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$CD44^{high}CD24^{low}$ 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.

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

Abbreviations: CD, Cluster of differentiation; CSCs, Cancer Stem Cells; EMT, Epithelial to mesenchymal transition; OSCC, Oral Squamous Cell Carcinoma; SP, Side Population; ABC, ATP-binding Cassette.

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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