MEDICAL SCIENCE MONITOR

Identification and Characterization of CD133^{pos} Subpopulation Cells From a Human Laryngeal Cancer Cell Line

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Recent research indicates that CD133 are expressed in several kinds of stem cells, among which, its high expression in laryngeal carcinoma has caused wide concern. To further explore efficaciously targeting drugs to laryngeal carcinoma stem cells (CSCs), we transplanted a solid tumor from CSCs into abdominal subcutaneous tissue of nude mice, and then compared the biological characteristics of laryngeal solid tumors with or without cisplatin intervention.

Material/Methods: In this study, the expression of CD133 was detected in the Hep-2 cell line by flow cytometry. By applying magnetic cell sorting (MACS) technology, we reported the results of purifying CD133-positive cells from a Hep-2 cell line. Cell proliferation, colony formation, and tumor-forming ability were examined *in vitro* and *in vivo* to identify the marker of CSCs in Hep-2 cell line.

Results: Upon flow cytometry analysis, CD133 was expressed constantly on 40.12±1.32% in Hep-2 cell line. Cell proliferation and colony formation ability were higher in CD133-positive cells compared to CD133-negative cells, and the *in vivo* tumorigenesis experiment showed the same results as *in vitro* assay. The 2 subpopulations cells were both sensitive to DDP, among which, the effect of DPP on proliferation ability and tumor-forming ability of CD133-positive cells was obviously greater than that of CD133-negative cells.

Conclusions: Above all, our study revealed that CD133-positive cells have properties of higher proliferation, colony formation, and tumorigenesis in Hep-2 cell line, indicating that CD133 could be a marker to characterize laryngeal cancer stem cells.

MeSH Keywords: **Carcinoma, Squamous Cell • Cell Line, Tumor • Cisplatin • Laryngeal Neoplasms • Mice, Nude**

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/895645

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Background

Laryngeal carcinoma, a kind of malignancy with high prevalence, accounted for approximately 1–5% of all malignant tumors and 7–35% in eye, ear, nose, and throat (ENT) malignant tumors [1]. Although rapid progress has recently been made in treatment, the prognosis for laryngeal carcinoma patients remains unsatisfactory. Even though in clinical therapy radiochemotherapy can save the throat structure and function, it has limited efficacy when complications and resistance emerge after patients received lengthy treatment [2]. Therefore, understanding the mechanism underlying laryngeal carcinoma is essential for better management of laryngeal cancer patients. Furthermore, a tumor is considered a kind of stem cell disease, and the cancer stem cells (CSC) play a key role in tumor formation and growth [3–5].

CD133 is a 5-transmembrane glycoprotein with a molecular weight of 120 kDa. It is a kind of early antigen and it was identified as a marker of normal hematopoietic stem cells [6]. The expression of CD133 decreases dramatically as cells differentiate, and CD133 is selectively expressed in marrow, peripheral blood hematopoietic stem cells, and endothelial progenitor cells, but not in the mature endothelial cells [7]. Accumulating evidence shows that CD133 is a stem cell surface marker of many tumors. It can be an independent factor that plays a crucial role in separating and identifying stem cells and progenitor cells in some tumors [8–12].

In this study we focused on detecting the expression of CD133 in the Hep-2 cell line. The 3 subpopulations were separated by MACS technology according to the different surface antigen in Hep-2: CD133+ (CD133-positive cells), CD133– (CD133-negative cells), and unsorted cells, and their tumor formatting ability were compared. We detected the proliferation and differentiation ability of CD133 *in vitro* and the resistance for cisplatin (DDP) of laryngeal cancer stem cells to pinpoint the marker of laryngeal cancer stem cells and find a more effective laryngeal carcinoma targeted therapy.

Material and Methods

Reagent and instrument

Laryngeal cancer cell line Hep-2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China); double-antibody RPMI1640 culture medium and fetal bovine serum (FBS) were obtained from Gibco™ (Invitrogen Co, Carlsbad, CA, USA); 0.25% trypsin was from TaKaRa (Dalian, China); immunomagnetic beads CD133, MTT, DMSO, and paraformaldehyde were purchased from Sigma (St. Louis, MO, USA); CD133 antibody was obtained from GeneTex (San Antonio, TX, USA);

serum-free medium (SFM) is RPMI1640 culture medium with epidermal growth factor (EGF), basic fibroblast growth factor (bEGF), and insulin, which was also from Gibco™ (Invitrogen Co, Carlsbad, CA, USA).

Automatic CO_2 constant temperature incubator, clean benches, inverted microscope, and fluorescence microscope purchased from Olympus (Japan), and ELIASA was purchased from Takara Shuzo (Otsu, Shiga, Japan).

Laboratory animal

The laboratory animals are 4–6-year-old healthy male nude mice (BALB/c-nu/nu) with weight of 18–20 gram, which were purchased from Shanghai Slac Laboratory Animal Centre. They were raised in a SPF laminar flow room with constant 40–50% humidity and at constant 22–25°C temperature. Sterilized feed and water were provided.
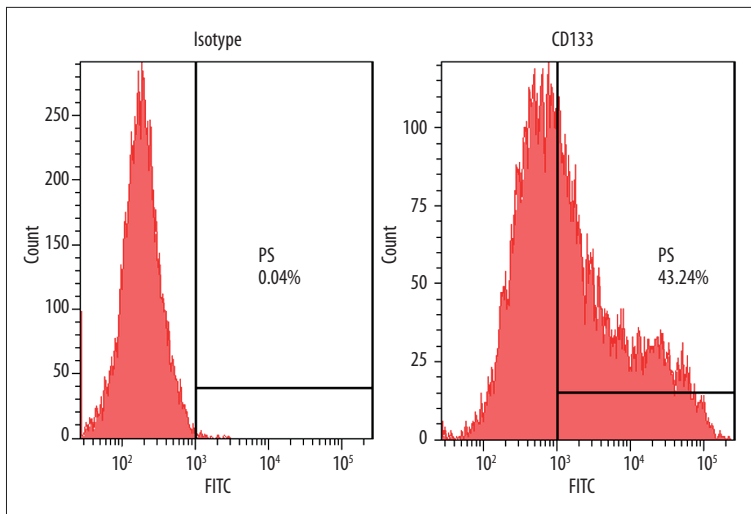
Culture of the Hep-2 cell line

Hep-2 cell line was obtained from the ENT Department of Shanghai Tongji Hospital. The cells were cultured in RPMI-1640 immediately after removal from patients. At first, we cleaned the surrounding dead and fatty tissue and rinsed the specimen in D-Hanks solution. We soaked the fresh tissue in another petri dish using double-antibody RPMI-1640, and then cut the specimen into 1-mm³ pieces. Then we moved the specimens to 0.25% trypsin and oscillated them for 30 min at 37°C. The cell suspension was filtered through 0.15-mm nylon mesh. The solution was centrifuged at 1000 rpm for 10 min, and then the sediment was suspended in D-Hanks solution. Cells were then suspended again in medium containing 50% calf bovine serum after centrifugation twice, as before.

Magnetic cell sorting

After primary culture, laryngeal cancer cells were made into 100-µl suspension with concentration of 10^6 cells per ml. The suspension was kept at room temperature for 30 min after we added 10 µl of CD133-FITC antibody, and then washed the cells 3 times. Afterwards, percentage of CD133+ in laryngeal cancer cells was detected by using a flow cytometer to confirm the purity of the sorted cells.

Cell proliferation detection

CD133+ and unsorted cells were plated at a density of 2000 cells adjusted to 100-µl culture solution per well in 96-well plates to cultured at 37°C. The absorbance of different laryngeal cancer cell subpopulations was detected using MTT after continuous inoculation for 7 days. Three duplications were set to detect the absorbance at 490-nm wavelength using a

Figure 1. Expression of CD133 in Hep-2 cell lines. Flow cytometry analysis of CD133 in Hep-2cell lines. Data are representative of 3 independent experiments.

microplate reader to draw a cell growth curve of mean absorbance value and culture time.

Cell clonogenicity assays

Soft agar colony-formation assay was performed as previously reported [13]. Briefly, sorted CD133+ and CD133− cells in logarithmic phase were dissociated into single cells by incubation in 0.05% trypsin and then cells were seeded into semisolid agarose medium (RPMI-1640 medium containing 10% heat-inactivated FCS and agarose-base layer, 0.6%; upper layer, 0.3%) at a density of 1×10^4 cells per well in 60-mm plates. Several drops of fresh medium were placed directly onto the surface of the upper layer each week. After 2–3 weeks of incubation at 37°C and 5% CO_2 atmosphere, foci were viewed and counted. Photographs were taken under a microscope for analysis of representative colonies.

Cell cycle analysis

CD133+ and unsorted cells inoculated to 6-well plates separately, and then cell cycle was detected by using a flow cytometer when cell concentration was 80%. The cells were suspended to 2×10^5/ml in preheated RPMI-1640 culture solution. Afterward, 2 ml of solution was centrifuged and cells were suspended again in PBS after washing twice in PBS. Then the cells were added into precooled 70% ethyl alcohol for 30 min, and dyed with 1 ml of DNA dye in the dark for 30 min after being washed in PBS. Cell cycle analysis was done using flow cytometry after the cells were filtered through 48-μM nylon meshes. Experiments were performed in triplicate.

In vivo tumorigenicity study

Ninety nude mice were used in this study. CD133+ cells and CD133− cells were injected subcutaneously into the left upper abdomen of nude mice at densities of 10^6 cells, 10^5 cells, 10^4 cells, and 10^3 cells per mouse, and 10 mice were injected at each density. Unsorted Hep-2 cells were injected into 10 nude mice at densities of 10^6 cells per mouse as control. Tumor formation of these mice was observed each week for 4 weeks. Four weeks later, 30 mice inoculated at 10^6 cells per mouse were sacrificed, and the presence of each tumor nodule, tumor weight, and tumor volume were confirmed.

Statistical analysis

Comparison among the 3 groups was made using software SPSS 11.0 using the chi square test. Statistical significance was defined as $P < 0.05$. Data are shown as mean ±SD.

## Results

Isolating CD133+ Cells from the Hep-2 Cell Line by MACS

To evaluate the efficiency of magnetic separation, harvested cells were tested by flow cytometry analysis. Before isolation, the CD133+ fraction was 40.12±1.32% (Figure 1) and was raised to 94.16±2.31% (Figure 2) after isolation. The results verified that CD133+ cells and CD133− cells were effectively isolated by this method.

In vitro proliferation of CD133+ cells and CD133− cells

The absorbance at 490 nm in 2 subpopulations of cells 1 day, 2 days, and 3 days after injection was detected by MTT assay. By comparing the proliferation of CD133+ cells and CD133− cells, we found that absorbance of CD133+ cells and CD133− cells showed a significant difference from the first day (P<0.05) (Figure 3). The results indicated that the cell proliferation ability in 2 populations showed significant difference at the first day after injection.
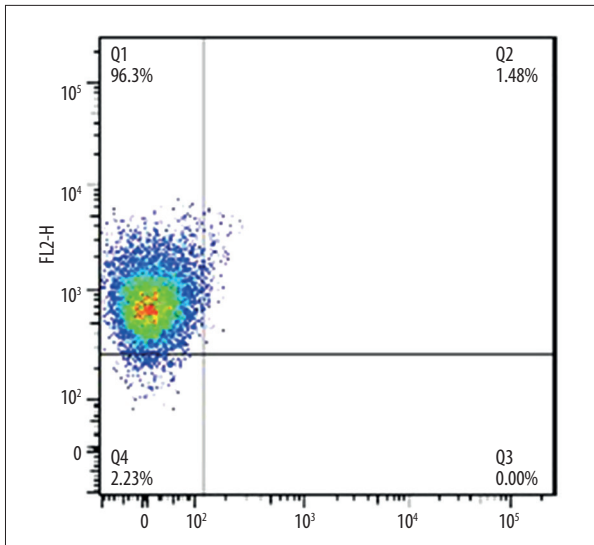
Figure 2. Hep-2 cells isolation situation. Purity of CD133 positive cells were confirmed after magnetic cell sorting assessed by flow cytometry. Data are representative of triplicate experiments.

In vitro cell colony forming capacity of CD133+ cells

Colony-forming assay confirmed the high self-renewal ability of CD133+ cells; in comparison to CD133− cells, CD133+ cells were able to form more tumor colonies (Figure 4).

Tumor-forming ability of CD133+ cells, CD133− cells and unsorted cells *in vivo*

Only 30% of CD133+ cells injected mice (3 of 10) formed tumors at a density of 10^3 cells per mouse. Five of 10 mice from the CD133+ cells group and 3 of 10 mice from the CD133− cells group formed tumors when 10^4 cells were injected into each mouse. All of the CD133+ cells and CD133− cells formed nodules
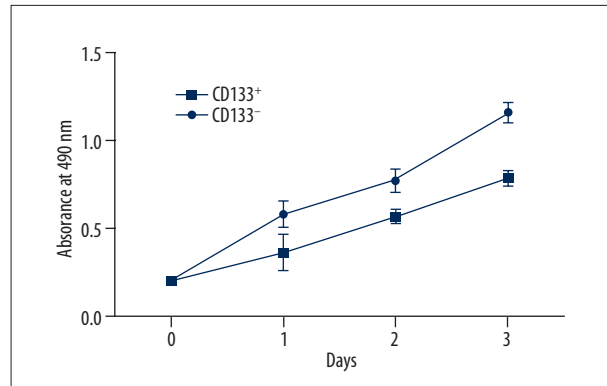

Figure 3. Growth curve of CD133+ cells and CD133− cells. Cell proliferation assay was performed by MTT analysis. Data are shown as mean ±SD and P<0.05 was considered as statistical significance.

at a density of ≥10^5 cells per mouse. Four weeks later, the presence of each tumor nodule, tumor weight, and tumor volume in mice injected with CD133+ cells were confirmed. As shown in Table 1, the tumor weight and tumor volume of the CD133+ group were greater than in the 2 other groups (P<0.05), but there was no significant difference within the CD133+ and the unsorted cells group (P>0.05).

Proliferation ability of CD133+ cells and CD133− cells under DDP treatment

MTT analysis was detected after 48 h of culturing in the presence of 5, 10, 15, and 20 μM DDP and the absorbance at 490 nm in 2 subpopulations of cells is shown in Figure 5. As a result, at the presence of DDP, CD133+ cells showed stronger proliferation ability than CD133− cells, indicating the cells in these 2 subpopulations are significantly different (P<0.05).
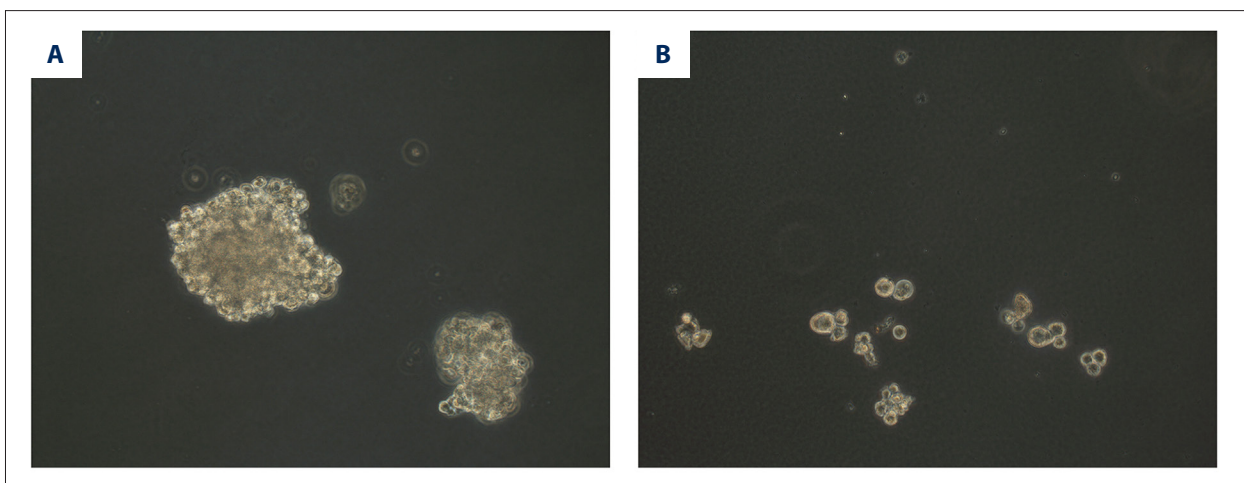
Figure 4. Colony formation assay of sorted CD133+ cells and CD133− cells. Photographs are representative of triplicate experiments. (**A**) CD133+ cells; (**B**) CD133− cells.

**Table 1.** Tumor weight, volume and forming time among three groups (mean ±SD).

Groups	No. of nude mice	Tumor weight (g)	Tumor volume (mm³)	Tumor forming time (d)
CD133⁺ cells	10	3.523±0.36	62.35±2.13	2–4
CD133⁻ cells	10	2.961±0.29	53.47±2.51	4–6
Unsorted cells	10	3.024±0.31	55.26±2.85	4–6

Tumor forming ability of different subpopulations cells under DDP treatment

All of the CD133⁺ cells, CD133⁻ cells, and unsorted cells formed nodules at a density of ≥10⁵ cells per mouse and there was no change after DDP was injected. Four weeks later, all mice were sacrificed, and the presence of each tumor nodule, as well as the tumor weight and tumor volume, were confirmed (Table 2). The results show that the tumor weight and tumor volume of the CD133⁺ group were greater than in the 2 other groups (P<0.05).

Discussion

The cancer stem cells (CSCs) hypothesis holds that tumors originate from a few cancer stem cells. In correlation studies started from hematological cancer, Florian et al. reported that acute myelogenous leukemia (AML) stem cells have the same phenotype as CD34⁺/CD38⁻ [14]. Recent research indicates that the cells composing a tumor are hierarchically organized with respect to their potential to initiate and sustain tumor growth. Only rare subsets of tumor cells, defined as CSCs, have the capacity to form tumors [15]. Cells phenotypes as CD44⁺CD24⁻/low were confirmed as breast cancer stem cells [16]; cells that express the human neural stem cells marker CD133 were reported as brain tumor stem cel l [17,18]; and cells CD133⁺CD44⁺α2β1ʰⁱ were proved as prostatic tumor stem cells [19]. Recent advances in stem cell research have facilitated the demonstration of the existence of CSCs in kidney cancer, liver cancer, lung cancer, pancreatic cancer, and various solid tumor cell lines [3]. The origin of CSCs is unclear, so researchers prefer to define CSCs by functions and characteristics. Generally speaking, the characteristic of CSCs are: 1) their capacity for self-renewal and multipotent differentiation;
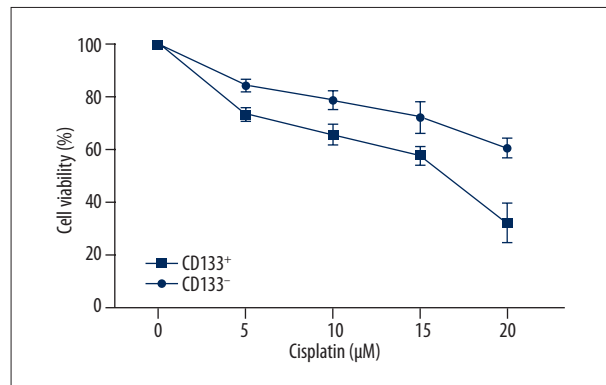
Figure 5. Sensitivity of CD133⁺ cells and CD133⁻ cells to chemotherapy agents. MTT analysis is detected after 48 h of culture in the presence of 5, 10, 15, and 20 µM DDP. Data are expressed as mean ±SD and P<0.05 was considered as statistical significance.

2) not belonging to a terminally differentiated cell, in the state of undifferentiation or poorly differentiated, or keeping constant number and strong migration ability; 3) at the silent stage; 4) could prevent apoptosis; and 5) inherent resistance to drugs. Recent studies were focused on screening and identifying specificity marker of CSCs [20].

Studies on laryngeal cancer stem cells are at the initial phase relative to other cancer stem cells. Whether laryngeal cancer stem cells have heterogeneity and whether any cancer stem cells exist among the heterogeneous populations remain to be determined. We studied the heterogeneous mechanism of laryngeal cancer stem cells by combining the primary culture and single-cell culture *in vitro*. The primary culture can reflect the biological characteristics *in vivo* because the cells, tissue, and organs were cultured directly and tumor cells were cultured *in vitro* before the first subculture. The tumor forming

Table 2. Tumor weight, volume and forming time among three groups under DDP treatment (mean ±SD).

Groups	No. of nude mice	Tumor weight (g)	Tumor volume (mm³)	Tumor forming time (d)
CD133⁺ cells	10	2.963±0.31	54.61±0.28	2–4
CD133⁻ cells	10	2.547±0.28	46.31±0.35	4–6
Unsorted cells	10	2.612±0.24	48.34±0.42	4–6

ability is the most important factor in CSC identification, so we chose the Hep-2 cell lines with stem cells characteristics and strong tumor forming ability as the study object. In combined recent studies on solid cancer stem cells, we investigated the expression of CD133 in Hep-2 cell line, the proliferation ability and tumor formation ability of CD133+ cells, and explored the possibility that CD133 acts as the marker of laryngeal cancer stem cells by observing the biological characteristics of tumor cells under DDP treatment. Our results indicated that CD133+ cells showed stronger tumor forming ability, shorter tumor forming time, and faster tumor growth than CD133− cells and unsorted cells.

Drug resistance is one of the characteristics of CSCs, and it has been reported several times that CSC is the key reason for limited effect of chemotherapy [2]. Drug-resistance molecules such as Brcp1/ABCG2, MRP1/2 are normally expressed to different degrees in lung, digestive tract, liver, and kidney, and these molecular play key roles in maintaining organism physiologic barriers such as the blood-brain barrier, blood-testis barrier, maternal fetal barrier, and blood cerebrospinal fluid barrier [21]. ABC overexpression transporters on tumor stem cell membranes can effectively attenuate the inhibition effect and lethal effect of antitumor drugs due to its ability to discharge metabolite, toxic substance, nucleotide, sterols analogues, and drugs. Our study revealed that CD133+ cells possess stronger proliferation ability than CD133− cells under DDP treatment, which indicates that the lethal effect of antitumor drugs on laryngeal cancer stem cells were weakened significantly.

Conclusions

In conclusion, we found CD133 could be a marker useful in characterizing laryngeal cancer stem cells because of the CSC characteristic of CD133+ cells. Moreover, CD133− cells also possess proliferation ability, which indicates that there are other undiscovered stem cell markers in the Hep-2 cell line, and more research is needed to find them for use in further combinations of those various markers in laryngeal cancer cells.

Conflicts of interest

The authors declare that they have no conflicts of interest concerning this article.

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