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Regular paper

Clinical significance of cancer stem cell markers in lung carcinoma

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The identification of side population (SP) cells in several cancer studies has been proved to be involved in the treatment failure (chemotherapy) and tumor relapse. Here we have sorted 7% of side population (SP) cells from lung adenocarcinoma by Hoechst 33342 dye expulsion method. Further, the characterization of sorted SP cells showed cancer stem like properties such as transcriptional upregulation of stemness genes (*OCT-4, SOX2* and *NANOG*), ATP binding cassette (ABC) transporter protein (*ABCG2*) and enhanced level of stem cell surface markers such as CD133 and CD44. Therefore, the aforesaid properties of lung adenocarcinoma SP cells play a significant and functional role in tumor invasion, metastasis, chemotherapeutic drug resistance and tumor recurrence in lung cancer.

Keywords: ABC transporters, cancer stem cells, drug resistance, self-renewal, tumor recurrence.

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Abbreviations: ABC, TP binding cassette; AC, adenocarcinoma; CSCs, cancer stem cells; SP, side population

INTRODUCTION

Cells that are present in small groups within a tumor bulk and have the ability to initiate cancer growth and metastasis (Clarke *et al*., 2006; Visvader *et al*., 2008; Rosen *et al*., 2009;) are known as cancer stem cells (CSCs). Studies in several tumors visualized the properties of CSCs, namely overexpression of cell surface proteins, ability to differentiate into different cell types, transcriptional upregulation of stemness genes and increased cell proliferate rate (Zhou *et al*., 2001; Haraguchi *et al*., 2006; Wu *et al*., 2008; Schoenhals *et al*., 2009).

Further, the CSCs are capable of pumping out the several chemotherapeutic drugs or DNA targeting drugs and become resistant as they express various membrane spanning ATP binding cassette proteins (ABC) such as ABCB1, ABCG1 and BCRP1. CSCs can be sorted either by CD133 overexpression or FACs based Hoechst 33342 dye efflux with lower intensity of fluorescence signal (Goodell *et al*., 1996; Kondo *et al*., 2004; Hirschmann *et al*., 2004; Patrawala *et al*., 2005). These small groups of cells, which can efficiently pump the dye out of the cell owing to the overexpression of ABC transporter proteins, are designated as Side Population (SP) cells (Goodell *et al*., 1996; Ho *et al*., 2007; Seo *et al*., 2007; Das *et al*., 2008; Salcido *et al*., 2010). The cancer stem cells-like properties of SP cells might play a major role in chemotherapy failure and tumor recurrence (Gao *et al*., 1998; Wang *et al*., 2007; Bourguignon *et al*., 2008). However, the precise signaling pathways and molecular mechanisms behind the side population cells mediated tumorigenesis are still unknown.

Worldwide, lung adenocarcinoma is one of the leading cause for cancer related deaths in all genders. Upon diagnosis, the average life span of lung cancer patients is about 5 years (Parkin *et al*., 2005). Different studies in lung cancer described that the occurrence of CSCs is responsible for inhibiting the uptake of different therapeutic drugs which leads cancer cells to resist apoptosis (Singh *et al*., 2004; Seo *et al*., 2007; Salnikov *et al*., 2010; Singh *et al*., 2010; Yue *et al*., 2014). As like in other solid tumors, 'lung CSCs' are also highly clonogenic, capable of self-renewal, and mutlidrug apoptosis-resistant (Salnikov *et al*., 2010; Singh *et al*., 2010). Accordingly, the sorting and further functional characterization of lung cancer SP cells (Das *et al*., 2008) will definitely pave the way for developing novel anti-cancer drugs that could effectively target CSCs. Therefore, we have performed functional characterization of lung adenocarcinoma SP cells.

MATERIALS AND METHODS

Cancer samples and culture

All the procedures (sample collection and surgery) were followed according to the ethical rules approved by The Second Affiliated Hospital of Nantong University (License number: SEC013244). The cancer samples were obtained in agreement with patients oral consent. According to The Second Affiliated Hospital of Nantong University rules and regulations, we do not provide any data for the identification of patients in this manuscript.

Lung carcinoma samples were obtained during surgery. Sample details: n=15 (Male-8; female-7), region – adenocarcinoma (AC), stage: pT1pN2pMX(IIIA) and pT3pN2pMX(IIIA). The obtained tumor samples were washed several times and subjected to overnight incubation in DMEM–F12 medium with antibiotics such as penicillin and streptomycin. Tissues were enzymatically digested by using collagenase II (20 mg/mL) for 2 hours at 37°C. Recovered cells were cultured in T75 flask in DMEM–F12 medium (Gibco-Invitrogen) supplemented with 10% FBS and antibiotics (penicillin and streptomycin). Cells were incubated in 5% CO_2 at 37°C. Once

the cells became confluent, they were washed and transferred to the flask containing fresh medium.

Hoechst 33342 dye labeling

Study groups were assigned as Control: cells labelled with Hoechst dye alone (n=7) and Verapamil treatment (n=7): cells labelled with Hoechst dye and 75 μM of Verapamil drug as well. The propidium iodide staining and labelling of Hoechst 33342 dye are followed exactly as per (Liu *et al.*, 2015). The method of sorting of side population cells by flow cytometry method was exactly followed by the procedure as described previously (Hirschmann *et al.*, 2004; Liu *et al.*, 2015).

Biochemistry

Cell lysates extraction, protein preparation and separation by SDS-PAGE were performed as described previously (Yue *et al.*, 2014). Primary rabbit antibodies (rabbit anti-human ABCG2, CD133 and CD44) and secondary antibody anti-rabbit-IgG HRP conjugated (Santa Cruz) were used. Protein signals were detected by ECL kit with Biorad densitometer.

Real-time PCR

Using Reverse Transcriptase kit (Fermentas), the total RNA was extracted, complementary DNA was synthesized and subsequently RT-qPCR was performed in iCycler (Biorad). The sequences of the primers and the signal quantification were performed as described previously (Yue *et al.*, 2014). The PCR parameters applied for this study were as follows: initial denaturation, 93°C for 40 sec; followed by 40 cycles of denaturation, 93°C for 5 min.; annealing, 58°C for 45 sec, and extension, 72°C for 30s. Final extension was executed at 72°C for 10 min. The amplified PCR products were electrophoresed on 1.2% agarose gel and stained with ethidium bromide. The band intensity was measured using ImageJ software and the relative RNA expression level was adjusted with GAPDH level, which was used as housekeeping gene.

In vitro assays

Matrigel invasion assay. D six-well matrigel invasion chambers (BD Biosciences) were applied for this assay. 10^4 cells were plated per insert in serum free medium while the insert outlets contained DMEM-F12 supplemented with 10% FBS as chemoattractant. Cells were grown for 48 hours and further subjected to washing and wiping the top layer of Matrigel with cotton swabs. The membrane containing invading cells was stained with hematoxylin for 5 minutes and then visualized under a light microscope using a 60× objective for counting the number of cells invaded. The quantitative graph represents the average values from three independent experiments.

Cell proliferation assay. This experiment was performed in triplicate. Both SP and non-SP cells were seeded in a 96-well plate at a density of 2×10^6 cells/well. The optical density (OD) at 450 nm was measured to determine cell proliferation activity. This measurement was taken for 7 days continuously and the growth graph presented the average value of three different experiments. Differentiation and Matrigel assays protocols were followed as per (Ho *et al.*, 2007). Similarly, the sphere formation assay was carried out exactly by the protocol descried in the literature (Yanamoto *et al.*, 2011).

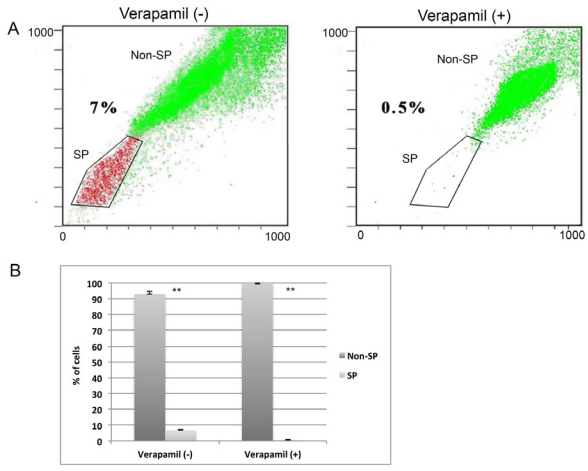

Figure 1. Side population cells in metastatic lung adenocarcinoma.
(A) Representative flow cytometry graph of dot plot analysis demonstrating the existence of SP cells which efflux Hoechst 33342 dye in the gated region. **(B)** Quantitative graph of dot plot analysis from the FACs profile. Error bar – standard deviation; (**P<0.01).

Statistical analysis

Student's t-test was performed for comparing the significance of differences between two different groups and the values denoted in the quantification charts are mean ± S.D. The values *P<0.05 and **P<0.01 are considered as significant.

RESULTS

Isolation of side population cells from lung adenocarcinoma

By Hoechst dye exclusion assay we have found that side population cells accounted for 7% (Fig. 1A) of lung adenocarcinoma samples and that their rate was significantly diminished to 0.5% by subsequent treatment of verapamil, which inhibits the function of ABC transporters (Fig. 1B). Hence, this result confirms the presence of ABC transporter proteins and its exclusive function of drug expulsion of SP cells.

Expression profile of SP and non-SP cells from lung adenocarcinoma

Expression profile of stemness and ABC transporter genes were analyzed by RT-qPCR. The transcriptional regulation of stemness and ABC transporter genes such as *OCT-4*, *NANOG*, *SOX2* and *ABCG2* were found to be significantly accelerated in SP cells (Fig. 2A). Subsequently, we have performed western blot analysis to monitor the expression pattern of cell surface proteins (CD133 and CD44) and ABCG2 as well. Interestingly, SP cells overexpressed CD133, CD44 and ABCG2 while the parental population cells did not (Fig. 2B). In addition, we observed that the number of total spheres formed by SP cells increased much faster and reached higher values than that formed by non-SP cells (Fig. 3A). The SP cells grew faster, formed tumor spheres more rapidly and more efficiently – spheres produced by SP cells were all augmented in size compared to non-SP cells (Fig. 3B). Therefore, the SP cells can be regarded as able to self-

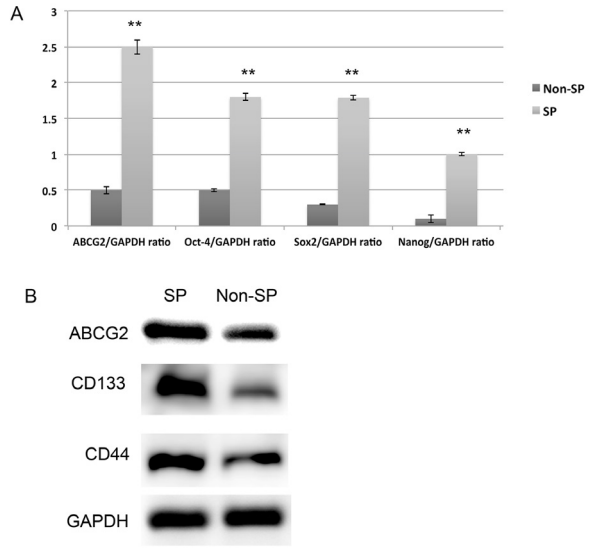

Figure 2. Expression profile of lung adenocarcinoma SP cells.
The RT-PCR evaluation (**A**) and western blots (**B**) showing the transcriptional upregulation of genes and expression pattern of surface proteins in SP cells, respectively. For both the analysis, the house keeping gene/protein used here is GAPDH. Error bar – standard deviation; (**P<0.01).

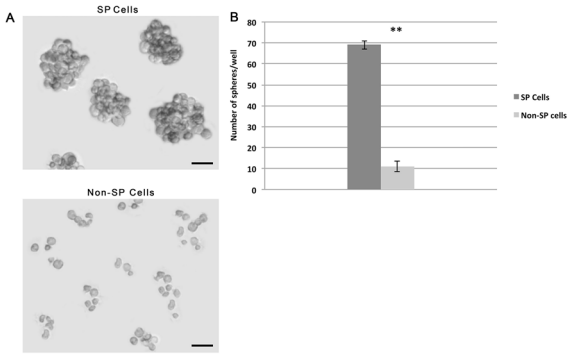

Figure 3. Efficiency of sphere formation in SP cells.
(**A**) Bright field microscopic pictures showing the size and morphology of the spheres generated by SP and parental non-SP cells. (**B**) Comparison of quantity of spheres formed by two different population of cells. Error bar – standard deviation; **P<0.01.

renew, highly tumorigenic, as well as crucial for tumor recurrence and metastasis.

SP cells are highly proliferative and resist DNA targeting drugs

To evaluate the *in vivo* tumorigenesis potential, we have performed *in vitro* cell proliferation assay for lung adenocarcinoma SP cells. The FACs sorted SP cells were proliferating much faster on the third day itself (D3) and reached 90% confluence on the seventh day. The growing rate and confluence abilities of non-SP cells were significantly lower than that of SP cells (Fig. 4A). Similarly, the cell differentiation assays revealed that the morphology of SP cells was remarkably changed. SP cells lost their original identity and were extended to form filamentous structures similar to ones present in fibroblasts (Fig. 4B). In addition, the SP cells became resistant to anti-cancer drugs and had prolonged cell survival rate once treated with drugs such as Paclitaxel, Etoposide,

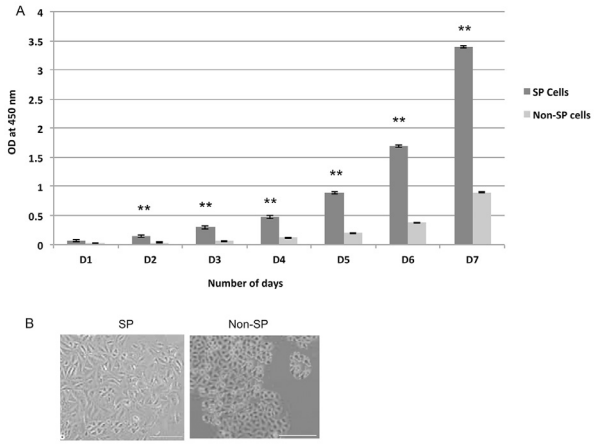

Figure 4. Cell proliferation and differentiation capacity of SP cells.
(**A**) Measurement of cell growth rate at optical density (OD) 450 $_{nm}$. (**B**) The FACs sorted SP cells underwent morphological changes and long filaments like in fibroblasts were formed. Error bar – standard deviation; **P<0.01.

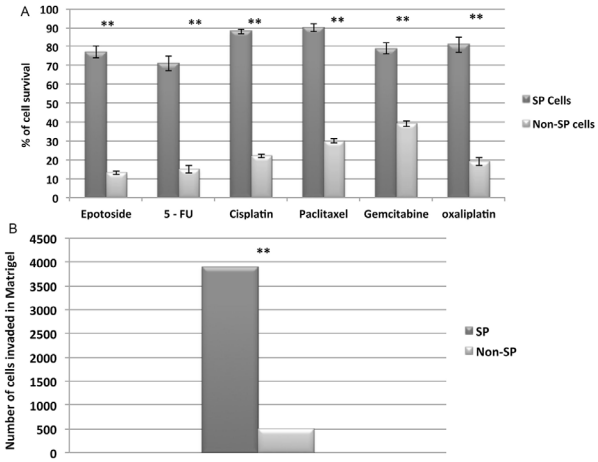

Figure 5. Chemoresistance and high invasion of SP cells.
(**A**) Quantification graph showing the cell survival rate of SP cells and parental non-SP cells after the DNA targeting drugs treatment. (**B**) Matrigel quantification showing the rate of invasiveness between SP and non-SP cells. Error bar – standard deviation; (**P<0.01).

Cisplatin, Gemcitabine, Oxaliplatin and 5-Flurouracil (5-FU) (Fig. 5A). The number of SP cells able to invade through the matrigel was significantly higher when compared to non-SP cells (Fig. 5B). The matrigel invasion assay data suggest SP cells do have higher invasion potential. Hence, all our findings suggest that lung adenocarcinoma SP cells play an essential role in the resistance to chemotherapeutic targets that might be involved in treatment failure, recurrence of tumor and invasion.

DISCUSSION

In the present study, we have identified a small, rare group of cancer initiating cells from lung adenocarcinoma samples called "side population (SP)" cells from lung adenocarcinoma samples, which showed the properties of CSCs. In line with previous findings (Ho *et al.*, 2007; Nakatsugawa *et al.*, 2011), our results also demonstrated the properties of SP cells which facilitate the en-

hanced cell proliferation rate, self-renewal capacity and metastasis of lung adenocarcinoma. We have found that approximately 7% of the cells from the tumor of lung adenocarcinoma samples were SP cells, which efflux the Hoechst 33342 dye by the over expression of ABCG2 pumps. Once treated with verapamil (ABCG2 inhibitor), the drug efflux activity was compromised and therefore the proportion of SP decreased to 0.5%. High expression profile of ABCG2 in SP cells contributes to severe resistance to several anti-cancer drugs and promotes prolonged cell survival as well. Therefore, these results confirm the presence and active role of ABC transporter protein in drug resistance of cancer stem cells which leads to the attenuation of apoptosis in cancer cells.

The FACs assorted SP cells were characterized phenotypically and functionally. They exhibited high cell proliferation rate and self-renewing capacity (Chiou *et al*., 2010; Nakatsugawa *et al*., 2011; Singh *et al*., 2012). They were highly capable of forming several-fold higher numbers of tumor spheres all of which were significantly bigger in size over the time course when compared to parental non-SP cells. The high potent sphere formation efficiency is regulated and driven by up-regulation of stemness genes such as *OCT-4*, *SOX*2 and *NANOG*, and is crucial for metastasis of lung adenocarcinoma patients (Salnikov *et al*., 2010; Nakatsugawa *et al*., 2011; Singh *et al*., 2012; Yue *et al*., 2014). Similar studies in lung cancers reported that the transcriptional upregulation of embryonic stemness genes is highly tumorigenic, may lead to epithelial mesenchymal transition (EMT) like features and might also involve high differentiation properties of SP cells. The possible explanation could be that *SOX2* upregulation is due to elevated EGFR signaling mechanism whereas the *OCT-4* and *NANOG* are upregulated by different independent pathways, which remains to be elucidated (Amann *et al*., 2005; Gazdar *et al*., 2008; Salnikov *et al*., 2010; Singh *et al*., 2012). Recent reports also showed that overexpression of *OCT-4* and *NANOG* increases the tumor initiating property of A549 cells (Chiou *et al*., 2010).

Further, the enhanced expression of exclusive markers for CSCs such as CD133 and CD44 in SP cells are responsible for chemo-and radiotherapy resistance (Honeth *et al*., 2008). These cell surface proteoglycans play a major role in cancer relapse, progression, and metastasis in several tumors (Honeth *et al*., 2008). Consequently, we identified that SP cells are highly invasive in matrigel assay. However, the complexity of mechanisms and receptor signaling pathways involved in SP cells function still needs to be studied in more detail. Taken together, our findings strengthen the hypothesis that elevated levels of ABC transporters and stemness genes promote stem like properties of SP cells which are significantly sufficient for tumor initiating, self-renewing, tumor invasion, metastasis, chemotherapy resistance and tumor recurrence. Further, more research is needed to elucidate the pathways and mechanisms involved in stemness properties and tumorigenic nature of SP cells, as this might lead to the development of a unique chemotherapeutic drug which eradicating CSCs to preclude the tumor recurrence.

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