Elevated expression of Nrf2 mediates multidrug resistance in CD133+ head and neck squamous cell carcinoma stem cells

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Abstract. Enhanced expression of the ATP-binding cassette (ABC) transporter protein ABC sub-family G member 2 (ABCG2) in cancer stem cells (CSCs) plays a major role in chemotherapeutic drug efflux, which results in therapy failure and tumor relapse. In addition to downregulating apoptosis in CSCs, it has been reported that the transcriptional upregulation of the redox sensing factor Nrf2 is involved in the upregulation of ABCG2 expression and consequent chemoresistance. The current study investigated the presence of cancer stem-like side population (SP) cells from head and neck squamous cell carcinoma (HNSCC) samples, and evaluated the Nrf2 expression profile and multidrug resistance properties of HNSCC stem cells. Fluorescence-activated cell sorting was used for SP cells detection, while reverse transcription-polymerase chain reaction was used for the analysis of Nrf2 expression. The present study identified ~2.1% SP cells present in HNSCC specimens, which were positive for cluster of differentiation (CD)133 expression and displayed significantly elevated messenger RNA expression of Nrf2, compared with non-SP cells. These data suggest that the ABC transporter ABCG2 is highly upregulated in SP cells, and this results in multidrug resistance. In addition, these CD133+ cells underwent rapid proliferation and exhibited high self-renewal and tumorigenic properties. Taken together, the present findings suggest that elevated expression of Nrf2 mediated drug resistance in HNSCC CSCs, which may be one of the causative factors for cancer treatment failure. Therefore, novel anti-cancer drugs that downregulate the Nrf2 signaling pathway could effectively improve the treatment and survival rate of patients with HNSCC.

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Introduction

The cancer stem cell (CSC) theory hypothesizes the presence of a small population of cells within the heterogenous cancer cell population termed CSCs or tumor-initiating cells, which are responsible for therapy failure and tumor recurrence in cancer (1,2). Previous studies conducted in several types of cancer reported that these CSCs exhibit a high potential for differentiation and self-renewal, as they possess increased expression of stem cell surface proteins and are highly tumorigenic *in vivo* and *in vitro* (1,2). It has also been reported that aberrantly regulated Wnt/beta-catenin and Notch1 signaling in CSCs are involved in multidrug resistance in these cells (3-6). The multidrug resistance properties of CSCs are due to the overexpression of the ATP-binding cassette (ABC) transporter protein ABC sub-family G member 2 (ABCG2), which acts as a drug efflux pump for DNA-targeting drugs, and causes cancer cells to escape from conventional cancer treatment strategies (7-9). Therefore, it is crucial to improve and design novel therapeutic drugs that could effectively eradicate refractory CSCs.

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies worldwide, and the life span of patients following diagnosis at metastatic stage is only 4 months (10). Despite recent innovations in cancer treatment strategies (6-8), the overall survival rate of HNSCC patients has not improved. Previous studies on HNSCC reported the persistence of a small population of CSCs that are the major cause for therapy failure (5,6). These CSCs have a high differentiation potential, are highly tumorigenic, and express stem cell surface markers, including cluster of differentiation (CD)44, CD133 and octamer-binding transcription factor 4 (10-12). Another remarkable feature of these CSCs is the fact that they are highly resistant to apoptosis and exhibit multidrug resistance, which confers them immortality (5-7). It has been previously reported that increased expression of stress-inducible transcription factors such as Nrf2 is also involved in ABC transporter-mediated drug efflux in CSCs (13). The current study attempted to identify and characterize CD133+ SP cells present in HNSCC samples, and to evaluate the expression of Nrf2 and ABCG2 in HNSCC CD133+ CSCs.

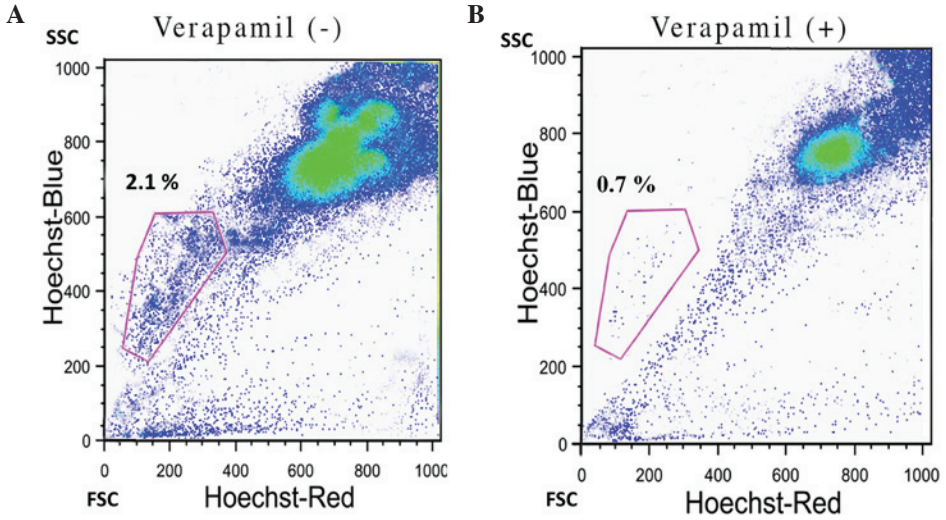

Figure 1. Representative dot plot images of fluorescence-activated cell sorting analysis. Live cells were selected by propidium iodide staining. (A) A total of 2.1% SP cells were identified in head and neck squamous cell carcinoma samples. (B) Upon treatment with the ATP-binding casette transporter inhibitor verapamil, the percentage of SP cells in the samples was reduced to 0.7%. SP, side population; FSC, forward scatter; SSC, side scatter.

Materials and methods

Cell culture from primary HNSCC samples. Human HNSCC samples were obtained from patients with HNSCC during surgery performed from March 2015 until December 2015 at The First Affiliated Hospital of Xinxiang Medical University (Weihui, China). The present study was approved by the ethics committee of The First Affiliated Hospital of Xinxiang Medical University. The primary tumor samples were minced with blades into small pieces, and then enzymatically digested with collagenase, hyaluronidase and DNase (all obtained from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), prior to be incubated for 2 h at 37˚C in the presence of 5% CO_2. Cells were disaggregated in phosphate-buffered saline and centrifuged at 350 x g for 20 min. The pellet was then resuspended in serum-free Dulbecco's modified Eagle's medium/F-12 (Gibco; Thermo Fisher Scientific, Inc.) containing human recombinant epidermal growth factor (20 ng/ml; Gibco; Thermo Fisher Scientific, Inc.) and human basic fibroblast growth factor (20 ng/ml; Gibco; Thermo Fisher Scientific, Inc.).

Fluorescence-activated cell sorting (FACS) analysis of HNSCC samples. Cells (~10^6 cells/ml) were subjected to FACS analysis. For that purpose, cells were divided into two groups: Group I, corresponding to cells labeled with 5 μl/ml Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO, USA) alone (n=7); and group II, corresponding to cells treated with verapamil drug (0.8 μl/ml; Sigma-Aldrich) in addition to Hoechst 33342 dye (n=7). Cells were next counterstained with propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.) at a concentration of 2 μg/ml, and subjected to FACS analysis in a FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ, USA), using a 610-nm dichroic short-pass filter, while the red and blue emissions were collected using 670/30-nm and 450/65-nm band-pass filters, respectively. Images of the cells were obtained using a fluorescence microscope at x40 magnification (BX63; Olympus Corporation, Tokyo, Japan).

In vitro proliferation, chemoresistance and sphere formation assay. These assays were performed as previously described (14). The DNA-targeting drugs used in these assays, including etoposide, gemcitabine, 5-fluorouracil, cisplatin, paclitaxel and oxaliplatin, were obtained from Sigma-Aldrich.

Tumor cell implantation. A total of ~$2x10^4$ SP and non-SP cells were administered to non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice by subcutaneous injection, as previously described (13,15). All mice (6-8 week-old males, n=7) were maintained in dedicated housings at a temperature of 25±1˚C, with constant access to food pellets and water *ad libitum*. Mice were sacrificed 4-5 weeks later, and the tumor size was measured according to the following formula: $V=(ab^2/2)$, where a is the long diameter and b the short diameter of the tumor (13). The maximum diameter noted was ~2.1 cm.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using TRIzol (Life Technologies; Thermo Fisher Scientific, Inc.) and subjected to RT with a Reverse Transcriptase kit (Fermentas; Thermo Fisher Scientific, Inc.). The sequences of the human specific primers used for PCR were as follows: ABCG2, forward 5'-TCAATCAAA GTGCTTCTTTTTTATG-3' and reverse 5'-TTGTGGAAG AATCACGTGGC-3'; Nrf2, forward 5'-ACACGGTCCACA GCTCATC-3' and reverse 5'-TGCCTCCAAAGTATGTCA ATCA-3'; and glyceraldehyde 3-phosphate dehydrogenase, forward 5'-ATGTCGTGGAGTCTACTGGC-3' and reverse 5'-TGACCTTGCCCACAGCCTTG-3' (IDT Shanghai Co. Ltd., Shanghai, China). The PCR parameters were as follows: Initial denaturation at 95˚C for 2 min, followed by 35 cycles of annealing at 58˚C for 45 sec, extension at 72˚C for 2 min and a final extension at 72˚C for 7-10 min. The reaction was performed in an Applied Biosystems thermocycler (Thermo Fisher Scientific, Inc.). The amplified products were analyzed on 1.5% agarose gel electrophoresis and visualized with ethidium bromide, using a gel image documentation device (Bio Basic

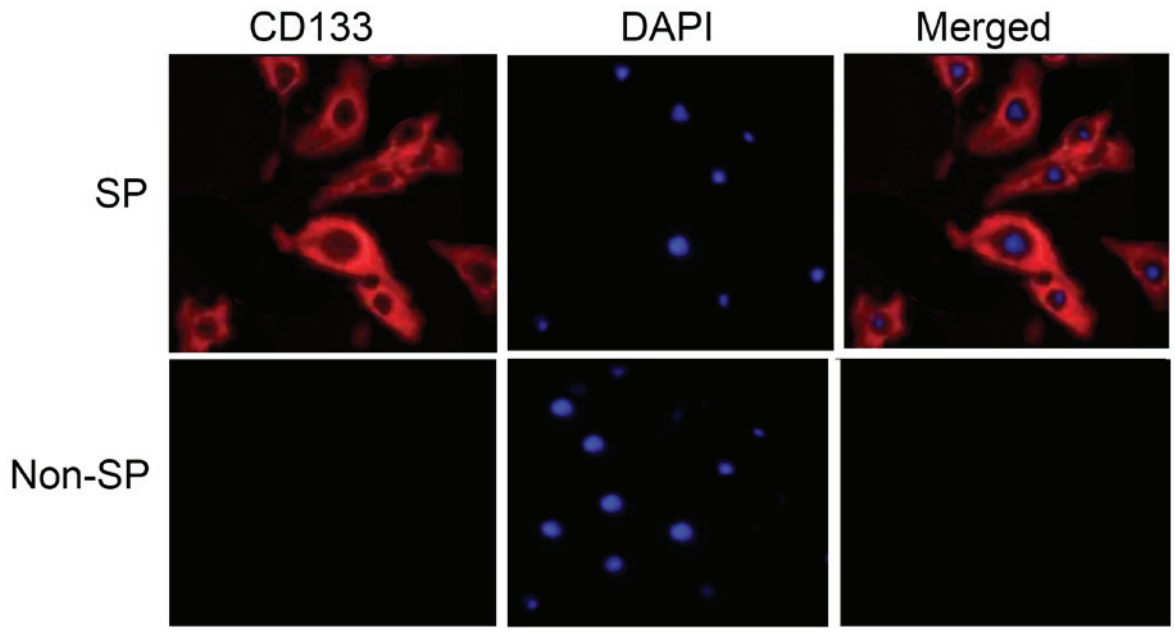

Figure 2. Immunocytochemistry was used to evaluate the expression of CD133 in SP and non-SP cells. Fluorescence-activated cell-sorted head and neck squamous cell carcinoma SP cells exhibited a higher positive immunofluorescence signal than non-SP cells regarding the expression of the stem cell surface protein CD133 (magnification, x40). SP, side population; CD, cluster of differentiation; DAPI, 4',6-diamidino-2-phenylindole.

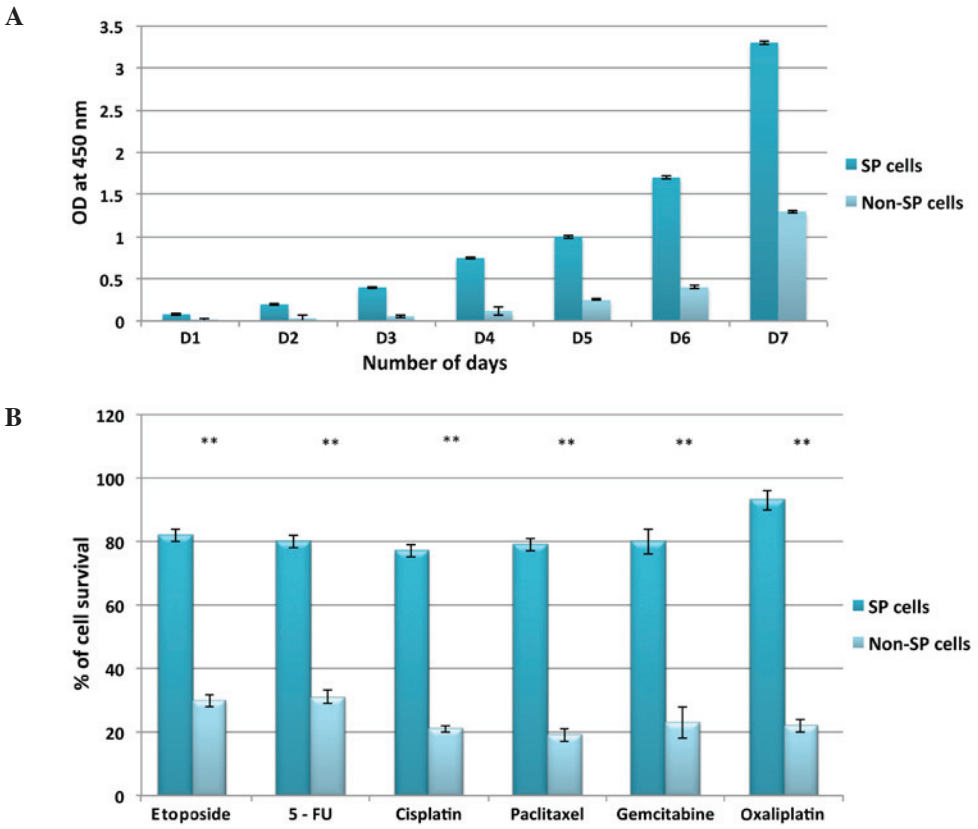

Figure 3. (A) Cell proliferation rate for SP and non-SP cells. Fluorescence-activated cell-sorted SP cells underwent a rapid proliferation rate, compared with non-SP cells. (B) Comparison of the cell survival rate between SP and non-SP cells following treatment with several DNA-targeting drugs. SP cells were highly drug resistant and displayed increased survival rate upon treatment, compared with non-SP cells. Data represent the mean ± standard deviation. **P<0.01 vs. control. SP, side population; OD, optical density; D, day; 5-FU, 5-fluorouracil.

Canada, Inc., Markham, ON, Canada). The intensity of the DNA bands from three independent experiments was measured with ImageJ version 3.2 (https://imagej.nih.gov/ij/).

Statistical analysis. One-way analysis of variance and Student's *t*-test were performed with GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA) in

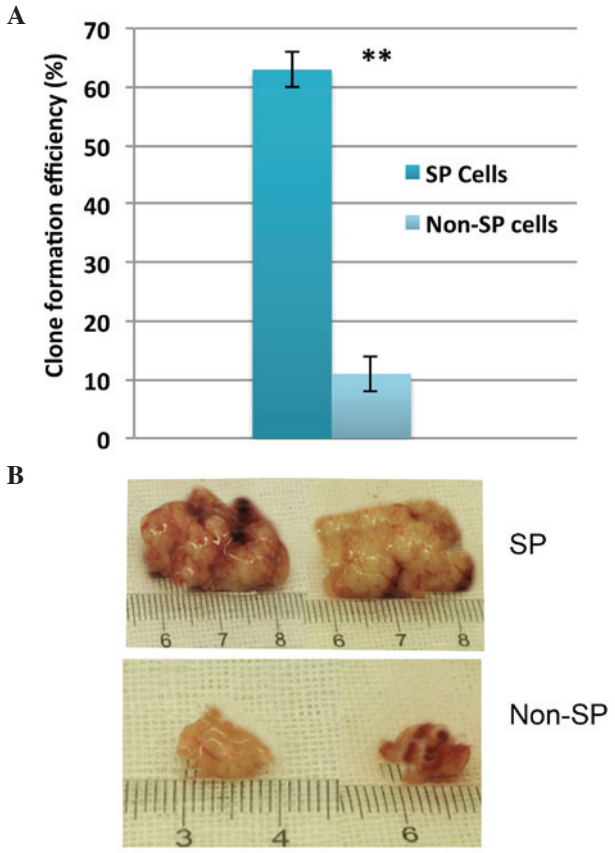

Figure 4. Comparison of the tumorigenic potential of SP vs. non-SP cells. (A) The total number of tumor spheres generated by head and neck squamous cell carcinoma SP cells was significantly higher than that generated by non-SP cells. (B) Images of the tumors generated following 22 days of subcutaneous injection of SP and non-SP cells into non-obese diabetic/severe combined immunodeficiency mice. Data represent the mean ± standard deviation. **P<0.01 vs. control. SP, side population.

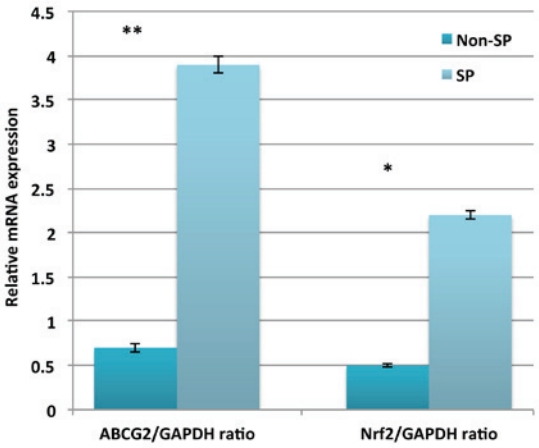

Figure 5. Relative expression of Nrf2 and ABCG2 in SP and non-SP cells. Elevated expression of Nrf2 and ABCG2 in SP cells was detected by reverse transcription-polymerase chain reaction. The bar graph corresponds to the quantification of three independent experiments. Data represent the mean ± standard deviation. *P<0.05; **P<0.01 vs. control. ABCG2, ATP-binding cassette sub-family G member 2; SP, side population; mRNA, messenger RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

order to evaluate the significance of the differences between SP and non-SP cells when comparing various or a single parameter, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification and characterization of CD133$^+$ cancer stem-like cells from HNSCC. FACS analysis identified ~2.1% SP cells in the HNSCC samples (Fig. 1A). In order to confirm the presence of SP cells, the samples were treated with the ABC transporter inhibitor verapamil. As indicated in Fig. 1B, the percentage of SP cells was significantly reduced to 0.7% upon treatment with verapamil (P=0.024). Furthermore, the FAC-sorted SP cells were positive for the stem cell surface protein CD133 (Fig. 2). These data suggest that HNSCC stem cells are able to resist the effect of chemotherapeutic drugs, and the overexpression of ABC transporter proteins is critical for the process of drug expulsion from the cell.

HNSCC CSCs are multidrug resistant and tumorigenic. To further characterize the HNSCC CD133$^+$ SP cells, the FAC-sorted SP and non-SP cells were subjected to an *in vitro* cell proliferation assay in order to determine the rate of cell proliferation. As revealed in Fig. 3A, the growth rate of SP cells was significantly higher, and the cells became confluent more rapidly (on day 6), than non-SP cells (day 15). Notably, the chemoresistance assay demonstrated that FAC-sorted SP cells were not susceptible to DNA-targeting drugs, including etoposide, gemcitabine, 5-fluorouracil, cisplatin, paclitaxel and oxaliplatin (Fig. 3B). The survival rate of SP cells was >70% following treatment with the above drugs, whereas the survival rate of non-SP cells was <30%.

Next, the self-renewal capacity of SP cells was investigated. HNSCC CD133$^+$ SP cells were able to generate larger tumor spheres (Fig. 4A), whose size markedly increased in a time-dependent manner (data not shown). In addition, injection of the lowest cell density of SP cells tested ($4x10^3$ cells) into NOD/SCID mice could efficiently induce tumor growth *in vivo* (Fig. 4B). Taken together, these data suggest that enhanced cell proliferation and self-renewal, alongside high multidrug resistance, contribute to therapy failure, tumor recurrence and invasion.

Elevated expression of Nrf2 and ABCG2 contributes to chemoresistance in HNSCC SP cells. In order to explore the molecular mechanism and signaling pathways involved in the enhanced expression profile of ABC transporters and the phenomenon of multidrug resistance, the expression of molecules associated with Nrf2 signaling was evaluated. Previous reports have indicated that Nrf2-dependent ABCG2 expression in SP cells is responsible for the chemoresistance exhibited by these cells. Furthermore, depletion of Nrf2 expression suppressed the ABCG2-mediated multidrug resistance of CSCs (16-18). In the present study, the relative messenger (m)RNA expression of Nrf2 was significantly upregulated in SP cells (P=0.018), which also displayed upregulation of the ABCG2 gene (Fig. 5). Therefore, these data suggest that a link exists between Nrf2 and ABC transporters regarding their contribution to the phenomenon of multidrug resistance in SP cells.

Discussion

Currently, the major challenge in the treatment of cancer is the presence of a small population of CSCs that could escape the current conventional treatment strategies and is capable of reinitiating tumor growth, metastasis and invasion following treatment (19). Therefore, the development of novel anticancer drugs to prevent tumor recurrence is an ultimate essential goal in the field of cancer therapy. Numerous studies concerning the characterization of CSCs revealed that these cells undergo rapid proliferation and generation of tumor spheres, possess a high potential for differentiation, and exhibit multi-drug and apoptosis resistance (19,20).

In the present study, the most common Hoechst dye exclusion assay (21) was used to purify CSCs from HNSCC specimens, and identified ~2.1% SP cells in the samples. Similar to previous findings, the present characterization experiments demonstrated that HNSCC cancer stem-like SP cells were capable of generating rapid tumor spheres, displayed an enhanced cell proliferation rate and were highly resistant to DNA-targeting drugs (17-19). Notably, these HNSCC SP cells possessed enhanced expression of the stem cell surface protein CD133, compared with non-SP cells. Previously, it was reported that the HNSCC cell lines M3a2 and M4e also contained SP cells, which were highly tumorigenic and chemoresistant (22). In the current study, it was also demonstrated that HNSCC SP cells were able to induce tumor growth rapidly *in vivo* in NOD/SCID mice.

These findings suggest that overexpression of ABC transporter genes and the stem cell surface protein CD133 may be involved in chemoresistance and maintenance of self-renewal in HNSCC SP cells. These observations were further confirmed by the reduction in the number of SP cells following treatment with verapamil. These data clearly indicates that ABC transporter genes are actively involved in pumping the above drug out of the cells.

Nrf2 is a basic leucine zipper domain transcription factor, which is known to protect cells from oxidative stress and other foreign pathogens by accelerating the expression of several antioxidant enzymes and ABC transporter proteins (23-25). Nrf2 is subjected to proteasomal degradation by Kelch-like ECH-associated protein 1 (KEAP1), and previous studies in primary cell cultures demonstrated that this ocurred either by loss of Nrf2-KEAP1 interaction or by mutations in the KEAP1 or Nrf2 genes (16-18); however, the mechanism of proteasomal degradation of Nrf2 requires further investigation. Recent studies in lung cancer cells reported that overexpression of Nrf2 leads to transcriptional upregulation of ABC transporter genes such as ABCG2, which contributes to drug resistance (13). Similarly, the present study also demonstrated that the relative mRNA expression of Nrf2 in HNSCC stem cells was significantly elevated compared with non-SP cells. Therefore, it is possible to speculate that the multidrug resistance properties of HNSCC SP cells are regulated by the Nrf2-mediated overexpression of ABCG2. However, it is worth investigating whether the depletion of Nrf2 by small interfering RNA could lead to the attenuation of ABCG2 expression, thus enhancing the sensitivity of SP cells towards drug treatment. In addition, the precise molecular mechanism involved in the regulatory pathways and other causative factors that result in Nrf2-mediated multidrug resistance remain to be studied in detail.

In summary, the present data suggest that HNSCC contains $CD133^+$ cancer stem-like SP cells that are highly resistant to a variety of drugs due to the overexpression of drug efflux pumps (such as ABCG2), which is induced by the stress-inducible factor Nrf2 and is ultimately responsible for treatment failure and tumor recurrence. Therefore, developing novel therapeutic drugs that efficiently suppress the function of Nrf2 and its downstream activating factors will aid to prevent Nrf2-mediated resistance to chemotherapeutic drugs and tumor recurrence in HNSCC.

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References

1. Schoenhals M, Kassambara A, De Vos J, Hose D, Moreaux J and Klein B: Embryonic stem cell markers expression in cancers. Biochem Biophys Res Commun 383: 157-162, 2009.
2. Chiou SH, Wang ML, Chou YT, Chen CJ, Hong CF, Hsieh WJ, Chang HT, Chen YS, Lin TW, Hsu HS and Wu CW: Coexpression of Oct4 and Nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial-mesenchymal transdifferentiation. Cancer Res 70: 10433-10444, 2010.
3. Lino MM, Merlo A and Boulay JL: Notch signaling in glioblastoma: A developmental drug target. BMC Med 8: 72, 2010.
4. Zhang XP, Zheng G, Zou L, Liu HL, Hou LH, Zhou P, Yin DD, Zheng QJ, Liang L, Zhang SZ, *et al*: Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells. Mol Cell Biochem 307: 101-108, 2008.
5. Kawaguchi-Ihara N, Murohashi I, Nara N and Tohda S: Promotion of the self-renewal capacity of human acute leukemia cells by Wnt3A. Anticancer Res 28: 2701-2704, 2008.
6. Khan NI, Bradstock KF and Bendall LJ: Activation of Wnt/beta-catenin pathway mediates growth and survival in B-cell progenitor acute lymphoblastic leukaemia. Br J Haematol 138: 338-348, 2007.
7. Salnikov AV, Gladkich J, Moldenhauer G, Volm M, Mattern J and Herr I: CD133 is indicative for a resistance phenotype but does not represent a prognostic marker for survival of non-small cell lung cancer patients. Int J Cancer 126: 950-958, 2010.
8. Singh A and Settleman J: EMT, cancer stem cells and drug resistance: An emerging axis of evil in the war on cancer. Oncogene 29: 4741-4751, 2010.
9. Ho MM, Ng AV, Lam S and Hung JY: Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Res 67: 4827-4833, 2007.
10. Burkert J, Wright NA and Alison MR: Stem cells and cancer: An intimate relationship. J Pathol 209: 287-297, 2006.
11. Rehman AO and Wang CY: CXC12/SDF-1 alpha activates NF-kappaB and promotes oral cancer invasion through the Carma3/Bcl10/Malt1 complex. Int J Oral Sci 1: 105-118, 2009.
12. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF and Ailles LE: Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 104: 973-978, 2007.
13. Singh A, Wu H, Zhang P, Happel C, Ma J and Biswal S: Expression of ABCG2 (BCRP) is regulated by Nrf2 in cancer cells that confers side population and chemoresistance phenotype. Mol Cancer Ther 9: 2365-2376, 2010.

14. Yang B, Ma YF and Liu Y: Elevated Expression of Nrf-2 and ABCG2 involved in multidrug resistance of lung cancer SP cells. Drug Res (Stuttg) 65: 526-531, 2015.
15. Shi Y, Fu X, Hua Y, Han Y, Lu Y and Wang J: The side population in human lung cancer cell line NCI-H460 is enriched in stem-like cancer cells. PLoS One 7: e33358, 2012.
16. Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K and Yamamoto M: Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol 24: 7130-7139, 2004.
17. Zhang DD, Lo SC, Cross JV, Templeton DJ and Hannink M: Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol Cell Biol 24: 10941-10953, 2004.
18. Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, Herman JG, Baylin SB, Sidransky D, Gabrielson E, *et al*: Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. PLoS Med 3: e420, 2006.
19. Dean M, Fojo T and Bates S: Tumour stem cells and drug resistance. Nat Rev Cancer 5: 275-284, 2005.
20. Morrison SJ and Kimble J: Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441: 1068-1074, 2006.
21. Song J, Chang I, Chen Z, Kang M and Wang CY: Characterization of side populations in HNSCC: Highly invasive, chemoresistant and abnormal Wnt signaling. PLoS One 5: e11456, 2010.
22. Bunting KD: ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 20: 11-20, 2002.
23. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, *et al*: An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236: 313-322, 1997.
24. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P and Kensler TW: Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci USA 98: 3410-3415, 2001.
25. Kwak MK, Kensler TW and Casero RA Jr: Induction of phase 2 enzymes by serum oxidized polyamines through activation of Nrf2. Effect of the polyamine metabolite acrolein. Biochem Biophys Res Commun 305: 662-670, 2003.