Characterization of a stem-like population in hepatocellular carcinoma MHCC97 cells

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Abstract. A small stem-like subpopulation was isolated from human hepatocellular carcinoma (HCC) MHCC97 cells, characterized by their high efflux ability of the Hoechst 33342 dye. These side population (SP) cells were able to generate a heterogeneous mixture of SP and main population (MP) cells, while the MP cells rarely generated SP cells. Cell cycle analysis also revealed that more SP cells were in the G0 phase. They express higher levels of BCRP1, AFP and CK19 than MP cells. SP cells showed significantly higher viability than MP cells following treatment with doxorubicin or methotrexate. Actin polymerization and migration assays indicate that SP cells have a higher migration capacity and *in vivo* tumorigenicity of these cells is also higher. Collectively, we conclude that the SP is an enriched source of stem-like cells and may be an effective target for therapy and a useful tool to investigate the HCC tumorigenic and metastatic process.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. Despite advances in the detection and treatment of HCC, the mortality rate remains high because the majority of HCC patients present at an advanced stage or with metastasis for which most potentially curative therapies have limited efficacy (1). Hence, understanding the mechanism underlying carcinogenesis and metastasis formation is essential for the management of liver malignancies.

It is presumed that an accumulation of genetic alterations contributes to processes which are critical for multistep hepatocarcinogenesis and metastasis formation (2,3). Therefore, only cells which have a high proliferative potential and a long life span, such as stem cells, would have the ability to accumulate the necessary mutations to transform into cancer cells. There is growing evidence that cancerous tissues, including HCC, are heterogeneous and may include long-lived cancer stem cells (CSCs). CSCs are responsible for tumor formation and progression as they can perpetuate themselves through self-renewal and to generate mature cells of a particular lineage through differentiation (4). CSCs have been identified and isolated from leukemia (5), breast cancer (6), glioblastoma (7), prostate cancer (8), gastric cancer (9), lung cancer (10) and colon cancer (11). The side population (SP) phenotype, first described in hematopoietic malignancies (12), is defined as the population of cells that have the blockable ability to efflux the nucleic acid dye Hoechst 33342 via ATP-binding cassette transporters such as ABCG2/BCRP1. The SP is thought to be enriched for stem-like cells in several normal human tissues, cancers and cell lines and thus may be useful for the identification and isolation of CSCs and the mechanism of tumor progression.

Up to now, the whole mechanism underlying hepatocarcinogenesis has not been clearly documented. The identification of CSCs and elucidation of the hierarchy in HCC cells might contribute to the understanding of hepatocarcinogenesis, metastasis formation and the exploration of novel therapeutic approaches.

In the present study, we report the identification and isolation of SP cells phenotype in human HCC cell lines that demonstrate specific characteristics of CSCs when compared with MP cells, such as quiescence, elevated chemoresistance, increased tumorigenicity, higher actin polymerization ability and increased migration capacity towards the chemokine CXCL12, whose receptor (CXCR4) has been linked to HCC dissemination and poor prognosis (13). These data demonstrate that the SP in human HCC contains stem-like cells and may provide novel diagnostic tools and strategies for controlling this malignancy.

Materials and methods

Cell culture. The human HCC cell lines MHCC97, Huh-7 and HepG2 were cultured in RPMI-1640 (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin G (Invitrogen). In all experiments, cells were cultured at 37˚C in a humidified 5% CO_2/95% air atmosphere.

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Flow cytometry analysis. To identify and isolate the SP and MP fractions, cells were detached from culture flasks using Trypsin-EDTA (Invitrogen), pelleted by centrifugation, washed with PBS, and resuspended at $1x10^6$ cells/ml in Hanks' balanced salt solution (HBSS) supplemented with 2% FBS and 10 mM HEPES (Invitrogen). These cells were then preincubated at 37˚C for 15 min and stained with Hoechst 33342 dye (Sigma Chemical, St. Louis, MO) at 6 μg/ml (MHCC97) or 10 μg/ml (Huh-7 and HepG2). Cells were then incubated for 90 min at 37˚C either alone or with 50 μM verapamil (Sigma), before resuspension in HBSS containing 2% FBS, 1% penicillin-streptomycin G, 10 mM HEPES and 2 μg/ml of propidium iodide (BD Pharmingen, San Diego, CA) to label dead cells. Cells were then filtered through a 40 μm strainer (Becton-Dickinson Falcon) and maintained at 4˚C before analysis and sorting using a FACSAria flow cytometer (Becton-Dickinson).

Cell cycle analysis. Sorted SP or MP cells were fixed with 4% PFA and stained with 5 μg/ml Hoechst 33342 in HBSS containing 2% FBS, 10 mM HEPES and 0.004% saponin (Sigma) at 37˚C for 30 min. Without washing, cells were stained with 1 μg/ml Pyronin Y (Sigma) on ice for 10 min. Cells were washed and analyzed by flow cytometry.

RNA extraction and real-time PCR analysis. Total RNA was extracted using the RNeasy Micro kit (Qiagen, Hilden, Germany), treated with DNase I (Invitrogen) and reverse transcribed using random hexamers and SuperScript II reverse transcriptase enzyme (Invitrogen) according to the manufacturer's protocol. Real-time PCR was carried out using SYBR-Green Real-Time Core Reagents (Applied Biosystems) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Primers were designed to generate a PCR product of <200 bp. GAPDH was used as a control. The expression levels of each gene in the SP were determined relative to gene expression in the MP.

Chemo-resistance assay. Sorted SP or MP cells were cultured with the chemotherapeutic agent doxorubicin (DOX, 50 nM) or methotrexate (MTX, 100 nM). After 72 h exposure, cell viability was examined by an MTS-based Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Transplantation experiment. Sorted SP or MP cells were resuspended in HBSS and mixed with Matrigel (1:1) (Becton-Dickinson). These suspensions were subcutaneously injected into 6-week-old nude mice under anesthesia. Mice were inoculated with $1x10^5$ or $1x10^6$ non-sorted cells, $1x10^3$ or $1x10^4$ SP cells, or $1x10^3$ or $1x10^4$ MP cells (five mice per group). Tumor growth was monitored every week. Pieces of subcutaneous tumors were fixed in formalin, embedded in Tissue-Tek OTC compound and frozen at -20˚C. Cryostat sections (8 μm) were cut and subjected to hematoxylin and eosin staining.

Actin polymerization. Sorted SP or MP cells were resuspended and kept at 37˚C. Human CXCL12 (PeproTech) or PBS were added to cell suspensions and aliquots were taken at the indicated times and immediately fixed in 4% paraformaldehyde for 10 min. After washing, samples were stained with FITC-Phalloidin (Molecular Probes) stain F-actin and analyzed by flow cytometry. The relative F-actin index was determined as the ratio of the F-actin level of SP or MP cells treated with CXCL12 to SP or MP cells treated with PBS.

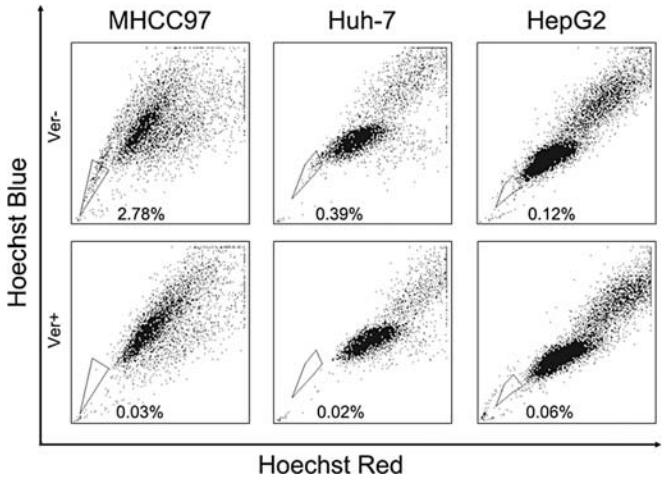

Figure 1. Identification of SP and MP cells in the HCC cell lines MHCC97, Huh-7 and HepG2. Cells were stained with Hoechst 33342 either alone or in the presence of verapamil. SP cells were gated and shown as the percentage of total cells. SP cells disappeared in the presence of verapamil (bottom panel).

Transwell migration assay. Sorted SP and MP cells were resuspended in 0.1% fatty acid-free BSA. Cell suspensions were added to transwells (8-μm pores, BD Biosciences) in 24-well plates containing the chemokine CXCL12. After incubation for 24 h, membranes containing migrating cells were stained with hematoxylin for 3 min, washed and mounted on slides. The relative migrating index was determined as the ratio of the number of migrated cells in each group to the number of migrated cells in total cell group.

Statistical analysis. The data are expressed as the mean ± SD. Statistically significant differences were determined using the Student's t-test. P-values <0.05 were considered significant.

Results

SP phenotype in HCC cell lines. Using flow cytometry, we were able to identify and successfully isolate populations of SP and MP cells from the three human HCC cell lines (Fig. 1). The SP gate was defined as the region where cells were absent in the presence of verapamil, an agent which blocks the efflux of Hoechst 33342. The three cell lines, MHCC97, Huh-7 and HepG2, contained 2.64±0.31, 0.32±0.09 and 0.09±0.03% SP cells, respectively. Since MHCC97 contained the highest percentage of SP cells further experiments were performed using these cells.

SP cells regenerated both SP and MP cells. To compare the repopulation capabilities of the sorted SP or MP cells, they were cultured separately under the same conditions for 2 weeks before staining with Hoechst 33342 and analysis (Fig. 2A). Both fractions were viable in culture, but SP cells generated

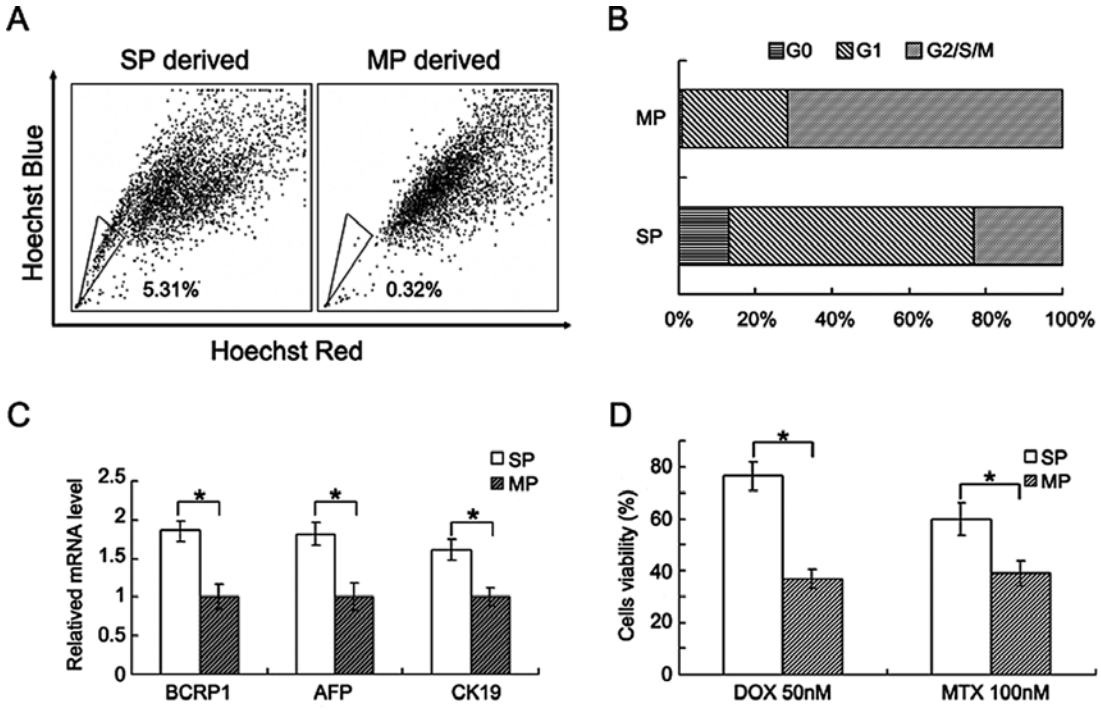

Figure 2. *In vitro* characteristics of MHCC97 SP and MP cells. (A) SP and MP repopulation ability was determined by Hoechst 33342 staining after 2 weeks in culture. (B) The cell cycle status of SP and MP cells was examined by Hoechst 33342 and pyronin Y staining. (C) Relative mRNA expression of BCRP1, AFP and CK19 in SP and MP cells was determined using real-time PCR analysis. (D) Chemo-resistance of SP and MP cells to anticancer drugs. Cells were incubated with DOX (50 nM), or MTX (100 nM) for 72 h. Cell viability was then determined using the MTS assay. Each value represents the mean ± SD. *P<0.05.

both SP and MP cells with a SP fraction size (4.82±0.32%) that was larger than in the original population, whereas MP cells produced predominantly MP cells.

More SP cells remained quiescent. Cell cycle analysis indicated that 13.03±2.51, 63.90±1.95 and 23.07±2.60% of the SP cells were in the G0, G1 and S/G2/M phase, respectively. In MP cells, 0.73±0.31, 27.63±5.59 and 71.63±5.62% cells were in the G0, G1 and S/G2/M phase, respectively. Therefore, cells in the G0 phase were more abundant in SP than MP cells, consistent with previous studies that a significant fraction of stem cells are in the quiescent G0/G1 phase (Fig. 2B).

Gene expression analysis. Expression of the hepatocyte-specific marker AFP and the cholangiocyte-specific marker CK19 has been associated with subpopulations showing bipotential stem/progenitor properties in HCC. BCRP1 has the capacity to efflux a broad range of cytotoxic substances and is characteristic of the SP phenotype. These genes were expressed in both SP and MP cells but up-regulated in SP relative to MP cells; AFP (1.81±0.15-fold), CK19 (1.61±0.13-fold) and BCRP1 (1.85±0.13-fold) (P<0.05) (Fig. 2C).

Resistance to chemotherapeutic agents. The chemo-resistance ability of SP cells has been reported to depend mainly on ABC transporters (14). To determine whether SP cells are able to resist ABC transporter-independent anticancer drugs more than MP cells we tested DOX and MTX which are used for the treatment of HCC (Fig. 2D). SP cells were more resistant than MP cells, especially to DOX, exhibiting a viability rate of 76.6±5.6% compared with 36.8±3.6% after 72 h incubation (P<0.05). Cells were then stained with Hoechst 33342 and reanalyzed. A higher percentage (5.2±0.36%) of SP cells was seen after 72 h of DOX treatment. Following 72 h of MTX treatment, cell viability was 59.8±6.2% in SP cells and 39.1±4.8% in MP cells. This suggested that SP cells might be more resistant to anticancer drugs than MP cells.

Tumorigenicity of SP and MP cells in vivo. Tumorigenicity was examined by the subcutaneous transplantation of unsorted, SP or MP cells into nude mice. Transfer of $1x10^5$ or $1x10^6$ unsorted MHCC97 cells led to tumor formation in nude mice. Transplantation of $1x10^3$ SP or $1x10^4$ SP cells produced tumors in 60 or 80% of the mice. In contrast, transplantation of $1x10^3$ MP cells consistently failed to form tumors, while $1x10^4$ MP cells showed tumor formation in only 20% of the mice. This indicated that SP cells showed significantly higher tumorigenicity than MP cells. In addition, histological analysis of SP or MP origin tumors showed similarities to primary tumors (Fig. 3).

Cell polarization. Cell polarization requires actin polymerization (15). As shown in Fig. 4A, the F-actin levels of sorted MP cells after 3, 5, 10 min stimulation with CXCL12 were 1.12±0.15, 1.14±0.18 and 1.10±0.17-fold relative to PBS. Sorted SP cells showed 1.27±0.17, 1.43±0.16 and 1.29±0.13-fold F-actin staining intensity at the same time points. This suggests that CXCL12 treatment increased the extent of actin polymerization in SP cells more than MP cells at the studied time points.

To examine the possible differences in migration between SP and MP cells, we performed a transwell-based migration assay. Untreated SP and MP cells showed similar migration abilities after 24 h incubation. Following treatment with

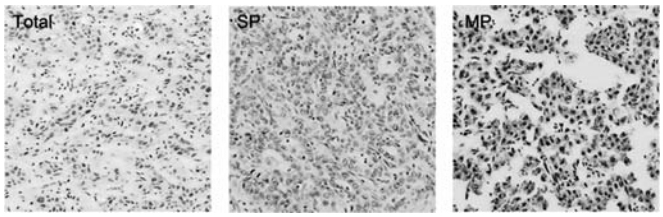

Figure 3. *In vivo* tumorigenicity of MHCC97 SP and MP cells. Tumors were formed by injection of $1x10^5$ total cells, $1x10^3$ SP cells and $1x10^4$ MP cells subcutaneously. Hematoxylin and eosin staining showed cancers derived from total cells, SP or MP cells (magnification x200).

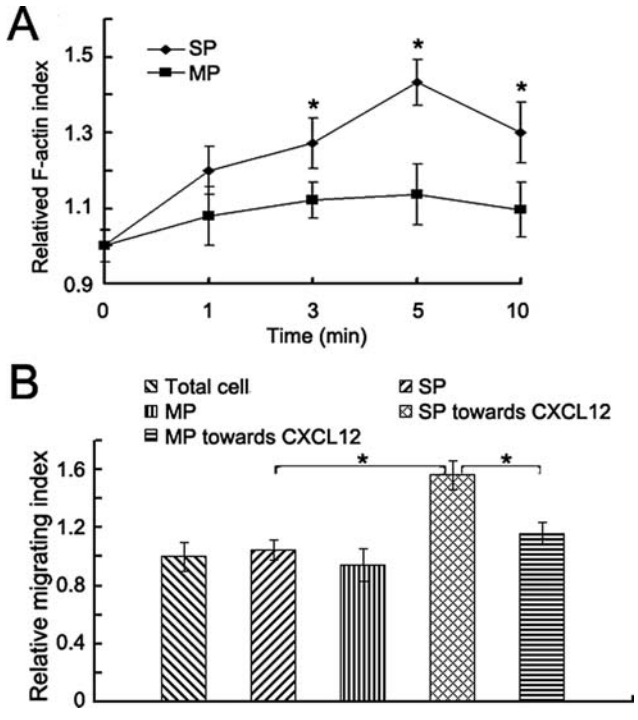

Figure 4. *In vitro* migration characteristics of MHCC97 SP and MP cells. (A) The effect of CXCL12 on actin polarization. F-actin levels were examined at the indicated time points after CXCL12 stimulation. Phalloidin staining was then analyzed by flow cytometry. The relative F-actin index was determined as the ratio of the F-actin levels of cells treated with CXCL12 to cells treated with PBS. (B) The effect of CXCL12 on cell migration. A transwell system was used to examine cell migration. The relative migrating index was determined as the ratio of the number of migrated cells in each group to that of total cell group.

CXCL12, the relative migrating index of SP cells (1.56±0.10%) was higher than in untreated SP cells (1.05±0.07%) ($P<0.05$) and MP cells (1.16±0.08%) ($P<0.05$) (Fig. 4B).

Discussion

Current evidence suggests that solid carcinomas in many tissues may originate from somatic stem or progenitor cells that have non-differentiated properties with cancer cell potential. SP cells have been reported to be enriched for stem cells in tumors as well as normal tissues. All three human HCC cell lines analyzed in this study contained SP cells, with MHCC97 cells containing the highest percentage of SP cells, making them the focus of this study.

SP cells showed repopulation capacities, generating an SP and MP cell mixture with a slightly higher SP percentage than the original cell line after 2 weeks culture, whereas the MP cells yielded only a small fraction of SP cells. This is in accordance with previous observations that the SP fraction can divide both symmetrically and asymmetrically and display a self-renewal capacity whereas MP cells use only symmetric division (16,17).

As previously reported by several investigators, an important characteristic of hematopoietic and other tissue-specific stem cells is that they remain dormant or quiescent, being arrested in the G0 phase of the cell cycle, and thus protected from depletion or exhaustion. We hypothesized that if there are stem cells in the SP fraction, but not in the MP fraction, SP cells will contain quiescent cells whereas most MP cells would be in the cell cycle. Entry into G1, following exit from G0 is associated with an increase in transcription, which can be measured by pyronin Y, an RNA-specific dye. Simultaneous staining of pyronin Y and Hoechst 33342 revealed more SP cells were in the G0 phase, which suggested that the SP fraction was enriched for stem cells.

It is believed that some cancers, including HCC, arise from subpopulations with bipotential stem or progenitor properties (18). The MHCC97 cell line is both AFP and CK19-positive and our study showed that AFP and CK19 mRNA were expressed in both SP and MP cells, but their expression was up-regulated in SP cells relative to MP cells. Liver oval cells, considered to be putative hepatic stem cells or progenitor cells (19), express many molecules, including AFP and CK19. The similarity between the phenotype of the SP and oval cells led us to speculate that SP cells might exist in the upstream hierarchy of HCC cells and have a bipotential capacity to differentiate into hepatocytes and cholangiocytes under appropriate conditions.

ABCG2/BCRP1 expression has already been found in SP cells in breast cancer (20), lung cancer (21) gastric cancer (9) and in normal hematopoietic SP cells (22). Moreover, it has been demonstrated that increased expression of ABC transporters in cancer SP cells is associated with resistance to chemotherapeutic agents (14). Consistent with studies showing BCRP1 to be a molecular determinant of the SP phenotype, BCRP1 mRNA expression was markedly higher in MHCC97 SP cells compared with MP cells in our study. Furthermore, chemo-resistance to anticancer drugs in HCC treatment, DOX and MTX, was markedly higher in SP cells than MP cells. These results suggest a potential cause of drug-resistance in HCC through the increased expression of BCRP1 in SP cells.

When injected into nude mice, $1x10^3$ SP cells developed tumors whereas MP cells, which constitute the majority of cancer cells, rarely formed tumors despite the injection of increased numbers of cells. This demonstrates that SP cells are more tumorigenic than MP cells, indicating a significant enrichment of stem-like cells in this small subpopulation. This is potentially important because effective curative therapy most likely depends on the successful eradication of stem-like cells.

CXCL12 is highly expressed by endothelial cells and in some tissues that can undergo metastatic growth, such as the lung, liver and lymph nodes (23). CXCR4 expression is associated with tumor dissemination in liver, colorectal, breast and oral squamous cell carcinoma (13,24-27). Further data also

underlined the importance of CXCR4 induced migration, invasion and angiogenesis (28,29). To explore the effects of CXCL12 on MHCC97 SP and MP cells, we examined actin polymerization, an early event which induces membrane protrusions and stabilizes new membrane extensions at the leading edge of migrating cells. We found that CXCL12 increased actin polymerization levels in SP cells. Considering that the regulation of lymphocyte chemotaxis and trafficking by cytokines is largely dependent on regulators of actin polymerization (30), CXCL12 might at least partly direct SP cell migration by promoting actin polymerization. When we compared the migratory ability of SP and MP cells towards CXCL12 using a transwell system, we found that CXCL12 increased SP cell migration *in vitro*. This indicated that SP cells may play a key role in metastasis formation in HCC.

In conclusion, we isolated a tumorigenic SP cell population by sorting the MHCC97 cell line and propose that these SP cells exhibit stem-like characteristics. Our data suggest that the SP cells show tumorigenic potential, chemo-resistance and migratory ability, which might lead to relapse and metastasis formation, which may have important clinical implications in HCC treatment. Further studies on the identification and characterization of SP cells using clinical HCC specimens will contribute to the understanding of the mechanism underlying hepatocarcinogenesis and metastasis formation and aid the development of effective therapeutic strategies.

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