Oral Oncology 62 (2016) 139–146

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Circulating tumor stem like cells in oral squamous cell carcinoma: An unresolved paradox

Shanaya Patel [a], Kavan Shah [a], Sheefa Mirza [b], Kanisha Shah [a], Rakesh Rawal [a,*]

[a] Division of Medicinal Chemistry & Pharmacogenomics, Department of Cancer Biology, The Gujarat Cancer & Research Institute, Gujarat, India
[b] Department of Life Sciences, Gujarat University, Gujarat, India

ARTICLE INFO

Article history:
Received 19 August 2016
Received in revised form 26 September 2016
Accepted 22 October 2016

Keywords:
Oral cancer
Cancer stem cells
Circulating tumor cells
Self renewal
Chemoresistance
CD44v6 and Nanog

ABSTRACT

Objective: Circulating tumor cells (CTCs) are increasingly gaining importance due to their immense potential in enhancing diagnosis, prognosis and response to therapy in solid malignancies. Therefore, we aimed to comprehend the molecular diversity and critical role of this disseminated tumor population in OSCC.
Methodology: CD44+ subpopulation was isolated using immuno-magnetic cell separation and their purity was validated using flow cytometry. Characterisation of self renewal potential and resistance to chemotherapy was assessed using tumor sphere forming and cytotoxicity assay. Gene expression profile of pertinent CSC (CD44s, CD44v3, CD44v6) and stemness markers (Bmi1 and Nanog) was carried out in CD44+ cells using Real Time PCR. Predominantly expressed markers and their association with clinico-pathological conditions were substantiated in 30 OSCC patients.
Result: Flow cytometry analysis depicted a predominant population of CD44+CD24−CD45− cells suggesting that circulating tumor cells had a subpopulation of CSC like cells in the circulation. These cells demonstrated increased sphere forming capability and intrinsic chemo-resistance compared to non-CSC, thus indicating the CSC features of self-renewal and chemo-resistance. Additionally, CD44+ cells showed significantly increased expression levels of CD44v6 and Nanog compared to CD44− cells. Clinically, expression pattern of CD44v6 and Nanog correlated with different anatomical subsites, loco-regional aggressiveness of the disease and recurrence, thus opening newer avenues that can be explored for better prognostic and therapeutic implications.
Conclusion: This study explored the inevitable role of CD44v6 and Nanog as circulating stem like cell markers in assessment of loco-regional aggressiveness, detection of relapse and therapeutic response and resistance.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the major causes of morbidity and mortality worldwide with an estimated incidence of half a million cases diagnosed annually [1]. Amongst these, nearly 50% of patients suffer from tumor-related complications due to heterogeneity and complex behavioural pattern of this malignancy [2]. Despite the recent advances in diagnosis and therapeutic modalities, prognostic rate remains dismal with over half of the oral cancer patients suffering from loco-regional relapses while 1/4th of the patients developing distant organ metastases [3–6]. Thus, it is becoming increasingly important to develop methods for monitoring disease progression and detecting early metastatic spread in order to facilitate individual therapeutic interventions.

Circulating tumor cells (CTCs), a subpopulation of rare atypical precursor cells released in the circulation by primary tumors, are believed to have acquired somatic mutations and genomic rearrangements identical to that found in primary tumors. This characteristic of CTCs would aid in understanding the mutational behavioural pattern of tumors without the need for an invasive tissue biopsy [7]. Recent evidences have demonstrated significant correlations between occurrence of CTCs and disease progression, poor prognosis and disease free survival in breast, prostate, colorectal, and lung cancers; however the prognostic value and clinical utility of CTCs in OSCC is yet to be elucidated [8–10]. In spite of the impending potential of CTCs, there are certain challenges that need to be considered such as - (i) Reusability of the processed

* Corresponding author at: Div. of Medicinal Chemistry & Pharmacogenomics, Dept. of Cancer Biology, The Gujarat Cancer & Research Institute, NCH Campus, Asarwa, Ahmedabad 380016, Gujarat, India.
E-mail address: rakesh.rawal@gcriindia.org (R. Rawal).

http://dx.doi.org/10.1016/j.oraloncology.2016.10.019
1368-8375/

samples (ii) Short life span of CTCs in circulation and (iii) Plausible spreading of disseminating cells during surgery or incision biopsy [10,11]. Thus, overcoming these obstacles would not only facilitate monitoring the therapeutic response in patients but would also help identify potential targets for individual patient therapy.

Recent reports have observed a great discrepancy in correlation of CTC status with clinico-pathological characteristics of cancer patients due to usage of different techniques such as - immunomagnetic separation, microfluidic platforms and cellular-marker based enrichment [12–15]. Amongst these, EpCAM based enrichment technique has been one of the most widely used techniques till date; however it could not detect CTCs in all metastasized or advanced disease patients of OSCC. This unpredictability is consistent with other reports and could be attributed to either low incidence of CTCs or to exclusion of non-EpCAM and/or non-keratin expressing CTCs which needs serious contemplation as EMT is characterised by loss of epithelial features [16]. Hence, there was a paradigm shift from EpCAM based enrichment techniques to technologies capable of capturing non-EpCAM based CTC population by using other cell surface markers (N-cadherin and EGFR) or cancer stem cell (CSC) markers (CD44) [17,18]. Mani et al. and Yu et al. observed that amongst the probable set of markers, stem like cells demonstrated significant dynamic changes and their ability to survive in circulation was considerably higher owing to its self renewal property [19,20]. Furthermore, culturing CTCs based on CSCs markers has become an imperative aspect to develop *in-vitro* models for assessing drug sensitivity and improvising personalized treatment; however this approach is less explored in OSCC [10,21]. Therefore, this study aims to understand the molecular diversity and critical role of this disseminated tumor population in OSCC by culturing patient derived CD44+ circulatory tumor stem like cells *ex-vivo*. Furthermore, we sought to assess the cytotoxic effect of Cisplatin on this rare subpopulation, evaluate the gene expression profile of pertinent CSC markers and analyze their clinical significance in OSCC patients, thus providing a better model to monitor disease progression, therapeutic response and identify potential targets for personalised therapy in future.

Methodology

Sample characteristics

Peripheral Blood Samples of 30 OSCC Patients (23 tobacco habituated and 7 non habituated) and 15 healthy controls were collected for the study using EDTA vacuum tubes (Becton Dickinson Vacutainer Systems Europe, Meylan, France). Whole blood samples were processed fresh on the same day for RNA isolation using RNA blood mini kit (Qiagen) following manufacturer's instructions. This study was approved by the Ethical Committee of The Gujarat Cancer & Research Institute, Gujarat and written informed consent were duly taken from the patients for participation in the study.

Immunomagnetic cell sorting

Mononuclear cells were isolated from peripheral blood of OSCC patients by density gradient centrifugation. These cells were exposed to FITC-conjugated anti-CD44 mouse antibody (Stem Cell Technologies) to identify the CD44+ cells from the heterogeneous tumor population while the remaining cells apart from the positive subpopulation was considered as the CD44− subpopulation. Further, EasySep® FITC positive selection kit (Stem Cell Technologies) was used to identify the FITC labelled CD44 positive cells. These sorted cells were cultured in RPMI (HiMedia), 10% fetal bovine serum (FBS; HiMedia), and 100 U/ml Penicillin-streptomycin (HiMedia) in low attachment conditions. All experiments were performed in duplicates to verify the reproducibility of the data.

Flow cytometry

The purity of immuno-magnetically sorted CD44+ subpopulation was validated by gating the sorted cells with conjugated primary antibodies of CD44-FITC (Stem Cell Technologies), CD24-PE and CD45-PE (BD Biosystems) phenotypes using flow cytometry analysis. These cells were exposed to anti-mouse secondary antibodies and percentage of each population was calculated by acquiring the sample in FACs Canto II instrument using FACS Diva software.

Tumor sphere formation assay

The immuno-magnetically-sorted cells were seeded in 6-well ultra-low attachment plates (Corning; New York, NY, USA) at a density of 5×10^3 cells/well and cultured in RPMI-1640, 10% FBS and 100 U/ml Penicillin-streptomycin at 37 °C and 5% CO_2. The percentage of orosphere was calculated by dividing the number of spheres by the number of cells seeded per well.

Cytotoxicity assay

CD44+ and CD44− subpopulations were seeded at a density of 1×104 in 96-well plate. These cells were exposed to Cisplatin drug for 24 h in various concentrations ranging from 0.25 to 1.0 μg/ml. Further, 10 μl of 5 mg/ml MTT (Hi-Media) was added to each well and the cells were incubated at 37 °C for 4 h. 200 μl of DMSO was added and mixed thoroughly on orbital shaker for 15 min to dissolve the formazon crystals. Absorbance was estimated at 590 nm with a reference filter of 620 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.

Quantitative gene expression of pertinent CSC markers

Total RNA was extracted from blood of OSCC patients and healthy individuals using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg RNA of each sample using High Capacity cDNA Reverse transcription kits (Applied Biosystems). Furthermore, real-time PCR reaction mix was prepared in a total volume of 20 μl comprising of 2X Kapa SYBR Fast qPCRMastermix Universal (Kappa Biosystems), 200 nM of each primer, 2.5 mM of $MgCl_2$ and 1 μl of cDNA. β-actin gene expression was measured as endogenous control and all the primers were custom ordered using the following sequences [Supp Table 1]. The amplification run was performed on the AriaMx Real-time PCR System (Agilent technologies) and the PCR reactions were subjected to 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 3 s and annealing at 60 °C for 20 s, for 40 cycles. The experiment was performed in triplicates and the relative mRNA levels were analyzed using the ddCt method after normalization with β-actin values.

Statistical analysis

Statistical analysis of gene expression profile with clinicopathological parameters was done by Student's *t*-test using SPSS version 13. Differences were expressed as mean ± SD and data was considered statistically significant when p-values was lower than 0.05.