

Cancer stem-like side population cells in the human nasopharyngeal carcinoma cell line CNE-2 possess epithelial mesenchymal transition properties in association with metastasis

DAN GUO¹, BEN-LING XU², XU-HUA ZHANG² and MING-MIN DONG¹

¹Department of Otolaryngology, Head and Neck Surgery, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052; ²Department of Biotherapy, Laboratory Center, Affiliated Cancer Hospital, Zhengzhou University, Zhengzhou, Henan 450008, P.R. China

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Abstract. It has been recently reported that side population (SP) cells in nasopharyngeal carcinoma (NPC) cell lines display characteristics of cancer stem-like cells. However, the biological behavior and the significance of these cells for NPC progression remain unclear. In this study, we isolated SP cells from the NPC cell line CNE-2 by flow cytometry and investigated their biological characteristics. We discovered that SP cells had stronger colony forming abilities compared to the non-side population (NSP) cells, and observed that some SP cells looked more like the shape of mesenchymal cells when cultured in the common polyHEMA-coated flask. When checked by quantitative real-time PCR, the SP cells expressed higher levels of stemness-related genes Oct4, Sox2 and Nanog, and mesenchymal cell-related genes N-cadherin, vimentin and Snail, while they expressed lower levels of the epithelial cell-related gene, E-cadherin. Western blot and immunofluorescence staining methods further verified that SP cells expressed higher vimentin and expressed lower E-cadherin levels. Finally, Transwell invasion assay results indicated that the SP cells had higher invasive potential compared to NSP cells. Collectively, our data reveal that SP cells in the CNE-2 cell line not only possess the properties of cancer stem cells, but also have more mesenchymal cell characteristics which are associated with epithelial mesenchymal transition (EMT) and cancer cell invasion and metastasis. These findings are helpful for developing novel targets for effective clinical treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is fairly rare among Caucasians in Western Europe and North America (1/100,000),

but it is the most frequent head and neck tumor in Guangdong, south China, where the incidence peaks at 50/100,000 (1,2). NPC is a highly malignant tumor which often invades adjacent regions and metastasizes to regional lymph nodes and distant organs. Thirty to sixty percent of patients with NPC will eventually develop distant metastases. Although advances in therapy have improved the quality of life, survival rates have remained unchanged over the past decades. Mortality from this disease remains high because of the development of distant metastases and the emergence of local and systemic recurrences resistant to chemoradiotherapy. It is therefore essential to develop a deeper understanding of the cellular and molecular mechanisms of NPC metastasis and their recurrence in order to develop more effective therapeutic approaches.

Evidence has recently been accumulating to support the hypothesis that tumors contain a small subpopulation of cells called cancer stem cells (CSC), which exhibit self-renewing capacities and are responsible for tumor maintenance and metastasis (3). Currently, there are mainly two methods used to identify and isolate cancer stem cells. One is to use the molecular markers expressed on the cell surface by flow cytometry or immune magnetic beads. Several molecules have been identified as potential cancer stem cells markers, such as CD133 in brain tumors, colorectal carcinoma, prostatic carcinoma and pancreatic carcinoma (4-7) and CD44 in head and neck, breast, and ovarian cancer (8-10). However, these findings are still controversial. For example, CD133 has been challenged as a cancer stem cell marker in colon cancer (11), while CD44 has been questioned as a head and neck cancer stem cell marker (12). In head and neck squamous cell carcinoma (HNSCC), Prince *et al* first demonstrated that a CD44⁺ population of cells possessed the properties of CSC (8), but a relatively high number of these cells (5,000 cells) were needed to generate new tumors in immunodeficient mice, indicating either a low frequency of CSC or a low specificity of CD44 as a CSC-marker in HNSCC. The latter hypothesis is supported by the observation that CD44s and CD44v6 expression levels do not distinguish normal from benign or malignant epithelia of the head and neck. CD44s and CD44v6 are abundantly expressed in the majority of cells in head and neck tissues, including carcinomas (12). Thus, the identification of more

Correspondence to: Professor Ming-Min Dong Department of Otolaryngology, Head and Neck Surgery, the First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052, P.R. China
E-mail: dmmmt@163.com

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specific CSC markers for HNSCC is desirable. The second method using the nucleic acid dye Hoechst 33342 has identified so-called side population cells (SP) with stem cell characters. Stem cells can pump out the dye from the cytoplasm due to the activity of ABC transporter transmembrane proteins. SP cells are a distinct, small cell population composed of unstained cells in the left lower quadrant of a flow activated cell sorter (FACS) profile (13). This method is mainly used for cultured tumor cells without known stem cell markers.

The epithelial-mesenchymal transition (EMT) is characterized as a switch from a polarized epithelial phenotype to a highly motile fibroblast or mesenchymal phenotype. During EMT, epithelial cells break down cell to cell and cell extracellular matrix contacts and migrate to other locations in the body (14). EMT is critical to metazoan embryogenesis, chronic inflammation and fibrosis, and has been demonstrated to be a central mechanism in cancer invasiveness and metastasis (15). It has been reported that EMT generates cells with stem cell-like properties (16), and that EMT often occurs in cancer stem cells (17,18), which suggests that metastases are sometimes caused by cancer cells that acquire stem cell characteristics.

Wang *et al* have reported that SP cells in the CNE-2 human NPC cell line display stem cell characteristics (19). However, the molecular mechanisms underlying the regulation of SP cells in NPC remain unclear. In the present study, we examined whether SP cells of the CNE-2 NPC cell line have both characteristics of cancer stem cells and EMT. We report evidence that SP cells enrich CSC-like cells and have the properties of mesenchymal cells.

Materials and methods

Analyzing and sorting of SP cells by flow cytometry. The human NPC cell line CNE-2 was cultured in the RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin G, and 100 µg/ml streptomycin, maintained at 37°C in a humidified 5% CO₂ incubator. When the cells grew about 80% confluent in the flask, they were analyzed by flow cytometry (FACS Aria II, BD Biosciences). Cells were digested with 0.25% trypsin and 1 mM EDTA, washed with calcium/magnesium free phosphate-buffered saline (PBS), resuspended in RPMI-1640 (supplemented with 2% FBS) with a concentration of 1×10⁶ cells/ml. Subsequently, the DNA-binding dye Hoechst 33342 (Sigma, St. Louis, MO) was added at a final concentration of 5 µg/ml, with or without verapamil (Sigma) as a confirmation at a final concentration of 100 µmol/l. Both cell treatment groups were incubated at 37°C in a 5% CO₂ incubator for 90 min in the dark with every 30 min interval mixing. After incubation, cells were washed with ice-cold PBS and filtered through a 45 µm cell strainer to obtain single-suspension cells. Five minutes before the analysis of cells and sorting using FACS AriaII, a final concentration of 1 µg/ml propidium iodide (PI) (Sigma) was added. Hoechst 33342 was excited with a UV laser at 350 nm and fluorescence emission was measured with 405/BP309 (Hoechst blue) and 570/BP20 (Hoechst red) optical filters. PI labeling was measured through the 630/BP30 filter for the discrimination of dead cells. PI-negative cells were sorted into two subpopulations, Hoechst 33342 negative cells, SP cells, whose ability of fluorescent efflux could be blocked

by verapamil, and Hoechst 33342 positive cells (non-SP cells). Finally, the two groups were collected for sorting purity evaluation and further experiments.

Clone formation assay. After FACS sorting, the SP and NSP cells were incubated in 6-well plates at a density of 500 cells per well in RPMI-1640 medium supplemented with 10% FBS. Cells were cultivated under standard culture conditions. After most cell clones had expanded to more than 50 cells (about 12 days), they were washed twice with PBS, fixed in methanol for 15 min, and stained with crystal violet for 15 min at room temperature. After washing out the dye, the number of clones which contained more than 50 cells was counted microscopically and the results were compared. The clone formation efficiency (CFE) was the ratio of the formation of clone numbers to the planted cell numbers.

Quantitative real-time RT-PCR. Total-RNA was extracted by using the RNAsimple total-RNA kit (Invitrogen Life Technologies, Carlsbad, CA), then converted to cDNA with the Quantscript RT kit (Invitrogen). qRT-PCRs were carried out in a 20 µl final volume using Power SYBR-Green Mix (Takara, Bio, Inc., Shiga, Japan) and run on a 7300 Real-Time PCR System (Applied Biosystems, USA). Reactions were carried out in triplicate as controls and the housekeeping gene GAPDH was used as an internal control to normalize the expression levels of different genes. Their reaction condition was: initiation with a 10 sec denaturation at 95°C, followed by 40 cycles of amplification with 10 sec of denaturation at 95°C, 5 sec of annealing according to the melting temperatures of each pair of primers and 30 sec of extension at 60°C. Then data were analyzed using the modified $\Delta\Delta C_t$ method (20). The genes examined and their PCR primers are listed in Table I.

Western blotting. Fresh-sorted cells of SP and NSP were collected respectively. Cells were lysed in the Nonidet P-40 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 mM DTT, 10 U/ml aprotinin, and 20 µg/ml leupeptin. Cell lysates were cleared by centrifugation, and protein concentrations were determined using the BCA protein assay (Pierce, USA). Equal amounts of protein sample (20 µg) were separated on 4% SDS acrylamide gel (Bio-Rad, USA) for 0.5 h at 60 V and on 12% SDS acrylamide gel for 1.5 h at 150 V. The samples were then transferred onto a nitrocellulose membrane for 1.5 h at 20 V (Whatman, UK). After blocking in 5% fat-free milk, the membrane was incubated with the primary antibody (anti-E-cadherin mouse monoclonal antibody, 1:3,000; anti-vimentin mouse monoclonal antibody, (BD Biosciences, USA) 1:4,000 overnight at 4°C, followed by incubation with the secondary HRP-conjugated goat anti-mouse IgG antibody for 1 h at room temperature (1:5,000). The antibody was diluted in PBS containing 5% Blotto (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and 0.1% Tween-20. The stained membranes were visualized by enhanced chemiluminescence reaction using the ECL Plus kit (GE Healthcare, USA). The mouse monoclonal β -actin antibody (BD Biosciences) was used as the loading control for western blot analysis at a dilution of 1:5,000. The band intensities of the PCR products were analyzed using the UVP VisionWorks LS 6.6 α (UVP), and were expressed as the means \pm SD.

Table I. Examined genes and their PCR primers.

Gene	Primer sequence (5'→3')	Size of product (bp)
ABCG2	F: GGATGAGCCTACAACCTGGCTT R: CTCCTGAGGCCAATAAGGTG	162
Oct4	F: TCGAGAACCGAGTGAGAGGC R: CACACTCGGACCACATCCTTC	121
Sox2	F: CACACTGCCCCTCTCACACAT R: CATTTCCCTCGTTTTTCTTTGAA	81
Nanog	F: CCAACATCCTGAACCTCAGCTAC R: GCCTTCTGCGTCACACCAIT	121
Vimentin	F: ATGCGTGAGATGGAAGAGAATTTTGC R: TTATTCAAGGTCATCGTGATGCTGAGA	372
E-Cadherin	F: TCGACACCCGATTCAAAGTGG R: TTCCAGAAACGGAGGCCTGAT	192
Snail	F: TATGCTGCCTTCCCAGGCTTG R: ATGTGCATCTTGAGGGCACCC	143
N-Cadherin	F: CCGGAGAACAGTCTCCAACCTC R: CCCACAAAGAGCAGCAGTC	156
GAPDH	F: TCTGCTCCTCCTGTTTCGACA R: AAAAGCAGCCCTGGTGACC	141

Immunofluorescence analysis of the expression of E-cadherin and vimentin in SP and NSP cells. Fresh-sorted cells were grown on sterile glass cover slides overnight at 37°C, washed twice with PBS, and fixed in 4% paraformaldehyde for 30 min at 4°C. The cells were washed twice more with PBS, and incubated with 5% bovine serum albumin for 2 h at room temperature to block nonspecific binding of IgG. After twice further PBS washes, the cells were incubated overnight at 4°C with primary antibodies against E-cadherin and vimentin (BD Biosciences) that had been diluted in PBS according to the manufacturer's instructions. Subsequently, the slides were washed twice with PBS containing 0.02% Tween-20 (PBS-T) and added fluorochrome-conjugated secondary antibodies (BD Biosciences, USA) at room temperature for 2 h in a dark chamber. The cells were washed thrice with PBS-T and cover slipped with aqueous mounting medium (containing 0.5 mg/ml 4,6-diamidino-2-phenylindole to stain the nuclei). Random photos (Leica TCS SP5II, Germany) of eight visual fields were acquired using 100-fold magnification. The total and positive cell numbers were counted in order to calculate the expression percentage.

Matrigel-Transwell invasion assay in vitro. Matrigel invasion chambers (BD Biosciences) were used to compare the invading capacity between SP and NSP cells. The first step was to prepare artificial basilar membrane. Matrigel was thawed (Sigma, USA) at 4°C overnight. Matrigel was diluted (1:3) in serum-free cold RPMI-1640. Diluted Matrigel (100 µl/well) was gently added to ensure homogeneity. The Transwell was incubated at 37°C for 3 h for gelling. SP or NSP fresh sorted cells were collected, washed and resuspended in RPMI-1640 culture medium (containing 2% FBS)

at a density of 5x10⁵ cells/ml. The gelled Matrigel was gently washed with warmed serum free-culture media. RPMI-1640 (600 µl) supplemented with 10% FBS was added to the lower compartments according to the manufacturer's instructions. Cell suspensions (100 µl) were respectively put into the upper compartments. Then the Transwell was kept for 48 h under standard culture conditions. After incubation, the Transwell was removed from 24-well plates and were scrapped off the non-invading cells on the top of the Transwell with a cotton swab. The Transwells were fixed with 95% alcohol for 15 min and then stained with crystal violet for 15 min. Images (Nikon, TE2000-S, Japan) of 5 visual fields per membrane of triplicate membranes were captured using 200-fold magnification and the total cells were counted. The test was repeated three times.

Statistical analysis. SPSS 17.0 was used for statistical data analyses. All data were presented as mean ± SEM from at least three independent experiments. The paired t-test was used for analyzing the difference in colony formation efficiency, Transwell invasion assay and gene expression between the SP and NSP cells. P-values <0.05 were considered to indicate significant differences.

Results

FACS analysis and cell sorting. When examined using FACS, the small population of CNE-2 cells which had the strongest dye efflux ability in the left lower quadrant of the FACS, were gated as SP cells (P3 gate). The main population of cells which were Hoechst 33342 positive were gated as NSP cells (P4 gate). SP cells occupied 16.2% of the total cells (Fig. 1A). In the control group with verapamil, the percentage of SP

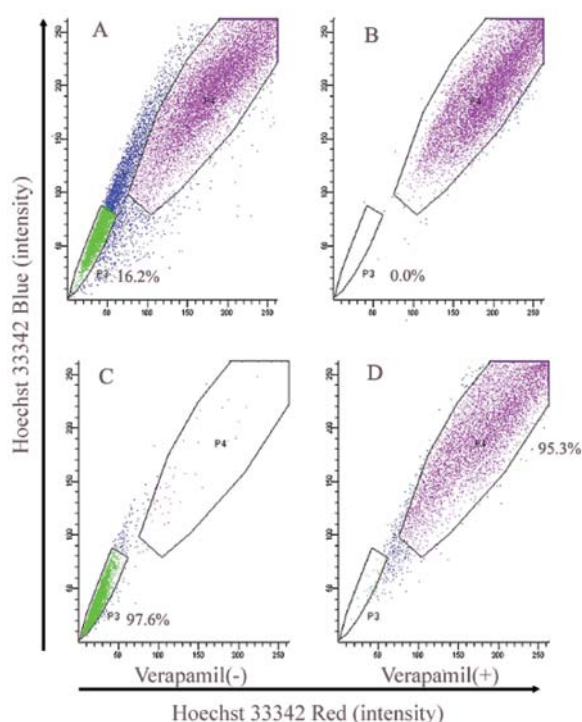


Figure 1. Cell sorting results and sorting purity. (A) Sorting of CNE-2 cells using Hoechst 33342. The P3 gate represents the SP cells (16.2% of total cells) and the P4 gate represents the NSP cells. (B) The SP proportion was decreased to 0.0% when the cells were preincubated with verapamil to block the ATP transporter. (C and D). The sorting purity of the fresh sorted SP and NSP cells was 97.6% and 95.3% respectively.

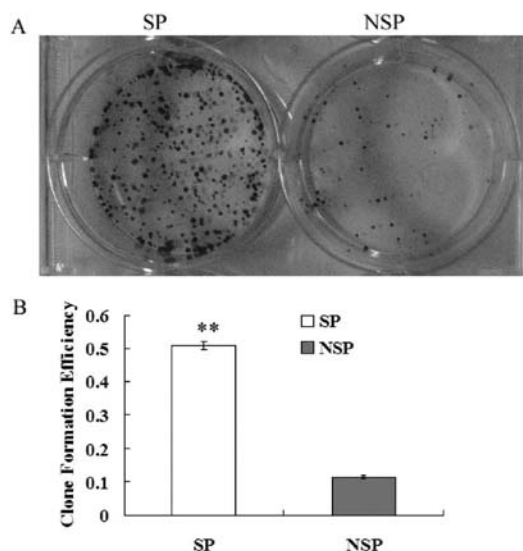


Figure 2. Results of colony formation assay. (A) SP cells could form more and generally bigger colonies (left plate) compared to NSP cells (right plate). The Clone formation efficiency of the SP cells was significantly higher than that of the NSP cells (B) (** $P < 0.001$).

cells dropped to 0.0% of the total cell population (Fig. 1B), which is consistent with reports that Hoechst 33342 exclusion is verapamil sensitive. The purity of collected SP (P3) and NSP (P4) after sorting were 97.6% and 95.3% respectively (Fig. 1C and D), indicating that these cells qualified for further study.

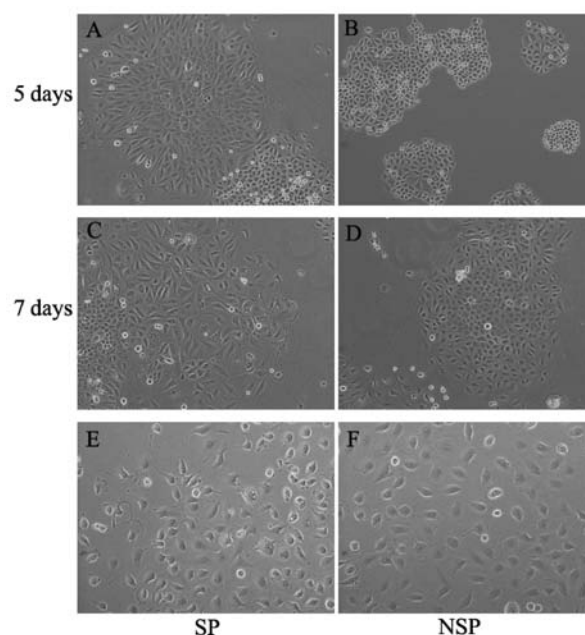


Figure 3. Morphological changes of SP and NSP cells in RPMI-1640 and UltraMEM culture; (A and B) SP and NSP cells cultured 5 days in RPMI-1640; Larger clone formation in SP cells and polygonal squamous epithelial cells began to change to fusiform cells (A); (C and D) SP and NSP cells cultured for 7 days in RPMI-1640; SP cells grew many long filaments, causing them to resemble fibroblasts (C); (E and F) SP and NSP cells in UltraMEM culture; SP cells lost their normal appearance and began to extend fibroblast-like filaments (E); NSP cells also displayed morphological changes, although these were not as noticeable as those of SP cells (F).

Colony formation assay. Colony formation assays were repeated thrice in triplicate. After culture for about 12 days at which time most clones reached more than 50 cells, we counted the clone numbers and found that the mean CFE of SP cells ($50.8 \pm 1.06\%$) was much higher compared to that of the non-SP cells ($11.4 \pm 0.4\%$). A t-test was performed in SPSS 17.0 and there were significant differences in CFE between them, ($P < 0.001$) (Fig. 2).

Cell morphological changes assay. We seeded the fresh-sorted SP and NSP cells in RPMI-1640 (with 10% FBS) and UltraMEM (with 5% FBS) for contrast and observed the cell morphologic changes dynamically. We found that SP cells grew faster than NSP cells in RPMI-1640 and the formed clones were greater and larger compared to those of NSP cells (Fig. 3A and B). As the cultured time increased, the SP cells began to change shape, from polygonal squamous epithelial cells to fusiform cells and grew many long filaments, causing them to resemble fibroblasts (Fig. 3C), whereas the NSP cells did not change to a greater extent. When maintained in UltraMEM, SP cells grew faster than NSP cells (Fig. 3E and F). Moreover, cell morphology changed markedly in UltraMEM (Fig. 3E and F). SP cells lost their normal appearance in UltraMEM culture and began to extend fibroblast-like filaments (Fig. 3E). NSP cells also displayed morphological changes, although these were not as noticeable compared to the SP cells (Fig. 3F).

Quantitative real-time RT-PCR analysis of stemness and EMT related genes. To determine whether SP cells of NPC have the characteristics of stem cells and EMT, we obtained

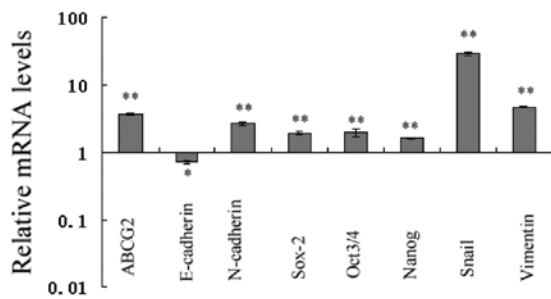


Figure 4. Quantitative PCR analysis of the expression of stemness-related and EMT-related genes. GAPDH mRNA was used to normalize the variability in template loading. The relative expression levels in SP cells to NSP cells were given. The data were reported as mean \pm SEM. Significant differences were * $P < 0.05$ and ** $P < 0.01$. The mRNA levels of stemness-related genes ABCG2, Nanog, Oct3/4, N-cadherin and Sox2 were increased remarkably in SP cells. The key EMT related genes Snail, N-cadherin and vimentin were also increased in SP cells, whereas E-cadherin was decreased in SP cells.

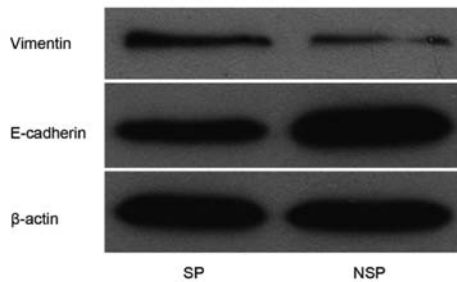


Figure 5. Western blot analysis of E-cadherin and vimentin in the SP cells and NSP cells. A reduced expression of E-cadherin and an increased expression of vimentin in SP cells compared to NSP cells.

RNA from the fresh-sorted SP and NSP cells and analyzed ABCG2, Oct4, Sox2, Nanog, vimentin, E-cadherin, Snail and N-cadherin expression using real-time RT-PCR. Epithelial cell special gene E-cadherin was found to be downregulated in SP cells, whereas the expression levels of the EMT-related genes vimentin, Snail and N-cadherin were higher in SP cells than in NSP cells of NPC. The stemness-related genes, Oct3/4, Sox2, Nanog, ABCG2 were also found to be more highly expressed in SP cells compared to NSP cells (Fig. 4). All differences were statistically significant.

Western blot analysis. Since the enhanced migratory/invasive ability of epithelial cells is often caused by EMT, we analyzed a panel of representative epithelial and mesenchymal markers by immunoblotting to determine whether this process occurs in SP cells of NPC. The results displayed that the SP cells of NPC caused an EMT-like marker shift, which was a dramatic downregulation of the epithelial marker E-cadherin and upregulation of the EMT-associated transcription factor vimentin (Fig. 5).

Immunofluorescence. Immunofluorescence staining further revealed that the expression of E-cadherin dramatically decreased in SP cells contrasting to NSP cells (Fig. 6), whereas the levels of vimentin were strongly increased in SP cells (Fig. 7). These results thus demonstrate that SP cells of NPC have the characteristics of EMT.

Matrigel-Transwell invasion assay in vitro. CSC has the capability to initiate and drive the growth of the primary tumor and of the invasion and metastasis. In analogy to this, we analyzed whether SP cells of NPC could display a similar invasive potential. By using a Matrigel invasion chamber, we compared the invading capacity of SP and NSP cells. The SP cells of the CNE-2 cell line displayed a significantly increased invading capacity (105.40 ± 16.118) compared to the NSP cells (33.40 ± 10.21) (Fig. 8) ($P = 0.003$).

Discussion

It has been extensively reported that SP cells represent normal stem cells or cancer stem cells in recent years (13,19,21,22). In the present studies we established that SP cells in the nasopharyngeal carcinoma cell line CNE-2 contained SP cells. The ratio of SP cells was on average 16.6%, higher than the ratio reported Wang *et al* (19), although both studies utilized the same cell line. Many reasons may be causing this difference. As we reported earlier, the SP cell ratio of a cell line is effected by many factors during Hoechst 33342 dyeing, such as cell concentration, Hoechst 33342 concentration and staining time. The same cell line with different passage generations stored in different laboratories may also contain a different SP cell ratio. Although the SP ratio in our study was 16.2%, the cell line was very sensitive to the ABCG2 inhibitor verapamil, which is used to block the ABCG2 pumping Hoechst 33342 function. Corresponding to these results, qRT-PCR further tested that ABCG2 levels in SP cells was much higher compared to NSP cells. ABCG2 is a stem cell marker, and it always has high expression in stem cells, whose function is to efflux many drugs and toxic substances out of the stem cells in order to prolong their lifetime and generate genetic stability. Sox2, Nanog and Oct4 are transcription factors (TFs) that are always highly expressed in embryonic stem cells, and they are also found to be highly expressed in many cancer stem cells (23-26). In our study, these TFs were more highly expressed in SP cells, indicating that SP cells highly express stemness-related genes. In addition, our results of the colony formation test *in vitro* indicated that the SP cells had much higher colony formation ability. All these results verified that the SP cells we isolated were enriched with more cancer stem-like cells.

Another capability of CSCs is that they undergo epithelial mesenchymal transition (EMT), a key step during embryogenesis (27-29) and wound healing (30). During EMT, epithelial cells lose their typical epithelial cell shape and change to fibroblast-like cells. Additionally, they break down cell-cell and cell extracellular matrix contacts, and migrate to other locations in the body (14). Evidence suggests that genetic programs relevant to EMT are also transiently activated in epithelial cancer cells through which epithelial cancer cells are able to invade and metastasize. In the present study, we cultured the fresh-sorted SP cells and NSP cells in 10% FCS medium in a polyHEMA-coated flasks, and found that after several days of culture, some of the SP cells acquired fibroblast-like, mesenchymal appearances, which indicated that SP cells may undergo EMT. Next we cultured sorted SP and NSP cells in an UltraMEM medium containing 5% FCS, 10 ng/ml EGF and 5 ng/ml FGF. Wang *et al* (19) reported that SP cells grew resembling fibroblasts in this medium. Similar

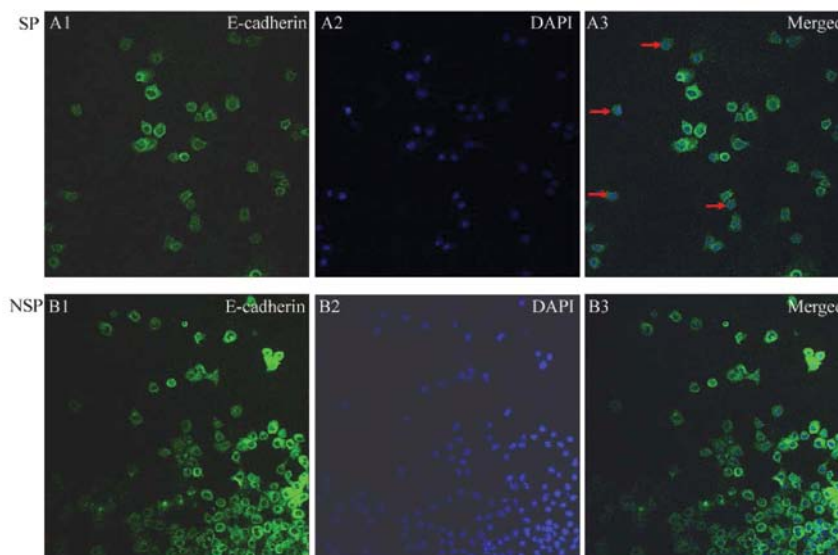


Figure 6. Immunofluorescence images of SP and NSP cells stained using antibody against E-cadherin. Immunofluorescence staining reveals a reduced expression of E-cadherin in SP cells (A1 and A2) in comparison with the NSP cells (B1 and B2)

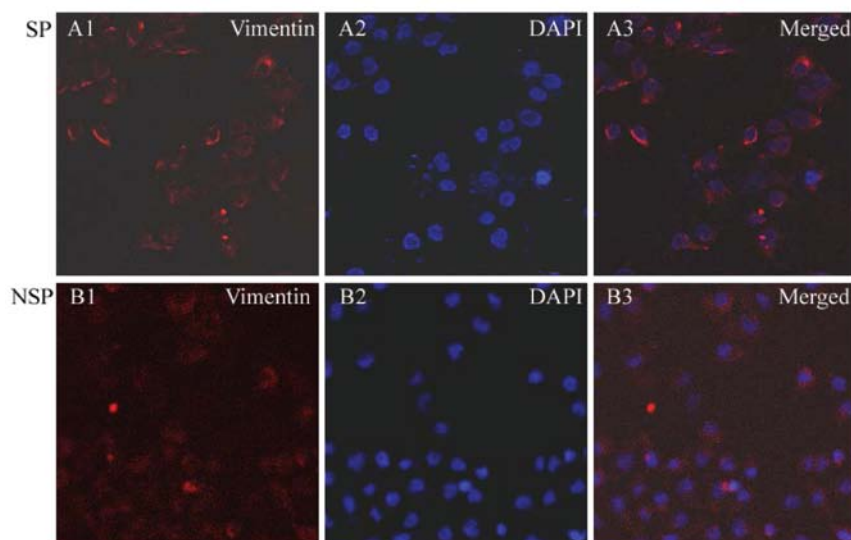


Figure 7. Immunofluorescence images of SP and NSP cells stained using antibody against vimentin. Immunofluorescence staining reveals an increased expression of vimentin in SP cells (A1 and A2) compared to the NSP cells (B1 and B2).

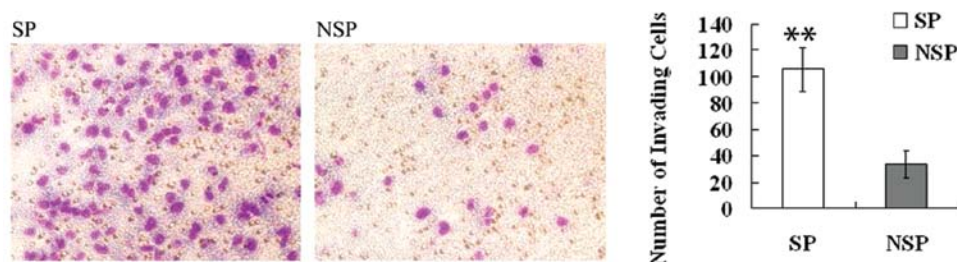


Figure 8. Invasion assay. Matrigel invasion chambers were used to compare the invading capability between SP and NSP cells. SP cells displays stronger invading activity *in vitro* than NSP cells (** $P < 0.01$)

to their results, we also observed that SP cells grew comparable to fibroblast, further confirming the results we observed in the 10% FCS medium. It has been reported that cancer stem

cells possess many mesenchymal characteristics, and they are more amenable to EMT (16). To further test whether SP cells more readily undergo EMT compared to NSP cells, we

analyzed EMT-related genes expressed in SP and N SP cells by qRT-PCR. Consequently, SP cells expressed higher levels of mesenchymal-related genes N-cadherin, vimentin and Snail, while they expressed low levels of the epithelial-related gene, E-cadherin. These results indicate that genetic programs relevant to EMT are more activated in SP cells. To verify the qRT-PCR results, we performed western blot analysis to confirm the epithelial-typical expressed gene, E-cadherin and the EMT-related gene, vimentin. Consistent with the PCR results, western blot results also demonstrated that E-cadherin was expressed more in NSP cells, and lesser in SP cells; for vimentin, the results were vice versa. When analyzed by immunofluorescence, the expression results of E-cadherin and vimentin were in accordance to western blot analyses. In collaboration, our results support the conclusion that SP cells in the CNE-2 cell line not only enrich cancer stem-like cells, but also possess mesenchymal cell characteristics which make them undergo EMT more readily.

There are several reports indicating how cancer stem cells have the strongest invasion and metastasis ability and how the main cell population causes cancer invasion and metastasis. Cancer metastasis consists of a sequential series of events: the epithelial-mesenchymal transition (EMT) and the mesenchymal-epithelial transition (MET). They are recognized as critical events for metastasis of carcinomas (15). In our present study, we discovered that SP cells had a stronger invading activity compared to NSP cells by using their Matrigel-Transwell invasion assay *in vitro*, indicating that SP cells may be the main cell population causing nasopharyngeal carcinoma invasion and metastasis. In conclusion, our data indicate that SP cells in CNE-2 cell line possess the properties of cancer stem cells, and they have more mesenchymal cell characteristics related to EMT and cancer cell metastasis. Invasion and metastasis are the main causes leading to death, while EMT is the first step of cancer metastasis. Therefore the study on EMT may be equally important to that of nasopharyngeal carcinoma stem cells.

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References

- Spano JP, Busson P, Atlan D, Bourhis J, *et al*: Nasopharyngeal carcinomas: an update. *Eur J Cancer* 39: 2121-2135, 2003.
- Ong YK, Heng DM, Chung B, *et al*: Design of a prognostic index score for metastatic nasopharyngeal carcinoma. *Eur J Cancer* 39: 1535-1541, 2003.
- Visvader JE and Lindeman GJ: Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 8: 755-768, 2008.
- Singh SK, Clarke ID, Terasaki M, *et al*: Identification of a cancer stem cell in human brain tumors. *Cancer Res* 63: 5821-5828, 2003.
- O'Brien CA, Pollett A, Gallinger S, *et al*: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 445: 106-110, 2007.
- Collins AT, Berry PA, Hyde C, *et al*: Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 65: 10946-10951, 2005.
- Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, *et al*: Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 1: 313-323, 2007.
- Prince ME, Sivanandan R, Kaczorowski A, *et al*: 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.
- Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al*: Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 100: 3983-3988, 2003.
- Zhang S, Balch C, Chan MW, *et al*: Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 68: 4311-4320, 2008.
- Shmelkov SV, Butler JM, Hooper AT, *et al*: CD133 expression is not restricted to stem cells, and both CD133⁺ and CD133⁻ metastatic colon cancer cells initiate tumors. *J Clin Invest* 118: 2111-2120, 2008.
- Mack B and Gires O: CD44s and CD44v6 expression in head and neck epithelia. *PLoS One* 3: e3360, 2008.
- Goodell MA, Brose K, Paradis G, Conner AS and Mulligan RC: Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* 183: 1797-1806, 1996.
- Radisky DC and LaBarge MA: Epithelial-mesenchymal transition and the stem cell phenotype. *Cell Stem Cell* 2: 511-512, 2008.
- Thiery JP and Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 7: 131-142, 2006.
- Mani SA, Guo W, Liao MJ, *et al*: The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133: 704-715, 2008.
- Chao C, Yan W, Michael H, *et al*: Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. *PLoS One* 6: e16466, 2011.
- Yang L, Ping YF, Yu X, *et al*: Gastric cancer stem-like cells possess higher capability of invasion and metastasis in association with a mesenchymal transition phenotype. *Cancer Lett* 310: 46-52, 2011.
- Wang J, Guo LP, Chen LZ, Zeng YX and Lu SH: Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. *Cancer Res* 67: 3716-3724, 2007.
- Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- Scharenberg CW, Harkey MA and Torok-Storb B: The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99: 507-512, 2002.
- Alvi AJ, Clayton H, Joshi C, *et al*: Functional and molecular characterization of mammary side population cell. *Breast Cancer Res* 5: R1-R8, 2003.
- Boyer LA, Lee TI, Cole MF, *et al*: Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122: 947-956, 2005.
- Loh YH, Wu Q, Chew JL, *et al*: The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* 38: 431-440, 2006.
- Rodda DJ, Chew JL, Lim LH, *et al*: Transcriptional regulation of Nanog by OCT4 and SOX2. *J Biol Chem* 280: 24731-24737, 2005.
- Chew JL, Loh YH, Zhang W, *et al*: Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol* 25: 6031-6046, 2005.
- Hay ED: An overview of epithelial-mesenchymal transformation. *Acta Anat (Basel)* 154: 8-20, 1995.
- Perez-Pomares JM and Munoz-Chapuli R: Epithelial-mesenchymal transitions: a mesodermal cell strategy for evolutive innovation in Metazoans. *Anat Rec* 268: 343-351, 2002.
- Thiery JP: Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 15: 740-746, 2003.
- Savagner P, Kusewitt DF, Carver EA, *et al*: Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes. *J Cell Physiol* 202: 858-866, 2005.