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

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Abstract: ***Background:** The use of D-240 and p53 as a prognostic markers to predict early lymph node metastasis in amputated specimens of penis so as to avoid unnecessary lymphadenectomy. **Aim:** To detect early metastasis in amputated specimens of penis, by using two immuno markers, D2-40 and p53 and correlation of these with the histopathological variables and clinical stage. Also to compare which marker is the better predictor for metastasis. **Materials and Methods:** Retrospective study of 49 cases of partial /total amputation with lymphadenectomy of penile cancers were divided into metastatic and non metastatic groups on H&E sections in which 16/49 had metastasis. Histological slides from all cases were processed with immunohistochemical technique using anti-p53 and anti-D2-40 antibodies. 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. **Results:** 13 /49 cases showed increased p53 tumor density and 11/49 showed increased LVD by D2-40. pvalue of 0.05 was considered significant in our study. 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. **Conclusions:** 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.*

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

1. Introduction

Squamous cell carcinoma of the penis is common in developing countries like India and its incidence is similar to that of cervical cancer. It forms 20% of all malignancies in Asian countries. 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 and 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}. The traditional elective lymphadenectomy accounts for overtreatment in up to 80% of patients and is associated with high morbidity (30-90%)^{4,5}. 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 nodes⁷. 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.

2. Materials and Methods

The data and histologic slides of a 49 cases of carcinoma of penis done by partial/Total amputation of penis from year 2006-2014 were retrieved from the archives of Christian Medical College, Vellore. 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 8 & 1/2 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

Volume 7 Issue 4, April 2018

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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. Then 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 malfunctioned, small with collapsed lumen, in contrast with the widely open lymphatics in peritumoral regions⁵¹. This also helped us to detect tumor emboli within small lymphatics that were 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 4 μ 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).

Specific protocols were selected according to the marker. A standard protocol was used for most of the markers with minimal variation in certain cases.

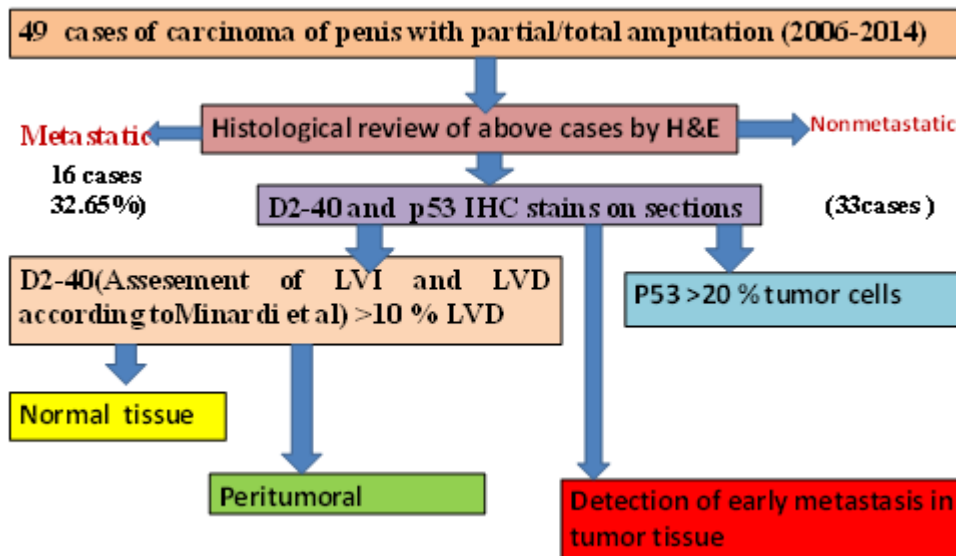


Figure 1: Algorithm of assessment of D2-40 and p53 in Carcinoma of penis

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.
 $N = 4 \times p \times q / d^2$
 $P = \text{sensitivity/specificity}$
 $Q = 1 - P$ $d = \text{precision} - 10\%$

3. 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. 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 %).

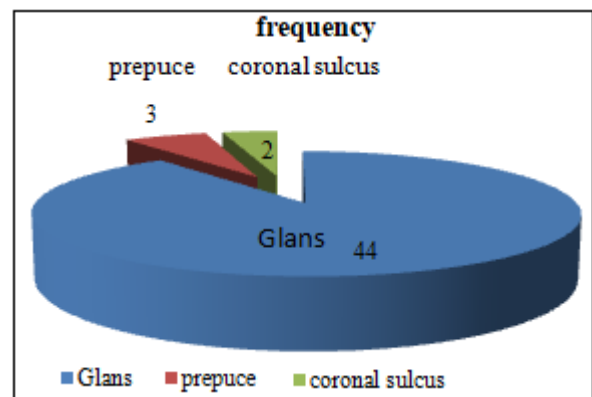


Figure 1: Distribution of tumour site in study cohort

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.

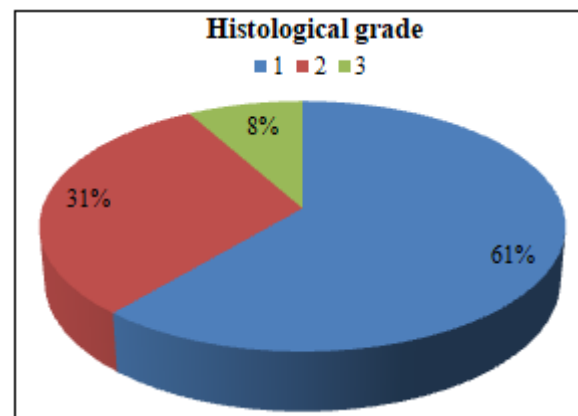


Figure 2: Distribution of histological grade

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.

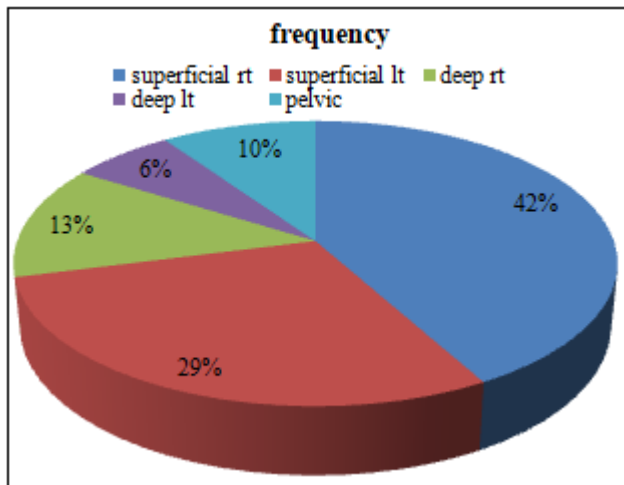


Figure 3: Distribution of pathological node positive status.

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%).

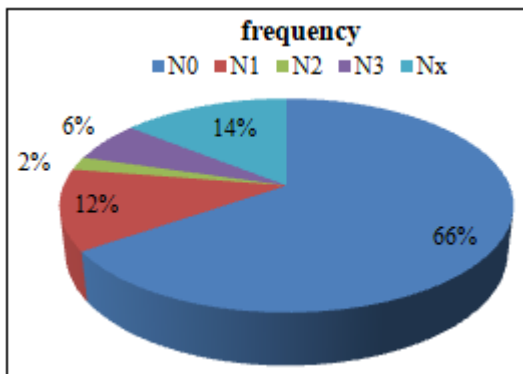


Figure 4: Distribution of clinical node

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

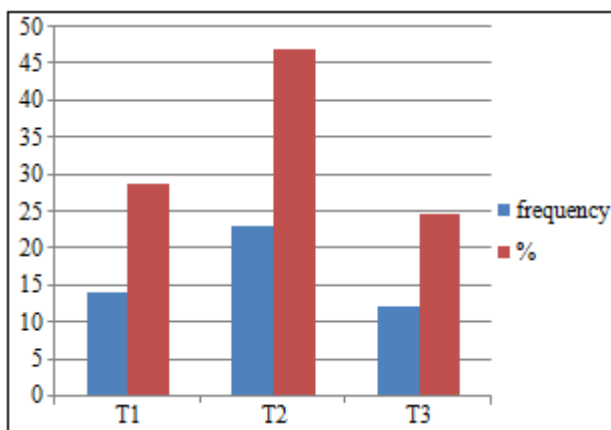


Figure 5: Distribution of T stage

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.

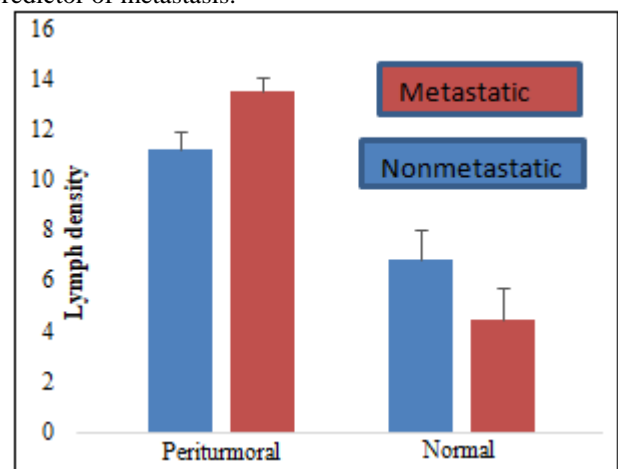


Figure 6: Comparison of lymphatic density in normal and peritumoral tissue in metastatic and Nonmetastatic groups in carcinoma penis

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 positive 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 amputated specimens. 19/33 negative lymph node cases 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.

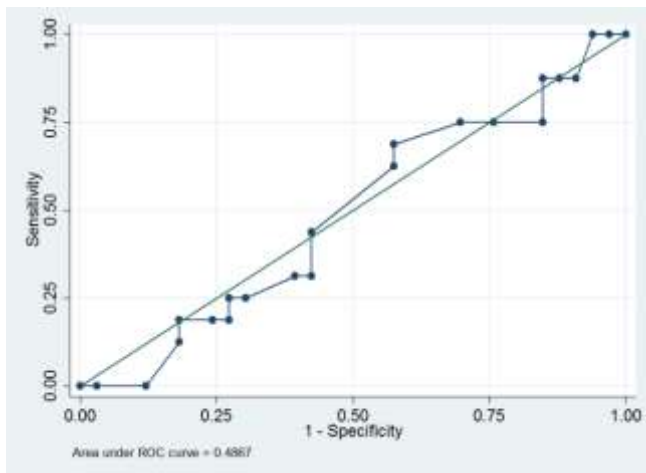


Figure 8: ROC for D2-40

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), histopathological lymph node status (p 0.4).

4. 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 histopathological 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 histopathological 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 tract²⁹⁻³⁵ 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. 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 histopathological 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 studies²⁵⁻²⁸. Of these 49 patients, histopathological 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 stage²⁵. The commonest site of involvement was glans penis (44/49), which was similar to findings in other studies²⁵⁻²⁸.

The predominant histological type in our study was conventional squamous cell carcinoma which was also similar to other studies²⁵⁻²⁷. 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 al 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 spread⁵¹

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 metastasis²⁵. 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 prostate³⁸

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 stage⁵².

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 patients⁵³

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 be. 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 metastasis³.

Histopathological 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), circumcision (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.

5. 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 correlate 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 amputated 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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Abbreviations

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