Original Article

Role of hypoxia and epithelial-mesenchymal transition in the formation and maintenance of oral cancer stem cells in oral squamous cell carcinomas and metastatic lymph node: An immunohistochemical analysis

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Abstract

Background: Inspite of having advanced treatment modalities the overall survival rate in oral squamous cell carcinoma (OSCC) remains poor. This is considered to be mainly due to local recurrence and distant metastasis. Various studies have concentrated on the role of oral cancer stem cells (OCSCs) in the progression and metastasis of OSCC. However, the role of tumor microenvironment components has been less delved into. Hence clarity on cell biology and metastatic potential OCSCs is essential for the development of more effective anti-cancer treatment.

Aim: To establish the role of OCSCs in different grades of OSCC and metastatic lymph nodes through the expression of cluster of differentiation 44 (CD44). To demonstrate and correlate the role of hypoxia and Epithelial mesenchymal transition (EMT) in the various grades and metastatic lymph nodes in the formation and maintenance of OCSCs by employing Hypoxia-inducible factor-1 Alpha (HIF 1α) and Snail respectively.

Method and Material: A total of 36 cases of OSCC, 12 from each grade and 12 normal oral mucosal tissues were included in the study. Immunohistochemical staining was performed for the demonstration of CD44, HIF1α, and Snail.

Statistics: Descriptive analysis, Chi-square, and Spearman's rank correlation were used to analyze frequency and proportion, to compare expression and correlate between lesion proper and lymph node in each group respectively.

Results: Significant expression of CD44, HIF1 α, and Snail among advancing grades of OSCC and their metastatic lymph node were observed. A positive correlation was seen between them.

Conclusions: The prognosis of OSCC can be improved by better understanding and targeting the molecules involved in the formation and maintenance of OCSCs.

Keywords: Cancer stem cells, CD44, HIF1α, oral squamous cell carcinoma, Snail

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for 95% of all oral cavity neoplasms and sixth most common cancer. Despite significant improvement achieved during the last decades in its detection, prevention, and treatment, outcome and prognosis related to cure and survival have still been poorer due to tragic events of treatment resistance and tumour recurrence. More than 50% of patients eventually develop local recurrence or metastasis usually within the first two years following completion of treatment.[1] Metastasis is the key prognostic indicator and a critical determinant of cancer management and therapy. One of the most recent factors suggested to have a vital role in metastasis is cancer stem cells (CSCs) and their niche.[2]

CSCs are the heterogeneous group of the population of cells in cancer characterized by self-renewal and the ability to differentiate. These are drivers of key processes in cancer progression, such as tumour growth, recurrence, metastasis and treatment resistance.[3] Role of CSCs in metastasis to secondary organs is not clear. Various studies have demonstrated that CSCs have more metastatic ability in breast carcinoma, colorectal cancer and pancreatic cancer.[4-6]

The origin of CSCs remains controversial; there is increasing evidence to support that CSCs arise by either mutation from normal stem/progenitor cells or deregulation of genetic programmes regulating these cells. These acquired mutations allow normal stem cells to transform from quiescent and tightly regulated phenotypes to constitutively activated ones.[7] There is a recent suggestion in the literature that cellular processes such as autophagy, hypoxia, and epithelial-mesenchymal transition (EMT) in combination or alone can lead to the enrichment of CSCs in oesophageal carcinoma.[8] Need of the hour is to prevent the progression and metastasis of OSCC which can be brought about by preventing the formation of oral CSCs (OCSC). It is proposed in breast carcinoma cases that targeting breast CSCs has a greater implication on prognosis.[9] Clarity on cell biology and metastatic potential of OCSCs is essential for developing more effective anti-cancer treatment.

Thus, this study aims to establish the role of OCSCs in different grades of OSCC and metastatic lymph nodes through the expression of cluster of differentiation 44 (CD44) and to demonstrate the role of hypoxia and EMT in the various grades and metastatic lymph nodes in the formation and maintenance of OCSCs by employing hypoxia-inducible factor-1 Alpha (HIF1α) and Snail, respectively.

The CD44 is a transmembrane cell adhesion molecule involved in cell-to-cell and cell-to-matrix interactions by binding with hyaluronan, extracellular matrix proteins and growth factors. It consists of a cytoplasmic domain and a transmembrane domain. It is constituted by 20 exons, in which the first five and the last five exons are constant, and the remaining 10 exons result in the generation of a variable region. Various isoforms exist due to the splicing of variable exons, which encode the proximal portions of the extracellular cytoplasmic domain.[10]

HIF is a key regulator of the cellular response to hypoxia. It is a heterodimer composed of two subunits: HIF1α and HIF1β. HIF1α functions as a transcriptional activator in hypoxia and binds specifically to the promoters or enhancers of more than 100 genes involved in multiple aspects of tumour biology.[11]

Snail is a prominent inducer of EMT. It contains an N-terminal SNAG domain and a C-terminal zinc finger domain. The N-terminal SNAG domain interacts with several co-repressors and epigenetic remodelling complexes, and the C-terminal zinc finger domains are responsible for DNA binding. The serine-rich domain and nuclear export sequence control the Snail protein stability and subcellular localization. Snail superfamily members have been implicated in various important developmental processes, including neural differentiation, cell fate, survival decisions and left–right identity.[12]

MATERIALS AND METHODS

The laboratory-based study involved the use of paraffin-embedded tissue blocks of histopathologically confirmed cases of different grades of OSCC and metastatic lymph nodes, retrieved from the Department of Oral Pathology, RRDCH; Bangalore. A total of 36 cases of OSCC, 12 in each grade, their metastatic lymph nodes, and 12 normal oral mucosal tissues as control were included in the study. The hospital medical records obtained these patients' clinical and demographic details. Samples were categorized into four groups as follows: Group I—well-differentiated squamous cell carcinoma (WDSCC), Group II—moderately differentiated OSCC (MDSCC), Group III—poorly differentiated OSCC (PDSCC), and Group IV— normal mucosa. Ethical approval for this study was obtained from the Ethics Committee of RRDCH, Bangalore, on 12.07.2018 (Ref.no.RRDCH ET/01/ORALP/2018-19). Recurrent/secondary cases of OSCC and cases with any therapeutic interventions were excluded from the study sample. Each section was stained with haematoxylin and eosin and also for immunohistochemical stain.

Immunohistochemistry

The 4 μm formalin-fixed, paraffin embedded sections were taken on charged slides and dried overnight at 60°C. These sections were deparaffinized and rehydrated, and antigen retrieval was carried out according to standard protocols. The sections were then incubated with the primary antibodies. The antibodies used were CD44 monoclonal antibody (clone: SP37), HIF1α monoclonal antibody (clone: EP1215Y), and Snail (clone: OTI5E12). Sections were then washed twice with an appropriate buffer, stained with a secondary detection kit Master polymer plus detection system (HRP) (Vitro master diagnostic) counterstained with haematoxylin, and mounted with DPX Mountant. Two observers scanned all the slides at 200x and evaluated them at 400x independently.

Evaluation of Immunohistochemical staining

Positive immunoexpression was seen as a brown stain at the site of the target antigen cell membrane for CD44, cytoplasm, and nucleus for HIF1α and nucleus for Snail.

Assessment of positive immunoexpression of all three antibodies among four groups was based on the immune reactive score (IRS). The evaluation of distribution, staining intensity, and percentage of positive cells were evaluated by selecting the most reactive areas on the respective slide at ×10 and then counting at ×40 magnification. The IRS was calculated by combining the quantity score (percentage of positively stained cells) with the staining intensity score. The quantity score ranged from 0 to 4, and the staining intensity score ranged from 0 to 3. The scoring method is described in Table 1.[13]

Statistical analysis

The collected data were entered in the Excel spreadsheet, and statistical analysis was performed using the Statistical Package for Social Sciences [SPSS] for Windows Version 22.0 Released in 2013, Armonk, NY: IBM Corp. Descriptive analysis of CD44, HIF1α & Snail in frequency and proportions in each study group. Chi-square test was used to compare the expression of CD44, HIF1α and Snail between four groups. Spearman's rank correlation test was used to estimate the relationship between lesion proper & lymph node between different antibodies in each group. The level of significance was set at $P < 0.05$.

RESULTS

Clinical characteristics

OSCC patients ranged in age from 30 yrs to75 yrs (mean age: 54.6 yrs), most patients were females (58.3%), and most cases were located on the buccal mucosa (52.7%), and others were located on the retromolar area (33.3%), tongue (8.3%) and floor of mouth (5.5%).

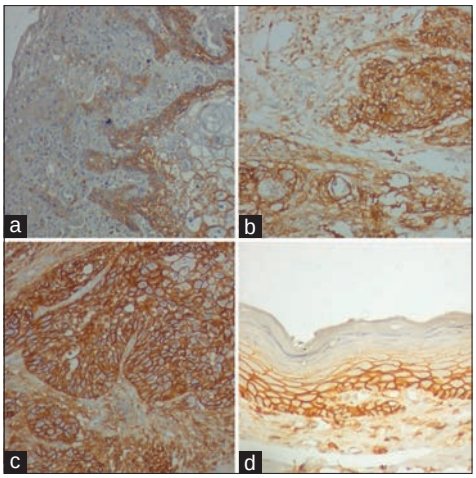

Figure 1: Photomicrograph showing expression of CD44 in lesion proper of well-differentiated oral squamous cell carcinomas (a), moderately differentiated oral squamous cell carcinomas (b), poorly differentiated oral squamous cell carcinomas (c) and normal epithelium (d). (100x)

Table 1: Immune reactive score (IRS) A (percentage of positive cells) B (intensity of staining) IRS score (multiplication of A and B)

A (percentage of positive cells)	B (intensity of staining)	IRS
0=no positive cells	0=no colour reaction	0-1=negative
1= <10% of positive cells	1=mild reaction	2-3=mild
2=10–50% positive cells	2=moderate reaction	4-8=moderate
3=51–80% positive cells	3=intense reaction	9-12=strongly positive
4 = >80% positive cells		

Table 2: Comparison of CD44 expression in lesion proper amid four groups

CD44 expression	Group 1		Group 2		Group 3		Group 4		*P*
	n	%	*n*	%	*n*	%	*n*	%	
Negative	1	8.3%	0	0.0%	0	0.0%	4	33.3%	<0.001*
Mild	6	50.0%	2	16.7%	0	0.0%	8	66.7%	
Moderate	5	41.7%	10	83.3%	4	33.3%	0	0.0%	
Strongly Positive	0	0.0%	0	0.0%	8	66.7%	0	0.0%	

*Significance was set at $P < 0.05$

Table 3: Comparison of CD44 expression in lymph node amid three groups

CD44 expression	Group 1		Group 2		Group 3		*P*
	n	%	*n*	%	*n*	%	
Negative	4	33.3%	0	0.0%	0	0.0%	0.001*
Mild	6	50.0%	3	25.0%	0	0.0%	
Moderate	2	16.7%	9	75.0%	10	83.3%	
Strongly Positive	0	0.0%	0	0.0%	2	16.7%	

*Significance was set at $P < 0.05$

Immunohistochemical findings

Comparison of CD44 expression in lesion proper among four groups by the Chi-square test showed a statistically

significant *P* value of <0.001. A moderate CD44 expression was seen in 50% of WDSCC and 83.3% of MDSCC. 66.7% of PDSCC showed strong expression, and 66.7% of normal mucosa had mild expression [Figure 1 and Table 2].

CD44 expression among metastatic lymph nodes of various grades of OSCCs was mild in 50% of WDSCC, moderate in 75% and 83.8% of MDSCC and PDSCC, respectively, with a significant *P* value of 0.001 [Figure 2 and Table 3].

Table 4: Comparison of HIF1α expression in lesion proper amid four groups

HIF1α expression	Group 1		Group 2		Group 3		Group 4		*P*
	n	%	*n*	%	*n*	%	*n*	%	
Negative	7	58.3%	0	0.0%	0	0.0%	9	75.0%	<0.001*
Mild	5	41.7%	4	33.3%	0	0.0%	3	25.0%	
Moderate	0	0.0%	8	66.7%	12	100.0%	0	0.0%	

*: Significance was set at $P < 0.05$

Table 5: Comparison of HIF1α expression in lymph node amid three groups

HIF1α expression	Group 1		Group 2		Group 3		*P*
	n	%	*n*	%	*n*	%	
Negative	9	75.0%	6	50.0%	5	41.7%	0.23
Mild	3	25.0%	6	50.0%	7	58.3%	

Table 6: Comparison of Snail expression in lesion proper amid four groups

Snail expression I	Group 1		Group 2		Group 3		Group 4		*P*
	n	%	*n*	%	*n*	%	*n*	%	
Negative	1	8.3%	0	0.0%	0	0.0%	5	41.7%	<0.001*
Mild	7	58.3%	4	33.3%	1	8.3%	7	58.3%	
Moderate	4	33.3%	8	66.7%	11	91.7%	0	0.0%	

*: Significance was set at $P < 0.05$

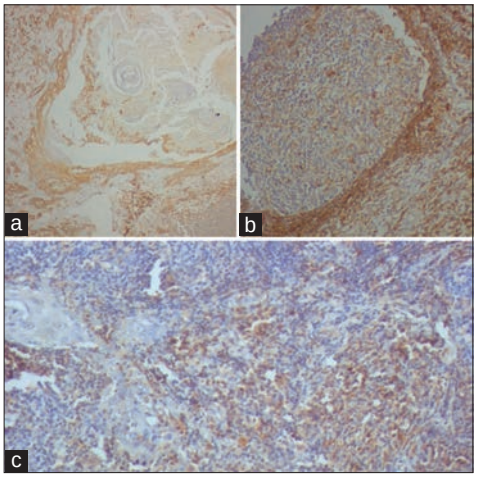

Figure 2: Photomicrograph showing expression of CD44 in metastatic lymph node of well-differentiated oral squamous cell carcinomas (a), moderately differentiated oral squamous cell carcinomas (b), poorly differentiated oral squamous cell carcinomas (c) (100x)

Comparison of HIF1α expression in lesion proper among four groups using the Chi-square test showed a statistically significant *P* value of <0.001. A negative expression of HIF1α was seen in 58.3% and 75% of WDSCC and normal mucosa, respectively. 66.7% and 100% expressions were seen in MDSCC and PDSCC, respectively [Figure 3 and Table 4].

HIF1α expression among metastatic lymph nodes of various grades of OSCCs was negative in 75% and 50% of WDSCC and MDSCC correspondingly. A 58.3% of PDSCC showed mild expression of the HIF1 α with a *P* value of 0.23 [Figure 4 and Table 5].

Comparison of Snail expression in lesion proper among four groups using the Chi-square test showed a statistically significant *P* value of <0.001. A mild Snail expression was seen in 58.3% of WDSCC and normal mucosa. A 66.7% and 91.7% of MDSCC and PDSCC, respectively, showed moderate expression [Figure 5 and Table 6].

Snail expression among metastatic lymph nodes of various grades of OSCCs was mild and seen in 91.7% of PDSCCs. [Figure 6] A negative expression was seen in most of the cases in the other two groups. Chi-square test showed a *P* value of 0.007 [Table 7].

The CD44 expression in Group I for lesion proper showed a significant very strong positive correlation with Snail expression scores at rho = 0.88, $P < 0.001$. Similarly, HIF1α expression showed a borderline significant moderate positive correlation with Snail expression rho = 0.50,

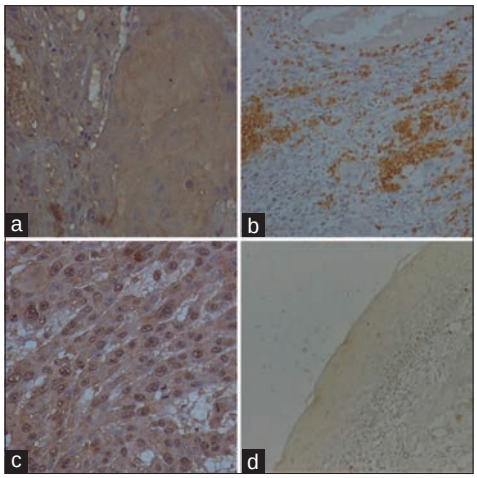

Figure 3: Photomicrograph showing expression of HIF1α in lesion proper of well-differentiated oral squamous cell carcinomas (a), moderately differentiated oral squamous cell carcinomas (b), poorly differentiated oral squamous cell carcinomas (c) and normal epithelium (d). (100x)

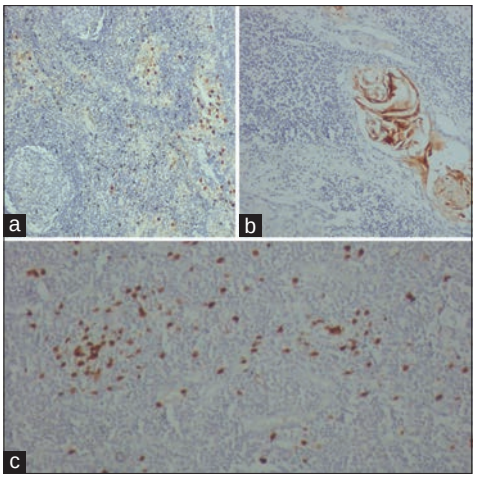

Figure 4: Photomicrograph showing expression of HIF1α in metastatic lymph node of well-differentiated oral squamous cell carcinomas (a), moderately differentiated oral squamous cell carcinomas (b), poorly differentiated oral squamous cell carcinomas (c) (100x)

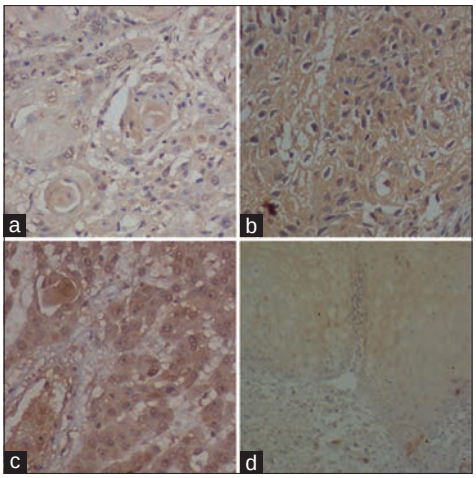

Figure 5: Photomicrograph showing expression of Snail in lesion proper of well-differentiated oral squamous cell carcinomas (a), moderately differentiated oral squamous cell carcinomas (b), poorly differentiated oral squamous cell carcinomas (c) and normal epithelium (d). (100x)

Table 7: Comparison of Snail expression in lymph node amid three groups

Snail expression	Group 1		Group 2		Group 3		*P*
	n	%	*n*	%	*n*	%	
Negative	7	58.3%	6	50.0%	1	8.3%	0.007*
Mild	5	41.7%	6	50.0%	11	91.7%	

*: Significance was set at $P < 0.05$

Table 8: Spearman's rank correlation for a lesion proper between different antibodies in each group

Groups	Antibody	Values	CD44	HIFα	Snail
Group I	CD44	rho	1	0.35	0.88
		P	.	0.26	<0.001*
	HIF1α	rho	0.35	1	0.50
		P	0.26	.	0.10
	Snail	rho	0.88	0.50	1
		P	<0.001*	0.10	.
Group II	CD44	rho	1	-0.23	-0.30
		P	.	0.47	0.35
	HIF1α	rho	-0.23	1	0.31
		P	0.47	.	0.33
	Snail	rho	-0.30	0.31	1
		P	0.35	0.33	.
Group III	CD44	rho	1	0.16	-0.54
		P	.	0.63	0.07
	HIF1α	rho	0.16	1	0.05
		P	0.63	.	0.87
	Snail	rho	-0.54	0.05	1
		P	0.07	0.87	.
Group IV	CD44	rho	1	-0.41	-0.24
		P	.	0.19	0.45
	HIF1α	rho	-0.41	1	0.10
		P	0.19	.	0.76
	Snail	rho	-0.24	0.10	1
		P	0.45	0.76	.

*: Significance was set at $P < 0.05$

$P = 0.10$. Contrastingly, in Group III, CD44 expression showed a borderline significant moderate negative correlation with Snail expression scores at rho = -0.54, $P = 0.07$. However, no significant correlation was observed between the expressions of antibodies in Group II & Group IV [Table 8].

The CD44 expression in Group II for lymph nodes showed a significantly strong negative correlation with Snail expression at rho = -0.62, $P = 0.03$. However, no significant correlation was observed between the expression of antibodies in Group I and Group III [Table 9].

DISCUSSION

Each year approximately 263000 new cases of oral cancer are detected worldwide despite various and advanced treatment modalities. India has the largest prevalence of oral cancer patients among South Asian countries, accounting for almost 40% of deaths.[14] This increased incidence of OSCC is attributed to the development of local and regional recurrences that are resistant to therapies and also due to the development of distant metastasis. Many clinical and molecular factors have been postulated to have a role in this.

One of the breakthrough events in the understanding of cancer biology was the discovery of CSCs by John Dick in acute myeloid leukaemia. It has been suggested that these play an important role in tumour development and progression.[15] Prevalence of tumorogenic stem cells in head and neck SCC has been proved by Prince.[16] These CSCs can divide asymmetrically and expand into heterogeneous cells, which is believed to be a critical role in oral carcinogenesis, field cancerization, cancer progression, recurrence, metastatic dissemination and tumour resistance.[2,3,17]

Table 9: Spearman's rank correlation for a lymph node between different antibodies in each group

Groups	Antibody	Values	CD44	HIFα	Snail
Group I	CD44	rho	1	-0.43	0.27
		P	.	0.17	0.40
	HIF1α	rho	-0.43	1	-0.10
		P	0.17	.	0.76
	Snail	rho	0.27	-0.10	1
		P	0.40	0.76	.
Group II	CD44	rho	1	-0.26	-0.62
		P	.	0.42	0.03*
	HIF1α	rho	-0.26	1	0.00
		P	0.42	.	1.00
	Snail	rho	-0.62	0	1
		P	0.03*	1	.
Group III	CD44	rho	1	-0.23	0.21
		P	.	0.46	0.52
	HIF1α	rho	-0.23	1	0.36
		P	0.46	.	0.26
	Snail	rho	0.21	0.36	1
		P	0.52	0.26	.

*: Significance was set at $P < 0.05$

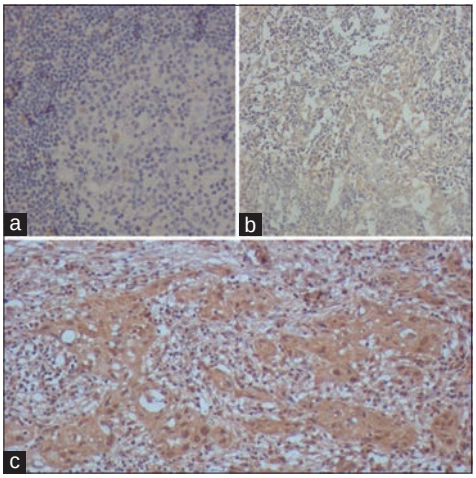

Figure 6: Photomicrograph showing expression of Snail in metastatic lymph node of well-differentiated oral squamous cell carcinomas (a), moderately differentiated oral squamous cell carcinomas (b), poorly differentiated oral squamous cell carcinomas (c) (100x)

Various hypotheses have been suggested for the origin of CSCs in head and neck SCC. It has been proposed that they can arise from stem cells, progenitor cells or differentiated cells.[11] Normally, stem cells follow a signalling pathway that directs differentiation into mature cells and governs self-renewal. Any alteration in the microenvironment can cause signalling pathways to give rise to tumours.[3] Brabletz *et al*.[18] hypothesized that there were two types of CSCs: stationary CSCs (SCSCs) and migrating CSCs (MCSCs). SCSCs are embedded in the epithelia and are non-mobile, while MCSCs mediate tumour cell metastasis. They proposed that MCSCs were derived from SCSCs which undergo EMT.

In our study, the expression of CD44 was observed in all grades of OSCC and their metastatic nodes thus confirming the presence of CSCs. The expression of CD44 progressed from lower to higher scores as the grades advanced from WDSCC (group I) to PDSCC (group III). Adnan Y *et al*.[19] also suggested the presence of CSCs in OSCC and correlated increased expression of CD44 with poor prognosis. A study by Surendran *et al*.[20] also suggested that increased expressions of CSCs are associated with an increase in the severity of dysplasia.

Aya k stated that CD44 is expressed in all layers of dysplastic epithelium, and invasive OSCC and soluble serum levels of CD44 were higher in PDSCC.[21] Invasion and metastasis of OSCC require epithelial cell migration by remodelling the intercellular junctions through extracellular matrix/EMT. It has been stated that CD44 undergoes such alterations which render the CSC to detach, migrate and invade the surrounding tissue.[10]

The role of CSCs in metastasis to secondary organs is not clear. Various studies have demonstrated that CSCs have more metastatic ability. Shuang Li stated that CSCs can be closely correlated with tumour metastasis as they can induce metastasis through multiple pathways and participate in angiogenesis and lymphangiogenesis by increasing the production of growth factors.[2]

We observed CD44 expression in metastatic lymph nodes thus demonstrating the migratory capacity of OCSCs to lymph nodes in various grades of OSCC. Demonstration of the migratory capacity of OCSCs to lymph nodes can help us prove that oral CSCs can be employed as a predictor of the prognosis of the disease. To date, no study has demonstrated the presence of CSCs in metastatic lymph nodes of OSCC. Jing Hu *et al*.[4] provided evidence of metastasis of CSCs to lungs in breast carcinoma and proposed that increased CD44 positivity can be correlated with distant metastasis. Gao *et al*.[5] demonstrated that CSCs of colorectal cancer engage liver and lung metastasis. Metastatic CSCs of pancreatic cancers were seen liver.[6] Microenvironment and cellular processes such as autophagy, hypoxia, anti-cancer therapy, and EMT alone or in combination have a role to play in controlling the pool of CSCs in oesophageal carcinoma.

We at the molecular level confirmed the role of hypoxia and epithelial-mesenchymal transition in the formation and maintenance of OCSCs by perceiving the expression of HIF1α and Snail in various grades of OSCC and their metastatic lymph node. Our results were by various data published which showed increased expression of HIF1α[22] and Snail in lesion proper[23] with advancing grades of OSCC. Mild-to-moderate expression of HIF1α

and Snail was seen in only the metastatic part of the lymph node.

Hypoxia is one of the hallmarks of cancer and reflects the imbalance between oxygen consumption by the rapidly proliferating cancer cells.[21] A hypoxic microenvironment plays a critical role in tumour development and progression including head and neck tumours. It also has the potential to regulate cell differentiation by facilitating the maintenance and evolution of cancer stem cell characteristics[24] Chiro Ota *et al*.[25] demonstrated that Snail induces CSC-like properties through EMT and maintains stemness by upregulating various CSC markers. It could be involved in the maintenance of CSCs and the microenvironment. Ayob *et al*.[26] proposed that CSCs have cross-talk with the tumour microenvironment to errand their survival and resistance to chemotherapy.

Although the coefficient correlation between CD44, HIF1 α and Snail expression among the lesion proper and lymph nodes of various grades of OSCC were not statistically significant, there was a positive correlation among them thus suggesting that there was a cross-talk between CSCs, hypoxia, and EMT and they have a role in the formation and maintenance of OCSCs. The activation and acquisition of chemoresistant CSCs can be prevented by targeting the tumour microenvironment.

Management of OSCC is challenging because of locoregional recurrence and metastasis. CSCs and their niche are stated to play a vital role in the prognosis of cancer. Furthermore, there is lack of extensive research related to the formation, maintenance and metastatic potential of OCSCs in OSCC. The novelty of present study lies with an evaluation of OCSCs and its niche, specifically hypoxia and EMT in the OSCC and metastatic lymph nodes using CD44, HIF1α and Snail. We observed that there is a definite amplified expression of these immunohistochemical markers among advancing grades of OSCC and their metastatic lymph node. Thus, we hypothesize that OCSCs will migrate to lymph nodes and have migratory capacity. Hypoxia and EMT, components of the tumour niche, have a role in the maintenance of OCSCs. However, a statistically significant correlation between CD44, HIF1α and the Snail could not be established due to the smaller sample size. Thus, we conclude that by establishing the role of hypoxia and EMT in the formation and maintenance of OCSCs helps in targeting the microenvironment, and thus, the activation and acquisition of chemoresistant CSCs can be prevented, and prognosis of OSCC can be improved.

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Conflicts of interest

There are no conflicts of interest.

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