Targeting CD133 in the enhancement of chemosensitivity in oral squamous cell carcinoma–derived side population cancer stem cells

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ABSTRACT: Background. Oral squamous cell carcinoma (OSCC) is one of the most common cancers in the world. Previously, we enriched a subpopulation of OSCC-derived cancer stem cells (OSCC-CSCs), and identified CD133 as an OSCC-CSC marker.

Method. We determined the function of CD133 on chemosensitivity of oral cancer CSCs by silencing CD133.

Results. Initially, we observed that the expression profile of CD133 in OSCC-side population (OSCC-SPs) cells, which exerted properties of CSCs, was significantly upregulated than that of major population (MPs) cells of OSCCs. The cell viability experiments showed that SPs were more chemoresistant compared with major populations. Importantly, targeting CD133 ameliorated the drug resistance of OSCC-SPs to cisplatin treatment. Targeting CD133 and cisplatin co-treatment led to the maximal inhibition on tumor initiating properties in OSCC-SPs.

Conclusion. Side population cells with CSCs properties existed in OSCCs, and silencing CD133 exhibited a prominent therapeutic effect in enhancing the sensitivity of chemotherapy in OSCC through elimination of CSCs. © 2015 Wiley Periodicals, Inc. Head Neck 38: E231–E238, 2016

KEY WORDS: oral squamous cell carcinomas, chemosensitivity, CD133, cancer stem cells, side population

INTRODUCTION
Oral squamous cell carcinoma (OSCC) is a lethal cancer with clinical, pathological, phenotypical, and biological heterogeneity.1 In spite of improvements in the diagnosis and management of OSCC, long-term survival rates have improved only marginally over the past decade.2 The increasing interest in the cancer stem cell (CSC) model is dramatically altering the current directions in cancer treatments and drug developments.3 We identified a subpopulation of oral cancer-derived CSCs from OSCC cells by sphere formation assay.4 Oral cancer-CSCs have been known to have the capacity to promote tumor progression and metastasis, and also contribute to chemoresistance.5–11 Side population technology by Hoechst dye exclusion property is among the successful methodologies for CSC isolation.12 However, the molecular mechanisms by which to regulate the biological properties of side population cells from OSCCs have not been well characterized.

CD133, a 5-transmembrane glycoprotein, is a hematopoietic stem cell and endothelial progenitor marker and seems to be involved in angiogenesis.13,14 CD133 expression has been suggested to serve as a prognostic signature for tumor regrowth, malignant progression, and tumor stages in leukemia, brain tumors, retinoblastoma, renal tumors, pancreatic tumors, colon carcinoma, prostate carcinoma, hepatocellular carcinoma, thyroid carcinoma, melanoma, and oral cancer.6,15–20 CD133 has been shown to negatively correlate with the survival prognosis of patients with OSCC.6 However, the role of CD133 in OSCC-CSCs has yet to be explored.

Herein, we demonstrated a critical role of CD133, which was upregulated in side population cells and previously identified OSCC-CSCs,6 in the regulation of chemosensitivity of OSCC-CSCs. Overall, downregulation CD133 ameliorated the drug resistance of OSCC-CSCs to cisplatin treatment. Synergistic effects of targeting CD133 and cisplatin chemotherapy treatment attenuated tumor initiating cells property in OSCC-CSCs. Thus, our study implicates that targeting CD133 would be a valuable therapeutic clinically in combination with conventional chemotherapy treatment modalities for malignant OSCC.

MATERIALS AND METHODS
Side population analysis
OSCC cell lines were suspended at 1 × 10⁶/mL in prewarmed Dulbecco modified Eagle’s medium (DMEM) with 2% fetal bovine serum (FBS). Hoechst 33342 dye was added at a final concentration of 5 μg/mL in the presence...
or absence of verapamil (50 μM; Sigma, St Louis, MO) and was incubated at 37°C for 90 minutes with intermittent shaking. Then the cells were washed with ice-cold Hanks buffered salt solution with 2% FBS and centrifuged at 4°C, and resuspended in ice-cold Hanks buffered salt solution containing 2% FBS. Propidium iodide at a final concentration of 2 μg/mL was added to the cells to gate viable cells. The Hoechst 33342 dye was excited at 357 nm and its fluorescence was dual-wavelength analyzed (blue, 402–446 nm; red, 650–670 nm). Analyses were done on a FACS Vantage (BD Biosciences, San Diego, CA).

Tumor spheres assay

Spheres from OSCC cells were isolated in medium consisting of serum-free DMEM/F12 medium (Gibco, Grand Island, NY), N2 supplement (Gibco), 10 ng/mL human recombinant basic fibroblast growth factor, and 10 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN). Cells were plated at a density of 10^4 live cells/10-mm low attachment dishes, and the medium was changed every other day until the tumor sphere formation was observed in about 1 week.21

In vitro cell invasion assay

Invasion assays were done in 24-well Transwell units with an 8.0 μm porous transparent polyethylene terephthalate membrane. Cells (1 × 10^5 per well) were added to upper chambers (filter coated with 1 mg/mL Matrigel) in 100 μL of the low serum medium. The lower chambers were filled with 500 μL high serum medium. After 24 hours of incubation at 37°C, cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton swabs. Cells that had migrated through the membrane to the lower surface were stained with Hoechst 33258 and counted in 5 different fields under a fluorescence microscope.22

Soft agar colony forming assay

Each well (35 mm) of a 6-well culture dish was coated with 2 mL bottom agar (Sigma–Aldrich) mixture (DMEM, 10% [v/v] fetal calf serum, 0.6% [w/v] agar). After the bottom layer was solidified, 2 mL top agar-medium mixture (DMEM, 10% [v/v] fetal calf serum, 0.3% [w/v] agar) containing 2 × 10^7 cells was added, and the dishes were incubated at 37°C for 4 weeks. Plates were stained with 0.005% Crystal Violet then the colonies were counted. The number of total colonies with a diameter ≥100μm was counted over 5 fields per well for a total of 15 fields in triplicate experiments.

Subcutaneous xenografts in nude mice

The animal study was approved by the Institutional Animal Care and Use Committee in Chung Shan...
Medical University (permit number: 935). All procedures were performed with the mice under anesthesia, and efforts were made to minimize animal suffering during experiments by following the guidelines. OSCC cells mixed with Matrigel (1:1; BD Bioscience) were injected subcutaneously into BALB/c nude mice (6–8 weeks). Tumor volume was calculated using the following formula: tumor volume (mm$^3$) = (length $^3$ width$^2$)/2.

Flow cytometry for cell surface marker analysis

Cells were stained with anti-CD133 antibody (Miltenyi Biotech, Auburn, CA) and anti-ABCG2 antibody (Chemicon, Temecula, CA) with labeling according to the manufacturer’s instructions. Fluorescence emission from 10,000 cells was measured with an FACS Calibur (Becton Dickinson, San Jose, CA) using CellQuest software.

ALDEFLUOR assay

An ALDEFLUOR assay kit was purchased from StemCell Technologies, Inc. (Vancouver, British Columbia, Canada). For this assay, $1 \times 10^5$ cells were suspended in 50 $\mu$L of assay buffer, and ALDEFLUOR was added to the cell suspensions for a final concentration of 1 $\mu$M. For aldehyde dehydrogenase 1 (ALDH1) inhibitor control, diethylamino-benzaldehyde was added to a final concentration of 150 $\mu$M. Cells were then incubated at 37°C for 45 minutes and were stained with 7-amino-actinomycin D on ice for 5 minutes. After washing the cells with phosphate-buffered saline, live cells (7-amino-actinomycin D)-positive for green fluorescence were analyzed by flow cytometry to compare the
fluorescence intensity of the diethylaminobenzaldehyde-treated samples. High fluorescence was associated with high ALDH activity (ALDH\textsuperscript{+} cells).

**Stable silencing of CD133 by lentiviral vector**

The pLV-RNAi vector was purchased from Biosettia (Biosettia, San Diego, CA). The method of cloning the double-stranded short hairpin RNA (shRNA) sequence is described in the manufacturer’s protocol. Lentiviral vectors expressing shRNA that targets human CD133 (oligonucleotide sequence: Sh-CD133-1:5'–AAAAAGCAAGGCGTTCAAGATTGGATACACAAGGCTGCTCC-3'; Sh-CD133-2:5'–AAAAAGATACACCCACTTACTTGGATACCAAAGGCTGCTCC-3') were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. Lentivirus production was performed by transfection of plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells using lipofectamine 2000 (LF2000; Invitrogen, Calsbad, CA). The green fluorescent protein positive cells were cells expressing the shRNA for silencing CD133.

**Cell proliferation assay**

Methyl thiazolyl-tetrazolium (MTT) assay kit (Sigma–Aldrich, Oakville, Ontario, Canada) was used to analyze the cell proliferation. Specifically, 1 × 10\textsuperscript{5} cells were seeded in each well of a 24-well plate, and then 10 μL of MTT solution was added to the cells, which were then incubated at 37°C for 3 hours. The supernatant was removed, and 200 μL of dimethyl sulfoxide were added directly to the cells. The MTT color reaction was analyzed using a microplate reader set at A560 nm.

**Statistical analysis**

Statistical Package of Social Sciences software version 13.0 (SPSS, Chicago, IL) was used for statistical analysis. Student’s t test was used to determine statistical significance of the differences between experimental groups; p values < .05 were considered statistically
significant. The level of statistical significance was set at .05 for all tests.

RESULTS

Existence of side population cells in oral squamous cell carcinoma cells

Side population cells have been found to have characteristics of CSCs. We also found that about 0.1% to 6.0% of side population cells existed in 6 OSCCs (Figure 1A and 1B). Previously, we and others have isolated OSCC-CSCs through tumor spheres assay. To elucidate the relationship between side population cells and sphere-forming CSCs, the amount of side population cells in OSCCs-derived tumor spheres was first examined. Apparently, OSCCs-derived tumor spheres contained more side population cells than the parental OSCCs (Figure 1C). To further understand whether side populations have the tumor initiating characteristics, OECM1 and SCC25 cells were sorted into side population cells and major population cells by flow cytometry (Figure 1D).

OSCC-derived side population (OSCC-SP) cells display tumorigenic potentials cancer initiating cells properties and elevated expression of CD133.

We next performed tumor sphere assays to evaluate the self-renewal ability of OSCC-SP and OSCC-derived MP (OSCC-MP) cells, respectively. Interestingly, OSCC-SP cells from OECM1 cells displayed higher tumor sphere-forming capability than OSCC-MP cells did (Figure 2A). Next, the in vitro and in vivo tumorigenic activities between OSCC-SP and OSCC-MP cells were compared. The invasiveness/colony formation abilities of OSCC-SP cells were also significantly higher than those of the OSCC-MP cells (Figure 2B and 2C). Furthermore, injection of \(10^5\) OSCC-MP cells into nude mice did not lead to tumor formation, but nude mice administrated with \(10^4\) OSCC-SP cells displayed visible tumors 3 weeks after injection (Figure 2D). Interestingly, the xenograft-derived cells were enriched for side population cells more than the parental cell line (Figure 2E).

Oral squamous cell carcinoma-side population cells elevated chemoresistance and expression of CD133

Because resistance to chemotherapy treatment is a major clinical criterion for characterizing CSCs in OSCC, we then evaluated the chemosensitivity of side population and major population cells, respectively. Cell viability assays showed that side population cells were more
chemoresistant to cisplatin, 5-fluorouracil or doxorubicin treatment compared with the major population cells of both OECM1 (Figure 3A) and SCC25 (Figure 3B) cells. Finally, the side population cells expressed higher a level of the drug resistance-related gene and specific surface markers (ABCG2 and CD133; Figure 3C and 3E). A similar upregulation of CSCs marker ALDH1 activity was also observed in side population cells when compared with major population cells (Figure 3D and 3E). Together, we hypothesized that upregulation of CD133 might be crucial for modulating chemosensitivity of OSCC-CSCs.

**Diminished side population and in vivo tumorigenicity in oral squamous cell carcinoma-side population by targeting CD133**

To further investigate the functional role of CD133 on oncogenicity, subsequently, approach of loss-of-function of CD133 through lentiviral-mediated transduction was conducted in OSCC-SP. Real-time reverse transcriptase-polymerase chain reaction and immunoblotting analyses confirmed that lentivirus expressing both sh-CD133-1 and sh-CD133-2 markedly reduced the expression level of CD133 mRNA (Figure 4A, upper panel) and protein (Figure 4A, lower panel) in transduced OSCC-SP. Flow cytometry analyses confirmed that both sh-CD133-1 and sh-CD133-2 markedly reduced the expression of CD133 protein in OSCC-SP (Figure 4B). Silencing CD133 decreased the percentage of side population cells in both OECM1 and SCC25 cells (Figure 4C). Furthermore, inhibition of CD133 expression significantly attenuated the in vivo tumor growth of nude mice mediated by OSCC-SP cells (Figure 4D).

**Downregulation of CD133 enhanced chemosensitivity in oral squamous cell carcinoma-side population**

The combination CD133-knockdown and cisplatin treatment showed a synergistic effect in abrogating proliferation rate in OSCC-SP (Figure 5A). Single cell suspension of CD133-knockdown OSCC-SP treated with or without cisplatin treatment was used for analysis of their invasion/colonogenicity in vitro as described in the Materials and Methods section. Treatment with cisplatin alone did not affect the invasion ability in OSCC-SP cells (Figure 5B), the combination of silencing CD133 and cisplatin treatment enhanced the efficacy of these treatments (Figure 5B). Meanwhile, similar synergistic effect of downregulation of CD133 and chemotherapy treatment was
also observed in colony formation assay (Figure 5C). Taken together, targeting CD133 exhibited a prominent therapeutic effect in enhancing the sensitivity of chemotherapy in OSCC-CSCs.

**DISCUSSION**

During the last decades, the existence and the identity of CSCs have been identified in hematopoietic tumors as well as a variety range of solid tumors, including breast, brain, lung, colon, prostate, head and neck, and others. Breast cancer was the first solid tumor in which CSCs were isolated: the breast CSCs was characterized as a minority population (≤5%) of cells expressing high levels of CD44 and low levels of CD24 cell surface markers. Colo, lung, and hematopoietic CSCs are isolated by cell sorting with the expression of CD133, although the function of CD133 is still unclear. These cells grow indefinitely as spheres in vitro and were tumorigenic in vivo. Hoehst 33342 staining CSCs are enriched in the side populations of cancer cells, excluding intracellular Hoehst 33342 in vitro, and CSCs are enriched in the side populations of cancer cells, tumor suppressors, cell cycle regulators, and transcription treatment.

**TARGETING CD133 IN CHEMOSENSITIVITY ENHANCEMENTS**

**CONCLUSION**

Overall, our present research showed OSCC-derived side population cells displayed CSCs characteristics and targeting CD133 sensitized CSCs to chemotherapies. Our results provide insights into the clinical prospect of CD133-based therapies for OSCC.

**REFERENCES**


