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ORIGINAL ARTICLE

Multidrug resistance and tumor-initiating capacity of oral cancer stem cells

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Summary

Purpose: *Recent studies in several tumors showed that presence of cancer stem like side population (SP) cells are responsible for chemotherapeutic drugs resistance and tumor relapse. In our present study, we have analyzed the role of SP cells in oral squamous cell carcinoma cell (OSCC) line OSCC-77.*

Methods: *The oral cancer cell line OSCC-77 was analyzed for the presence of SP cells by FACS using Hoechst 33342 dye exclusion method. Further the FACS-sorted SP and non-SP cells were subjected to drug resistance and sphere formation assays.*

Results: *We identified that the presence of SP cells in OSCC-77 cell line was 3.4%, which was reduced to 0.6% in the presence of verapamil, an inhibitor of ABC transporter. Furthermore, we showed that these SP cells were highly drug-resistant, had increased survival and were highly potent for self-renewal. Also, the clone formation efficiency of SP cells was significantly higher compared to non-SP cells ($p<0.01$).*

Conclusion: *Our data suggest that cancer stem-like SP cells of OSCC-77 cell line contribute to multidrug resistance and are highly involved in tumor relapse. However, further characterization of SP cells at gene expression level and their signaling pathways might provide new insights into the development of novel anticancer drugs.*

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Key words: *oral cancer, OSCC-77, side population, stem cell*

Introduction

Oral cancer is the sixth most common cancer and affects 3% of World's population [1]. Oral cancer is responsible for 7% of cancer deaths in males and 4% in females anually. The proposed possible risk factors include tobacco use, smoking, alcohol and areca nut chewing [2]. There are two kinds of oral cancer: i) Oral cavity cancer, originating from the mouth and ii) Oropharyngeal cancer which develops from the oropharynx. OSCC is the most common oral cancer that affects the tissue lining of the oral cavity [3,4].

Despite recent advances in cancer treatment, the difficulty in eradicating tumors may be due to the persistence of cancer stem cells (CSCs) which maintain the tumor tissue. These CSCs are responsible for the treatment failure (minimal residual disease), evading drug treatment and causing tumor recurrence [5,6]. The population of CSCs possesses characteristics of self-renewal, exhibits high tumorigenicity *in vivo*, high differentiation potential, and multidrug and apoptosis resistance [7].

Cell that exclude Hoechst 33342 dye are designated as SP cells. These cells share the fundamental characteristics of CSCs, express stem-like genes, and they are resistant to chemotherapeutic drugs. Furthermore, SP cells overexpress the ATPase binding cassette (ABC) transporters such as

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ABCB1 (MDR1), ABCC1, ABCG2 (BCRP1), which contribute to multidrug resistance and express stem cell surface markers such as CD133, CD44, CD34, CD29, and CD24 [8]. Recently, SP cells were identified in several solid tumors including breast cancer, brain tumors, glioblastoma, gastrointestinal cancers, head and neck SCC, hepatocellular carcinoma cell lines and even primary cultures from neuroblastoma patients [9].

Therefore, identification and characterization of SP cells is crucial for understanding the mechanism of drug resistance and tumor recurrence, which could lead to efficient targeting of CSCs. Consequently, the present work was designed to analyze the prevalence of SP cells using the OSCC cell line OSCC-77, based on Hoechst 33342 efflux. Furthermore, the sorted SP cells were subjected to drug resistance and sphere formation assays. The results are presented and discussed in detail below.

Methods

Cell line and cell culture

OSCC-77 cell line was obtained from cancer growth at the mandibular region (Grade 4; recurrent type). OSCC-77 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), supplemented with antibiotics (1% Gibco penicillin-streptomycin) and maintained in T-75 flasks at 37°C in a humidified 5% CO^2 and 95% air atmosphere. Once 90% confluent, cells were removed from the culture flasks using Trypsin-EDTA (0.25%-53mM EDTA), washed, resuspended in 10% DMEM and centrifuged at 5000 rpm for 6 min. Then, cells were resuspended in 10% DMEM and cell count was performed using hemocytometer.

Labeling of Hoechst 33342 dye

Study groups

Group I: Control: cells labeled with Hoechst 33342 dye alone (N=5)
Group II: Drug-treated cells: cells treated with verapamil and Hoechst 33342 dye (N=5).

Cells were counted by hemocytometer ad approximately 10^6 cells/ml of 10% DMEM were labeled with Hoechst 33342 stock (sigma)-bis-benzimide (5 µl/ml) either with dye alone or in combination with verapamil 0.8 µl/ml. Then, cells were counterstained with propidium iodide (PI) 2 µg/ml. The cells were sorted using a flow cytom¬eter and the sorted cells were cultured and maintained in DMEM/F-12 supplemented with 10% FBS. The Hoechst 33342 dye was excited at 355 nm and its dual wavelength fluorescence was analyzed (blue, 450 nm; red, 675 nm).

Sphere formation assay

Sphere formation assay was carried out as previously described [10]. The FACs-sorted SP cells and non-SP cells were seeded at a density of 1000 cells/ml, resuspended in tumor sphere medium consisting of serum-free 1:1 mixture of Ham's F-12/DMEM, N2 supplement, 10 ng/ml human recombinant basic fibroplast growth factor (bFGF), and 10 ng/ml epidermal growth factor (EGF) and subsequently cultured in ultra-low attachment plates for about 2 weeks. For the quantification of generated tumor spheres, SP and non-SP sorted cells were seeded at a low density of 20 cells/L and after the 7^{th} day of culture the number of generated spheres was counted and quantified.

Immunofluorescent staining

Immunofluorescent staining of the spheroids was performed as previously described [11]. Tumor spheres generated by SP cells were fixed in 4% paraformaldehyde (4^0C, 10 min) and blocking was done with normal serum for 30 min. Cells were incubated with the mouse monoclonal anti-Oct4 (1:200; Chemicon, Japan) overnight. After washing 3 times with PBS, overnight-incubated cells were assayed for FITC-conjugated chicken anti-rat IgG in the dark. Spheroids were counterstained with Hochest 33342 for nuclei staining and viewed under fluorescence microscopy. The images were processed by using adobe photoshop CS4 and Image J.

Drug resistance assay

Drug resistance assay was performed as previously described [12]. Approximately $1x10^4$ cells/plate were cultured in 96-well plates and treated with different drugs. Drug concentrations: 5-fluorouracil (5-FU) 10 µg/ml, cisplatin 20 µmol/L, paclitaxel 2 µmol/L and oxaliplatin 100 mM. The mean value of optical density (OD)450 obtained was represented as a graph. The rate of cell resistance in both SP and non-SP groups was calculated as previously described [10].

Statistics

Data were expressed as mean±standard error. The statistical significance was determined using one way analysis of variance (ANOVA) and a p value<0.05 was considered as statistically significant.

Results

Identification of cancer stem cell like side population cells containing MDR1 using Hoechst 33342

During FACs analysis of OSCC-77, live cells were selected against PI as P1 gated population. SP cells were identified and sorted out from the gated P1 using Hoechst 33342, which is a DNA binding dye. The fluorescence emission of Hoechst

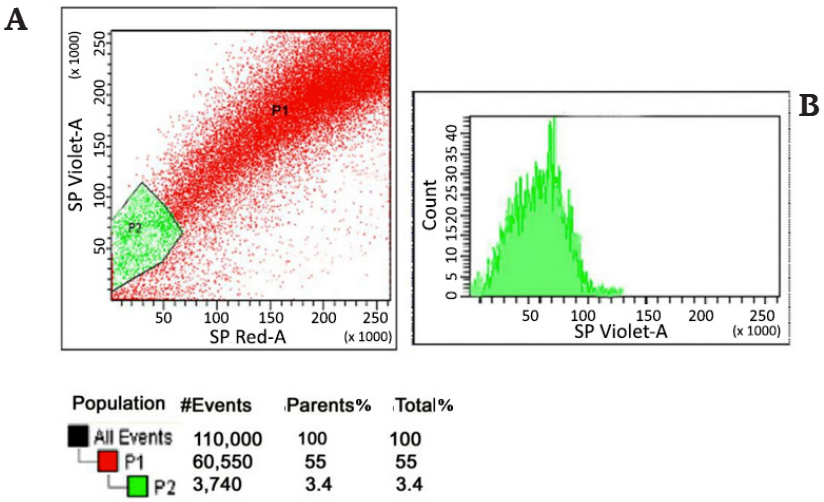

Figure 1. A: Dot plot analysis of FACS showing 3.4% of SP cells from oral cancer cell line OSCC-77 in the P2 gated region. P1 is the main population. **B:** Histogram showing the events of P1 population..

33342 was monitored at approximately 450 nm (SP-Violet) and at 675 nm (SP-Red) followed by UV excitation, and a group of cells were observed that displayed low blue and red fluorescence (Figure 1A,B). The right higher quadrant (PI) of the FACS profile was designated as main population (non-SP cells) (Figures 1 and 2). The left lower quadrant (P2 gated) of the FACS profile was defined as SP cells. As shown in Figure 1, we identified 3.4% of SP cells in OSCC-77 cells, that excluded Hoechst 33342 dye. Treatment with verapamil reduced the percentage of SP cells (P2 gated region) from 3.4% to 0.6%. These data suggest that dye exclusion process of oral cancer SP involved mainly MDR1 transporter protein as verapamil treatment can efficiently block the Hoechst 33342 dye exclusion by SP cells (Figure 2A,B).

Multidrug resistance and tumor recurrence properties of side population cells

The FACS-sorted SP and non-SP cells were subjected to drug resistance and sphere formation assays to examine their multidrug resistant properties and the self-renewing capacity. We observed that the SP cells were highly resistant to 5-FU, cisplatin, paclitaxel and oxaliplatin. Also, the survival rates of SP cells were significantly higher compared with non-SP cells after treatment with these drugs (Figure 3A). Sphere formation assay showed that the number of tumor

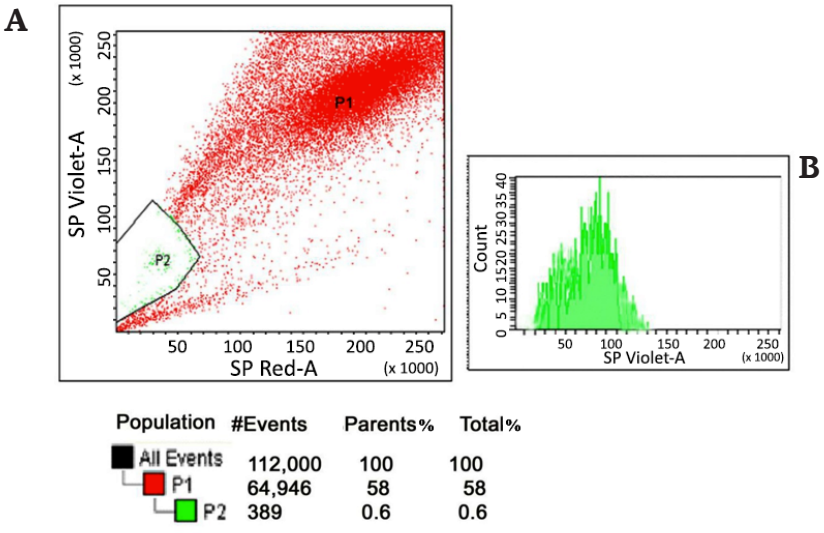

Figure 2. A: Dot plot analysis of FACS showing that OSCC-77 cells contain reduced SP cells (0.6%) in P2 gated region after treatment with verapamil. **B:** Histogram showing the events of P1 population..

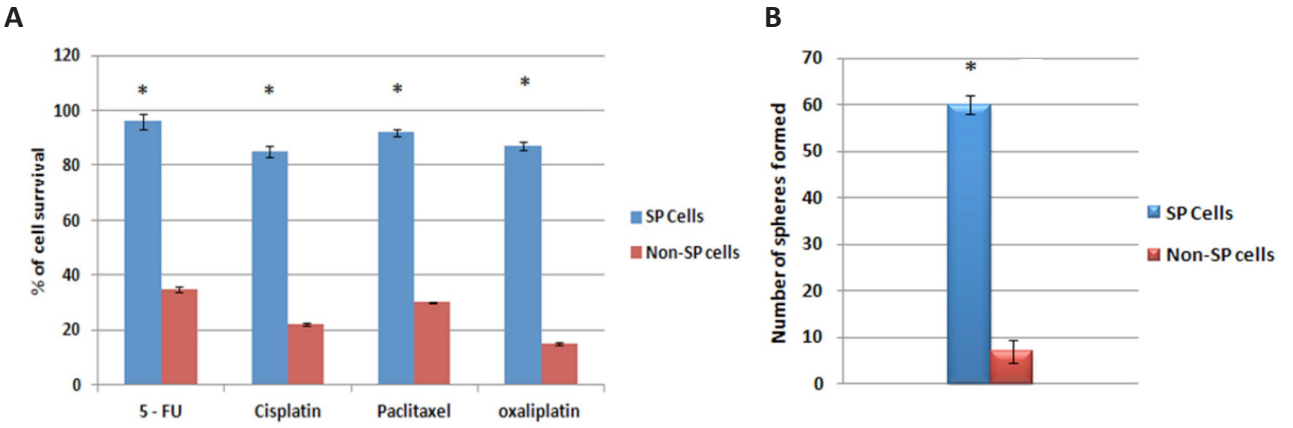

Figure 3. A: Comparison of cell survival rate of side population (SP) cells and non-SP cells upon treatment with 5 fluorouracil (5-FU), cisplatin, paclitaxel and oxaliplatin. **B:** Quantification of number of tumor spheres generated by SP and non-SP cells, counted after 7 days of culturing. The number of spheres generated by SP cells are significantly higher than those of non-SP cells. *p<0.05

spheres generated by OSCC SP cells in serum free medium was significantly higher than in non-SP cells (Figure 3B). Interestingly, the tumor spheres formed by SP cells showed Oct-4 immunofluorescence positivity (Figure 4A-D). Hence, these data clearly suggest that oral cancer SP cells possess CSCs-like properties, responsible for multidrug resistance, treatment failure and tumor relapse.

Discussion

The presence of CSCs appears to be a major cause for resisting DNA damaging agents due to overexpression of efflux pumps. This specific property of CSCs leads to failure of conventional drug treatment modalities which kill most of the neoplastic cells but leave CSCs unaffected. Hence, CSCs are the main target for achieving complete tumor eradication, and offering to patients the chance for long-term disease free survival. So far several studies reported that Hoechst 33342 dye exclusion is a valuable technique to identify and isolate CSCs. Cells that exclude this dye are named SP cells [8] which share the fundamental features of CSCs such as self-renewal, high tumorigenicity, differentiation potential, chemotherapeutic resistance etc. Recently, the presence of SP cells has been demonstrated in several solid tumors which include breast cancer, ovarian cancer, nasopharyngeal cancer, central nervous system cancers and their contribution in chemotherapeutic resistance has been revealed [13-16].

In the current study we used Hoechst 33342 dye to isolate SP cells from OSCC-77 cells by FACs analysis. We identified 3.4% SP cells in oral cancer, which was reduced to 0.4% upon treatment with verapamil. Most likely, the dye exclusion properties of SP cells is due to overexpression of MDR transporters (such as ABCB1, ABCG2), like in other tumors [9,13] which might be responsible for drug resistance and treatment failure. Subsequently, the sorted SP cells showed high resistance to different drugs such as 5-FU, cisplatin, oxaliplatin and paclitaxel. In addition, the survival rates of SP cells after treatment with the above mentioned drugs were significantly higher when compared to non-SP cells. There are two different

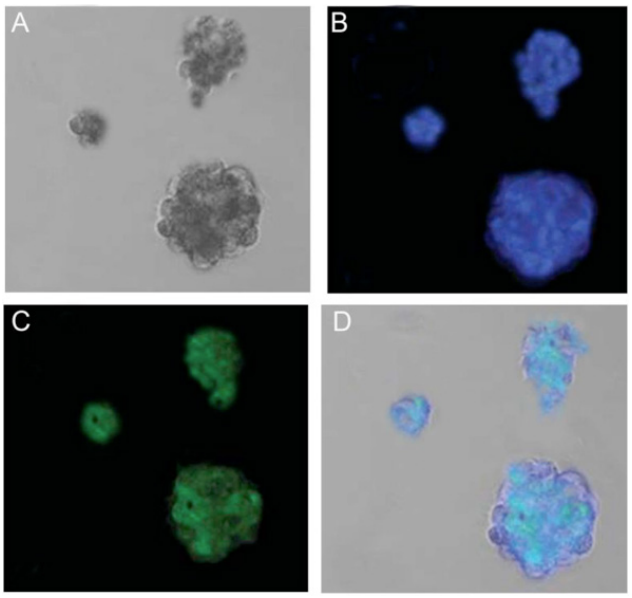

Figure 4. Representative images of immunofluorescence staining with Oct-4 in tumor sphere assay generated by OSCC-77 SP cells. **A:** Bright field micrographs; **B:** Nucleus fluorescence staining with Hochest33342 (blue). **C:** Immunofluorescence staining of anti-Oct-4 (green fluorescence). **D:** Overlay of (**B**) and (**C**).

mechanisms responsible for the increased survival: i) activation and overexpression of adenosine triphosphate binding cassette (ABC) transporters (ABCB1, ABCG2) which blocks the entry of chemotherapeutic drugs into the cell and thus DNA damage of cancer cells is prevented and ii) increased expression of anti-apoptotic factor bcl-2 which leads to reduced apoptosis [17]. Possibly these two mechanisms might occur in OSCC-77 SP cells but this has to be further studied in detail.

CSCs are highly potent to form tumor spheres. Our data clearly demonstrated that the sorted SP cells are able to form tumor spheres and they are positive to Oct-4 staining. Therefore, Oct-4 expression might be involved in the self-renewal process of SP cells. Recently Wanshan Li et al. showed that SP cells sorted from SCC-55 cell line were immunofluorescence-positive to CD147 (EMMPRIN) and CD44 (EpCAM) compared to non-SP cells (personal communication). Therefore, the enriched expression of CD147 and CD44 on the surface of SP cells might play a major role in tumor growth, metastasis and tumor recurrence. We also hypothesized that these SP cells might have an increased expression of the anti-apoptotic factor bcl-2 which confers properties of apoptosis-resistance. Hence, identification and characterization of SP cells is crucial for designing new therapeutic drugs to eventually target CSCs and block ABC transporters. However, further studies at mRNA level elucidating signaling pathways in SP cells might provide valuable information for eliminating minimal residual disease and prevent tumor relapse.

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