ORIGINAL ARTICLE

Tumor hypoxia and cancer stem cell markers expression in oral squamous cell carcinoma- An Immunohistochemical analysis

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ABSTRACT

Background: Oral cancer is the common reason for the poor prognosis in head and neck carcinomas and the increase in morbidity and mortality rates. The biological behavior of cancer is a complex process. About 50-60% of solid tumors exhibit hypoxic areas within the tumor stroma, which was influenced by the transcriptional activity of hypoxia inducible factor (HIF). HIF promotes stemness and the proliferation of vessel-like structures in tumors, which leads to invasion and metastasis. **Aim:** To evaluate and correlate the expression of HIF- 1α, MCT1, NESTIN, and SALL2 in the tumor proper and tumor periphery of non- metastatic, metastatic, and recurrent OSCC. **Materials and Methods:** A Total of 60 proven OSCC cases with proper tumor center and periphery were collected. Among them, 25 were nonmetastatic, 25 were metastatic, and 10 were recurrent cases of OSCC. Immunohistochemical analysis of HIF- 1α, MCT1 NESTIN, SALL2, and CD31/PAS double staining was done. **Results:** Depending on the extent of stained tumor cells, the intensity of staining, and the index score, the expressions of both MCT1, SALL2, and NESTIN were highly significant in the periphery of OSCC with a *P* value of 0.001. The total number of vessels expressed in non-metastatic, metastatic, and recurrent OSCC were not significant, but overall expression of CD31/PAS was statistically significant in the periphery of the tumor with *P* value -0.024. **Conclusion:** Based on the above results, it is observed that the role of hypoxia helped in cancer stem cell (CSC) maintenance with the formation of vessel-like structures by tumor cells at an early stage of cancer promotes its development and recurrence.

KEY WORDS: Cancer stem cells, hypoxia, oral squamous cell carcinoma, prognosis, vasculogenic mimicry

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INTRODUCTION

The commonest cancer of the head and neck region that contributes 84-97% of total cancers occurring in the oral cavity is oral squamous cell carcinoma.[1] It ranks sixth among all the cancer types with a high mortality rate.[2] In India, new cases of oral cancer are around 77,000, with 52,000 deaths reported annually, which accounts for about one-fourth of the global incidence.[1] To understand the pathogenesis of OSCC, many researches and investigations were done related to smoking and alcohol consumption, but the specific pathogenesis remains unclear.[3,4] One such pathway that seeks attraction for investigation is tumor hypoxia, which plays several roles in tumor progression.[5,6] Recently, its role has been extended in the evolution and maintenance of CSCs, which promotes cancer progression and affects its progression.[7]

Under normoxic conditions, the cells are dependent on oxidative phosphorylation for an efficient source of energy. In tumorous, due to hypoxia, there is a shift in the metabolic pathway by inhibiting oxidative

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phosphorylation by mitochondria and activating glycolysis as a prime source of energy.[8] The hypoxia and acidosis are responsible for promoting the genetic alteration which are involved in cell proliferation and in glucose metabolism and, they are involved in promoting the angiogenesis, macrophage polarization into tumor-associated macrophages which may lead to invasion and metastasis.[9-11] Monocarboxylate transporters work as an important route for lactate uptake by the tumor cells, and MCT has a high affinity for lactate during the metabolic shift.[12] Low oxygen level stimulates the expression of HIF-1α, which regulates the expression of more than 100 genes, which vary in different tissues and cells.[13,14]

In a highly aggressive tumor, the cancer cells will form a structure resembling a capillary and a network forming a matrix-rich pattern like structures that mimic embryonic vasculogenic network, which are described as vasculogenic mimicry (VM).[15-17] In 1999, Maniotis proposed VM as vessel-like structures lined with tumor cells without endothelial cells. VM is a newly defined mechanism for nutrient and oxygen supply to tumor cells, and its increased expression carries an increased risk for poor prognosis and low survival of cancer patients.[18]

In solid tumors, the microenvironment at the tumor periphery is different from the core of the tumor. Most stromal cells do not favor the hypoxic region, but macrophages thrive in the oxygen-deprived areas and facilitate angiogenesis. Hence, in the tumor periphery, hypoxia promotes cancer invasion, and in tumor center, hypoxia contributes to the escape of cancer cells, which leads to the aggressive behavior of resilient stem-like tumor cells that migrate to the margins.[19] In gliomas, tumor cells in the periphery showed increased expression of stem cell markers.[20] Thus, in this study, the biological behavior of solid tumor in center and periphery of metastatic, nonmetastatic and recurrent oral squamous cell carcinoma was analyzed through the immune expression of hypoxia and cancer stem markers.

CSCs are dependent on HIFs for survival and self-renewal, which promotes tumor growth. Therefore, cancer stem cells and hypoxic tumor cells can be used as a potential therapeutic target to prevent further progression and metastasis. In oral cancer, minimal studies were done to show the correlation between hypoxia and cancer stem cells in OSCC. Hence, in this study, a correlation was done between HIF1α, MCT1and Nestin, Sall2 were done in tumor center and periphery of OSCC.

MATERIALS AND METHODS

Data collection

A retrospective in-vitro study was done in formalin-fixed paraffin-embedded tissue of 10 normal mucosa, 25 non-metastatic, 25 metastatic, and 10 recurrent OSCC cases, which were reported between January 2019 and December 2021 in the Department of Oral Pathology and Oral Microbiology. From each case of non-metastatic, metastatic, and recurrent OSCC, 1 sample from the invasive front and 1 sample from the center of the tumor were taken. A total of 130 samples were included in the study. This study was approved by the Institutional Ethical Committee of VMRF(DU), (VMSDC / IEC/185).

Excised specimens of OSCC confirmed by biopsy and located in any area of the mouth with only lymph node metastasis were included in the study. Other variants of OSCC, like basaloid and spindle cell carcinomas and specimens from patients who underwent chemo and radiotherapy, were not included. Primary Antibody: HIFI-A antiserum is used with 1:50 dilution, MCT1 antiserum with 1: 100 dilutions, Nestin monoclonal antibody is used with 1:400, Sall2 antiserum is used with 1:100, and CD31 ready -to-use monoclonal antibody were used. Secondary Antibody: Polyexcel –HRP/DAB detection system which is non biotin, polymerbased detection system, CAT NO- PEH-002 (Pathnsitu) was used.

Methodology and scoring

Serial sections of about 3 micrometre thickness using a standard rotary microtome were obtained from the archival blocks. The sections were prepared and used for both routine H and E and Immune-Histochemical Study. For the IHC study, the sections were fixed on the positively charged hydrophobic poly-L-Lysine coated microscopic slides. The double staining procedure was used for CD31/PAS; $CD31^{-ve}$ and PAS^{+ve} vessels are considered as VM-positive vessels. $CD31^{+ve}/PAS^{-ve}$ or $CD31^{+ve}$ and PAS^{+ve} are VM negative. IHC stained sections were evaluated by the brown stain observed under 40x magnification of light microscope. The data were analyzed semi-quantitatively by Klein"s scoring criteria. The final score of compiling the proportion and intensity is graded as negative = 0–1, mild = 1–2, moderate = >2–4, and severe = >4–6.

Statistical analysis

The compiled data was transferred to an Excel sheet and subjected to statistical analysis, SPSS version 20.0 using mean, standard deviation. A Chi-square test was used for comparison of the tumor center and periphery while analysis of variance (ANOVA) was used for comparison between non-metastatic, metastatic, and recurrent OSCC. Pearson correlation coefficient test was used to arrive the *P* value (cut off < 0.05 is considered statistically significant).

RESULTS

All the 130 samples collected from 25 non-metastatic OSCC, 25 metastatic OSCC 10 recurrent OSCC cases, and 10 normal oral mucosa were tested for immunoexpression of HIF1-α, MCT1, Nestin, and SALL2. Percentage of tumor cells stained for HIF1-α, MCT1, Nestin and SALL2 were shown as follows; [Figure 1].

Expression of HIF1-α

When comparing the overall expression, staining intensity, and index score in the center and periphery of non-metastatic, metastatic, and recurrent OSCC, periphery of the tumor showed a significant *P* value of 0.001**. In tumor center the *P* value of extent, staining intensity and index score were 0.043*, 0.004**, 0.026* respectively.

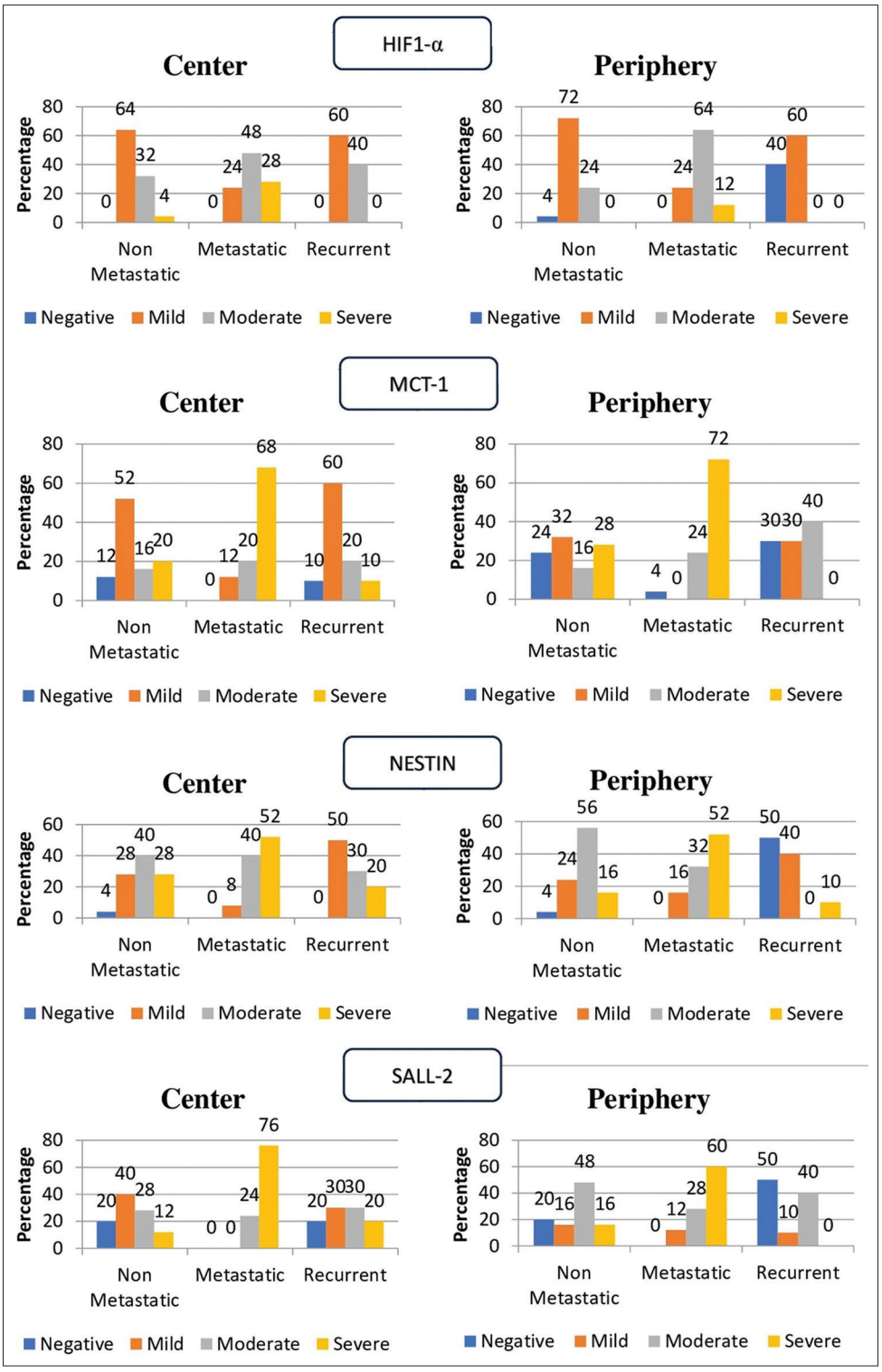

Figure 1: Percentage of tumor cells stained with HIF1-α, MCT-1, NESTIN and SALL-2 in tumor center and periphery of OSCC

When comparing the overall expression of HIF1-α in non-metastatic, metastatic, and recurrent OSCC, metastatic OSCC showed significant expression with a *P* value of 0.001**. Similarly, when comparing the overall expression of HIF1-α in the tumor center and periphery of oral squamous cell carcinoma, the center of the tumor showed significant results with a *P* value of 0.003** [Figure 2].

Expression of MCT-1

When comparing the overall expression of MCT1, staining intensity and index score, both center and periphery showed significant differences. When comparing the overall expression of MCT1 in non-metastatic, metastatic, and recurrent OSCC, metastatic OSCC showed significant expression with a *P* value

of 0.001**. When comparing the overall expression of MCT1 in the tumor center and periphery, no significant differences were observed [Figure 3].

Expression of Nestin

When comparing the overall expression, staining intensity, and index score in the center and periphery of non-metastatic, metastatic, and recurrent OSCC, the periphery of the tumor showed a highly significant difference with a *P* value of 0.001**. In the tumor center, only the extent of tumor cells stained was significant with a *P* value of 0.017* respectively.

When comparing the overall expression in the center and periphery of oral squamous cell carcinoma, Nestin expression was significant in both the center and periphery. Still, the periphery showed a highly significant *P* value of 0.001** respectively. When comparing the overall expression of Nestin in non-metastatic, metastatic, and recurrent OSCC, metastatic OSCC showed a highly significant *P* value of 0.001** [Figure 4].

Expression of sall2

When comparing the overall expression in the center and periphery of non-metastatic, metastatic, and recurrent OSCC, both center and periphery showed significant differences in percentage of cells stained, staining intensity, and index score with a significant *P* value of 0.001**, respectively.

When comparing the overall expression of SALL2 in non-metastatic, metastatic, and recurrent OSCC, metastatic OSCC showed a significant *P* value of 0.001**. When comparing the expression of SALL2 in the center and periphery of non-metastatic, metastatic, and recurrent OSCC, both center and periphery showed significant results with a *P* value of 0.001** respectively [Figure 5].

Expression of cd31/pas

In the tumor center of non-metastatic OSCC, 52% of vessels showed positive for VM, in metastatic OSCC, 48% showed positive VM vessels. In recurrent OSCC, 40% showed positive VM vessels. In the periphery, non-metastatic OSCC showed 40% positive VM vessels, metastatic showed 60% of positive VM vessels, and recurrent showed 10% of positive VM vessels.

The total no. of CD31-/PAS+ vessels was not significant among non-metastatic, metastatic, and recurrent OSCC. When comparing the overall CD31-/PAS+ vessels in the tumor center and periphery of non-metastatic, metastatic, and recurrent OSCC, the periphery of the tumor showed significant results with a *P* value *0.024** [Figure 6].

Both hypoxia-related markers and cancer stem cell-related markers show significant expression in metastatic OSCC when compared to non-metastatic and recurrent OSCC. On comparing the expression of Nestin in the tumor center of OSCC with SALL2 and MCT1, both the markers showed a significant result for severe expression with a *P* value of 0.001** respectively. In the tumor periphery, Nestin showed a significant expression compared to SALL2 with a *P* value of 0.001**. The overall expression of CD31/PAS in the tumor center, NESTIN, and SALL2 showed significant results with a *P* value of 0.031* and 0.003**, respectively. In the periphery, no significant difference was noted.

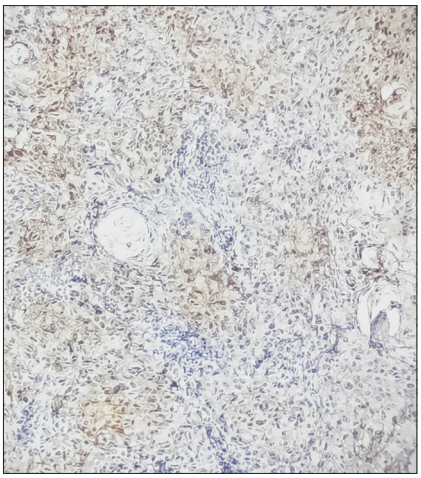

Figure 2: Shows HIF 1-α expression in the tumor center of metastatic OSCC under 40X magnification

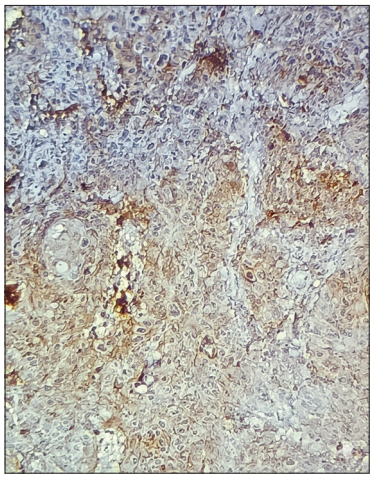

Figure 3: Shows MCT-1expression in tumor periphery of metastatic OSCC under 40X magnification

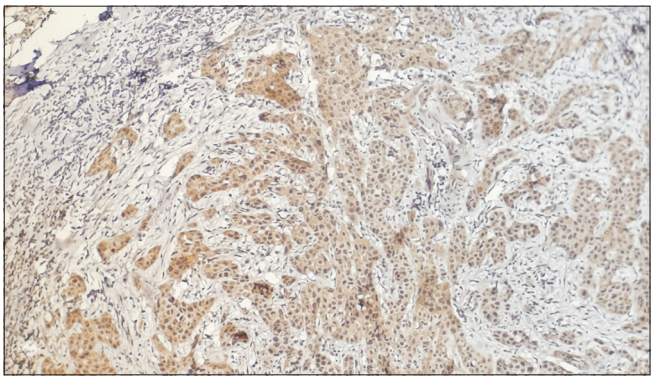

Figure 4: Shows SALL-2 expression in tumor periphery of metastatic OSCC under 40X magnification

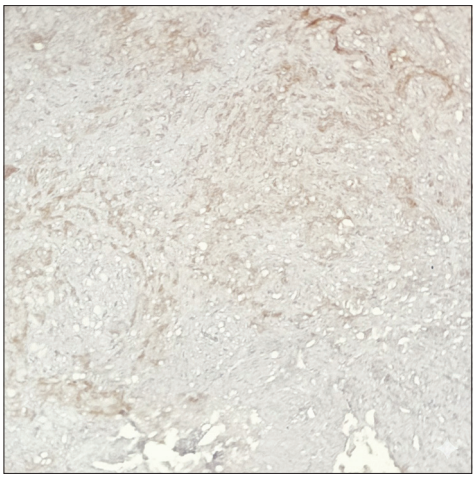

Figure 5: Shows Nestin expression in the tumor periphery of metastatic OSCC under 40X magnification

Correlating the overall marker expression in the tumor center and periphery using Pearson correlation coefficient showed a positive relation exist between the hypoxia and cancer stem cell markers with significant *P* value [Tables 1 and 2].

Correlating the marker expression in non-metastatic OSCC, there was a positive correlation between HIFI-α and MCT1 - *P* value (0.006**), HIFI- α Nestin – *P* value (0.026*), Nestin and SALL2 –*P* value (0.002**). In metastatic OSCC, a positive correlation existed between MCT1 and Nestin – *P* value (0.038*), Nestin and SALL2 – *P* value (0.001**), and MCT1 and SALL2 – *P* value (0.006**). In recurrent OSCC, a positive correlation existed between MCT1 and Nestin – *P* value (0.032*). Also, there is a positive correlation between HIF1-α and Nestin with CD31/PAS with a significant *P* value of 0.042* and 0.001** using Pearson correlation co-efficient in the periphery of non-metastatic OSCC. In multivariate analysis, Nestin-positive, MCT1-positive, and SALL2 positive cases are significant, suggesting that they can be used as individual prognostic markers for OSCC [Table 3].

DISCUSSION

Epigenetic/genetic changes in tumor cells and rearrangements of cellular and stromal components in TME through mutual/dynamic crosstalk influence tumorigenesis and its progression. The unabatable growth and invasiveness into the surrounding structures characterize OSCC.[21] Cancer stem cells, a subpopulation of cells, play a decisive role in tumor recurrence and metastasis and contribute to radiochemoresistance. Hypoxia in the formation of CSC formation, its pluripotency, and its maintenance has been reported in previous studies.[13-15] In addition, hypoxia in solid tumors leads to a decrease in pH and creates an acidic environment, which contributes to multi-drug resistance.

In solid tumors, the surrounding microenvironment is different from the tumor center. In the tumor center, the oxygen level is low, which drives the immune cells to recruit in the periphery of the tumor to support cancer invasion. Hypoxia also contributes to the tumor cell escape from the center of the tumor and provides aggressive selection pressure for resilient stem-like tumor cells, which migrate to the tumor periphery, eventually leading to tumor invasion and metastasis.[19]

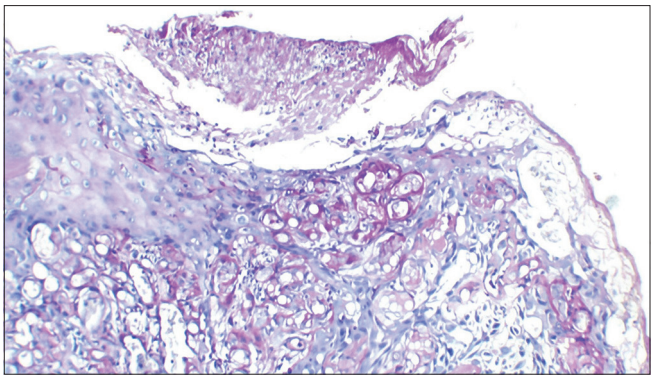

Figure 6: PAS-positive vessel with basement membrane-like substance stained in magenta colour surrounded by tumor cells under 40X magnification

Hypoxia regulates the CSC niche and promotes the transformation of CSC into endothelial cell -like structures. In this process, a tube-like structure that mimics the embryonic vascular network is formed by losing the surface marker for the epithelium and gaining an endothelial-like cell. This structure resembles tubular structures containing a matrix rich in collagen, heparan sulphate proteoglycan, and plasma. The glycoprotein in these structures contains type I, IV, and VI collagen. Expression of VM in a tumor denotes aggressive and metastatic potential.[22]

In the present study, the expression of HIF1-α and MCT1 are correlated with the expression of Nestin and SALL2 in the tumor center and periphery of non-metastatic, metastatic, and recurrent OSCC. A positive correlation exists between the markers in the tumor center and periphery of OSCC. In non-metastatic OSCC, HIF1-α showed a positive correlation with MCT1 and Nestin with a significant *P* value of 0.006** and 0.026*. In metastatic OSCC, a positive correlation existed between MCT1 and Nestin, Nestin and SALL2, and MCT1 and SALL2 with a *P* value of 0.038*, 0.001**, and 0.006**. In recurrent OSCC, a positive correlation existed between MCT1 and Nestin with a *P* value of 0.032*, respectively.

In this study, HIF1-α shows a positive correlation with MCT1 with a significant *P* value of 0.006*, which was like the study conducted by Miranda-Gonçalves V *et al*.[23] who reported that hypoxia-induced MCT1 expression in glioblastomas supports the glycolytic pathway, which is responsible for lactate efflux and mediates cell survival and aggressiveness.

MCT1 and Nestin showed a positive correlation in metastatic and recurrent OSCC (0.038* and 0.032*), and a positive correlation

existed between MCT1 and SALL2 in metastatic OSCCC (0.006**). Similarly, in a study by Sandforth *et al.*[24] who investigated that MCT1 expression seen in pancreatic adenocarcinoma along with increased in expression of stemness marker Nestin, OCT4, KLF4, Nanog and concluded that MCT1 dependant lactate efflux leads to reverse Warburg effect in PDAC and efficiently drives metabostemness.

A positive correlation existed between the two cancer stem cell markers Nestin and SALL-2 in non-metastatic and metastatic OSCC with a *P* value of 0.002** and 0.001** but not in recurrent OSCC. This was similar to the study by Sachdeva R *et al.*[25] who analysed the tumor cells positive for pSmad1 and pSmad2, which showed an increase in expression of Nestin, OLIG1, SALL2, OCT3/4, and SOX2 of glioblastoma tumor cells when compared to normal brain cells, and suggested that quiescent cancer stem cells in tumors act as a reservoir for tumor recurrence.

Table 1: Shows the overall Correlation of hypoxia and cancer stem cell markers in tumor center of OSCC

	Center	*HIF*	*MCT*	*NESTIN*	*SALL*
HIF	Pearson Correlation	1	0.373 (**)	0.265 (*)	0.344 (**)
	Sig. (2-tailed)	.	0.003	0.041	0.007
	n	60	60	60	60
MCT	Pearson Correlation	0.373 (**)	1	0.425 (**)	0.475 (**)
	Sig. (2-tailed)	0.003	.	0.001	0.001
	n	60	60	60	60
NESTIN	Pearson Correlation	0.265 (*)	0.425 (**)	1	0.461 (**)
	Sig. (2-tailed)	0.041	0.001	.	0.001
	n	60	60	60	60
SALL	Pearson Correlation	0.344 (**)	0.475 (**)	0.461 (**)	1
	Sig. (2-tailed)	0.007	0.001	0.001	.
	n	60	60	60	60

Table 2: Shows the overall Correlation of hypoxia and cancer stem cell markers in tumor periphery of OSCC

	Periphery	*HIF*	*MCT*	*NESTIN*	*SALL*
HIF	Pearson Correlation	1	0.572 (**)	0.517 (**)	0.482 (**)
	Sig. (2-tailed)	.	0.001	0.001	0.001
	n	60	60	60	60
MCT	Pearson Correlation	0.572 (**)	1	0.438 (**)	0.556 (**)
	Sig. (2-tailed)	0.001	.	0.001	0.001
	n	60	60	60	60
NESTIN	Pearson Correlation	0.517 (**)	0.438 (**)	1	0.613 (**)
	Sig. (2-tailed)	0.001	0.001	.	0.001
	n	60	60	60	60
SALL	Pearson Correlation	0.482 (**)	0.556 (**)	0.613 (**)	1
	Sig. (2-tailed)	0.001	0.001	0.001	.
	n	60	60	60	60

In this study, a positive association existed between HIF1-α and Nestin in non-metastatic OSCC with a *P* value of 0.026*, which was similar to the study by Shentu. Y *et al.*[26] who isolated Nestin positive cells in peritonea and detected the relationship between Nestin and HIF1-α – VEGF pathway. They found that knock-down of Nestin reduced the level of HIF1-α level, where Nestin directly binds to HIF1-α and protects from proteosomal degradation.

Our study showed that Nestin expression was highly significant in metastatic OSCC with a *P* value of 0.001**. Similarly, in Singh *et al.*[27] study and Elser *et al.*[28] where there is a positive association of Nestin in increasing grades of tumor and neoangiogenesis.

In this study where HIF1-α shows a positive correlation with Nestin and VM with a *P* value of 0.042* and 0.001** which was like the study conducted by Sun *et al.*,[29] who studied the association of CSC in VM formation in ALDH+ and CD133+ cells isolated from mouse breast cancer and reported that CSC provide more VM-related molecules to form vascular channels.

Apart from stemness and differentiation potential, HIF enhances plasticity to tumor stem cells; these transformed mobile tumor cells through epithelial endothelial transition induced by hypoxia. Upregulation of Twist and Snail in the tumor cell leads to downregulation of molecules which promotes components of angiogenesis such as VE- cadherin and fibronectin. Through a series of intercellular pathways, degradation of laminin and remodeling of ECM occurs that cause tumor cell migration which expresses high level of matrix metalloproteinase. The arrangement of tumor cells in duct-like structures with remodeled ECM leads to the formation of a VM network, which extends into vascular channels and transports red blood cells and nutrients to the network.[30]

However, definitive mechanism in which CSC differentiate into endothelial-like cells is still not clear. Based on recent researches in HIF signaling pathways, it is said that hypoxia directly/indirectly induces CSC to differentiate into non-CSC cell phenotype to form endothelial cell-like structures in tumor.[22] The mystery remains unraveled and must be explored by further analysis.

Table 3: Shows Multivariate analysis of 60 patients for HIF1- α, Nestin, MCT1 and SALL2

	n	*Mean*	*Std. Deviation*	*Std. Error*	*95% Confidence Interval for Mean*		*Minimum*	*Maximum*	*Sig*
					Lower Bound	*Upper Bound*			
HIF 1- α	60	1.08	0.279	0.036	1.01	1.16	1	2	0.308
CD31/PAS	60	1.57	0.500	0.065	1.44	1.70	1	2	0.161
MCT	60	1.15	0.360	0.046	1.06	1.24	1	2	0.047
NESTIN	60	1.10	0.303	0.039	1.02	1.18	1	2	0.020
SALL	60	1.17	0.376	0.049	1.07	1.26	1	2	0.013

This study shows that HIF1- α, MCT1, Nestin, Sall2, and VM have a definitive part in tumor progression, invasion, and metastasis. Association with VM denotes its increased tendency to undergo tumor recurrence and therapy resistance. Hence, understanding the mechanism behind tumor biology and targeted therapy against CSC and VM channels will prevent tumor recurrence.

The limitations of the study include: more samples from center and periphery of the tumor must be studied to know the accurate impression of the tumor morphology. More representative immunohistochemical markers related to HIF pathways have to be done to find out the exact mechanism of cancer.

The uniqueness of this study includes,

- Investigating the correlation of HIF and cancer stem cell markers in the tumor center and periphery of non-metastatic, metastatic, and recurrent OSCC.
- Our study is the first to investigate the immunohistochemical expression of MCT1 and SALL2 and its association with hypoxia and cancer stem cells in the tumor center and periphery of non-metastatic, metastatic, and recurrent OSCC.

CONCLUSION

High expression of HIF 1- α, Nestin, and VM could be a possible reason for increased metastasis in OSCC. Significant changes seen in the periphery of the tumor may denote that hypoxia-mediated escape of tumor cells to the periphery facilitates the cross-talk between tumor cells and ECM, which promotes cancer progression, invasion, and metastasis. Hence, understanding the cross-talk between the different signaling pathways mediated by HIF will help to elucidate the potential target in oral cancer to prevent treatment resistance.

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Conflicts of interest
There are no conflicts of interest.

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