Research Article

Notch 1 signaling pathway is the potential target of novel anti-cancer drugs for the treatment of human nasopharyngeal cancer

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Guo Fang Guan[1], De Jun Zhang[1], Ding Xin[1], Lian Ji Wen[1], Duo Jiao Yu[1], Yan Qing Lu[1], Yan Zhao[1] and Tie Jun Wang[2]

[1]Department of Otolaryngology, Head and Neck Surgery, The Second Hospital of Jilin University, Changchun 130 041, China; [2]Department of Radiation Oncology, The Second Hospital of Jilin University, Changchun 130 041, China.

Article Info

Abstract

Activation of Notch signaling pathway in cancer stem cells plays a crucial role in the regulation of self–renewal and maintenance of side population cells. In the present study, we have identified cancer stem like 2.7% side population cells from nasopharyngeal carcinoma samples whose prevalence was signifi-cantly reduced to 0.3% upon verapamil treatment. The protein level of Notch1 and Hes-1 are highly up-regulated in fluorescence-activated cell sorting puri-fied side population cells and thus leads to the elevated expression of stem cell surface proteins (Oct-4, Sox2 and Nanog), which are essential for side population cells self-renewal. In addition, these nasopharyngeal carcinoma side population cells are CD133 and CD44 positive and they possess enhan-ced cell proliferation rate, highly tumorgenic and invasive. Our findings suggest that Notch1 signaling is a potential target of novel anti-cancer drugs, which could efficiently target and eradicate the cancer stem cells.

Introduction

Nasopharyngeal carcinoma is one of the most common cancers originate from the upper region of pharynx, occurs in both men and women among worldwide. Despite recent advances in treatment strategy, still patients are suffering from therapy failure, tumor recurrence and metastasis.

According to the concept of cancer stem cell theory, tumors are heterogeneous and they contain small sub-population of cells, which have indefinite life span, are termed as cancer stem cells. These cancer stem cells play a major role in tumorigenesis and also responsible for resistance to cancer therapies and tumor recurrence (Kawasaki et al., 2008). Several studies showed that cancer stem cells are highly self-renewal, possess differentiation potential, express stem cell surface pro-teins (e.g., CD24, CD44 and CD133), chemo- and apoptosis resistance. However, the molecular mecha-nism and signaling pathways behind the cancer stem cells mediated tumorigenesis is not clear. So far, studies in different cancers showed that Wnt/β-catenin and Notch signaling play a crucial role in self-renewal and maintenance of cancer stem cells. Notch signaling is highly activated in cancer stem cells and thus leads to the over expression of stem cell proteins which contri-buted to rapid propagation of cancer stem cells (Pannuti et al., 2010; Wang et al., 2005; Zhang et al., 2010; Teng et al., 2010; Bao et al., 2006). Studies also demonstrated in nasopharyngeal carcinoma cell line that inactivation of Notch signaling leads to increased sensitivity of nasopharyngeal carcinoma side popula-tion cells towards to radio- and chemotherapy resis-tance (Phillips et al., 2006; Woodward et al., 2007; Yu et al., 2012). Therefore, identification of the factors and signaling pathways involved cancer stem cells media-ted therapy failure and tumor relapse is a crucial one to improve the treatment regimen.

Side population cells have been identified and charac-terized in several solid tumors, including glioblastoma, gastrointestinal system tumor, head and neck squa-mous carcinoma, hepatocellular cell lines, and also in primary cultures from neuroblastoma patients (Kondo et al., 2004; Haraguchi et al., 2006; Hirschmann-Jax et al., 2004; Chiba et al., 2006; Chen et al., 2006). However, the studies concerning the side population cells of human nasopharyngeal carcinoma is very less. There-fore, exploration of cancer stem like side population cells from nasopharyngeal carcinoma and targeting its key signaling pathways will be worth to develop new anti-cancer drugs to eliminate the minimal residual disease. The presence of side population cells from nasopharyngeal carcinoma for activation of Notch signaling pathways was investigated.

Materials and Methods

Sample collection and cell culture

Nasopharyngeal carci-noma samples were collected at the time of surgery according to the ethical approval. The details of samples: number of patients (n=15); stage –III-IV; region – infratemporal. The cancer tissues were washed extensively in PBS solution containing anti-biotics and incubated overnight in DMEM/F12 (GIBCO) containing penicillin (500 U/mL), streptomy-cin (500 µg/mL), and amphotericin B (1.25 µg/mL) (GIBCO). Enzymatic digestion was performed using collagenase (1.5 mg/mL) (GIBCO) and hyaluronidase (20 µg/mL) in PBS for 1 hour. Cells were cultured in DMEM with 10% FBS, supplemented with antibiotics and maintain in T-75 flasks at 37°C in a humidified 5% CO_2 and 95%air atmosphere. Cells on becoming 90% confluent are removed from culture flask using Trypsin-EDTA (0.25% - 53 mM EDTA) washed, cells suspended in 10% DMEM. Cell count was taken using hemo-cytometer.

Labeling with Hoechst 33342

Study groups

Control – cells + Hoechst 33342 (n=3); Drug treated – cells + verapamil + Hoechst 33342 (n=3). Cells in staining medium (approx 10^6 cells/mL of 10% DMEM) are labeled with Hoechst 33342 stock (sigma)-bis-benzimide (5 µL/mL) either with dye alone or in combination with drug (verapamil - 0.8 µL/mL). The cells were mixed and placed in water bath at 37°C for 90 minutes exactly. After 90 min, cells were spin down (2,000 rpm for 10 min at 4°C) and resuspended in 500 µL of HBSS containing 10 mM HEPES. Finally, counter stained the cells with PI (propidium iodide) 2 µg/mL sample at 4°C. Cells were filtered through a 50 µm nylon mesh (BD) to remove cell clumps into labeled FACS tubes. Separate tubes with medium (10% DMEM) were kept for sterile sorting of side population cells and

main population cells. The cells were sorted using a flow cytometer (FACS Aria II; BD Biosciences; Franklin Lakes, NJ, USA). The Hoechst 33342 dye was excited at 355 nm and its dual-wavelength fluorescence was analyzed (blue, 450 nm; red, 675 nm).

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

Total RNA was extracted and complementary DNA was prepared using reverse transcriptase kit (Fer-mentas). 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). Reactions were carried out in triplicate. The primer sequence used fir Notch1 and Hes-1 and methods were used as described previously (Yu et al., 2011). The primer sequences for other gene (Guo et al., 2012) were used 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); Sox2 – (F: CAC ACT GCC CCT CTC ACA CAT and R: CAT TTC CCT CGT TTT TCT TTG AA); Nanog – (F: CCA ACA TCC TGA ACC TCA GCT AC and R: GCC TTC TGC GTC ACA CCA TT); GAPDH – (F: TCT GCT CCT CCT GTT CGA CA and R: AAA AGC AGC CCT GGT GAC C). GAPDH is used as a house keeping genes. The PCR parameters used to set PCR reactions are: Initial denaturation -95°C for 15 sec; annealing -58°C for 45 sec; extension -60°C for 30-45 sec; cycles – 35. The data represented in the graph are the average values of three independent experiments.

Western blot analysis

For Western blot analysis, proteins were extracted from the side population and non-side population cells, and protein concentration was determined using the Bradford assay. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to a membrane, the gels were treated with the primary antibodies (rabbit anti-human) for Notch1 intracellular domain (NICD) antibody (1:5000), ABCG2 (1:10000), GAPDH (1:10000) and mouse anti-human Hes-1 antibody (1:500). Subsequently, the cells were treated with the secondary antibody (goat anti-rabbit IgG with alkaline phosphatase markers) and a chemiluminescence reagent. Blots were detected and scanned by using a densitometer (Biorad GS-710). Equal concentration of the proteins was loaded per lane and GAPDH is used as a loading control.

Clone formation efficiency

The sorted side population and non-side population cells were placed at a density of 1,000 cells/mL resuspended in tumor sphere medi-um consisting of serum-free 1:1 mixture of Ham's F-12/DMEM, N2 supplement, 10 ng/mL human recombi-nant bFGF, and 10 ng/mL EGF and subsequently cultured in ultra-low

attachment plates for about 2 weeks. SP and non-SP cells sorted 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 average values of three independent experiments.

Immunofluorescent staining

The sorted side population cells and main population cells were seeded in 35 mm culture plates (approximately 100 µL). Kept in incubi-tor for 3 hours and 1 mL of medium (10% DMEM) was given. After overnight incubation, the cells were rinsed with PBS and fixed in 4% PFA (Para formaldehyde) in 1x PBS, for 5 min at 4°C. After washing with 1x PBS, cells were blocked with 1% BSA-TBS with RNase (conc: 10 µL/1000 µL of 3% BSA-TBS). After 1 hour incubation at room temperature, rinsed with PBS and primary antibody in 1% BSA-TBS was given (dilution: 1:100; 2 µL/200 µL), incubated overnight at 4^0C. After washing with 1x PBS cells were incubated with secondary antibody conjugated with FITC (dilution: 1:100 in 1% BSA-TBS), at room temperature for 1 hour. Again PBS wash was given and propidium iodide was added (1 µL/200 µL of PBS). Then cells were viewed under confocal laser scanning microscope (Leica TCS). Image analysis and figures were prepared using Adobe Photoshop CS4.

In vitro proliferation activity

The sorted side population and non-side population cells were seeded in a 96-well plate at 2×10^6 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 (10 µL) and incubated in CO_2 incubator for 2-3 hours. The optical density 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 (Ho et al., 2007). After 16-18 days of cell sorting, cells were cultured in normal RPMI 1640 and they were subjected to determine the differentiation ability of the two subpopulations under the microscope.

Martigel invasion assay

Cellular invasiveness of side population and non-side population cells was determined by using six-well Matrigel invasion chambers (BD Biosciences Discovery Labware). Cells were seeded in serum free medium at a density of 2×10^5 per insert. Outer wells were filled with DMEM containing 5% FBS as chemoattractant and incubated at 37°C for 48 hours. Subsequently, the non-invading cells were washed by swabbing top layer of Matrigel with Q-tip (Ho et al., 2007). Membrane

containing invading cells was stained with hematoxylin for 3 min, washed, and mounted on slides. The entire membrane with invading cells was counted under light microscope at 40× objective. The values represented in the graph are the average value of three independent experiments.

Cell resistance assay

Approximately 1×10^3 cells/plate were cultured in 96-well plates and treated with the chemotherapeutic drugs at the following concentrations: 5-fluorouracil (5-FU) with a concentration of 10 µg/mL, gemcitabine 250 mM, oxaliplatin (100 mM), paclitaxel 30 ng/mL, cisplatin 5 mg/mL, etoposide 10 mg/mL and oxaliplatin (2 µg/mL 100). 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. The values presented in the graph are the average of three independent experiments.

Statistical analysis

One-way analysis of variance (ANOVA) and student t-test was performed to determine the significant difference between the treatment and control groups. A probability level of $p<0.05$ or 0.01 was considered as statistically significance. The difference was considered statistically significant at $p<0.05$ or $p<0.01$.

Results

The distinct side population cell population of about 2.7% (Figure 1A, gated region) found towards the side population-violet region of the dot plot of the FACS profile. After treatment with verapamil (ABC transporter inhibitor), the percentage of side population cells was significantly reduced to 0.3% (Figure 1B, gated region). These data clearly indicates that the property of dye exclusion by side population cells actively involves ABC transporter proteins and that was confirmed by treatment of side population cells with verapamil where the side population cells were significantly diminished (Figure 1C). Therefore, the side population cells are highly resistant to drug uptake due to the over expression of ABC transporter proteins (Figure 1D and E).

The Western blot analysis revealed that NCID and Hes-1 are highly expressed in side population cells than non-side population cells (Figure 2A). Further, by RT-PCR. The relative mRNA expression of Notch1 and Hes-1 were significantly higher in side population cells (Figure 2B).

As shown in the graph (Figure 3A), the side population cells can able to generate more number of spheres rapidly than non-side population cells. The size of the
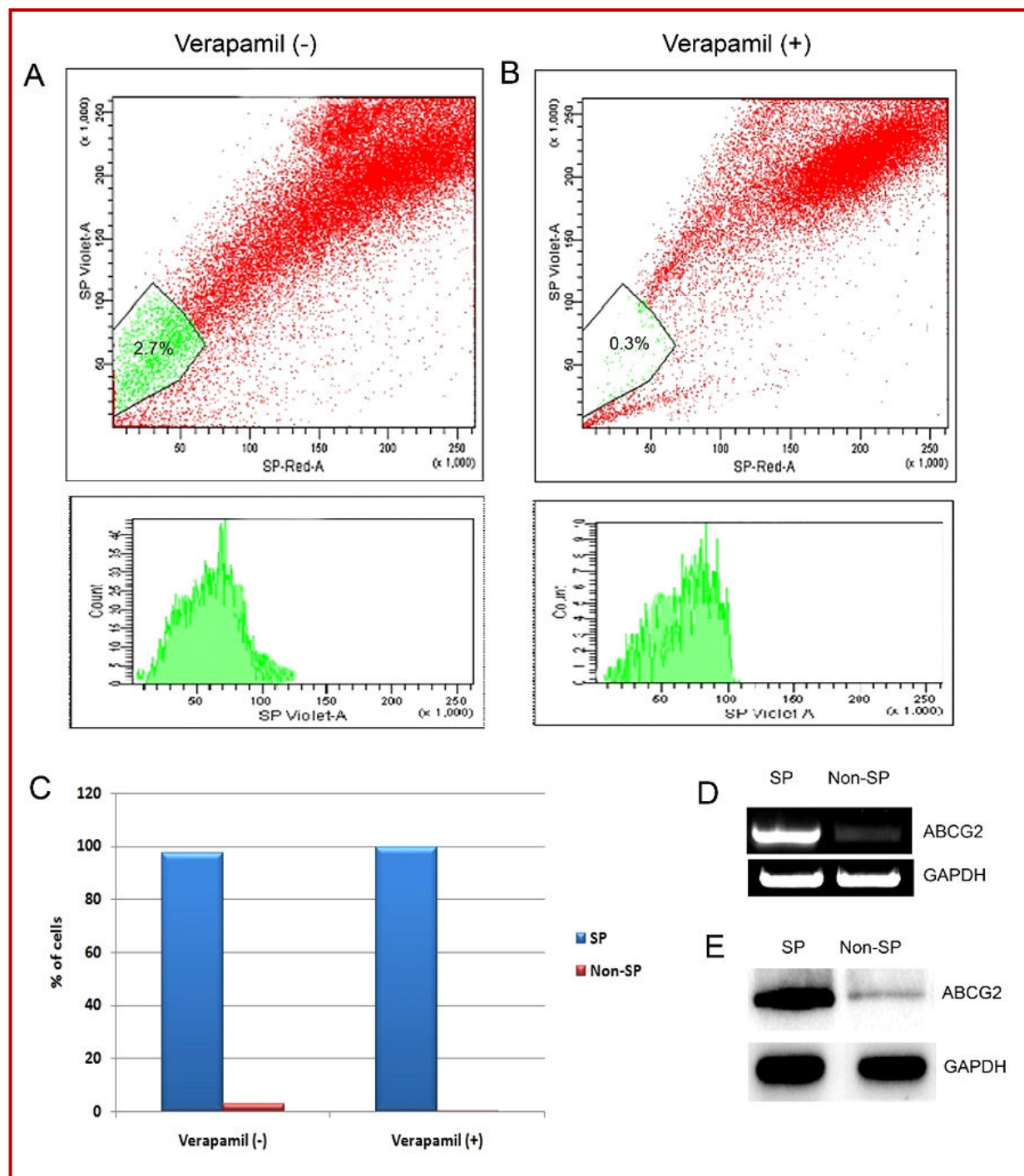
Figure 1: Analysis of side population (SP) cells in nasopharyngeal carcinoma (NPC). A. Cells were stained using Hoechst 33342 dye and analyzed using flow cytometry. SP cells are outlined (gated population) and also shown as a percentage of the total cell population. B. The percentage of SP cells was significantly reduced to o.3% in the presence of verapamil. C. Graphical representation for presence of SP cells with and without verapamil treatment. Quantitative graph made from the dot blot analysis by FACS. D. RT-PCR and western blot (E) analysis showing elevated expression of ABCG2 in SP cells

tumor spheres was gradually increased with time (data not shown). Thus, confirms that side population cells were highly self-renewal. The expression of Oct-4, Nanog and Sox2 were found to be up-regulated in SP cells (Figure 3B). The immunofluoresence assay revealed that these side population cells were highly positive to stem cell proteins CD133 and CD44 than non-side population cells (Figure 3C).
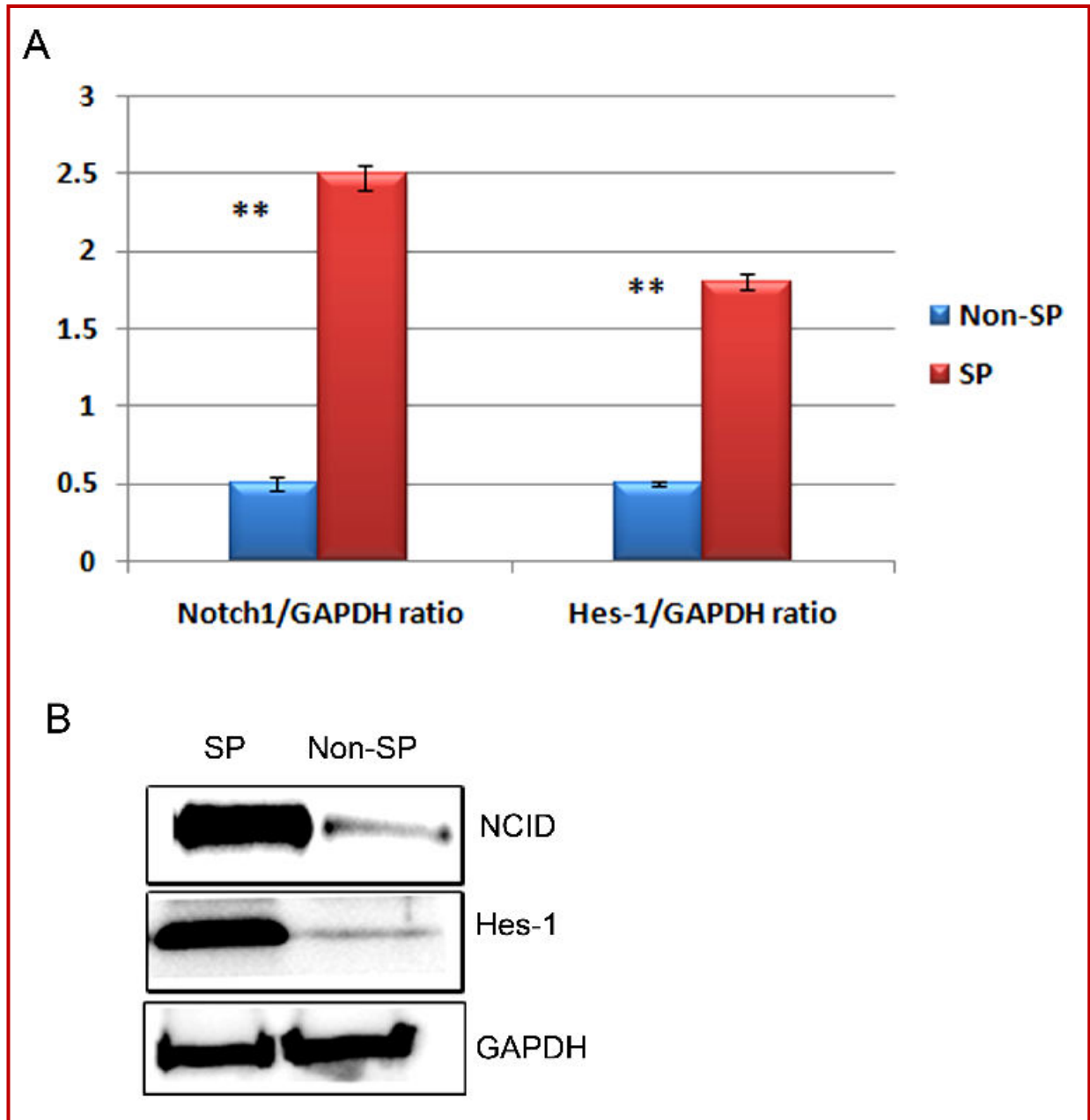
Figure 2: Notch signaling activation in side population (SP) cells. A. The quantification graph from RT-PCR analysis showing the elevated mRNA expression of Notch1 and Hes-1 in SP cells. B. Western blot showing the increased protein expression of NCID and Hes-1 in SP cells than non-SP cells. Equal concentration of proteins was loaded per lane. GAPDH was used as a house keeping gene and loading control

The sorted side population cells (Figure 4A) underwent rapid cell proliferation starting from the 3rd day itself and become more confluent on 7th day. However, the growth rates of non-side population cells are significantly low when compared side population cells (Figure 4A). Similarly, the side population cell morphology was changed remarkably and started to lost their normal appearance after day 5, and after 7 days side population cells began to extend fibroblast-like filaments. These striking features were not observed significantly in non-side population cells (Figure 4B).

In vitro matrigel invasion assay revealed that FACs purified side population cells are significantly more invasive than non-side population cells (Figure 5A). Subsequently, the side population cells showed increased resistance to multi-drugs such as etoposide, gemcitabine, 5-flurouracil, cisplatin, paclitaxel and oxaliplatin. Upon treatment with these drugs, the survival rate of side population cells were significantly higher than non-side population cells (Figure 5B) due to over expression of ABCG2 and also possibly by reduced apoptosis.
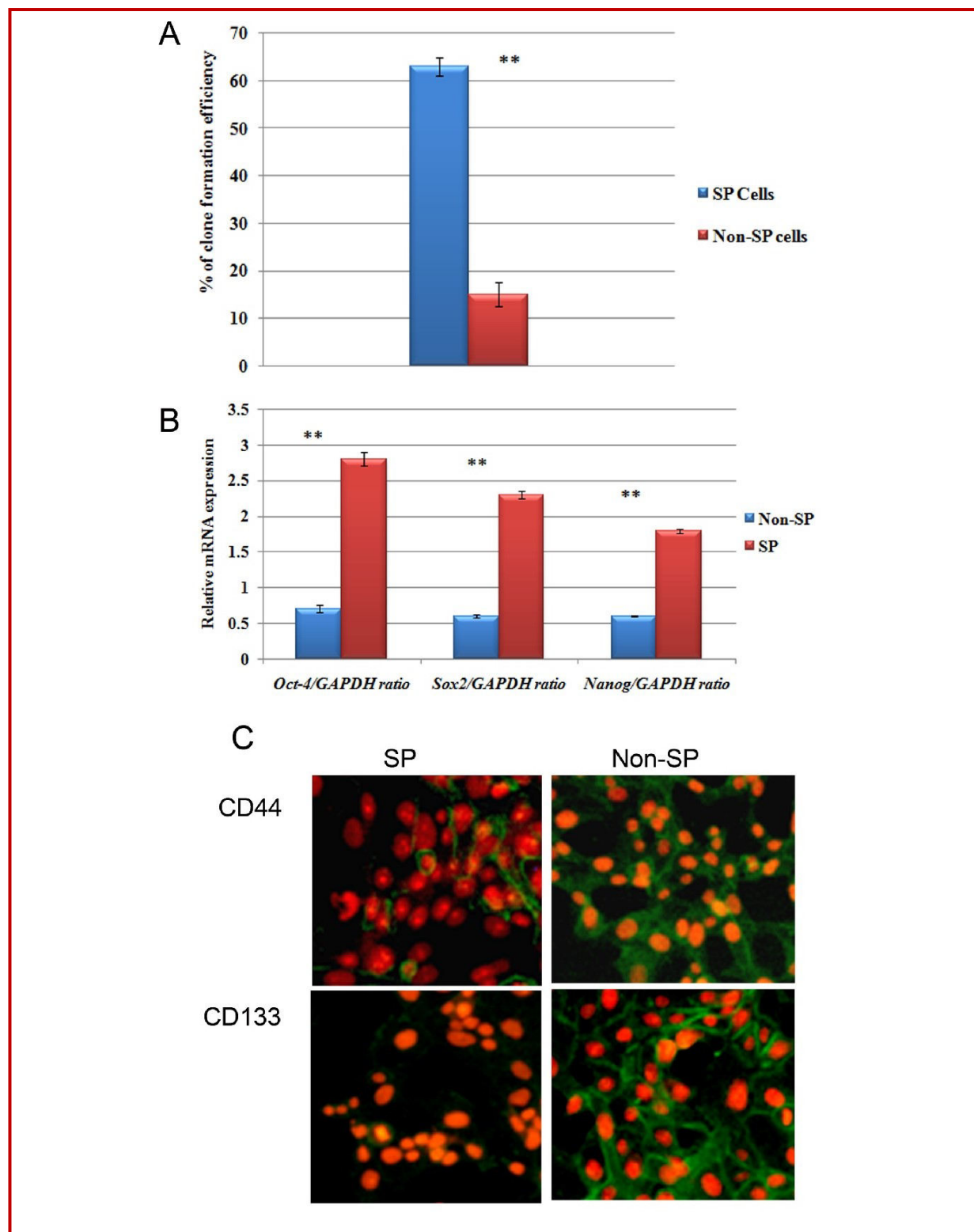
Figure 3: Side population (SP) cells were more tumorigenic and self-renewal. A. Clone formation efficiency of SP cells. The total number tumor spheres generated by NPC SP cells are significantly higher than non-SP cells. B. The quantification graph of RT-PCR analysis showing that the relative mRNA expression of Oct-4, Sox2 and Nanog are significantly higher in SP cells compared to non-SP cells. C. Fluorescence microscopic analysis of stem cell surface markers. SP cells (almost entire population) showed fluorescence positive for the presence of CD133 and CD44 whereas this fluorescence are not enriched in non-SP. The bar represents the standard deviation (**p< 0.01).
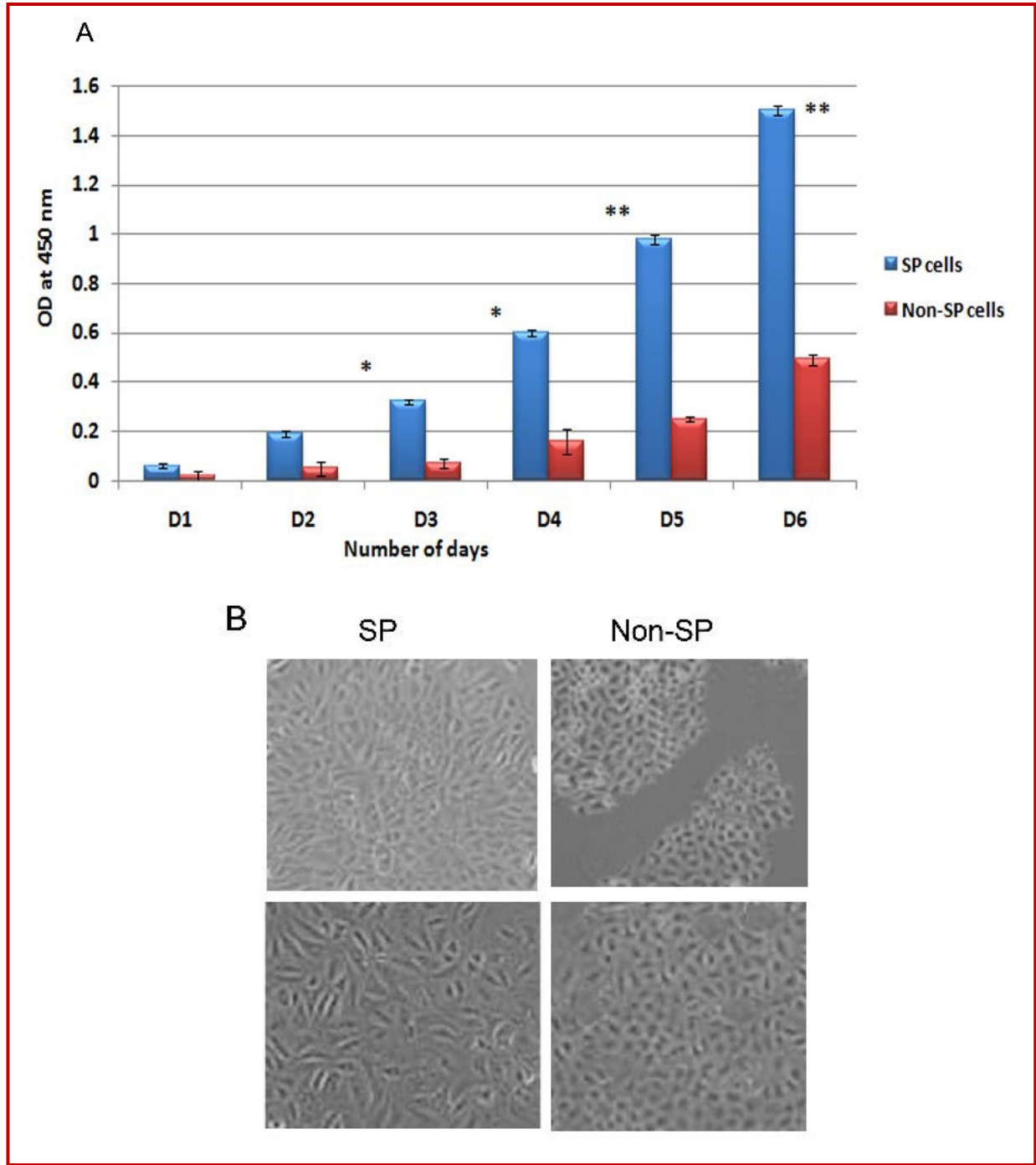
Figure 4: Side population (SP) cells are high potent of proliferation and differentiation. A. Cell proliferation rate of SP cells are significantly higher than and non-SP cells. The x-axis represents time, while the y-axis indicates the corresponding optical density (OD) value at 450 nm. B. The cell morphology of FACs sorted SP cells changes rapidly after culturing of 5 days. Later (day 7), the SP cells developing many long filaments resembling fibroblast but not in non-SP cells. The bar represents the standard deviation (*$p<0.05$; **$p<0.01$)

Discussion

Cancer stem cells are the major cause for chemotherapy resistance, tumor relapse and metastasis. Accumulating evidences suggested that identification and characterization of rare, small population of cancer stem like side population cells helps to target the cancer stem cells in order to eliminate the tumor refractory. Side population cells of NPC cell line are highly
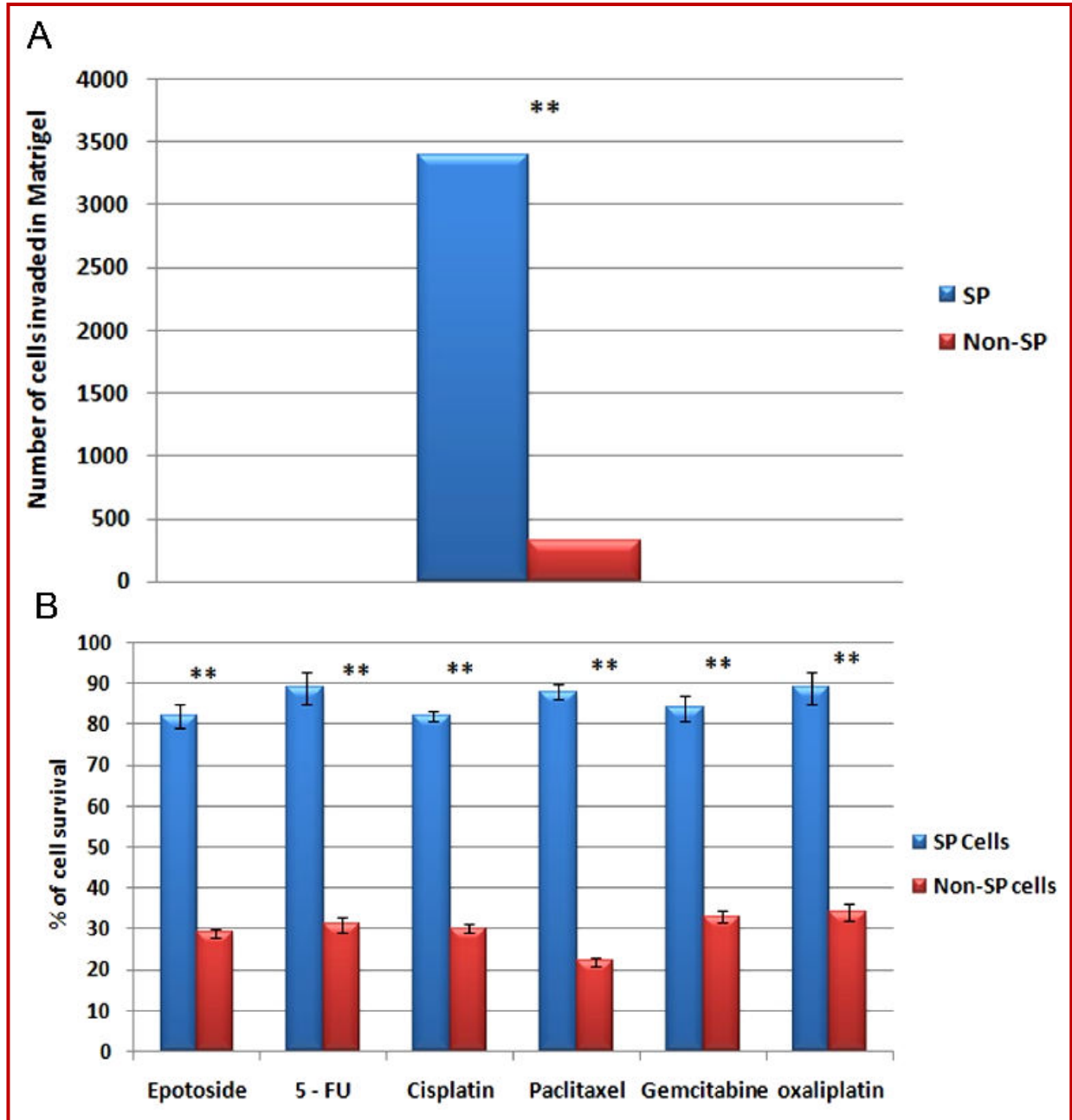
Figure 5: Side population (SP) cells are chemoresistance and highly invasive. A. Comparison of cell survival rate of side population (SP) cells and non-SP cells after treatment with the drugs such as etoposide, gemcitabine, 5-fluorouracil (5-FU), cisplatin, paclitaxel and oxaliplatin. The SP showed increased resistance to these drugs and their survival rate are higher (more than 80%) than non-SP cells. B. SP cells invasiveness as measured by the Matrigel assay. SP and non-SP cells (4×10^5) were seeded and incubated for 72 hours. Graph representing the number of cells invaded across the membrane. The bar represents the standard deviation, $**p<0.01.*p<0.05$

proliferative, self-renewal, differentiation and tumorigenic (Wang et al., 2007) and therefore they are considered as enriched side population. The present study showed the presence of side population cells in nasopharyngeal carcinoma samples and they strongly resist several chemotherapeutic drugs due to enhanced expression of ABC transporter ABCG2 and possibly by reduced rate of apoptosis. The multidrug resistance

phenomenon shown by these cells enables it to survive the chemotherapy regimen and hence result in poor prognosis in patients suffering from NPC. Further, this study showed that side population cells are highly self-renewal, enhanced cell proliferation rate, tumorigenic and invasive. These side population cells showed elevated mRNA levels of oct-4, sox2 and nanog. In addition, side population cells showed enhanced immuno-

fluorescence for CD44 and CD133 staining. Previous studies in different types of solid tumors showed that enriched CD147, CD133 on tumor cells induces tumor invasion by over stimulating and stimulates production of several matrix metalloproteinases by adjacent stromal cells (Guo et al., 2000). Similarly, CD44 also proved in the study of head and neck squamous cell carcinomas (HNSCC) that CD44+ cells are highly tumorigenic and they are able to propagate tumor formation in mice, whereas CD44− cells were not (Yanamoto et al., 2007). Notch signaling pathway has been shown to be involved in the self-renewal properties and they are crucial for maintenance of NPC CSCs (Yu et al., 2012). Further, in the Notch depleted cells, the SP cells are more sensitive to chemotherapies and the rate of apoptosis was enhanced. In addition, the self-renewal and high proliferation rate was compromised in the Notch depleted cells. In consistence with the previous studies (Zhang et al., 2004; Fan et al., 2008; Sikandar et al., 2010; Zhen et al., 2010), we also demonstrated that sorted NPC SP cells possess elevated Notch1 pathways as the expression level of NCID, Notch1 and Hes-1 are up-regulated in side population cells. Simultaneously, the activation of Notch1 pathways leads to the over expression of stem cell genes such as Oct-4, Sox2 and Nanong and thus essential for self-renewal property of SP cells. Therefore, Notch1 plays a pivotal role in tumorigenesis, self-renewal and maintenance of side population cells in addition to stem cell maintenance and differentiation during embryonic and adult development (Artavanis et al., 1999; Lino et al., 2010). Similar to Notch1, it was shown that Hedgehog (Hh) and Wnt, are the other key factors involved in the regulation of self-renewal of cancer stem cells in different cancers (Kawasaki et al., 2008). Most likely, all these pathways collectively elevated and function together in side population cells. Therefore, the good therapeutic approach is to design novel anti-cancer drugs that could able to inhibit all these critical pathways essential for the regulation of cancer stem cells in nasopharyngeal carcinoma.

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Author Info
Tie Jun Wang (Principal contact)
e-mail: wangtiejun2218@hotmail.com