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Full Length Research Paper

Epithelial-mesenchymal transition is associated with increased invasiveness of side population cells from hepatoma SMMC-7721 cells

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To study the *in vitro* invasiveness of side population (SP), cells purified from hepatoma SMMC-7721 cells and the expressions of epithelial-mesenchymal transition (EMT) markers of SP cells and non-SP (NSP) cells, fluorescence-activated cell sorting (FACS) was used to sort SP cells and NSP cells from SMMC-7721 cells. The *in vitro* invasiveness of both subpopulations was tested by Transwell chamber assay. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot were applied to evaluate the expressions of Vimentin, E-cadherin, N-cadherin and Snail in SP cells and NSP cells, respectively. In Transwell invasion assay, the number of SP cells (66.3 ± 7.4) penetrating the basilar membrane was significantly higher than that of NSP cells (27.5 ± 6.5) ($P < 0.01$). The mRNA expressions of Vimentin, N-cadherin and Snail was significantly increased in SP cells (9.527-fold, 6.834-fold and 8.173-fold, respectively) when compared with that in NSP cells ($P < 0.01$). In contrast, the decrease of E-cadherin mRNA expression in SP cells (5.353-fold) was markedly higher than that in NSP cells ($P<0.01$). The protein expressions of E-cadherin, Vimentin, N-cadherin and Snail in SP cells were markedly different from those in NSP cells (0.174 ± 0.014 and 0.935 ± 0.012; 1.117 ± 0.012 and 0.314±0.011; 0.975 ± 0.017 and 0.179 ± 0.013; 0.917 ± 0.014 and 0.202 ± 0.013; $P < 0.01$). The results suggest that SP cells from hepatoma SMMC-7721 cells are highly invasive *in vitro* which may be related to the changes in expressions of EMT markers.

Key words: SMMC-7721, cancer stem cells, side population cells, invasion, epithelial-mesenchymal transition.

INTRODUCTION

Invasion and metastasis are the most important biological characteristics of cancer and also the leading causes of death in cancer patients. Therefore, to illustrate the molecular mechanisms underlying the invasion and metastasis of cancers has become one of the focuses in cancer research. Cancer stem cells (CSCs) are a small population among cancer cells and possess the ability to self-renew and differentiate into a variety of cell population making up the cancer mass (Reya et al., 2001). CSCs are not only associated with cancer initiation and growth, but play a crucial role in the cancer metastasis. Matrix invasion is one of the earliest steps in the metastasis in which epithelial-mesenchymal transition (EMT) has been found to play critical roles. EMT is a biologic process that allows polarized epithelial cells, which are adherent to the basement membrane via its basal surface and undergo multiple biochemical changes. These changes enable these cells to possess a phenotype of

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Abbreviations: SP, Side population; **EMT,** epithelial-mesenchymal transition; **FACS,** fluorescence-activated cell sorting; **NSP,** non-SP; **RT-PCR,** reverse transcriptase-polymerase chain reaction;

mesenchymal cells, which includes enhanced capacity of migration and invasiveness, elevated resistance to apoptosis and markedly increased production of extracellular matrix (ECM) (Kalluri and Neilson., 2003). EMT was originally identified as a crucial differentiation and morphogenetic physiological process during embryogenesis (Thiery et al., 2006). Klarmann et al. (2009) found that a subpopulation of $CD44^+$ CSC-like cells invaded the Matrigel through EMT, while $CD44^-$ cells were non-invasive. Furthermore, the genomic profile of the invasive cells closely resembles that of $CD44^+CD24^-$ prostate CSCs and shows evidence of increased Hedgehog signaling. Finally, invasive cells from DU145 cells and primary prostate cancer cells are highly tumorigenic in NOD/SCID mice when compared with non-invasive cells. All these results strongly suggest that CSCs are highly invasive and metastatic, which are related to EMT.

Previous studies have shown that adult stem cells can be identified by a side population (SP) phenotype. SP cells were originally reported as an enriched population of murine hematopoietic stem cells identified using Hoechst 33342 dying and fluorescence-activated cell sorting (FACS) (Goodell et al., 1996). Recently, SP cells have been identified and isolated from various solid tumors, and highly express stem cell markers (Wu et al., 2008; Haraguchi et al., 2006a). Moreover, SP cells from cancers have been reported to have stem cell-like functions, such as resistance to anticancer drugs, clonogenic ability, and tumorigenicity. In other words, SP cells from cancers are promising CSCs (Haraguchi et al., 2006b; Song et al., 2010; Inowa et al., 2010). In the present study, SP cells were purified from hepatoma SMMC-7721 cells, and the *in vitro* invasiveness and the expressions of EMT markers were investigated.

MATERIALS AND METHODS

Cell culture

Human hepatoma SMMC-7721 cell line (Chinese Academy of Science, Shanghai, China) was maintained in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/mL penicillin G and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. Cells were passaged every 3~4 d.

Fluorescence-activated cell sorting (FACS) for SP cells

SMMC-7721 cells in a logarithmic growth phase were analyzed using FACS (Ma et al., 2009). Cells were washed with phosphate buffer solution (PBS), digested with 0.025% trypsin and 1 mM EDTA, and the re-suspended in RPMI-1640 containing 2% FBS at a density of 1×10^6 cells/mL. The cells were incubated with Hoechst 33342 (Sigma, USA) at 2.5 µg/mL either alone or in combination with ATP-Binding Cassette (ABC) transporter inhibitor verapamil (Sigma, USA) at 100 µg/mL for 60 min at 37°C. Then, these cells were centrifuged and re-suspended in ice-cold RPMI-1640 containing 1 µg/mL propidium iodide (Sigma, USA) and maintained at 4°C for flow cytometry and sorting. Cell sorting was performed on a FACS Aria Ⅱ FCM (B.D, USA). The Hoechst dye was excited with UV laser and the fluorescence measured with both 675/20 filter (Hoechst Red) and 450/50 filter (Hoechst Blue). Finally, the two types of cells were collected for evaluation of the purity and further experiments.

Transwell invasion assay

The invasiveness of SP and non-SP (NSP) cells was determined using 12-well Transwell plates (Corning, USA) coated with matrigel (B.D, USA). Cells were seeded into the upper inserts at 2×10^5 per insert in serum-free RPMI-1640. The lower wells were filled with RPMI-1640 containing 10% FBS as a chemoattractant. Cells were incubated at 37°C for 48 h, and then non-invading cells were removed by swabbing the top layer of Matrigel with a cotton swap. Membranes containing invading cells were stained with Giemsa. The number of invading cells was counted under light microscope. The invasion assay was done in triplicates.

Quantitative real-time RT-PCR

Total RNA was isolated from cells using Trizol (Invitrogen, USA), followed by first strand cDNA synthesis using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to the manufacturer's instructions. The following primers were used: Vimentin (372 bp): 5'-ATGCGTGAGATGGAAGAGAATTTTGC-3' (forward) and 5'-TTATTCAAGGTCATCGTGATGCTGAGA-3' (reverse); E-cadherin (192 bp): 5'-TCGACACCCGATTCAAAGTGG-3' (forward) and 5'-TTCCAGAAACGGAGGCCTGAT-3' (reverse); Snail (143 bp): 5'-TATGCTGCCTTCCCAGGCTTG-3' (forward) and 5'-ATGTGCATCTTGAGGGCACCC-3' (reverse); N-cadherin (156 bp): 5'-CCGGAGAACAGTCTCCAACTC-3' (forward) and 5'-CCCACAAAGAGCAGCAGTC3' (reverse); GAPDH (141 bp): 5'-TCTGCTCCTCCTGTTCGACA-3' (forward) and 5'-AAAAGCAGCCCTGGTGACC-3' (reverse). All primers were synthesized in Shanghai Invitrogen Biotechnology Co., Ltd. Real-time RT-PCR was performed using a SYBR Premix Ex Taq Perfect real-time kit (TaKaRa Bio, Japan) in a 7300 Real-time PCR system (A.B, USA). The PCR conditions were predenaturation at 95°C for 10 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. To quantify the gene expression, the ΔΔCt method was used to calculate the relative fold change after normalization by the expression of GAPDH. PCR was done in triplicates.

Western blot assay

SP cells and NSP cells were lysed with cell-lysis buffer (50 mMTris-HCl, (pH8.0), 150 mM NaCl, 0.02% NaN3, 0.1% SDS, 100 µg/mL PMSF, 1 µg/mL Aprotinin and 1% NP-40). Total proteins were measured using the BCA kit (Pierce, USA) according to the manufacturer's instructions. Totally 20 µg of proteins were separated on the 10% SDS-PAGE gels and then transferred to nitrocellulose membrane. After blocked with 5% non-fat milk, the membranes were incubated with anti-E-cadherin antibody (Abcam, UK), anti-Vimentin antibody (Abcam, UK), anti-Snail antibody (Abcam, UK), anti-N-cadherin antibody (Abcam, UK), and anti-α-actin antibody (Sigma, USA). Then, the membranes were washed with TBS-T thrice and treated with horseradish peroxidase-conjugated secondary antibody before developing using the ECL kit (Pierce, USA) by enhanced chemiluminescence.

Statistical analysis

Data were expressed as mean ± standard deviation (SD).

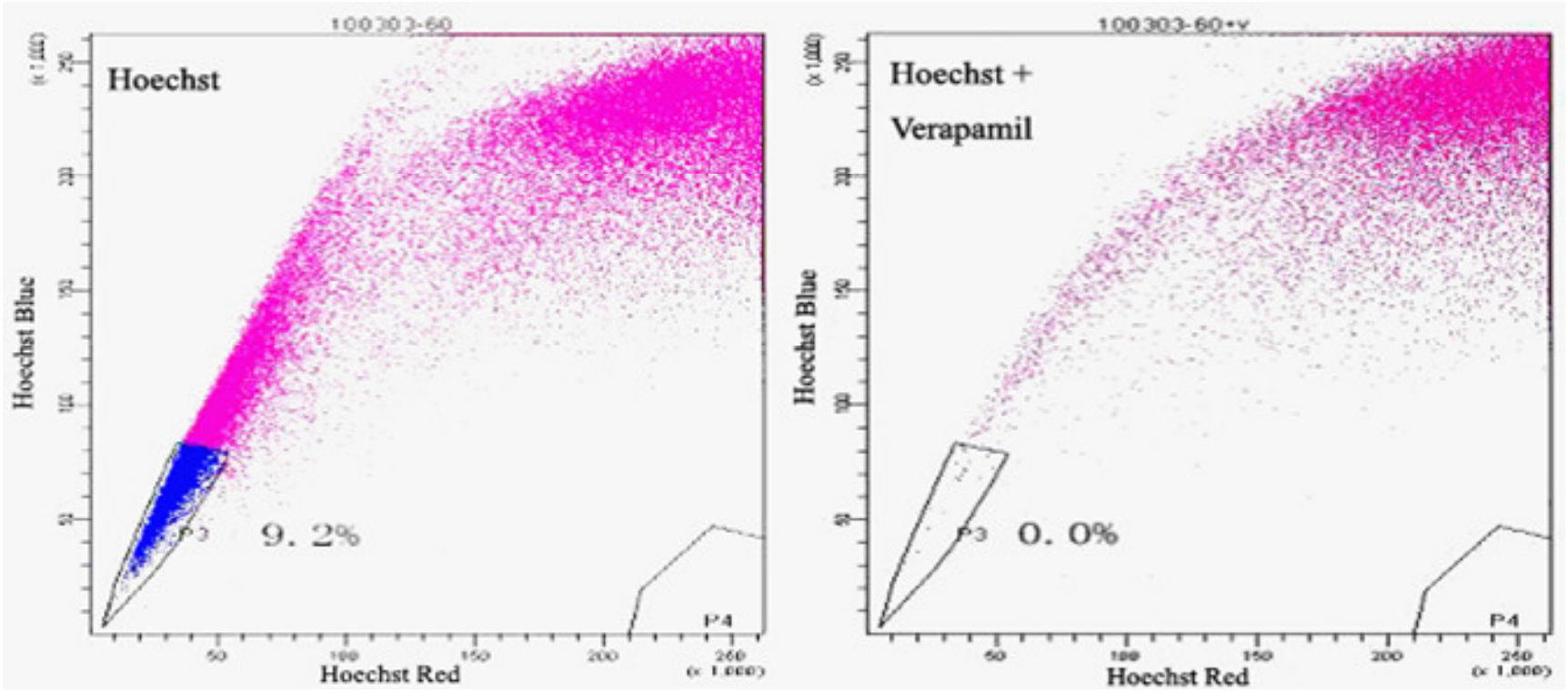

Figure 1. SP cells in hepatoma SMMC-7721 cells. SMMC-7721 cells were stained with Hoechst 33342 dye in the absence or presence of Verapamil, followed by fluorescence-activated cell sorting (FACS). The distinct region of SP fraction disappeared by the transporter inhibitor, Verapamil.

Comparisons between two groups were performed using the *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

SP cells purified from hepatoma SMMC-7721 cells

We examined SMMC-7721 cells by flow cytometry of live cells for exclusion of Hoechst33342 dye, and defined the gating of these cells by disappearance of this fraction in cells pre-incubated with the transporter inhibitor, Verapamil. The percentage of SP cells in SMMC-7721 cells was 9.2 ± 0.2% (Figure 1).

High *in vitro* invasion of SP cells

The *in vitro* invasion capacity was expressed as the number of cells penetrating the polycarbonates coated with matrigel. The number of SP cells (66.3 ± 7.4) penetrating the basilar membrane was significantly higher than that of NSP cells (27.5 ± 6.5) ($P < 0.01$) (Figure 2).

mRNA expressions of EMT markers in SP cells and NSP cells

The mRNA expressions of Vimentin, N-cadherin and Snail were significantly increased by 9.527-, 6.834- and 8.173-fold in SP cells, which were significantly higher than those in NSP cells ($P < 0.01$). In contrast, the decrease of E-cadherin expression in SP cells (5.353- fold) was significantly higher than that in NSP cells ($P < 0.01$) (Figure 3).

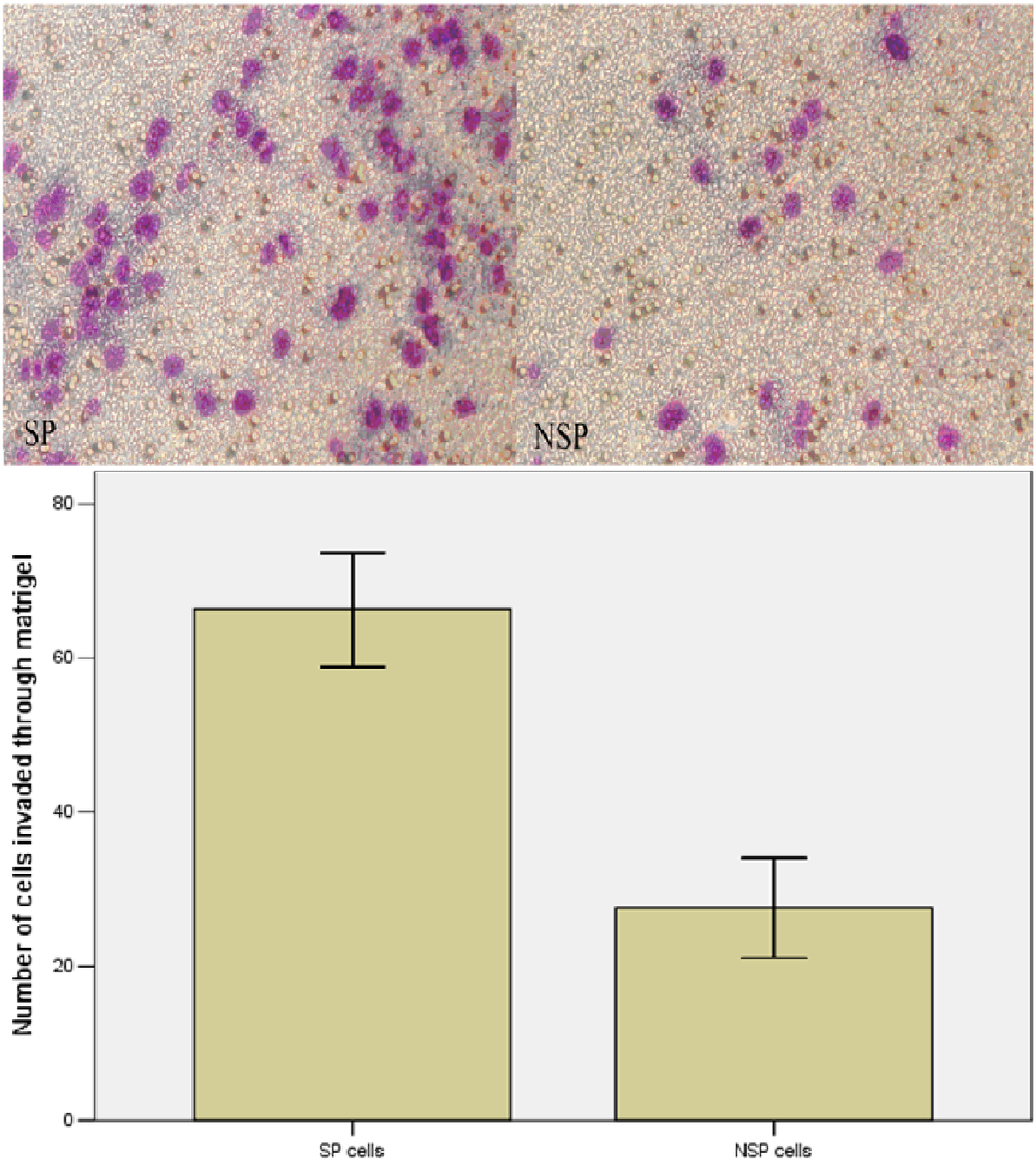

Figure 2. Invasive ability of SP cells and NSP cells detected by transwell invasion assay. The number of SP cells (66.3±7.4) penetrating the basilar membrane was significantly higher than that of NSP cells (27.5±6.5) (P<0.01).

Protein expressions of EMT markers in SP cells and NSP cells

The protein expressions of E-cadherin, Vimentin, N-cadherin and Snail proteins in SP cells were 0.174 ± 0.014, 1.117 ± 0.012, 0.975 ±0.017 and 0.917 ± 0.014, respectively which were remarkably different from those in NSP cells (0.935 ± 0.012, 0.314 ± 0.011, 0.179±0.013 and 0.202 ± 0.013) (P < 0.01) (Figure 4).

DISCUSSION

Metastasis is a multistage process and mainly includes the following steps: Cancer cells escape from the primary tumor, penetrate into the blood vessels and/or lymphatic

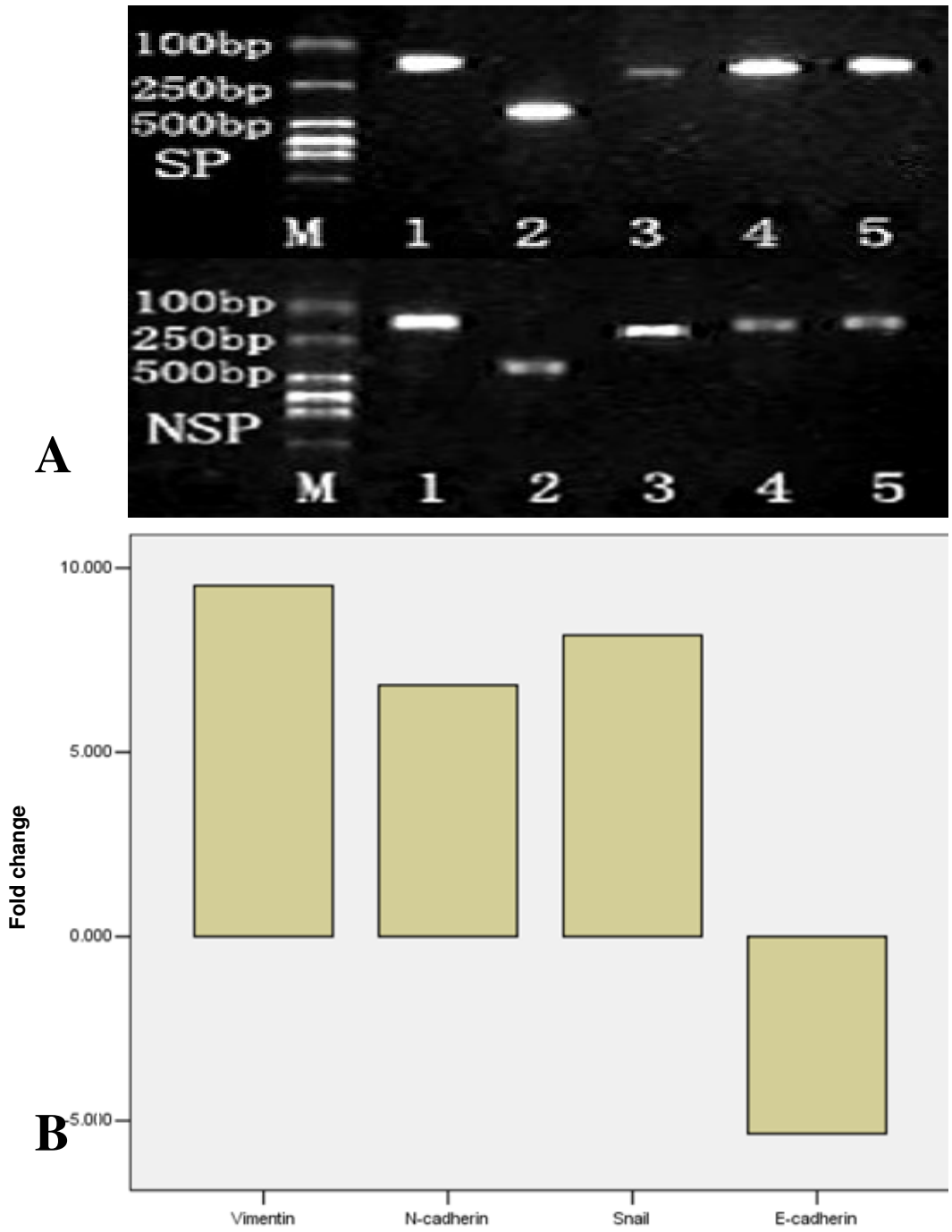

Figure 3. mRNA expressions of EMT markers in SP cells and NSP cells. **(A)** Lane M: Marker; lanes 1: GAPDH; lane 2: Vimentin; lane 3: E-cadherin; lane 4: N-cadherin; lane 5: Snail; (**B)** The mRNA expressions of Vimentin, N-cadherin and Snail were significantly higher than those in NSP cells (P<0.01). In contrast, the mRNA expression of E-cadherin in SP cells was significantly lower than that in NSP cells (P<0.01).

vessels, and migrate through the walls of vessels into surrounding tissues where they settle, proliferate and induce angiogenesis, creating metastases (Brábek et al., 2010). Only a small percentage of cells released from the primary tumor can successfully complete the metastatic process and eventually form distant metastatic lesions (Luzzi et al., 1998). Thus, one can hypothesize that those cells participating in the invasion and metastasis and ultimate formation of metastatic lesions may be CSCs. Brabletz et al. (2005) found that cells in the invasive front of tumor-host possessed the biological characteristics of stem cells and acquired the ability of metastasis through

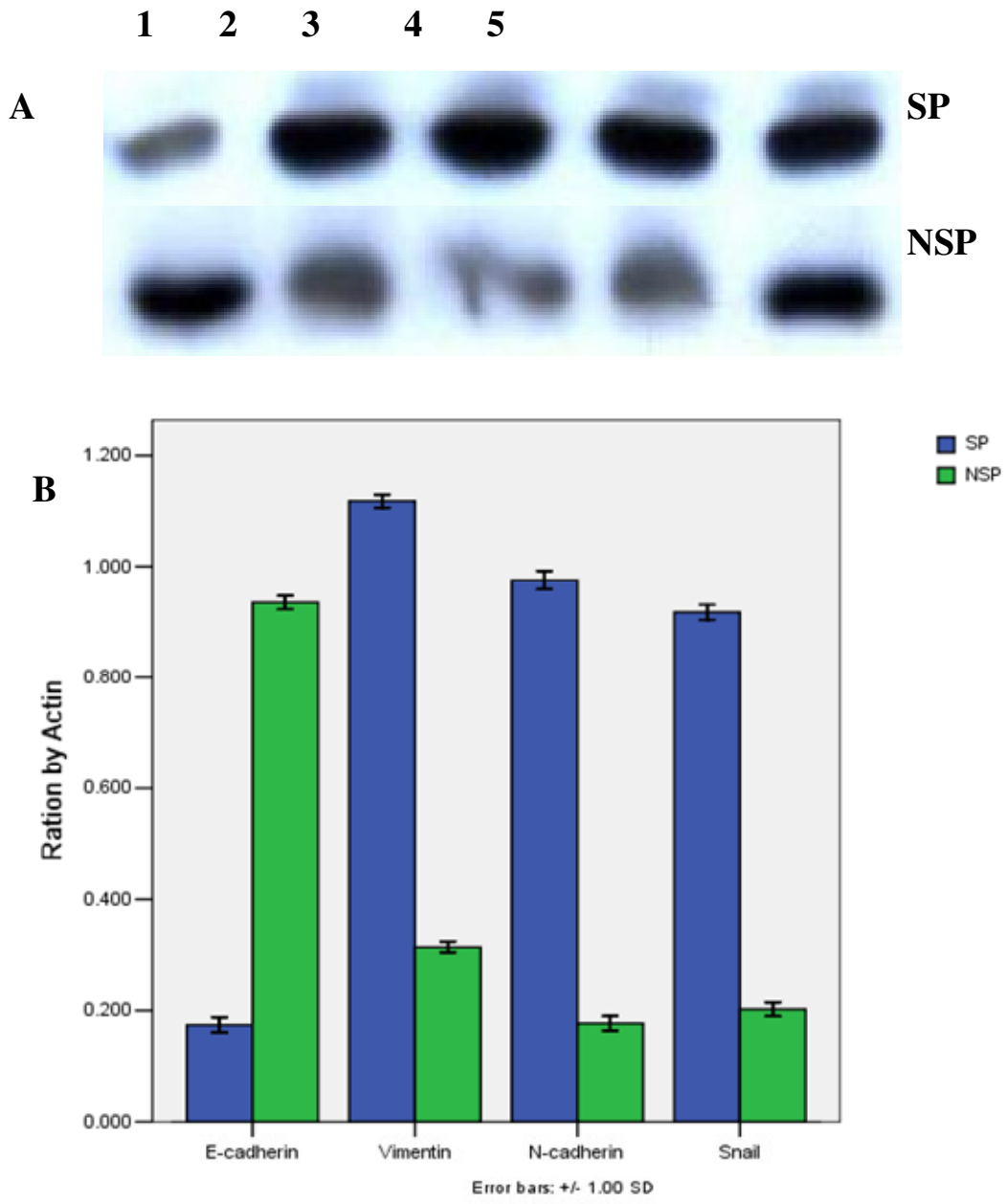

Figure 4. Protein expressions of EMT markers in SP cells and NSP cells. **(A)** Lane 1: E-cadherin; lane 2: Vimentin; lane 3: N-cadherin; lane 4: Snail; lane 5: α-actin; (**B)**The protein expressions of Vimentin, N-cadherin and Snail were significantly higher than those in NSP cells (P<0.01). In contrast, the protein expression of E-cadherin in SP cells was significantly lower than that in NSP cells (P < 0.01).

EMT. These cells are named as migrating cancer stem cells (MCSCs). Hermann et al (2007) identified a distinct subpopulation of $CD133^{+}/CXCR4^{+}$ CSCs in the invasive front of pancreatic cancers, suggesting the metastatic phenotype of the individual cancer cells. Depletion of the CSC pool for these migrating CSCs virtually abrogated the metastatic phenotype of pancreatic cancer cells without affecting their tumorigenic potential. These findings demonstrate that pancreatic CSCs contribute to the metastasis. A method for the isolation of potential CSCs based on their invasive properties has been examined by Yu and Bian (2009). The results demonstrated that the inva-sive population of the U87 glioma cell line highly expressed the stem cell markers (nestin and Oct4), and more spheres formed when cultured in serum-free medium than the non-invasive population.

Currently, EMT is recognized as pathological mechanism in the progression of various diseases including inflammation, fibrosis and cancers. Alterations in the expressions of critical molecules have been observed

during the acquisition of EMT phenotype, which is consistent with their association in cellular signal transduction pathways. In the process of EMT, cells lose the epithelial cell-cell junction, actin cytoskeleton reorganization and the expressions of proteins that promote cell-cell contact such as E-cadherin, γ-catenin, and zonula occludens-1 (ZO-1), and gain mesenchymal molecular markers such as vimentin, fibronectin, α-smooth muscle actin (SMA), fibroblast-specific protein-1, and Ncadherin (Christiansen et al., 2006). For epithelial malignancies, the EMT is considered to be a crucial event in the metastasis, which involves the disruption of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype allowing these cells to travel to the metastatic site without being affected by conventional treatment (Thiery, 2002). More recently, it is proposed that isolated CSCs are in fact generated by this EMT event (Mani et al., 2008). SP cells can rapidly efflux lipophilic fluorescent dyes and are characterized by a special profile in the fluorescence-activated flow cytometric analysis. Although, SP cells only represent a small fraction of the whole population, they appear to be enriched in the stem cells. Thus, they can provide a useful tool and a readily accessible source for CSCs studies (Taga, 2008).

In the present study, we isolated CSC-like SP cells from hepatoma SMMC-7721 cells, using fluorescent dye Hoechst 33342 and flow cytometry cell sorting. The *in vitro* invasion capacity of these cells was evaluated by Transwell chamber assay. Our results confirmed that SP cells were more invasive than NSP cells. Furthermore, in SP cells, the expression of E-cadherin was significantly decreased and those of Vimentin, N-cadherin and Snail were markedly elevated when compared with those in NSP cells. Cadherin is a kind of calcium dependent transmembrane glycoprotein, mainly including E-cadherin in the epithelial tissues, N-cadherin in the nerves and muscular tissues, and P-cadherin in the placenta (Nelson, 2008). E-cadherin is an essential component of cell-cell junctions, and its loss contributes to invasive behavior and allows the β-catenin to enter the nucleus where it regulates the transcription of EMT- and proliferation-regulatory genes (Baranwal et al., 2009). Contrary to E-cadherin, N-cadherin may promote the invasion and migration of carcinoma cells. N-cadherin has been shown to enhance cell migration during EMT (Hazan et al., 1997). In epithelial carcinoma, E-cadherin is down-regulated in most cases, sometimes accompanied by the up-regulation of N-cadherin (Hazan et al., 2000). These findings suggest that there may be a shift from E-cadherin to N-cadherin in the expression. This shift and their mutually exclusive expression pattern in these invasive cancer cells strongly reflect that the dedifferentiation from epithelial phenotype to mesenchymal phenotype is often associated with an increase of invasiveness (Hazan et al., 2004).

Vimentin, a kind of intermediate filament protein, is one of the important characteristics of EMT, and Vimentin in the epithelial cancers can promote the invasion and metastasis of cancers (Jin et al., 2010). Snail is a zinc-finger transcription factor consisting of a highly conserved C-terminal region with 4~6 zinc fingers which serve as the DNA-binding domains recognizing the consensus E2-box type elements (CAGGTG) (Peinado et al., 2007). Snail transcriptionally represses the adherens junction protein, E-cadherin, by binding to these CAGGTG sequences within its promoter, ultimately inducing EMT (Cano et al., 2000). Yang et al (2009) have found that hepatocellular carcinoma Mahlavu cell line with increased expressions of Snail and Twist exhibited an increase of invasiveness and metastasis when compared with hepatocellular carcinoma Huh-7 cell line with low expressions of endogenous Twist and Snail. Over-expression of Snail or/and Twist in Huh-7 cells can induce EMT and invasiveness as well as metastasis, whereas knockdown of Twist or Snail in Mahlavu cells reverses the EMT and inhibits the invasiveness and metastasis. In our study, the differences in the expressions of EMT markers between SP cells and NSP cells indicate that SP cells may undergo EMT leading to great invasiveness. The mechanisms underlying the relation between CSCs and EMT require further investigation.

Conclusion

In conclusion, we confirm the existence of a small SP in SMMC-7721 cells, and these SP cells possess greater *in vitro* invasiveness than NSP cells. Moreover, these SP cells exhibit the characteristics of EMT. Thus, we speculate that the invasiveness of SP cells may be related to EMT.

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