Tumor initiating potential of side population cells in human gastric cancer

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Abstract. Side population (SP) cells are a small subpopulation of cells with enriched source of gastric tumor-initiating cells (TICs) with stem-like cell property that are characterized by high efflux ability of Hoechst 33342 dye, reflecting high expression of several subtypes of the ATP-binding cassette transporter family that is characteristic of stem cells. The present study is the first to discover and characterize SP cells within gastric cancer (GC) tumors. In this study, human GC cell lines (MKN45, KATOIII, MKN74, MKN28 and MKN1) were analyzed using flow cytometry for SP cell isolation, and all GC cell lines showed a distinct fraction of SP cells, ranging from 0.02±0.001 to 1.93±0.16%. Among these cell lines, MKN45 cultures possessed the highest percentage of SP cells. Using MKN45 cells, we demonstrated stem cell-like characteristics of SP cells of the cell lines as a possible subpopulation with enriched TICs, as indicated by ABC transporter gene expression (MDR1 and BCPR1), chemo-resistance and tumorigenicity *in vivo*. In addition, we report the first identification and isolation of SP cells from clinical GC tissues as well as human GC cell lines. These SP cells demonstrate higher tumorigenicity *in vivo* than does the overall cell population in the parent tissue. In conclusion, we demonstrate that solid tumor tissue such as human GC contains TICs, with the existence of heterogeneity and distinct hierarchy in malignancy, suggesting the future possibility of a novel therapeutic tool targeting TICs for overcoming this malignant disease.

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Abbreviations: SP, side population; GC, gastric cancer; TIC, tumor-initiating cell; NOG, NOD/Shi-*scid*,IL-2Rγ^{null}

Key words: side population, gastric cancer, tumor-initiating cell, mdr1, bcrp1, NOD/Shi-*scid*,IL-2Rγ^{null} mice

Introduction

Although the incidence of gastric cancer (GC) is decreasing in many countries, GC remains one of the most common cancers and the second most common cause of cancer deaths worldwide (1). Since early-stage GC is often asymptomatic and difficult to diagnose, many patients tend to be diagnosed with advanced GC even in the developed countries. Given this diagnostic background, GC is recognized as a malignant disease with a poor prognosis, with a 5-year survival rate of <30% (2). The emerging field of biology of tumor-initiating cells (TICs) promises to be a critical focus of basic research that could yield important new tools for overcoming GC.

Recent progress in the identification and isolation of stem cells in normal organs has been utilized in several biological and medical fields to explore new procedures and therapies, such as tissue regeneration. In the cancer field as well, a 'stem cell theory' concept is being enthusiastically investigated, and several lines of evidence have accumulated suggesting the existence of TICs, even in solid tumors such as brain, breast or colon cancer (3,4), as well as in hematopoietic malignancies, already well confirmed (5). However, while several researchers have demonstrated the existence of TICs with molecular markers such as CD133 in isolated cells possessing tumor initiating-like properties (6,7), there are still major questions about solid-tumor TICs that are important to the understanding of carcinogenesis, proliferation, invasion, and metastasis in cancer biology.

'Side-population' (SP) cells, first described by Goodell *et al* (8), are a small subpopulation of cells with enriched stem cell activity and a distinctive expression profile of ATP-binding cassette (ABC) transporters. These cells are refractory to Hoechst 33342 dye-staining due to the ABCG2 (BCRP1) transporter (9), and are resistant to certain drugs due to other ABC transporters (10). The SP phenotype was identified in each normal adult tissue, suggesting that SP is enriched in primitive and non-differentiated cells (11,12). On the other hand, concurrent studies have shown isolation of SP cells from various kinds of human solid cancers such as lung cancer, mesenchymal neoplasms, acute myelogenous leukemia, neuroblastoma, and glioma (13-17). Thus, SP characterization derived from cancer cells will reach a novel concept 'stem cell theory', as well as normal stem cells, and many studies

have already suggested that the SP will be enriched fraction of TICs in cancer cells.

The present study is the first to search for GC cells exhibiting the SP phenotype, which would possibly constitute TICs with stem-like cell properties. In the study, we report identification and isolation of the SP phenotype in clinical GC tissues as well as human GC cell lines, demonstrating specific characteristics such as elevated tumorigenicity *in vivo*, compared with 'main population' (MP) cells. These data demonstrate that SP in human GC contains TICs, and could provide new diagnostic tools and novel strategies for controlling this malignant disease.

Materials and methods

Cell culture. Human GC cell lines (MKN45, KATOIII, MKN74, MKN28 and MKN1) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). MKN45 and KATOIII were derived from poorly differentiated adenocarcinomas. MKN74 and MKN28 were from a moderately and well differentiated adenocarcinoma, respectively. MKN1 is a gastric adenocarcinoma cell line obtained from a metastatic lymph node and has the ability to differentiate in two directions: to adenomatous or to squamous cells.

These cell lines were cultured in RPMI-1640 (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 U/ml penicillin-streptomycin G, and 100 μg/ml streptomycin (Invitrogen). In all experiments, cells were cultured at 37˚C in a humidified 5% CO_2/95% air atmosphere.

Flow cytometry analysis. To identify and isolate the SP and MP fractions, the cells were removed from the culture dish using trypsin and EDTA, pelleted by centrifugation, washed with PBS, and resuspended at 37˚C in Hanks' balanced salt solution (HBSS) containing 2% FBS and 10 mM HEPES (Invitrogen). The cells were then stained with Hoechst 33342 dye (Sigma) at a concentration of 4 μg/ml for MKN74, 5 μg/ml for MKN28 and KATOIII, and 10 μg/ml for MKN1, MKN45 and a clinical specimen of human GC. The cells were then incubated for 60 min at 37˚C either alone or with 50 μM of reserpine (Daiichi Pharmaceutical, Tokyo, Japan), and then suspended in HBSS containing 2% FBS, 100 U/ml penicillin streptomycin G, and 100 μg/ml streptomycin and 10 mM HEPES. Next the cells were passed through a 40-μm mesh filter, and maintained at 4˚C until flow cytometry analysis. They were subsequently counterstained with 1 μg/ml of propidium iodide (Sigma) to label the dead cells. Then, $1x10^6$ viable cells were analyzed and sorted using a FACSVantage™ SE (Becton-Dickinson, San Jose, CA). The Hoechst 33342 dye was excited at 357 nm, and its fluorescence was dual-wavelength analyzed (blue, 402-446 nm; red, 650-670 nm). The distribution of cells was analyzed using by FlowJo software (Tomy Digital Biology, Tokyo, Japan).

To examine the expression of the differentiation and epithelium markers CD15 and EMA (epithelial membrane antigen) in the MKN45 SP and MP cells, the cells were washed and incubated with mouse monoclonal antibodies (CD15-PE, EMA-FITC) for 30 min at 4˚C. They were then washed and counterstained with 1 μg/ml of propidium iodide to label the dead cells.

Quantitative RT-PCR. Total RNA from these cells was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of total RNA was determined using a Nano Drop (Nano Drop Technologies, San Diego, CA). Briefly, purified total RNA was reverse-transcribed to generate double-stranded cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster, CA), and the expression of human MDR1 and BCPR1 was analyzed using the Applied Biosystems 7500 Fast real-time PCR System (Applied Biosystems). TaqMan Gene expression assay primer and probe mixes were used for GAPDH, MDR1 and BCRP1 (assay IDs Hs99999905, Hs00184491 and Hs00184979, respectively; Applied Biosystems). GAPDH was detected using TaqMan primers and probes and was used as the control gene. The thermal cycling reaction was followed by incubation at 95˚C for 20 sec and 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. Data were collected using analysis software (Applied Biosystems). The expression level of each gene was determined relative to the value of the expression of gene in MP.

Immuno-cytochemistry staining. The cells were fixed with 4% paraformaldehyde/PBS for 30 min at 4˚C and then washed three times with cold PBS and with cold 0.2% Triton-X/PBS for 10 min at 4˚C. The chamber slide was washed with cold 0.2% Triton-X100/PBS for 5 min at 4˚C three times and with Blocking Reagent-N101 (Wako Pure Chemical Industries, Tokyo, Japan) for 30 min at room temperature. The primary antibodies were used simultaneously with anti SSEA-4 (stage-specific embryonic antigen-4; R&D Systems, Abington, UK). Isotype-matched mouse antibodies were used as controls. After washing, primary antibody binding was detected using the corresponding Alexa Fluor 568 conjugated secondary antibodies (Invitrogen). Staining was observed using fluorescence microscopy. The cells were counterstained with mounting medium containing Hoechst 33342 dye to identify all the nuclei.

Survival studies with anticancer agents. MKN45 SP and MP cells cultured in medium as described above were incubated and treated with cisplatin (6 mM), 5-fluorouracil (6 mM), or doxorubicin (0.25 mM). After 48 h of exposure to the chemotherapeutic agents, the viability of the cells was determined using a Cell Count Reagent SF (Nacalai Tesque, Inc., Kyoto, Japan) method, and the absorbance values were detected using a Micro Plate Reader Model 550 (Bio-Rad Laboratories, Hercules, CA).

Transplantation experiment. Sorted SP and MP cells were collected, and cells were resuspended in HBSS. Cell suspension was then mixed with Matrigel (1:1) (Becton-Dickinson). This cell-Matrigel suspension was then subcutaneously injected into 6-8-week-old immunodeficient NOD/Shi-*scid*,IL-2Rγ^{null} (NOG) mice (Central Institute for Experimental Animals, Kawasaki, Japan) under anesthesia. Groups of mice were inoculated with non-sorting cells at $1x10^6$, and $1x10^5$ or SP cells at $1x10^4$, $1x10^3$ and $1x10^2$ or MP cells at $1x10^4$, $1x10^3$

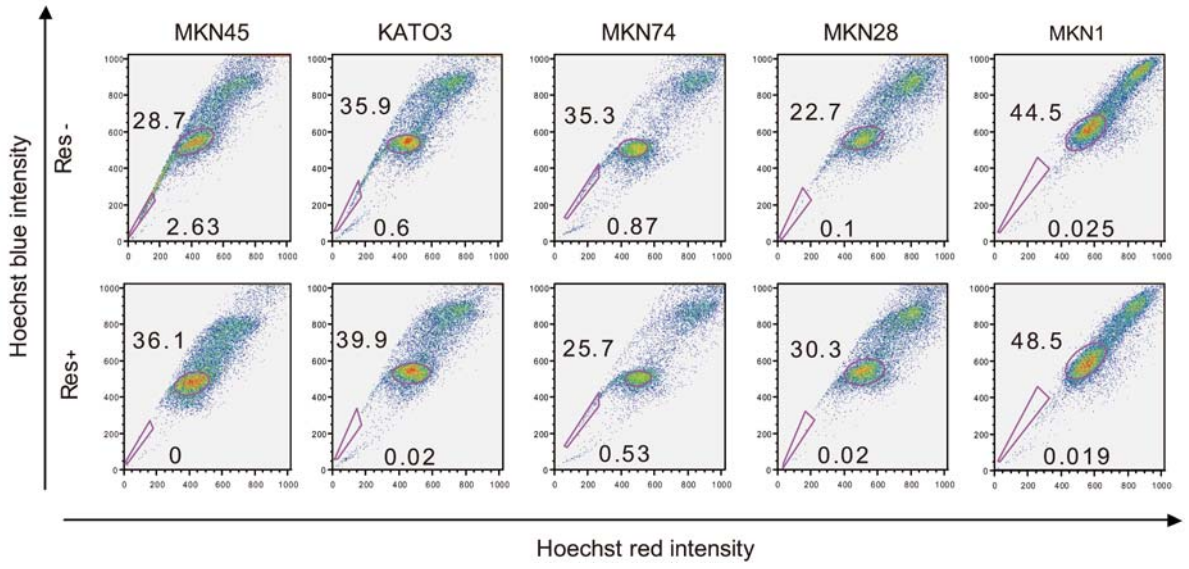

Figure 1. Analysis of side population (SP) cells in five human GC cell lines. These cell lines (MKN45, KATOIII, MKN74, MKN28 and MKN1) were stained using Hoechst 33342 dye and analyzed using flow cytometry. The SP and MP cells are outlines and shown as a percentage of the total cell population. The SP cells disappeared in the presence of reserpine (bottom panel).

and $5x10^2$ (five mice per group). Tumor growth was monitored every 2 days after second week of inoculation. Tumor volume was calculated by the weight, and re-analyzed by the Hoechst 33342 dye efflux assay as described above. 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-eosin staining.

Clinical specimen implantation. Human GC was obtained from patients undergoing surgical resection with informed consent and patients' agreement (approved by the ethics committee of Keio University; no. 17-47). Briefly, fresh primary GC specimens obtained within 60 min of surgery were rinsed and mechanically minced in HBSS. Because GC samples are very small fragments (<2 mm^3), these samples were implanted subcutaneously into NOG mice. GC engraftments were established and FACS analysis was performed as described above. SP and MP cells were harvested and suspended in HBSS. Both fractions were then injected into NOG mice for Tumor 1 at $2x10^5$ SP cells and $2x10^5$ MP cells, for Tumor 3 at $1x10^4$ SP cells and $1x10^4$ MP cells, for Tumor 5 at $1x10^4$ SP cells and $1x10^4$ MP cells. The mice were monitored twice a week for palpable tumor formation and were sacrificed at 8 weeks after transplantation for detecting the tumor formation.

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

Results

SP phenotype in GC cell lines. Flow cytometry analysis with Hoechst 33342 dye staining demonstrated that identification and isolation of SP and MP cells was successfully performed in five different kinds of human GC cell lines (Fig. 1). The SP gate was defined as the diminished region in the presence of reserpine, which blocked the activity of Hoechst 33342 dye transporter. All five human GC cell lines analyzed in this study contained a distinct fraction of SP cells ranging from 0.02±0.001 to 1.93±0.16% of the population (Table I). Interestingly, MKN45 representing an immature cell type derived from poorly differentiated adenocarcinoma, contained the highest percentage of SP cells of the five cancer cell lines studied. Thus, the size of the SP fraction within a tumor may correlate with the tumor's degree of differentiation. Further experiments were performed using MKN45 cell line, since MKN45 contained the highest percentage of SP cells among the five cancer cell lines.

Table I. Percentages of SP fractions in various human cancer cell lines of gastrointestinal organ system.

Human gastric cell lines	Percentage of SP fraction mean ± SD
MKN1	0.02±0.001%
MKN28	0.15±0.06%
MKN74	0.17±0.09%
KATOIII	0.81±0.29%
MKN45	1.93±0.16%

Results of flow cytometry analysis are shown in Fig. 1. SP, side population.

Gene expression analysis. Expression of ABC transporters: MDR1 and BCRP1 has been shown in primitive cells and associated with its capacity to export a broad range of cytotoxic drugs. In particular, BCRP1 has been implicated in the

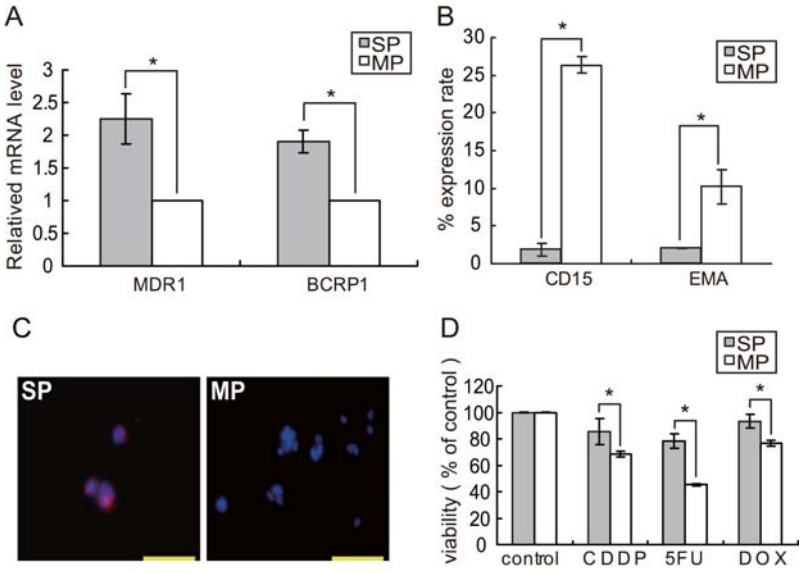

Figure 2. *In vitro* characteristics of MKN45-derived SP and MP cells. (A) Relative mRNA expression of genes MDR1 and BCRP1 in MKN45 SP and MP cells was determined using a quantitative real-time RT-PCR amplification analysis. The result was analyzed by the relative quantification ($\Delta\Delta C_T$) method. (B) Flow cytometry analyses showed the expression of the cell surface markers CD15 and EMA in the MKN45 SP and MP cells. (C) Immuno-cytochemistry showed the expression of stem cell marker SSEA-4. The cells were also labeled with Hoechst dye to identify the nuclei (scale bar, 100 μm). (D) Chemo-resistance of MKN45 SP and MP cells to anticancer drugs. The cells were maintained in supplemented medium for 48 h and then incubated with cisplatin (6 mM), 5-fluorouracil (6 mM) or doxorubicin (0.25 mM) for 48 h. Cell viability was then determined using a cell count assay (black bar, SP cells; white bar, MP cells). Each value represents the mean ± SEM (n=5). *P<0.05, t-test, statistical significance.

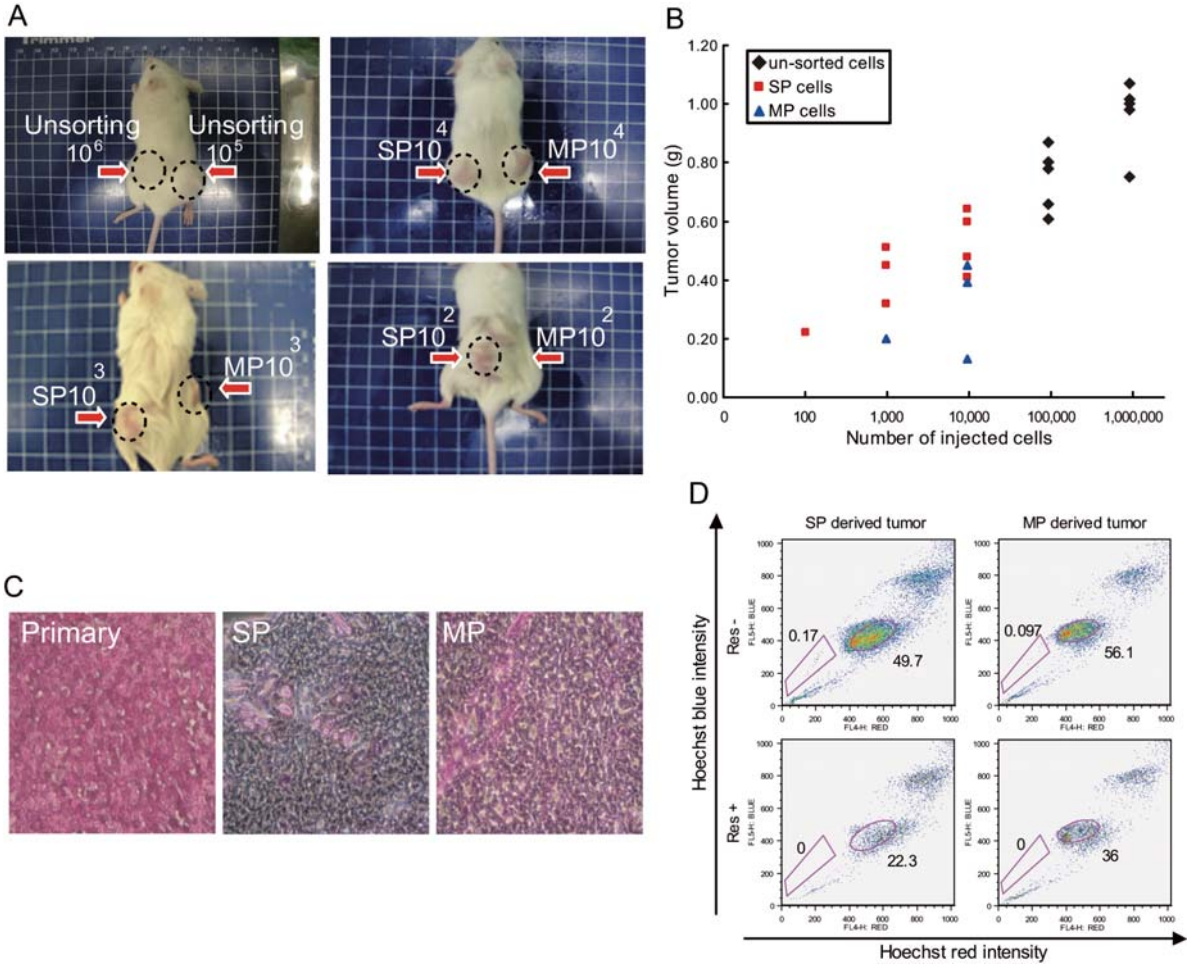

Figure 3. Tumorigenicity in MKN45 SP cells and MP cells. (A) Representative subcutaneous tumors (arrows) produced by the injection of $1x10^6$ unsorted cells (left) and $1x10^5$ unsorted cells (right), $1x10^4$ SP cells (left) and $1x10^4$ MP cells (right), $1x10^3$ SP cells (left) and $1x10^3$ MP cells (right), 100 SP cells (left) and 100 MP cells (right). (B) Growth of tumor in NOG mice with SP or MP cells (n=5). (C) Hematoxylin-eosin staining of cancers derived from SP, MP and primary cancer cells (magnification x200). (D) Re-analysis of SP-derived tumor by the Hoechst 33342 dye efflux assay.

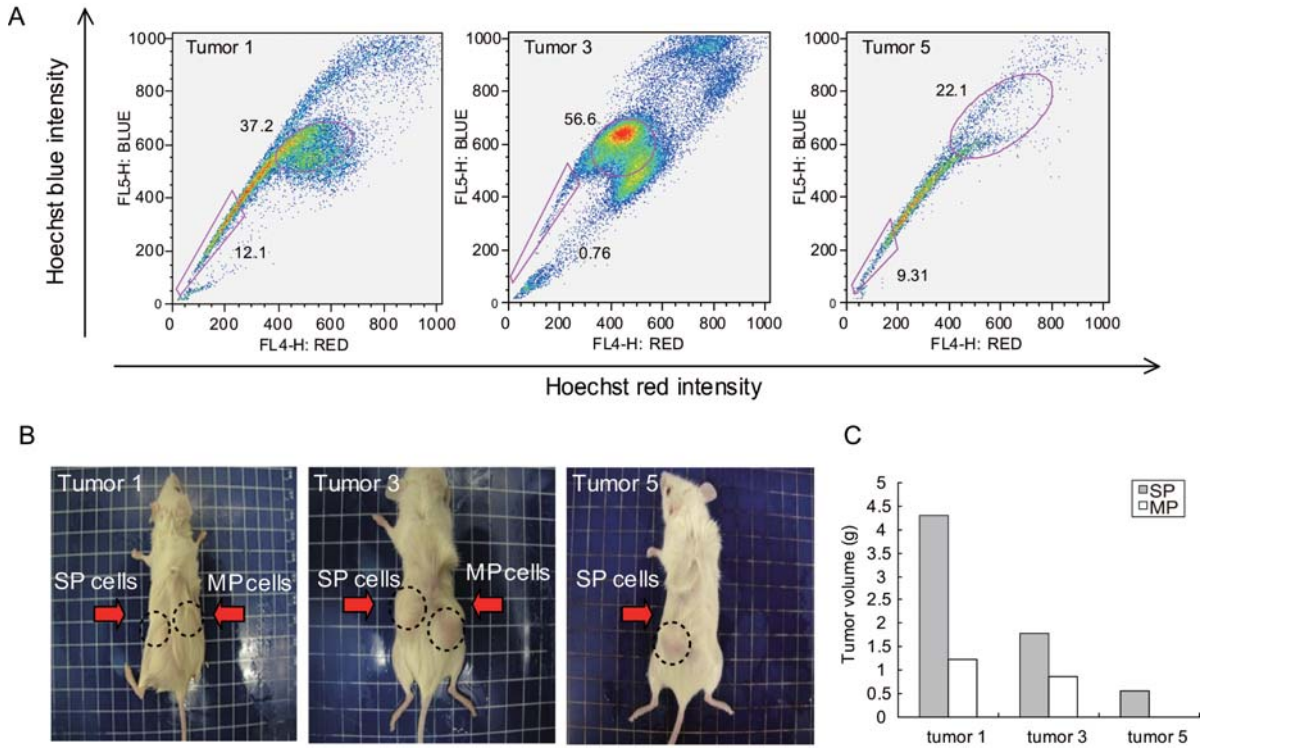

Figure 4. SP phenotype in clinical GC specimens. (A) Human GC specimens contain SP cells. Cells from three representative GC were stained with Hoechst 33342 and analyzed by flow cytometry. (B) Representative subcutaneous tumors (arrows) produced by the injection of Tumor 1 $2x10^5$ SP cells (right) and $2x10^5$ MP (left) cells, Tumor 3 $1x10^4$ SP cells (right) and $1x10^4$ MP cells (left), Tumor 5 $1x10^4$ SP cells (right) and $1x10^4$ MP cells (left). (C) The evaluation of SP-originated and MP-originated tumor size in NOG mice.

high Hoechst 33342 dye efflux capacity that marks the SP phenotype. These genes were up-regulated in SP relative to MP cells; MDR1 (2.21±0.47-fold) and BCPR1 (1.9±0.17-fold) ($P<0.05$) (Fig. 2A).

Cell surface marker expression profiles determined by FACS and immuno-cytochemistry. FACS analysis revealed that cell surface markers for differentiation are down-regulated in SP cells (CD15: 1.9±0.9%; EMA: 2.1±0.1%) relative to MP cells (CD15: 26.4±1.1%; EMA: 11.4±2.1%) with statistical significance ($P<0.05$) (Fig. 2B). Immuno-cytochemical staining for SSEA-4, a non-differentiation marker, was positive in SP and negative in MP (Fig. 2C). These cell surface marker results suggest that the SP phenotype might represent a non-differentiated state relative to the MP phenotype.

Chemo-resistance of SP cells to anticancer agents. The chemo-resistant ability of SP cells has been reported to depend mainly on ABC transporters (18). To determine whether SP cells resist ABC transporter-independent anticancer drugs more than MP cells do, we tested cisplatin, 5-fluorouracil (5-FU) and doxorubicin because they are generally used for the treatment of GC (Fig. 2D). SP cells were more chemo-resistant than MP cells, especially to 5-FU, exhibiting a cell survival rate of 78.5±5.4% after 48-h incubation, compared to 45.4±0.9% in MP cells ($P<0.05$). These data suggest that SP cells may be more resistant to anticancer drugs generally than are MP cells.

Tumorigenicity of SP phenotypic subpopulations in vivo. Tumorigenicity was examined using immune-deficient NOG mice (19), into which $1x10^2$-$1x10^4$ SP or MP cells of MKN45 origin were subcutaneously transplanted (Table II). Non-sorted MKN45 cells formed xenografts in NOG mice at $1x10^5$ or $1x10^6$ cells. Transplantation of $1x10^2$ MP cells consistently failed to form tumors in all mice (n=5), while $1x10^3$ or $1x10^4$ MP cells showed tumor formation in one of five or three of five mice. In contrast, transplantation of $1x10^2$ SP cells produced tumors in one of five mice. $1x10^3$ or $1x10^4$ SP cells transplantation into NOG mice showed tumor formation in three of five or four of five mice (Fig. 3A). Tumor volume of each xenograft is described in Fig. 3B. The mean weight of tumors formed by SP cells was 0.47±0.13 g, in comparison, the mean weight of tumors by MP cells was 0.29±0.14 g, ($P<0.05$). This result indicates that xenografts derived from SP cells showed significantly higher tumor-initiating capacity after transplantation than those from MP cells.

Histological analysis of the SP-originated tumors and MP-originated tumors showed similar to primary tumor (Fig. 3C). In addition, FACS analysis for these xenografts suggested that the tumors derived from SP cells might generate both SP and MP cells; however, those derived from MP cells generated almost exclusively MP cells (Fig. 3D).

Tumorigenicity of SP cells derived from clinical GC specimens. Five clinical human GC specimens were transplanted into NOG mice, yielding tumor-forming xenografts (Table III). SP cells from three xenografts (Tumor 1, Tumor 3 and Tumor 5) were found to constitute average of 7.39% in total cells (Fig. 4A). When SP and MP cells were transplanted subcutaneously into NOG mice, tumor formation was also

Table II. Tumorigenicity of SP and MP cells in NOG mice.

	Cell numbers of injection				
	100	$1x10^3$	$1x10^4$	$1x10^5$	$1x10^6$
Unsorted cells				5/5	5/5
SP cells	1/5	3/5	4/5		
MP cells	0/5	1/5	3/5		

SP and MP cells were isolated separately and injected subcutaneously into NOG mice. Tumor formation was observed for 8 weeks after injection.

confirmed within 8 weeks. SP cells formed a tumor of average of 3.15 times in comparison with the MP cells, suggesting that SP cells even from a clinical sample might have higher tumorigenic potential compared to MP cells (Fig. 4B and C). These results suggest that heterogeneously distributed tumors with various functions even in clinical GC specimens may consist of a distinct tumorigenic population amid a dominant population of differentiated tumors that might have decreased tumorigenic potential.

Discussion

The recent finding of SP cells in human normal and cancer tissues led to the hypothesis that solid carcinomas may originate from somatic stem or progenitor cells having non-differentiated properties with cancer cell potential, and several studies have already suggested the existence of TICs in malignant tumors in brain, breast, colon etc. Our finding that TICs can be enriched by isolating SP cells from GC has several important implications. All five human GC cell lines analyzed in this study contained SP cells, and MKN45, representing an immature cell type derived from poorly differentiated adenocarcinoma, contained the highest percentage of SP cells of the five cancer cell lines studied. Thus, the ratio of the SP fraction within a tumor may correlate with the tumor's degree of differentiation.

In this study, over-expression of two ABC transporter genes, MDR1 and BCRP1, is suggested in SP cells relative to MP cells. Consistent with studies that show BCRP1 to be a molecular determinant of the SP phenotype, expression of BCRP1 mRNA was markedly higher in SP cells. In addition, chemo-resistance of SP cells to anticancer drugs was observed, suggesting a cause of drug-resistance through increased expression of MDR1 (20-22) in gastrointestinal tumors or BCRP1 in breast cancer (23-25) or lung cancer (26). BCRP1 expression has also been found in SP cells derived from adult human pancreatic islets of Langerhans (27) and in normal hematopoietic SP cells (28,29).

SP cells showed expression of SSEA-4, a stem cell marker in SP cells, and low levels of both an epithelial marker (EMA) and a differentiated marker (CD15), suggesting a non-differentiated state. In addition to cell proliferation *in vitro*, tumorigenic activity of SP cells was also significantly higher than MP cells in NOG mice, as indicated by the significantly different weights of their respective tumors. Furthermore, 100 SP cells enabled tumor formation in NOG mice, but 100 MP cells could not form tumors. As reported by several investigators using cancer cell lines *in vitro* and *in vivo* (30), we observed repopulation from SP cells to a mixture of MP/SP cells with almost the original proportion of these two cell types, whereas repopulation from MP cells yielded only a small fraction of SP cells. These results indicate that a tumor hierarchy exists in which SP cells can generate both SP and MP cells; this is in accordance with previous observations that the SP fraction can divide asymmetrically and display a capacity of self-renewal (31). The consistency between our findings *in vitro* and *in vivo* may offer insights into TICs characteristics. Preliminary investigation using three clinical GC specimens in NOG mice revealed tumorigenic potential in GC SP cells. Our experiments with GC-derived SP and MP cells also demonstrated high tumorigenicity and repopulation ability of SP cells, suggesting clinical usefulness of this system for the study of TICs, even if the clinical specimens came from a few patients at present.

We conclude that SP cells demonstrate TICs capacity in GC cell lines and possibly in clinical GC specimens. Our data suggest a hierarchy that TICs proliferate with differentiation to become differentiated GC cells, pathologically recognized as cancer cells. Further studies on the identification and characterization of TICs using clinical GC specimens are required for this valuable method, which might contribute to the development of novel therapeutic strategies.

Table III. Engraftment of human gastric cancers into NOG mice.

Sample	Origin	Mouse tumor formation	Diagnosis	Stage	TNM
Tumor 1	Primary tumor	Yes	tub2+por1	IV	T1(SM)N1H1
Tumor 2	Primary tumor	No	por2>tub2	IV	T3N2CY1
Tumor 3	Primary tumor	Yes	por2>muc	IIIB	T3(SE)N2M0CY0
Tumor 4	Primary tumor	No	tub2>pap	IIIA	T3(SE)N1
Tumor 5	Primary tumor	Yse	por2	IIIB	T3(SE)N2M0

In vivo growth characteristics of human clinical GC specimen. Mice were injected with human clinical GC specimen minced into <2 mm^3 pieces. Cells from three xenografts were isolated by flow cytometry as described in Fig. 4.

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