CD13, CD133, CD24, CD44, EpCAM, ABCG2, Nanog, and Bmi-1. The expression of these molecules was slightly higher in SP cells than in NSP cells. In addition, the differences in expression levels of CD133, CD24, and CD44 between SP and NSP cells were slightly larger in HAK-1B than HAK-1A cells (Fig. 4a and 4b). The other putative CSC markers, such as CD90 and Oct-4, were not expressed. As to hepatocyte markers, CYP3A4 was expressed in HAK-1A and HAK-1B, but albumin was not expressed in either cell line.

**Discussion**

The present study utilized two HCC cell lines showing clonal dedifferentiation that were established at our department from a single nodule-in-nodule HCC (HAK-1A, HAK-1B), and which are unique in the world. Our aim was to study the SP cell fractions, which are considered universal markers for CSCs [15-17], in these two cell lines to clarify the relationship between CSCs and clonal dedifferentiation

SP cells from HAK-1A, which was established from a part of the well-differentiated HCC, represented only 0.2% of total cells, an extremely low percentage. However, in SP cells from the HAK-1B cell line, which is a part of the poorly-differentiated HCC derived from dedifferentiation of HAK-1A, the SP fraction was 0.9%, 4.5 times higher than in HAK-1A. Further, our analysis of the putative CSCs markers CD133, CD90, EpCAM and CD13 found no expression of CD90 or EpCAM in either HAK-1A or HAK-1B, while CD13 and CD133 was expressed in HAK-1B alone. In addition, while there was no difference in the expression of CD133 between SP and NSP cells in HAK-1B, CD13 expression was apparently higher in HAK-1B SP cells (21.7%) than HAK-1B NSP cells (8.9%). Haraguchi et al. [20] have reported that CD13 was an abundantly expressed marker in SP cells from the HCC cell lines HuH7 and PLC/PRL/5. This fraction existed primarily during the $G_0$ phase of the cell cycle, and exhibited high tumorigenicity and drug resistance. Our findings suggest the possibility that CD13 could also be a CSC

marker for HAK-1B cells. The significance of CD13 in HAK-1B should be further studied.

Analysis of biological features revealed that HAK-1B SP cells possess some properties of CSC, such as higher sphere forming ability and tumorigenicity, as compared with NSP cells. However, HAK-1B SP cells lacked the following four features of CSC. Firstly, HAK-1B SP cells lacked high drug resistance and colony forming ability. Drug treatment usually increases the percentage of CSC, but our results did not always show such tendency, i.e., PEG-IFN-α2b treatment increased the percentage of SP cells, but 5-FU treatment increased the percentage of SP cells. The reason for this contradictory result is not clear and should be further elucidated. Secondly, the percentage of $G_0/G_1$ phase is usually higher in CSC, but our cell cycle analysis revealed no difference in rates between SP and NSP cells. Thirdly, differentiated cells do not generate CSC, but in our study, NSP cells could generate SP cells. Fourthly, no difference in microarray analysis and slight difference in qRT-PCR analysis were observed in stemness gene expression between SP and NSP cells.

These results suggest that HAK-1B SP cells do not fulfill the criteria to be considered CSCs.

As described above, it is recognized that the SP cell fraction in a variety of tumors is rich in CSC [15-17], but some reports question whether there is a relationship between SP cells and CSCs. Burket et al. [30] examined SP and NSP cells in 4 gastrointestinal cancer cell lines and found that CD34 was expressed in the NSP fraction but not in the SP fraction, however no significant differences were observed in any other category, including colony formation, tumorigenicity, or multi-lineage differentiation.

Two main theories are still being debated with regard to the histogenesis of HCC. For many years the observation of preneoplastic nodules in HCC induced experimentally by exposure to chemicals supported the dedifferentiation hypothesis, i.e. the theory that HCC was derived from the dedifferentiation of adult hepatocytes. Further, the recent

discovery of the role of small oval cells in the process of carcinogenesis has led to development of the maturation arrest hypothesis, which suggests that HCC derives from the maturation arrest of hepatic stem cells, and analysis of HCC cells has indicated the presence of cells with stem-cell-like properties [31]. It is also suspected that dedifferentiation may cause CSCs. As of yet there have been no reports on the relationship between dedifferentiation and CSCs. The present study suggested a relationship between dedifferentiation and expression of CSC markers, but many aspects of the mechanisms of dedifferentiation remain unclear, and many different mechanisms have been reported to explain the abnormal proliferation and dedifferentiation of liver cells in HCC pathogenesis. Among these the most common are (i) inactivation of p53, p14 and p16, (ii) overexpression of cyclin D1/Cdk4, insulin-like growth factor-II or c-MET, or (iii) activation of the Ras/mitogen activated protein kinase (MAPK), transforming growth factor (TGF)-β signaling or Wnt/β-catenin signaling [32-37]. Further studies are required to clarify the mechanisms underlying dedifferentiation, including the relation between dedifferentiation and CSCs.

In conclusion, the present study found that the SP cell fraction was 4.5 times higher in the HAK-1B cell line than in the HAK-1A cell line, and that the expression of CD13 and CD133, which are considered to be CSC markers, was observed only in HAK-1B. Also, a comparison of HAK-1B SP and NSP cells found that CD13 expression was higher in the SP fraction, suggesting a possible relationship between the expression of CSC markers and dedifferentiation. Moreover, HAK-1B SP cells showed more malignant biological features, such as higher sphere forming ability and tumorigenicity as compared with NSP cells. However, with the exception of these biological features, no other CSC characteristics were clearly observed in the HAK-1B SP cells. Thus, the concept of the SP cells as a universal marker for CSC may not apply to HAK-1A and HAK-1B. We plan to examine the relationship between dedifferentiation and CSC using other CSC markers, such as CD13 and CD133.

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Figure legends

Fig. 1a. SP cells are considered to be abolished by reserpine. The ratio of SP cells in the HAK-1A cell line was 0.207%. There was almost no expression of CD133, CD90, EpCAM or CD13 in SP and NSP cells from HAK-1A.

Fig. 1b. The ratio of SP cells in the HAK-1B cell line was 0.9%. In the HAK-1B cell line, CD133 was expressed in 4.6～6.4% of SP cells and 3.9～5.3% of NSP cells. CD13 expression was higher in SP cells (21.7%) than in NSP cells (8.9%). CD90 and EpCAM expression was low in both SP and NSP cells. The experiments were repeated twice, and almost identical results were obtained.

Fig. 2. After culturing HAK-1A SP cells or HAK-1B SP cells for 1 week, the percentage of HAK-1A SP cells and HAK-1B SP cells decreased to 1.9% and 7.3%, respectively. In contrast, culture of HAK-1A and HAK-1B NSP cells generated a small population of SP cells in HAK-1A (0.1%) and HAK1B (0.7%). The experiments were repeated twice, and almost identical results were obtained.

Fig. 3a. Cell proliferation was significantly higher in SP cells than in NSP cells at 24 h, 48 h, 72 h, 96 h, or 120 h. The experiments were repeated twice, and almost identical results were obtained.

Fig. 3b. Cell cycle analysis found no apparent difference in $G_0$-$G_1$/ S/ $G_2$-M ratios between HAK-1B SP and NSP cells. The experiments were repeated twice, and almost identical results were obtained.

Fig. 3c. Drug resistance to CDDP, 5-FU or PEG-IFN-α2b was compared between SP and NSP cells from HAK-1B at 48 h and 96 h. The viability of SP cells was significantly lower than NSP cells after 96 h treatment with 0.75 μM or 1.5 μM 5-FU, or 96 h treatment with 500 IU/mL PEG-IFN-α2b. No other significant differences were observed between SP and NSP cells. The experiments were repeated at least three times, and almost identical results were obtained.

Fig. 3d. After exposure of HAK-1B cells to PEG-IFN-α2b for 72 h, the percentage of SP cells decreased as compared with control. Conversely, the percentage of SP cells increased when HAK-1B cells were treated with 5-FU for 72 h. The experiments were repeated at least three times, and almost identical results were obtained.

Fig. 4a. Colony formation assay found no significant difference between HAK-1B SP cells and NSP cells. The experiments were repeated at least three times, and almost identical results were obtained.

Fig. 4b. Sphere formation was significantly higher in HAK-1B SP cells than NSP cells. The experiments were repeated at least three times, and almost identical results were obtained.

Fig 4c. Injection of 1, 5, or 10 x $10^3$ SP or NSP cells produced no tumors in NOD/SCID mice. In contrast, four mice that received 5 x $10^4$ SP cells and five mice that received 10 x $10^4$ SP cells developed tumors at 8 weeks. In addition, one mouse that received 5 x $10^4$ NSP cells and two mice that received 10 x $10^4$ NSP cells also developed small tumors

Fig. 5a and b. SP and NSP cells of HAK-1A and HAK-1B expressed mRNAs of CSC markers, such as CD13, CD133, CD24, CD44, EpCAM, ABCG2, Nanog, and Bmi-1, and hepatocyte marker, CYP3A4. The expression of CSC markers was slightly higher in SP cells than in NSP cells.

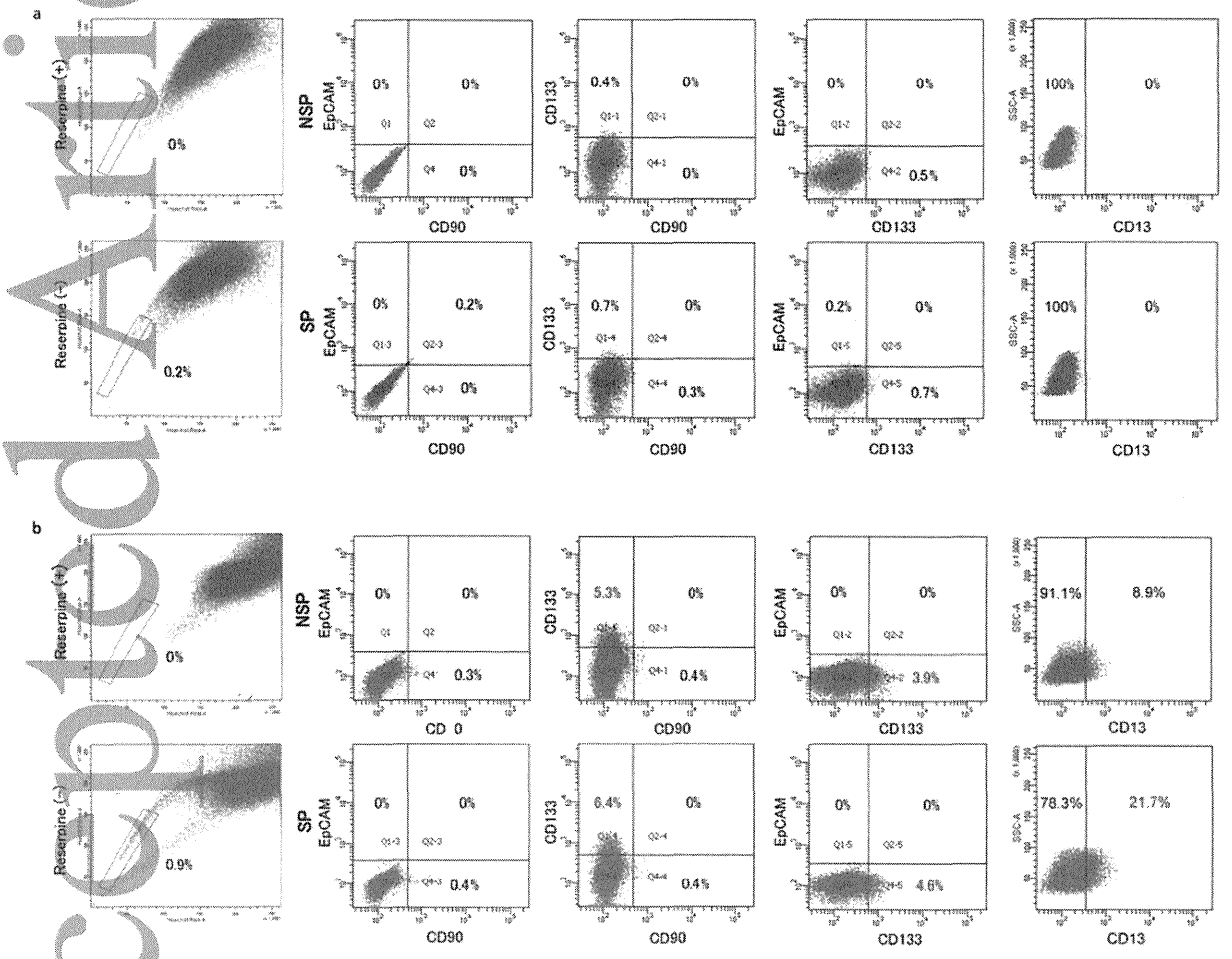
Table 1 Primer and probe mixes list for qRT-PCR analysis

| Gene name | Assay IDs |
|---|---|
| CD13 | Hs00174365_m1 |
| CD133 | Hs00195682_m1 |
| CD24 | Hs03044178_m1 |
| CD44 | Hs01075861_m1 |
| CD90 | Hs00174816_m1 |
| EpCAM | Hs00158980_m1 |
| ABCG2 | Hs01053790_m1 |
| Oct-4 | Hs03666771_m1 |
| Nanog | Hs04260366_m1 |
| BMI1 | Hs00201350_m1 |
| Alb | Hs00910225_m1 |
| CYP3A4 | Hs00604506_m1 |
| β-actin | Hs99999903_m1 |

Table 2. Summary of cDNA microarray of SP and NSP cells from HAK-1A and HAK-1B

Gene expression in SP cells and NSP cells sorted from HAK-1A or HAK-1B cells was analyzed by cDNA microarray, but no significant differences were observed between SP cells and NSP cells of either cell line with regard to stemness gene expression.

| | **HAK-1A** | **HAK-1B** |
|---|---|---|
| **Top Associated Network Functions** | RNA Post-Transcription Modification, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair (e.g., ↑FAM124A, C1ORF35, etc.) | Cancer, Drug Metabolism, Molecular Transport (e.g., ↑ALDH3A1, ATF7, IL33, etc.) |
| **Top 5 up-regulated molecules in SP vs. NSP** | GBP5 (×10.5), BMP3 (×8.4), SLITRK2 (×8.1), TMEM90B (×7.8) CUGBP2 (×7.6) | FGF2 (×13.1), ZNF311 (×12.6), ADH6 (×8.2), HPCA (×7.9) AKR1B10＊ (×7.6) |
| **Top 5 down-regulated molecules in SP vs. NSP** | ZNF646 (×11), SAA3P (×10.1), HTR2C (×7.5), UGT2B7 (×7.4), CCR9 (×7.4) | CACNG3 (×47.1), HNMT (×20.3), GAK (×11.6), C14ORF126 (×11.5), GGT5 (×10.7) |
| **Stemness gene expression in SP vs. NSP** | CD44 (×1.04), Oct-4 (×0.95), Bmi-1 (×0.87), ABCG2 (×0.83), CD24 (×0.54), EpCAM (×1.02) | CD44 (×0.93), Oct-4 (×0.84), Bmi-1 (×0.97), ABCG2 (×1.47), CD24 (×1.27), EpCAM (×0.84) |

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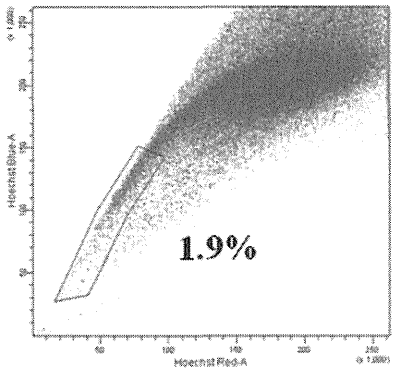
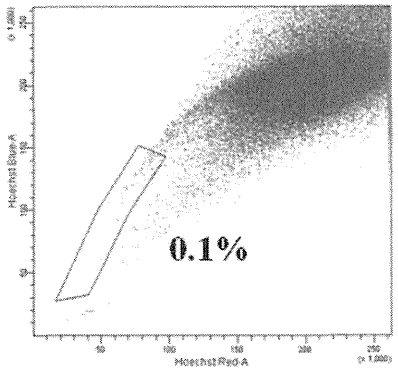
HAK-1A

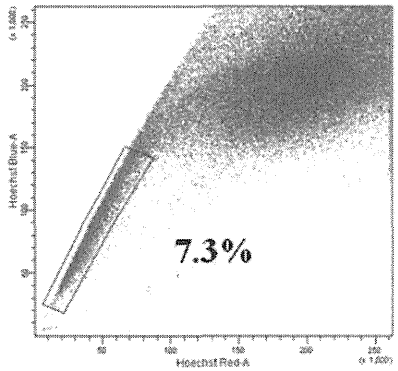



Sorted SP cells




Sorted NSP cells

HAK-1B

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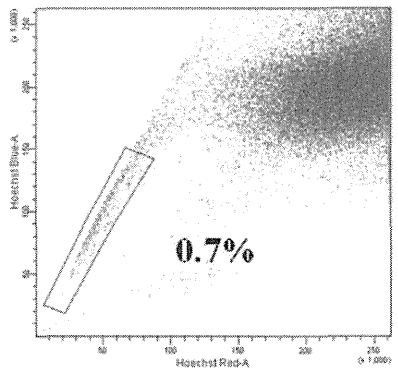


Sorted NSP cells

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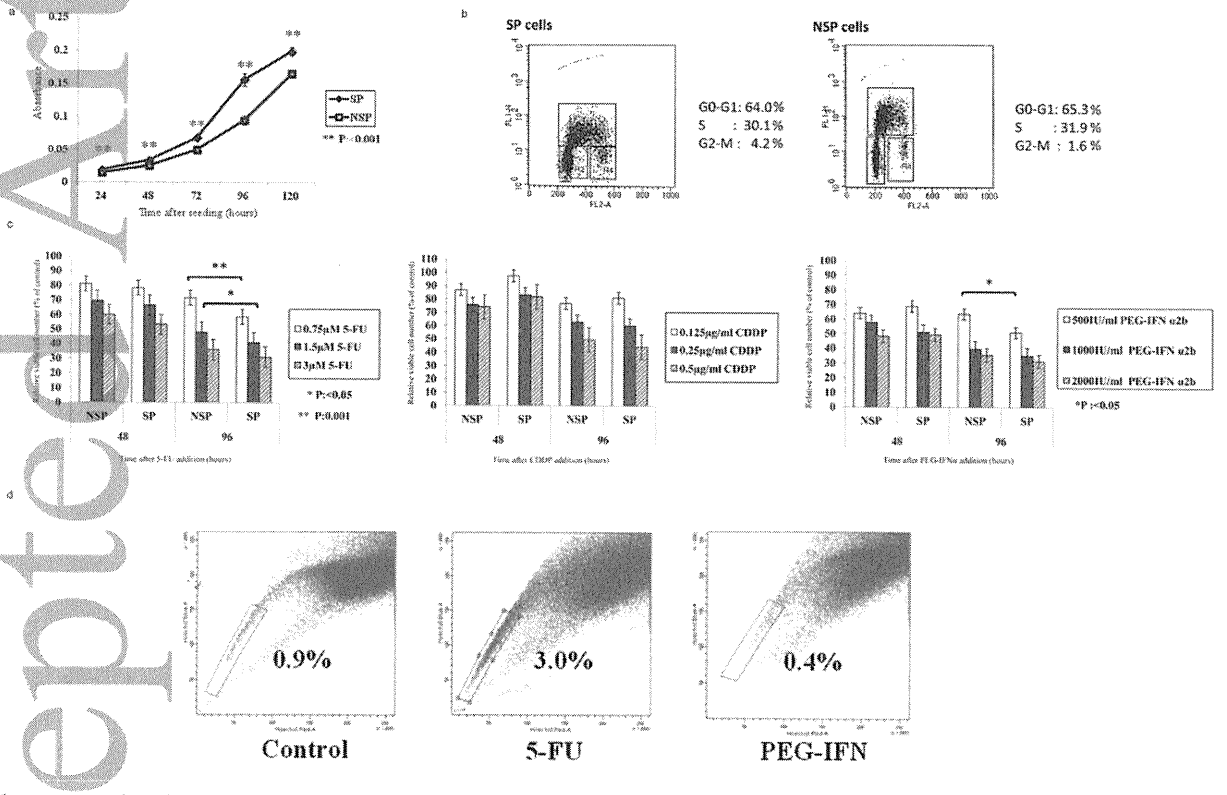


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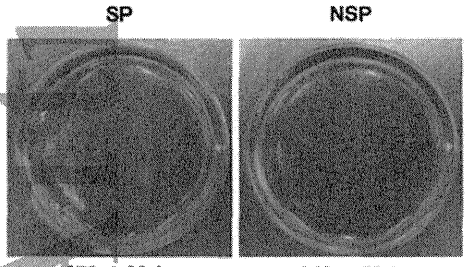
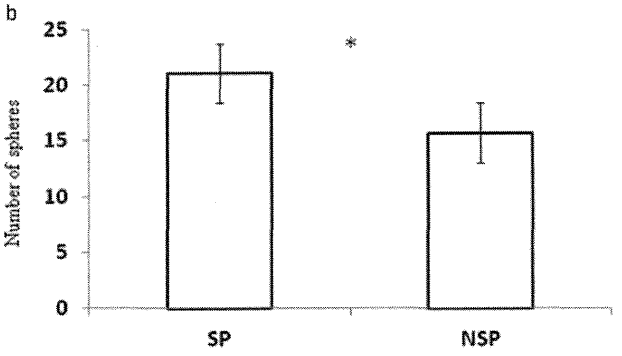
a



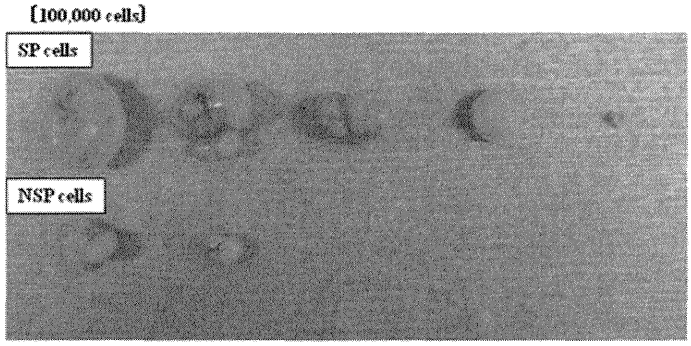

b




c

Tumorigenicity in NOD/SCID mice

| Cell type | Injected cell number | | | | |
|---|---|---|---|---|---|
| | 1,000 cells | 5,000 cells | 10,000 cells | 50,000 cells | 100,000 cells |
| SP | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 4/5 (80%) | 5/5 (100%) |
| NSP | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) | 1/5 (20%) | 2/5 (40%) |

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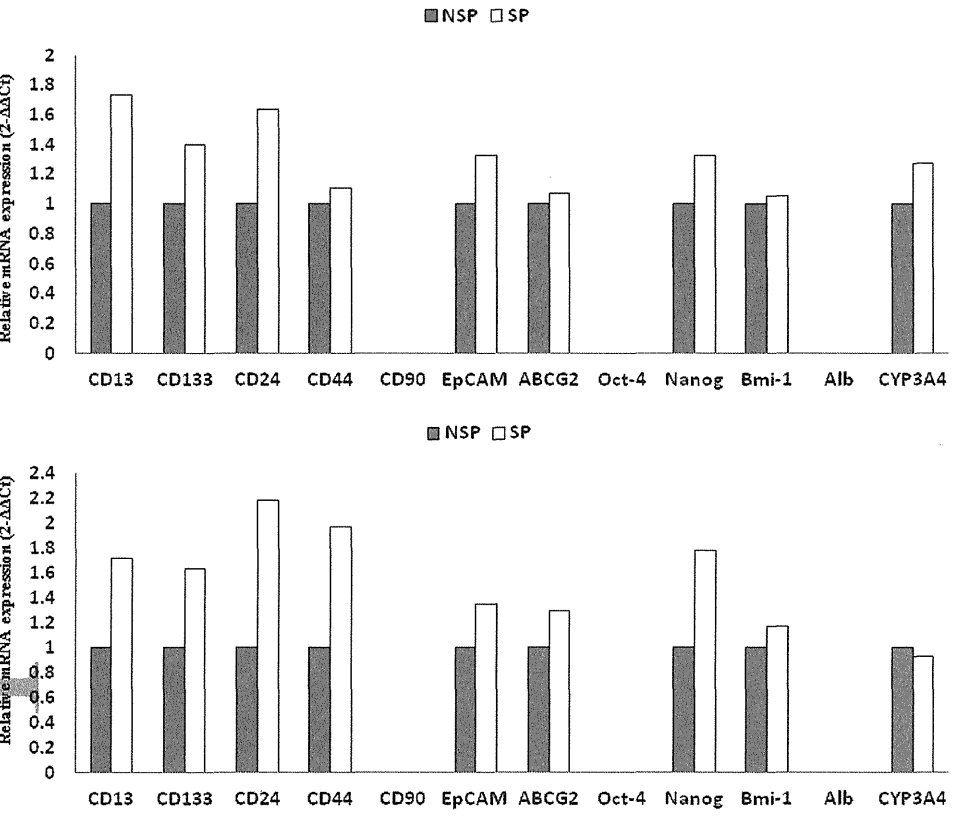


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# Antiproliferative effects of sorafenib and pegylated IFN-α2b on human liver cancer cells *in vitro* and *in vivo*

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**Abstract.** Novel therapeutic strategies are needed to treat patients with advanced hepatocellular carcinoma (HCC). Combination therapy of sorafenib and type I interferon (IFN) has substantial activity in patients with metastatic renal cell carcinoma. We investigated the antiproliferative effects of sorafenib in combination with pegylated interferon-α2b (PEG-IFN-α2b) on human hepatocellular carcinoma (HCC) cells *in vitro* and *in vivo*. A poorly differentiated HCC cell line derived from a patient with hepatitis C virus infection, HAK-1B and the moderately differentiated HCC cell line KIM-1 were used in this study. We demonstrated a synergistic antiproliferative effect of combination therapy on HAK-1B cells *in vitro*. In the *in vivo* study, a significant reduction of tumor volume and weight were observed in the combination group in both HAK-1B and KIM1 tumors, although synergistic effects were not clearly observed. The density of CD34-positive microvessels was significantly lower and cleaved caspase-3-positive apoptotic cell numbers were higher, in the sorafenib group and the combination group compared to the control or PEG-IFN-α2b group in both HAK-1B and KIM-1 tumors. Ki67 labeling index was significantly lower in the combination group compared to the control group in KIM-1 tumors. In conclusion, our results suggest that the combination therapy may be more effective for the treatment of HCC cases with variable sensitivity to antitumor effects of single therapy with either sorafenib or PEG-IFN-α2b.

## Introduction

Primary liver cancer, of which hepatocellular carcinoma (HCC) represents the major subtype accounting for between 85 and 90%, is the sixth most common tumor globally and the third most common cause of cancer-related death (1). Systemic treatment options for advanced HCC are limited and most deaths occur within 1 year of diagnosis (2-4).

Sorafenib is an oral multikinase inhibitor that was approved by the US Food and Drug Administration in December 2005 for the treatment of advanced renal cell carcinoma (RCC) and in November 2007 for the treatment of HCC. It has been shown to inhibit the activity of Raf kinase and several receptor tyrosine kinases, including vascular endothelial growth factor receptors (VEGFR)-1, 2 and 3, platelet-derived growth factor receptor (PDGFR)-α and β, FLT3, Ret and c-Kit. The intracellular signaling pathway Raf/MEK/ERK and the extracellular receptors VEGFR and PDGFR have been implicated in the molecular pathogenesis of HCC (5).

The Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial revealed efficacy of sorafenib in the treatment of HCC, i.e., both median survival and time to progression showed 3-month improvements by sorafenib therapy (6). Cheng *et al* (7) also reported the efficacy of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma. Combination therapy with sorafenib has a potential to improve the outcome of sorafenib monotherapy. Phase II trial of combination therapy of sorafenib and IFN-α has substantial activity in patients with metastatic RCC (8,9). The combination therapy of IFN-α and 5-fluorouracil is partly or completely effective in about 50% of the patients with advanced HCC (10). Type I interferon (IFN) has various effects, including anti-viral effects, antiproliferative effects and anti-angiogenic effects (11), and our laboratory previously reported the antiproliferative effect of IFN-α on human liver cancer cells *in vitro* and *in vivo* (12-14). In addition, type I IFN has suppressive effects on the occurrence of HCC, and the recurrence of HCC after curative treatment in patients with chronic hepatitis C virus infection (15-20). On the basis of above-described background, our current study examined the growth inhibitory effects of combination treatment of sorafenib and Pegylated IFN-α2b (PEG-IFN-α2b) on human HCC cell lines *in vitro* and *in vivo*.

## Materials and methods

*Cell line and cell cultures.* This study used two HCC cell lines [KIM-1 (21) and HAK-1B (22)], which were originally established and characterized in our laboratory and previously confirmed to retain morphological and functional characte-

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*Key words:* hepatocellular carcinoma, pegylated interferon-α2b, sorafenib, combination therapy, microvessel density

ristics of the original tumor. Both of these two cell lines were established from surgically resected HCC nodules. KIM-1 is a moderately differentiated HCC cell line, and HAK-1B is a poorly differentiated HCC cell line which was derived from a patient with hepatitis C virus (HCV) infection.

The cells were grown in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56˚C, 30 min) fetal bovine serum (FBS, Bioserum, Victoria, Australia), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco-BRL/Life Technologies Inc., Gaithersburg, MD, USA) and 12 mmol/l sodium bicarbonate, in a humidified atmosphere of 5% $CO_2$ in air at 37˚C.

*Sorafenib and pegylated IFN-α2b.* Sorafenib, kindly provided by Bayer Pharmaceutical Corporation (West Haven, CT, USA), was dissolved in dimethyl sulfoxide (DMSO) to create a 10 mM stock solution and stored at -20˚C for *in vitro* study. For the *in vivo* study, we prepared the solution at time of use.

PEG-IFN-α2b (PEG Intron®) was kindly provided by MSD K.K. (Tokyo, Japan). The specific activity of PEG-IFN-α2b was $6.4x10^7$ IU/mg protein.

*Effect of sorafenib alone or combination treatment of sorafenib and PEG-IFN-α2b on the proliferation of HCC and CHC cell lines in vitro.* The effects of sorafenib and/or PEG-IFN-α2b on the growth of the cultured cells were examined with colorimetry using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kits (Chemicon International Inc.) as described (12-14). Briefly, the cells ($1.5\text{-}5.5x10^3$ cells per well) were seeded on 96-well plates (Nunc Inc., Roskilde, Denmark), cultured for 24 h, and the culture medium was changed to a new one containing 0.2% DMSO (control) or sorafenib (0.3125, 0.625, 1.25, 2.5, 5, 10 or 20 μM), or both sorafenib (0, 1.25, 2.5 or 5 μM) and PEG-IFN-α2b (0, 2,000, 4,000, 8,000 IU/ml) (constant-ratio combination). After culturing for 72 h, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength at 570 nm and the reference wavelength at 630 nm. To keep the optical density within linear range, all experiments were performed while the cells were in the logarithmic growth phase.

Combination analysis was performed by using the method as described by Chou and Talalay (23), and the CalcuSyn software program (Biosoft, Cambridge, UK) for automated analysis. This program calculates the combination index (CI). A CI of 0.9-1.1 indicates a nearly additive effect, a CI of <0.9 a synergistic effect, a CI of >1.1 an antagonistic effect.

*Morphological observation.* For morphological observation under a light microscope, cultured HAK-1B cells were seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with or without 1.25 μM of sorafenib for 72 h, fixed for 10 min in Carnoy's solution, and stained with hematoxylin and eosin (H&E).

*Quantitative analysis of apoptotic cells induced by sorafenib and/or PEG-IFN-α2b.* HAK-1B and KIM-1 were cultured with the culture medium containing 0.02% DMSO or 2 μM of sorafenib for 72 h. For a study of combination therapy, HAK-1B cells were cultured with sorafenib (1.25 μM) or PEG-IFN-α2b (2,000 IU/ml), or both sorafenib (1.25 μM) and PEG-IFN-α2b (2,000 IU/ml) for 72 h. After incubation, the cells were stained with the Annexin V-EGFP (enhanced green fluorescent protein) using Apoptosis Detection Kits (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol. After staining, the cells were analyzed using a FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA), and the rate of Annexin V-EGFP-positive apoptotic cells was determined.

*Effects of sorafenib and/or PEG-IFN-α2b on HCC cell proliferation in nude mice.* This experiment was approved by the institutional committee for animal experiments and conducted according to the 'Guide for the Care and Use of Laboratory Animals' published and revised by the National Institute of Health in 1985.

Cultured HAK-1B or KIM-1 cells ($1.0x10^6$ cells/mouse) were transplanted subcutaneously (s.c.) to 4-week-old female BALB/c athymic nude mice (Clea Japan Inc., Osaka, Japan). On the 7th day when tumor size became 5 to 10 mm in diameter (day 0), the mice were divided into four groups (n=8 each) in a manner to equalize the mean tumor diameter of every group. Each group was assigned to one of the four treatments: i) control; ii) PEG-IFN-α2b alone; iii) sorafenib alone; and iv) sorafenib + PEG-IFN-α2b (combination).

Sorafenib was diluted with 12.5% Cremophor EL/12.5% ethanol/75% water for oral dosing in mice. Sorafenib (200 μg/day) was administered by tube feeding once a day for 14 days. PEG-IFN-α2b (1,920 IU) was subcutaneously injected twice a week for 14 days (days 1, 4, 8 and 11). In the control and the sorafenib alone groups, 0.1 ml of medium as the replacement of PEG-IFN-α2b was injected subcutaneously twice a week. In the control and the PEG-IFN-α2b alone groups, 0.2 ml of Cremophor EL/ethanol/water (12.5/12.5/75) as the replacement of sorafenib was administered by tube feeding once a day. The dose of sorafenib (200 μg) in the ratio to the average bodyweight of a mouse (20 g) was 10 mg/kg and this is almost comparable to a clinical dose (800 mg total daily dose). The clinical dose of PEG-IFN-α2b in chronic hepatitis C is 96,000 IU/kg per week. Because of species difference and different target which is not virus, but tumor, we used twice the dose per week in nude mice.

Tumor size was measured in two directions using calipers, and tumor volume ($mm^3$) was estimated by using the equation: length x $(width)^2$ x 0.5. This measurement was performed every two days. Mouse body weight was measured on days 0, 7 and 14. Mouse was sacrificed and the tumor was resected the next day after the completion of the 14-day treatment (day 15). The resected tumor was fixed in formalin after the weight measurement, prepared into paraffin sections, and underwent HE staining and immunohistochemistry.

*Immunohistochemistry.* Paraffin-embedded tissue samples were cut into 4-μm sections. Anti-mouse CD34 (Rat monoclonal, MEC14.7, Abcam, Cambridge, UK) (1:50 dilution) and Ki67 (Rabbit monoclonal, SP6, Abcam, Cambridge, UK) (1:100 dilution) staining were performed by standard avidin-biotin-peroxidase complex method and 3,3'-diaminobenzidine (DAB) solution was used for color development. Cleaved caspase-3 (rabbit polyclonal antibody, Cell Signaling Technologies, Beverly, MA, USA) (1:250 dilution) staining was