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ORIGINAL PAPER

Isolation and identification of a distinct side population cancer cells in the human epidermal squamous cancer cell line A431

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Abstract Side population (SP) cells have been suggested to be multipotent cancer stem cells. To address whether SP cells exist in epidermal squamous cancer cell line A431, A431 cells dyed with Hoechst 33342 were sorted through flow cytometry. The SP cells were then analyzed by colony-forming and cell proliferation assay. Further, tumorigenicity and microarray analysis were used to compare biological difference between SP and non-SP (NSP) cells. Our results showed that SP cells existed in the A431 cell line, showing higher proliferating and colony-forming ability than NSP cells. Tumors generated from SP cells were larger than those from the NSP cells in NOD/SCID mice. The mRNA microarray profiling revealed that five cancer marker gene expressions were up-regulated and one tumor suppressor gene expression was down-regulated. These findings suggest that SP cells in A431 could contribute to self-renewal, neoplastic transformation, and cancer metastasis of human epidermal squamous cell carcinoma.

Keywords Side population · Squamous cell carcinoma · A431

Introduction

Solid tumors are characterized by the presence of poorly differentiated cells that originate from the affected tissue and are presumably responsible for the long-term tumor maintenance, renewal, and drug resistance. Cells with these properties have been termed as cancer stem cells (CSC) or tumor-initiating cells (TIC) [11, 24]. The CSC hypothesis suggests that only the CSC within the tumor can self-renew and proliferate extensively to form new tumors, whereas the remaining cells do not [29]. CSCs have been identified within acute myeloid leukaemia, breast, and brain solid tumors [1, 24]. There is also an evidence that squamous cell carcinoma (SCC) is maintained by CSC [15, 22].

Identification of CSC could be crucial for mechanistic studies of tumor formation and maintenance. Different approaches have been used for CSC/TIC isolation from tumor cells, such as an immunophenotype-based selection [20], cell growth as non-adherent spheres [21], Hoechst33342 dye-based side population (SP) sorting [5, 12–14], and high aldehyde dehydrogenase (ALDH) enzymatic activity [10].

SP was defined originally in flow cytometric analyses by the ability to efflux the vital DNA dye, Hoechst 33342, resulting in Hoechst-negative SP cells and Hoechst-positive non-SP (NSP) cells. Previous studies of cancers in vitro and primary tumors in vivo have showed that SP cells are uniquely capable of generating both SP and NSP cell populations, exhibiting properties consistent with cancer stem or stem-like cells. All these results suggest that SP cells are multipotent "cancer stem cells" [5, 12–14, 31]. SP

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cells are sensitive to ATP-binding cassette (ABC) transporter blockade and more chemotherapeutic drug resistance than NSP cells in many solid tissue cancer and cell lines, such as rat glioma line C6, human breast cancer line MCF-7, and the human squamous cell lines H357, SCC4, SCC13, and SCC15 [16, 17].

A431 is an appropriate cell line capable of recapitulating human SCC with high proliferation rate and poor differentiation. Epidermal malignant cell lines including A431 could develop a range of colony morphologies including holoclone, meroclone and paraclone morphologies. Holoclones are found to be small, rapidly adhesive and highly clonogenic. Cells with holoclone morphology are similar to tumor-initiating cells and possess the essential properties of malignant stem cells. Meroclone and paraclone are considered to be generated by early and late proliferating cells, respectively [3, 19].

Bortolomai et al. [4] previously isolated the SP cells from A431 and demonstrated that SP cells represented about 0.13% in A431 cells. However, the biological difference in tumorigenicity and global gene expression between SP and NSP cells in A431 remains to be determined. In this study, we successfully identified and isolated SP cells from A431. These SP cells grown significantly faster in vitro and formed larger tumour in vivo than the NSP cells. Gene expression profiling by gene chip analysis showed that five potential oncogene and/or tumor therapeutic drug targets were up-regulated and one tumour suppressor gene was down-regulated in SP cells compared to the NSP cells. Our results confirmed that the SP cells in A431 cell line have CSCs properties and suggested that differentially expressed genes could be the targets for therapies of human epidermal squamous cell carcinoma.

Methods

Cells and culture conditions

The human epidermal SCC cell line A431 was obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. The cells were cultured in DMEM medium (Invitrogen, CA) supplemented with 10% fetal bovine serum (Hyclone) and 100 U/ml penicillin/streptomycin (Gibco). Cultures were maintained in a humidified incubator at 37°C in 5% CO_2 air atmosphere.

Hoechst staining and SP cells sorting

Hoechst 33342 labeling and flow cytometric sorting of A431 SP cells were done following a procedure by Loebinger et al. [17] with minor modifications. Cells at 80% confluence were rinsed twice with PBS, which were lifted from culture dishes with 0.05% trypsin–0.02% EDTA (Sigma) for 10 min, washed with DMEM containing 10% FBS and harvested by centrifuging at 300*g* for 3 min. The cells were resuspended at 1×10^6 cells/mL in cold PBS buffer, labeled with 10 μM Hoechst 33342 (Sigma, USA) for 30 min at 37°C, either alone or in the presence of 50 μM verapamil (Sigma) as the SP cell negative control. Then the cells were resuspended in serum-free DMEM and counterstained with 2 μg/mL propidium iodide (Sigma) to label and exclude dead cells from the analysis and sorting. Hoechst 33342 was excited at 375 nm, and the fluorescence emission was detected through 450/40-nm band-pass (Hoechst blue) and 695/40-nm long filters (Hoechst red), respectively. Propidium iodide was excited at 488 nm and the fluorescence mission was detected through a 616/23-nm band-pass filter. The cells showing fluorescence were collected as NSP, whereas the cells showing no or reduced fluorescence of both blue and red were collected as SP cells.

Colony-forming assay

Six-well plate (corning) was coated with gelatin and 1,000 SP or NSP A431 cells were seeded per well. The cells were allowed to grow for 2 weeks for colony formation. The colonies were observed under microscope and counted. The assays were repeated three times.

In vitro proliferation assay

A total of 5,000 SP cells, NSP and unsorted A431 cells (passed through the flow cytometric sorter once as the control) were plated per well in six-well plates and cultured in a CO_2 incubator. The Cells were harvested at days 2–7 and cell numbers were counted by cell counter. To calculate the clonogenic cell doubling time T_d, the logarithmic value of the cell number against the linear value of the cell growth time was plotted first, and then any two points on the straight line section (exponential growth period) of the growth curve were taken. To calculate T_d, two integrals of log values of the cell numbers (N_i = cell number at an earlier time; N_i (ΔT) = cell number at a later time) would be taken, and the following formula would be used to calculate T_d: $T_d = \Delta T \times \log 2/(\log N_i (\Delta T) - \log N_i)$. ΔT is the time taken for the N_i cells to grow to N_i (ΔT). If N_i (ΔT) is twice that of N_i, the formula becomes $T_d = \Delta T$.

RNA isolation and microarray analysis

The total RNAs from 5×10^5 SP and NSP cells were prepared with NucleoSpin® RNA clean-up Mini kit according to the instruction of the manufacturer (Macherey–Nagel, Germany). The RNA was subjected to GeneChip®

expression array analysis with two-cycle target labeling (implemented by CapitalBio Corp., Beijing, China). Briefly, cDNA was synthesized from total RNA using T7-Oligo (dT) primers, and biotinylated cRNA was synthesized using the cDNA. Two micrograms of the labeled cRNA was hybridized to the 22K Human Genome Array. The array image was scanned and analyzed using LuxScan 10KA.

Mouse tumorigenicity assay

Six-week-old male NOD/SCID mice were purchased from Institute of Zoology, Chinese Academy of Medical Sciences (Beijing, China) and were used for the experiments. All mouse procedures were performed in accordance with Chinese animal procedural and ethical guidelines. The animals were housed in pathogen-free conditions with filtered air, autoclaved food and water. A431 SP cells and NSP cells (both sorted and passed once) were suspended in sterile PBS at a concentration of 1×10^6 cells/mL. A total of 200 μL of the suspension containing 2×10^5 cells were injected subcutaneously in the armpit of NOD/SCID mice (SP cells in the right and NSP cells in the left) with a 29G needle. The xenograft tumors were measured for every 3–4 days with calipers and the volume was calculated as $ab^2/2$ (a and b represent the length and width of a tumor, respectively). The tumor value doubling time was calculated using the following formula: $T_d = \Delta T \times \log 2/(\log V_i(\Delta T) - \log V_i)$. V_i and $V_i(\Delta T)$ are the tumor values measured at times i and i + ΔT. At 4 weeks after transplantation, the xenografts were removed and processed for paraffin-embedded sections.

Statistical analyses

The data were analyzed by statistics soft SPSS 11.0. P values less than 0.05 were considered to be significant.

Results

SP cells were present in the A431 cell line

To determine whether SP cells exist in the human SCC A431, we stained A431 cell with Hoechst 3342 and analyzed the cells by flow cytometry. Using this procedure, we identified a very small subpopulation of cells (1.73 ± 0.42%) being SP cells (Fig. 1a). As expected, addition of 50 μM verapamil (a known inhibitor of Hoechst 3342 efflux) converted these Hoechst 3342-negative into Hoechst 3342-positive cells (Fig. 1b) confirming that these cells were indeed SP cells.

SP cells had greater proliferative capability than NSP cells in A431

To compare the growth rate between SP and NSP cells isolated from the A431 cell line, we established growth curves for these two cell populations. The linear growth curves exhibited that the SP cells proliferated much faster than both the mock-sorted A431 cells and NSP cells (Fig. 2a). During the early stage (0–2 days), all the cells grew at a similar rate. However, during the middle stage (2–5 days), the growth of the SP cells surpassed both the mock-sorted A431 and NSP cells. During the late stage (5–7 days), the number of the SP cells became much larger than those of A431 and NSP, with the mock-sorted cells growing faster than the NSP cells. Plotting the above proliferation data into semi-log cell curves revealed that the cell number doubling the times of the SP and mock-sorted cells were the same during exponential growth period (24 h), while the doubling time for the NSP cells was much longer (50 h) (Fig. 2b). These results showed that the SP cells grow faster and have greater proliferation potential.

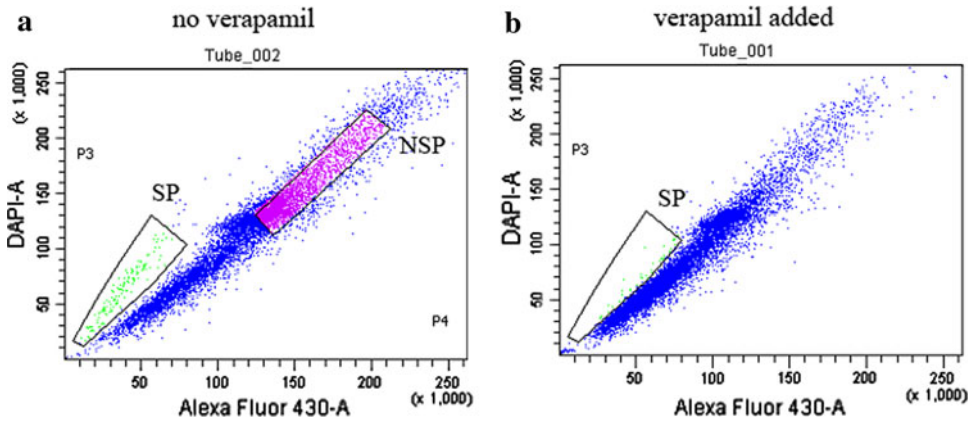

Fig. 1 SP cells in the human skin carcinoma cells A431. Suspended A431 cells were stained with Hoechst 33342 and sorted using the flow cytometer. A small subpopulation of cells (1.73 ± 0.42%) was identified (**a**). Addition of verapamil drastically reduced the staining of SP cells (**b**)

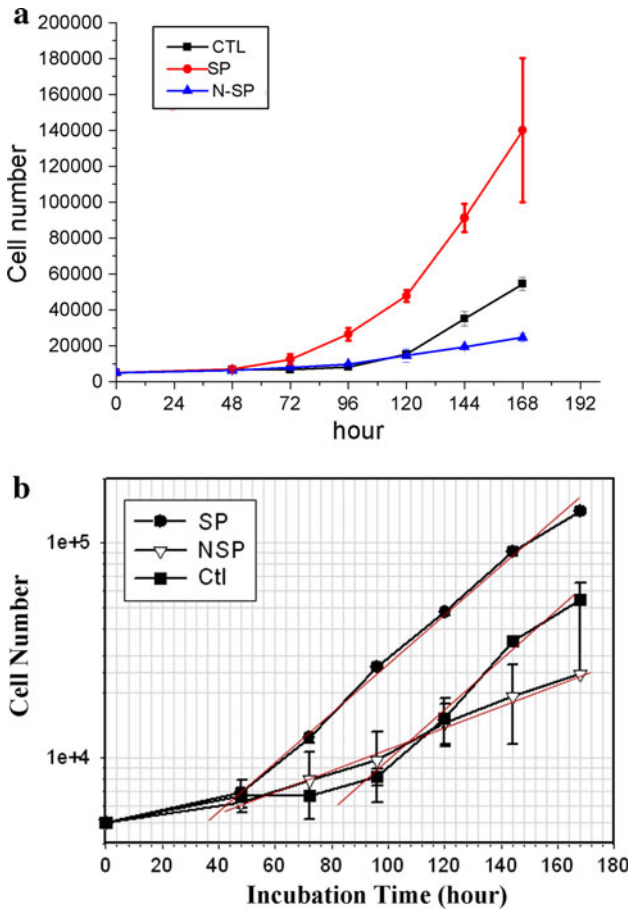

Fig. 2 Comparison of proliferations between SP and NSP cells. Sorted SP and NSP cells were cultured and counted in due time. Each datum point was the mean value of three experimental results. The linear growth curves indicated that the SP cells proliferated much faster than both the A431 mock-sorted and NSP cells (**a**). The data in (*a*) were replotted in semi-log scale to evaluated the growth rates (**b**)

The SP cells formed more holoclone colonies than NSP cells in vitro

After 2 weeks growth, most of the colonies formed by SP cells were holoclone colonies and larger (Fig. 3a, c), while NSP cells formed more paraclones (Fig. 3b, d). The SP cells formed significantly more colonies (about fourfolds) than NSP cells (Fig. 3e).

Comparison of tumorigenicity between SP and NSP A431 cells

To determine whether the SP cells with greater cell growth rates could contribute to a faster growing tumor in vivo, 6-week-old male NOD/SCID mice ($n = 4$) were injected subcutaneously in the armpit with sorted SP cells and NSP cells from A431 (2×10^5 cells in 200 μL of PBS). The mice were examined for tumor formation up to 29 days post cell injection. At the day 19 after the injections, all the four sites injected with SP cells formed tumors with the volume larger than 450 mm^3, while only one sites injected with NSP cells had a tumor with a volume 30.25 mm^3 (Fig. 4a). On day 29, the mean volume of tumors grown from NSP cells was smaller than the tumors grown from SP cells (Fig. 4b, c). In addition, on day 29, SP cell-formed tumors weighed statistically significantly more than those formed from NSP cells (SP tumors $= 2.57 \pm 0.68$ g, NSP tumors $= 0.63 \pm 0.44$ g, $n = 4$, $P = 0.003$).

Comparison of gene expression profiles of SP and NSP cells in A431 cell line

The mRNA expression profiles of the SP and NSP cells isolated from A431 cell line were analyzed by gene chips (Fig. 5a). This microarray analysis identified 32 differentially expressed (Fig. 5b), with 16 genes being up-regulated and 16 genes being down-regulated in the SP cells compared to the NSP cells. Interestingly, five up-regulated genes are potential cancer markers and/or therapeutic targets, including CoAA, ADAMTS1, AKR1C, LUM, SPP1 and one down-regulated potential tumor suppressor gene MGMT (Table 1). The fold changes in up-regulation of the five potential oncogenes and the down-regulation of this tumor suppressor gene were statistically significant between SP and NSP cells.

Discussion

CSCs have been identified in many cancers. These stem cells could be isolated as a specific cell population that had the capability to strongly efflux Hoechst 33342 DNA-binding fluorescent dye [6, 12, 30]. SP cells sorting method is suitable for CSCs research, mainly for the cancers whose specific surface markers are unknown. Bortolomai et al. [4] previously isolated SP in A431 with a ratio of 0.13%. In our study, we identified 1.73% SP cells in our cultured A431 cell line. A possible cause for this difference may be the difference in cell density. In our studies, 80% confluent A431 cells were used for SP sorting, while Bortolomai et al. [4] used 70% confluent A431 cells for SP sorting. This notion is supported by observations made by Loebinger et al. [17]. These authors found that increased cell density drastically increased the SP cell population in human oral squamous cell carcinomas H357 from 0.47 ± 0.16 to $4.53 \pm 0.61\%$.

The SP cells isolated from human oral SCC cell lines H357, SCC4, SCC13, and SCC15 proliferate significantly faster than their corresponding NSP cells [17]. Our data showed that isolated SP cells from the human skin A431 cells also grew significantly faster than the NSP cells sorted out simultaneously as well as the unfractionated A431

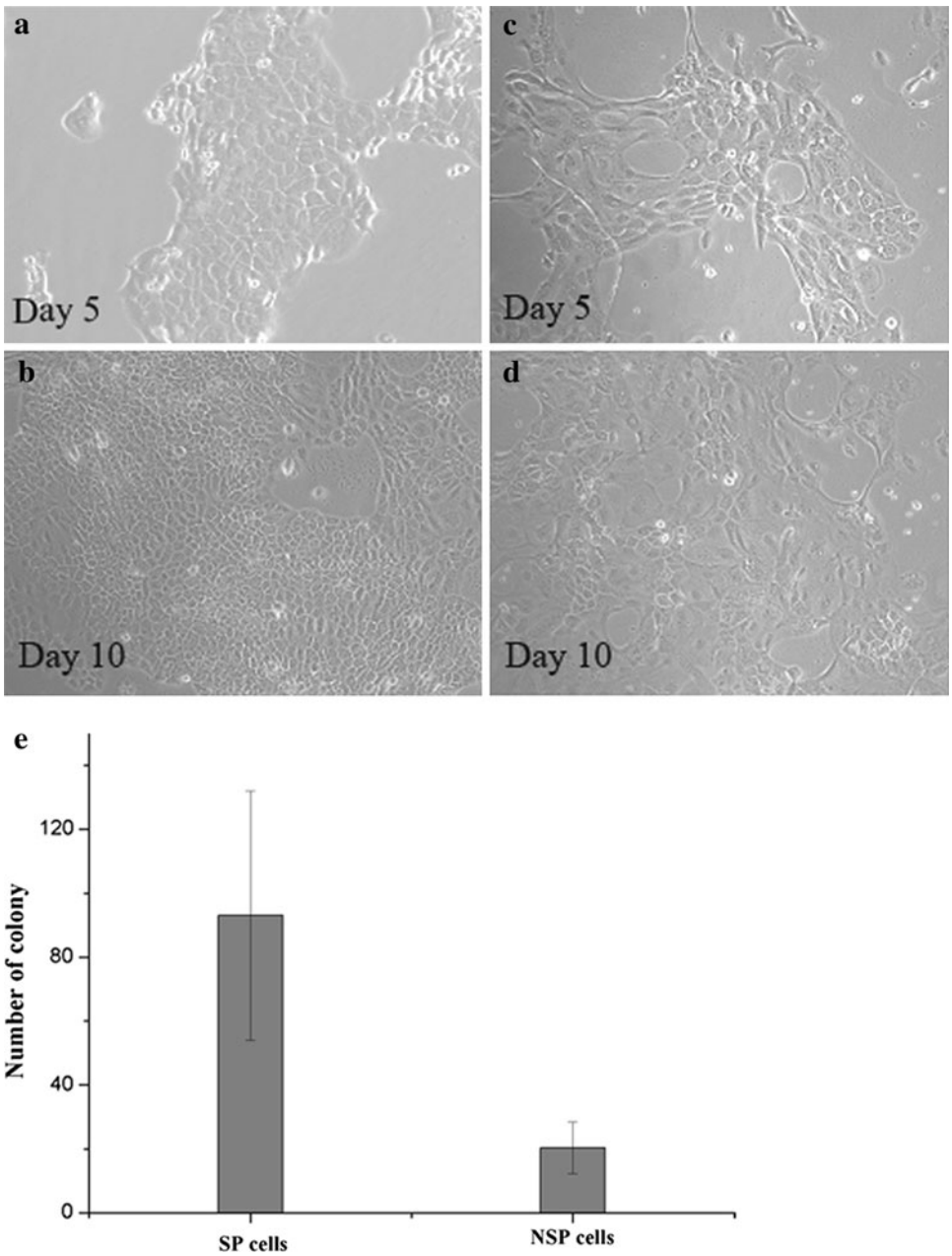

Fig. 3 Comparison of colony-forming ability between SP cells and NSP cells. In dishes of SP cells seeding, the most of colonies was holoclone colonies characterized by its round colony outline and small, closely packed and slightly spindle-shaped cells (**a**, **c**). While in dishes with NSP cells seeding, paraclones with larger, more flattened, cells should be seen (**b**, **d**). SP cells formed more colonies than NSP cells (**e**)

cells. We further demonstrated that SP cells formed more holoclones than NSP cells, while NSP cells formed more paraclones. The colonies derived from SP were larger than those from NSP cells. It is a feature of malignant cell lines including epidermal malignant cell lines characteristically show marked morphological heterogeneity [19]. A holoclone is characterized by its round colony outline and small, closely packed and slightly spindle-shaped cells; a meroclone has larger, more flattened, central cells that remain in contact with each other unlike cells at the periphery of the colony which have separated and acquired an ovoid outline; A paraclone is one in which few flattened cells remain in contact and the colony consists largely of scattered ovoid cells [3]. Differences in colony morphologies are readily distinguishable. Research has proved that cells with holoclone morphology are similar to tumor-initiating cells and possess the essential properties of malignant stem cells, while paraclone are considered to be generated by late proliferating cancer cells [19]. Bortolomai et al. [4] suggested A431 could also develop a range of colony morphologies including holoclone, meroclone and paraclone morphologies. Taken together, these data suggest that the A431 SP cells have greater proliferating potential than the NSP cells.

To examine whether the difference in cell growth rates between the SP and NSP cells in vitro leads to the different

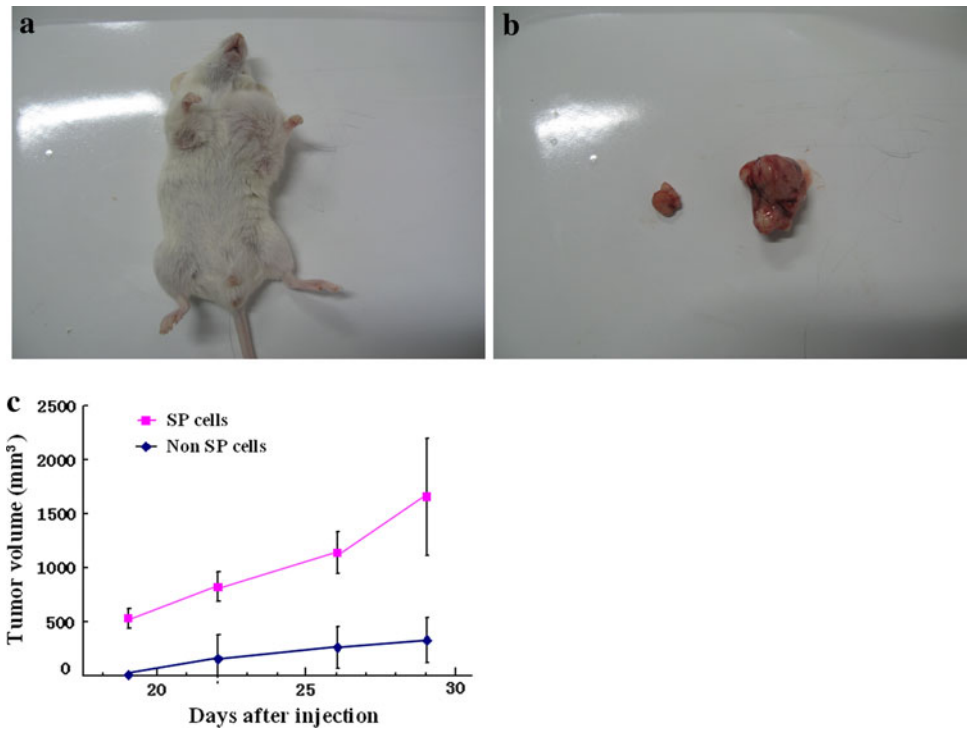

Fig. 4 Comparison of tumor formation between SP and NSP cells. Male mice were injected with NSP (*left*) and SP (*right*) into the armpits and then observed for 29 days for tumor formation (**a**). The average volume of tumors grown from NSP cells (*left*) was smaller than the tumors grown from SP cells (*right*) at the end of the experiment (**b**). Tumor growth curves were plotted in linear scale (**c**)

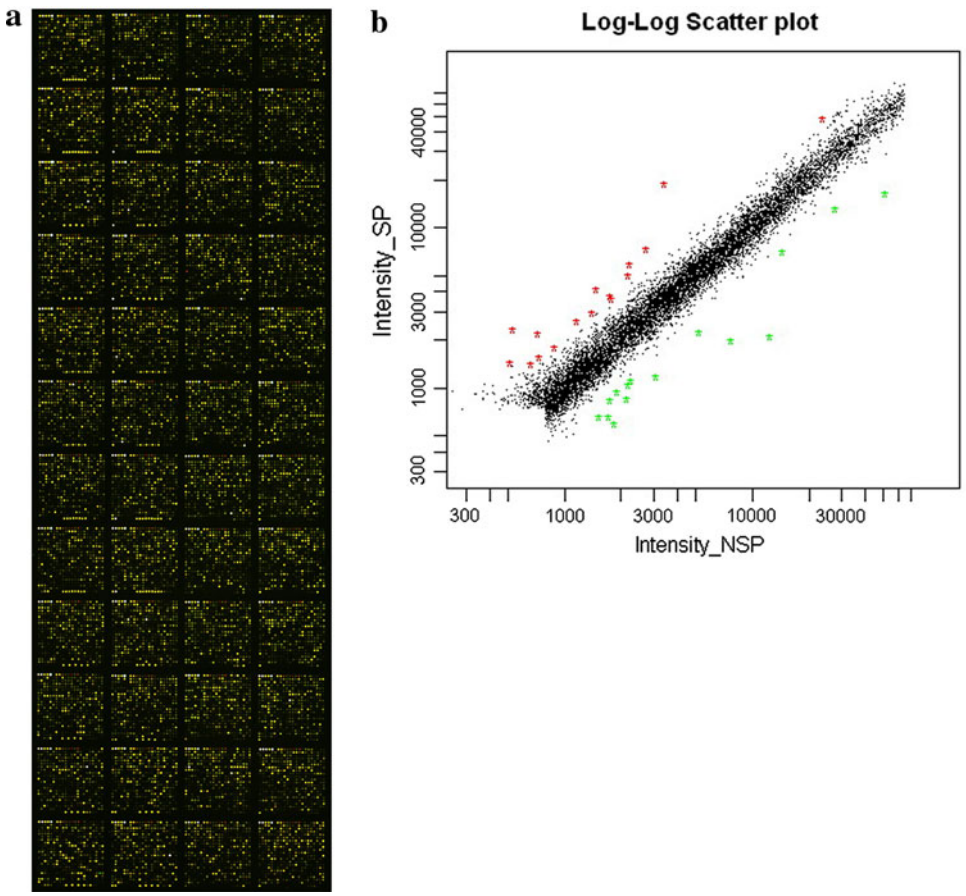

Fig. 5 Comparison of gene expression profiles SP and NSP cells (**a**). Thirty-two genes were found to be differentially expressed in scatter plot (**b**)

Table 1 Differential genes identified using microarray analysis between SP and NSP in A431

ID	Gene-symbol	Ratio (SP/NSP)	Description
6059	KLK5	0.4937	Serine protease, implicated in carcinogenesis
1091	**MGMT**	0.4300	Methylated in 29 of 101 glioblastoma tumors, also methylated in other tumors
15994	LCN2	0.4826	Potential oncogene
2386	KRT23	0.4794	Karatin 23
22270	FGA	0.7394	Alpha component of fibrinogen
20542	EXT1	2.8303	Mutations in this gene cause the type I form of multiple exostoses
3309	*CoAA*	2.1480	Amplified in human cancers
12825	S100A7A	0.1709	Overexpressed in in chronic inflammatory skin diseases
6237	TAF15	0.3198	Pro-oncogene
15211	EXT1	2.6752	
12823	S100A9	0.3234	Malignant progression
2322	*ADAMTS1*	2.0552	Cancer cachexia
16050	PIGR	0.4014	
4529	GPR137	0.4997	No info
9437	RNF215	0.4950	No info
10889	CHP	0.4793	No disease-related info
4853	HIST1H2AC	2.1173	No disease-related info
6329	PER1	2.1177	No disease-related info
19085	*AKR1C1*	2.0482	A novel target of jasmonates in cancer cells
772	FOLR3	0.3908	
14272	IRS2	2.1262	Potential oncogene
12748	MGC10981	4.4022	No info
8727	LOC148709	0.4310	No info
8919	AKR1C4	2.3473	No disease-related info
7622	*LUM*	2.2426	Marker for multiple cancer
19009	SPANXF1	5.5578	No disease-related info
15205	ANXA10	3.0284	No info for its function
1105	*SPP1*	2.7137	Potential markers and tagets for multiple cancers

The tumor suppressor gene is marked by *bold* and the potential oncogenes are marked by *italics*

potential of tumor formation in vivo, we inoculated NOD/SCID mice with the same number of the SP and NSP cells sorted from A431 cell line. The mice injected with the SP cells developed more and faster growing tumors with significantly increased tumor size and weight in comparison to the NSP cell-injected mice. In other study, 2×10^6 cells sorted SP and NSP cells from human oral squamous carcinoma H357 were allowed to grow subcutaneously in NOD/SCID mice for 49 days and only SP cells were able to form tumors, while NSP cells formed no tumors [17]. Compared to H357 SP cells, much fewer SP cells from A431 and much shorter time were needed to form tumors and NSP from A431 also formed tumors in our results. The results suggest that the biological features of A431 cells are different from H357 cells. The A431 cell line is characterized in vitro by a high proliferation rate and poor differentiation, and by in vivo growth capability with a relatively low number of cells. A431 is an appropriate cell line capable of recapitulating human SCC with high proliferation rate and poor differentiation [4]. Recently, this cell line was used as a model to identify Podoplanin/CD44 high as new markers of a tumor stem cell [2].

To understand the mechanisms underlying the difference in cell proliferation and tumor formation in vitro and in vivo between the SP and NSP cells in A431, we performed a gene chip analysis and identified five up-regulated oncogenes CoAA (RBM14), ADAMTS1, AKR1C, LUM, SPP1 (OPN). CoAA is overexpressed in a subset of primary human cancers including non-small cell lung carcinoma, squamous cell skin carcinoma and lymphoma. CoAA has transforming activities [27]. ADAMTS-1 overexpression in tumors is associated with increased production of matrix metalloproteinase-13, fibronectin, transforming growth factor-beta (TGF-beta), and interleukin-1beta (IL-1beta). ADAMTS-1 contributes to tumor development and has been established as promising therapeutic targets for inhibiting cancer metastasis [18, 26]. AKR1C significantly enhanced foci formation and plays crucial roles in induction of neoplastic

transformation of mouse NIH3T3 cells [7, 8]. SPP1 is a secreted matricellular protein produced by epithelial, mesenchymal, and immune system cells. By interaction with several integrin and non-integrin receptors, SPP1 acquires a proinvasive role and could upregulate expression of MMP-7 and CD44, which are known as TCF/LEF transcription targets and are important in tumorigenesis and cancer progression [23, 25]. Increased SSP1 expression in A431 cells would be involved in the autocrine/paracrine control of the A431 CSC/TIC component inside the niche. The above up-regulated oncogenes reflected various differences between SP and NSP, such as nuclear transcription, neoplastic transformation, and cancer metastasis.

The gene array analysis also identifies one down-regulated tumor suppressor gene MGMT in the SP cells. The critical role of MGMT is DNA repair and MGMT promoter methylation is significantly associated with overall poorer and disease-free survival. Genomic stability declines as a result of decreasing MGMT expression which could lead to poorer prognosis and tumor recurrence [9, 28]. In our study, down-regulated MGMT in SP suggested that defective DNA repair may be correlative with tumor recurrence. Having increased expression of oncogenes and reduced expression of antioncogene in the SP cells should make these tumor stem cells more proliferative and tumorigenic.

In summary, our study confirmed the existence of CSCs in A431 cells line and revealed that the SP and NSP cells in A431 are different at molecular and cellular levels both in vitro and in vivo. Further characterization of the SP cells is required to fully exploit the genes which could be used as a therapeutic target of squamous cell carcinoma.

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Conflict of interest None.

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