

Identification of Stem-like Cells in Head and Neck Cancer Cell Lines

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Abstract. *This study investigated the existence of stem-like cells in established head and neck squamous cell carcinoma (HNSCC) lines, HSC3 and HSC4. Flow cytometric analysis confirmed the presence of side population (SP) cells excluding Hoechst 33342 in HSC4 cells (0.37±0.06%) but not HSC3 cells in a reserpine-sensitive manner. After sorting, the SP cells generated both SP and main population (MP) cells in culture while MP cells generated MP cells only. Higher expression of stem cell markers was detected in SP than in MP cells. These results suggest that cancer stem-like cells exist in head and neck squamous cell carcinoma.*

Head and neck squamous cell carcinomas (HNSCC) are the sixth most prevalent type of malignancy worldwide (1). The most common type of HNSCC is oral squamous cell carcinoma (OCC). The overall five-year survival rate in patients with HNSCC is still lower than 50% (2, 3). HNSCC frequently shows local recurrence after the initial surgical or radiological treatment at the primary site, and even after complete resection (2, 3).

Recent data have demonstrated that tumours contain a small subpopulation of cells called cancer stem-like cells (CSCs), which are responsible for tumour maintenance and metastasis (4, 5). CSCs have the ability to self-renew and are responsible for tumorigenesis, progression, metastasis, and relapse after treatments (6-8). Some groups have demonstrated that in HNSCC a small population of CD44⁺ cancer cells obtained from fresh tumour tissue (9, 10, 11) or

permanent cell lines (12, 13) behave like CSCs and give rise to new tumours (9).

Side population (SP) cells, characterized by the efficient efflux of Hoechst 33342 dye, are thought to be the enriched in stem cells in many normal tissues (14-16) as well as in cancer (17, 18). SP cells express various adenosine triphosphate-binding cassette (ABC) transporter family members that are responsible for drug resistance, including ABCG2 (BRCP1) (19-21). Previous studies show that CSCs of HNSCC can be identified by SP phenotype from established cancer cell lines (22-25).

In this study, the existence of SP cells in HNSCC cell line was investigated by fluorescence-activated cell sorting (FACS) analysis and their characteristics identified.

Materials and Methods

Cell lines and cell culture. The HNSCC cell lines used were HSC3 and HSC4, from JCRB cell bank (Osaka, Japan). The cells were cultured in MEM (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS; JRH Biosciences, Lenexa, Kansas, USA), 1% antibiotic-antimycotic mixture stock solution (100×) (nacalai tesque, Kyoto, Japan), and maintained at 37°C in a humidified 5% CO₂/95% air atmosphere.

SP analysis and cell sorting. The single-cell suspension (10⁶ cells/ml) was incubated in Hank's solution containing 3 µg/ml Hoechst 33342 (Sigma-Aldrich, Saint Louis, Missouri, USA) at 37°C for 60 min with intermittent mixing. The control cells were incubated in the presence of 20 µg/ml of reserpine (Daiichi-sankyo, Tokyo, Japan). After incubation, cells were washed in Hank's solution. Propidium iodine (1 µg/ml) was added to discriminate dead cells. Analysis and sorting were performed using Beckman Coulter flow cytometry. SP and main population (MP) cells (1×10⁶ cells separately) were cultured in MEM with 10% foetal calf serum (FCS) for 5-days. Each cell was stained with Hoechst 33342 and analysed using EPICS ALTRA HyPerSort (Beckman Coulter, Inc. Fullerton, CA, USA). Each experiment was performed at least 3 times.

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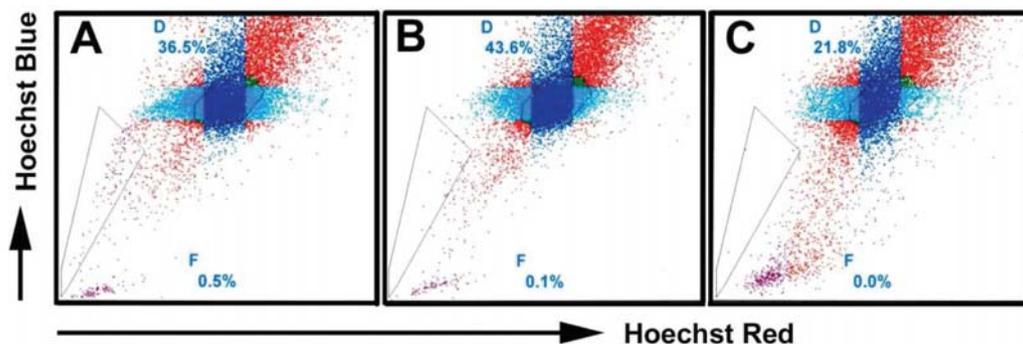


Figure 1. Identification of SP cells in HSC4 (A, B) and HSC3 (C) cells. In HSC4 cells, SP cells comprised almost 0.4% of the total cells (A), which disappeared in the presence of reserpine (B). No SP cell was detected in HSC3 cells (C). Similar results were obtained in at least three independent experiments.

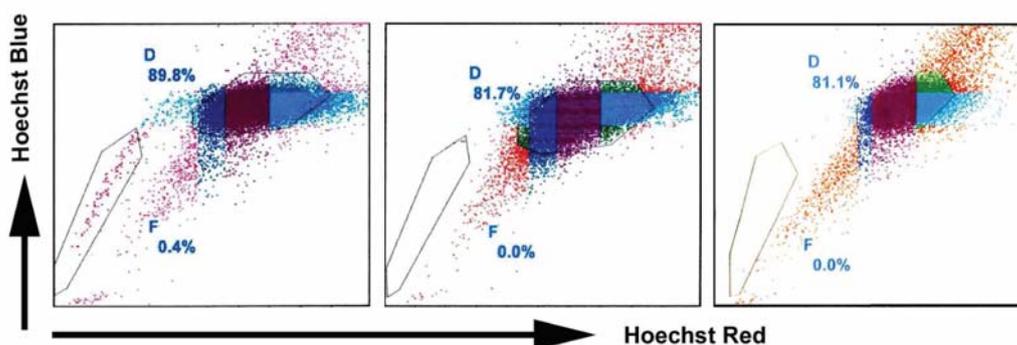


Figure 2. SP cells are able to generate both SP and MP cells. HSC4 cells were labelled with Hoechst33342 and analyzed by flow cytometry. SP cells and MP cells were sorted and cultured separately. After 10-days of culture, SP cells (A, B) and MP cells (C) were stained with Hoechst33342 and re-analysed by flow cytometry. SP cells disappeared in the presence of reserpine (B).

RT-PCR. Total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed with SuperScriptII Reverse Transcriptase (Invitrogen) and random primers (Takara Bio Inc., Shiga, Japan). The following primers were used: Notch-1, β -catenin, SMO, ABCG2, GAPDH (26); Oct3/4, Nanog, CD133 (27).

Clonogenic formation. Single cell populations of sorted SP and non-SP cells were plated in 96-well plates (1 cell/well, 10 plates for each cell, 3 times) and cultured with MEM with 10% FCS for 21 days. Clones with more than 50 cells were counted. The percentage of cells that formed colonies was presented as clone formation efficiency (CFE).

Patient samples. Poorly differentiated carcinomas (WHO classification 2005; IIa, n=4) were obtained from National Tokyo Medical Center, Tokyo, Japan, in accordance with institutional guidelines. Samples had been collected by standard procedures at diagnosis before treatment with chemotherapy, fixed in 10% formalin, embedded in paraffin wax, and then sectioned in the midmodiolar plane at 5.0 μ m. The slides obtained from peripheral and central part of tumours were used for immunohistochemical analysis.

Immunofluorescence analysis. Immunocytochemistry was carried out with antibodies against the following: CD44 (eBio, San Diego, CA, USA) and ABCG2 (BD, Franklin Lakes, NJ, USA). The appropriate species-

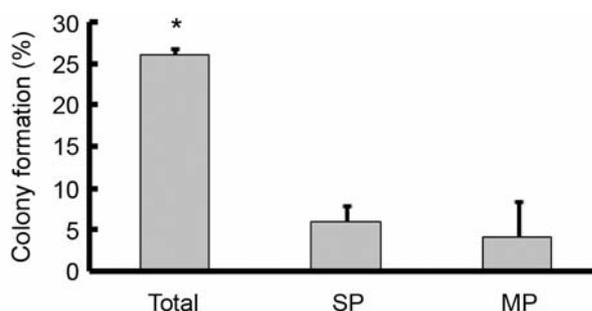


Figure 3. SP cells have similar clonogenic ability to MP cells. Single HSC4 cells, SP cells and MP cells were seeded in each well of 96-well plates. For each type cell, ten plates were used. The plates were cultured for 2 weeks, and the number of colonies in each plate was counted. * $p < 0.01$ vs. MP or SP. The data are representative of three independent experiments.

specific antibodies conjugated to either Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. For tissue sections, the DAKO Cytomation EnVision+ System-HRP kit (AEC) (Dako, Kyoto, Japan) was used according to the manufacturer's instructions. The nuclei were stained with haematoxylin and the cells were observed under an inverted light microscope.

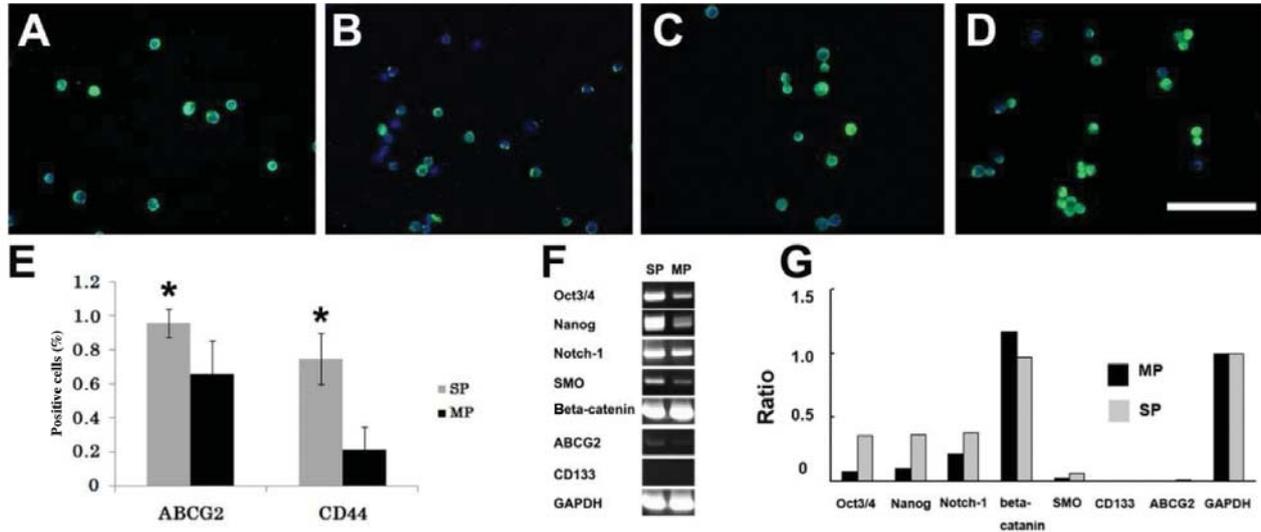


Figure 4. Expression of stem cell markers on SP and MP cells. SP (A, C) and MP (B, D) cells were labeled with CD44 (A, B), and ABCG2 (C, D). Nuclei in cells stained with DAPI. The percentage of positive cells of both antibodies is shown in E. mRNA expression of stem cell markers were analysed in SP and MP cells by using RT-PCR (F). Data analysed with ImageJ are shown in G. Scale bar: 50µm. * p<0.01 (MP vs. SP).

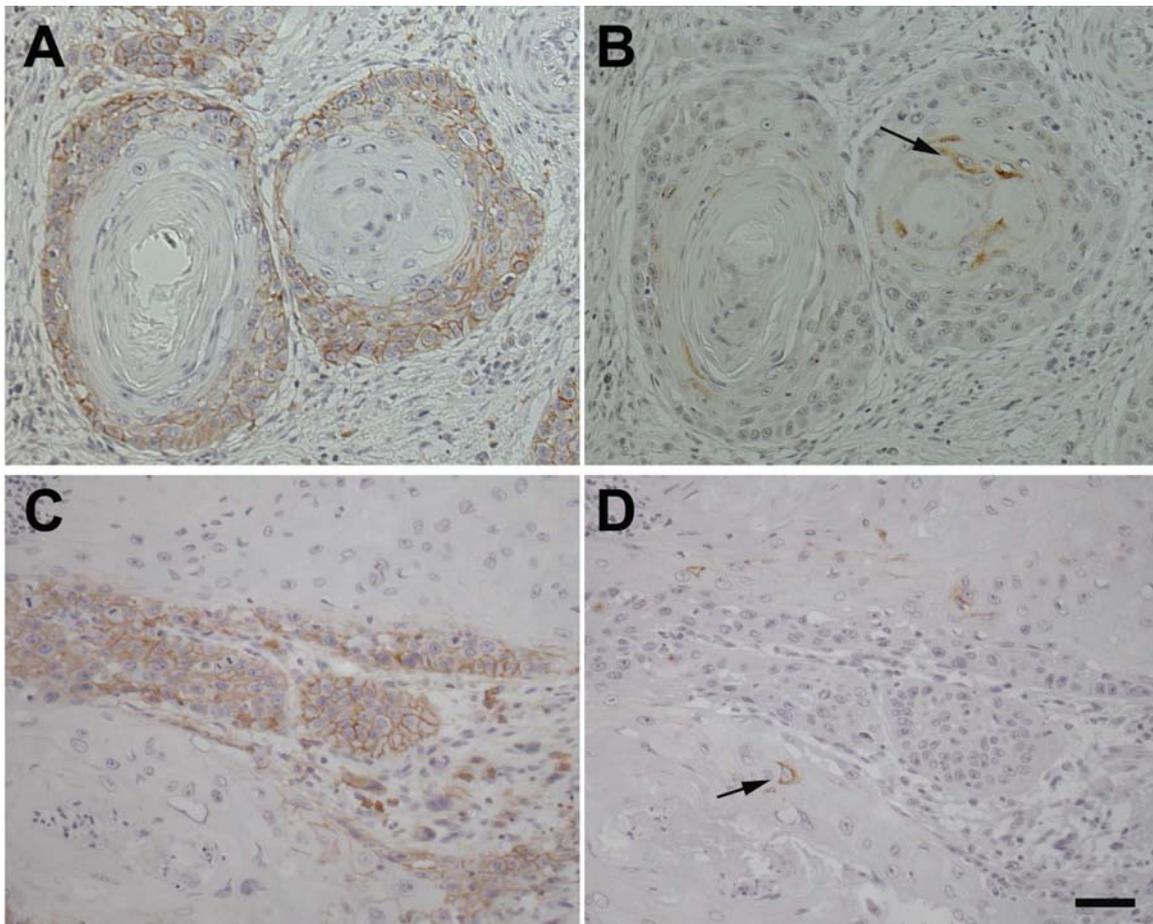


Figure 5. Immunohistochemical analysis of CD44 (A, C) and ABCG2 (B, D) expression in squamous cell carcinomas of the tongue. A and B show the central part and C and D show the peripheral part of the same sample. Scale bar: 100 µm.

Sorted cells were fixed for 10 min at room temperature in 4% (w/v) paraformaldehyde (PFA) in PBS. After being attached to slides by using cytospin, cells were blocked in PBS supplemented with 10% (v/v) normal goat serum for 1 h at room temperature prior to staining with a primary antibody overnight at 4°C. They were then washed three times (15 min each time) with PBS, followed by incubation with the secondary antibody for 40 min at room temperature. Nuclei were counter-stained with Hoechst 33342. The specimens were viewed with a laboratory microscope (DM 2500; Leica, Houston, TX, USA).

To evaluate immunolabelled populations, the relative number of positively staining cells was expressed as a percentage (mean±S.D.) of the total cell count. More than 500 cells were counted per experiment. Cell count was performed by using ImageJ, a java-based image analysis program developed at National Institute of Mental Health, Bethesda, MD, USA.

Statistical analysis. All statistical analyses were performed using one-way ANOVA (OriginPro7.5; Originlab Co., Northampton, MA, USA) was used for comparisons between experimental groups and control groups. $P<0.01$ was considered significant.

Results

Identification of SP cells in HNSCC cell lines. The existence of SPs in two HNSCC cell lines (HSC3 and HSC4) was examined by staining them with Hoechst 33342 dye to generate a Hoechst blue-red profile. As a control, reserpine was added to block the activity of the Hoechst 33342 transporter, and the SP gate was defined as the diminished region in the presence of reserpine. Flow cytometric analysis confirmed the presence of SP cells excluding Hoechst 33342 in HSC4 cells (0.37±0.06%) (Figure 1A) but not HSC3 cells (Figure 1C). The SP cells in HSC4 decreased significantly in the presence of reserpine (Figure 1B).

SP cells are able to generate both SP and MP cells. To examine whether SP cells can generate both SP and MP cells, SP and MP cells were sorted and cultured *in vitro*. After 10 days expansion, the cells were re-stained with Hoechst 33342 and reanalyzed by flow cytometry. The SP cells repopulated both SP and MP cells (Figure 2A), while MP cells repopulated MP cells only (Figure 2C). The proportion of the SP cells was 0.5%, similar to the first sorting. The SP cells decreased significantly in the presence of reserpine (Figure 2B). These results indicated the SP cells are the source of SP and MP and most of SP cells converted into MP cells during the culture.

Clonogenic ability of SP cells is similar to that of MP cells. To compare the colony formation ability of SP cells with MP cells, the clonogenic ability of SP and MP cells was examined. Single sorted SP and MP cells were cultured in 96-well plates. The number of colonies was counted on the 21st-day of culture. As shown in Figure 3A, there was no difference in clone number between SP cells and MP cells (Student's *t*-test, $p=0.46$). Colony formation from total HSC4 cells was higher than SP and MP ($p<0.01$ vs. SP and MP, separately) (Figure 3).

Higher expression of stem cell markers was detected in SP than MP cells. To ascertain whether SP cells contained stem cells, the expression of marker protein was identified by RT-PCR and immunohistochemical analysis.

Immunostaining data showed that compared with MP cells, SP cells showed higher expression of CD44 (0.75±0.15% (SP) vs. 0.22±0.13% (MP), $p<0.01$) (Figure 4A, B and E) and ABCG2 (0.96±0.08% (SP) vs. 0.66±0.20% (MP), $p<0.01$) (Figure 4C-E), which are known to be markers of CSCs (9, 23).

The results of RT-PCR confirmed that SP cell expressed higher level of Oct3/4, Nanog, which is a marker of embryonic stem cells. Recently, expression of Oct3/4 and Nanog was reported to be positively associated with late-stage progression and worse prognosis of oral cancer patients (5). In addition, ABCG2 gene expression was particularly up-regulated in SP cells, at a rate 5.56 times higher than in MP cells (Figure 4F and G).

These results indicate that although SP and MP cells had similar clonogenic ability, the up-regulated expression of stemness genes suggests a possibility that the SP population was enriched in CSCs. Thus the extension of stem-like cells in HSC4 cells *in vitro* was shown.

ABCG2-positive cells were detected in tumour tissue. To examine the subcellular localization of CD44 and ABCG2 in OSCC, immunohistochemical analysis of CD44 and ABCG2 was performed. Both ABCG2 and CD44 were expressed mainly in the membrane of the tumour cells. CD44 expression was found predominantly in the peripheral areas of the tumour nest, while ABCG2 expression was observed in the centre of the cancer nest (Figure 5). CD44 positive cells were also detected in the mesenchymal cells. No cell was detected to be double-positive for CD44 and ABCG2. No different expression of CD44 and ABCG2 between central and peripheral of tumour had been detected (Figure 5).

Discussion

Like somatic stem cells, CSCs have the properties of self-renewal, producing heterologous descendent cells and slow cell cycling (28). In this work, it was shown that SP cells are capable of self-renewal and they also generated MP cells by asymmetric division. This is consistent with previous studies showing that SP cells are able to generate MP cells *in vitro* or *in vivo* (26, 29, 30).

In the present study, no significant differences in clonogenic ability between SP and MP cells were detected. Although several previous studies (22, 26) showed the clonogenic ability of SP cells was higher than that of MP cells, Burkert *et al.* recently reported a study analyzing for gastrointestinal cancer cell lines and found both SP and MP cells showed similar clonogenicity and *in vivo* tumour formation capacity (31). In the present study, lower clonogenic ability of SP and MP cells compared with total HSC4 cells may have been a result of longer treatment with Hoechst dye, which is potentially cytotoxic (32).

In the present study, SP expression was higher in several markers that have been considered as stem cell markers (Figure 4E-G). No expression of CD133 was detected by RT-PCR, nor by immunohistochemistry analysis. In a number of recent studies, CD133, a transmembrane pentaspan protein, has been used for identification and isolation of a putative cancer stem cell population from malignant tumours of brain (33), prostate (34), colon (35) and HNSCC (25). The genetic background of the cell used in this study may account for this difference. In addition, data in this study showed β -catenin gene expression was unregulated in MP than SP cells. β -catenin is a multifunctional protein involved in two independent processes: cell-cell adhesion and signal transduction. Reduced and lack of expression of β -catenin in tumours results in cell proliferation, migration, and invasion, and is associated with poor prognosis (36-38). Therefore reduced expression of β -catenin in SP cells may be related to the tumorigenic ability of SP cells.

ABCG2 is an ABC transporter member and a characteristic of SP cells (39). ABCG2 is widely present in untreated human solid tumours and may represent a clinically relevant mechanism of drug resistance. Positive tumours showed membranous and cytoplasmic staining and ABCG2 immunoreactivity in over 10% of cancer cells. In HNSCC specimens, ABCG2 showed heterogeneous staining. In general, squamous tumours stained more weakly than other tumour types (40). In the present study, ABCG2 was expressed mainly in the membrane of the tumour cells of specimens weakly and heterogeneously, consistent with the previous study (40).

CD44 is a cell surface-expressed molecule that is currently used to identify CSCs as one of the cell surface markers for solid tumours (9, 34, 41). With respect to HNSCC, several studies have demonstrated that CD44 presence identifies a population of human HNSCCs that possess properties of CSCs (9, 12, 13, 22).

However, several observations are inconsistent with the above data. For ABCG2, there is evidence to suggest that ABCG2 expression alone does not define the stem cell population (21, 26, 42, 43). For CD44, Mack and Gires (44) showed CD44 was abundantly expressed in head and neck carcinomas. The expression does not distinguish normal from benign or malignant epithelia of the head and neck. Hence, the authors indicated that the value of CD44 as a marker for the definition of a small subset of cells (*i.e.* fewer than 10%) representing head and neck cancer stem cells may need revision (44). In the present study, no CD44 and ABCG2 double-positive cells were detected in tumour tissue. Further studies are needed to fully elucidate the regulatory mechanisms of CD44 and ABCG2 expression, and further studies using a large clinical sample and longer follow-up are necessary to verify these results.

In the present study, the existence of a similar small SP fraction, as defined by the Hoechst 33342 dye efflux assay, in

HSC4 cells was reported. It was confirmed that these SP cells also exhibit characteristics of a tumour-initiating, CSC phenotype. Although the SP does not necessarily represent pure CSC or all of the malignant stem cells from the whole population, it is a significant enrichment of these rare cells that is responsible for initiating and maintaining cancer. Further studies are needed to identify CSCs of HNSCC precisely. The elucidation of the key tumorigenic population might contribute not only to clarification of the mechanism of HNSCC but also to the establishment of a novel therapy for HNSCC.

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