·Basic Research·

Side population cells from HXO -Rb44 retinoblastoma cell line have cancer -initiating property

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Abstract

• AIM: To ascertain whether side population (SP) cells in HXO-Rb44 retinoblastoma cell line have cancer stem cell-like property *in vitro* and *in vivo*.

• METHODS: We analyzed and sorted SP from HXO-Rb44 retinoblastoma cell line by Hoechst 33342 staining on flow cytometry. SP and NSP cells were determined their ability of proliferation and self-renewal by SP reanalysis, soft agar assay and tumor sphere assay *in vitro*. Clone formation was detected by seeding HXO-Rb44 and HXO-Rb44 -RFP cells into soft agar. The expression of *ABCG2, MDR1, Bmi-1* and *Oct-4* was determined by RT-PCR between SP and non-SP (NSP) cells. Moreover, they were injected into nude mice to determine their tumorigency *in vivo*.

• RESULTS: SP from HXO-Rb44 retinoblastoma cell line could grow clonally in soft agar assays and form tumor spheres from single cells in conditioned media. The expressions of *ABCG2, MDR1, Bmi-1* and *Oct-4* were significantly higher in SP than NSP cells. As few as SP cells resulted in tumor formation in 6 of 12 injected sites, however, the injection of NSP cells failed to form new tumor.

• CONCLUSION: SP cells isolated by Hoechst 33342 from the HXO-Rb44 retinoblastoma cell line had property of high tumorigency *in vivo* and *in vitro*. Therefore, SP might be a target while developing retinoblastoma therapies.

• KEYWORDS: side population; self-renewal; retinoblastoma; HXO-Rb44

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INTRODUCTION

Tumors have long been viewed as a population in which all cells have the equal propensity to form new tumors, the so-called conventional stochastic model. Recently, the hierarchical model claimed that there was a small subset of cancer stem cells (CSCs) that were responsible for tumor initiation and growth. Cancer stem cells have been identified by a specific cell surface marker in several solid tumors including breast cancer [1], brain tumor [2], prostate cancer[3], lung cancer [4] *et al*. Most markers which were selected to isolate CSC isolation are chosen from normal stem cells or other malignancies. The disadvantage of procedure is that the CSCs marker was usually unknown, moreover, the expression level (high versus low) used for identifying CSCs sometimes differs between tissue types [5].

ABC transporters are membrane pumps that use ATP to efflux endogenous small molecules such as bile acids, cholesterol, ions and peptides across cell membranes. The drug-resistance property of stem cells, as conferred by these ABC transporters, is extremely useful in the isolation and the analysis of stem-like populations. While most cells accumulate the fluorescent dye Hoechst 33342, stem cells do not due to ABC pump-mediated efflux. CSC often expressed

ATP-binding cassette (ABC) transporter activities highly and showed chemo-resistance and phenotype of Side Population cells which allowed for FACS sorting of stem cells as the so-called "SP"[6].

Retinoblastoma usually occurs on child under five years, a majority of patients relapse and become drug-resistant [7]. Various types of ABC Transporters, especially ABCG2, contribute to drug resistance in cancers including retinoblastoma, by pumping chemotherapy drugs out of cancer cells[8]. Therefore, SP cells might be a target for therapy in order to prevent relapse and drug-resistance in retinoblastoma. To verify this hypothesis, SP and NSP cells were sorted out separately from the HXO-Rb44 retinoblastoma cell line and estimated whether the SP fraction had property of cancer stem cells *in vivo* and *in vitro*.

MATERIALS AND METHODS

SP and NSP presentation The HXO-Rb44 retinoblastoma cell line is procured from Xi'an Jiaotong University. It is grown in DMEM supplemented with 100mL/L fetal bovine serum (FBS) and penicillin/streptomycin at 37℃ in a humidified atmosphere with 50mL/L CO_2. To analyze SP and NSP fractions, the cells were removed from their dishes with 2.5g/L trypsin and 0.5g/L EDTA, centrifuged, washed with PBS and resuspended at 37℃ in HBSS containing 20mL/L FBS. Cells (1×10^6) were labeled in HBSS with 5.0mg/L Hoechst33342 dye either alone or in combination with 50mol/L verapamil at 37℃ for 90 minutes. After washing three times with PBS, the cells were resuspended in HBSS containing 20mL/L FBS and 1mmol/L HEPES, passed through a 40-um mesh filter, then maintained at 4℃ until flow cytometric analysis. Then, 1×10^6 viable cells were analyzed and sorted using a FACSVantage SE Cell Sorter (BD Biosciences, CA). The Hoechst dye was excited with a UV laser and its fluorescence was measured with both 675/20 (Hoechst red) and 424/44 filters (Hoechst blue).The analysis were repeated three times.

Clone formation assays Following melting of 10g/L agar (DNA grade) in the microwave and warming 2×DMEM supplemented with 200mL/L FBS to 40℃ in a water bath, equal volumes of the two solutions were mixed to yield a new solution of 5g/L agar, 1×DMEM and 100mL/L FBS. Next, 1.0mL of mixed solution was added to each well of a 6-well plate to form the base agar. Then, 7g/L agar was melted in the microwave and cooled to 40℃ in a water bath; similarly, 2×DMEM and 200mL/L FBS were warmed to the same temperature. Freshly sorted SP and NSP cells from HXO-Rb44 were passed through a 40μm filter to provide a single cell suspension and were counted. Following this, 3mL DMEM, 1.5mL 2×DMEM containing 200mL/L FBS and 1.5mL agar including 400 cells were mixed together, and a 1.5mL cell suspension of this solution was placed into each well of a 6-well plate as the top agar. Finally, 100 cells were seeded into each well, and were incubated at 37℃ in a humidified incubator for 2-3 weeks. Colonies were either left unstained, or were stained with 5g/L MTT for no more than one hour, and counted using a dissecting microscope. The procedure was repeated three times. After SP cells were seeded in soft agar assays, colonies containing more than 50 cells (primary colony) were removed from soft agar with sterile Pasteur pipettes, treated with trypsin and mechanically dissociated into single cells. Then, 100 cells were seeded into each well at 37℃ in humidified incubator for another 2-3 weeks. Colonies (secondary colony) were stained with 5g/L MTT for no more than one hour, and counted using a dissecting microscope. The procedure was repeated three times.

Lentivirus infection Plasmid DNA was kept at a 3:3:1 ratio [Vector (ERFP): ⊿8.91: VSV-G] according to the lipofectamine protocol (Invitrogen). Next, 2.5μg of plamid DNA was diluted in 500μL of serum-free DMEM for each well, and 2.5μL of Mix PLUS reagents and 6.25μL lipofectamine LTX reagent were added to the diluted DNA and incubated for 30 minutes at room temperature. Then, approximately 500μL of the DNA-lipofectamine LTX complex was added to each well containing 293FT cells, which were 40-50% confluent. The cells were incubated at 37℃ in a CO_2 incubator for six hours and the medium was changed for transfected cells. After 24 hours, the media from 293FT cells was collected, and 8mg/L of polybrene was added and transferred to target cells. The transfer of media from 293 FT to HXO-Rb44 cells was repeated after 24 and 48 hours.

Sphere formation assays To test sphere formation in suspension, sorted SP and NSP from HXO-Rb44 cells were passed through a 40μm filter to provide a single cell suspension. Next, 4mL of medium containing 100 cells were added to 6-well plates with serum-free media including F12 with EGF (10μg/L), insulin (20mg/L) and bFGF (10μg/L). Aliquots of EGF, insulin and bFGF were added twice per week. After 10-14 days, plates were visually assayed for the formation of floating spheres. Spheres were counted using a dissecting microscope.

Gene expression detection 110 SP and NSP cells from the HXO-Rb44 total population were collected in a separate centrifuge tube with 350μL RLT buffer containing 10g/L 2-mercaptoethanol, total RNA was extracted from these cells using a RNeasy Mini Kit (Qiagen, CA) according to protocol. RNA was transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, CA). RT-PCR was performed using a SuperScript One-Step kit

(Invitrogen, CA). The primers were as follows: GAPDH, 5-CTG CAC CAC CAA CTG CTT AG-3 and 5-AGG TCC ACC ACT GAC ACG TT-3; ABCG2, 5-GGG TTC TCT TCT TCC TGA CGA CC-3 and 5-TGG TTG TGA GAT TGA CCA ACA GAC C-3; MDR1, 5-GCC TGG CAG CTG GAA GAC AAA TAC-3 and 5-ATG GCC AAA ATC ACA AGG GTT AGC-3; Bmi-1, 5-AGC AGA AAT GCA TCG AAC AA-3 and 5-CCT AAC CAG ATG AAG TTG CTG A-3; Oct-4, 5-GAG AAT TTG TTC CTG CAG TGC-3 and 5-GTT CCC AAT TCC TTC CTT AGT G-3. The PCR products were separated by electrophoresis on a 20g/L agarose gel.

Tumor formation assays Sorted SP and NSP from HXO-Rb44 cells were resuspended in 50μL HBSS, ranging in density from 10 to 10 cells, and then mixed with 50μL Matrigel to prevent injected cell dispersion and loss. Then, cells were subcutaneously injected into 6- or 7-week old nude mice on the day of sorting. Nude mice were obtained from the Animal Institute of the Xi'an Jiaotong University, China (XJTU). All experiments were approved by the Animal Care Committee of XJTU. Mice were monitored every day to assess tumor formation for six to eight weeks after transplantation.

Statistical Analysis All values were expressed as mean ± SD. Any significant difference among mean values was further evaluated by the Student's *t* test.

RESULTS

Identification of SP in HXO-Rb44 retinoblastoma cell line SP were identified as a characteristic tail separated from the whole population by flow cytometry (Hoechst red 675/20; Hoechst blue 424/44). The percentage of SP cells was 1.04% which decreased in number while treated with verapamil in HXO-Rb44 retinoblastoma cell line. SP and NSP cells were analysis and sorted out to determine their proliferation, self-renewal and tumorigenic property in the next step(Figure 1).

Clone formation While seeded as single cells in soft agar assay, the clonogenic ability of SP and NSP were examined. The clonogenic efficiency of SP was significantly higher than NSP cells in soft agar assays (Figure 2A, $P<0.05$). Furthermore, SP cells could divide into colonies of more than 30 cells in soft agar after three weeks. The clonogenic efficiency was not apparent difference between NSP and whole population. It is a slightly improvement of clonogenic efficiency from primary to secondary colonies in detecting the self-renewal ability of SP cells. The shape of SP colonies is similar between secondary and primary, which clarifying that SP cells can maintain and expand themselves progressively. To clarify whether the colony came from a single cell, HXO-Rb44 were infected with enhanced red

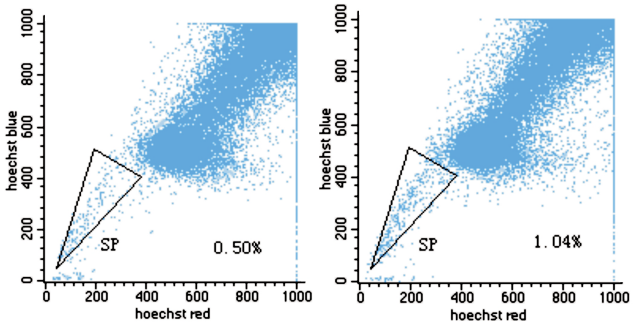

Figure 1 SP and NSP cells from the HXO-Rb44 cells were analyzed through uptake of the DNA binding dye Hoechst33342 with or without the presence of verapamil

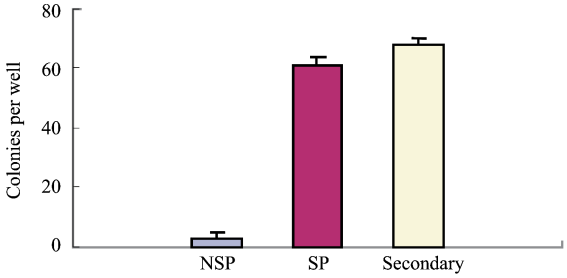

Figure 2 Soft agar assays for SP and NSP cells isolated from HXO-Rb44 cells, SP cells were more clonogenic than NSP cells. After repeated 3 times, column diagram data indicate that there was a statistically significant difference in clonogenic efficiency between SP and NSP cells ($P<0.05$)

fluorescent protein-expressing (ERFP) lentivirus. Equal numbers of HXO-Rb44-RFP and HXO-Rb44 cells were seeded from primary to second passage into serial soft agar assays. It is revealed that HXO-Rb44-RFP cells expressed strong red fluorescence and showed a similar growth rate compared to non-infected HXO-Rb44.

Sphere formation The ability of SP and NSP cells to generate spherical clones and self-renewal was evaluated by sphere formation assays. It was difficult to observe floating spheres coming from NSP cells (Figure 3A, $P<0.05$). SP cells started to form floating spheres after 2-3 days of seeding, and became primary spheres, including more than 30 cells, after 10-14 days, which suggesting that SP cells have self-renewal ability(Figure 3B).

Gene expression To clarify whether SP have stem cell-like property, SP and NSP cells were detected their gene expression of stem cell markers by RT-PCR. The expression of ABC transporters, including *ABCG2*, *MDR1*, *Oct-4* and *Bmi-1*, were significantly higher in SP than NSP cells, which suggested that SP cells have some stem cell like property (Figure 4).

Tumor formation To compare tumorigenic ability *in vivo*, we subcutaneously injected SP and NSP sorted from HXO-Rb44 cells into nude mice. Each mouse was injected

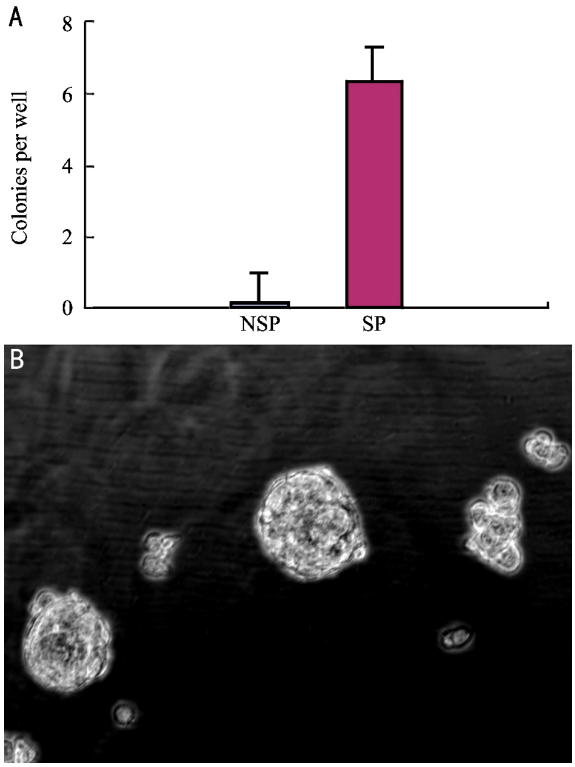

Figure 3 Serial sphere assays A:After repeated 3 times, column diagram data indicate there was a statistically significant difference in clonogenic efficiency between NSP and SP cells in sphere assays, $P<0.05$; B:Sphere assay for SP cells

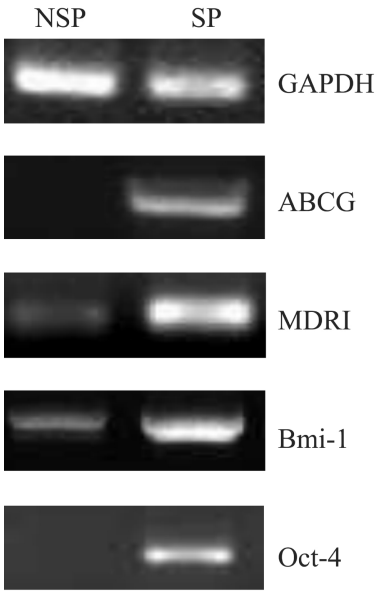

Figure 4 ***GAPDH, ABCG2, MDR1, Bmi-1*** **and** ***Oct-4*** **RNA expression of SP and NSP cells isolated from HXO-Rb44 by RT-PCR**

with different amounts of SP and NSP separately. 70 days after injection, as few as SP cells resulted in tumor formation in 6 of 12 injected sites, however, the injection of NSP cells failed to form new tumor (Table 1), although the observation period had to be increased to 90 days.

Table 1 The tumor diameter for injected SP and NSP sorted from the HXO-Rb44 cells in nude mice after six weeks

Cell type	Cell dose	Tumor/injected sites
SP	1×10^5	Not tested
	1×10^4	4/8
	1×10^3	6/12
	1×10^2	1/12
NSP	1×10^5	1/8
	1×10^4	0/8
	1×10^3	0/12
	1×10^2	Undetected

DISCUSSION

It was formerly believed that cancer is a homogenous disease and that each cancer cell can give rise to the entire tumor. However, recent studies have demonstrated that only a rare and biologically distinct subset of cancer cells (cancer stem cells) is capable of extensive proliferation [9]. Although rapid progress have be received in the field of cancer stem cells recently, there have no reports identifying SP cells from the HXO-Rb44 retinoblastoma cell line. Therefore, we analyzed SP which was 1.04% in the HXO-Rb44 cells. Further studies are needed to compare the relationship between SP prevalence and tumorigenicity.

The clonogenic assay is a soft agar system designed for growing single cells *in vitro*. The assay has been extensively used in studies both of stem cell biology and for screening new agents. Either stem cells or malignant cells can grow independently in soft agar. Thus, soft agar assays can be used to investigate this tumorigenic ability *in vitro* [10]. It is proved that the colonies came from single cells by seeding the mixed cells of HXO-Rb44-RFP and HXO-Rb44 into soft agar assay. The clonogenic ability of SP isolated from HXO-Rb44 was higher than that of NSP cells in soft agar and sphere forming assays, which suggested SP cells have the property of self-renewal.

The tumor forms in mice by injecting 10(4 of 8) and 10 (6 of 12), even 10 (1 of 12) SP cells in animal transplant assay. Mice did not form tumor by injecting 10(0 of 8) and 10(0 of 12) NSP until 90 days. Therefore, it was significant difference in tumorigenicity *in vivo* between SP and NSP cells, which suggests that SP cells enriched stem cell like characteristics.

Cancer stem cells are defined by their ability to: be serially transplanted in a xenograft assay thereby showing the ability to self-renew (regenerate); generate daughter cells that possess some proliferative capacity but are unable to initiate or maintain the cancer because they lack intrinsic regenerative potential [11]. A stem cell could undergo

asymmetric division continuously, producing one cell that retains self-renewal ability, as well as another that differentiates into a mature cell [12]. Therefore, soft agar and sphere formation assays can be used to test the self-renewal ability of stem cells *in vitro*[13, 14]. The data of soft agar assays and sphere formation assays suggested the SP from HXO-Rb44 cells have self-renewal ability.

However, it is the first step to identifying and understanding the role of self-renewal property of CSCs, the eventual goal is to generate targeted therapeutics that inhibit these essential characteristics of CSC fraction. The targeting pathways are pivotal in normal and cancer stem cell function[15]. *Bmi-1* is a polycomb gene family member, plays an important role in cell cycle regulation, cell immortalization, and cell senescence. Recently, numerous studies have demonstrated that Bmi-1 is involved in the regulation of self-renewal and differentiation of stem cells [16]. The *Oct-4* POU transcription factor is expressed in mouse totipotent embryonic stem and germ cells. Differentiation of totipotent cells to somatic lineages occurs at the blastocyst stage and during gastrulation, simultaneously with *Oct-4* downregulation. Stem cell lines express *Oct-4* only if undifferentiated. Cellular expression of *Oct-4* is believed to have the capacity for self-renewal [17]. *Oct-4* and *Bmi-1* overexpression in SP cells isolated from HXO-Rb44 were observed, which might reflect the property of self-renewal of SP cells. SP could efflux the DNA binding dye Hoechst 33342 via *ABCG2/MDR1* transporter which contribute to the chemotherapeutic resistance. Therefore, it is induced that SP might be a target for retinoblastoma therapy.

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