Identification of ABCG2\(^*\) cells in nasopharyngeal carcinoma cells

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Abstract. Tumor stem cells are a small subset of tumor cells with the ability of self-renewal and differentiation and are regarded as a cause of tumor growth and recurrence. Previously we have shown that stem-like label-retaining cells (LRCs) can be detected in nasopharynx, tongue, esophagus and xenograft tumors formed by nasopharyngeal carcinoma (NPC) cell lines (5-8F, 6-10B and TMNE). The present study aimed to identify ABCG2\(^*\) cells in 5-8F NPC cells and compare their tumorigenic potential with ABCG2\(^+\) cells, expecting that we can obtain insight into the mechanism of the differential phenotypes of ABCG2\(^*\) and ABCG2\(^+\) cells. By using magnetic cell sorting (MACS) method, we isolated ABCG2\(^*\) cells and ABCG2\(^+\) cells from 5-8F cells. Among these two subpopulations and unsorted 5-8F cells, the rate of ABCG2\(^*\) cells at G1 phase was highest, while the rate of ABCG2\(^+\) cells at S phase was highest, indicating that ABCG2\(^*\) cells were mostly quiescent. However, ABCG2\(^+\) cells showed lower cloning efficiency and tumorigenicity than ABCG2\(^*\) cells. We also used Affymetrix U133 plus 2.0 human whole genome expression chip to identify the gene expression profile of ABCG2\(^*\) and ABCG2\(^+\) cells and found that both subpopulations expressed some stem cell associated genes, e.g., PSCA, ABCG2 and ALPI were expressed in ABCG2\(^*\) cells, and K19, integrin \(\alpha\)_6, integrin \(\beta\)_4, CD44 and K14 were expressed in ABCG2\(^+\) cells, suggesting there were stem cells in both ABCG2\(^*\) and ABCG2\(^+\) cells. Our data demonstrated that there exist ABCG2\(^*\) cells in NPC cells, but ABCG2\(^+\) alone is not sufficient for isolating cancer stem cells in 5-8F NPC cells.

Introduction

Stem cells, with the ability to proliferate infinitely through self-renewal and differentiation, can be isolated and cultured from inner cell mass of blastocyst (1), primordial germ cells (2), bone marrow (3), brain (4), skin (5), digestive canal (6), respiratory tract (7), cornea (8), muscle (9), liver (10), pancreas (11) and lung (12). Many tumors contain a sub-population of stem cells known as cancer stem cells (CSCs). CSC has unlimited potential for self-renewal and can drive tumorigenesis and develop multidrug resistance (13,14). To date, CSCs have been identified in human leukemia (15) and in solid tumors including breast (16), bladder (17), colorectal (18), gastric (19), hepatocellular (20) and lung carcinomas (21), malignant melanoma (22), nasopharyngeal (23), pancreatic (24), prostate (25) and renal carcinomas (26). However, the characterization of CSC remains insufficient and CSC has not been isolated from some tumors. CSC is regarded as the root of cancer, and thus should be more important for cancer therapy than other tumor cells. Therefore, CSC might be a good therapeutic target for cancer treatment.

Side population (SP) cells, originally isolated from murine hematopoietic stem cells using their characteristic to efflux Hoechst 33342 dye and FACS method (27), have been sorted from many normal human tissues such as heart (28), prostate (29), limbal epithelium (30), skin (31), mammary gland (32) and kidney (33), and have also isolated from human cancer cells such as small cell lung cancer (34), glioma (35), prostate cancer (36), leukemia (37), neuroblastoma (38), hepatoma (39), nasopharyngeal carcinoma (23), colorectal cancer (39), thyroid cancer (40) and lung cancer (41). Cancer SP cells exhibit stem cell-like functions such as resistance to chemotherapy drugs, clonogenic ability and tumorigenicity. Therefore, SP cells can be regarded as a kind of enriched CSCs.

The phenotype of SP cells depends on the expression of ABCG2, a member of ATP binding cassette (ABC) transporters which belong to one of the largest transmembrane protein families. They use ATP to transport various substrates across cell membranes. The substrates include chemotherapy drugs, metabolites and other compounds such as Hoechst 33342 dye. To present, about 50 ABC transporters have been identified (42) and are divided into seven subfamilies (from

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A to G), among which ABCG2 is the second member of the G subfamily. ABCG2 was first identified in doxorubicin-resistant human MCF-7 breast cancer cells and thus also named as breast cancer resistance protein (BCRP) (43). It is widely distributed in normal tissues and stem cells including SP cells. High expression of ABCG2 has been detected in CSCs isolated from embryonic cancer (44), retinoblastoma (45), lung (41), liver (46), pancreas (47) and gallbladder cancer (48).

Previously we have identified LRCs in nasopharyngeal epithelia and NPC xenograft tissues with bromodeoxyuridine (BrdU) (49). In this study, we isolated ABCG2+ and ABCG2 cells from 5-8F NPC cells by using MACS and then characterized their biological properties and expression profiles. Our results suggest that ABCG2 alone is insufficient to identify CSCs in 5-8F NPC cells.

Materials and methods

Ethics statement. All animal work was performed under the institutional guidelines approved by the Animal Care and Use Committee of Central South University. The present study was also approved ethically by the institutional review board of Central South University.

Double labeling detection for LRC and ABCG2 expression in 5-8F cells. We used immunofluorescence method to detect LRCs and ABCG2 expression in 5-8F cells. Briefly, 5-8F cells was labeled with BrdU, inoculated into nude mice and traced for 8 weeks. Then, the tissue sections were made from formed tumor blocks, hydrated, treated with 3% H2O2 for 10 min to remove endogenous peroxidase, with 2 N hydrochloric acid for 30 min at 37°C, with 0.1 M sodium borate for 4 min and then treated with 0.25% trypsin and washed with PBS after each above treatment. The treated tissue sections was added with antibody for BrdU (Sigma, St. Louis, MO) at 4°C overnight.

After washing with PBS, the sections were labeled with mouse anti-mouse IgG-FITC antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated for 30 min at RT. After washing with PBS, the sections were blocked with normal goat serum, added with mouse anti-human ABCG2 antibody (BD Pharmingen, USA), for 30 min at RT; then added with Texas Red conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) and incubated for 30 min at RT. After washing with PBS, the sections were observed and photographed with a fluorescence microscope.

Separation of ABCG2+ cells by MACS. 5-8F NPC cells were harvested, prepared into single cell suspension and counted. Less than 10^3 cells were obtained for cell sorting. The volume of cell suspension was adjusted to 200 μl with 1X PBS containing 0.5% bovine serum albumin (BSA). Mouse anti-human ABCG2 antibody (20 μl) was added to the cell solution, mixed and incubated for 20 min at 4-8°C. The cells were washed with 1X PBS containing 0.5% BSA three times and the cell volume was adjusted to 200 μl. The goat anti-mouse IgG2a bound with magnetic beads (Miltenyi Biotec, Germany) was added to the cell solution, mixed and incubated for 20 min at 4-8°C and then washed with 1X PBS containing 0.5% BSA. The cells were harvested and the volume of cell suspension was adjusted to 0.5 ml. The sorting column was fixed on magnetic sorting stand (Miltenyi Biotec) and equilibrated by applying 0.5 ml 1X PBS containing 0.5% BSA. The bound cell suspension (0.5 ml) was applied to the column and the effluent was harvested. 1X PBS (0.5 ml) containing 0.5% BSA was applied to the column two times. The effluent was ABCG2 cell fraction. Another 1 ml of 1X PBS containing 0.5% BSA was applied to the column, the effluent was repeated for application to new columns and the ABCG2+ cells were subsequently enriched. The final effluent was centrifuged for 5 min at 4°C, 1,000 rpm to obtain the cells.

Identification of sorting effect. We used immunocytochemistry method to identify the purity of ABCG2+ cells. Briefly, enriched ABCG2+ were made into cell pellets. The cell pellets were treated with 3% H2O2 for 10 min, with 0.25% trypsin for 15 min and blocked with normal goat serum for 20 min. The cells were added with mouse anti-human ABCG2 antibody and incubated at 4°C overnight. After washing with PBS, the cells were added with goat anti-mouse IgG-HRP and incubated for 30 min at RT. After washing with PBS, the cells were developed with AEC (Zhongshan Goldenbridge Biotech Co., Ltd., China), counterstained with hematoxylin and mounted with Glycerol vinyl alcohol aqueous mounting solution (GVA, Zymed Laboratories, Inc., USA). The red ABCG2+ cells were observed with an optical microscope and the purity was calculated.

We also used flow cytometry to identify the purity of ABCG2+ cells. The MACS-sorted ABCG2+ cells were resuspended, added with anti-human ABCG2 antibody-FITC (1:200), incubated for 30 min at 4-8°C and washed with 1 ml of 1X PBS containing 0.5% BSA three times. Then the purity of ABCG2+ cells was measured with flow cytometry (FACS Calibur, BD, USA). Unsorted 5-8F cells added with IgG-FITC were used as control.

Cloning efficiency determination. Colony formation assay was performed as previously described (50). Briefly, single cell suspension was prepared from ABCG2+ and ABCG2- and unsorted 5-8F cells and counted. Each cell type was seeded in 12-well plates (200 cells/well) and cultured at 37°C for 14 days in an incubator with 5% CO2. Then the cells were fixed with methanol and stained with 0.4% crystal violet. Colonies containing at least 50 cells were counted under an inverse microscope. Cloning efficiency (%) = cell colony amounts/200 x 100%.

Cell cycle analysis. ABCG2+, ABCG2- and unsorted 5-8F cells (2x10^6) were harvested, respectively, washed with PBS, fixed with 70% ice-cold ethanol for 30 min. The ethanol was discarded and the cells were resuspended in 500 μl PBS, added with RNase A to a final concentration of 100 μg/ml, incubated at 37°C for 30 min, stained with 20 μg/ml of propidium iodide (PI) for 30 min, measured with flow cytometry and analyzed with Mod Fit LT software.

Analysis of tumorigenesis in NOD/SCID mice. ABCG2+, ABCG2- and unsorted 5-8F cells (10^5, 10^6, 10^7 per each type) were injected s.c. into three 4-6 weeks old female NOD/SCID mice with body weight of 17-24 g (Shanghai Slac Laboratory Animal Co., Ltd., Shanghai, China), respectively.
All the mice were sacrificed 6-16 weeks after injection and examined for tumors. The tumor blocks were dissected and made into tissue sections for inspection. Tumor blocks were dissected and fixed by immersion in 4% paraformaldehyde phosphate buffer. After fixation for 2-4 h, tissues were dehydrated, paraffin-embedded, sectioned at 4 µm and stained with haematoxylin and eosin (HE) for histological examination.

Microarray analysis. Microarray analysis was performed as previously described (51). GeneChip Human Genome U133 Plus 2.0 was used to analyze the gene expression profile of ABCG2+ cells and ABCG2- cells. The chip covers 47,400 transcripts and contains 38,500 known human genes. A probe hits only one genomic location; probes that can be mapped to the same target sequence in the correct direction are grouped together in the same probe set; each probe set consists of 10-20 pairs of 25 mer probes; each probe pair consists of two probe cells, one of which is perfect match and another of which is mismatch containing one base mismatch. The gene sequences are selected from GenBank, dbEST and RefSeq.

Total RNA of ABCG2+ cells and ABCG2- cells was extracted and used to purify polyA+ mRNA. The cDNA, double strand DNA and biotin-labeled cRNA were synthesized in turn. After fragmented, the labeled cRNA was loaded on the gene chip for microarray analysis. Hybridization, elution and staining of the chip were conducted with Affymetrix Hybridization Oven 640 and Affymetrix Fluidics Station 450 according to the manufacturer's instruction. After the chips were scanned, GCOS data processing software was used to calculate and process the obtained data. Before comparison of the results of two chips, the data of each chip were normalized to obtain reporter signal value. For screening the differentially expressed genes between ABCG2+ and ABCG2- group, signal log ratio ≥1.0 or ≤-1.0 (indicating 2-fold upregulation or downregulation of gene expression level) was set as screening criterion.

Verification of differentially expressed genes by RT-PCR. ABCG2+ and ABCG2- cells were harvested, respectively. Total RNAs were extracted from the harvested cells with TRIzol reagent according to the manufacturer's protocols and subsequently digested with DNase I to remove the residual amount of genomic DNA. RT-PCR was carried out with AMV reverse transcriptase system to detect the expression of selected genes. The PCR conditions were as follows: 3 min at 95˚C; 40 sec at 94˚C, 30 sec at 55-58˚C and 50 sec at 72˚C for appropriate cycles; 10 min at 72˚C for extension. GAPDH was used as internal control. The used PCR primers and cycle number are shown in Table I. RT-PCR product bands were scanned with image analyzer (Pharmacia, USA) and the accumulated optical density value (IA) of each band was analyzed with Imagemaster VDS software.

Geneontology analysis. GOSTAT (http://gostat.wehi.edu.au) was used to analyze and annotate the differentially expressed genes. GO provides three kinds of specifying terminology to describe the characteristics of gene products, including molecular function, biological process and cellular component.

Statistical analysis. SPSS13.0 statistical software and one-way ANOVA were used to analyze the cloning efficiency data. GCOS was used to test gene expression level of ABCG2+ and ABCG2- group and rank-test was applied to define the determinant interval. A P-value <0.05 was considered to be statistically significant.

Results

Double labeling detection of LRC and ABCG2 expression in 5-8F cells. 5-8F NPC cells were labeled with BrdU, inoculated into nude mice, traced for 8 weeks and then detected for LRC and ABCG2 expression. In LRCs, there was 61.69±8.31% (n=3) of ABCG2+ cells, while in ABCG2- cells, there was 12.05±2.80% (n=3) of LRCs (Table II, Fig. 1).
ABC2+ CELLS IN NPC

Sorting ABC2+ cells by MACS from 5-8F cells. 5-8F NPC cells were cultured and harvested, labeled with ABC2 antibody and magnetic beads, and then sorted through MS sorting column. The rate of ABC2+ cells was 2.11±0.36% (n=5). The ABC2+ and ABC2- cells were smeared on slides, respectively and detected for ABC2 expression with immunocytochemistry methods. ABC2 was highly expressed in ABC2+ cells, the positive signals located on the cell membrane and the purity of ABC2+ cells reached 90.73%. ABC2 was weakly expressed in only a minority of ABC2- cells (Fig. 2A and B).

After labeled with IgG-FITC, ABC2+ and ABC2- cells were analyzed by flow cytometry. It was shown that the purity of ABC2+ cells was 95.93% (Fig. 2C-E). These data showed that we had successfully enriched ABC2+ cells.

Identification of biological characteristics of ABC2+ cells. ABC2+, ABC2- and unsorted 5-8F cells were analyzed by flow cytometry, respectively. As seen in Fig. 3A-C and Table III, the rate of G0/G1 phase cells was the highest (73.74%) in ABC2+ cells among these three types of cells and the rate of S phase cells was the highest (32.56%) in ABC2- cells, indicating that ABC2+ cells were mostly quiescent and more ABC2- cells were in DNA synthesis period. Therefore, some of the ABC2+ cells might be the transient amplifying cells that could proliferate rapidly.

Cloning efficiency was analyzed among ABC2+, ABC2- and unsorted 5-8F cells. The ABC2+ cells formed smaller number of colonies compared with ABC2- cells and unsorted 5-8F cells (P<0.05), while the formed colony number of ABC2+ cells was higher than that of 5-8F cells (P<0.05) (Fig. 3D and E, Table IV).

Table II. Results of double labeling detection for LRCs and ABC2 expression.

|                     | DLCs/LRCs (%) | DLCs/ABC2+ cells (%) |
|---------------------|---------------|----------------------|
| Xenografted nude mouse 1 | 60.12         | 9.64                 |
| Xenografted nude mouse 2 | 54.28         | 11.38                |
| Xenografted nude mouse 3 | 70.67         | 15.12                |
| Mean ± SD           | 61.69±8.31    | 12.05±2.80           |

Table III. Cell cycle distribution of ABC2+, ABC2- and unsorted 5-8F cells.

|                     | G0+G1 (%) | S (%) | G2+M (%) |
|---------------------|-----------|-------|----------|
| ABC2+               | 73.74     | 20.92 | 5.34     |
| ABC2-               | 60.37     | 32.56 | 7.07     |
| Unsorted 5-8F       | 62.92     | 28.43 | 8.65     |

Table IV. Comparison of colony-forming capacity of ABC2+, ABC2- and unsorted 5-8F cells.

| Colony no. | ABC2+ | ABC2- | 5-8F |
|------------|-------|-------|------|
| 42, 48, 49 | 115, 129, 132 | 77, 86, 88 |
| 46, 49, 57 | 104, 122, 129 | 69, 78, 82 |
| 33, 48, 49 | 112, 118, 125 | 80, 83, 91 |
| Mean ± SD  | 46.78±6.48  | 120.67±9.22 | 81.56±6.58 |

To compare the tumorigenicity of ABC2+, ABC2- and unsorted 5-8F cells, we inoculated these cells into NOD/SCID mice, respectively. When inoculated with 10^2-10^4 cells, tumor formation could not be observed even after 113 days in either group. When inoculated with 10^5 cells, tumor block could be seen after 12 days in ABC2- cell group and 5-8F cell group and after 20 days in ABC2+ cell group. The weight of tumor blocks was highest in ABC2- cell group and lowest in ABC2+ cell group (Fig. 3F, Table V). Tumor formation rate was lowest in ABC2+ cell group (2/3) and was 100% (3/3) in other two groups. Paraffin sections were prepared from these tumor blocks and detected by H&E staining. The morphology of tumor cells from these three groups exhibited no significant difference (Fig. 3G-I).

Gene expression profile of ABC2+ and ABC2- cells. Affymetrix oligonucleotide microarray (Human Genome U133 Plus 2.0 Array) was used to monitor gene expression of about
47,400 transcripts containing 38,500 known genes in ABCG2+ and ABCG2− cells. After the differential gene expression profiles between ABCG2+ and ABCG2− cells were constructed, differentially expressed genes or ESTs which were upregulated (for 2 fold) or downregulated (for 2 fold) were screened. There were 353 genes or ESTs upregulated significantly in ABCG2+ cells and 590 genes or ESTs upregulated significantly in ABCG2− cells out of the 47,400 transcripts. The 80 most significantly differentially expressed genes in ABCG2+ and ABCG2− cells are listed in Table VI.

Differentially expressed genes were analyzed by Gene Ontology. A group of genes generally involved in negative regulation of cell cycle progression were discovered in ABCG2+ cells, whereas this functional classification could not be found in ABCG2− cells, which can explain the fact that most ABCG2+ cells were in G0/G1 phase of cell cycle. The stem cell associated genes PSCA, ABCG2 and ALPI were upregulated significantly in ABCG2+ cells, while another set of stem cell related genes including K19, integrin α6, integrin β4, CD44 and K14 were upregulated significantly in ABCG2− cells.

Table V. Tumors formed by dorsal subcutaneous inoculation of ABCG2+, ABCG2− and unsorted 5-8F cells into NOD/SCID mice.

| Cells      | Cell no. | Tumor formation rate | Tumor weight (g)     | Latency period (day) |
|------------|----------|----------------------|----------------------|----------------------|
| ABCG2+     | 1x10^2   | -                    | -                    | -                    |
|            | 1x10^3   | -                    | -                    | -                    |
|            | 1x10^4   | -                    | -                    | -                    |
|            | 1x10^5   | 2/3                  | 0.93, 0.17           | 20, 29               |
| ABCG2−     | 1x10^2   | -                    | -                    | -                    |
|            | 1x10^3   | -                    | -                    | -                    |
|            | 1x10^4   | -                    | -                    | -                    |
|            | 1x10^5   | 3/3                  | 2.44, 3.95, 2.29     | 12, 14, 14           |
| Unsorted 5-8F | 1x10^2 | -                    | -                    | -                    |
|            | 1x10^3   | -                    | -                    | -                    |
|            | 1x10^4   | -                    | -                    | -                    |
|            | 1x10^5   | 3/3                  | 2.96, 1.43, 1.69     | 12, 13, 15           |

Figure 2. Verification of sorted cells by immunocytochemistry (A and B) and flow cytometry (C-E). ABCG2 is expressed only in ABCG2+ cells (A) but not in ABCG2− cells (B). (C-E) Indicate measurement of ABCG2 expression by flow cytometry in 5-8F cells (C), ABCG2+ cells (D) and ABCG2− cells (E), respectively. The purity of sorted ABCG2+ cells is 95.93%.
### Table VI. Eighty most differentially expressed genes in ABCG2+ and ABCG2- cells.

| Gene symbol | UniGene ID | Chromosomal location |
|-------------|------------|----------------------|
| A2M         | 6.4 Hs.212838 | α-2-macroglobulin | chr12p13.3-p12.3 |
| ALPI        | 5.9 Hs.37009  | Alkaline phosphatase, intestinal | chr2q37.1 |
| CGA         | 5.7 Hs.119689 | Glycoprotein hormones, α polypeptide | chr6q12-q21 |
| SLC16A6     | 5.3 Hs.42645  | Solute carrier family 16, member 6 | chr17q24.2 |
| DAB2        | 5.2 Hs.481980 | Disabled homolog 2, mitogen-responsive phosphoprotein | chr5p13 |
| C1QTNF6     | 4.9 Hs.22011  | C1q and tumor necrosis factor related protein 6 | chr2q13.1 |
| SNAP25      | 4.6 Hs.167317 | Synaptosomal-associated protein, 25 kDa | chr20p12-p11.2 |
| DIO2        | 4.4 Hs.202354 | Deiodinase, iodothyronine, type II | chr14q24.2-q24.3 |
| CTGF        | 4.1 Hs.410037 | Connective tissue growth factor | chr6q23.1 |
| ECG2        | 4.1 Hs.244569 | Esophagus cancer-related gene-2 | chr5q32 |
| PDE3A       | 4.1 Hs.386791 | Phosphodiesterase 3A, cGMP-inhibited | chr12p12 |
| TFPI        | 4.0 Hs.516578 | Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) | chr2q31-q32.1 |
| ADAM12      | 4.0 Hs.386283 | ADAM metallopeptidase domain 12 (meltrin α) | chr10q26.3 |
| C6orf176    | 3.9 Hs.31917  | Chromosome 6 open reading frame 176 | chr9q27 |
| EID3        | 3.9 Hs.171695 | E1A-like inhibitor of differentiation 3 | chr12q3-q24.1 |
| DUSP1       | 3.8 Hs.78977  | Dual specificity phosphatase 1 | chr5q34 |
| PCSK1       | 3.6 Hs.1908   | Proprotein convertase subtilisin/kexin type 1 | chr5q15-q21 |
| PRO0132     | 3.6 - PRO0132 protein | chr2q34 |
| SLC16A6     | 3.6 Hs.463838 | Solute carrier family 16, member 6 | chr17q24.2 |
| MRS2L       | 3.4 Hs.533291 | MRS2-like, magnesium homeostasis factor | chr9p22-3.3-p22.1 |
| SLC29A3     | 3.3 Hs.438419 | Solute carrier family 29, member 3 | chr10q22.1 |
| PRG1        | 3.2 Hs.1908   | Proteoglycan 1, secretory granule | chr10q22.1 |
| CES1        | 3.2 Hs.535486 | Carboxylesterase 1 | chr16q13-q22.1 |
| UGT1A8      | 3.2 - UDP glucuronosyltransferase 1 family, polypeptide A8 | chr2q37 |
| APOC3       | 3.1 Hs.534984 | Apolipoprotein C-III | chr1q23.1-q23.2 |
| ABCG2       | 3.1 Hs.480218 | ATP-binding cassette, sub-family G, member 2 | chr4q22 |
| FYN         | 3.1 Hs.390567 | FYN oncogene related to SRC, FGR, YES | chr6q21 |
| WNT5A       | 3.1 Hs.152213 | Wingless-type MMTV integration site family, member 5A | chr3p21-p14 |
| FO8B        | 3.0 Hs.75678  | FBJ murine osteosarcoma viral oncogene homolog B | chr19q13.32 |
| PAPSS2      | 3.0 Hs.524491 | 3'-Phosphoadenosine 5'-phosphosulfate synthase 2 | chr10q23-q24 |
| VTN         | 3.0 Hs.2257   | Vitronectin | chr17q11 |
| CPS1        | 3.0 Hs.149252 | Carbamoyl-phosphate synthetase 1 | chr2q35 |
| RHOBT1      | 3.0 Hs.148670 | Rho-related BTB domain containing 1 | chr10q21.2 |
| FTO         | 3.0 Hs.528833 | Fatso | chr16q12.2 |
| TBX3        | 3.0 Hs.129895 | T-box 3 | chr10q24.1 |
| C20orf100   | 3.0 Hs.26608  | Chromosome 20 open reading frame 100 | chr20q13.12 |
| BMP2        | 2.9 Hs.73855  | Bone morphogenic protein 2 | chr20p12 |
| PPP1R15A    | 2.8 Hs.76556  | Protein phosphatase 1, regulatory (inhibitor) subunit 15A | chr19q13.2 |
| MX1         | 2.8 Hs.517307 | Myxovirus resistance 1 | chr2q22.3 |
| PAPSS2      | 2.8 Hs.524491 | 3'-Phosphoadenosine 5'-phosphosulfate synthase 2 | chr10q23-q24 |
| IF          | -3.2 Hs.312485 | I factor (complement) | chr4q25 |
| TBX18       | -3.2 Hs.251830 | T-box 18 | chr6q14-q15 |
| ITGB4       | -3.2 Hs.370255 | Integrin, β 4 | chr17q25 |
| SLCO1B3     | -3.2 Hs.504966 | Solute carrier organic anion transporter family, member 1B3 | chr12p12 |
| CD300LG     | -3.3 Hs.147313 | CD300 antigen like family member G | chr17q21.31 |
| GJA1        | -3.3 Hs.74471  | Gap junction protein, α 1, 43 kDa (connexin 43) | chr6q21-q32.2 |
| FLI1        | -3.4 Hs.504281 | Friend leukemia virus integration 1 | chr11q24.1-q24.3 |
| IGFBP3      | -3.4 Hs.450230 | Insulin-like growth factor binding protein 3 | chr7p13-p12 |
| GABRB1      | -3.4 Hs.27283  | γ-aminobutyric acid (GABA) A receptor, β 1 | chr4p12 |
| LUM         | -3.5 Hs.406475 | Lumican | chr12q21.3-q22 |
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Table VI. Continued.

| Gene symbol | UniGene ID | Chromosomal location |
|-------------|------------|----------------------|
| CALB1       | Hs.65425   | chr8q21.3-q22.1      |
| TP73L       | Hs.137569  | chr3q28              |
| DSG3        | Hs.1925    | chr18q12.1-q12.2     |
| PPP1R14C    | Hs.486798  | chr6q24.3-q25.3      |
| OR5K1       | Hs.531371  | chr3q12.1            |
| GPR87       | Hs.58561   | chr3q24              |
| RSAD2       | Hs.17518   | chr2p25.2            |
| EGLN3       | Hs.135507  | chr1q13.1            |
| CLCA2       | Hs.241551  | chr1p31-p22          |
| FST         | Hs.9914    | chr5q11.2            |
| LOC196264   | Hs.15396   | chr1q23.3            |
| DSC3        | Hs.41690   | chr1q12.1            |
| KRT14       | Hs.355214  | chr17q12-q21         |
| PDCD8       | Hs.424932  | chrXq25-q26          |
| PRSS35      | Hs.98381   | chr6q14.2            |
| BNC1        | Hs.459153  | chr1q25.2            |
| ITGB6       | Hs.470399  | chr2q24.2            |
| SCEL        | Hs.492938  | chr1q22              |
| LAMC2       | Hs.530509  | chr1q25-q31          |
| PDZK3       | Hs.481819  | chr5p13.3            |
| GABRA2      | Hs.116250  | chr4p12              |
| LAMB4       | Hs.62022   | chr7q22-q31.2        |
| KRT19       | Hs.514167  | chr17q21.2           |
| DSG3        | Hs.1925    | chr1q25-q26          |
| ITGB4       | Hs.370255  | chr1q25              |
| ROCK1       | Hs.306307  | chr1q11.1            |
| IGFBP7      | Hs.479808  | chr4q12              |
| IGFBP3      | Hs.450230  | chr7p13-p12          |
| LIFR        | Hs.133421  | chr5p13-p12          |
| IL1A        | Hs.1722    | chr2q14              |

aSLR, ABCG2⁺ vs. ABCG2⁻ signal log ratio.

cells, suggesting that there are stem cells in both ABCG2⁺ and ABCG2⁻ cells.

From the chip analysis results, we selected 6 meaningful genes such as ALPI, ABCG2 and WNT5A which were highly expressed in ABCG2⁺ cells, and BNC1, IGFBP3 and SCEL which were highly expressed in ABCG2⁻ cells to perform RT-PCR verification. The expression of these genes was consistent with the results of chip analysis (Fig. 4A and B).

Fig. 4C exhibited the scatterplot of average expression value in ABCG2⁺ and ABCG2⁻ cells. The x-axis showed the signals of ABCG2⁺ cell group and the y-axis showed the signals of ABCG2⁻ cell group. Red plots indicated genes whose detection results were P (present) in two groups, blue blots indicated genes whose detection results were P in only one of the two groups and yellow plots represented genes whose detection results were A (absent) in both groups. From top to bottom, the green oblique lines represented expression difference at 30, 10, 4, 2, 1/2, 1/4, 1/10 and 1/30-fold between ABCG2⁺ and ABCG2⁻ cells, respectively. The result demonstrated that the expression difference of most genes between ABCG2⁺ and ABCG2⁻ cells was 2- to 4-fold and that only a minority of the genes could reach 10- to 30-fold or above.

Discussion

Previously we have shown that LRCs exist in nasopharynx, tongue, esophagus and xenograft NPC tissues (49). One of the characteristics of adult stem cells is that they can be labeled for a long time and therefore are known as LRCs (52). Label retaining experiment is an effective method to label and detect stem cells in tissue of living organism (53). BrdU and 3H-thymine deoxyribose (3H-TdR) are commonly
used labeling markers. The mechanism underlying the label retaining of a marker in stem cells is unclear. One explanation is that stem cells exhibit slow cell cycle progression, therefore the marker can remain in DNA of LRCs after tracing for a long time, while the marker in other cells will be gradually diluted with rapid cell division. Cairns (54) raised another explanation for LRC. Because of the asymmetry of stem cell division, labeled DNA is always allocated to daughter stem cells and the newly synthesized DNA is always allocated to daughter differentiated cells. We consider that the above explanations can partly explain the mechanism of the label retaining characteristic of stem cells.

In this study, we first labeled the cells in NPC tissue formed by inoculation of 5-8F cells into nude mice. Although scarce, the existence of LRCs indicated that there were cancer stem cells in NPC tissue. To further identify these LRCs, we detected the expression of ABCG2 in the same NPC tissue. ABCG2, a member of ABC transporter superfamily, is a transmembrane
protein in charge of the efflux of chemotherapy drugs, metabolites and other compounds such as Hoechst 33342 dye, thus it is responsible for the phenotype of SP cells and has been widely used as a marker in CSCs isolated from retinoblastoma (45), embryonic (44), lung (46), pancreas (47), gallbladder (48) and head and neck cancers (55) and NPC (23). Our results showed that there were approximately 62% of ABCG2+ cells in LRCs, suggesting that LRCs may represent a group of CSCs in NPC cells. This result is similar to that of research work of Welm et al (56). They found that the number of LRCs in SP cells was 4 times of those in non-SP cells.

Currently it is technically impossible to isolate LRCs from tumor tissue. We tried to sort ABCG2+ cells by MACS from 5-8F cells. The ABCG2-positive rate was 2.11%, which is similar to those in other tumor cells (57). Subsequently, we identified the biological characteristics of these ABCG2+ cells, and found that among ABCG2+, ABCG2- and unsorted 5-8F cells, the rate of ABCG2+ cells were highest at G0/G1 phase, while the rate of ABCG2- cells were highest at S phase, indicating that ABCG2+ cells were mostly quiescent, and more ABCG2- cells were in DNA synthesis period. Therefore, some of the ABCG2- cells might be the transient amplifying cells that could proliferate rapidly. Among the three kinds of cells, the cloning efficiency of ABCG2+ cells was lower than that of ABCG2- cells and unsorted cells, and the tumorigenicity of ABCG2- cells was also the lowest. We suppose that there may be several possibilities leading to the above results. One is that ABCG2 alone can not sufficiently enrich CSCs from 5-8F cells, therefore there are non-CSCs in ABCG2+ cells and there are some CSCs in ABCG2- cells. Another is that ABCG2+ cells are enriched in SP cells, but are not equal to SP cells, thus may not exhibit typical properties of CSCs. Our results are similar to those of Patrawala et al (57). They found that side population isolated from prostate cancer, breast cancer and glioma was enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells were similarly tumorigenic.

To explore the molecular mechanism underlying the biological characteristics of ABCG2+ cells, Affymetrix oligonucleotide microarray was used to monitor expression of about 47,400 transcripts containing 38,500 known genes in ABCG2+ and ABCG2- cells. There were 353 genes and ESTs upregulated significantly and 590 genes downregulated significantly in ABCG2+ cells. As analyzed by Gene Ontology, a group of genes generally involving in negative regulation of cell cycle were discovered in ABCG2+ but not in ABCG2- cells. The stem cell associated genes PSCA, ABCG2 and ALPI were upregulated significantly in ABCG2+ cells, while K19, integrin α6, integrin β4, CD44 and K14 were upregulated significantly in ABCG2- cells. Together with the fact that the rate of LRC in ABCG2+ cells is only 12%, we suppose the most likely possibility is that ABCG2 alone is insufficient to mark CSCs in 5-8F cells. Further study waits to be conducted to isolate and identify CSCs from NPC cells and NPC tissue.

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