Constitutive secretion of Interleukin-4 dictates CD133+ side population cells to resist drug treatment and cell death

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Summary

Purpose: In the present study, we made an attempt to elucidate the role of oversecretion of interleukin-4 (IL-4) in cancer stem cells (CSCs) of head and neck squamous cell carcinoma (HNSCC).

Methods: HNSCC samples were analyzed for the presence of CSCs by flow cytometry. In addition, we have performed drug and apoptosis resistance assays to determine the role of IL-4 in CSCs.

Results: HNSCC samples contained 3.3% of CD133+ cancer stem like side population (SP) cells in HNSCC which displayed infinite cell proliferation and they had high self-re-newal capacity. These CD133+ cells displayed enhanced expression of IL-4, which promoted multidrug and apoptosis resistance. After neutralizing IL-4, the CD133+ SP cells became more sensitive to drug treatment and apoptosis.

Conclusions: Our data suggest that the autocrine secretion of IL-4 is a potential target for the development of novel anticancer drugs to prevent the CSCs-mediated therapy failure and tumorigenesis.

Key words: apoptosis, CD133, chemotherapy, interleukin-4, tumor recurrence

Introduction

HNSCC is one of the most common malignancies worldwide. Despite recent advances in treatment strategies, the patients still develop disease relapse within 2-5 years and therefore the overall survival rate remains very low [1-3]. According to the CSC theory, the presence of a small population of tumor-initiating cells are termed as "cancer stem cells", responsible for chemotherapy failure and tumor recurrence [1-3]. These CSCs are highly potent of self-renewal, differentiation, overexpression of stemness genes (such as CD133, CD44, etc), drug/apoptosis resistance and they are highly tumorigenic [4-6]. Therefore, it is essential to understand the precise moleculat mechanism of CSC-mediated tumorigenesis for the development of novel anticancer drugs.

One of the most popular methods used so far to isolate CSCs is Hoechst 33342-dye exclusion assay. During fluorescence-activated cell sorting (FACs), a small population of cancer cells which efflux the Hoeschst 33342 dye out of the cell fall on the left side quadrant of the dot plot analysis of FACs [7]. These cells are designated as SP cells which share all the remarkable features of CSCs [8-12]. Interestingly, these SP cells have enhanced expression of ATP binding cassette pro-

tein (ABCG2) involved in drug efflux function and thus responsible for therapy failure [8,9]. Studies have reported that autocrine expression of IL-4 is a T cell response, involved in accelerating antiapoptotic and cell cycle machineries in different cell lines [13,14]. Very recently, it was reported that autocrine secretion of IL-4 in CSCs protects from apoptosis and thereby it promotes the CSCs survival rate [15,16]. Based on these interesting findings, in the present work we made an attempt to address the possibly significant role of overproduced IL-4 in CSCs-mediated tumorigenesis in HNSCC. The experimental design, results and conclusion of this paper are described in detail below.

Methods

Cell culture from primary HNSCC samples

Human HNSCC samples were collected from the patients at the time of surgery in the Department of Otolaryngology, according to the approved Institute Ethics Committee of the Tumor Hospital of Jilin Province. Patients details: number of patients: 17; age range: 35-49 years; type: recurrence; region: oral cavity, mucous membrane and nasopharynx; stage: metastases to the lungs. The obtained primary tumor samples were minced with blades into small pieces and then enzymatically digested with collagenase (Sigma-Aldrich, St.Louis, MO, USA), hyaluronidase (Sigma-Aldrich), and DNase (Sigma-Aldrich), and then incubated for 2 hrs at 37°C supplied with 5% CO_2. After pipetting every 15 min, cell disaggregates were washed twice with phosphate buffered saline (PBS) (Sigma-Aldrich), and then subjected to centrifugation in order to remove the cell debris. Cell suspension was filtered and the resulting single cells were placed under stem cell suspension culture conditions, consisted of serum-free Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) F12 medium supplemented with N2, B27, human recombinant epidermal growth factor (EGF 20 ng/ml) (Sigma-Aldrich), and human basic fibroplast growth factor (bFGF 20 ng/ml, Sigma-Aldrich).

FACS analysis

Cells were cultured in DMEM with 10% fetal bovine serum (FBS, Sigma-Aldrich), supplemented with antibiotics (penicillin 100 U/ml and streptomycin 50 μg/ml) and maintained in T-75 flasks at 37° C in a humidified 5% CO_2 and 95% air atmosphere. Cells becoming 90% confluent were removed from the culture flask using Trypsin-EDTA (0.25% - 53mM EDTA, Sigma-Aldrich), washed, and suspended in 10% DMEM. For cell count hemocytometer was used.

Study groups: Control: cells labeled with Hoechst 33342 dye (Life Technologies, Carlsbad, CA, USA) alone (N=9); Drug-treated cells: cells treated with verapamil (Life Technologies) and Hoechst 33342 dye (N=9). Approximately 10^6 cells/ml of 10% DMEM were labeled with Hoeschst 33342 stock (sigma)-bis-benzimide (5 μl/ml) either with dye alone or in combination with ABC transporter inhibitor verapamil – 0.8 μl/ml. Then, cells were counterstained with 2 μg/ml PI (propidium iodide, Sigma-Aldrich). The cells were sorted using flow cytometer and were cultured and maintained in DMEM/F-12 supplemented with 10% FBS. The Hoechst 33342 emission was first split by using a 610-nm dichroic short-pass filter, and the red and the blue emissions were collected through 670/30- and 450/65-nm bandpass filters, respectively.

In vitro proliferation activity assay

The sorted SP and non-SP cells were seeded in a 96-well plate at $2x10^6$ cells/well and then cultured in a CO_2 incubator. Each group was set up in triplicate. Cell proliferation activity was measured every day for 7 days. Each well was supplemented with CCK-8 solution (10μl) and incubated in CO_2 incubator for 2-3 hrs. The optical density (OD) was determined at 450 nm. These data were used to calculate cell growth graphs based on the mean value of OD_{450} and standard deviation values for each well.

Differentiation assay

The differentiation assay was performed as per the previously described protocol [17].

Western blot analysis

For Western blot analysis, proteins were extracted from the SP and non-SP cells, and protein concentration was determined using the Bradford assay [18]. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to a membrane, the gels were treated with the primary antibody (rabbit anti-human IL-4:1000) and subsequently incubated with secondary antibody (goat anti-rabbit IgG with alkaline phosphatase markers) and a chemiluminescence reagent for Western blot (Enhanced chemoluminescence to agent/ECL, Western Blotting Detection Reagents).

Blots were detected and scanned by using a densitometer (Biorad GS-710). An equal concentration of the proteins was loaded per lane and GAPDH was used as loading control.

Clone formation efficiency

The sorted SP and non-SP cells were resuspended at a density of 1000 cells/ml in tumor sphere medium consisting of serum-free 1:1 mixture of Ham's F-12/DMEM, N2 supplement, 10 ng/ml human recombinant bFGF, and 10 ng/ml epidermal growth factor (EGF) and subsequently cultured in ultra-low attachment plates for about 2 weeks. Sorted SP and non-SP cells were

seeded at a low density of 20 cells/L and the number of generated spheres (>100 mL) was counted after 7 days of culture. The values represented in the graph are the mean of three independent experiments.

Real-time reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was extracted and complementary DNA was prepared using Reverse Transcriptase kit (Fermentas, Burlington, Canada). Real-time RT-PCR analysis was subsequently performed on an iCycler IQ real-time detection system (Bio-Rad), using IQ Supermix with SYBR-Green (Bio-Rad). The sequences of human specific primers used were as follows: ABCG2 – (F: GGA TGA GCC TAC AAC TGG CTT and R: CTT CCT GAG GCC AAT AAG GTG); OCT4 – (F: TCG AGA ACC GAG TGA GAG GC and R: CAC ACT CGG ACC ACA TCC TTC); EpCAM – (F: CTG CCA AAT GTT TGG TGA TG and R: ACG CGT TGT GAT CTC CTT CT); CD133: (F: TCT TGA CCG ACT GAG AC and R: ACT TGA TGG ATG CAC CAA GCA C). BCL-2: (F: ACA CTG TTA AGC ATG TGC CG and R: CCA GCT CAT CTC ACC TCA CA); GAPDH – (F: TCT GCT CCT CCT GTT CGA CA and R: AA AGC AGC CCT GGT GAC C). GAPDH was used as a house keeping gene. The PCR parameters used to set PCR reactions are: Initial denaturation – 90°C 45 sec; annealing – 60°C for 45 sec; extension -72°C for 30-45 sec; cycles – 30. The data represented in the graph are the average values of three independent experiments. The amplified products were separated by electrophoresis on ethidium bromide-stained 1.2% agarose gels. Image J was used to measure the band intensity. The values presented in the graph are the mean from three independent experiments.

Immunofluorescence staining

The sorted SP and non-SP cells were fixed onto glass slides in ice-cold 4% paraformaldehyde (4°C,10 min), blocked with normal serum for 30 min, and incubated with mouse monoclonal CD133 (1:200) for one hour. After washing the slides with PBS, they were incubated with FITC-conjugated chicken anti-rat lgG overnight in the dark. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and viewed under fluorescence microscopy. All images were processed by Adobe Photoshop version CS6.

TUNEL assay

The rate of apoptosis was analyzed by TUNEL assay by using detection kit (Boehringer Mannhein, Germany).

Cell resistance assay

Approximately 1x10³ cells/plate were cultured in 96-well plates and treated with the chemotherapeutic drugs at the following concentrations: 5-FU with a concentration of 10 μg/ml, oxaliplatin (100 mM), paclitax-

el 30 ng/ml and cisplatin 5 mg/ml. The mean value of OD_{450} obtained was represented as a graph. Cell resistance in both groups was calculated using the following formula: Cell resistance rate (%) = (experimental group OD_{450} value/control group OD_{450} value) x 100. Similarly, the cells were pretreated for 24 hrs or not with 10 mg/ml of anti-IL-4 and subsequently the cell viability was measured after treatment with the drugs. The values presented in the graph are the mean of three independent experiments.

Statistics

One-way analysis of variance (ANOVA) and Student's t-test were performed to determine significant differences between the treatment and control groups. A probability level of $p<0.05$ or <0.01 was considered as statistically significant.

Results

Existence of CD133+ cancer stem cell in HNSCC

By FACS sorting, 3.3% of cancer stem-like SP cells from HNSCC have been purified (Figure 1A), which were further subjected to immunocytochemistry. As shown in Figure 1B, the FACs-sorted SP cells displayed CD133 overexpression when compared to the main population (non-SP) cells. In addition, RT-PCR analysis revealed that the transcriptional regulations of CD133 in SP cells were significantly upregulated (Figure 1C).

Phenotypic characterization of CD133+ SP cells in HNSCC

The *in vitro* cell proliferation assay revealed that CD133+ SP cells possessed infinite cell proliferation capacity compared to non-SP cells (Figure 2A) and became more confluent on the 7th day. Further, the morphology of SP cells was aberrantly changed and started to form fibroblast-like filaments produced on the 7th day (Figure 2B). However, these changes were not observed in non-SP cells. The sphere formation assay revealed that production of CD133+ cells was highly efficient to generate more tumor spheres compared with non-SP cells (Figure 3A). Next, we evaluated the expression level of stem cell surface genes in CD133+ cells by RT-PCR analysis. As shown in Figure 3B, the transcriptional regulation stemness genes, such as Oct-4 and EpCAM, was significantly upregulated in CD133+ SP cell cells when compared to non-SP cells. From these data, it is obvious that CD133+ SP cells possessed elevated stemness proteins, and might actively be involved in the regulation of self-renewal and invasion of
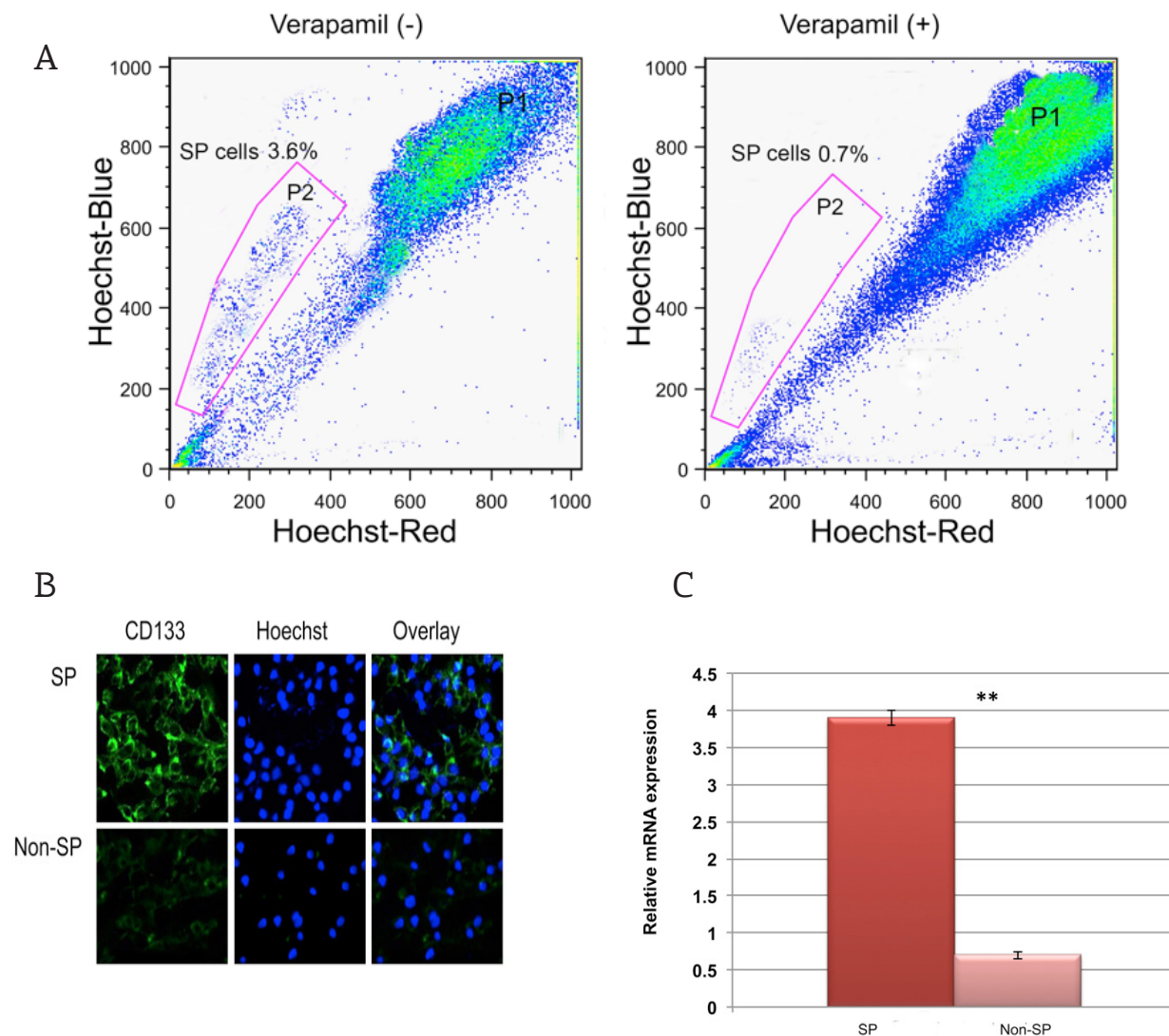
Figure 1. Analysis of cancer stem like side population (SP) cells in HNSCC. **A:** Cells were stained using Hoechst 33342 dye and analyzed using flow cytometry. SP cells of 3.3% are outlined as gated population from main population (non-SP cells). The prevalence of SP cells was significantly diminished to 0.7% upon treatment with verapamil. **B:** Representative images of immunofluorescence staining of SP and non-SP cells. **C:** RT-PCR analysis, showing elevated mRNA expression of CD133 in SP cells. Quantification graph from the data of three separate independent experiments. GAPDH was used as a house-keeping gene. Bars represent standard deviation. **p<0.01.

SP cells.

CD133+ SP cells resist multidrug treatment and apoptosis

The CD133+ SP cells were treated with several anticancer drugs (5-FU, oxaliplatin, cisplatin and paclitaxel). After treatment with the above mentioned drugs, the survival rate of CD133+ SP cells was much higher (more than 70%) compared with non-SP cells (Figure 4A). Subsequently, the number of CD133+ SP cells that underwent apoptosis was significantly less than in non-SP cells (Figure 4B). In addition, the relative mRNA expression of ABCG2 was significantly elevated in conjunction with the elevation of antiapoptotic gene bcl-2 (Figure 4C). These data clearly suggested that the presence of CD133+ SP cells was responsible for chemotherapy failure and tumor recurrence.

Oversecretion of IL-4 in SP cells are the major factors involved in chemo/apoptosis resistance

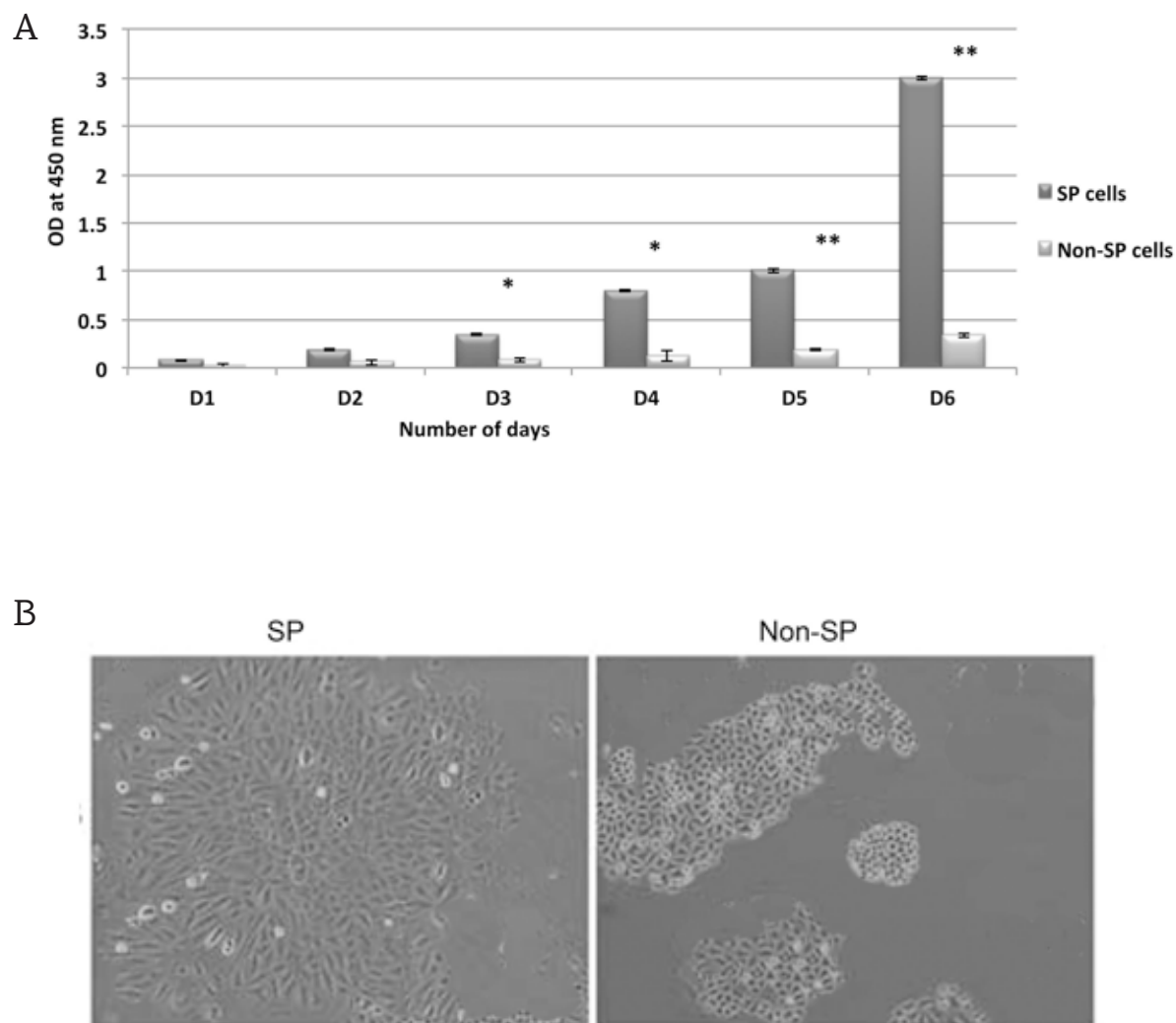We found that autocrine production of IL-4

Figure 2. SP cells displayed rapid proliferation and high differentiation. **A:** In vitro proliferation asaay showing that cell proliferation rate of SP cells was significantly higher than non-SP cells. **B:** The morphology of CD133+ SP cells changed on the 5th day, showing filamentous structures like fibroplasts. Bars represent standard deviation. *p<0.05;**p<0.01.

was much higher in CD133+ SP cells than in non-SP cells (Figure 5A). In addition, in order to address the significant role of IL-4 in CD133+ SP cells, we have presented them in IL-4 neutralizing antibody [19] and subsequently evaluated the drug resistance and apoptosis rate of CD133+ SP cells. After neutralization of IL-4, the CD133+ SP cells became more sensitive to multidrug treatment and their survival rate was significantly reduced (Figure 5B). Similarly, the positive apoptosis number of CD133+ SP cells was increased after IL-4 neutralization (Figure 5C). Hence, these data suggested that hypersecretion of IL-4 in CD133+

SP cells was crucial for multidrug and apoptosis resistance in HNSCC SP cells.

Discussion

To date, the presence of CSCs is of great concern since they are responsible for treatment failure and tumor recurrence. Current conventional therapies are able to kill most of the neoplastic cells whereas CSCs escape, remain dormant during treatment and constitute the minimal residual disease (MRD) [20]. Hence, it is crucial to characterize the CSCs in order to elucidate the factors
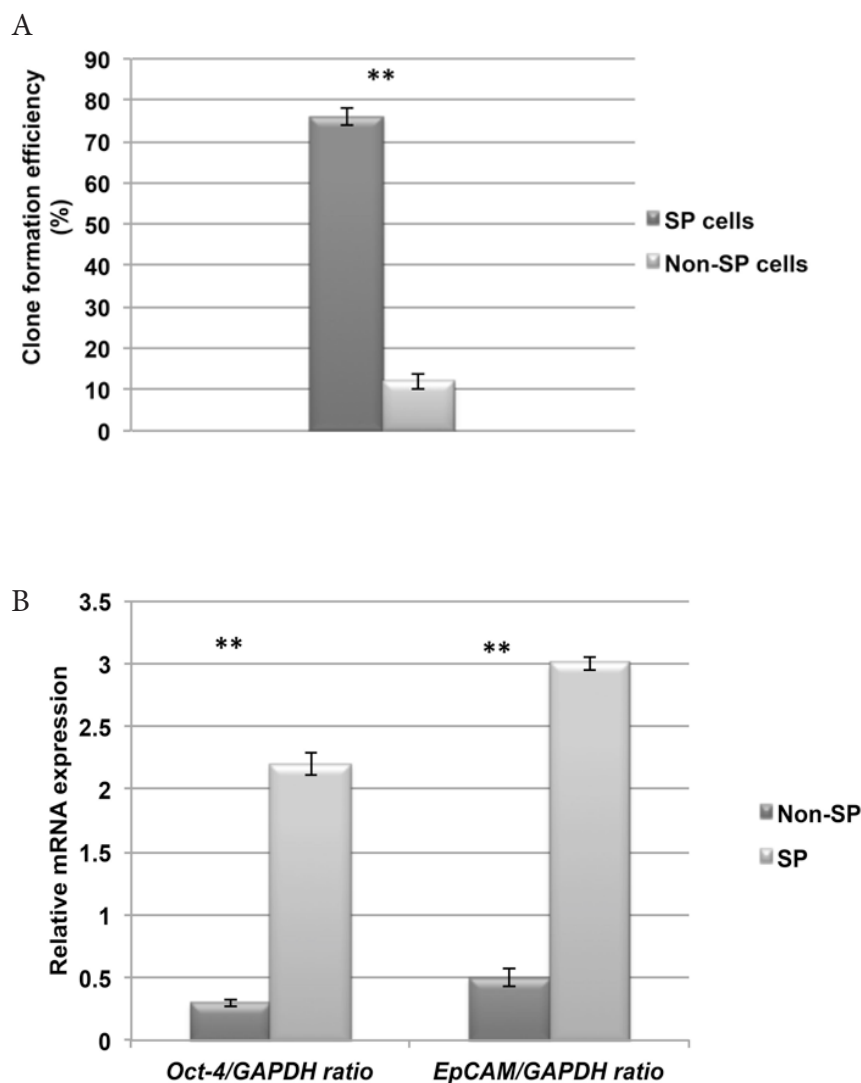
Figure 3. HNSCC SP cells showed high self-renewal capacity. **A**: Clone formation efficiency revealed that SP cells could rapidly generate more tumor spheres than non-SP cells. **B**: Quantification graph from RT-PCR analysis showing elevated transcriptional regulation of stemness genes in SP cells than in non-SP cells. Bars represent standard deviation. **p<0.01.

and signaling pathways involved in the CSCs-mediated tumorigenesis and invasion.

Most common methods for the purification of CSCs are Hoechst 33342 dye exclusion, based on the expression of stem cell surface proteins [7,8]. Several studies reported that upregulation of drug efflux proteins and downregulation of cell death signaling factors are involved in the CSCs-mediated multidrug and apoptosis resistance [21,22]. Based on Hoechst 33342 dye exclusion assay, 2.7% cancer stem like SP cells were isolated from HNSCC cell lines, such as M3a2 and M4e [23], and they were able to initiate more tumor spheres *in*

vitro. By using the same technique, we also identified about 3.3% SP cells in HNSCC samples whose prevalence was reduced to 0.9% upon treatment with verapamil (inhibitor of ABC transporter protein), confirming thus the role of ABC transporter protein in drug efflux. Similar to previous findings, these SP cells displayed increased expression of CD133, Oct-4 and EpCAM, which are essential for the maintenance of self-renewal and metastasis [24,25].

Another remarkable feature of cancer stem like SP cells are resistance to DNA targeting drugs. In the present work, the FACs purified HN-
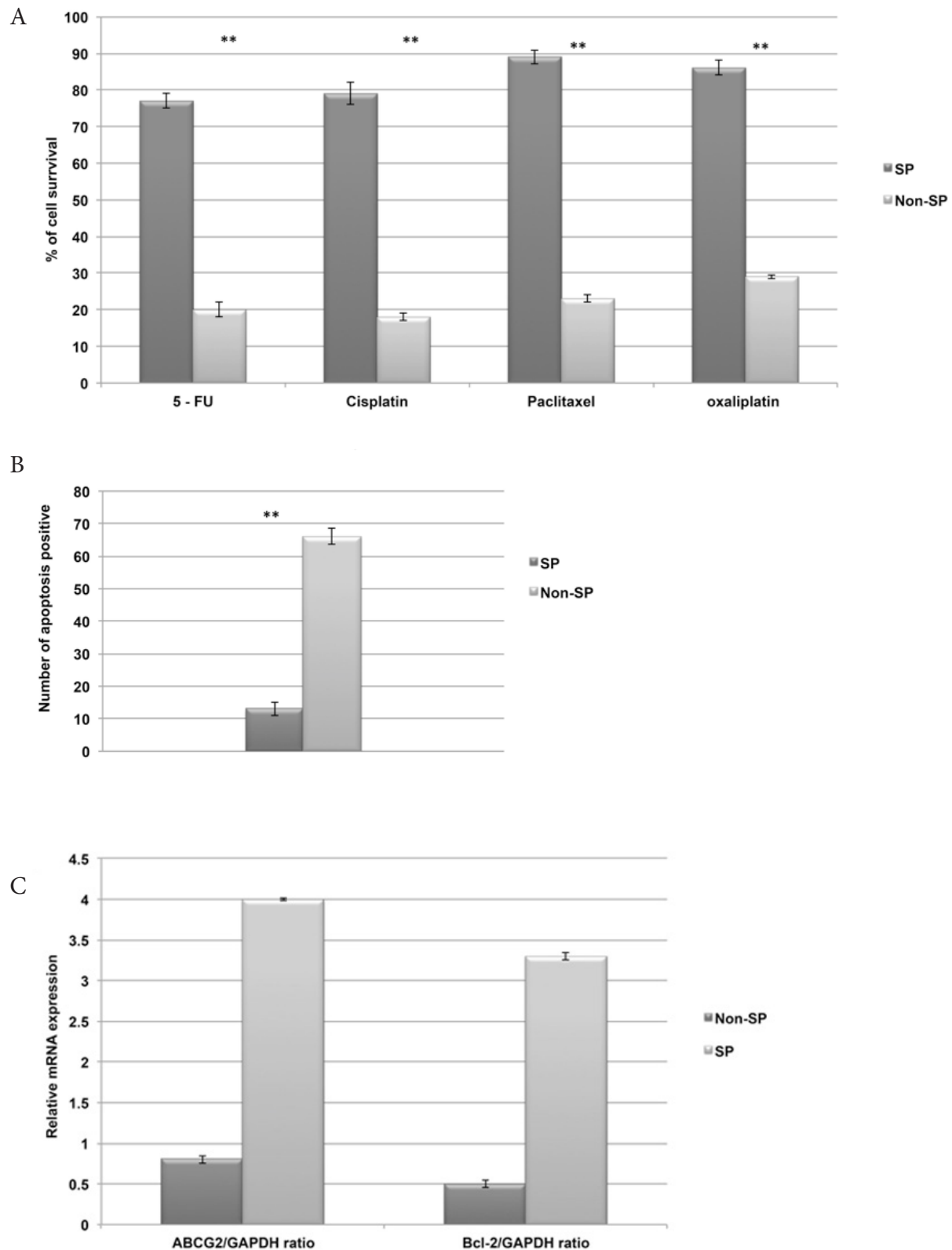
Figure 4. HNSCC SP cells showed high multidrug and apoptosis resistance. **A:** Comparison of cell survival rate between SP and non-SP cells after treatment with the DNA targeting drugs (5-FU, oxaliplatin, cisplatin and paclitaxel). **B:** The number of SP cells that underwent apoptosis was significantly lower than in non-SP cells. **C:** Quantification graph of RT-PCR analysis showing that the relative mRNA expression of ABC transporter gene ABCG2 and antiapoptotic gene Bcl-2 were significantly upregulated in SP cells. Bars represent standard deviation. **p<0.01..
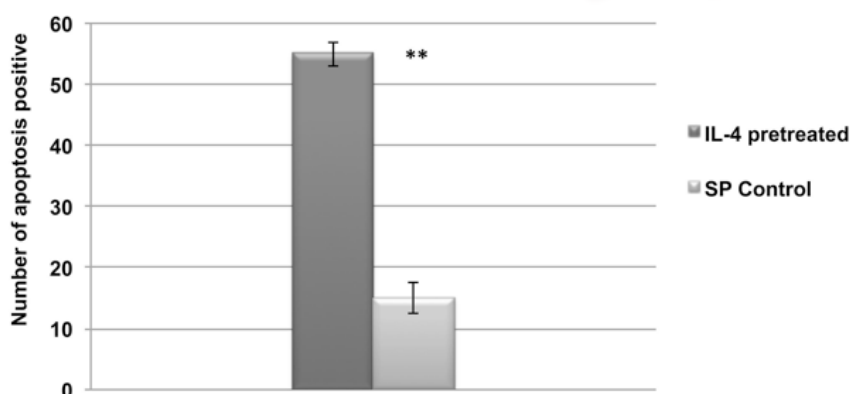
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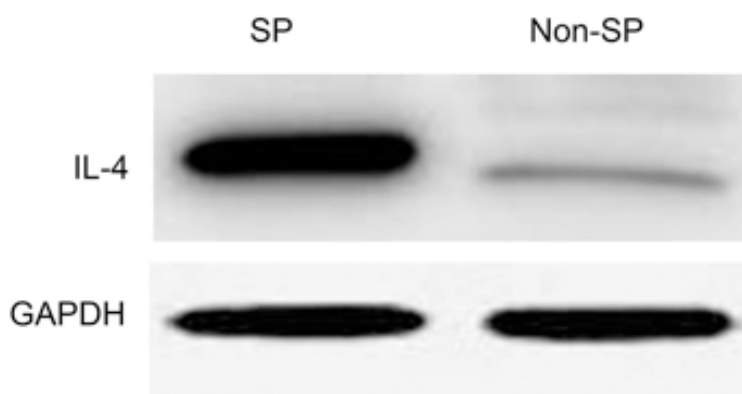
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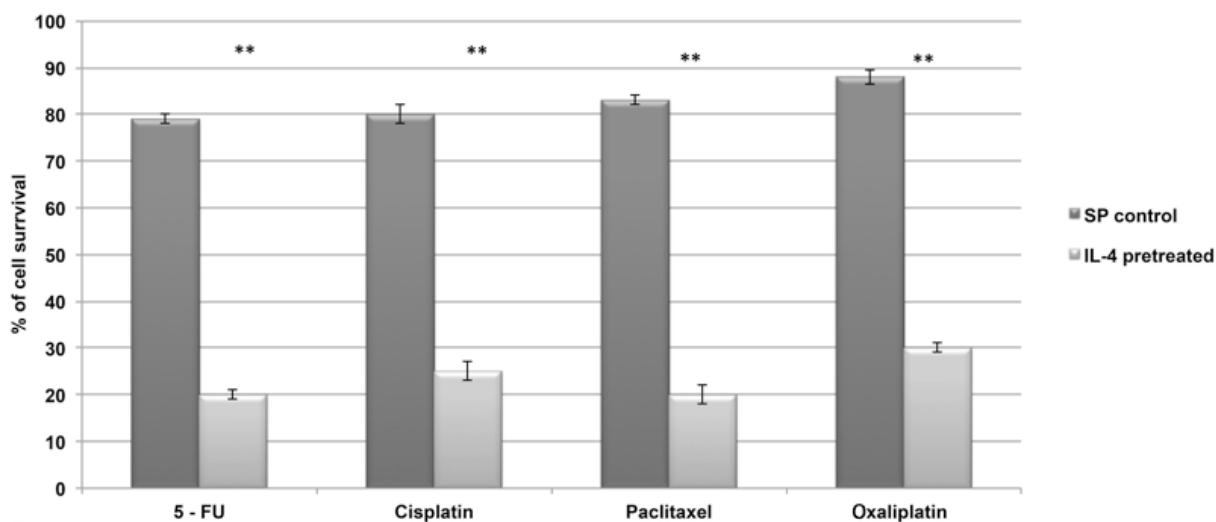
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Figure 5. Secretion of IL-4 was the cause for drug and apoptosis resistance of SP cells. **A:** Western blot analysis showing elevated secretion of IL-4 in SP cells. Comparison of cell viability (**B**) and apoptosis rate (**C**) of SP cells after pretreatment with anti-IL-4 for 24 hrs. Bars represent standard deviation. ****p<0.01.

SCC SP cells showed increased resistance to several chemotherapeutic drugs (5-FU, oxaliplatin, cisplatin and paclitaxel) and consequently the rate of apoptosis was significantly reduced. Possibly this is due to upregulation of ABC genes and antiapoptotic genes as we showed that the relative mRNA expression of these genes was significantly elevated in HNSCC SP cells. But the molecular links between these two different mechanisms are not clear so far. However, we speculate that there might be other additional factors involved in drug and apoptosis resistance of CSCs.

Recent reports in colon cancer showed that autocrine secretion of IL-4 alters the apoptosis rate and promotes cell survival of SP cells [15,16]. Similarly, we also found increased autocrine secretion of IL-4 in HNSCC SP cells. Upon neutralization of IL-4, the SP cells showed reduced cell proliferation and became more sensitive to multidrug treatment and apoptosis. In line with these findings, there are reports showing that oversecretion of IL-4 and IL-10 are involved in promoting cell survival by accelerating cell death resistance signals [13,14]. Therefore, these findings clearly indicate that IL-4 secretion is the crucial factor for the enhanced cell survival rate of CSCs.

In summary, our data demonstrated that HNSCC SP cells could be considered as enriched CSCs since they are highly tumorigenic and invasive. Overproduction of interleukin-4 in HSNCC SP cells is the driving force to resist cell death, promote enhanced cell viability, and thus favoring tumor recurrence. Therefore, future studies concerning the stimulating factor for IL-4 production and the downstream pathways involved in IL-4-mediated ABC genes and antiapoptotic factors regulation would help develop new anticancer drugs that could completely eradicate tumor refractoriness.

Acknowledgement

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