RESEARCH ARTICLE

Clinical implications of cancer stem cell-like side population cells in human laryngeal cancer

Dan Yu · Chunshun Jin · Yan Liu · Jingpu Yang · Yin Zhao · Haitao Wang · Xue Zhao · Jinzhang Cheng · Xueshibojie Liu · Caigang Liu

Abstract In this study, we try to detect and isolate the cancer stem cell-like side population cells (SP) from the laryngeal carcinoma cell line and primary laryngeal carcinoma and explore the clinical implications of SP cells in laryngeal carcinoma. The SP cells and non-side population cells (NSP) cells were sorted by Hoechst 33342 through FACS. The proliferation capacity, invasion ability, migration ability, and tumorigenic activity of the SP cells were evaluated. In addition, the association between the SP cells ratio and the prognostic factors of laryngeal cancer was analyzed. As a result, the percentage of the SP cells in Hep-2 cells was 5.1 %. The SP cells depicted float colonies, but the NSP cells failed to generate the typical cell spheres. The clone formation ratios were 47.47 ± 10.20 % vs. 4.98 ± 1.41 % in the flat plates and 46.82 ± 5.67 % vs. 12.53 ± 3.51 % in the soft agar for SP and NSP cells ($P=0.01$ and 0.01). The SP cells depicted a higher migrating potency than the NSP cells in both the transwell assay and scarification test (all $P<0.05$). The matrigel invasion assay showed that the artificial basement membrane penetration rate of SP cells was 39.04 ± 4.78 %, which was higher than 25.16 ± 4.63 % of the NSP cells ($P<0.05$). Only 10^3 SP cells were able to form tumors in mice, whereas 10^4 NSP cells failed to form tumors. The SP cells were correlated with the differentiation, lymph node metastasis, and clinical stage of the laryngeal cancers. In conclusion, SP cells may be a potential prognostic factor of laryngeal cancer.

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Introduction

Laryngeal cancer is one common disease in head and neck cancers, and its incidence has increased year by year [1]. In recent years, comprehensive treatment measures such as functional laryngeal surgery, radiotherapy, chemotherapy, concurrent chemoradiotherapy, and gene therapy have gained a higher 5-year survival rate for patients with laryngeal cancers, but 30 % to 40 % of them still died of tumor recurrence or metastasis [2, 3]. Hence, there is an urgent need to explore the mechanism of origin, invasion, as well as metastasis of the laryngeal cancer in order to design new treatment methods [4].
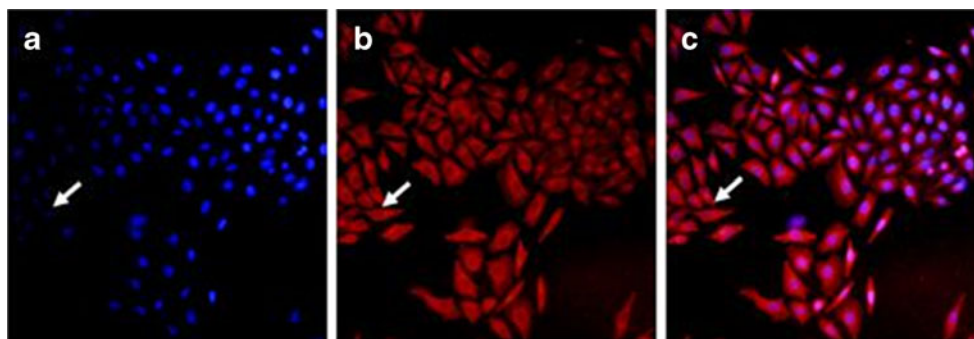
Stem cells, which represent only a very small percentage of the total tumor mass, have been found to be the source of some, and possibly most, cancers [5]. The cancer stem cell hypothesis states that certain stem cells remain in tissues to replenish them after injury or disease, yet because they are self-renewing and can survive for a longer period of time, the adult stem cells can also accumulate mutations, which would cause them to produce cells that divide uncontrollably, forming a tumor [6–8]. Laryngeal cancer stem cell research is only in the initial stage, but the cancer stem cell concept provides a new perspective method for overcoming tumors, which could have an immeasurable impact on the future of cancer research.

Due in part to the better understanding of cancer stem cell theory, stem cell related genes in malignant tumors have gained more academic attention. Currently, studies addressing laryngeal SP cells, especially based on the primary solid tumors, are still rare. In the study herein, we try to sort and identify the laryngeal SP cells in order to lay a foundation for the management of laryngeal cancer.

Fig. 1 Fluorescence microscope showed Hoechst33342- Hep-2 cells (×400). **a** The cell nucleus showed blue fluorescence stained by Hoechst33342; **b** the cell membrane showed red fluorescence stained by CM-DiI; **c** an overlay of panels **a** and **b** is shown (*arrow*)

Materials and methods

Patients and materials

The laryngeal cancer cell line Hep-2 was provided by China Center for Type Culture Collection (Wuhan, Hubei province). BALB/c mice were 4–6 weeks of, weighed 15 to 18 g, and were provided by the Experimental Animal Center of Lake Hayes. We selected 120 cases that were treated at the Jilin University between Jan 2003 and Dec 2008 for the prognostic analysis. The inclusion criteria were as follows: (a) curative operations were performed; (b) resected specimens were pathologically examined; (c) more than ten lymph nodes were pathologically examined after operation; and (d) a complete medical record was available. The study protocol was approved by the Ethics Committee of Jilin University.

Western blot analysis

Cells were prepared from 100 mg of SP and NSP cells. Approximately 1×10^7 cells were harvested by trypsinization. Cells were lysed in 200 µl ice-cold lysis buffer for 30 min, and the lysates were centrifuged at $12,000 \times g$ for 15 min. Protein concentration was quantified using a protein measure Kit (Xian RunDe Biotechnology Ltd.), then protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12 % SDS-PAGE) and transferred onto nitrocellulose membranes. For immunodetection, the membranes were blocked in tris-buffered saline (TBS) containing 5 % nonfat milk powder for 2 h at 4 °C, incubated with primary antibodies to mouse anti-human ABCG2 monoclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA), mouse anti-human beta-actin monoclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) overnight at 4 °C, and then incubated in tris-buffered saline Tween (TBST) containing horseradish peroxidase-labeled antibody against IgG for 90 min. Immunoreactive proteins were visualized using the enhanced chemiluminescent detection system (BestBio, Shanghai, China) according to the manufacturer's instruction.

Extraction of total RNA and reverse transcription followed by PCR (RT-PCR)

Total RNA was extracted from approximately 1×10 [6] cells using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. cDNA was then synthesized from 2 µg total RNA using oligo dT as the primer along with the MMLV reverse transcriptase (Takara Inc, Japan). PCR was performed with the following primers: ABCG2: sense 5′-AACGAACGG ATTAACAGGG-3′, antisense, 5′-AAGGTGAGGCTATCAA ACA-3′; GAPDH: sense, 5′-TGTCATCAATGGAAATCCC-3′, and antisense, 5′-GAGACCACCTGGTGCTCA-3′. After the PCR reaction, the products were loaded on 1 % agarose gel and visualized by ethidium bromide staining.

Flow cytometry

The Hep-2 cells in logarithmic growth phase were added Hoechst33342 to a final concentration of at 5 µg/ml and cultured at 37 °C for 90 min. Violet, blue, and red-fluorescent Hoechst33342 dye was excited when bound to 407, 450/40, and 675/40 nm. PI excitation was at 488 nm blue and 633 nm red light. Two-dimensional plot was drawn by Hoechst Red as *x*-axis and Hoechst Blue as *y*-axis, and the verapamil missing region is set to SP cells gate (gated), sorted SP cells, and NSP cells. Each experiment was repeated three times.

Cell proliferation assay

Cells were seeded in 96-well plates and incubated at 37 °C in 5 % CO_2 for 24, 48, or 72 h. The growth rates of the cells were

Table 1 ABCG2 protein expression in SP and NSP groups SP ($\bar{x} \pm S$, $n=3$)

Groups	ABCG2/GAPDH absorbance ratio	P
SP	0.391 ± 0.058	0.003
NSP	0.04 ± 0.006	

determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide (MTT) assay [9]. The absorbance was read at 540 nm. Each experiment was repeated three times.

CCK-8 proliferation assay

The new sorting SP and NSP cells were centrifuged and washed with PBS one time/per well in 100 μl (5×10^3/well) and were seeded in 96-well plates. The 96-well plates were cultured at 5 % CO_2, 37 °C, and the medium was changed every other day. One orifice was randomly selected at 1, 3, 5, and 7 days after inoculation, and every well was added 10 μl CCK-8 reagent. Then, the cells were measured for absorption values at a wavelength of 450 nm after 3 h incubation on a microplate reader. The growth curves were drawn by the growth days as abscissa and the absorbance as vertical axis.

Soft agar colony formation assay

The anchorage-independent growth of SP and NSP cells was monitored by the soft agar colony formation assay. In brief, cells (100/well) re-suspended in 1.5-ml mixture of 1.2 % low-melt agarose and 2× RPMI 1640 (v:v=1:1) were loaded in triplicate on the top of the solidified bottom agar comprising equal-volume mixture of 0.7 % low-melt agarose and RPMI 1640 in 12-well plates. The cells were incubated at 37 °C, 5 % CO_2 for 2 weeks. The colonies composed of more than 50 cells were counted.

In vitro migration and invasion assay

Cells growing in the log phase were treated with trypsin and re-suspended as single-cell solution. Then, the cells were counted, and 1×10^5 cells in 1 mL of serum-free RPMI 1640 medium were split into the upper chamber of Transwell Boyden chamber (8-μm pore size, BD Biosciences, USA), where the transwell membrane was coated either with (for invasion) or without (for migration) matrigel. Serum-free medium was also added to the lower chamber. The migration assay proceeded in 37 °C, 5%CO_2 tissue culture incubator for 12 h, and the invasion assay for 18 h. The non-migrated/invaded cells in the upper chamber were removed using a cotton swab, and the migrated/invaded cells fixed in methanol and stained with eosin for migrated cells or Giemsa for invaded cells. The cells that were trapped in or attached to the reverse side of the porous membrane were photographed through ×200 microscope objective, the numbers of migrated cells in at least five random fields were counted under phase contrast microscope, and the average was calculated [9].

Limiting dilution injection experiment

The sorted SP and NSP cells were re-suspended in serum-free DMEM into a cell suspension containing 10^5, 10^4, 1×10^3 cells per 200 ul. Cells to be injected were then suspended in RPMI1640/Matrigel mix (1:1 volume) and injected into the appropriate area of the mammary fat pad.

Statistical analysis

All data were analyzed with SPSS statistics software (Version 13.0, Chicago, IL, USA). $\Delta\Delta Ct$ Method was employed in the gene chip data analysis. The ΔCt was calculated for each pathway-focused gene in each treatment group. ΔCt (group 1)=average Ct−average of HK genes' Ct for group 1 array. ΔCt (group 2)=average Ct−average of HK genes' Ct for
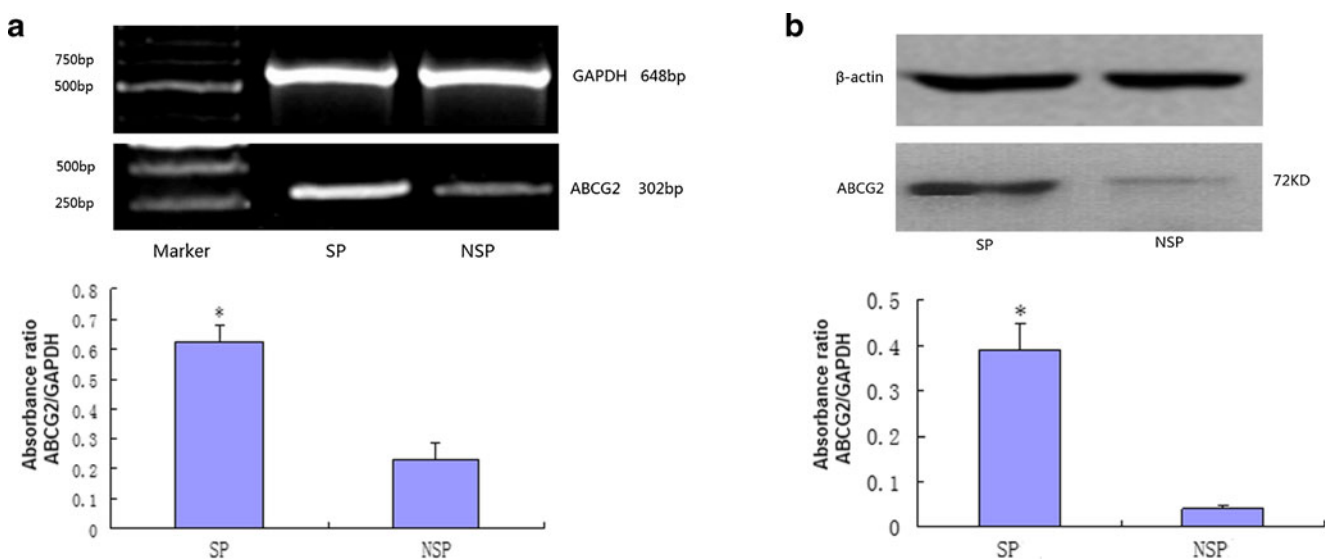

Fig. 2 ABCG2 mRNA expression status of SP and NSP cells. ABCG2 mRNA was higher in NP cells compared to NSP cells (**a**, $P<0.05$). ABCG2 protein expression status of SP and NSP cells. ABCG2 protein expression was higher in NP cells compared to NSP cells (**b**, $P<0.05$)
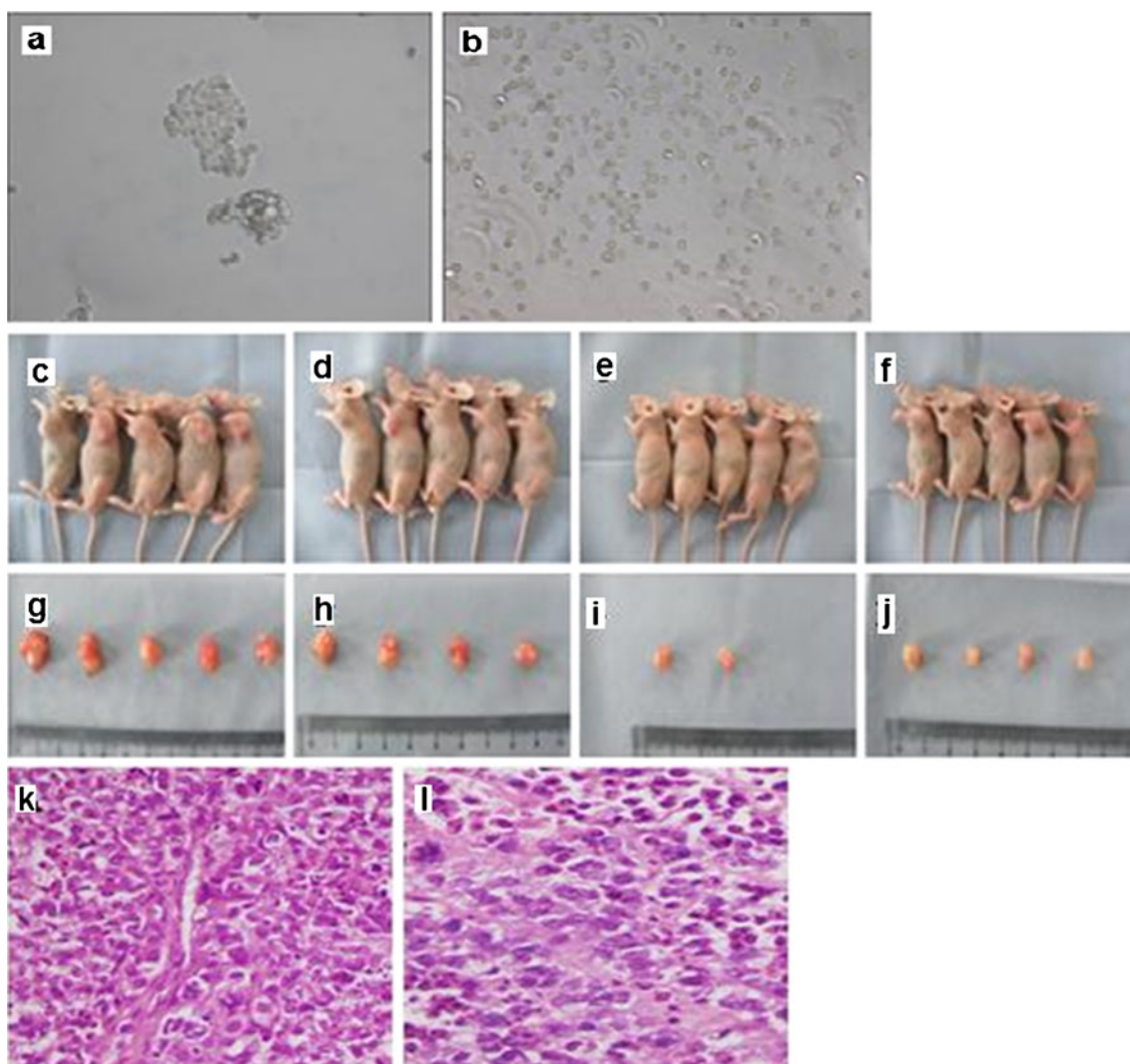
Fig. 3 After 7 days of culture, SP tumor cells could produce viable mammospheres in serum-free suspension culture condition (**a**), while NSP cells could not (**b**). SP tumor cells got a higher node mice group 2 array. ΔΔCt=ΔCt (group 2)−ΔCt (group 1). Relationships between SP ratio and clinic-pathological parameters were studied using the chi-square test, Fisher's extract test, or independent *t* tests. One-way ANOVA was applied to test the differences between groups for all in vitro analyses. ANOVA test was used for the in vivo xenograft experiment. A *P* value of less than 0.05 was considered statistically significant.

tumorigenicity ability compared to NSP cells (**c**, **g** 105SP; **d**, **h** 104SP;**e**, **i** 103SP; **f**, **j**105 NSP). SP and NSP cell transplantation tumor HE staining was shown (**k** SP cells, **l** NSP cells; ×400)

verapamil was added. The two group cells showed similar characteristics with Hep-2 cells after cultured in DMEM with 10 % FBS. The expression of ABCG2 in SP cells was significantly higher than NSP cells ($P<0.05$) (Table 1; Fig. 2).

The identification of the stemness of SP tumor cells

After 7 days of culture, single-cell suspensions of SP tumor cell produced viable mammospheres (20 to 100 μm), which could be passaged further in serum-free suspension culture

Results

The sorting of SP tumor cells of laryngeal cancer

The percentage of the Hoechst33342− cells (SP cells) was 5.1±0.25 % in Hep-2 cell line in immunofluorescence staining (Fig. 1). The proportion of the sorted SP cells was 4.4±0.85 % in FACS test, which decreased to 0.63±0.31 % after the

Table 2 Limiting dilution xenograft formation experiment

Groups	10^5	10^4	10^3
SP	5/5	4/5	2/5
NSP	4/5	0/5	0/5

condition, while no mammosphere was produced by NSP cells in the same culture condition (Fig. 3). As few as 10^3 SP cells were able to form tumors in mice, whereas 10^4 NSP cells failed to form tumors, which indicated that the tumorigenic activity of the SP cells were stronger than the NSP cells ($P<0.05$) (Table 2) (Fig. 3).

The SP cells depicted float colonies, but the NSP cells failed to generate the typical cell spheres. Furthermore, the SP cells were integrated with big nucleolus and little cytoplasm under the transmission electron microscope, while the NSP cells showed the small pyknosis of the nucleolus, with significantly expanded endoplasmic reticulum and heterochromatin (Fig. 4). In the CCK-8 proliferation assay, the absorbance of the SP cells was higher than that of the NSP cells in the third cultured day. The clone formation ratios were 47.47 ± 10.20 % vs. 4.98 ± 1.41 % in the flat plates and 46.82 ± 5.67 % vs. 12.53 ± 3.51 % in the soft agar for SP and NSP cells ($P=0.01$ and 0.01).

In the differentiation potency assay by FACS, the proportion of the sorted SP cells was 4.2 ± 0.47 % on the 14th day, which was similar to that of the first time sorting (4.4 ± 0.85 %). Both the SP and NSP cells displayed a similar cell cycle distribution in the cell cycle assay. The SP cells depicted a higher migrating potency than the NSP cells in both the transwell assay and scarification test (all $P<0.05$) (Fig. 5). The matrigel invasion assay showed that the artificial basement membrane penetration rate of SP cells was 39.04 ± 4.78 %, which was higher than 25.16 ± 4.63 % of the NSP cells ($P<0.05$).

The relationship between SP cells and clinic-pathological factors of the primary laryngeal cancers

SP cells existed in most of the primary laryngeal cancer cells, and the average proportion of the SP cells from the 120 cases of laryngeal carcinoma specimens is 3.1 ± 2.36 %. SP cells were correlated with gender, differentiation, lymph node metastasis, and clinical staging of the laryngeal cancer ($P=0.021$, 0.001, 0.001, and 0.001, respectively, Table 3). Survival analysis revealed that SP cells ratio was associated with laryngeal cancer-specific survival in 120 cases (87.77 % vs. 63.49 %, $P=0.001$, log–rank test) (Fig. 6). After performing a Cox regression, differentiation, lymph node metastasis, clinical staging, and SP cells ratio were shown to be the independent prognostic factors of laryngeal cancer.

Discussion

Recurrence and metastasis are two of the primary causes for the death in patients with malignant tumors, which is the biggest challenge for the tumor treatment [10]. Until now, the fundamental causes for the genesis, recurrence, and metastasis of the tumor have not been identified, although some progress
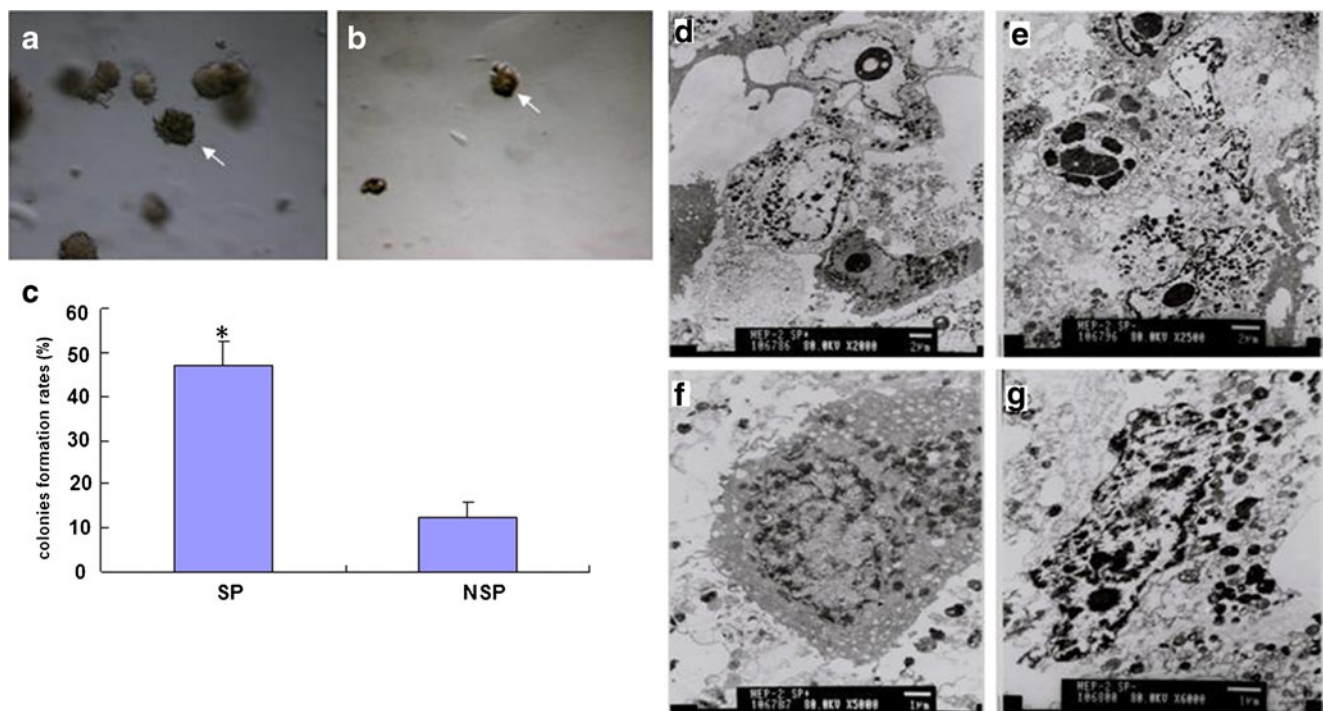

Fig. 4 The SP cells depicted float colonies as proliferating (**a**), but the NSP cells did not generate the typical cell spheres (**b**). There was significant difference in colonies' formation rates between the two groups (**c**). SP cells were integrated with big nucleolus and little cytoplasm (**d** ×2,000, **f** ×5,000), while the NSP cells showed the small pyknosis of the nucleolus under transmission electron microscope (**e** ×2,000, **g** ×5,000)

has been made in the study of biology. The concept of the tumor stem cell has supplied a new thread for the study of the biological properties of the tumor, which make it possible for targeting the tumor stem cell to kill them and thus prevent the recurrence and metastasis of the tumor [11, 12]. This concept has become the hot topic, but because most of the specific makers of the cells are unknown, the isolation and identification of the tumor stem cell is quite difficult [13, 14]. The side population (SP) is a special type of the cell subpopulation, and the isolation of SP cells is easy and mature [15].

Many researchers have checked out SP cells from various normal and tumorous tissues [16, 17]. It was confirmed that

Fig. 5 The SP cells depicted a higher migrating potency than the NSP cells in both the transwell assay (**a** SP cells; **b** NSP cells) and scarification test (**c** and **d**)
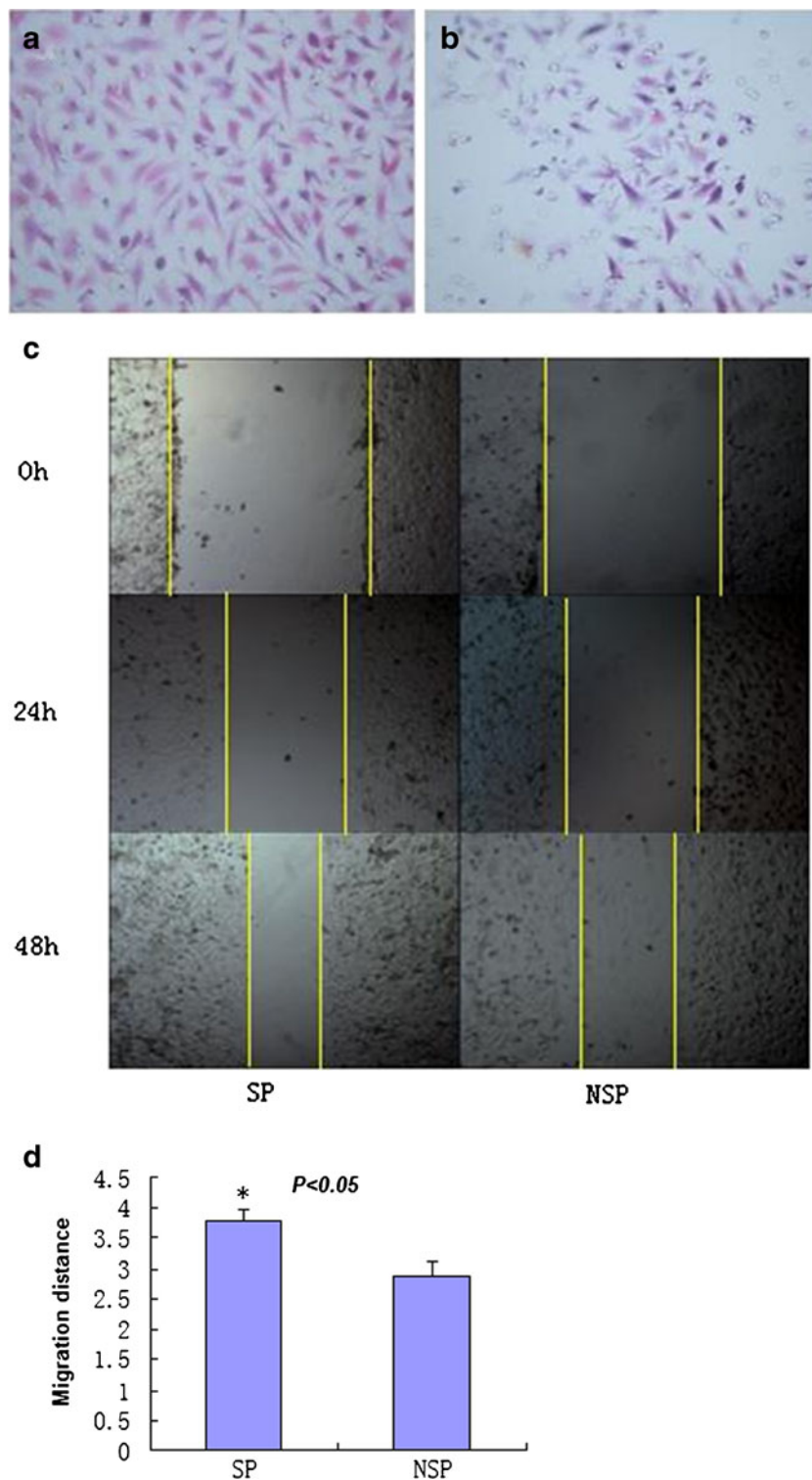
Table 3 The relationship between SP cells ratio and clinical–pathological factors of laryngeal cancer

Factors	Cases	SP cells ratio		P
		<3.1 %	≥3.1 %	
Gender		57	63	0.021
Male	96	51	45	
Female	24	6	18	
Age				0.278
<60	57	24	33	
≥60	63	33	30	
Differentiation				0.001
High degree	99	39	60	
Middle-low degree	24	18	3	
Lymph node status				0.001
Positive	39	30	9	
Negative	81	27	54	
Clinical stage				0.001
I–II	81	54	27	
III–IV	39	3	36	

compared with the NSP cells, SP cells from most tumor tissues possessed faster proliferation and self-renewal properties; they formed tumors easier in immunodeficient mice and had stronger drug resistance, for which the stem cell characteristic cells were considered to be enriched in the SP cells.

At present, the study of cancer stem cells in laryngeal carcinoma is still in the primary stage. Wang et al. [7] observed that SP rates were 2.6 %, 0.1 %, 6.8 %, 1.8 %, and 0.7 % in nasopharyngeal carcinoma cell lines CNE-2, C-666-l, SUNE-1, HONE-1, and CNE-1, and the CNE-2 SP cells have a stronger proliferation, differentiation capacity, and tumorigenicity compared to the control group. In another study, Zhang et al. [8]
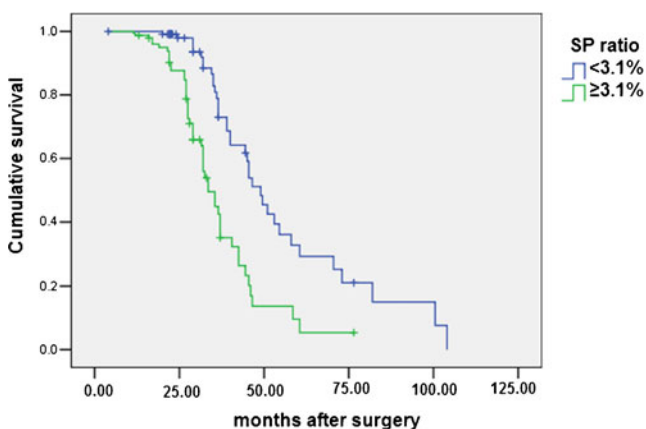

Fig. 6 The laryngeal cancers with <3.1 % SP cells ratio got a better post-operative prognosis compared to those with ≥3.1 % SP cells ratio in 120 cases (87.77 % vs. 63.49 %, P=0.001, log–rank test)

selected SP cells from oral cancer cell lines and 11 solid tumor specimens and found that SP cells play an important role in the incidence of oral cancer.

Our study investigated the biological properties of the SP cells from the laryngeal cancer cell line Hep-2 and the primary cultured laryngeal carcinoma cells, desiring to support a new approach and some experimental reference for the study of laryngeal TSCs. In the study, the percentage of SP cells was about 5.1 %, and the cells got high ABCG2 protein expression compared to NSP cells. The sorted SP cells had self-renewal properties and got strong proliferation and invasion abilities. The SP cells formed tumors easier in immunodeficient mice compared to the control groups.

Furthermore, the SP cells existed in most of the primary laryngeal carcinoma cells and correlated with the differentiation, lymph node metastasis, and clinical stage of the laryngeal carcinoma.

Conclusion

The SP cells sorted from the laryngeal cancer cell line Hep-2 are tumor stem cell-like subpopulation that displays characteristics of high proliferative, clone formation, differentiation capacities, strong tumorigenic ability, and resistance of chemotherapy drugs. The SP cells exist in most primary laryngeal carcinoma specimen cultured cells, which were correlated with clinical pathological characteristics of the laryngeal carcinoma.

Conflicts of interests No competing interests are declared by any of the authors.

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