Distinct Subpopulations of Head and Neck Cancer Cells with Differential Intracellular ROS Exhibiting Diverse Chemoresistance, Stemness and Proliferative Activity

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Running title: ROS$^{low}$ cell with CICs property and chemoresistance

Keywords: ROS; Stemness; Chemoresistance; Proliferative activity; Head and neck cancer

Grant Support: This study was supported by grants from the National Science Council (NSC 101N050, 102N031 and 102N446), Taipei Veterans General Hospital (VGHTPE: V102E2-003) and Ministry of Education, Aim for the Top University Plan, National Yang-Ming University (101ACT513 and 102AC-TC14).

The authors declare no conflict of interest

Total number of figures and tables: 7 figures, 11 supplementary figures, 1 supplementary Materials and Methods and 1 supplementary table
Abstract

Head and neck squamous cell carcinoma (HNSCC) is a lethal cancer. Emerging evidence supports cancer-initiating cells (CICs) are responsible for tumor growth, chemoresistance, etc. However, the physiological mechanisms by which to maintain the characteristics of CICs in HNSCC (HN-CICs) remain elusive. For hematopoietic stem cells, low intracellular reactive oxygen species (ROS_Low) level helps sustain their stemness properties. Therefore; to understand the role of ROS_Low cells, which are speculated to be the HN-CICs, may reveal physiological dependencies for head and neck tumorigenesis. Previously, we have identified the existence of HN-CICs from sphere cells. Herein, we initially demonstrated that there were more ROS_Low cells in sphere cells than in parental HNSCCs. Further, the ROS_Low cells mostly co-expressed CICs surface markers (memGrp78, Glut3, etc.). Next, we exploited flow cytometry to sort the ROS_High, ROS_Midi and ROS_Low cells. The isolated ROS_Low cells exerted more CICs properties, quiescence, chemoresistance, in vitro malignancy and in vivo tumorigenicity; however, the ROS_High cells were the most proliferative. Lastly, the pharmacological depletion of ROS modulators in cisplatin-treated HN-CICs was performed to reduce the CICs properties by enhancing cell differentiation and to sensitize the cisplatin cytotoxicity by promoting cell death. Overall, we identified the subpopulations of cells with differential intracellular ROS level harboring diverse chemoresistance, proliferative activity and stemness properties. We also demonstrated that the ROS_Low cells not only exert CICs properties but mainly contribute to tumor growth and chemoresistance in HNSCCs. Modulation of low intracellular ROS level should be a future alternative HNSCC treatment in combination with conventional chemotherapy.
Introduction

Emerging evidence supports the hierarchical model of cancer initiating cells (CIC; also referred to as cancer stem cells (CSC)), in that, each tumor formation is generated from a distinct subset of cells with characteristics of self-renewal and differentiation capacity (1). Clinically, conventional chemotherapeutics generally affect proliferative cells, potentially eliminate proliferating cancer cells but not target slow dividing cells (2). Like normal tissue stem cells, CICs also exhibit quiescent slow-cycling phenotype (3). Additionally, CICs have been shown to be involved in tumor progression, cancer recurrence and metastasis because of their therapeutic resistance (4,5). Therefore, to uncover the regulatory physiological mechanisms by which to sustain the slow-growing CICs warrants an important study for future therapy development.

Head and neck squamous cell carcinoma (HNSCC) ranks the sixth most common cancer worldwide (6). Previously, we have enriched and identified the existence of a subpopulation of head and neck cancer initiating cells (HN-CICs) from sphere cells. The enriched HN-CICs from HNSCC cells display both the enhanced properties of stemness and malignancy (7). In addition, the cell surface markers such as CD133 (8) and cell membrane anchoring GRP78 (memGRP78) (9) for identifying the HN-CICs have also been reported in our previous studies. However, the unique physiology and regulatory mechanisms by which to mediate the HN-CICs properties of clinical refraction remain elusive. Therefore, the studies on targeting the mechanism(s) in HN-CICs to contribute to the therapeutic resistance of HNSCC would benefit future HNSCC therapy.

Reactive oxygen species (ROS) including superoxide (O$_2^•$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (HO$^•$), play a major role to cellular proliferation, differentiation and survival (10). It has recently been shown that stem cells have a unique mechanism, which is important to cope with the accumulated ROS, to increase antioxidant defenses and to play a crucial redox regulator on self-renewal and differentiation, (11-13). For instance, hematopoietic stem cells maintain a low intracellular ROS status that in turn facilitates quiescence; whereas, a higher ROS state activates cell proliferation and differentiation but exhausts self-renewal in these cells (14). Achuthan S. et al demonstrate that drug-induced senescence generates chemoresistant stem-like cells with low ROS in breast cancer (15). In addition, a lower level of intracellular ROS is also present in breast CSCs, by which may
contribute to radioresistance (16). Overall, these studies have led to the hypothesis that the maintenance of low ROS levels within the CICs is important for regulating chemoresistance and quiescent state.

Cancer cells are capable of maintained at an oxidation-reduction reactions (redox) homeostasis state by up regulating ROS scavenging enzymes, which can confer drug resistance (17). Superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), and peroxiredoxin are major intracellular ROS scavenging enzymes (17). Further, accumulating evidence supports that elevated activity of SOD and catalase are associated with promoting cancer cell resistance to anticancer agents (15,18,19).

In the current study, we determined the tumorigenic potential and stemness properties of subpopulation cells with the differential intracellular level of ROS in HNSCCs and HN-CICs. We showed that the HN-CICs possessed more ROS\textsubscript{Low} cells than HNSCCs did. Then, we demonstrated the cells of low level of ROS highly co-expressed the stem cell markers (CD133, memGrp78 and Glut3) and the subpopulation of ROS\textsubscript{Low} cells was expanded in ALDH\textsuperscript{+} cells than in ALDH\textsuperscript{-} cells. Further, three types of cell with differential level of ROS: the high, middle, and low level of ROS (termed “ROS\textsuperscript{High}”, “ROS\textsuperscript{Mid}”, and “ROS\textsuperscript{Low}”) were isolated, respectively. The isolated ROS\textsubscript{Low} cells displayed CICs properties and possessed high tumorigenicity. Lower ROS level in CICs was also associated with chemoresistance; and pharmacological depletion of ROS scavengers enhanced the chemosensitivity but diminished the clonogenicity of HN-CICs. In summary, our studies suggest that distinct subpopulation of cells with differential ROS level exerted diverse stemness properties, chemoresistance and tumorigenicity in HNSCC; and eliminating ROS\textsuperscript{Low} cells should be considered for further exploration on therapeutic development for HNSCC.

Together, abrogation of drug-resistant mechanisms by depletion of ROS scavengers in CICs could have significant therapeutic implications in the future.

**Materials and Methods**

**Cell lines**

Human HNSCC cell lines, tongue carcinoma cells (SAS), obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) (20) were cultured in DMEM medium with 10% FBS (Grand Island, NY). Human gingival squamous carcinoma cells (OECM-1) were provided from Dr. C. L. Meng (National Defense Medical College, Taipei, Taiwan) and
grown in RPMI supplemented with 10% FBS. Cells were cultured at 37°C containing 5% CO2.

Establishment of the primary cells from HNSCC patient
This research follows the tenets of the Declaration of Helsinki and all samples were obtained after informed consent from the patients. The clinical samples were approved and in accordance with the institutional review board (IRB No: 2012-07-035BCY), Taipei Veterans General Hospital. Primary HN-CICs were established from HNSCC patients that derived from specimens of oral surgical resection. The primary HN-CICs were cultured in serum-free DMEM/F12 medium (GIBCO), N2 supplement (GIBCO), 10 ng/mL Epidermal Growth Factor (EGF) and 10 ng/mL human recombinant basic fibroblast growth factor-basic (FGF) (R&D Systems, Minneapolis, MN). Otherwise, the primary HNSCC were cultured in RPMI supplemented with 10% FBS (Grand Island, NY).

Cell lines cultivation and enrichment of HN-CICs
The two cell lines SAS and OECM1 were plated at a density of 7.5×10⁴ live cells/10-mm dish, then cultured in tumor sphere medium consisting of serum-free DMEM/F12 medium (GIBCO), N2 supplement (GIBCO), 10 ng/mL human recombinant basic fibroblast growth factor-basic (FGF) and 10 ng/mL Epidermal Growth Factor (EGF) (R&D Systems, Minneapolis, MN). The medium was changed every other day until the tumor sphere formation was achieved to enrich the SAS-HN-CICs or OECM1-HN-CICs in about 4 weeks (7).

Establishment of cisplatin-resistant cell line
The cisplatin resistant (SAS-cisPtR) cells were generated by serially fractionated exposure of the parental SAS cell lines to cisplatin over a time period of 3 month. At 5 µM of cisplatin killed more than 50% of cells for 48 h, cells were allowed to recover over 20 days with fresh medium and repeated three times. The cisplatin resistant (SAS-cisPtR) cells were for further experiments (Supplementary Figure S1).

Cell viability and chemo-resistance assay
Cells were seeded onto 24-well culture plates at 1 × 10⁴ cells/well for 24 hours. Then the test drugs were added to the culture medium for 72 hr. Subsequently, 5 µl of MTT solution (4 mg MTT/ml PBS) was added to each well and the cells were further incubated at 37°C for 3 hours until a purple Formazan was visible. The staining solution was removed and 100 µl DMSO was added at room temperature in the dark for 30 min. The absorbance of DMSO solution was detected with a microtiter plate reader at 560 nm. The cell viability ratio is
calculated as OD560 of experimental groups/OD560 of control groups.

**ROS assay and cell sorting**

To detect the intracellular ROS levels, single-cell suspension was resuspended in PBS contain 2% FBS at 10⁶ cells/mL, and stained with 10 μM DCF-DA, 5 μM CM-H2DCFDA, 2.5 μM CellROX Deep Red or 5 μM Carboxy-DCFDA (Invitrogen) at 37°C for 30 min, respectively. Cells were washed and resuspended in 10 μM propidium iodide (PI) solution for analysis or sorting by using FACSARia cell sorter (Becton Dickinson). For all FACS experiments, the dead cells were excluded by PI staining.

**Anchorage independent growth assay**

Each well (35 mm) of a six-well culture dish was coated with 2 ml bottom agar (Sigma-Aldrich) mixture (DMEM, 10% (v/v) FBS, 0.6% (w/v) agar). After the bottom layer was solidified, sorted cells were cultured in 2 ml top agar-medium mixture (DMEM, 10% (v/v) FBS, 0.3% (w/v) agar), and the dishes were incubated for 2 weeks at 37°C. Subsequently, plates were stained with 0.005% Crystal Violet then the colonies were counted. The number of total colonies was counted over five fields per well for a total of 15 fields in triplicate experiments.

**In vivo tumorigenic assay**

All animal studies were approved and in accordance with the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University, Taipei, Taiwan (IACUC approval No. 1001223). Equal volume of cells and Matrigel (BD bioscience, San Diego, CA, USA) was mixed and subcutaneously injected into the back of nude mice (6–8 weeks). Tumor volume (TV) was calculated using the following formula: (Length × Width²) / 2.

**Results**

**Quantification of intracellular ROS level and co-expression of CIC surface markers in parental HNSCCs cells, sphere cells and primary cells established from HNSCC tumor tissue.**

It has been demonstrated that cells with low intracellular ROS concentration are more likely to maintain their stem properties in hematopoietic stem cells (14). In addition, the association of low ROS level and radioresistance in breast cancer stem cells has been reported (16). Previously, we have successfully enriched a subpopulation of HN-CICs derived from both SAS and OECM1 HNSCCs (7,9). Herein, we first aim to understand whether the intracellular level of ROS is relatively lower in HN-CICs. To measure the
intracellular ROS level of HN-CICs, the cells were stained with oxidation sensitive fluorescent probe (DCFDA, CM-DCFDA or CellROX Deep Red, respectively) followed by flow cytometry analysis (14,16). Compared to parental HNSCCs, we observed that a subpopulation of cells with low intracellular level of ROS was significantly increased in HN-CICs (for both SAS-S and OECM1-S) (Figure 1A, Figure 1B and Supplementary Fig. S2A). In addition, primary cells (Primary-S) freshly established from HNSCC patient tumor tissue grown in defined serum free medium with bFGF and EGF growth factors (the medium to grow the SAS-S and OECM1-S cells) also displayed more cells with low ROS content in comparison to the same cells cultivated in medium supplied with 10% fetal bovine serum (Primary-P) (the medium to grow the SAS-P and OECM1-P cells) (Primary-S: 12.2% vs Primary-P: 4.3%)(Figure 1A). We next sought to determine whether the fluorescence change were attributable to altered by the dye oxidation and not altered by uptake, ester cleavage, or efflux of the probe. As shown in Supplementary Fig. S2B, these results demonstrated that no significant difference of SAS-P and SAS-S cells on staining of the oxidation-insensitive analog of Carboxy-DCFDA as the negative control (21) (Supplementary Fig. S2B).

Our previous data demonstrates that both memGRP78 and CD133 could be used as a surface marker for enrichment of HN-CICs (8,9). Next, we wanted to determine whether there was a positive correlation between the lower level of ROS and the abovementioned cancer initiating cell surface markers of HN-CICs. Examined by FACS analyses, the HN-CICs showed elevated co-expression of either memGRP78 or CD133 with lower level of ROS in comparison to the parental HNSCCs (Figure 1C and 1D). Further, the expression of Glut3, a recently identified cell surface marker of brain tumor stem cells (22), was also highly correlated to the cells containing low intracellular ROS amount (Figure 1E). Finally, it has been shown that cells harboring higher aldehyde dehydrogenase (ALDH) activity display the characteristics of cancer stem cells (23). As expected, the sorted ALDH+ cells also displayed more low ROS cells than the sorted ALDH− cells in HNSCCs (Figure 1F). To further clarify the correlation between ALDH activity and representative CIC markers of cells, SAS sphere cells or cisplatin resistant SAS cells (SAS-cisPtR) were selectively double-stained with CD133/Glut3 or Grp78/Glut3. The ALDH activity of the double-stained cells was also examined by ALDEFLUOR assay. Interestingly, we found that cells subpopulation which was positively double-stained with the selected CIC markers (such as the CD133+Glut3+ or memGrp78+Glut3+ subpopulation) also containing the highest level of ALDH activity in comparison to the rest individual subpopulations, respectively (Supplementary Fig. S1). The abovementioned observation suggests that the ALDH+ subpopulation cells mainly overlap with the same subpopulation of cells which are Grp78+CD133+Glut3+ in HNSCC cells. Collectively, these results suggest that lower level of ROS is closely related to cancer stemness properties in HN-CICs.

Interestingly, we also observed a subpopulation of cells containing higher level of
endogenous ROS in HN-CICs (Figure 1G). The high ROS level may be reflective of an “activated” state to mimic the proliferative activity of progenitor cells in neural stem cells (24). Characterization of the ROS\textsuperscript{High} cells was further examined (see text later).

**ROS\textsubscript{Low} cells display cancer initiating cells properties**

To characterize the CICs properties of cells with differential level of intracellular ROS amount, we stained the HN-CICs with ROS-indicator dye DCFDA to separate cells into ROS\textsubscript{Low}, ROS\textsubscript{Medi} and ROS\textsubscript{High} subpopulations followed by flow cytometry (Figure 2A), and the stemness properties of the three isolated cell subpopulations were further assessed. Initially, we observed that the ROS\textsubscript{Low} cells maintained a spheroid-growing phenotype; however, both the ROS\textsubscript{Medi} and ROS\textsubscript{High} cells grew as an adherent phenotype under the cell culture condition containing 10% fetal bovine serum (Figure 2B). Further, the freshly isolated ROS\textsubscript{Low}, ROS\textsubscript{Medi} and ROS\textsubscript{High} cells cultivated within the medium containing 10% fetal bovine serum for 5 days, afterward; the intracellular ROS level of the cultivated cells was analyzed, respectively. We observed that the ROS\textsubscript{Low} cells remained as ROS\textsubscript{Low} cells whereas cells with low level of ROS were not detectable from the further cultivated ROS\textsubscript{Medi} and ROS\textsubscript{High} subpopulation cells by FACS analyses (Figure 2C).

Additionally, the expression level of stemness genes (Oct4 and Nanog) within the ROS\textsubscript{Low}, ROS\textsubscript{Medi} and ROS\textsubscript{High} cells was examined transcriptionally and translationally. As shown in figure 3A and 3B, we observed enhanced expression of mRNA transcript and protein level of stemness genes (Oct4 and Nanog) in ROS\textsubscript{Low} cells in comparison with those of ROS\textsubscript{Medi} and ROS\textsubscript{High} cells, respectively. Next, we performed tumor spheres formation assay for evaluating the self-renewal ability of ROS\textsubscript{Low}, ROS\textsubscript{Medi} and ROS\textsubscript{High} cells. Again, the ROS\textsubscript{Low} cells displayed better spheres-forming capability than ROS\textsubscript{Medi} and ROS\textsubscript{High} cells did (Figure 3C). Of note, the ROS\textsubscript{High} cells displayed an intermediate spheres-forming ability (Figure 3C and Supplementary Fig. S3A).

In reference to stem cell phenotype, stem cell can be further identified by property of smaller cell size (25-27). Additional evidence also suggests that terminally differentiated keratinocytes progressively increase their cell size during human keratinocyte maturation (28). We therefore analyzed cell size of ROS\textsubscript{Low}, ROS\textsubscript{Medi} and ROS\textsubscript{High} cells by physical parameters forward and side scatter (FSC: cell size and SSC: cell granularity). As shown in figure 3D, the size of ROS\textsubscript{Low} cells was smaller than that of ROS\textsubscript{Medi} and ROS\textsubscript{High} cells.

**A low endogenous ROS status of HNSCCs maintains the quiescent state of cancer initiating cells**

Stem cells are present in a quiescent state but are able to exit quiescence by ROS activation; in addition, the quiescent state appears to be necessary for retaining the self-renewal of stem cells, and is a critical factor in the resistance of CICs to chemotherapy.
and targeted therapies (29). Next, we sought to determine whether the sorted ROS\textsuperscript{Low} cells possess the abovementioned stem cell perspectives. To assess the quiescent state of cells, we performed cell cycle analysis by double staining the cells with proliferative marker Ki-67 and PI for DNA content. As expected, we found that the sorted ROS\textsuperscript{Low} cells were present in a relatively quiescent state (Ki-67\textsuperscript{+}/PI\textsuperscript{+}: 2.5%), whereas the sorted ROS\textsuperscript{High} cells displayed the highest percentage of proliferative activity (Ki-67\textsuperscript{+}/PI\textsuperscript{+}: 49.5%) (Figure 3E). The above observation was further confirmed by immunoblotting with cell-cycle exit index (BrdU and Ki67). As shown in Figure 3F, we discovered that the sphere cells expressing high oxidative state (yellow arrows) exhibited a significantly higher proliferative activity in comparison to the cells expressing lower oxidative state (white arrows). We next sought to determine if increasing of intracellular superoxide level by NADPH oxidase activation could trigger cell cycle entry of the quiescent ROS\textsuperscript{Low} cells which were largely resided in sphere cells? Strikingly, the sphere cells treated with NADPH oxidase activator H\textsubscript{2}O\textsubscript{2} (30) or arsenic (31) displayed more adhesive cells but less sphere cells under microscopic observation (Supplementary Fig. S3B). These data indicate that the low ROS state is associated with the quiescent state of stem cells; on the contrary, the high ROS state is correlated to proliferative activity in HNSCCs.

\textit{ROS\textsuperscript{Low} cells show enhanced malignant potentials in vitro and in vivo}

To evaluate the differential malignancy among ROS\textsuperscript{Low}, ROS\textsuperscript{Medi} and ROS\textsuperscript{High} cells, the in vitro anchorage independent growth ability, and the in vivo xenografts assay were performed. The sorted ROS\textsuperscript{Low} cells significantly grew larger and more colonies than the ROS\textsuperscript{Medi} cells and ROS\textsuperscript{High} cells did in soft agar (Figure 4A). In vivo, the ROS\textsuperscript{Low} cells also showed the best tumor-initiating ability, being able to form tumor with only 500 inoculated cells, while the ROS\textsuperscript{High} cells needed at least 1X10\textsuperscript{4} cells to grow the tumors; that concludes the ROS\textsuperscript{Low} cell is 20 fold more tumorigenic than the ROS\textsuperscript{High} cells. Nevertheless, the ROS\textsuperscript{Medi} cells did not generate any tumors under the examined conditions (from 5X10\textsuperscript{2} to 1X10\textsuperscript{4} cells) (Figure 4B and 4C).

To further verify the hypothesis that low level of ROS is pivotal for maintenance of cancer stemness properties and responsible for tumor growth in nude mice, the stemness properties within the newly established primary cells derived from ROS\textsuperscript{Low} and ROS\textsuperscript{High} generated tumors were further analyzed, respectively (see Materials and Methods for establishment of primary cells from xenografted tumors). In the beginning, the newly established primary culture cells derived from ROS\textsuperscript{Low} generated tumor formed spheroids promptly. Empirically, after 3 days of cultivation, the sphere cells became adherent when the non-serum culture condition was switched to DMEM medium containing 10% serum (Figure 4D). However, we did not observe any spheroid formation from the primary culture cells, which were derived from tumors generated by ROS\textsuperscript{High} cells. Interestingly, the
primary culture cells established from ROS\textsuperscript{Low} derived tumors kept more ROS\textsuperscript{Low} cells (11.5\%) than the cells established from ROS\textsuperscript{High} derived tumors did (7.3\%)(Figure 4E). Additionally, we observed more\textsuperscript{mem}GRP78 positive cells in primary culture cells established from ROS\textsuperscript{Low} derived tumors (10.0\%) than the cells established from ROS\textsuperscript{High} derived tumors (5.5\%)(Figure 4F).

Collectively, these results suggest that the sorted ROS\textsuperscript{Low} cells are more tumorigenic and possess CICs properties in comparison with the ROS\textsuperscript{High} and ROS\textsuperscript{Mid} cells.

**HN-CICs are more chemoresistant and cisplatin treatment enriches the ROS\textsuperscript{Low} cells**

To further understand whether HN-CICs are more chemoresistant than the HNSCC cells, we examined the cytotoxic effect of cisplatin treatment on both HNSCC and sphere cells with enriched HN-CICs. Being treated with cisplatin for 72 hours, both the parental SAS and OECM1 cells gave an IC50 around 7.5 μM, however, the SAS-sphere cells or OECM1-sphere cells showed a similar IC50 of more than 20 μM to the cisplatin treatment (Figure 5A). Further, the SAS-sphere cells displayed an enhancement of ROS\textsuperscript{Low} cells in a dose-dependent manner under cisplatin treatment (Figure 5B). In addition, we found an approximately 4-fold reduction in the percentage of ROS\textsuperscript{High} cell in cisplatin treatment group versus the untreated group (Figure 5C; untreated: 20\% vs. cisplatin treated: 5\%), suggesting that ROS\textsuperscript{High} cell are relatively chemosensitive in comparison to ROS\textsuperscript{Low} cells, in SAS-sphere cells.

Cancer stem properties are enhanced during the selection of cisplatin resistance in HNSCC (32). To further evaluate the correlation between low ROS level and cancer stemness properties, we established a cisplatin resistant HNSCC cell line (SAS-cisPt\textsuperscript{R}) from SAS cells by progressive cisplatin treatment (Supplementary Fig. S4; and see details from Material and Methods). Obviously, the cell subpopulation with low level of ROS was significantly enhanced in cisplatin resistant HNSCCs (SAS-cisPt\textsuperscript{R}) (Figure 5D). It has also been shown that cells harboring higher aldehyde dehydrogenase (ALDH) activity display the characteristics of cancer stem cells (23). Therefore, we wanted to understand the relationship between ALDH activity and cisplatin resistance in HNSCCs. Empirically, we found that SAS-cisPt\textsuperscript{R} cells increased the percentage of ALDH positive (ALDH\textsuperscript{+}) cells (Figure 5E).

**ROS scavenging and pharmacological modulation of ROS level affect the chemosensitivity of HN-CICs**

In light of recent findings that conventional chemotherapy may not be in sufficient effect, where the increased expression of ROS defense genes is accompanied within CICs (16,33,34). Given our observations of HN-CICs containing more ROS\textsuperscript{Low} cells, we proposed that these cells may possess elevated level of ROS-scavenging enzymes compared to HNSCCs. Therefore; we compared the expression profile of ROS scavenger genes of the enriched
HN-CICs (from SAS and OECM1) to that of parental cells (SAS and OECM1) by Affymetrix microarray analyses. As noticed, the transcripts of some ROS scavenger genes were up-regulated in enriched HN-CICs (Fig. 5F and Supplementary Fig. S5). Further, this Affymetrix microarray analysis showed significant increasing trend of superoxide dismutase 2 (SOD2) catalase (CAT) and peroxiredoxin 3 (PRDX3) in the enriched HN-CICs (Fig. 5F). Both SOD2 and CAT are antioxidant enzymes, and are associated with regulation of cellular ROS and the acquired drug resistance in cancer (15,17,35). Consistent to microarray results, RT-PCR analyses showed that the expression of SOD2 and CAT transcript was increased in enriched HN-CICs than that in parental HNSCCs (Fig. 5G). Further, the catalase enzymatic activity of HN-CICs was measured by catalase activity assay. As shown in Supplementary Fig. S6, HN-CICs exhibited a higher catalase activity as compared with HNSCC. However, we did not see significant difference of the PRDX3 transcript among the HN-CICs and HNSCCs (Fig. 5G). We observed that low ROS level was in association with upregulation of antioxidant enzymes such as SOD2 and CAT in HN-CICs (Figure 5G). Next, we aimed to pharmacologically inhibit or genetically diminish the activity of ROS scavenger enzymes, then to abrogate the chemoresistance and stemness properties of CICs’. Pharmacological and genetic inhibition of SOD2 or catalase, respectively, both reduced the ROS\textsuperscript{Low} subpopulation cells in SAS-sphere cells (Figure 5H; around 8%, Supplementary Figure S7A and S8A). In opposite, single cisplatin treatment enhanced the ROS\textsuperscript{Low} cell subpopulation in HN-CICs (Figure 5H; 22% and Supplementary Figure S7A). However, combined treatment with cisplatin plus 2-ME or 3-AT significantly lowered the ROS\textsuperscript{Low} cell subpopulation compared to single treatment with cisplatin in HN-CICs (Figure 5H; and Supplementary Figure S7A). Further, triple treatment with 2-ME, 3-AT plus cisplatin showed the most significant effect on reducing the ROS\textsuperscript{Low} cell subpopulation in HN-CICs (Figure 5H; around 2% and Supplementary Figure S7A). Next, we wanted to address whether SOD2 and/or CAT inhibition in combination with/without cisplatin treatment would reduce the cell viability in HN-CICs. As shown in figure 6A, the cell viability of CICs singly or combinatorially treated with 2-ME, 3-AT and/or cisplatin was analyzed by MTT assay. Along with the reduction of ROS\textsuperscript{Low} cell subpopulation in HN-CICs under triple treatment of 2-ME, 3-AT plus cisplatin (Fig. 5H), we also observed that the cell viability of the triple treated HN-CICs was the most significantly diminished (left with around 35%) by MTT assay (Fig. 6A).

Finally, to further examine whether pharmacological modulation of ROS levels contributed to cell death in HN-CICs, SAS-sphere cells were singly or combinatorially treated with 2-ME, 3-AT or cisplatin. The treated cells undergoing cell death was determined by co-stained with Annexin V and PI and examined by flow cytometry. Single treatment of 2-ME or 3-AT caused a slight increase of cell death in HN-CICs. Additionally, combined treatment with 2-ME or 3-AT sensitized the cytotoxicity of cisplatin treatment in HN-CICs.
Moreover, the triple treatment with 2-ME and 3-AT plus cisplatin was the most effective and induced around 45% of the cell population undergoing cell death (Figure 6B and Supplementary Figure S7B). Together, these data demonstrate the importance of antioxidant defense to maintain the low ROS level, cell viability and chemosensitivity of HN-CICs.

**Antioxidant ability is required to maintain the stemness properties and malignancy of HN-CICs.**

To further evaluate whether the antioxidant ability is important to sustain the stemness properties of HN-CICs, the SAS-sphere cells were treated with 2-ME, 3-AT or cisplatin alone or in combination. As shown in figure 6C, the sphere cell singly treated with 2-ME or 3-AT displayed decreased expression of “cancer stemness” proteins (Oct-4 and Nanog). In opposite, we observed that sphere cell singly treated with cisplatin showed higher protein levels of Oct-4 and Nanog but did not affect the expression of epithelial differentiation marker, cytokeratin 18 (CK-18) and Involucrin (Fig. 6C, Fig. 6D, Fig. 6E and Supplementary Figure S7C). However, pharmacological and genetic inhibition of SOD2 or catalase caused the increase of CK18+ subpopulation cells but the decreased expression of CIC marker CD44 in HN-CICs (Figure 6D, Supplementary Figure S8B and S8C). In addition, the sphere/anchorage independent colony formation abilities of HN-CICs under genetic inhibition of SOD2 or catalase were also significantly abolished (Supplementary Figure S8D and S8E). Interestingly, HN-CICs undergoing pharmacological or genetic inhibition of catalase rather than inhibition of SOD2 displayed a more potent efficiency to cause the cell differentiation (Fig. 6D; 3-AT: 40%, 2-ME: 20%, Fig. 6E and Supplementary Figure S8B). Nevertheless, the triple treatment of 3-AT, 2-ME plus cisplatin showed the most potential to enhance the CK18 positive cells (Figure 6D; around 56% and Fig. 6E). In the mean times, triple treatment of 3-AT, 2-ME plus cisplatin significantly inhibited the sphere formation ability of HN-CICs, whereas relatively larger size spheres were observed from single cisplatin treated HN-CICs (Fig. 6F).

We next sought to determine whether inhibition of the activity of ROS scavenger enzymes could attenuate the malignancy of HN-CICs both in vitro and in vivo. As shown in figure 6G, the anchorage independent growth abilities of SAS-sphere cells under triple treatment were significantly abolished. In addition, triple treatment of 3-AT, 2-ME plus cisplatin to SAS sphere cells or SAS-cisPtR cells significantly reduced the tumor volumes by xenograft transplantation assay (Fig. 6H and Fig. 6I). Overall, combined treatment with 2-ME and 3-AT reduced the self-renewal ability of HN-CICs, and sensitized the in vitro cytotoxicity mediated by cisplatin treatment on HN-CICs.

Catalase has been reported to be highly correlated with tumor malignant grade (36). We therefore proceeded to evaluate whether overexpression of catalase could enhance stemness
properties. Treatment of SAS sphere cells with PEG-CAT displayed not only elevated expression of catalase activity but also increased percentage of ROS$_{low}$ cells (Supplementary Figure S9A and S9B). Furthermore, we demonstrated that catalase overexpression also resulted in increased expression of CIC markers ($^{\text{mem}}$Grp78, CD44 and ALDH activity). (Supplementary Figure S9C, S9D and S10A). To further understand whether treatment of cisplatin, 3-AT or 2-ME can cause significant cytotoxicity to normal stem cells, the hematopoietic stem cells (HSC) were first treated with the above drugs and subjected to MTT assay, respectively. We observed that single treatment of cisplatin or 2-ME decreased the cell viability of HSC cells. However, there was no significant inhibition of cell viability on 3-AT treated HSC cells (Supplementary Fig. S11).

Discussion

Patients with HNSCC are still very likely to relapse within months after therapy (37) that may be because conventional treatments cannot efficiently eliminate CICs, which are involved in the tumor progression, metastasis, and chemo/radio resistance (2). Most of the anticancer drugs kill cancer cells by induced ROS generation, but prolonged treatment with the drugs result in reduced ROS level as a result of therapy resistance (38,39). Caraglia M. et al. provide evidence that the determination of oxidative stress status could be a marker of drug efficacy in cancer patients (40). Thus, we hypothesized CICs are resistant to therapies due to their lower intracellular ROS content compared to the differentiated cancer cells. Moreover, understanding the relationships between ROS and drug resistance of CICs is important to improving the efficacy for future chemotherapies.

In the present study, we show that HN-CICs contain lower concentrations of ROS than parental cancer cells. Consequently, cell subpopulations including ROS$_{low}$, ROS$_{medi}$ and ROS$_{high}$ with differential ROS level were isolated. Notably, ROS$_{low}$ cancer cells are closely associated with CD133, $^{\text{mem}}$Grp78, Glut3 and ALDH positive cells (Figure 1). We observed that the ROS$_{low}$ cells displayed a sphere-growing phenotype, in contrast, both the ROS$_{medi}$ and ROS$_{high}$ cells grew as an adherent phenotype under the same culture condition (Figure 2). Further, ROS$_{low}$ HN-CICs cells displayed CICs properties in comparison to ROS$_{medi}$ and ROS$_{high}$ subpopulation cells. Furthermore, cell-cycle studies indicated that ROS$_{low}$ cells existed in lower Ki-67 activity (Figure 3). Importantly, an in vivo nude mice model demonstrates that ROS$_{low}$ population possessed high tumorigenic potential (Figure 4). Additionally, HN-CICs seem able to regulate ROS to exhibit chemoresistance by increasing production of antioxidant genes (Figure 5). Pharmacological depletion of ROS scavengers in CICs reduced their clonogenicity and resulted in chemosensitization to cisplatin both in vitro and in vivo (Figure 6). Collectively, our data first demonstrated the crucial role of low ROS level for the tumorigenicity and chemoresistance of HN-CICs. In addition, distinct subpopulation of cells with differential intracellular ROS level in HNSCC exhibit diverse
proliferative activity, CICs properties and chemoresistance.

Cells containing ROS mediated high chemically reactive byproducts are implicated in stress and disease (41). Increasing evidence suggests that low levels of ROS are critical for maintaining the self-renewal and stemness, while high levels of ROS effectively shut down self-renewal and confer potent capacity for stem cell differentiation (12,42). In addition, CICs have similar redox properties as normal stem cell (43). Diehn M. et al. have reported that CD44^+CD24^- breast CICs have a unique mechanism that protects themselves from ROS through increased antioxidant defenses and unique redox-dependent effects on tumor radioresistance. They also found that breast CIC-enriched population is association with genes involved in glutathione synthesis, including Gclm, Gss, and FoxO1 (16). For glioblastoma, it has been reported that glioblastoma stem cells (GSCs) are assumed to constitute a radio-resistant fraction by HIF-2α-mediated ROS status (33,44). However, a relationship between ROS and chemoresistance property in HN-CICs remains elusive. In the present study, we demonstrated that lower ROS levels in HN-CIC enriched population are associated with increased expression of ROS scavenger such as catalase and SOD2. Pharmacological depletion of ROS scavengers (SOD2 and/or catalase) in CICs markedly enhanced the cytotoxicity of cisplatin (Figure 6). Notably, it is evident that catalase and SOD2 has been implicated in chemotherapy resistance of cancer cells (15,45). Based on these findings, we proposed that different cancer stem cells in diverse antioxidant systems have conserved this attribute, which probably helps protect their genomes from ROS-mediated damage. Previous studies also show that signaling molecules such as FoxOs, APE1/Ref-1, NrF2, ATM, HIFs, p38 and p53 are involved in the regulation of stem cell self-renewal and differentiation through modulation in antioxidant enzyme systems (11). Therefore; future research delineating the details of what ROS signaling molecules are essential to maintaining stemness properties of CICs in these different cell types are remained to be determined.

CICs in colon, breast and ovaries have been shown to demonstrate have the ability to maintain quiescent state to evade therapy (46-48). In addition, Dey-Guha I. et al. report that rapidly proliferating cancer cells can produce “G0-like” progeny through asymmetric division which are enriched following chemotherapy in breast cancer. These G0 phase cells show lower intracellular ROS level and exhibit suppressing AKT expression (49). Herein, we also found that low endogenous ROS status of HNSCCs maintains the quiescent state of CICs. Surprisingly, we observed ROS^{high} cells exhibit high proliferation index (Figure 3E and 3F). Recently, many reports demonstrate higher ROS state is essential for proliferation of stem/progenitor cells (14,50). For example, ROS can play roles as second messengers in tightly neural stem cell proliferation and survival by driving PI3K/AKT signaling (24). Additionally, it is evident that ROS generation is essential for K-ras mediated cell proliferation and tumorigenesis in lung cancer (51). Based on these findings, we proposed that CIC-enriched populations are partially differentiated due to persistent ROS redox stress.
on the basis of tumor microenvironment changes, which leading to a heterogeneous mixture of CICs and non-CICs in the tumor. It would be of interest to determine the regulations of these signaling pathways of ROS<sub>Low</sub> and ROS<sub>High</sub> HN-CICs, respectively. These studies also suggest that combination of inhibitors for ROS<sub>Low</sub> and ROS<sub>High</sub> regulator of the signaling pathway might be more effective compared with blockade of single regulating HN-CICs.

Most conventional anticancer drugs such as cisplatin and 5FU are preferentially toxic to proliferating cells, decreases tumor size (52). Herein, we have also confirmed that long term treatment of cisplatin can enhance the ROS<sub>Low</sub> cells of CICs properties, and make them prone to survive (Figure 5D and 5E). Further, combinatorial treatment of scavenger inhibitors for ROS<sub>Low</sub> and conventional anticancer drugs reduced the self-renewal ability and survival in HN-CICs (Figure 5 and 6). In the mean times, it is effective to target the proliferative ROS<sub>High</sub> cells by long term treatment of cisplatin (Figure 5C). Therefore, it will be reasonable that co-treatment with a ROS<sub>Low</sub> inhibitor along with a ROS<sub>High</sub> inhibitor as a chemotherapeutic regimen may improve the future treatment of HNSCCs.

Together, our present research shows HN-CICs contain distinct subpopulation cells and the elevated ROS<sub>Low</sub> cells in HN-CICs mainly contribute to tumor progression and chemoresistance (Fig. 7). Clinical therapies could perhaps be optimized by overcoming low ROS levels and identification of regulatory ROS level mechanisms in CICs may be a useful prognostic factor for HNSCC patients.

**Acknowledgements**

The Authors thanks Dr. K-W Chang (Department of Dentistry, National Yang-Ming University) for providing critical comment.
References
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Figure legends

Figure 1. Quantification of intracellular ROS level and co-expression of cancer stem cell surface markers in parental HNSCCs (SAS-P and OECM1-P), sphere cells (SAS-S and OECM1-S, derived from parental HNSCCs) and primary cell lines established from HNSCC patient tumor tissue.

(A) Single-cell suspension from parental HNSCCs, sphere cells or Primary HNSCC cells was stained with DCFDA. Then, the intracellular level of ROS in parental HNSCCs, sphere cells and Primary cells was determined by FACS analyses. (B) The percentage of CM-DCFDA+ cells in parental HNSCCs and sphere cells was analyzed by flow cytometry. The co-expression profile between low ROS level and (C) memGrp78, (D) CD133 and (E) Glut3 in HNSCCs and sphere cells was examined by FACS. (P: Parental HNSCCs; S: HNSCCs derived sphere cells; Primary-P: primary HNSCC cultivated in serum containing medium; Primary-S: primary HNSCC cells cultivated in defined medium without fetal bovine serum (details seen in Materials and Methods)). (F) The percentage of low ROS level cells in isolated ALDH1+ and ALDH1+ HNSCCs, respectively. (G) Quantification of ROSHigh cells was determined by co-plotting the expression profile of ROS content of SAS-P and SAS-S cells stained by DCFDA and analyzed by flow cytometry. Results are means ± SD of triplicate samples from three experiments (***, p < 0.001).

Figure 2. Isolation of ROSLow, ROSMedi and ROSHigh cells.

(A) ROSLow, ROSMedi and ROSHigh cells were sorted using DCFDA staining as the intracellular ROS indicator from SAS sphere cells. (B) Representative images of freshly sorted ROSLow, ROSMedi and ROSHigh cells, grown under standard medium containing 10% fetal bovine serum for 1 and 5 days, were shown by phase contrast microscopy (arrow: spheroids). (C) At day 5, the intracellular ROS content of further cultivated ROSLow, ROSMedi and ROSHigh cells was re-analyzed by flow cytometry.

Figure 3. Stem cells properties and physiological characteristics of ROSLow, ROSMedi and ROSHigh cells.

Expression of pluripotent stemness genes (Oct4 and Nanog) in ROSLow, ROSMedi and ROSHigh cells was determined by (A) Q-RT-PCR and (B) immunoblot analyses. The amount of GAPDH was referred as loading control. (C) Representative images of tumor-sphere-forming ability of ROSLow, ROSMedi and ROSHigh cells grown under defined serum-free selection medium were shown. The numbers of tumor spheres were further calculated using microscope. (D) Representative plots according to the size (FSC) and structure (SSC) of the sorted ROSLow, ROSMedi and ROSHigh cells were shown by FACS analyses. (E) The proliferative activity of sorted ROSLow, ROSMedi and ROSHigh cells was stained with Ki-67/PI and further analyzed by flow cytometry. Results are means ± SD from three experiments (***,
p < 0.001). (F) Intracellular localization of BrdU labeling (green) and Ki-67 (red); and content of ROS (orange) were determined by immunofluorescence staining. Nuclei were stained with DAPI (blue) (yellow arrows indicating ROSHigh cells; white arrows indicating ROSLow cells).

Figure 4. Distinct in vitro malignancy and in vivo tumorigenic properties of ROSLow, ROSMedi and ROSHigh cells.

(A) To elucidate the anchorage independent growth ability, single cells suspension of ROSLow, ROSMedi and ROSHigh cells were plated onto soft agar and further analyzed. (B) Summary of the in vivo tumor growth ability of sorted ROSLow, ROSMedi and ROSHigh cells examined by xenotransplantation analyses (left panel). Representative tumors, which were generated by inoculating the ROSLow, ROSMedi and ROSHigh cells into the subcutaneous space of recipient nude mice, was recorded on day 56, respectively (right panel). (C) Tumor growth curves were measured after inoculation of ROSLow, ROSMedi and ROSHigh HNSCCs subcutaneously into nude mice (left panel: 1X10^4 cells; right panel: 1X10^3 cells). (D) Growth pattern of primary culture cells, which were derived from ROSLow or ROSHigh cell generated tumors, respectively, cultured under standard medium containing 10% serum. In the beginning, primary cells derived from ROSLow tumor formed spherical clusters of cells. In opposite, ROSHigh tumor derived cells immediately attached to the culture dish (upper panels). After incubation for 3 days, the ROSLow tumor derived cells were proliferative from spherically aggregated cell clusters (lower panels). Expression profile of the intracellular (E) ROS content and (F) memGrp78 of newly established cell lines, which were derived from ROSLow and ROSHigh xenografted tumors was examined by FACS analyses, respectively. Error bars correspond to SD. Results are means ± SD of triplicate samples from three experiments (n=3; ***, p < 0.001).

Figure 5. Cisplatin resistance and low ROS stress in HN-CICs were mediated by ROS scavenge enzymes.

(A) HNSCC parental (SAS-P and OECM1-P) cells and HNSCC-sphere (SAS-S and OECM1-S) cells were treated with cisplatin for 72 hours. Cell viability of the cisplatin treated cells was further determined by MTT assay. (B) SAS Sphere cells were treated with 5, 10 and 20 μM cisplatin, respectively, for 72 hours; then stained by DCFDA. The ROSLow cells in cisplatin-treated HN-CICs cultures were determined by flow cytometry. (C) The intracellular level of ROS in SAS-P and cisplatin-resistant (cisPtR) SAS cells was determined by FACS. (D) ALDH activity positive cells (ALDH+) in SAS-P and SAS-cisPtR cells were measured by FACS analyses (details seen in Materials and Methods). (E) Differentially expressed genes of ROS scavengers in parental HNSCCs (SAS and OECM1) and sphere cells (SAS-S and OECM1-S) under 2, 3, 5, or 9 weeks of cultivation with defined serum-free selection medium.
were analyzed and recorded (see Materials and Methods add (7)). (F) The expression of ROS scavenger mRNAs in parental HNSCCs (SAS and OECM1) or sphere cells was detected by real-time RT-PCR analysis. To further examine the physiological effect of cisplatin treatment in combination of scavenger inhibitors on ROS\textsuperscript{Low} or ROS\textsuperscript{High} cells, respectively, SAS-Sphere cells were either singly treated with cisplatin or co-treated with ROS scavenger inhibitors (2-ME 15 µM or 3-AT 25 mM) for 72hr, afterward; the percentage of ROS\textsuperscript{Low} cells (G) or ROS\textsuperscript{High} cells (H) were further analyzed. For intracellular ROS content measurement, the drugs treated cell were first stained with DCFDA, and then examined by flow cytometry. Data are means ± SD of triplicate samples from three experiments (***, p< 0.005). The same concentration (0.03%) of vehicle (DMSO) was added to all control groups.

**Figure 6. Combined treatment with ROS scavenger inhibitors and cisplatin diminished the cell viability, the sphere formation ability and anchorage independent growth, and enhanced the cell death and the differentiation ability of HN-CICs.**

SAS-Sphere cells were either singly treated with cisplatin or co-treated with ROS scavenger inhibitors (15 µM 2-ME or 25 mM 3-AT) for 72hr. Afterward; the cell viability (A), and the cells undergoing cell death (B) caused by the drugs treatment were further analyzed. For cell viability analysis, the drugs treated SAS-Sphere cells were further examined by MTT assay (see Materials and Methods). To evaluate the cells undergoing cell death, the drugs treated SAS-sphere cells were stained with Annexin V plus PI, and then examined by flow cytometry. (C) Expression of pluripotent stemness proteins (Oct4 and Nanog) in SAS-P, SAS-S, cisplatin-treated SAS, 2ME-treated and 3AT-treated cells was determined by immunoblot analyses. (D) The CK18 positive cells with the same treatment was determined by staining drugs treated cells with CK18 antibody, and then examined by flow cytometry. (E) Protein level of epithelial differentiation markers, CK18 and Involucrin in drugs treated cells was assessed by western blot. (F) Single cell suspension of SAS-Sphere cells was treated with cisplatin or co-treated with ROS scavenger inhibitors (2-ME and/or 3-AT) for 72 hr and the sphere formation ability of drugs treated cells were examined (see Materials and Methods). Arrows indicated the sphere cells. (G) In addition, the abovementioned SAS-Sphere cells were also plated onto soft agar for 12 day, and the colony formation ability of drugs treated SAS-sphere cells was examined (see Materials and Methods). Data are means ± SD of triplicate samples from three experiments (***, p< 0.005). Representative images of tumors generated on the recipient nude mice, which were inoculated with drugs treated SAS-sphere (H) and SAS-cisPt\textsuperscript{R} (I) cells into the subcutaneous space, were photographed (white arrows: control group; black arrows: drugs treated group). Tumor volume was also measured and recorded after inoculation of drugs treated SAS-sphere (H) and SAS-cisPt\textsuperscript{R} (I) cells in nude mice.
Figure 7. Schematic depicts physiological features of ROS$^{\text{Low}}$, ROS$^{\text{High}}$ and ROS$^{\text{Medi}}$ cells related to chemoresistance, stemness properties, tumorigenicity, proliferative activity, etc.

Our results (see contexts) indicate that ROS$^{\text{Low}}$ cells are in a quiescent state, and possess high tumorigenicity and enhanced stemness properties that may result in the chemoresistance of CICs to chemotherapy. On the other hand, ROS$^{\text{High}}$ cells are actively proliferate and more sensitive to therapy and differentiation. Further, the ROS$^{\text{Medi}}$ cells exhibit more differentiated and non-tumorigenic.
Figure 2. Chang et al.

A

B

C

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 5. Chang et al.
Figure 7. Chang et al.

- Quiescent
- Tumorigenicity
- Stemness gene expression
- Chemo-resistance

- High proliferation
- Moderate tumorigenicity

- Moderate proliferation
- Differentiation
- Non-tumorigenicity

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Distinct Subpopulations of Head and Neck Cancer Cells with Differential Intracellular ROS Exhibiting Diverse Chemoresistance, Stemness and Proliferative Activity


Cancer Res Published OnlineFirst September 12, 2014.