RATIONALE OF THE STUDY:

CD44+ cells have been identified as tumor-initiating stem cell subpopulation responsible for generating tumor heterogeneity with phenotypically discrete cells in head and neck cancers [40]. Expression of this designated cancer stem cell marker and its variant isoforms have been associated with various malignancies; however which isoform is predominant in OSCC needs to be elucidated. Moreover, embryonic stem cell marker - Nanog and oncogene Bmi1 have been found to regulate the self-renewal, stem cell maintenance and growth-promoting modulator of cancer stem cells in different tumor types [256, 257]. Propelling evidences strongly support the contribution of these CSCs in mediating tumorigenesis and thus targeting these CSCs could be a new conceptual approach which might facilitate in improvisation of the conventional therapeutic regimen of oral carcinogenesis [39]. However CSC shares many characteristics with normal stem cells, which makes specific targeting very complicated [258, 259]. Thus the need of the hour is to not only identify the disease specific CSC markers and understand the mechanism of CSCs in tumor development, metastasis and chemotherapeutic response but also highlight the underlying pathways regulating this subpopulation [258, 259].

Recent findings demonstrated the possibility of a complementary co-existence amongst the two CSC models, hence supporting the hypothesis that cancer is a genetic disease, regulated by epigenetic mechanisms which have no direct effect on the primary DNA sequence [43]. miRNAs demonstrate differential expression patterns in CSCs and non-CSC tumorigenic cells [260]. Attributed to their ability to modulate multiple genes simultaneously, miRNAs form prospective candidates having a vital role in regulating CSC self-renewal and maintenance of stemness property in a pathway dependent manner [261]. Furthermore, analogous to miRNA mediated regulatory changes that occur during carcinogenesis, CSCs interact with the tumor microenvironment via autocrine/ paracrine signals of cytokine networks that can modulate the CSC phenotype effectively [262, 263]. Collectively, miRNAs and dysregulated signals from microenvironment might have a plausible role in activating CSC dependent pathways leading to malignant transformation which is difficult to eliminate; however their exact mechanism needs to be elucidated. Hence designing strategies to specifically target the miRNA modulators and cytokine loops interacting
and regulating CSCs could be an important approach in improving the 5 year survival rate of OSCC.

Thus, in this study we aim to identify and characterize the highly tumorigenic CD44+ subpopulation in order to re-evaluate the prospects of CD44 as a defining Cancer Stem Cell marker of OSCC. Furthermore, on the basis of pathway prediction analysis we sought to conduct molecular characterization of CSC markers, miRNA expression profile and assess the impinging role of cytokines in CD44+ cells. This would aid in understanding the regulatory role of CSCs and its modulators in regulating tumor cell growth, anti-apoptosis, cell survival, and chemo resistance in OSCC. Further, we ought to correlate the expression pattern of this CSC mediated pathway with the difference in etiology and anatomic locations of the OSCC patients in a context dependent manner. Therefore, this study could be of clinical importance that may facilitate in the identification and development of strategies to target oral CSC subpopulation.
Chapter 3

METHODOLOGY:

**OSCC tissues collection and processing:**

Surgical tissue specimens from 30 OSCC patients (15 buccal mucosa and tongue tumor specimen each) were collected after obtaining written informed consent and this study was approved by The Institutional Review Board and Ethics Committee at The Gujarat Cancer Research Institute. Human primary OSCC carcinoma (T) tissues were obtained from surgical procedures sent to the pathology lab for diagnosis. Microscopically screened tumor tissues were cryopreserved in liquid nitrogen for further downstream applications. RNA extracted from Normal tongue (Agilent) and buccal mucosa swab of healthy individual were taken as controls for the study.

**Immuno-magnetic cell sorting of cancer stem cells and cell culture:**

Putative cancer stem cells were isolated from surgically resected primary tumors of HNSCC patients (tumor sites of buccal mucosa and tongue) by cutting them in to small pieces until they passed through a 25 ml serological pipette. These minced cells from the specimen were suspended in a 9:1 solution of DMEM (HyClone, Waltham, MA, USA), collagenase and hyaluronidase (Stem Cell Technologies; Vancouver, BC, Canada) and incubated at 37°C for one hour until they passed through a 10-ml pipette every 15 minutes for mechanical dissociation. Cells were filtered through a 40-μm nylon mesh (BD Falcon; Franklin Lakes, NJ, USA), washed with DMEM (Invitrogen) containing 10% FBS, and centrifuged at 800 rpm for 5 minutes. Single cell suspensions obtained from primary specimens were washed, counted, and re-suspended at 10^6 cells/ml in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100U/mL penicillin and 100 μg/mL streptomycin (complete medium). and cultured in 75 cm² tissue flasks (Corning, Stone Staffordshire, UK) at 37 °C and 5% CO₂. Cells were cultured in adherent flasks till the cultures achieved 75% to 85% confluence. Further, these adherent cells were selected for subculture by trypsinization with 0.25% trypsin and 0.02% EDTA (Life Technologies Inc.). The culture medium was changed twice a week and cellular homogeneity evaluated microscopically every 24–48 h. When possible, early passage and late passage primary cultures were frozen in 90% FBS and 10% DMSO and stored in liquid nitrogen for further experiments. The EasySep® FITC positive selection kit was used to identify the CD44 positive cells from the heterogeneous tumor population. The
target cells were exposed to anti-CD44 mouse antibody specifically labeled with FITC-conjugated (Stem Cell Technology) and were further labeled with dextran-coated magnetic nanoparticles using bispecific Tetrameric Antibody Complexes (TAC), thus separating magnetically labeled CD44+ cells from unlabeled cells which were defined as putative head and neck cancer stem cell population. These sorted cells were cultured in low glucose DMEM (Invitrogen), 10% fetal bovine serum, and 100 U/ml Penicillin-streptomycin in low attachment conditions. All experiments were performed in duplicates to verify the reproducibility of the data.

**Sphere forming assay:**

The immuno-magnetically-sorted cells were seeded in 6-well ultra-low attachment plates (Corning; New York, NY, USA) at a density of 5×10³ cells/well and cultured in low glucose DMEM, 10% fetal bovine serum and 100 U/ml Penicillin-streptomycin at 37°C and 5% CO₂. The medium was changed every alternative day until tumor sphere formation was observed after 2 weeks of incubation. The percentage of orosphere was calculated by dividing the number of spheres by the number of cells seeded per well. To verify the self-renewal property of the spheres, they were mechanically dissociated into single cell suspension in new ultra-low attachment plates for the generation of secondary and tertiary spheres.

**Drug cytotoxicity assay:**

The cytotoxicity of Cisplatin was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium Bromide) assay by preparing its dilutions in media. CD 44+ and CD44- cells were seeded in 96 well plate at a density of 5.0 × 10⁴ cells/well. Cisplatin was added to cultured cells in the range between 0.25 - 1.0 μg/ml to check their IC₅₀ (Inhibitory Concentration) value at 72 hrs exposure. After the removal of media, 10 μl of MTT (5 mg/mL, Hi-Media) was added to each well and plate was incubated at 37°C for 4 h. Then, 200 μl of DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. Absorbance was measured at 570 nm using an ELISA reader (Multiskan Spectrum Microplate Reader, Thermo Scientific). The experiments were performed in triplicates. All data were normalized to corresponding DMSO controls.
Pathway prediction analysis and quantification of identified CSCs markers and miRNA modulators in OSCC samples:

Pathway Prediction Analysis and Quantification of identified CSCs markers and miRNA modulators in OSCC Samples: The pathway prediction analysis was conducted with a special emphasis on identifying the epigenetic regulators and other modulators having a significant role in regulating the pertinent CSC and stemness markers. Bioinformatic tools such as KEGG pathway, Pathvisio and string protein-protein interaction were used in order to identify vital modulators having an intriguing role in governing the CSCs, thus giving the future studies clues of a plausible role of specific CD44 mediated pathway which might have prognostic and therapeutic relevance. Total RNA was extracted from cultured cell lysate and tumor tissues of the patients using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. cDNA was synthesized from 1µg RNA of each sample using High Capacity cDNA Reverse transcription kits (Applied Biosystems). miRNA specific cDNA was produced using first strand miRNA specific cDNA kit (Agilent technology). Real-time PCR reaction mix was prepared in a total volume of 20 µl containing 2X SYBR Fast qPCR Mastermix Universal (Kappa Biosystems), 200 nM of each primer, 2.5 mM of MgCl₂ and 1µl of cDNA. β-actin and U6 were used as endogenous control and all the primers were custom ordered using the following sequences. The amplification run was performed on the AriaMx Real-time PCR System (Agilent technologies). PCR reactions were prepared in duplicates and heated at 95°C for 3 min followed by 40 cycles of denaturation at 95 °C for 3 seconds and annealing at 60 °C for 20 sec, for 40 cycles. Results were analyzed using the ddCt method.

Bioplex cytokine profiling of OSCC samples:

Quantitative estimation of the concentrations of cytokine levels - IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN and TNF-α were analyzed in the matched serum samples of the patient's and healthy individuals using BioPlex Cytokine Assay as described in the manufacturer’s instructions. The cytokine concentration levels were calculated by BioPlex Manager Software on the basis of a standard curve derived from recombinant cytokine standards.
RESULTS:

(A) Identification and characterization of cancer stem like cells from primary tissue of OSCC patients:

OSCC primary tissue cells were subjected to CD44+ FITC Immuno-magnetic cell separation for isolation of CSC- like phenotypic cells which comprised of <10% of the total tumor population. We validated the CD44+ subpopulation using flow cytometry analysis and further subjected this pure population to various cellular and molecular assays in order to understand its underlying mechanism and role as tumor initiating cells in OSCC (Fig. 14).

![Flow Cytometry Image]

Figure 14: Validation of immunomagnetically isolated putative CD44 population using flow cytometry. Enriched CD44+ population was analyzed by flow cytometry and the image represents histograms. Image is representative of 3 independent experiments and presented graphically with mean ± SD.

(B) Increased self-renewal capability and tumorigenic potential of CD44+ cells:

We assessed the self renewal property of CD44+ and CD44- phenotypic cells by evaluating their sphere forming ability - a defining hallmark of CSC. Colony forming ability of CD44+ cells was found to be higher and the spheres were found to be significantly greater in number and diameter compared to CD44- subpopulation which seemed to form aggregates rather than true spheres (Fig. 15A). Moreover, these cancer stem-like cells (CD44+) formed non-adherent colonies indicating anchorage independent tumorigenic growth potential. Furthermore, CD44+ cells demonstrated a colony forming efficiency of <80% with the orospheres from buccal mucosa demonstrating a relatively increased ability of self renewal compared to tongue (Fig. 15B & 15C).
Figure 15: Self-renewal capability and tumorigenic potential of CD44+ and CD44- cell population. (A) Representative images of tumor spheroids derived from CD44+ and CD44- subpopulation on the 14th day of immunomagnetic cell separation. (B) Colony forming ability of CD44+ population counted against the number of days for 3 independent experiments and plotted as average ± SD. (C) Colony forming assay for respective population of buccal mucosa and tongue subtypes of OSCC with number of colonies calculated on the 7th and 14th day.

However, no significant difference was observed in the sphere forming potential of cells derived from tobacco habituated patients as compared to non-habituated counterparts. Collectively, these results highlighted the increased intrinsic self-renewal capability and high tumorigenic potential of CD44+ cells isolated from primary tumor of OSCC patients.

(C) CD44+ cancer stem like cell population demonstrated chemoresistance:

Cisplatin is one of the most conventional chemotherapeutic agents used as initial therapeutic regimen demonstrating a disease stabilization and associated with tumor regression in OSCC patients; however, many patients are resistant to these drugs leading to poor therapeutic response, recurrence and relapse of the disease. Recent evidences suggest that slow-cycling cancer stem cell population have the
potential to evade drug or radiation therapy than actively dividing cancer cells. To analyze this property of CSCs, CD44+ and CD44- distinct population of sorted cells were exposed to cisplatin at increasing concentrations for 72 h, followed by assessing their viability by MTT Cytotoxicity Assay. Our results demonstrated that CD44+ cells showed significantly higher survival rate than the CD44- subpopulation when treated with concentrations of 0.25µg/ml, 0.5 µg/ml and 1 µg/ml (p <0.001) (Fig. 16).

**Figure 16: Chemotherapeutic drug sensitivity/ Cytotoxic effect of Cisplatin on CD44+ and CD44- cells.** CD44+ and CD44- sorted cells were exposed to Cisplatin at increasing concentrations for 72 h, followed by MTT cytotoxicity Assay. This image is representative of differences in Percent survival between CD44+ and CD44- cells. Data shown as mean ± SD and shows statistical significance at p <0.001 for all concentrations.

In corroboration with the self renewal assay results depicting an increasing trend of CD44+ subpopulation in habituated and buccal mucosa site patients; the chemotherapeutic agent were more effective on these in-vitro models attributed to higher levels of CD44+ cells. However, this would not help us establish any association between the chemo-resistance property with any etiological or organ specific behaviour. These findings substantiated that CD44+ cells depicted increased survival rate compared to the CD44- population attributed to dose dependent drug resistance to Cisplatin.
(D) Gene expression profile of csc markers in CD44+ cells and its significance in OSCC patients:

Analysis of pertinent CSC markers showed a significantly increased expression patterns of CD44s, CD44v3, CD44v6, Nanog and Bmi1 in CD44+ spheres as compared to CD44- cells, corroborating that these genes might have a plausible role in regulating the stemness and self-renewal property of cancer stem like cells (Fig. 17A). Identical expression pattern of these CSC markers was observed in OSCC patients compared to healthy controls, establishing an inevitable role of CSC phenotype in OSCC progression and suggestive of their potential role as prognostic and therapeutic marker which might facilitate in improvising the 5 year survival rate of OSCC patients (Fig. 17B).
Further, on intricately analyzing the data, a considerable difference was found between the gene expression patterns of the 2 subtypes (Tongue and Buccal mucosa) of OSCC such as – i) Significantly higher levels of CD44s, CD44v3 and CD44v6 were observed in Buccal mucosa patients compared to Tongue cancer patients; (ii) Nanog expression was found to be radically high in tongue cancer patients while significantly increased/enhanced Bmi1 expression was observed in buccal mucosa patients (Fig. 18A). Additionally, increased CD44s and CD44v6 expression levels were observed in Tobacco habituated patients (Chewers) compared to non-habituated patients (Fig. 18B). Collectively, these findings prompted us to evaluate the key modulators of these CSC markers in order to unravel the underlying mechanism regulating this malignancy in CSC dependent pathway.
Figure 18: Differential expression of CSC markers with diverse anatomic locations and etiological behavioural pattern. (A) Figure depicts differences in relative expression patterns of CSC markers - CD44s, CD44v3, CD44v6, Nanog and Bmi1 for tongue and buccal mucosa population respectively. (B) Image is representative of relative expression of CD44s and CD44v6 in tobacco habituated and non-habituated patients respectively. The data is expressed as mean ± SD with their p values indicated in the graphs as *p<0.05, **p<0.01 and ***p<0.001.
(E) Identification and expression profiling of miRNA modulators regulating these CSC markers in pathway dependent manner:

CSC markers showing significant expression patterns in tumor-spheres and specimen were subjected to online Pathway analysis softwares. A hypothetical pathway was formed comprising of vital CSC/ stemness markers and their miRNA modulators which were validated on tumor specimen of OSCC patients - an unexplored arena which could improvise the prognostic and therapeutic potential in OSCC (Fig. 19).

![Pathway prediction analysis](image)

**Figure 19: Pathway prediction analysis of pertinent CSC markers and their pertinent miRNA modulators and cytokine profiles using computational tools**

miR542-3p, miR34a and miR9 were found to be significantly down regulated whereas oncogenic miR21 depicted significantly increased expression levels in patients compared to their healthy counterparts. There have been inconclusive reports on miR9 which are unable to determine its role as a tumor suppressor or oncogenic miRNA. Our results are suggestive of its potential role as a tumor suppressor miRNA in OSCC; however further studies are required to confirm our findings. Moreover, strong correlations were established between miR542-3p and miR34a with CD44v6 using Pearson correlation analysis, thus substantiating the findings of our pathway prediction analysis (Table 8).
**Table 8:** Pearson correlation analysis of miRNA542-3p, miR34a, CD44s and Nanog with CD44v6 CSC marker

<table>
<thead>
<tr>
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<th>R² value of CD44v6</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>miR542-3p</td>
<td>-0.85</td>
<td>&lt; 0.008</td>
</tr>
<tr>
<td>miR34a</td>
<td>-0.88</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>CD44s</td>
<td>1.00</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Nanog</td>
<td>0.99</td>
<td>&lt; 0.00001</td>
</tr>
</tbody>
</table>

Subsequently, miR542-3p demonstrated significant association with (i) non-habituated patients compared to patients with previous history of tobacco consumption (ii) buccal mucosa subsite compared to tongue; however, no significant correlation was established between expressions of any other miRNA with any other clinico-pathological conditions (Fig. 20). Therefore, upstream miR542-3p might have an imperative role in modulating miR34a and CD44v6, thereby initiating CSC mediated signaling cascade in OSCC.

**Figure 20:** Differential expression of miR542-3p with diverse anatomic locations and etiological patterns. The data is expressed as mean ± SD with their p values indicated in the graphs as **p<0.01.

(F) **Evaluating the Cytokine Network in OSCC Patients and Their Impinging Role In Regulating miRNA Mediated CSC Pathway:**

Pathway prediction analysis prompted towards the critical role of cytokine networks in regulating this miRNA mediated CSC pathway. Therefore, we analyzed Cytokines Levels of IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN and TNF-α in sera samples of
patients and healthy individuals. Our results indicated increased expression levels of IL-6 & IL-8 in patient’s cohort compared to controls (Table 9). Our findings highlighted that OSCC patients demonstrating an increased cytokine expression levels consequently demonstrated an increased expression pattern of CSC markers (CD44v6 and Nanog) and their miRNA modulators (miR542-3p, miR34a, miR21 and miR9). Further, relatively higher IL-6 expression levels in Buccal mucosa patients while increased IL-8 in Tongue cancer patients signified that cytokine profiles also depicted differential expression profiles in different subtypes of OSCC (Table 9).

**Table 9**: Serum Cytokine Levels of IL6 and IL8 in Tongue and buccal mucosa carcinoma patients compared to their normal counterparts

<table>
<thead>
<tr>
<th></th>
<th>Buccal mucosa</th>
<th>Healthy Control</th>
<th>T</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td><strong>IL-6 Levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>117.48</td>
<td>5.64</td>
<td>17.59</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SD</td>
<td>13.94</td>
<td>1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>3.59</td>
<td>0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-8 Levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>36.52</td>
<td>2.94</td>
<td>7.51</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SD</td>
<td>9.8</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>2.53</td>
<td>0.43</td>
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Hence, these results suggest that IL-6 and IL-8 might have a plausible role in regulating miRNA mediated CSC pathway; however the underlying mechanism by which circulating levels of cytokines govern miRNA mediated CSC marker needs to be elucidated.
DISCUSSION:

Accumulating evidences demonstrate that CSCs have the distinct capability to promote tumor progression and metastasis, and also contribute to radio and chemo-resistance [65]. However, it is indispensable to reinforce this hypothesis clinically and further unravel the underlying mechanisms of the CSC pathway in OSCC, thus opening newer avenues for CSC targeted therapy. In this study we aimed to identify CD44+ CSC like subpopulation from primarily derived tissue of the oral cavity and to elucidate the phenotypic characterization, functional relevance and underlying molecular mechanism of these tumor initiating cells in a pathway dependant manner. In view of the fact that EMT is a resultant repercussion of CD24 depletion in certain malignancies including OSCC, we considered CD44+ CD24- population over CD44+ CD24+ population for our study [264]. Our results observed an enhanced ability of CD44+ cells to form non-adherent colonies with exponentially increasing proliferation rate which was consistent with the findings of Ghuwalewala et al; however these results were contradictory to the findings of Han et al describing CD44+CD24+ cells as CSCs of HNSCC, [264-266]. Collectively, these results are indicative of the idiosyncratic characteristics of CD44+ cell subpopulation as tumor initiating cells with immense self renewal capability, anchorage independent tumorigenic growth potential and ability to undergo asymmetric division [267]. Further, results of cytotoxicity assay exhibited that CD44+ cells demonstrate strong intrinsic chemo-resistance towards Cisplatin - a conventional chemotherapeutic drug compared to CD44- fraction of cells. Nor et al reported that quiescent and slow cycling nature of CSCs and the ability of Cisplatin to enhance the CSC fraction could be some of the putative explanations for chemo-resistant nature of CSC towards Cisplatin [268]. These findings suggest that CD44+ cells are present in tumors as a distinct CSC subpopulation which is difficult to be eliminated by conventional therapeutic modalities, thus possibly causing poor therapeutic response and tumor recurrence in OSCC.

An inevitable role of CSC markers, their miRNA modulators and complex cytokine networks has been established in regulating CSC self-renewal and triggering a downstream signaling pathway which is responsible for the initiation, progression and invasiveness in OSCC. Therefore, a convoluted molecular characterization of CD44+ cells was conducted to identify vital CSC markers and their underlying
mechanism in OSCC which would facilitate in improvising therapeutic possibilities by targeting CSC clinically. These markers showed significantly high expression of CD44 variant isoforms - CD44v3 and CD44v6 in tumor spheres derived from primary tissue and in patients of OSCC, signifying an undisputable role of CSC phenotype in OSCC progression. Furthermore, enhanced expression pattern of stemness markers Nanog in CD44+ subpopulation, positive correlation between expression patterns of CD44v6 and Nanog in OSCC patients prompted towards the probable role of this embryonic stem cell marker in regulating the genetic profiles of CD44 variants. Further, CSC regulating factors are known to function in combinatorial complexes to regulate the CSCs. Our results highlighted increased expression patterns of oncogenic marker Bmi1 which has been previously reported to regulate stemness associated genes by upregulating Nanog via NFκB pathway [257]. Thus, these stemness markers may have an inevitable role in regulating the self-renewal, proliferation and differentiation of CSCs by modulating their expression patterns. Since pathway analysis depicted that these CSC markers were governed in a pathway dependent manner, it was pre-requisite to analyse the gene expression of downstream markers - PTEN and ATM which showed an inverse correlation with the CSC and stemness markers in OSCC. These findings substantiated that CSC specific phenotype along with stemness markers and downstream molecules form an exclusive CSC mediated pathway which might have a plausible role in governing the OSCC malignancy.

Additionally, pathway analysis impelled us to investigate the regulatory network of miRNAs which might have a role in governing this CSC mediated pathway. Amongst these, downregulation of miR542-3p and miR34a emerged as the master regulators modulating the expression of CD44v6 and Nanog. Wang et al observed that miR542-3p not only directly targeted p53 considered to be the guardian of the genome but also led to cell growth and tumor formation in vivo [269]. Till date, downregulation of miR542-3p has been documented in colon, prostate, and lung cancers; however to the best of our knowledge this one of the few studies reporting the reduced expression of miR542-3p in OSCC and its plausible role in modulating CSC mediated pathway [270]. Moreover, miR34a, a direct transcriptional target of p53, has been reported to directly repress CD44 and inhibit prostate CSC by induction of apoptosis and proliferation arrest [271]. Collectively these observations strengthen our findings suggesting that since these miRNAs directly target CD44v6 there might be strong functional relevance of miR542-3p and miR34a in regulating self-renewal
of CSC phenotype and triggering CSC mediated pathway responsible for the progression of the disease. Bourguignon *et al* have already established that stemness marker Nanog and STAT signaling have an inevitable role in promoting miR21 in CD44 activated cells but our results also observed that miR21 upregulation was significantly correlated with PTEN depletion in OSCC patients, which might be attributed to the fact that PTEN has a target site of this known oncogenic miRNA on 3’ UTR region based on bioinformatic observations [256, 272]. Our study portrayed miR9 as a vital tumor suppressive miRNA which is reported to modulate JAK/STAT3 pathway via targeting IL6 in cervical cancer [273]. Our findings speculate that miR9 may modulate CSC pathway by impinging Nanog/STAT3 axis via IL6 however further evidences and in-depth studies specifically focusing on the role of miR9 in regulating this axis need to be conducted. Thus, these miRNAs having tumor suppressive or oncogenic properties demonstrated their ability to directly target vital CSC markers in a pathway dependent manner clinically which could utilized to establish newer CSC and miRNA specific targeted therapies in OSCC.

Recent evidences highlight the vital role of autocrine/paracrine signaling of cytokine networks in self renewal and maintenance of stemness property in CSC markers by generating receptor-ligand interactions [263]. The speculated impinging role of cytokines in regulating this CSC mediated pathway, prompted us to analyse the cytokine profiles of OSCC patients with higher expression of CD44v6 and Nanog. Our results depicted significantly higher expression levels of IL-6 and IL8 in OSCC patients which is in strong corroboration with previous findings and a strong association of these two cytokines with CD44v6, Nanog, miR542-3p and miR34a markers [274]. Based on these findings we can hypothesize that these cytokines might have a possible role in self renewal and maintaining stemness of CSC in a direct or indirect approach. Role of cytokines in governing CSC phenotype can be speculated in OSCC based on the simultaneous increase in the expression and a significant correlation between CSC markers/their epigenetic regulators with IL6 and IL8 levels and evidences of significant downregulation of miR9 that directly targets IL6. Sansone *et al* suggested self renewal of breast CSCs could be regulated by the cytokines that were secreted as a result of activation of Nanog/STAT3 complex [275]. These cytokines generate a positive feedback loop between immune cells and tumor cells which further stimulates the tumor stem cell components accelerating metastasis
and therapeutic resistance which might be stimulating CSC self renewal, stemness maintenance and therapeutic resistance [263].

Importantly, we observed that diverse anatomic locations in OSCC had a significantly different expression pattern of CSC, miRNA modulators and cytokine profiles. This contemplation was based on evidences - (i) Relatively increased expression of CD44v3 and CD44v6 (CSC markers) was observed in Buccal mucosa patients whereas predominance of Nanog (stemness marker) expression was found in tongue cancer patients, suggestive of the fact that buccal mucosa can be termed as "CSC driven cancer" and tongue subsite can be designated as "Stemness driven cancer". (ii) our study revealed that only buccal mucosa patients showed significant association with Bmi1 expression contradictory to the findings of He et al demonstrating Bmi1 as a pro-tumorigenic factor leading to migration and invasion in tongue carcinomas [276]. This discrepancy could be attributed to factors such as - expression being analyzed at protein level, consideration of Side Population as CSC like cells and utilization of different techniques. (iii) IL6 was relatively higher in Buccal mucosa patients compared to IL8 expression levels being relatively higher in tongue cancer patients. These observations are indicative of the fact that different subsites of OSCC behave as independent entities and function with diverse context dependent role which might be attributed to their different embryogenic origins as these markers have a role in maintenance of pluripotency in ESCs. Moreover, higher expression levels of CD44v6 in habituated patients compared to non-habituated patients suggested that Tobacco might be contributing towards acquisition of CSC like state and long term consumptions might have a great impact on the CSC expression levels. Our findings are consistent with the findings of Andrew et al reporting the critical role of nicotine in regulating CSC characteristics in-vitro [129]. However, significant downregulation of miR542-3p was observed in non-habituated patients compared to their habituated counterparts prompting towards epigenetic mechanism having a probable role in governing non-etiological OSCC malignancies.
KEY FINDINGS:

- CD44+ subpopulation was markedly distinct from other tumor population displaying increased CSC properties, enhanced in-vitro tumorigenic potential and drug resistance ability that could be explicated by their gene expression profiles.

- Our data highlights the importance of CD44v6 and its plausible role in tumor progression via CD44/Nanog/Bmi1/PTEN signaling pathway.

- In addition, we demonstrated the regulatory role of miR542-3p and miR34a in enhancing the cancer stem like characteristics and downstream activation of CD44 axis which significantly correlated with patient outcome.

- Consequently, these expression profiles can be used as prognostic biomarkers and may be a candidate target for developing therapeutic strategies to overcome OSCC.

- Further, we can envisage the impinging role of circulatory cytokines in governing CSCs in a pathway dependent manner; however in depth studies are pre-requisite to substantiate these findings.

**Published in and as:**