RESEARCH COMMUNICATION

Identification of a Cancer Stem-like Population in the Lewis Lung Cancer Cell Line

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Abstract

Objective: Although various human cancer stem cells (CSCs) have been defined, their applications are restricted to immunocompromised models. Developing a novel CSC model which could be used in immunocompetent or transgenic mice is essential for further understanding of the biomolecular characteristics of tumor stem cells. Therefore, in this study, we analyzed murine lung cancer cells for the presence of CSCs. Methods: Side population (SP) cells were isolated by fluorescence activated cell sorting, followed by serum-free medium (SFM) culture, using Lewis lung carcinoma cell (LLC) line. The self-renewal, differentiated progeny, chemosensitivity, and tumorigenic properties in SP and non-SP cells were investigated through in vitro culture and in vivo serial transplantation. Differential expression profiles of stem cell markers were examined by RT-PCR. Results: The SP cell fraction comprised 1.1% of the total LLC population. SP cells were available to grow in SFM, and had significantly enhanced capacity for cell proliferation and colony formation. They were also more resistant to cisplatin in comparison to non-SP cells, and displayed increased tumorigenic ability. Moreover, SP cells showed higher mRNA expression of Oct-4, ABCG2, and CD44. Conclusion: We identified SP cells from a murine lung carcinoma, which possess well-known characteristics of CSCs. Our study established a useful model that should allow investigation of the biological features and pharmacosensitivity of lung CSCs, both in vitro and in syngeneic immunocompetent or transgenic/knockout mice.

Keywords: Side population - non-small cell lung cancer - lewis lung cancer cell line - cancer stem cells

Asian Pacific J Cancer Prev, **13**, 761-766

Introduction

Despite of the great improvements in reducing mortality rates and improving prognosis over the past decades, lung cancer remains the leading cause of tumor-related death in the world. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, which includes adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Petersen and Petersen, 2001; Collins et al., 2007). The current treatments for NSCLC are largely inadequate, and the survival for those patients is often measured in months (Jemal et al., 2004). Deeper understanding of the biomolecular basis of NSCLC is necessary to provide more effective clinical treatments.

Recently, there was increasing evidence indicating that the maintenance and spreading of a variety of tumors was sustained by a small subset of cancer cells - cancer stem cells (CSCs). These cells possess ability to self-renew, unlimited proliferation potential, and capacity to generate differentiated cells which constitute the major tumor population (Reya et al., 2001). CSCs may be also responsible for the functional heterogeneity that is commonly observed in solid tumors, and they are more resistant to conventional chemotherapy and radiotherapy (Dalerba et al., 2007; Visvader and Lindeman, 2008). Thus, isolation and characterization of CSCs may offer new therapeutic strategies against malignant tumors.

Currently, human CSCs have been identified in a variety of tumor types (Al-Hajj et al., 2003; Matsui et al., 2004; Singh et al., 2004; Collins et al., 2005; Fang et al., 2005; O'Brien et al., 2007; Prince et al., 2007; Yang et al., 2008); however, they could not be used in cancer researches requiring immunocompetent or transgenic/knockout mouse models. Accordingly, in this study, we wished to establish a murine lung CSC model. Prior reports have suggested that side population (SP) cells separated from diverse cancer cells possess stem cell-like properties (Ho et al., 2007; Prince et al., 2007; Wang et al., 2007; Sung et al., 2008; Zhou et al., 2008). On the contrary, several recently published studies have shown that SP cells were not enriched for a stem-like self-renewal phenotype (Broadley et al., 2011). In our experiments, we isolated SP cells from Lewis lung carcinoma cell line using Hoechst 33342 vital dye staining and fluorescence activated cell sorting (FACS), followed by serum free medium (SFM) culture. The characteristics of lung cancer SP cells were

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further investigated, and we found that SP cells had enhanced ability of self renewal, extensive proliferation, and chemoresistance in vitro, and higher tumorigenicity in vivo. In addition, we observed significantly up-regulated transcriptions of stem cell markers (Oct-4, ABCG2, and CD44) in SP cells. Taken together, this study convincingly showed that isolated SP cells from murine lung cancers exhibited cancer stem-like cell characteristics. Our findings provided a useful model for in vitro and in vivo researches of lung CSCs, especially in immunocompetent or transgenic/knockout mouse.

Materials and Methods

Cell culture

Lewis lung cancer cells (LLCs) were purchased from American Type Culture Collection, and were cultured in Dulbecco's modified Eagle's medium - high glucouse (DMEM; Hyclone), with 10% fetal bovine serum (FBS; PAA), 100 U/ml streptomycin, and 100 U/ml penicillin, in humidified atmosphere of 95% air and 5% CO_2 at 37°C, according to the supplier's instruction.

FACS analysis

Cells were resuspended at 10^6 cells per ml in prewarmed DMEM containing 2% FBS and 10 mM HEPES (Sigma), and incubated for 90 min at 37 °C plus 5 μg/ml Hoechst 33342 (Sigma), with or without 50 μM Verapamil (Sigma), which could inhibit ABC transporters. After the incubation period, cells were washed and resuspended in ice-cold Hoechst Buffer (Hanks Balanced Saline Solution; Hyclone) supplemented with 2% FBS and 10 mM HEPES. 2 g/ml propidium iodide was added to the cells to gate viable cells. Finally, side population analyses and sorting were performed on the FACS (BD Biosciences). The Hoechst dye was excited with the UV laser at 375 nm and its wavelengths were detected using a 450/20 BP filter (Hoechst blue) and a 675 EFLP optical filter (Hoechst red).

Confocal fluorescence imaging

Tumor cells were first washed in PBS 3 times, and incubated with 300 μL Hoechst 33342 in the dark at 37 °C for 30 min. The cells were then washed in PBS twice, and were finally added with 400 μl PBS. The images of living cells were observed on a microscope (Leica TCS SP5 confocal).

SFM culture

For SFM selection, SP cells were cultured at a concentration of 10^4 per ml on a plate. Cells were grown in DMEM / F12 medium (Hyclone) containing 5 ug/ml insulin supplements (Sigma), with 20 ng/ml epidermal growth factor (EGF; Pepro Tech), 10 ng/ml basic fibroblast growth factor (bFGF; Pepro Tech), and 0.4% BSA (Sigma).

Cell proliferation assay

SP cells and non-SP cells were counted and plated into 96-well plates at a concentration of 10^3 cells per well, and in total five identical plates were prepared. The cells were incubated for 5 days, with one plate assayed for growth each day. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) at a concentration of 0.5 mg/ml was added into each well, and the medium was replaced with 200 ul dimethylsulfoxide (DMSO; Sigma) after 4 hours, and then vortexed for another 10 minutes. Absorbance was recorded at 570 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments).

Colony formation assay

For this assay, SP cells and non-SP cells were plated in DMEM – high glucose with 10% FBS on six-well plates, at a concentration of 10^2 cells per well, and were cultured for 2 weeks. The number of the clones (> 50 cells) was counted using a microscope.

Chemotherapy resistance analysis

For this analysis, 5×10^3 cells were first plated into 96-well plate. 24 hours later, Cisplatin was added into each well at different concentrations (0.25, 0.5, 1, 2, 4, 8, and 16 ug/ml), with PBS served as control. Cell growth was assessed by MTT assay after cultured for 3 days.

Tumor generation experiment

5-week old male C57BL/6 mice were obtained from The Animal Facility of Third Military Medical University (Chongqing, China). Serial dilutions of tumor cells (down to as low as 1×10^4 cells) were subcutaneously injected, and the size and weight of mice xenografts were measured, in order to evaluate the tumorigenic activity of lung cancer SP cells. Histology analysis by Hematoxylin and eosin staining were further performed.

RNA preparation and real-time PCR

Total RNA isolated from SP cells, non-SP cells, and differentiated cells were used for cDNA synthesis with RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. The sequences of primers: mouse Oct-4 sense primer sequence: 5'-GGAAGCCGACAACAATGAGA-3', and antisense primer sequence: 5'-AGAGCAGTGACGGGAACAGA-3'; mouse ABCG2 sense primer sequence: 5'-GTGCCACCATGTTCAACTTA-3', and antisense primer sequence: 5'-CTGCCAGAGTAGTGGAAGATT-3'; mouse CD44 sense primer sequence: 5'-GTACATCAGTCACAGACCTAC-3', and antisense primer sequence: 5'-CACCATTTCCTTGAGACTTGCT-3'; mouse β-actin sense primer sequence: 5'-GACCCAGATCATGTTTGAGACC-3', and antisense primer sequence: 5'-ATCTCCTTCTGCATCCTGTCAG-3'.

Statistical analysis

All quantified data were presented as mean ± SD. The differences between experimental groups were assessed by an unpaired t test (SPSS 18.0), and $p < 0.05$ was considered statistical significant.

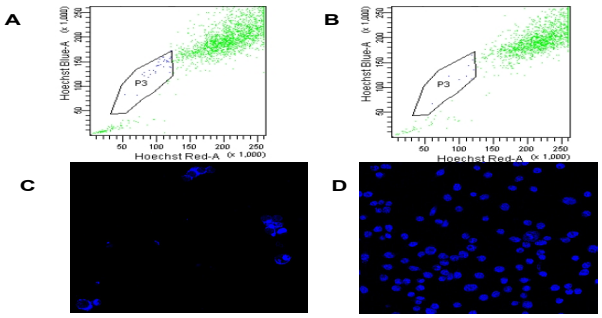

Figure 1. SP Analysis. (a and b) LLCs were labeled with Hoechst 33342, and then analyzed by flow cytometry with or without Verapamil. SP cells were gated and shown as a percentage of the whole cell population. (c and d) SP cells and non-SP cells were stained with Hoechst 33342, and observed using a confocal microscope (magnification × 400)

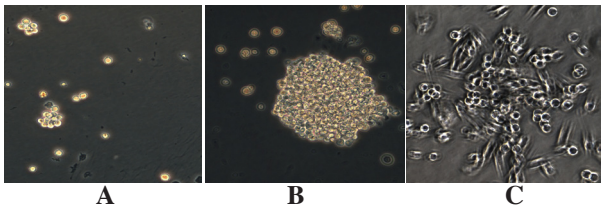

Figure 2. SP and Non-SP Cells were Cultured in SFM with EGF and bFGF. (a) SP cells were able to grow, and formation of multicellular spheres was observed at day 7 (magnification × 200). (b) The size of cell spheres had significantly increased after continuous subcultivation (magnification × 200). (c) Spherical clusters were cultured in complete medium, and acquired the typical morphologic characteristics of differentiated cells (magnification × 200)

Results

SP analysis

To examine whether LLCs contains SP cells, we performed a Hoechst dye exclusion assay. As most cells accumulate Hoechst 33342, SP cells could be isolated because of its distinct projection pattern by actively effluxing this dye. The SP cell fraction comprised 1.1% of the LLC population, which decreased significantly in the presence of the selective ABC transporter inhibitor Verapamil (Figure 1a, b). SP and non-SP cells were further isolated using FACS. Consistently, under a confocal microscope, we observed that the nucleus of SP cells could not be stained with Hoechst 33342 (Figure 1c, d).

SFM culture and sphere formation

Tumor cells were first cultured in SFM. SP cells were able to grow, and formation of non-adherent multicellular spheres in suspension was observed at day 7 (Figure 2a). After continuous subcultivation through 10 passages, the size of cell spheres had significantly increased, with more regular morphologic features (Figure 2b). We next examined the in vitro differentiation potential of spherical tumor cells. After cultured in complete medium, spherical clusters adhered to the plastic, and acquired the typical morphologic characteristics of differentiated cells (Figure 2c).

Proliferation and colony formation abilities were enhanced in Lewis lung cancer SP cells

To evaluate the proliferation ability of Lewis lung cancer SP cells, we performed a MTT analysis. The growth and survival of SP cells and non-SP cells were measured in complete medium. A significant increase in cell proliferation in SP cells was observed over a 5-day period ($p < 0.05$) (Figure 3a). In addition, through colony formation assay, we found a significant increase in colony formation ability compared with non-SP cells ($p < 0.05$) (Figure 3b).

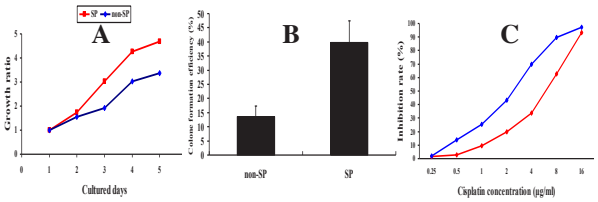

Figure 3. Growth Characteristics, Colony Formation, and Chemoresistance of SP Cells. (a) Growth curves of SP and non-SP cells. SP cells were associated with enhanced proliferative ability compared to non-SP cells ($p < 0.05$). (b and c) The ability of clone formation was significantly increased in SP cells ($p < 0.05$). (d) Growth curves of SP and non-SP cells treated with Cisplatin at various concentrations

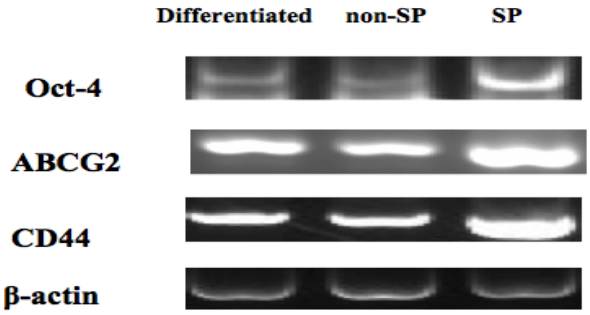

Figure 4. Expression of Stem Cell Markers. The results of RT-PCR analysis demonstrated elevated expressions of Oct-4, ABCG2, and CD44 genes in SP cells, as compared to non-SP and differentiated cells

Lewis lung cancer SP cells were resistant to conventional chemotherapy

Previous reports had shown that cancer stem cells were resistant to chemotherapy, which resulted in the poor clinical effects of conventional chemotherapeutic drugs. Therefore, we investigated the cytotoxic effects of the chemotherapeutic agents on Lewis lung cancer SP cells. Similarly, after treated with Cisplatin, SP cells showed enhanced drug resistance ability in all Cisplatin concentrations as compared to non-SP cells ($p < 0.05$) (Figure 3c).

Increased expressions of Oct-4, ABCG2 and CD44 in Lewis lung cancer SP cells

We next examined if the expression of Oct-4, which is a stem cell marker of embryonic stem cells and a biological marker of lung CSCs, might up-regulated in SP cells derived from Lewis lung carcinoma cell line. The results confirmed that there was a significantly up-regulated Oct-4 transcription in SP cells as compared to either non-SP cells or differentiated cells (Figure 4). Moreover, the expression of ABCG2 mRNA in SP cells was largely increased, which might contribute to the enhanced ability of chemoresistance in SP cells. Similarly, the expression

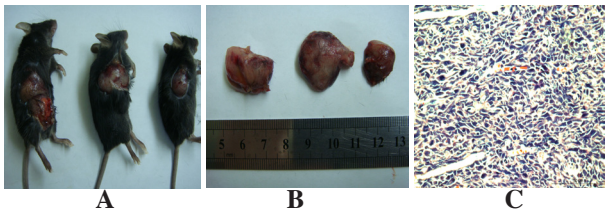

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Figure 5. In Vivo Growth Characteristic of SP Cells. (a and b)The injection of as low as 104 SP cells consistently generated xenografts in C57BL/6 mouse. (c) Histological analysis of the tumor xenografts was performed by HE staining (magnification × 400)

of CD44 was also positively associated with SP cells. Expression of CXCR4 was tested; interestingly, there were no significant differences between the two groups, which was different from the prior report (Nian et al., 2011). In addition, our findings demonstrated that Lewis lung cancer stem-like SP cells lost their phenotypic features upon differentiation.

Lewis lung cancer SP cells were tumorigenic in vivo

We next explored the tumorigenic potential of Lewis lung cancer SP cells in vivo. In this assay, SP cells or non-SP cells were subcutaneously injected into C57BL/6 mice separately, and tumor formation was evaluated 4 weeks after intervention. The injection of as low as 10^4 stem-like cells consistently generated xenografts, while non-SP cells with same cell number were unable to produce tumor in mice, which confirmed the tumorigenic ability of Lewis lung cancer stem-like cells (Figure 5a, b). The histological examine demonstrated that tumor xenografts formed by stem-like SP cells were equal to the typical Lewis lung cancer (Figure 5c).

Discussion

Cancer stem cells are a tiny subset population of tumor cells which retain the capacity to self-renew and continuously regenerate or add to the tumour. Furthermore, it has been reported that CSCs were resistant to chemotherapy and targeted therapy, which resulted in cancer relapse and metastasis. Therefore, it is widely believed that identification and characterization of CSCs may contribute significantly to the development of effective therapies.

SP cells refer to a unique cell population first identified in bone marrow cells by Goodell et al., which could efflux the DNA-binding dye Hoechst 33342 (Goodell et al., 1996). Thereafter, SP cells have been defined in multiple species and tissues. Previous studies have shown that SP cells from tumor cells possess the properties ascribed to cancer stem cells. SP analysis is now the most widely adopted technique for identification of CSC. Ho et al. (2007) demonstrated that side population in human lung cancers is enriched with stem-like cancer cells. Similarly, Sung et al. (2008) suggested that SP cells from A549 cell lines displayed cancer stem cell properties. However, several recent reports have indicated that side population cells were lack of stem cell characteristics, or were just associated with partly enriched CSCs (Mitsutake et al., 2007; Broadley et al., 2011).

In this study, we investigated whether murine lung cancer contains SP cells, and whether these cells possess CSCs' characteristics. SP cells were isolated using Hoechst staining and FACS, and further cultured in SFM, which has been shown to be very useful for CSC selection and expansion. SFM could maintain an undifferentiated stem cell status, because serum would lead to irreversible differentiation of stem cells (Dou and Gu, 2010). Moreover, the addition of EGF and bFGF was proved to be capable to induce proliferation of multipotent, self-renewing, and expandable stem cells (Reynolds and Weiss, 1996; Tropepe et al., 1999). Our in vitro experiments showed significantly higher growth ratio and enhanced colony formation ability in SP cells as compared with non-SP cells. Lung cancer SP cells displayed the capacity to generate differentiated lung cancer cells after cultured with complete medium, and were significantly more resistant to conventional chemotherapy in comparison to LLCs. In addition, SP cells had higher tumorigenic potential following subcutaneous injection into C57BL/6 mice. Thus, these isolated SP cells possessed the CSC properties and might provide a model for comparison with human lung cancer.

Stem cell marker is the key for detection and isolation of CSCs, which are the basis of CSC targeted therapy. Currently, surface markers for lung cancer CSCs are still poorly understood. Prior study has shown that only $CD133^+$ cells served as a tumor initiating population in lung cancer (Eramo et al., 2008). However, a recent report demonstrated that both the $CD133^+$ and $CD133^-$ lung cancer cells contain similar numbers of cancer stem cells, thus CD133 alone could not be used as a cell marker for lung cancer stem cells (Meng et al., 2009). In the present study, the expression of stem cell markers in isolated SP cells was explored, and we observed significantly up-regulated transcriptions of Oct-4 and ABCG2 genes in these cells. The transcriptional activator Oct-4, a member of the POU family which expressed in both embryonic and adult stem cells, is a key regulator of self-renewal and differentiation in stem cells. Previously published studies have shown that Oct-4 plays an important role in maintaining the characteristics of CSCs (Chiou et al., 2008). More recently, a new study demonstrated that the expression of Oct-4 in DDP-selected A549 cells was notably up-regulated, and it has been proved that drug selected cancer cells have the characteristics of CSCs (Teng et al., 2010). Therefore, Oct-4 is an important marker of CSCs. ABCG2/BCRP1, the second member of the ABC family of transporter proteins, is a molecular determinant of the side population phenotype. Elevated expression of ABCG2 has been observed in a number of putative CSCs from various cancers, which is correlated with the enhanced chemoresistance ability of CSCs (Seigel et al., 2005; Shi et al., 2008). ABCG2 is a well-known molecular marker useful for identifying and isolating CSCs. Similarly, we found that elevated expression of CD44 was also positively associated with SP cells. Interestingly, in contrary to the previously published report (Nian et al., 2011), there were no significant differences in CXCR4 expression between SP and non-SP cells. Thus, further researches screening for definitive markers of lung

CSCs are required.

Previous studies have shown significantly different chemosensitivity and radiosensitivity of the same tumors in immunocompetent and immunodeficient animals, emphasizing the inherent limitations of the immunocompromised models that they did not mimic the normal immunocompetent host (Luster and Leder, 1993; Jarm et al., 1997; Lukacs et al., 1999; Schreiber et al., 2002; Casares et al., 2005; Obeid et al., 2007). Compared to human CSCs, the murine lung cancer stem-like SP cells identified in our study could be applied in syngeneic immunocompetent mouse, which were very useful for either studying the underlying immunomodulating mechanism, or evaluating the treatment efficacy of novel chemotherapy and immunotherapy on the basis of lung CSCs. Furthermore, they could also be applied in transgenic/knockout mouse, which allow investigating the relevance of certain gene function in the host and the biological features of CSCs.

In conclusion, we isolated SP cells from Lewis lung cancer cell line by Hoechst staining and flow cytometry, followed by SFM selection. Our in vitro and in vivo experiments demonstrated that SP cells possessed the well-known CSC characteristics of self-regeneration, high proliferative capacity, and chemotherapy resistance. These findings may provide new insights for future CSC research and clinical anti-cancer therapy.

Acknowledgements

This work was supported by National High Technology Research and Development Program of China (No.2008AA02Z104).

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