CD44^{high}CD24^{low} molecular signature determines the Cancer Stem Cell and EMT phenotype in Oral Squamous Cell Carcinoma

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ABSTRACT

Almost all epithelial tumours contain cancer stem-like cells, which possess a unique property of self-renewal and differentiation. In oral cancer, several biomarkers including cell surface molecules have been exploited for the identification of this highly tumorigenic population. Implicit is the role of CD44 in defining CSCs but CD24 is not well-explored. Here we show that CD44^{high}CD24^{low} cells isolated from the oral cancer cell lines, not only express stem cell related genes but also exhibit Epithelial-to-Mesenchymal transition (EMT) characteristics. This CD44^{high}CD24^{low} population gives rise to all other cell types upon differentiation. Typical Cancer Stem Cell (CSC) phenotypes like increased colony formation, sphere forming ability, migration and invasion were also confirmed in CD44^{high}CD24^{low} cells. Drug transporters were found to be over-expressed in CD44^{high}CD24^{low} subpopulation thereby contributing to elevated chemo-resistance. To validate our findings in-vivo, we determined the relative expression of CD44 and CD24 in clinical samples of OSCC patients. CD44 expression was consistently high whereas CD24 showed significantly lower expression in tumour tissues. Further, the gene expression profile of the CSC and non-CSC population unravels the molecular pathways which may contribute to stemness. We conclude that CD44^{high}CD24^{low} represents cancer stem-like cells in Oral Squamous Cell Carcinoma.

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1. Introduction

Oral Squamous Cell Carcinoma (OSCC) is one of the most prevalent subtype of Head and Neck Cancers worldwide afflicting >300,000 people annually, with ~150,000 deaths. (http://globocan.iarc.fr/Pages/fact_sheets_population.aspx) (Felthaus et al., 2011). The 5-year-survival rate for the disease has not improved through decades, despite advances in treatment modalities that predominantly include surgery and sometimes chemo-radiotherapy (Davis et al., 2010; Chikamatsu et al., 2012). The major cause of failure to cure OSCC includes resistance to therapy, recurrence and metastasis, both local and distant (Mitra et al., 2011). Most of this phenomenon is attributed to the diverse architecture of this tumour that consists of functionally heterogeneous lineages of cancer cells (Kreso and Dick, 2014; Sayed et al., 2011). In this context, role of Cancer Stem Cells, a self-renewing subpopulation of

tumour cells that sustain the long-term clonal growth of cancers, is evident (Magee et al., 2012; Clarke et al., 2006). The CSCs escape chemotherapy and are responsible for the recurrence of even more aggressive tumours (Valiyaveedan et al., 2015; Chen, 2009). This hypothesis has gained support in a variety of tumours in addition to cultured cancer cell lines that are shown to harbour CSC-like cells (Chaffer et al., 2013; Yeung et al., 2010; Iacopino et al., 2014). It is advantageous to use malignant cell lines as they are not contaminated with normal stem cells or cancer associated stroma and available in large numbers. Hence cell lines serve as a promising model for exploring the biology of CSCs in multiple cancers.

Defining CSC with specific markers has become difficult due to its frequent phenotypic transitions (Clevers, 2011; Shah et al., 2014). Various markers independently or in combination have been investigated to study CSCs from various tumours. These include a series of CSC-entity specifying molecules based on surface marker expression, drug transporters, enzymatic activity, signalling pathways and so on (Routray and Mohanty, 2014; Major et al., 2013). A couple of studies evaluated ALdh1 activity as a functional marker for isolation of CSC-like cells in OSCC (Zou et al., 2012). It was also found to be a relevant CSC marker in closely related Esophageal SCC along with Bmi1 and Nanog (Hwang et al., 2014). The "Side Population" analysis was equally effective in

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sorting CSCs based on their ability to efflux out Hoechst 33,342 dye (Yanamoto et al., 2011; Golebiewska et al., 2011). Also, CSCs could be simply enriched by growing the cancer cells in serum free media under non-adherent conditions (Chiou et al., 2008). Drug treatment at intermittent and incremental doses has also been shown to successfully expand the CSC population (Xu et al., 2015). In addition, activation of the EMT programme has increasingly been shown to generate CSCs (Ansieau, 2013). Nonetheless, the utility of surface markers, remained the most popular of all isolation and characterization strategies.

Prince et al. have proposed CD44$^+$ as the tumour-initiating stem cell in Head and Neck Cancers that could re-establish the original tumour heterogeneity (Prince et al., 2007). Besides, other markers like CD29,CD133, CD24, CD166, and EpCAM in different types of cancers are also used (Keysar and Jimeno, 2010; Zhang et al., 2009; Hurt et al., 2008). CD24, a small membrane glycoprotein is of considerable importance in breast cancer and has recently emerged as a major determinant of stemness in various cancer (Sheridan et al., 2006; Jaggupilli and Elkord, 2012). Although evidences in breast cancer firmly established CD44^{high}CD24^{low} cells to be stem-like, studies in HNSCC have not been conclusive. Here we revisit the prospects of CD44 and CD24 in defining the Cancer Stem Cell markers of OSCC.

In the present study, we have used a set of OSCC cell lines and isolated the putative stem cell fraction (CD44^{high}CD24^{low}) along with CD44^{high}CD24^{high} and CD44^{low}CD24^{high} (non-stem) sub-populations. These populations were subjected to various molecular and cellular assays to determine whether CD24 has any relevant contribution in the context of CD44^{high} population towards defining CSC marker. We have clearly demonstrated CD44^{high}CD24^{low} cells as CSC-like cells within oral cancers.

2. Methods

2.1. Cell culture

Human OSCC cell lines, UPCI: SCC131, UPCI: SCC084 and UPCI: SCC036 were purchased from University of Pittsburg, USA (Table S5) (White et al., 2007). These were cultured in DMEM medium supplemented with 10% foetal calf serum, 1% Pen Strep and 0.006% Gentamicin (Life Technologies, Thermo Fisher Scientific Inc., MA USA). SCC25 was obtained from American Type Culture Collection and cultured in DMEM-F12 supplemented with 10% foetal bovine serum (FBS) and 400 ng/ml Hydrocortisone at 37 °C in the presence of 5% CO2.

2.2. MACS and flow cytometry

Magnetic Assisted Cell Sorting was used for the sequential separation of CD24^{low} followed by CD44^{high} population. The detailed protocol is provided in Supplementary material. The MACS sorted cell populations were tested for percentage purification using flow cytometry. About 1×10^6 cells were re-suspended in PBS containing 1% FBS and 0.02% sodium azide and double stained with CD44-PE and CD24-FITC (BD Pharmingen, San Jose, CA). Cytometry was performed on the BD LSRFortessa and the data was analysed using BD FACSDiva 6.2 software. Unstained cells were used to set up the size gate and avoid debris and clumps. Isotype controls were kept to eliminate non-specific staining. For differentiation assay, the CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cells isolated from SCC25 were followed for 3 weeks and subjected to FACS analysis at every week. Unsorted SCC25 were kept as control to normalize the staining intensity of CD44 and CD24. SCC25 was treated with 5-FU for 72 h and CD44/CD24 status was assessed for the enrichment of stem-like population upon drug exposure.

2.3. Real time PCR analysis

Total RNA was extracted from the sorted populations using Trizol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA

using Verso cDNA synthesis kit (Thermo Fisher Scientific Inc., MD). Real time PCR was carried out using start universal SYBR Green master mix (Roche, Basel, Switzerland) on the 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA). Data were normalized with β-actin. The list of primers and its sequence are given in Supplementary File S1.

2.4. Western blot

Cells were lysed in NP-40 lysis buffer (Invitrogen, Carlsbad, CA) supplemented with 1× protease inhibitor cocktail. Clear cell lysates were measured with Bradford reagent (Sigma-Aldrich, St. Louis, MO). Equivalent amounts of proteins were separated using SDS-PAGE (8%-10%), transferred on to PVDF membrane (Millipore, Billerica,) and immunoblotted with primary antibodies; polyclonal E-cadherin, Involucrin, CD44, CD24 (Santa Cruz Biotechnology, CA, USA), polyclonal Oct4, Nanog and Sox2 (Abcam, Cambridge, UK), polyclonal C-myc (Cell Signalling Technology, Beverly, MA, USA) followed by HRP-conjugated secondary antibody (Sigma). Bands were visualized using enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA).

2.5. Immunofluorescence

The sorted populations of SCC25 were stained with EMT markers for confocal microscopy. The Protocol is given in supplementary in details.

2.6. Sphere forming assay

MACS sorted cell populations were cultured overnight to eliminate dead cells. Next day, 5000 cells/ml per well of low adherent 6-well plate were plated in DMEM-F-12 serum free media reconstituted with 1% B27 supplement, 20 ng/ml of Epidermal Growth factor and 20 ng/ml of Basic Fibroblast Growth Factor (Invitrogen). Medium was added every 2–3 days. Formation of sphere-like structures was visible at 4–7 days and the photographs of comparable groups were captured under phase-contrast microscope (Leica CTR4000) at 20× magnification at 7–14 days. All experiments were done in triplicate.

2.7. Clonogeneic assay

In brief, 1000 cells were seeded on 6 cm dishes and cultured for 7–10 days with medium change every 2–3 days. The colonies were stained with 0.2% methylene blue for 30 min, washed with PBS to remove the extra stain and images were taken. Colonies for three independent experiments were counted.

2.8. Monolayer wound healing assay

Different populations of UPCI: SCC131 cell lines were plated at a density of 1 million cells per well of 6-well plate and allowed to form a confluent monolayer. A scratch was then made with a thin edge of a 200 microtip. Photographs were taken under phase contrast microscope (Olympus 1 × 51, camera Jenoptik) at 0 h and after 16–18 h at 10× magnification. The distance was measured at both the time points, to assess the migration of cells from the wounded regions. Percent distance migrated were calculated for three independent experiments.

2.9. Matrigel invasion assay

The upper chamber of the Matrigel coated trans-well cell culture inserts (BD Biosciences, SanDiego, CA) were seeded with cells at a density of 0.5×10^6 per well in serum free DMEM. The lower chamber was layered with 0.75 ml of DMEM with 10% FBS to serve as a chemo-attractant. After incubating for 36–48 h, the cells on both the upper and the underside of the tranwell inserts were fixed with 3.7% formaldehyde, permeabilised with methanol and stained with Giemsa (sigma).The

cells in the upper chamber were removed with the cotton swab and the invaded cells on the underside were photographed. Images were acquired using bright field microscope (Leica DM3000, Germany) at 10× magnification. Three independent fields were analysed using ImageJ software. The percentage of cells that invaded through matrigel matrix was calculated.

2.10. Hoechst dye exclusion assay

The CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cell populations of UPCI: SCC131 were resuspended in DMEM medium containing 2% FCS. Five micrograms of Hoescht 33342 dye (Invitrogen) was added per 10^6 cells/ml and incubated at 37 °C in dark with intermittent shaking for 90 min. ABC transporter inhibitor, Verapimil (50 μM, Sigma) was used to block Hoechst efflux to set the "Hoechst low" gate. Stained cells were washed and resuspended in ice-cold PBS containing 2 mg/ml of Propidium Iodide. Flow cytometry was performed on BD LSRFortessa

(Becton-Dickinson) with UV excitation of 355 nm and the emissions were captured at 450/50 nm (Hoechst Blue) and 675/50 nm (Hoechst Red). Gating strategy ensured analysis of only live (PI excluded) and single (doublets excluded) cells. From the remaining cells, the least fluorescent population was termed "Side Population" and the percentage was determined.

2.11. Immunohistochemistry

Matched oral tumour (n = 10) and normal (n = 10) tissues along with 15 dysplastic tissues were obtained from the hospital section, Chittaranjan National Cancer Institute (CNCI), Kolkata, India. Prior to sample collection, written informed consent was taken from each individual and approved by the Research Ethics Committee of CNCI. The pathological history of the tumours is provided in Supplementary File S2. CD44 and CD24 were stained according to the standard protocol.
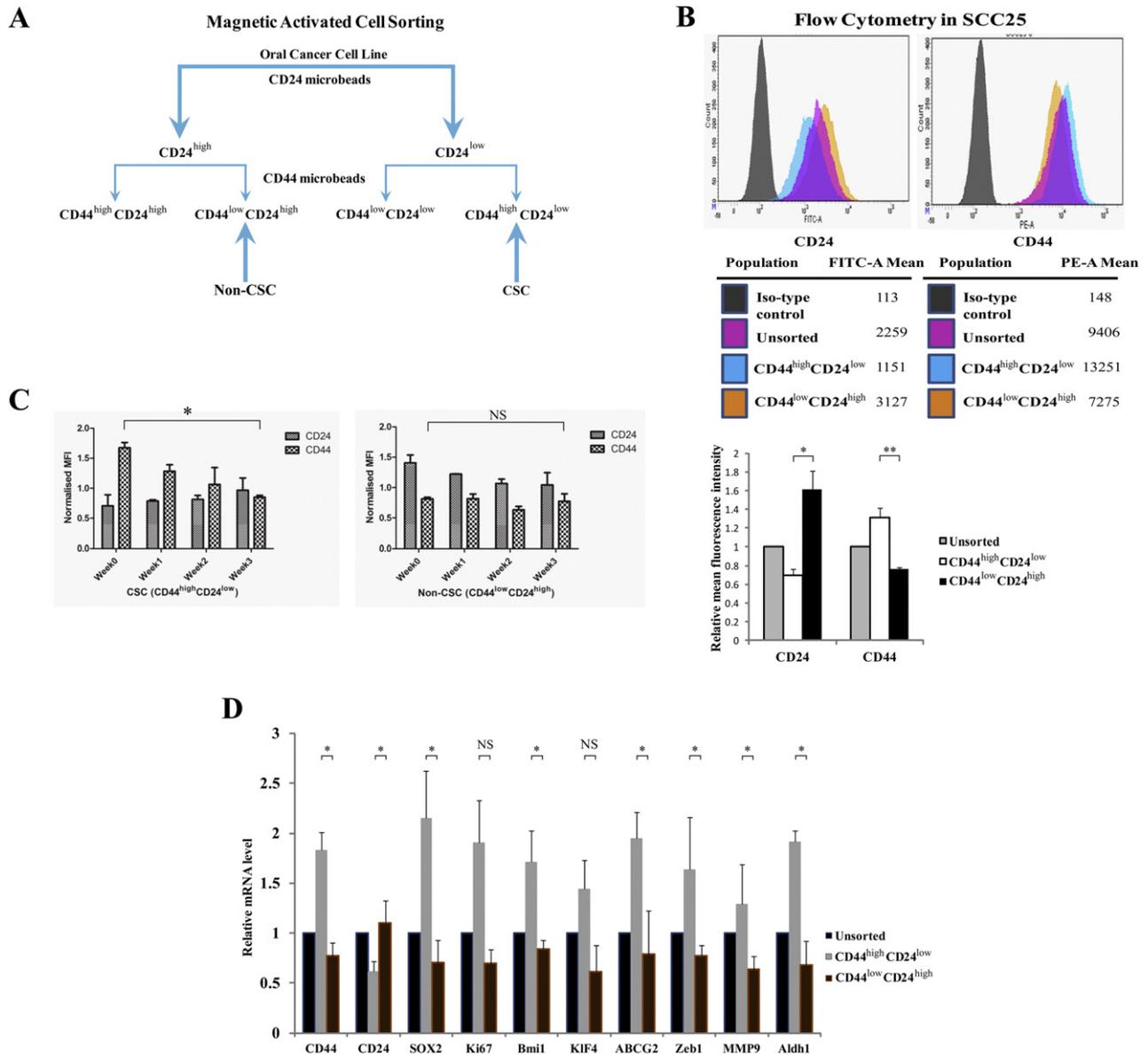

Fig. 1. Isolation of the putative CSC population (CD44^{high}CD24^{low}) using MACS and characterization of their stemness properties. (A) Flowchart of magnetic bead based sequential separation. (B) Each of the enriched cell population of SCC25 cell line was analysed by flow cytometry and the histograms indicated by colour codes. Mean fluorescence intensity (MFI) values of CD44 (PE) and CD24 (FITC) are given. Image is representative of 3 independent experiments and presented graphically with mean ± SD. (C) Relative MFI of CD44 and CD24 in the stem and the non-stem population of SCC25 cells subjected to FACS analysis every week. (D) qRT-PCR quantification of stem cell related genes in various sub population of SCC25 using specific primers (Supplementary File S1). Relative expression values were normalized to those of 18srRNA or β-actin. Data shown is average ± SD for three independent experiments. (* denotes p-value < 0.05, ** means p-value < 0.01, *** means p-value < 0.001).
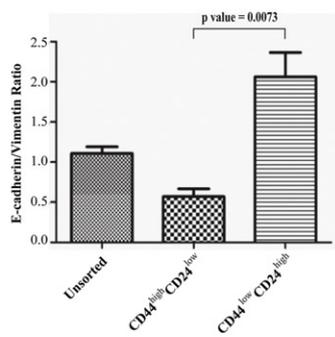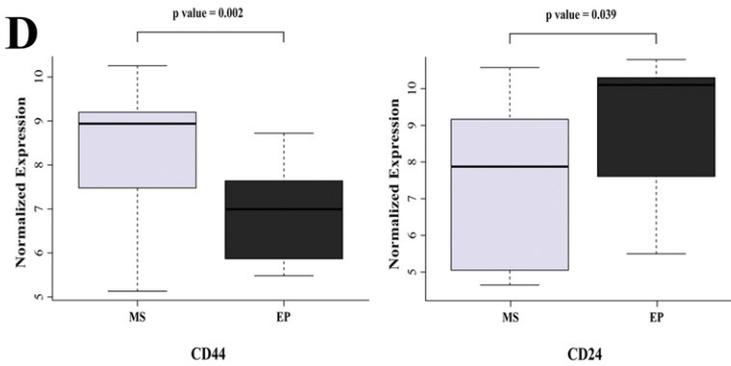
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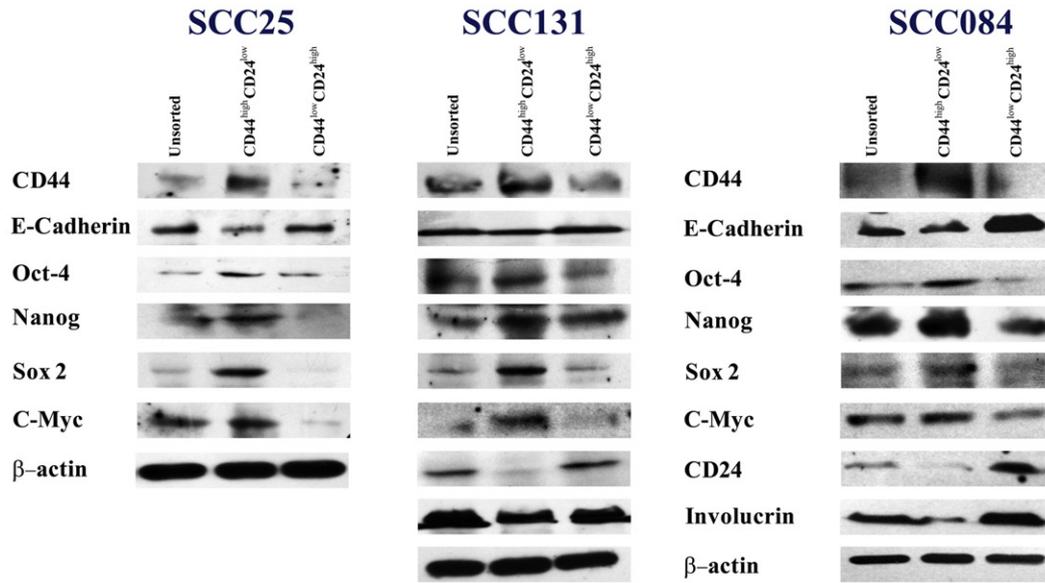
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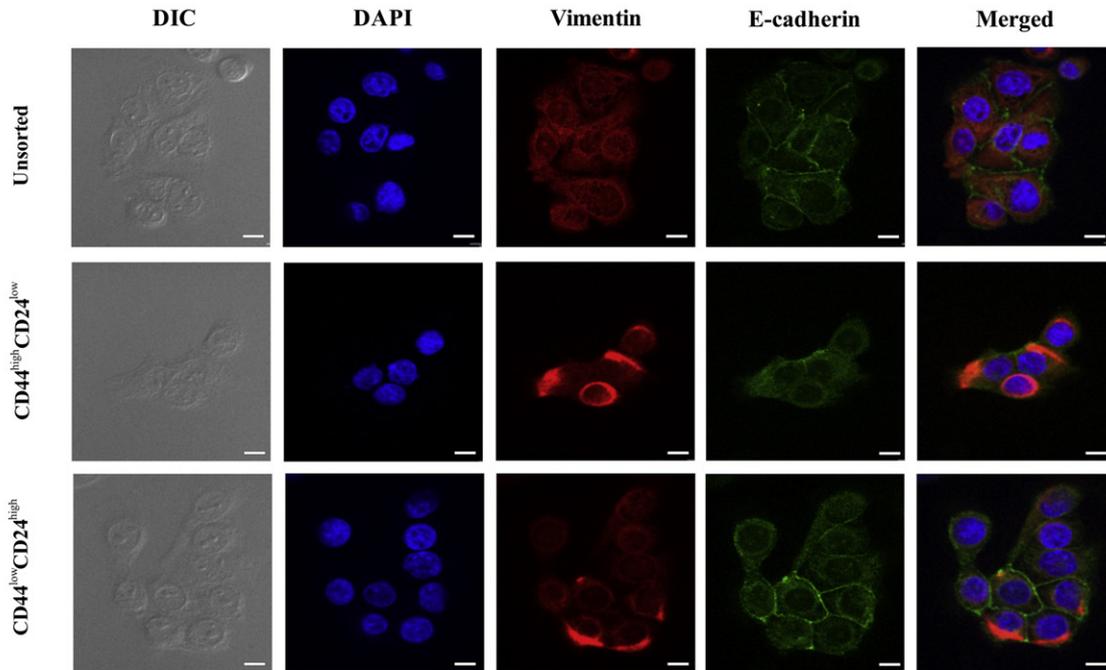
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Detailed Immunohistochemistry (IHC) protocol is given in supplementary material.

2.12. Microarray

Total RNA was extracted from the different fractions of SCC25 using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) using manufacturer's protocol. From the isolated RNA the mRNA was converted to biotinylated cRNAs and hybridized on Illumina HT12 V4.0 bead chip whole genome array (Illumina, San Diego, CA). The Illumina bioarrays were read in an Illumina iScan Reader (Illumina) and the primary intensity data were obtained in standard file format, using Genome Studio software. Raw data were pre-processed in R bioconductor lumi package (https://www.bioconductor.org/packages/release/bioc/html/lumi.html). The robust spline method was used for normalization. Using Benjamini and Hochberg method, the p-values were corrected for multiple testing. Genes with FC > 1.5 or FC < 1.5 were considered to be differentially expressed between different samples. These were subjected to Ingenuity Pathway Analysis (IPA; https://analysis.ingenuity.com).

2.13. Statistical analysis

For IHC, p-value was calculated using Mann–Whitney's U Test between median expression values. Survival curve was plotted using R package with significance calculated as Log Rank Test between two curves. Oncomine 4.4 research edition database (http://www.oncomine.org/resource/login.html) was used for the analysis. An independent two-tailed Student's t test was performed to determine the significance of difference between two experiments.

3. Results

3.1. Isolation and characterization of cancer stem-like cells from Oral Squamous Cell Carcinoma cell lines

A number of reports suggest that cultured human cancer cell lines contain various cellular subpopulations including CSCs that can be easily purified for different molecular analyses (Singh et al., 2003). In the present study, we subjected a number of oral cancer cell lines such as SCC25, UPCI-SCC131, UPCI-SCC084 to the isolation of CSC-like cell population based on CD44 and CD24 markers using Magnetic Assisted Cell Sorting. Accordingly, we identified three subpopulations CD44^{high}CD24^{low}, CD44^{low}CD24^{high}, and CD44^{high}CD24^{high} from these cell lines (Fig. 1A). The purity of these cell types was confirmed by flow cytometry in both SCC25 (Fig. 1B) and UPCI: SCC131 (Fig. S1A).

Each parental cell line showed strong CD44 expression; hence the purified CD44^{high} population was quite enriched whereas CD24 fold reduction was optimum (Fig. 1B, S1B). The sorted subpopulations were relatively enriched fractions and not absolutely pure. The different phenotypic cell types were studied within 4–5 days of isolation since they are known to change their characteristic properties to maintain equilibrium proportions of various subpopulations in adherent culture (Gupta et al., 2011). This was evident from the dynamic change in CD44 and CD24 profile upon prolonged culture that demonstrates the ability of CD44^{high}CD24^{low} cells to spontaneously propagate all other cell types (Fig. 1C). No such change was observed in CD44^{low}CD24^{high} cells (Fig. 1C). Nevertheless, only the freshly isolated CD44^{high}CD24^{low} cells were compared with the CD24^{high} cells in the subsequent experiments. Real Time PCR analysis showed significant up-regulation of various

stem cell markers like Sox2, Bmi1, C-myc in the CD44^{high}CD24^{low} population, although Klf4 expression was not statistically significant (Fig. 1D). We also checked Aldh1 expression at mRNA level and found it to be higher in CD44^{high}CD24^{low} population emphasizing enrichment of CSC population (Fig. 1D, S1C). In addition, EMT drivers like ZEB1, ATP binding Casette (ABC) transporters like ABCG2 and metastasis marker MMP9 were also remarkably higher in the CD44^{high}CD24^{low} compared with the CD44^{low}CD24^{high} cell populations. However, expression of Ki67, a proliferation marker was not significantly up-regulated in CD44^{high}CD24^{low} fraction (Fig. 1D). These results suggest that CD44^{high}CD24^{low} subpopulation in these cell lines probably possess the CSC-like property.

3.2. Acquisition of stemness and EMT phenotype in CD44^{high}CD24^{low} population

We next investigated the expression of well-known stem-related markers such as Oct4, Nanog, Sox2, C-myc at the protein level. Western blot analysis showed significantly higher expression of these genes along with depletion of differentiation marker Involucrin in the isolated CD44^{high}CD24^{low} population of all the cell lines compared with the CD44^{low}CD24^{high} ones (Fig. 2A, S1D). Moreover, we observed loss of E-cadherin, perhaps indicating EMT in concordance with increased stemness (Fig. 2A). Confocal microscopy also showed that E-cadherin to Vimentin ratio was significantly lowered in CD44^{high}CD24^{low} stem cell population in comparison to the CD44^{low}CD24^{high} non-stem cell counterparts substantiating the fact that CSC-like property coexists with EMT phenomenon (Fig. 2B, 2C). We also extrapolated our observation in Gene expression data sets of NCI-60 cell lines obtained from gene array analysis GC34162 at the developmental therapeutics programme of the NCI; http://dtp.nci.nih.gov (Scherf et al., 2000) (see supplementary methods). Differential expression of CD44 and CD24 were determined between epithelial and mesenchymal group of NCI-60 cell lines that were previously categorized on the basis of E-Cadherin/Vimentin expression ratio (Park et al., 2008). Interestingly CD44 was significantly higher and CD24 significantly lower in the mesenchymal set compared to the epithelial cell lines (Fig. 2D). These results confirmed that the CD44^{high}CD24^{low} population exhibits enhanced stem cell characteristics in conjunction with EMT.

3.3. Self-renewal, migratory and invasive properties of CD44^{high}CD24^{low} population

We assessed the stem like property of CD44^{high}CD24^{low} cell type by their intrinsic sphere-forming ability and indeed found the spheres to be greater in size and number than their non-stem cell counterparts (CD44^{low}CD24^{high}) in various cell lines (Fig. 3A, S2A). UPCI: SCC131 cell line was further explored to study the characteristics of the CD44^{high}CD24^{low} cell type. Colony forming ability of this population was higher indicative of their clonogenic potential (Fig. 3B, C). Also their morphology appeared to be mesenchymal-like, showing enhanced cellular spreading which comprise the initial steps of EMT process (Fig. 3D). Moreover, these cancer stem-like cells formed soft-agar colonies indicating anchorage independent growth ability of these cells (Fig. S2B). However, the proliferation rate of this subpopulation was not significantly different from the other population (Fig. S2C).

Wound healing assay was performed on all the isolated populations of UPCI: SCC131 cell lines to assess the motility of these cell types.

Fig. 2. Expression of Intracellular stem cell and EMT markers in CD44^{high}CD24^{low} cells. (A) Representative western blot images of CD44, CD24 and relative levels of Oct-4, Nanog, Sox2,C-myc, Involucrin and E-cadherin in the isolated populations of SCC25, UPCI: SCC131 and UPCI: SCC084 cell lines. Data has been normalized with β-actin. (B) Confocal immunofluorescence microscopy of E-cadherin (green) and Vimentin (red) counterstained with DAPI (blue) in SCC25 sorted populations. Images were taken at 60× magnification with scale bar equal to 10 μm. (C) Quantification of the E-cadherin to Vimentin ratio in each cell types with p-value indicated. (D) Box plots showing significant down-regulation of CD24 and up-regulation of CD44 in mesenchymal group compared with the epithelial group of NCI-60 cell lines. DAPI: 4′,6-diamidino-2-phenylindole.
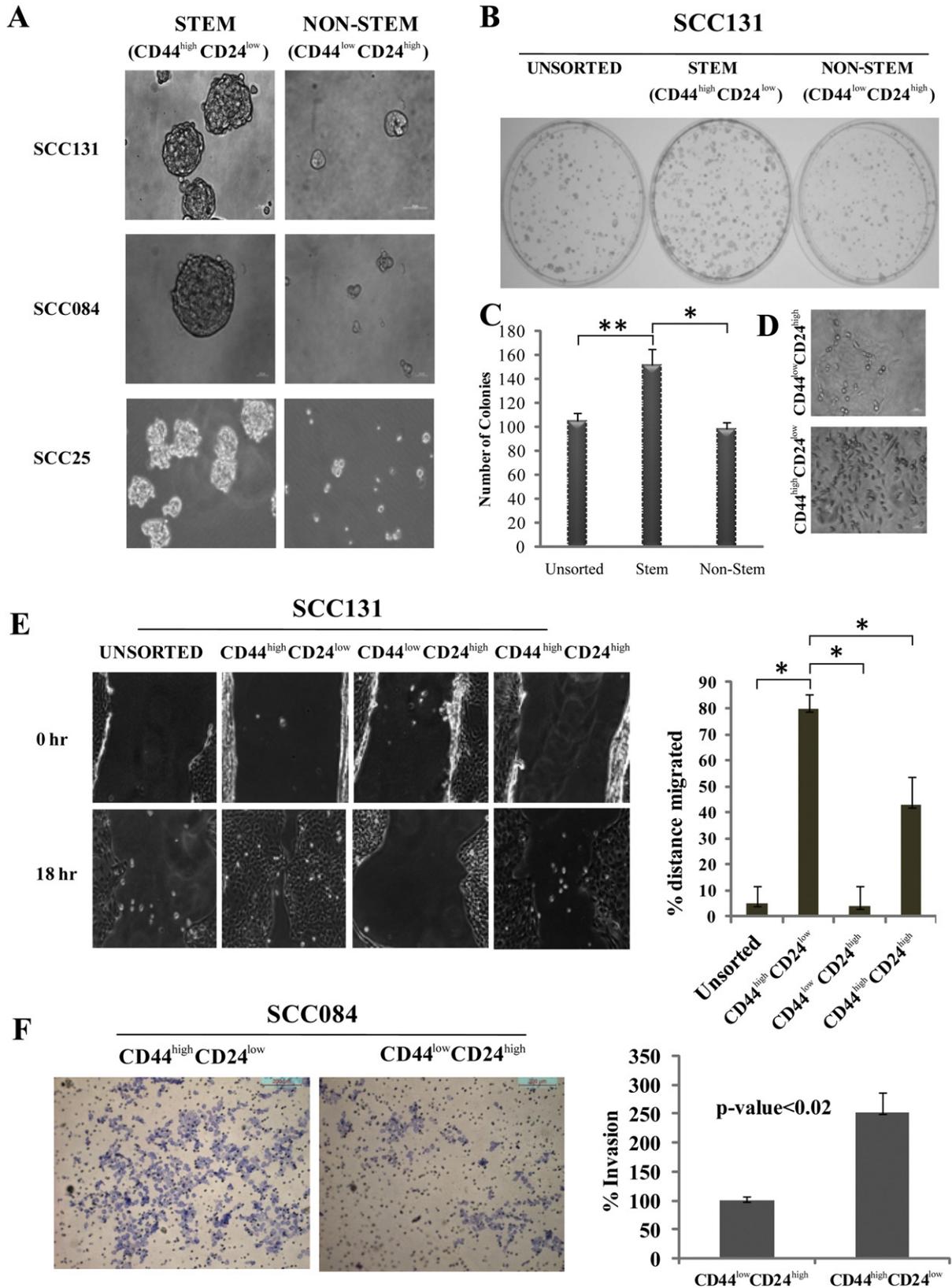
Fig. 3. Sphere forming ability, clonogenicity, migration and invasion of the CD44^{high}CD24^{low} cells. (A) Representative images of spheroids originated from CD44^{high}CD24^{low} and CD44^{low}CD24^{high} sub-population in each cell line. (B) Plate colony formation assay for the respective population of SCC131 with number of colonies counted for 3 independent experiments and plotted as average $\pm$ SD (C). (D) Colony morphology was found to be quite distinct in the two populations. (E) An in-vitro migration assay on the isolated fractions of SCC131 showing representative images at 0 h and 18 h of the scratch being made. The % distance covered after the wound healing has been graphically shown (right) as a mean of 3 independent values. (F) Cells invaded from the CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cell populations of SCC084 to the lower chamber of the insert were counted. Bright field images of invasion assay has been shown (left) and % invasion graphically represented.

The distance covered by the cells after the scratch, was highest in the CD44^{high}CD24^{low} cells and least in the CD44^{low}CD24^{high} cells. Interestingly, it was even relatively less in CD44^{high}CD24^{high} cells implying the contribution of CD24 negativity to the migratory abilities of CD44^{high} cancer cells (Fig. 3E). Matrigel Invasion assay was also performed on UPCI: SCC084 derived CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cell populations using trans-well inserts. The number of invading cells in CD44^{high}CD24^{low} was 2.5 fold higher than the CD44^{low}CD24^{high} cell type (Fig. 3F). Thus, the various cellular assays confirmed the stem-like features of CD44^{high}CD24^{low} cell type in comparison with the CD44^{low}CD24^{high} cell type in Oral cancer cell lines.

3.4. CD44^{high}CD24^{low} cells show enhanced chemo-resistance

We next investigated the chemo-resistance property of CD44^{high}CD24^{low} cells. It is known that the quiescent and slow-cycling stem cell population frequently evade drug or radiation therapy than actively dividing cancer cells (Pattabiraman and Weinberg, 2014; Dean et al., 2005). Towards this, we analysed the cell cycle status of the CSC and non-CSC population of SCC25 and found remarkable difference in G0/G1, S as well as G2/M phase cells (Fig. 4A). Upon synchronization, CD44^{high}CD24^{low} cells appear to progress slower than the CD44^{low}CD24^{high} ones (Fig. S3A). One of the mechanisms of enhanced chemo-resistance is their ability to pump out the excess drug with the help of P-glycoprotein or ABCG2. We, therefore, stained the live cell population with the Hoechst dye at a saturated dose and then compared the percentage of Hoechst^{low} cells in the CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cells. The Hoechst^{low} population is referred to as "Side Population" cells (SP phenotype) and considered as Cancer stem-like cells in various cancers (Golebiewska et al., 2011).We indeed observed that a higher proportion of CD44^{high}CD24^{low} population retained the SP phenotype (Fig. 4B).

Next, we determined the effect of drug on these two distinct populations. Cisplatin and 5-Flourouracil are two of the most popular drug regimens for the treatment of oral cancers (Andreadis et al., 2003). Studies have proved that existence of CSC-like features are responsible for the intrinsic drug resistance character in oral cancer (Valiyaveedan et al., 2015). WST-1 assay in the presence of different concentration of 5-Flourouracil confirmed that CD44^{high}CD24^{low} cells were significantly chemo-resistant than the CD44^{low}CD24^{high} subpopulation in SCC25, UPCI: SCC131 and UPCI: SCC084 cell lines (Fig. 4C and D). Besides, 5-FU treatment of the bulk cells led to the significant enrichment of CD44^{high}CD24^{low} population, suggesting survival of the resistant CSC population (Fig. 4E). However, since the difference in percentage survival was quite low, we tried to determine IC-50 values of 5-FU treatment for 72 h and found these cell lines to be inherently resistant (Fig. S3B). But, it was noteworthy that the resistance of SCC cell lines was directly proportional to the fraction of CD44^{high}CD24^{low} cells present in its unsorted population (SCC084 > SCC25 > SCC131) (Fig. S3B). We next, performed the similar assay with etoposide and consequently found notable difference in viability between the CSC and the non-CSC population of SCC131, SCC25 and SCC084 cells (Fig. 4F). Apoptotic analysis in SCC25 also showed that CD44^{high}CD24^{low} cells are more resistant to 5-FU treatment than the CD44^{low}CD24^{high} cells (Fig. S4A). Overall, the above results confirm that the CD44^{high}CD24^{low} cells are the drug-resistant population of the bulk tumour cells.

3.5. CD44^{high}CD24^{high} represents an intermediate population between CSC and Non-CSC

We then evaluated the role of CD24 in the background of high CD44 expression, whether or how it contributes to stem-like features. We used UPCI: SCC084 cell line to isolate the CD44^{high}CD24^{high} cell type and compared them with the putative stem cell counterpart (CD44^{high}CD24^{low})(Fig. 5A). It is notable that expression of Oct4, Sox2 and C-myc was relatively higher in the CD24^{low} population for both

UPCI: SCC131 and UPCI: SCC084 (Fig. 5B). Involucrin was moderately increased in CD44^{high}CD24^{high} population suggesting the onset of differentiation. As shown in Fig. 3E, migration of CD44^{high}CD24^{high} cell type in SCC131 was relatively less in comparison with CD44^{high}CD24^{low} implying CD24 in cellular motility indicative of EMT. This is further supported by inverse correlation of E-cadherin and β-catenin in CD24^{high} and CD24^{low} cells (Fig. 5B). Previous studies have shown activation of Wnt signalling pathway particularly in response to loss of E-cadherin that lead to Cancer Stem Cell expansion and maintenance (Su et al., 2015; Huang et al., 2015). All these data reasonably point to the fact that CD44^{high}CD24^{high} cells exhibit traits which are transitional between the stem-like CD44^{high}CD24^{low} and the non-stem CD44^{low}CD24^{high} cells.

### 3.6. Primary oral tumour tissues showed correlation of CSC frequency with tumour progression

To obtain an in vivo relevance of the observation we analysed the expression of CD44 and CD24 in primary oral tumour specimens. Previous studies in both oral and breast cancer have associated CD44 expression with stem cell phenotype but for CD24, expression has been quite heterogenous and found to be more in differentiated areas in breast cancer specimens (Park et al., 2010). In the present study conducted for oral cancer, CD44 immuno-localisation was preferably membranous during progression from normal epithelium to HNSCC, while that of CD24 was more nuclear-oriented with slightly membrano-cytoplasmic (Fig. 6A). Only membrane staining was considered for scoring CD44 and CD24 expression in all the samples. For the adjacent normal tissue samples, positive cells were counted only in basal/parabasal layers, as the highly proliferative cells therein, could only develop tumour. We found high expression of both CD44 and CD24 along the basal lining of the normal epithelium for 80% of the patients, gradually diminishing across the upper third layer of epithelial cells. However, with the advent of dysplasia, CD24 membrane intensity moderately decreased and was almost absent in the tumour (p < 0.001). On the other hand, CD44 staining score was constantly high and irregularly distributed in the OSCC tumour sections not varying much with tumour grade (Fig. 6B). From microscopic analysis of same sections, the CD44^{high} cells that showed low CD24 expression were determined to calculate CD44^{high}CD24^{low} cell numbers. We found its prevalence ranging from 10% in normal to 30% in dysplasia and 47% in tumour tissues implying higher CSC frequency with progression of the disease. Kaplan–Meier analysis did not reflect any significant co-relation between CD24^{low} status and the overall survival (Fig. 6C). This may be due to small sample size as only 4 samples showed high CD24 staining. In conclusion, we found patient's data to co-relate with the cell line observation in establishing CD44^{high}CD24^{low} as a putative CSC population in OSCC.

3.7. Gene expression profiles of OSCC derived CD44^{high}CD24^{low} cells

In our attempt to probe into the CSC-specific gene signature of CD44^{high}CD24^{low} cells, which may contribute to their stemness property, we performed whole-transcriptome based profiling of SCC25 cell line along with CD44^{high}CD24^{low} and CD44^{low}CD24^{high} subpopulations. We observed differential expression of 474 genes with ≥1.5 fold change between the CD44^{high}CD24^{low} cell and CD44^{low}CD24^{high} cell types (Supplementary File S3). Heatmap was generated using normalized gene expression values for only 108 genes with ≥2 fold change (Fig. 7A). Unsorted bulk SCC25 was more close to the non-stem cell gene signature. Among the most up-regulated genes, there were inflammation associated calcium binding proteins, S100A8, S100A9 whose upstream regulators like TNF-α, TGFβ1 and IL-8 could be involved in tumour progression and metastasis (Supplementary File S4). These factors are known to be secreted by cancer associated stroma cells which also stimulates angiogenesis. We indeed found changes in stem cell related genes like that of ALDH family proteins, differentiation associated

genes like Keratins, KRTDAP, KLK6 together with EMT related genes (SERPINB1, BGN, KLF9, CXCL10, JUNB, CTNNBIP1) (Tseng et al., 2009). Others include STAT1, TIMP1, IFIT1, OAS1, FGFR3, etc. that may act as

effectors in various oncogenic signalling cascades (Dubrovska et al., 2009). Interestingly, these genes were also discovered to be up-regulated in 12 HNSCC studies as analysed in Oncomine (Supplementary
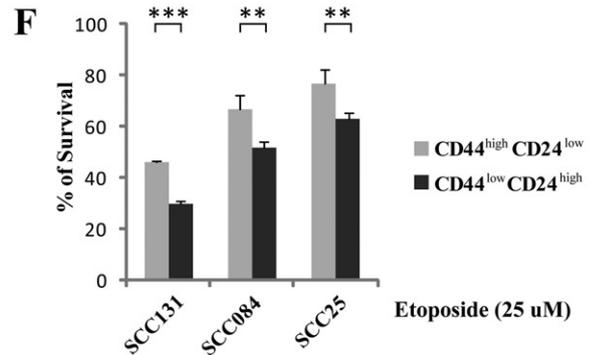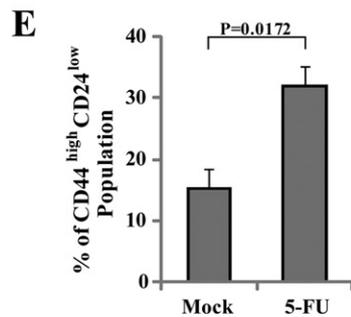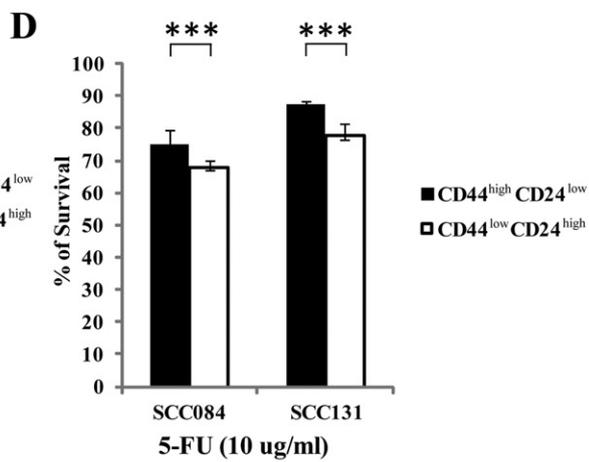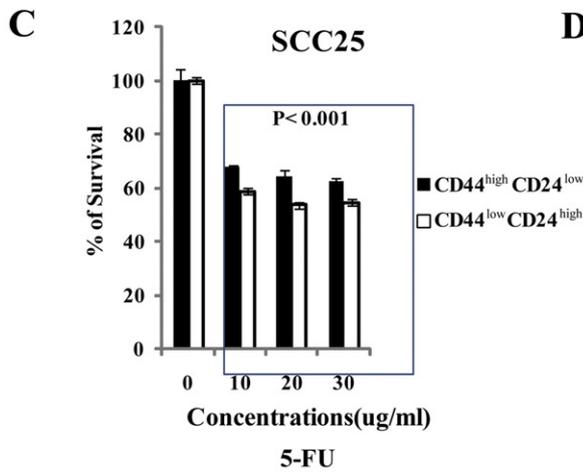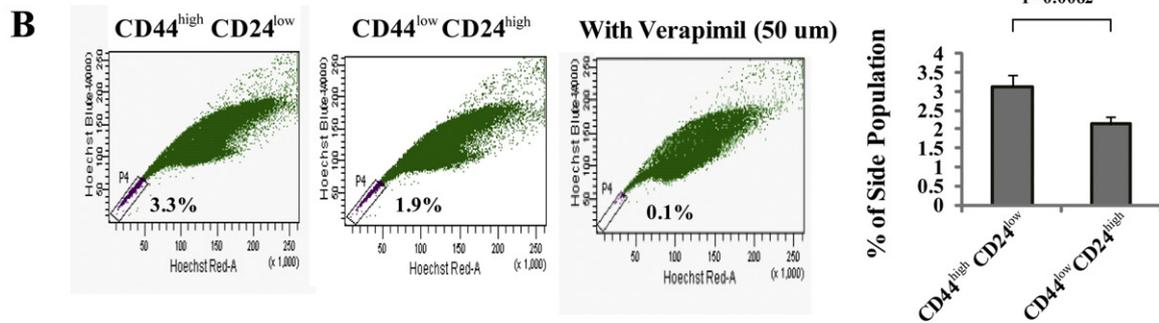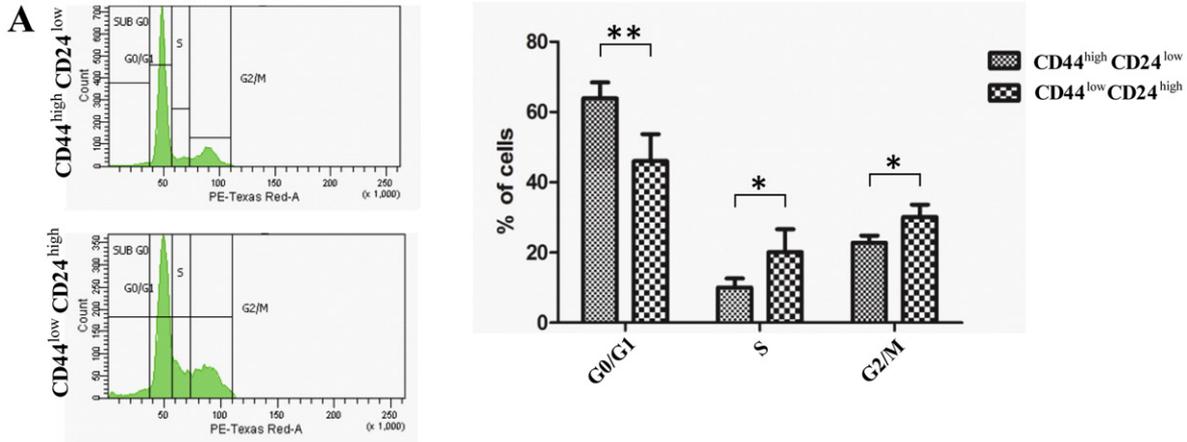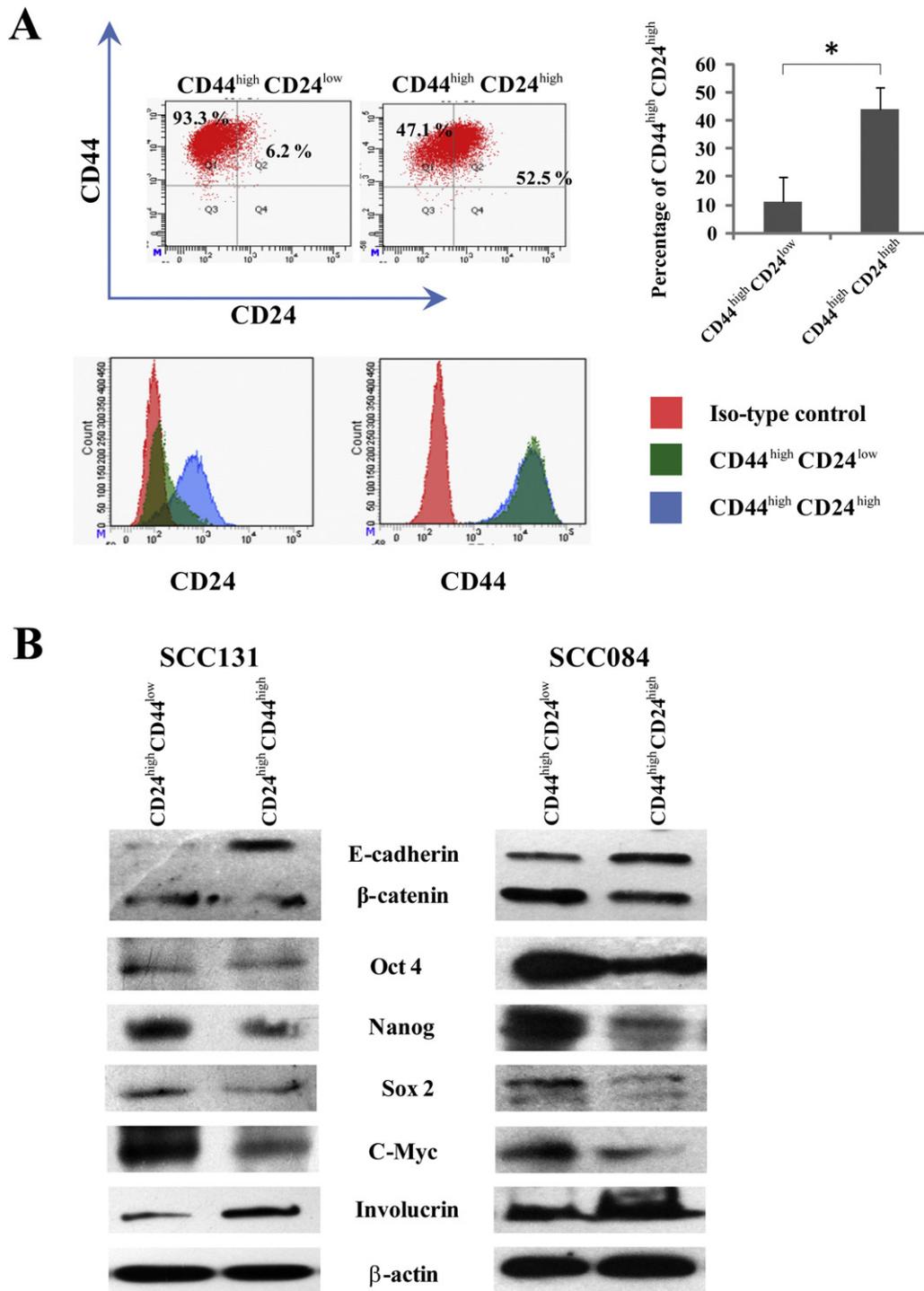
Fig. 5. CD44^{high}CD24^{low} are more stem-like than CD44^{high}CD24^{high} cells. (A) Representative FACS analysis in UPCI: SCC084 OSCC cell line. CD44^{high}CD24^{high} subpopulation is ~6%, and 52.5% in the purified CD44^{high}CD24^{low} and CD44^{high}CD24^{high} cell types. The graph shows mean of the percent of CD44^{high}CD24^{high} with ± SD of three independent sorting experiments. (B) Representative western blot images of Oct-4, Nanog, Sox2, Involucrin along with E-cadherin/β-catenin in the respective cell populations.

File S5). Down-regulated genes, however, were few such as CLDN1, DKK1, THBS1, CAV1, CDH2, AXL, CLDN1 etc. having diverse roles in cellular adhesion as well as Wnt signalling. Some of the genes like CDK6, SKP2 etc. were involved in cell cycle. To specifically predict the possible pathways involved in CSC progression, the differentially expressed 474 genes were subjected to Ingenuity Pathway Analysis. Functional Clustering of

Fig. 4. Drug sensitivity of CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cells. (A) CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cell populations of SCC25 were analysed for their cell cycle profile and the % population in each phase graphically represented. (B) Sorted population of UPCI: SCC131 was stained with Hoechst dye and side population analysed by flow cytometry. Mean Percent ± SD of Hoechst^{low} cells were plotted for three independent experiments. Gating was ensured with ABCG2 inhibitor, Verapimil (50 μm). (C) MACS sorted cells from SCC25 were exposed to 5-FlouroUracil (5-FU) at increasing concentrations for 72 h, followed by WST-1 Cell Viability Assay. Differences in Percent survival between CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cells were calculated. All experiments were performed in triplicate and data shown as mean ± SD. Data in inset show statistical significance at p < 0.001 for all concentrations. (D). Percent survival for UPCI: SCC131 and UPCI: SCC084 cell populations at 10 μg/ml 5-FU. (E) Percentage of CD44^{high}CD24^{low} population as determined by FACS, after 5-FU treatment in SCC25 cells. (F) % Survival of stem and the non-stem population in UPCI: SCC131, UPCI: SCC084 and SCC25 cell populations upon etoposide treatment (25 μM).
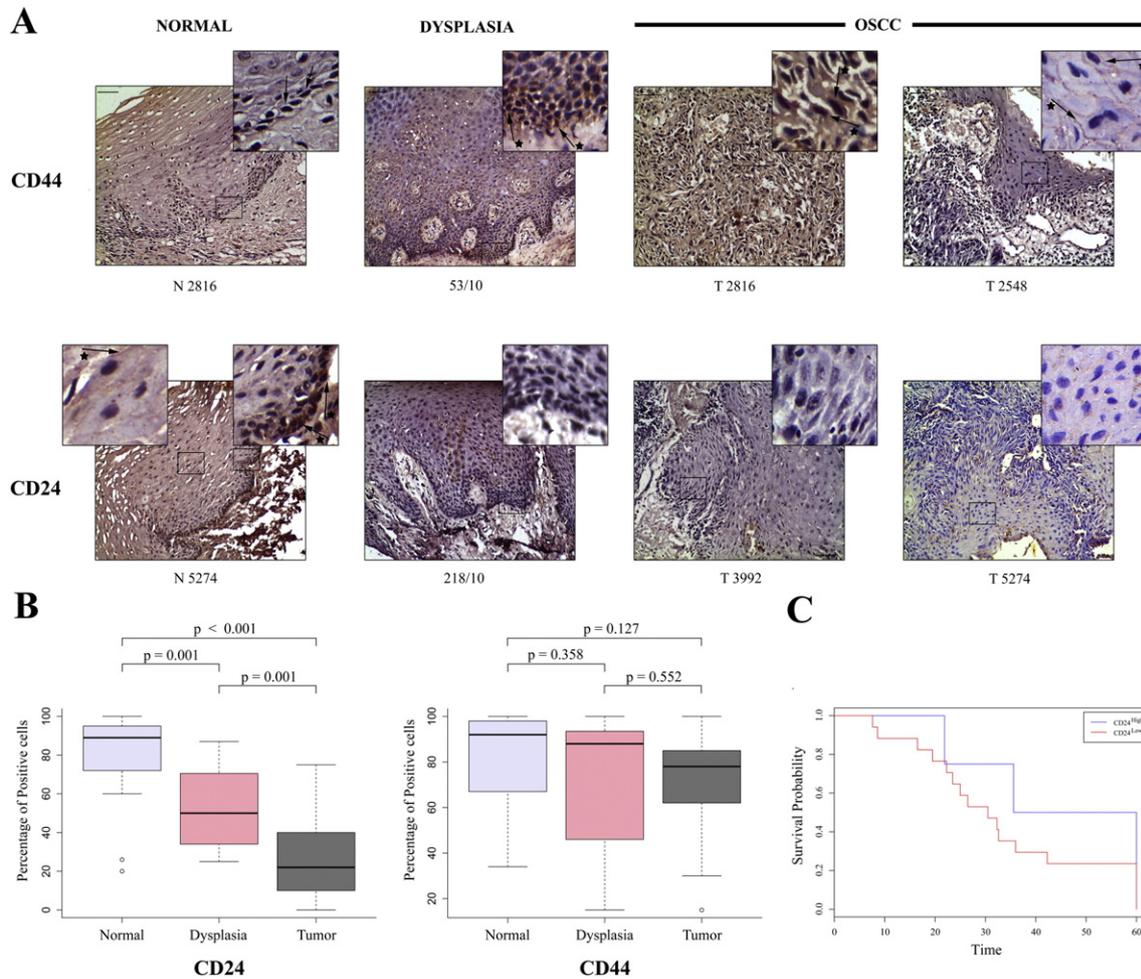
Fig. 6. Immunohischomical analyses of primary oral tumour tissues. (A) Representative pictures showing CD44 and CD24 staining of normal oral epithelium, dysplastic epithelium and OSCC tumour ($20\times$ magnification). Arrows represent expression of the protein. Arrows with star represent cytoplasmic/membrane expression that was scored. Inset is of $40\times$ and the scale bar is of length 50 μm. (B) Box plots showing percentage of positive cells for CD24 (left) and CD44 (right), with median difference calculated by Mann–Whitney-U-Test. P value revealed significance only for CD24 (C) Survival curves were plotted using Kaplan Meier method and p-value calculated by Log Rank test ($p = 0.335$) found to be insignificant. OSCC: Oral Squamous Cell Carcinoma.
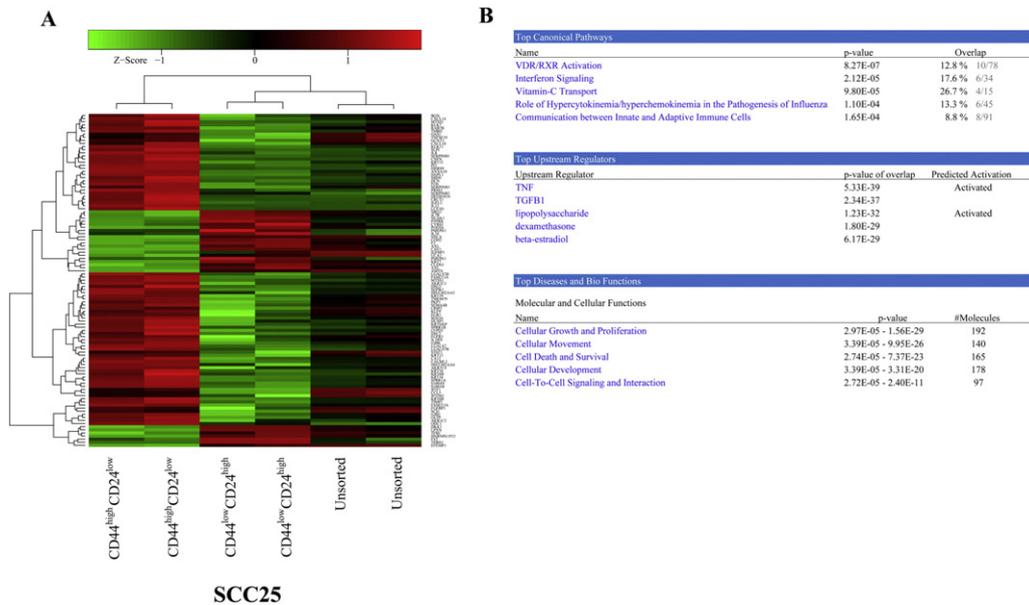

Fig. 7. Differential mRNA expression profile of CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cells of parental SCC25 line.(A) Heatmap was generated using the normalized expression values (z-score of ΔC_T) of significant (Adjusted $p \leq 0.05$, $n = 2$) differentially expressed genes between CD44^{high}CD24^{low} and CD44^{low}CD24^{high}. Hierarchical clustering of different cell types have been indicated. The scale for the heat map ranges from -1 = bright green to $+1$ = bright red. (B) List of Top Canonical pathways, top upstream regulators and biological functions enriched in the gene network analysis.

these genes revealed top canonical pathways and diverse molecular and cellular functions such as Cellular growth, movement and development, cell proliferation, cell death and survival and cell-to-cell interaction and signalling (Fig. 7B). Taken together, these findings put together a comprehensive picture of CSC gene signatures in OSCC.

4. Discussion

We have done an elaborate molecular and phenotypic characterization of the small percentage of CD24^{low} cells within the predominantly CD44 expressing OSCC cell lines. These cells showed relatively high expression of Sox2, Nanog, Oct4, the well-known embryonic stem cell markers, both at the RNA and protein level. ALdh1 and Bmi1 expression were also consistently up-regulated in CD44^{high}CD24^{low} cell population of all cell lines. Aldh1, Bmi1 and Nanog have already been established as prognostic stem cell markers in a closely related Esophageal Cancer (Hwang et al., 2014). Their importance in OSCC is also being recognized along with other cancers. Moreover, Nanog has been shown to be a therapeutic target controlling CSC self-renewal in various cancers including HNSCCs (Jeter et al., 2015). Studies in tongue carcinomas have denoted Bmi1 as a pro-tumorigenic factor with essential role in migration and invasion (He et al., 2015). Aldh1 activity has been found to overlap with CD44$^+$ population regulating common metabolic pathways in HNSCC (Zou et al., 2012). Collectively, these specific stemness markers were found to be enriched in the CD44^{high}CD24^{low} population. We also ascertained that the descendant population derived from the continually cultured CD44^{high}CD24^{low} cells consisted of other cell types whereas the non-stem population failed to generate the original symmetric condition within the stipulated time. This further emphasized that CD44^{high}CD24^{low} cells are indeed the CSC population in oral cancers.

CD44^{high}CD24^{low} cell types were found to be of mesenchymal nature, as reflected by loss of E-cadherin and gain of Vimentin. This observation was also supported by global analysis of NCI-60 cell line transcriptome dataset which showed expression of CD44 and CD24 to be inversely co-related in the mesenchymal vs epithelial group of cell lines. It might be that the over-expressed Oct4, a key determinant of CSCs, promotes tumour-initiating properties in OSCC by mediating EMT (Tsai et al., 2014). In this context, it must be mentioned that CD24 expression is down-modulated by one of the known EMT factor Twist, to enhance breast Cancer Stem Cell expansion (Vesuna et al., 2009). In OSCC also, it may be speculated that CD24 depletion is a prerequisite for induction of EMT. However, in several epithelial cancers like colorectal and nasopharyngeal, CD24$^+$ status has been observed as the definitive stem cell marker (Yeung et al., 2010; Yang et al., 2014). In view of this fact, one of the studies in HNSCC has described CD44^{high}CD24^{high} as the Cancer Stem Cells (Han et al., 2014). Conversely, we have found CD44^{high}CD24^{low} cells to be able to form larger spheres within a few days, indicative of its ability to undergo asymmetric division. These were endowed with enhanced self-renewal powers and could be perpetuated for many generations. There are several reports implicating the role of CD24 in promoting invasion and metastasis (Baumann et al., 2005). However, in our case, CD44^{high}CD24^{low} cells were indeed, more migratory and invasive under in vitro conditions. Although these cells did not proliferate any faster, despite altered cell cycle, but under clonal conditions, could give rise to holoclone-like colonies, a typical characteristic of stem cells with extensive differentiation potential. On the contrary, CD44^{high}CD24^{high} cells were not so enriched with stem-related markers.

"Side population" cells, those that effectively efflux out drugs, have always been shown to have CSC property (Golebiewska et al., 2011). In this study too, we found the presence of "SP" characteristics in the isolated CD44^{high}CD24^{low} cell types. This not only exemplifies the stemness characteristics of CD44^{high}CD24^{low} cells but also adds to the fact that these cells contribute to intrinsic chemo-resistance and hence difficult to eliminate. It eventually becomes clear with the sensitivity assays whereby CD44^{high}CD24^{low} cells exhibit enhanced survival upon

drug treatment owing to their quiescent slow cycling state and reduced apoptosis. Furthermore, as anticipated, the CD44^{high}CD24^{low} cells were particularly enriched upon drug treatment confirming that the drug itself can select for the CSCs. CD24 has been found to be positively associated with tumour grade in various cancers including breast cancer where CD44$^+$CD24$^-$ cells have already been well-characterized as CSCs (Wei et al., 2011). Though CD24 has shown prognostic value, it exerts contradictory roles particularly in response to CD44 mediated signalling (Ju et al., 2011). Moreover, in breast cancers, transitions between CD44$^+$CD24$^-$ and CD44$^+$CD24$^+$ have been evident upon drug exposure, which seemingly contributes to CSC-like chemotolerance owing to activated SFK signalling axis (Goldman et al., 2015). Therefore, distinguishing tumourigeneic properties of these two cell types may be difficult, depending on the tumour type and micro-environment. Although Han et al. found CD44$^+$CD24$^+$ to be more tumorigenic than CD44$^+$CD24$^-$ in cell lines derived from salivary gland neoplasms, we found the CD44^{high}CD24^{low} cell type to be CSC-like cells in cell lines primarily derived from oral cavity. Perhaps, the diverse anatomic locations of tumour along with other etiological factors account for context-dependent role of CD markers.

Immunohistochemical studies revealed increase in the proportion of CD44^{high}CD24^{low} cell type from low in normal tissues to quite high in tumours. This is interesting as we know that Cancer Stem Cells are the actual tumour initiating subpopulation and can expand depending on its evolutionary landscape during cancer development (Kreso and Dick, 2014). A number of studies have shown existence of CSCs in most of the histo-pathologically advanced tumours with lymph node metastasis. Under normal conditions, both CD44 and CD24 are involved in normal differentiation process, which is disrupted upon loss of CD24, and epithelial integrity is lost leading to tumorigenesis (Abdulmajeed et al., 2013) Despite this step by step procedure of tumour formation, extensive heterogeneity does exist from one individual to another (Park et al., 2010). Neither all the patients show similar CSC hierarchy nor do they respond to CSC-targeted treatments in a similar manner.

To derive specific gene signatures of CSCs in OSCC, we performed microarray based gene expression analysis of the various subpopulations of SCC25 cell line. Differential gene expression analyses between the CD44^{high}CD24^{low} and CD44^{low}CD24^{high} cell types implicated a number of genes in stemness, differentiation and EMT. We know that these features are generally high in cells at the tumour edges where it comes in contact with the stroma. Sometimes the cancer cells themselves take up the tasks of cancer associated fibroblasts via epithelial plasticity or vice-versa (Kinugasa et al., 2014). This is notable in our results as most of the genes up-regulated in CD44^{high}CD24^{low} cells have important roles in tumour-microenvironment. Inflammation at the tumour-stroma interface produces those ECM degrading enzymes contributing to invasion, metastasis and angio-genesis. A very interesting fact is that the CD markers used for conventional CSC identification are in reality determinants of autocrine/paracrine signalling whereby receptor-ligand interaction plays essential roles in stemness. Interaction with interleukins (IL-8) or chemokines (CXCL10, CXCL14) has important implications in the role of these CD receptors (van der Horst et al., 2012). In addition, stromal derived IFNs, TNF or TGF-β leads to selection of stress-resistant CSCs with constitutive Stat1 activation (Khodarev et al., 2012). Moreover, Claudin^{low} phenotype has been found to be linked with EMT and tumour initiation in breast cancers with TGF-β participation (Dave et al., 2012). Another secretory factor, Thrombospondin-1 inhibits self-renewal via its interaction with CD47 on endothelial cells (Kaur et al., 2013). From the literature survey, we thus put forward that several genes identified in the microarray analysis were indeed significantly associated with CSC features.

5. Conclusion

In conclusion, the CD44^{high}CD24^{low} cell population from oral cancer cell lines was notably distinct from other populations. They not only

displayed increased CSC and EMT properties but also enhanced in-vitro tumorigenic potential. Drug resistance, invasion and metastasis ability of these cell types were evident and could be partially explained by their gene expression profile. Correlation with patient sample was also manifested. Taken together, these cells certainly exhibit CSC phenotype and thus can be targeted for better treatment of the disease.

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Author contributions

Conceived and designed the experiments: SG, SR. Performed the experiments: SG, DG, SD, and SS. Analysed the data: SG, DG, PD, and SD. Contributed reagents and analysis tools: SR, CP, and NA. Wrote the manuscript: SG and SR. All authors reviewed the manuscript.

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Additional information

Micro-array data has been submitted to Gene Expression Omnibus with GEO accession number: GSE72118.

Competing financial interests

The authors declare no conflict of interest.

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