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Lymphovascular Density and Pro-Inflammatory Cytokines in Oral Squamous Cell Carcinoma

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I. INTRODUCTION:

Cancer of oral cavity is leading site of cancer in west and central region of India and contributing one third of burden globally with incidence of 19% and 2:1 male female ratio. About 90% of Oral cancers are Squamous Cell Carcinoma (OSCC) by histopathology; tumor size, cervical lymph node metastasis and distant metastasisare standard parameterswhichaffects prognosis of patients (1). Like other epithelial malignancies OSCC, favorablyspreads through lymphatic vessels to regional lymph node(cervical lymph node) and therefore, nodal metastasis is widely accepted and major prognostic factor in OSCC patients.Originally, lymphatic vessels werethought to work just like channels that act as way for tumor cells to metastasize to adjacent organ.Later, discontinuous structure of the lymph capillaries, a low lymph flow, minimal shear stress, and the composition of lymph with high hyaluronic acid content facilitate cell survival and providefavorable environment to tumor cells.Further, the discovery of some key lymphatic specific markers like lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1), Prospero-related homeobox-1 (Prox-1)and podoplanin (D2-40), an increased availability of in vitro and in vivo experimental systems to study lymphatic biology have high lightened a much more complex, active role for the lymphatic vasculature in metastatic tumor spread. This vasculature is regulated by a complex array of lymphangiogenic factors and chemokines secreted by tumor cells, stromal cells, tumor infiltrating macrophages, activated platelets and immune cells subsets (2-7).

Lymphangiogenesis is a basically absent under normal physiological conditions, it only takes place during certain pathological conditions such as inflammation, tissue repair and tumor growth,under such situationlymphatic vessels change their shape and size and allow proliferation and sprouting of new vessels from preexisting lymphatic vessels controlled by pro-lymphangiogenic factorslikevascular endothelial growth factor (VEGF) C and D. Such, pro inflammatory molecules thought to affect metastatic phenotype of tumor cells Also, collagen present in extra cellular matrix of tumor microenvironment strongly influences lymphangiogenesis by modulating VEGF level (6-7). Someexperimental work suggest lymphatic vessels expansion take place in two phases. In first phase of expansion of lymphatic vessels thought to be regulated by IL 7 and second phase by lymphotoxin (8). Moreover, in cases like receptor mismatch, difficulty in EMT where metastasis not possible via hematogeous route they assist tumor cells to move in peritumoral lymphatic vessles and help in dissemination, which then help tumor cells to reach sentinel lymph node, distant lymph node and finally in blood stream via thoracic duct and subclavian vein (8).

In present study, we try to find out association of Lymphatic vasculare with lymphangiogenic factor within tumor microenvironment in OSCC patients by assessing VEGF-C and inflammatory cytokine IL7expression,lymphovascular density (LVD), lymphatic vessel morphometry in tumor cells and its adjacent tissue environment.

II. PATIENTS AND METHODS

In this retrospective study, 67oral cancer patients who had been diagnosed and treated at Gujarat Cancer and Research Institute (GCRI) in the duration of 2015 to 2017 were included. The detailed clinical history such as patient's age, histopathological findings, treatment offered and disease status was recorded in the registers from the case file maintained at the Institutional Medical Record Department. Paraffin embedded tumor block of these oral patients were collected from Histopathology department of Institute. The study

was approved by Institutional Scientific Review Board and Ethics Committee.

Immunohistochemical localization:

Immunohistochemical localization of lymphatic vessels, VEGF-C, IL7was performed on formalin fixed paraffin embedded (FFPE) tissue blocks containing primary tumor evaluated by Hematoxylin and Eosin (H&E) staining, on Ventana Benchmark XT autoimmunostainer using Ventana reagents (Ventana, USA). The tissue blocks were obtained from the archives of the Pathology Department of the institute. 3-4 micron thin sections were cut on microtome (Leica, Germany) and taken on to 3-Aminopropyl triethoxyline (APES) coated slides. Briefly, the protocol includes following steps of deparafinization using EZ solution, antigen retrieval for 60 minutes using retrieval solution CC1, and incubation with Ultra View DAB Inhibitor for 4 minutes, 100µl of respective primary antibodies of podoplanin (D2-40)(1:20, Cell marque) for lymphatic vessels, anti-VEGF-C (1:75, Invitrogen), anti IL7 (1:50, Invitrogen) at 37°C for 32 minutes, Ultra View HRP Multimer for 8 minutes, Ultra View DAB Detection kit for 8 minutes, counterstained with hematoxylin for 8 minutes and mounted with DPX.

Scoring:

1. **Assessment of podoplaninin tumor cells**

Podoplanin expression on tumorcells were scored according to staining pattern as score 0-no staining, 1-only basal layer staining, 2-basal and suprabasal staining, 3-complete tumor staining (9). Individual tumor cells (ITC) were defined as dissociation of cells either in groups of cells (n<15) oras single cells according to the pattern of invasion defined by the Bryne's grading system taken up podoplanin antibody considered as positive and which that do not had dissociated cells considering as negative (10). The presence of individual tumor cells was considered as a separate criterion and scores were given as 0=absent, 1= present. The ITCs or tumor islands with positive expression of D2-40 were considered motile.

Morphometric Analysis of Lymphatic Vessels.

Podoplanin stains endothelial cell of lymph vessels, three different field with maximum Lymphatic vessels (hot spot) around tumor were counted and mean value were calculated. After identification of lymphatic vessels by podoplanin staining, each lymphatic vessel was manually selected using CellSens standard software to calculate surface area (µm) ^{2} and perimeter of vessel. The circularity (degree of roundness) of vessel was calculated as 4πArea / perimeter^2. At least five vessels in hotspot area were analyzed for its morphometric analysis.

2. Assessment of VEGF-C: Expression of VEGF-C in tumor and stromal fibroblasts were evaluated and score as 0 – no staining, 1 – weak staining, 2 – Moderate staining and 3 Strong staining.
3. Assessment of Interleukin IL7: Expression of IL7 in tumor, normal squamous epithelium and plasma cell were evaluated and score as 0 – no staining, 1 – weak staining, 2 – Moderate staining and 3 Strong staining.

Statistical analysis:

Statistical analysis was carried out using SPSS statistical software version 20 (SPSS Inc, USA). Mean, standard error (SE) of mean and median were calculated and Pearson's correlation was carried out for significance between the two parameters. Mann Whitney test was used to compare means between two groups. Univariate survival analysis was carried out by Kaplan and Meier method and Log Rank statistics was used to assess the prognostic significance of disease free survival (DFS) and overall survival (OS). Multivariate survival analysis was performed using Cox regression model with forward stepwise (likelihood ratio) method. The Wald statistics and relative risk [Exp(B)] with 95% confidence interval (CI) for Exp(B) were used to evaluate the prognostic significance. P values ≤ 0.05 were considered significant.

III. RESULTS:

Assessment of Podoplanin

Podoplaninis expressed by endothelial cell lining of lymphatic vessels. It is also expressed in epithelial cells of oral precancerous lesion and squamous cell carcinoma. In this study 81%(54/67) showed podoplanin expression and 19% patients (13/67) did not show podoplanin expression, out of 54 positive patients, 42% (28/54) patients had only basal layer expression, 34% (23/54) patients had basal and supra basal layer expression and 5% (3/54) patients had complete tumor expression. As only three patients had complete tumor staining this group was merge with basal and suprabasal layer expression group and considered as high podoplanin expression group and negative group was combine with only basal layer expression group considered as low podoplanin expression for analysis.

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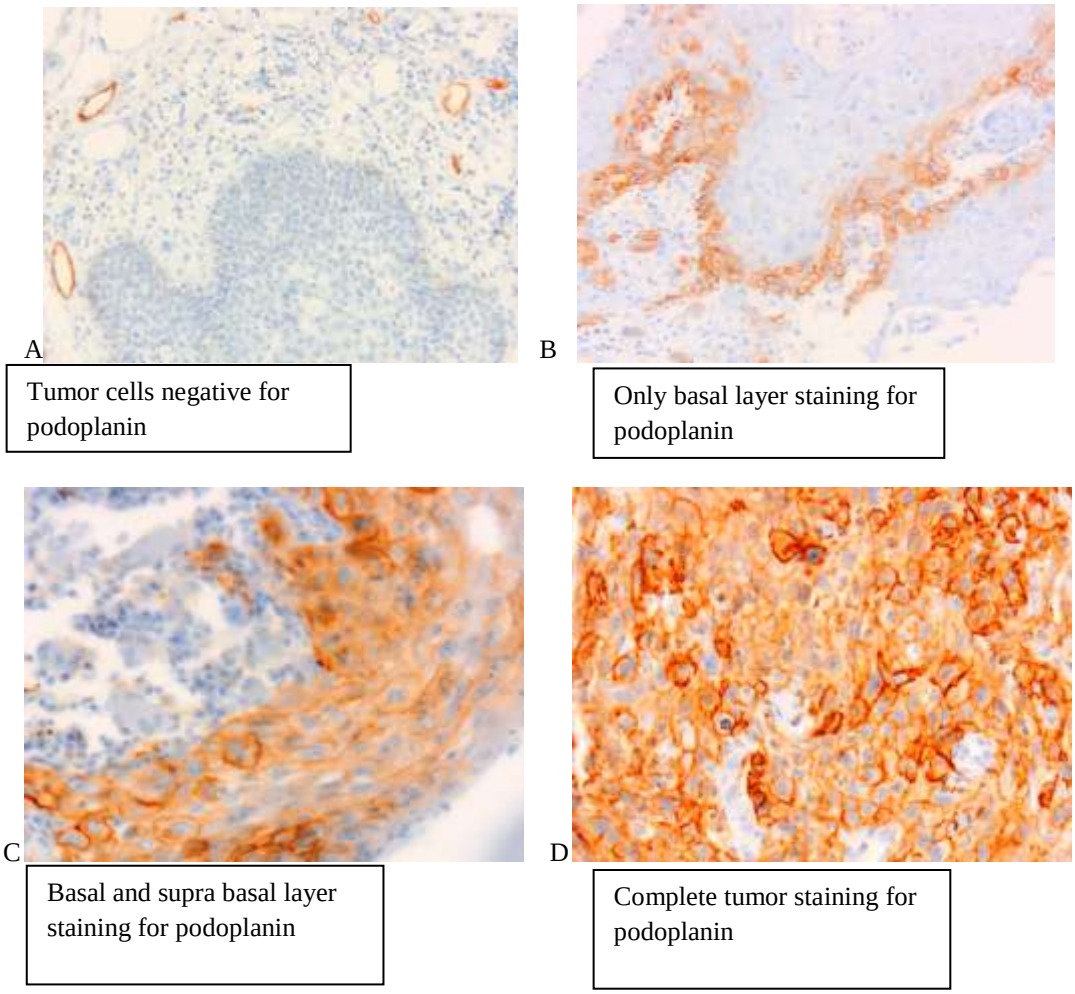

Figure 1: Expression of podoplanin in OSCC. A) Negative staining B) only basal layer staining C) Basal and suprabasal layer staining D) complete tumor staining

Correlation of Podoplanin expression with clinical parameters

In relation with clinical parameters, a significant high podoplanin expression was found in patients with <44 years of age (50.0%, 17/34, p=0.056) as compared to patients with > 45 years of age (27.3%, 9/33). Similarly, a trend of high podoplaninexpression was found in tongue (43.5%, 20/46); as compared to buccal mucosa (28.6%, 6/21) as tumour site. No such correlation was found with other clinical parameters like gender and habit (Table1).

Correlation of Podoplanin expression with pathological parameters

In relation with pathological parameters, a significant high podoplaninexpression was found in lymph node positive patients (51.7%, 15/29, p=0.058) as compared to Lymph node negative (28.9%, 11/38). A trend of high podoplaninexpression was found in poorly differentiated tumors (100.0%, 2/2) as compared to moderately (50.0%, 19/38) and well (18.5%, 5/27) differentiated tumors.Similarly, a trend of high expression of podoplanin was found in T3 (77.8%, 7/9); as compared to T1 (21.2%, 7/33) and T2 (48.0%, 12/25) tumors. No such correlation was found with other clinical parameters like stage, extra nodal extension Lymphatic permeation and perineural invasion (Table 2)

Individual tumor cells (ITC)

The tumor staging manual AJCC 8th edition emphasis more on tumor depth rather than tumor thickness and also includes depth of invasion as a criterion for tumor staging. Moreover, tumors with similar staging have differing growth patterns and clinical behavior. It is now well documented that several molecular events of significance for tumor spread, such as gain and loss of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and initiation of

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angiogenesis occur at the tumor–host interface which is known as invasive front, where the deepest and presumably most aggressive cells reside. The Invasive tumor front has been defined as the most progressed, three to six tumor cell layers or detachedtumor cell groups at the advancing edge of the OSCCs. Bryne's et al had developed simple morphological malignancy grading system that focus on pattern of tumor invasion and describe high grade tumor with wide spread of cellular dissociation of small groups of infiltrating cells (n>15) known as individual tumor cells (ITC). Podoplanin also stain these individual groups of cells.In this study, ITC stained by podoplaninwere evaluated and check for their present or absence at tumor front, of which,43% (29/67) patients were positive for of ITC while 56% (38/67) were negative.

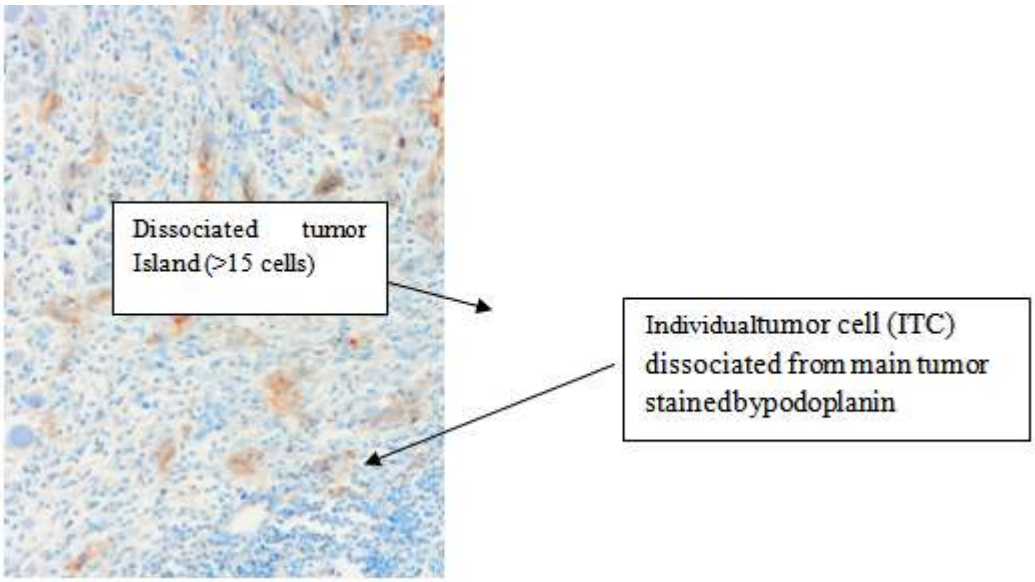

Figure 2: Individual Tumor cell (ITC)/ tumor nests stained by podoplanin

Correlation of Individual Tumor Cell with clinical parameters

In relation with clinical parameters,patients with tobaccohabit had individual tumor cell or tumor nest at invasive tumor front as compared to patients without habit (53%, 21/67, p=0.039) as compared to patients without habit (21%, 8/67). No such correlation was observed with other clinical parameters (Table 1).

Correlation of Individual Tumor Cell with pathological parameters

In relation with pathological parameters, patients with lymph node involvement had individual tumor cell or tumor nest at invasive tumor front (55%, 15/67) as compared to patients without lymph node involvement (34%, 13/67). No such correlation was observed with other pathological parameters.

Distribution of and morphometric analysis of Lymphatic Vessels.

Lymphatic vessels were stained by podoplanin staining, after identification of hot spot, surface area, perimeter and circularity of vessel were evaluated and compared in Lymph node positive and negative patients. Lymphatic vessels mainly observed in sub epithelial connective tissue and at periphery of tumor. Intratumoral vessels seen compressed due to invasive mass.

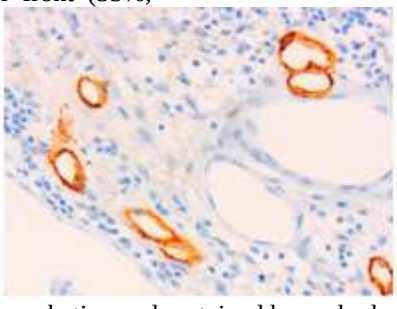

Figure 3: Lymphatic vessles stained by podoplanin (D2-40).

International Journal Dental and Medical Sciences Research
Volume 4, Issue 6, Nov-Dec 2022 pp 605-615 www.ijdmsrjournal.com ISSN: 2582-6018

In correlation with morphometric analysis, Node positive patients had significantly increased vessel perimeter (116.8±55 vs 91.37±51, P= 0.03) and vessels circularity (roundness) (0.78 ± 1.4 vs 0.68 ± 1.5) as compared to Node negative patients. Also, a trend of increased vessel area (834.6±655 μm^2 vs 586.1± 490 μm^2) was observed in node positive patients as compared to node negative patients. No significant difference in lymphatic vessels density was found between these two groups (Table 3).

Correlation of morphometric parameters of Lymphatic vessels with tumoralpodoplanin expression and ITCs.

High Lympahticvessles density was significantly associated with large vessels area (P=0.03). However, tumoralpodoplanin expression and ITC did not show significant correlation with morphometric parametrs (Table 2).

Correlation of morphometric parameters of Lymphatic vessels with IL7.

In our study, IL7 expresseion was found in tumor cells, incidentally, IL7 expression was also observed in plasma cells scattered in tumor and hence was recorded. Tumoral IL7 expression was significantly associated with large vessel area (P=0.015). Moreover, stromal plasma cell expression of IL7 is significantly associated with vessel circularity (0.026) and high Lymphovasluar density (0.036) (Table 3).

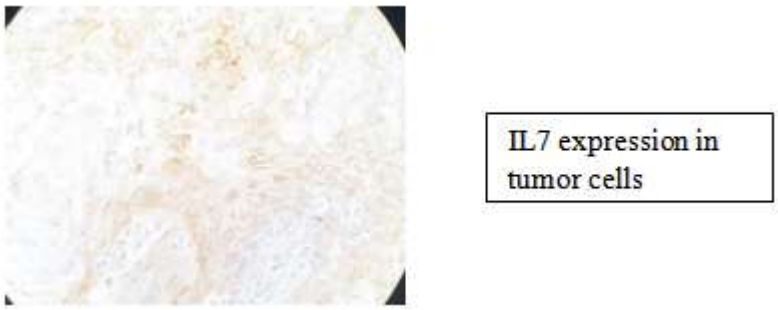

Figure 4: IL7 expression in OSCC

Correlation of morphometric parameters of Lymphatic vessels with VEGF-C.

In our study, VEGF-Cexpression was found in tumor cells, stromal fibroblasts and endothelial cells of blood vessels in periphery of tumor. We have analyzedtumoral as well as stromal fibroblasts expression of VEGF-C. TumoralVEGF-C expression was associated with large vessel area, however, value was not statistically significant (P=0.07) (Table 4).

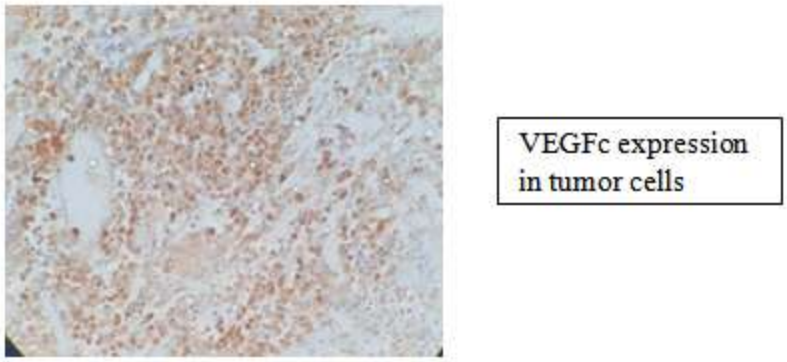

Figure 5:VEGF-C expression in OSCC

Intermarker Correlation

In intermarker correlation, Tumoral expression of podoplanin (0.001) and VEGF-C (0.07) was found to be significantly associated with formation of tumor nests or individual tumor cells. However, Tumoral (P=0.04) and stromal VEGF-C (P=0.02) expression was inversely correlated with Plasma cell IL 7 expression (Table 5).

Univariate Survival Analysis

According to Kaplan Meier Univariate survival analysis, with respect to DFS, patients with high podoplanin expression had significantly

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decreased mean months of DFS with high incidence of recurrence (50.0%, 13/26; 18.85±2.52 months; Log rank= 6.143, df=1, p=0.013) as compared to low podoplanin expression (24.4%, 10/41; 34.54±2.78 months).

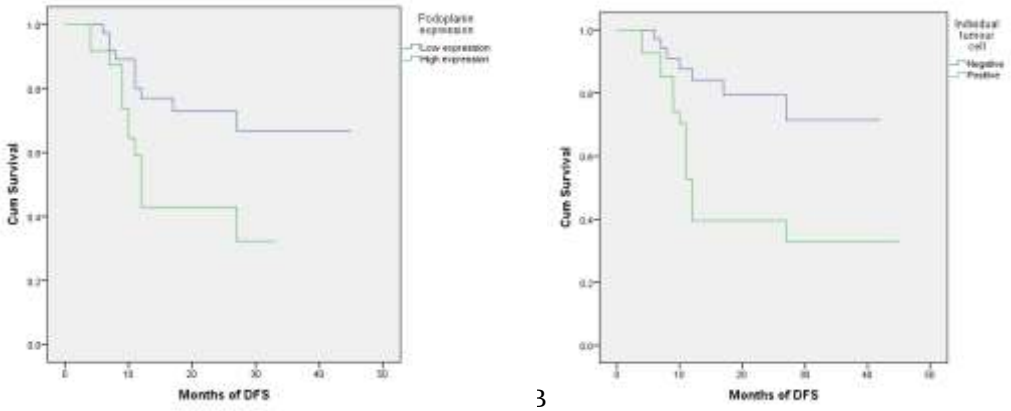

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Figure 6: A) Disease free survival of OSSC patients of tumoral podoplanain expression B) Disease free survival of OSSC patients with presence of ITC by D2-40

Similarly, patients with presence of ITC had significantly decreased mean months DFS (55.2%, 16/29; 22.32±3.44 months; Log rank= 9.118, df=1, p=0.003) as compared to patients who were negative for ITC (18.4%, 7/38; 34.35±2.52 months).

Similarly patients with early stage (Stage I and II) having presence of ITC byD2-40 had significantly reduced DFS (50%, 5/10; 20 6 ±6.4 months; log rank = 6.9, df=1, P = 0.08) as compare to patients with ITC negative.

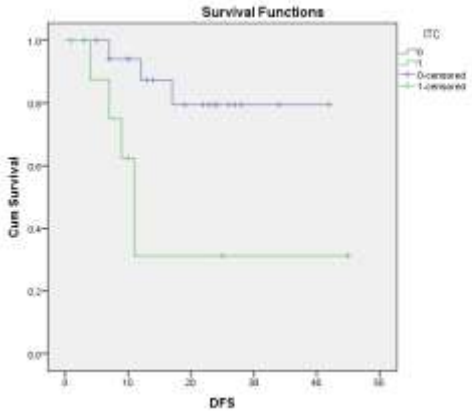

Figure 7: Disease free survival of early stage OSSC patients with presence of ITC by D2-40

Multivariate Survival analysis

Multivariate survival analysis by Cox's regressionmodel with forward stepwise (likelihood ratio) method was carried out to evaluate the prognostic significance of clinical and pathological parameters such as age, gender, tumor site, habit, disease stage, histopathology grade, Lymphatic permeation (LPVP), perineural invasion (PN), along withpodoplanin expression. Individual tumor cells entered step 1 as significant (Wald statistic=8.058, df=1, Exp(B)=3.640, p=0.005) and Lymphatic permeation entered a step 2 (Wald statistic=3.798, df=1, Exp (B) =2.567, P=0.051) for predicting DFS (Table 5).

IV. DISCUSSION:

In spite of multiple treatment options for Oral Squamous Cell Carcinoma, the frequency of recurrence is high with 50% 5-year overall survival rate. Manystudies have shown the process of hematogenous spread of tumor such as primary invasion into blood vessels, fixing, and proliferation to remote tissues via blood flow are known. In contrast, the details of lymphatic metastasis are much less well known than those of hematogenous spread. Inthepresentstudy, weevaluated lymphatic vessels using Podoplanin marker in OSCC and correlated its morphometric

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parameters with expression of pro inflammatory molecule VEGFC and IL7 to examine their relationship for lymphangiogenic activity for tumor progression.

Podoplanin stains endothelial cells of only lymphatic vessels, along with that Podoplaninis found to be expressed by malignant epithelial cells at tumor invasive front where they dissociate themselves from main tumor to form group or migrate individually. Therefore, in the present study we have assessed individual tumor cells or group of tumor cells (tumor nest) stained by Podoplanin at invasive tumor front.

In relation with clinicopathological parameters, patients with lymphnode metastasis had significantly high Podoplanin expression as well as individual tumor cells at invasive front as compared to patients without lymphnode metastasis suggesting alteration in lymphatic vascularate before tumor progression. In order to investigate this alteration, we have examined morphometric changes in two groups of patients one is lymph node positive and other is lymph node negative and we observed higher lymphatic vessels area and circularity in node positive patients, although the number of vessels have been similar. This suggests that the existing lymphatic vessels expand and the area of the lymphatic vessels increases. It appeared that lymph vessel endothelial cell adhesion became coarse in extended and expanded lymphatic vessels, and the gap became an environment where tumor cells are likely to invade, leading to lymphnode metastasis.

Further, correlation of morphometric parameters with tumoralpodoplanin expression, VEGF-C and IL7 expression suggest that tumoral VEGF and IL 7 promote the lymphatic vessels expansion as there is a significant association of these parameters with lymphatic vessels area.Moreover, tumoralpodoplanin and VEGF-C expression is found to be associated with formation of tumor nest and individual tumor cells (ITC), which facilitated tumor cell migration and its presence is significantly associated with recurrence of disease. In multivariate survival analysis, presence of ITC is found to be independent prognostic marker to predict recurrence or lymph node metastasis.

V. CONCLUSION:

Lymph node metastasis is one of the factors in OSCC affecting progression and prognosis. It can be suggested that inflammatory factors like VEGF-C and IL7 may affect existing lymphatic vessels morphology which may permit tumor invasion or migration.

Table1: Correlation of Podoplaninand Individual tumor cells with clinical parameters

Parameters		**Podoplanin Expression**		**P**	**Individual tumor cells**		**P**
Age	**N (67)**	**Low expression**	**High expression**		**Absent**	**Present**	
≤44	34(51%)	17(50.0%)	17(50.0%)	0.056	16(47.1%)	18(52.9%)	0.105
≥45	33(49%)	24(74.7%)	9(27.3%)		22(66.7%)	11(33.3%)	
Gender							
Male	47(70%)	29(61.7%)	18(38.3%)	0.896	25(53.2%)	22(43.8%)	0.372
Female	20(30%)	12(60.0%)	8(40.0%)		13(65.0%)	7(35.0%)	
Habit							
No habit	28(40%)	19(67.9%)	9(32.1%)	0.343	20(71.4%)	8(28.6%)	0.03
Tobacco	39 (58%)	22(56.4%)	17(43.6%)		18(46.2%)	21(53.8%)	

Tumor site							
Tongue	46 (69%)	26(56.5%)	20(43.5%)	0.245	24(52.2%)	22(47.8%)	0.26
Buccal Mucosa	21 (31%)	15(71.4%)	6(28.6%)		14(66.7%)	7(33.3%)	

Table 2: Correlation of Podoplanin expression with pathological parameters

Parameters		**Podoplanin Expression**		**p**	**Individual tumour cells**		**p**
Stage	**N 67(100%)**	**Low expression**	**High expression**		Absent	Present	
Stage I	19 (29%)	16(84.2%)	3(15.8%)	0.75	13(68.4%)	6(31.6%)	0.28
Stage II	13 (19%)	8(61.5%)	5(38.5%)		9(69.2%)	4(30.8%)	
Stage III	21 (31%)	11(52.4%)	10(47.6%)		9(42.9%)	12(57.1%)	
Stage IV	14 (21%)	6(42.9%)	8(57.1%)		7(50.0%)	7(50.0%)	
Lymph node							
Negative	38(57%)	27(71.1%)	11(28.9%)	0.05	25(65.8%)	13(34.2%)	0.086
Positive	29(43%)	14(48.3%)	15(51.7%)		13(44.8%)	16(55.2%)	
Extra nodal extension							
Negative	60(90%)	37(61.7%)	23(38.3%)	0.81	35(58.3%)	25(41.7%)	0.43
Positive	7(10%)	4(57.1%)	3(42.9%)		3(42.9%)	4(57.1%)	
Histological Grade							
PD	2(3%)	0(0.0%)	2(100.0%)	0.007	1(50.0%)	1(50.0%)	0.40
MD	38(57%)	19(50.0%)	19(50.0%)		19(50.0%)	19(50.0%)	
WD	27(40%)	22(61.2%)	5(18.5%)		18(66.7%)	9(33.3%)	
Tumor Size							
T1	33 (49%)	26(78.8%)	7(21.2%)	0.004	20(60.6%)	13(39.4%)	0.31
T2	25 (37%)	13(52.0%)	12(48.0%)		15(60.0%)	10(40.0%	

						)	
T3	09(14%)	2(22.2%)	7(77.8%)		3(33.3%)	6(66.7%)	
T4	00(00%)	00(00%)	00(00%)		00(00%)	00(00%)	
LPVP							
Negative	55(82%)	35(63.6%)	20(36.4%)	0.380	20(60.6%)	13(39.4%)	0.60
Positive	12(18%)	6(50.0%)	6(50.0%)		15(60.0%)	10(40.0%)	
PN							
Negative	60(90%)	38(63.3%)	22(36.7%)	0.125	36(60.0%)	24(40.0%)	0.11
Positive	7(10%)	3(42.9%)	4(57.1%)		2(28.6%)	5(71.4%)	

Table 3: Comparison of Lymphovascular density and morphometric parameters in node positive and negative patients

Parameter	Lymphnode involvement (N=67)	Mean	Standard deviation	P value
LVD	Absent (37)	11.05	6.054	0.8
	Present (29)	10.93	5.994	
Circularity	Absent (37)	.6827	.15270	0.01
	Present (27)	.7849	.14484	
Vessels area	Absent (37)	586.4800	490.74211	0.09
	Present (29)	834.6731	655.84331	
Vessel perimeter	Absent (37)	91.3754	51.59939	0.03
	Present (29)	116.8021	55.34156	

Table 4: Correlation of morphometric parameters of lymphatic vessels with tumoralpodoplanin expression

	N=67	**Tumoralpodoplanin**	**Individual tumor cells**	**Lymphatic vessels density (LVD)**
Vessel perimeter	Pearson Correlation	0.011	0.075	0.16
	Sig. (2-tailed)	0.92	0.54	0.187
Vessels area	Pearson Correlation	0.77	0.167	0.257
	Sig. (2-tailed)	0.53	0.176	**0.035**
Vessels roundness	Pearson Correlation	-0.083	-0.94	**-0.720**
	Sig. (2-tailed)	0.504	0.448	0.561

International Journal Dental and Medical Sciences Research
Volume 4, Issue 6, Nov-Dec 2022 pp 605-615 www.ijdmsrjournal.com ISSN: 2582-6018

Table 5: Correlation of morphometric parameters of lymphatic vessels with tumoral and stromal IL 7 expression

	N=67	Tumoral IL7	Stromal Plasma cells IL7
Vessel perimeter	Pearson Correlation	0.80	-0.02
	Sig. (2-tailed)	0.59	0.883
Vessels area	Pearson Correlation	0.357	0.211
	Sig. (2-tailed)	**0.015**	0.155
Vessels roundness	Pearson Correlation	0.157	0.32
	Sig. (2-tailed)	0296	**0.025**
Lymphatic vessels density (LVD)	Pearson Correlation	0.175	0.367
	Sig. (2-tailed)	0.24	**0.036**

Table 6: Correlation of morphometric parameters of lymphatic vessels with tumoral and stromal VEGF-C expression

	N=67	TumoralVEGF-C	Stromal fibroblasts VEGF-C
Vessel perimeter	Pearson Correlation	0.206	0.090
	Sig. (2-tailed)	0.128	0.512
Vessels area	Pearson Correlation	0.241	0.143
	Sig. (2-tailed)	**0.074**	0.297
Vessels roundness	Pearson Correlation	-0.129	0.064
	Sig. (2-tailed)	0.342	0.644
Lymphatic vessels density (LVD)	Pearson Correlation	-0.024	-0.096
	Sig. (2-tailed)	0.863	0.487

Table 7: Intermarker correlation

		VEGF-C (stromal fibroblasts)	Podoplanin (tumor)	Individual Tumor cell (ITC)	IL7 (Tumor)	IL7 (Plasma cells)
VEGF-C (Tumor)	Pearson Correlation	.819	-.035	.241	-.030	-.321
	Sig. (2-tailed)	**.000**	.796	**.073**	.855	**.041**
VEGF-C (stromal fibroblasts)	Pearson Correlation	-	-.033	.159	-.154	-.466
	Sig. (2-tailed)		.809	.247	.350	**.002**
Podoplanin (tumor)	Pearson Correlation	-	-	.522	.124	.194
	Sig. (2-tailed)			**.000**	.410	.190
Individual Tumor cell (ITC)	Pearson Correlation	-	-	-	-.041	.112
	Sig. (2-tailed)				.789	.454
IL7 (Tumor)	Pearson Correlation	-	-	-	-	.560
	Sig. (2-tailed)					**.000**

Table 8: Multivariate Survival analysis of podoplanin expression (DFS)

Parameter	B	SE	Wald	df	P	Exp (B)	95% CI for Exp (B)	
							Lower	Upper
Step 1 Individual tumor cells	1.292	0.455	8.058	1	0.005	3.640	1.4	8.88
Step 2 LPVP	0.943	0.484	3.798	1	0.051	2.567	0.99	6.62

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