Formation of spherical cancer stem-like cell colonies with resistance to chemotherapy drugs in the human malignant fibrous histiocytoma NMFH-1 cell line

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Abstract. Various human cancers have been revealed to contain cancer stem-like cells (CSCs) and the spherical colonies that possess stem-like properties and cancer-initiating abilities. Malignant fibrous histiocytoma (MFH) is a common soft-tissue sarcoma, and is considered to be a myxoma due to the observed high-grade lesions. In the present study, the spherical colonies were isolated from a human MFH cell line NMFH-1 using the sphere culture system. These colonies demonstrated stem-like properties, with the ability of self-renewal and strong drug-resistance to doxorubicin and cisplatin. In addition, verapamil, an adenosine triphosphate binding cassette protein transporter protein (ABCG2) inhibitor, enhanced the efficacy of the aforementioned chemotherapy agents. These colonies also demonstrated an increased expression of embryonic stem genes, including Oct3/4, signal transducer and activator of transcription 3, sex determining region Y-box 10 and ABCG2, and stem cell-associated surface markers, such as cluster of differentiation (CD)44 and CD133. These results indicated that NMFH-1 lesions contain cancer stem-like cell populations that demonstrate strong drug resistance, and verapamil enhanced the efficacy of the chemotherapy agents.

Introduction

The cancer stem-like cell (CSC) theory hypothesizes that tumors contain a small subpopulation of cancer cells that share numerous properties with normal stem cells, including proliferative potential and self-renewal (1). These rare stem-like tumor initiators are considered to be associated with initiating and maintaining the growth of tumors, and may be responsible for the local recurrence and distant metastasis of tumors (1). Previous studies have indicated that CSCs exist in numerous human tumors, including hematopoietic cancer (2), brain tumors (3), breast cancer (4), melanoma (5) and bone sarcoma (6,7). Advanced methods and techniques have contributed to the identification of CSCs (2). These methods include the detection of specific surface markers that are selectively expressed on CSCs, but not on the majority of tumor cells, serum-free suspension culture medium for colony formation *in vitro* and a unique pattern of staining with certain dyes, including Hoechst 33342, for detecting side population (SP) cells (2-5): The characteristics of side population cells include proliferative potential and self-renewal.

Previous studies have revealed that specific surface molecules, including cluster of differentiation (CD)133 and CD44, may be markers for certain CSC populations (8-11). Several studies have indicated that CSCs may demonstrate the ability of increased resistance to chemotherapy, due to the high expression of specific drug transporters, including multidrug resistance protein 1 and adenosine triphosphate (ATP)-binding cassette (ABC) sub-family G member 2 (ABCG2) (12,13). Certain cell enzymes have also been demonstrated to be useful molecules for the selection and detection of CSCs, and aldehyde dehydrogenase (ALDH) 1 is one of the possible candidates for a stem cell marker that may be used for the isolation of CSCs from tumors in cancers that include leukemia, breast cancer and sarcoma (14-16).

Although the mechanisms of drug resistance in CSCs are poorly understood, previous studies have revealed that these may be associated with the ABC drug transporters (17,18).

CSC markers remain limited, but the sphere culture system is particularly useful as a functional approach to enrich the potential CSC subpopulations which including proliferative potential and self-renewal. (6). The sphere culture system, which comprises stressful growth conditions of serum starvation and anchorage independence, is frequently used to identify and enrich stem and progenitor cells by eliminating the differentiated cells that are unable to survive (6). Reynolds and Weiss (19) first employed this system to demonstrate that the adult mammalian brain contained cells that gave

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rise to neurosphere clones. This method is also termed the neurosphere/sarcomaspere culture system. Previous studies have demonstrated that spherical forming colonies derived from various tumors demonstrated stem-like properties with the ability of self-renewal, increased expression of certain embryonic stem (ES) genes, and tumorigenicity in mouse models (6,7,20-23). The present study aimed to detect SP and $ALDH^+$ cell populations in human fibrosarcoma HT1080 and malignant fibrous histiocytoma (MFH) NMFH-1 cells.

Materials and methods

Cell lines and culture. The human synovial sarcoma SW982 cells and fibrosarcoma HT1080 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The novel myxofibrosarcoma NMFH-1 cell line, which was considered to be a myxoid variant of MFH, was provided by Dr. Akira Ogose (Division of Orthopedic Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan) (24). The SW982 cells were maintained in Leibovitz's L-15 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), the HT1080 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies) supplemented with 10% FBS, and the NMFH-1 cells were maintained in RPMI-1640 medium (Gibco Life Technologies) supplemented with 10% FBS. All cells were maintained at 37˚C in a 5.0% CO_2 atmosphere.

Identification of SP cells. The cell suspensions were labeled with Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO, USA), using the method described by Goodell *et al* (25). Briefly, the cells were trypsinized and re-suspended in pre-warmed Leibovitz's L-15 medium, DMEM or RPMI-1640 medium supplemented with 5% FBS at a concentration of $1x10^6$ cells/ml. Hoechst 33342 dye was added at a final concentration of 5.0 μg/ml in the presence or absence of 50 μM verapamil (Sigma-Aldrich), which acts as an inhibitor of the ABC transporter. The cells were incubated at 37˚C for 90 min with continuous agitation. At the end of the incubation, the cells were washed with ice-cold PBS supplemented with 5% FBS, centrifuged at 4˚C and resuspended in ice-cold PBS containing 5% FBS. Flow cytometry was performed using BD FACSAria II (BD Biosciences, Franklin Lakes, NJ, USA). The Hoechst 33342 dye was excited at 357 nm and the fluorescence was analyzed using a dual wavelength (blue, 402-446 nm; red, 650-670 nm).

Spherical colony formation assay. Monolayer cells at ~70% confluence in RPMI medium supplemented with 10% FBS were dissociated into single-cell suspensions using 0.25% trypsin and 0.05% EDTA (Sigma-Aldrich). The cells were then inoculated into B27-supplemented RPMI-1640 medium and 1% methylcellulose medium mixed with 10 ng/ml human recombinant epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (βFGF) at a cell density of $6x10^4$ cells per well in ultra low attachment 6-well plates (Corning Inc., Corning, NY, USA). EGF and βFGF were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA).

Fresh aliquots of EGF and βFGF were added every other day. Subsequent to 7-12 days of culture, the colonies that contained >40 cells were quantitated by inverted phase contrast microscopy (Olympus CKX41; Olympus, Tokyo, Japan). Spherical colonies were dissociated and re-introduced into 96-well ultra low attachment plates at least 5 times, in normal medium and in anchorage-independent methylcellulose medium, to investigate the self-renewal ability of the cells through secondary sphere formation.

Aldefluor assay and detection of the $ALDH^+$ subpopulations by FACS. The Aldefluor (StemCell Technologies, Inc., Vancouver, BC, Canada) was used to detect cell populations with high ALDH enzymatic activity. The cells were labeled with Aldefluor reagent, according to the manufacturer's instructions. Briefly, cultures of NMFH-1 and SP cells were harvested by trypsin-EDTA and resuspended in Aldefluor assay buffer, containing 1 μmol/l of the ALDH substrate BODIPY aminoacetaldehyde (StemCell Technologies, Inc.) per $1x10^6$ cells. The cells were then incubated for 30 min at 37˚C. As a negative control for each sample of cells, an aliquot was treated with 50 mmol/l 4-diethylaminobenzaldehyde (DEAB), an ALDH-specific inhibitor (StemCell Technologies, Inc.). Flow cytometry was performed using BD FACSAria.

Assay to determine sensitivity to chemotherapy drugs, with or without verapamil. To assess the sensitivity of NMFH-1 cells to cisplatin (CDDP) and doxorubicin (DXR), which are frequently used for chemotherapy in patients with sarcoma, the NMFH-1 cells were dissociated and inoculated into 96-well microtiter plates (Corning Inc.) at a concentration of 2,000 cells/90 μl/well. The cells were allowed to attach to the plates in RPMI-1640 supplemented with 10% FBS at 37˚C. Subsequent to 12 h incubation, the cells were then exposed to various concentrations of CDDP or DXR (1, 5, 10μM) with or without 50 μM verapamil. Following 48 h incubation, with or without chemotherapy drugs, the cell viability was measured by MTS assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. This was compared with the control cells, which were incubated without drugs. NMFH-1 cells were then inoculated into 96-well ultra low attachment microplates (Corning, Inc.) in RPMI-1640 supplemented with B27 with 1% methylcellulose medium at a concentration of 5,000 cells/90 μl/well for 10 days to allow sphere formation. The cells were then treated with CDDP or DXR at a final concentration of 1, 5 or 10 μM, with or without 50 μM verapamil, and subsequently cell viability was measured by MTS assay following 48 h of treatment.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from frozen packed cells using the RNeasy Total RNA system (Qiagen GmbH, Hilden, Germany) and first-strand cDNA was synthesized from 500 ng samples using the Superscript II RNase H Reverse Transcriptase system (Invitrogen Life Technologies). PCR was performed using 0.5 μl reaction mixture as templates. The primer sequences used for the amplification of the Nanog, Oct3/4, signal transducer and activator of transcription 3 (STAT3), sex determining region Y-box 10 (SOX10)

Table I. Primer sets for reverse transcription-polymerase chain reaction.

Gene	Forward, 5'-3'	Reverse, 5'-3'
Nanog	GCTGAGATGCCTCACACGGAG	TCTGTTTCTTGACTGGGACCTTGTC
Oct3/4	TGGAGAAGGAGAAGCTGGAGCAAAA	GGCAGATGGTCGTTTGGCTGAATA
STAT3	GGGTGGAGAAGGACATCAGCGGTAA	GCCGACAATACTTTCCGAATGC
SOX10	TATATACGACACTGTCCCGGC	AGTGTGGGTGCAACAGTCAAC
ABCG2	ACCTGAAGGCATTTACTGAA	TCTTTCCTTGCAGCTAAGAC
GAPDH	CAGCCGAGCCACATCG	TGAGGCTGTTGTCATACTTCT

STAT3, signal transducer and activator of transcription 3; SOX10, sex determining region Y-box 10; ABCG2, adenosine triphosphate-binding cassette sub-family G member 2.

and ABCG2 genes are listed in Table I. The GAPDH gene was used as an internal control to adjust the quantities of the template. Aliquots of amplification products (10 μl) were separated by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining (Qiagen GmbH, Hilden, Germany).

Western blotting. The cells were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1% Na-deoxycholate, 1 mM Na-vanadate, and protease inhibitors consisting of 5 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin and 1 mM NaF (Sigma-Aldrich) for 1 h in ice. Following centrifugation at 13,000 x g for 10 min at 4˚C, the protein concentration of the supernatants was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). The lysates were mixed with Laemmli buffer (dilution, 1:1; Sigma-Aldrich). In total, 50 μg of protein per lane was electrophoresed in 10% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Sigma-Aldrich). The membranes were blocked with non-fat milk for 1 h at room temperature and incubated overnight at 4˚C with rabbit monoclonal anti-CD44 (dilution, 1:2,000; ab51037, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-CD133 (dilution, 1:1,000; SAB2107606, Sigma-Aldrich) and rabbit polyclonal IgG anti-GAPDH (dilution, 1:5,000; sc-25778, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in 5% bovine albumin (Sigma-Aldrich), Tris-buffered saline (TBS) and 0.1% Tween-20 (Bio-Rad Laboratories, Hercules, CA, USA). Subsequent to being washed three times in TBS with 0.1% Tween-20, the blots were incubated with Goat anti-rabbit IgG (5366S; Cell Signaling Technology, Danvers, MA, USA; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Immunoreactive bands were detected by ECL Plus SuperSignal West Pico (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 60 sec.

Statistical analysis. The data are expressed as the mean ± standard deviation. The χ^2 test and Fisher's exact test were used where appropriate (n>40 and n<40, respectively). All statistical analyses were performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Detection of SP cells in human sarcoma cell lines. The present study aimed to detect the proportion of the SP cells in bone and soft tissue sarcoma cell lines. The NMFH-1 (Fig. 1A) and HT1080 (Fig. 1B) cell lines consisted of 0.3 and 1.08% SP cells, respectively. In each cell line, the percentage of SP cells was markedly diminished by treatment with verapamil, which is an inhibitor of the protein pumps, such as ABCG2, responsible for the exclusion of Hoechst 33342 dye, indicating that this population accurately indicated the proportion of SP cells. However, staining of the synovial sarcoma SW982 cells did not reveal the presence of SP cells, which was either due to inappropriate culture conditions or the cells lacking a stem cell population that was able to be defined. Therefore, the NMFH-1 cells, which contained the highest proportion of SP cells, was selected and underwent additional analysis.

$ALDH^+$ populations of NMFH-1 cells. The presence and size of the cell populations that demonstrated ALDH enzymatic activity were assessed in NMFH-1 cells using an Aldefluor assay. The results revealed that NMFH-1 cells contained populations of cells exhibiting ALDH activity, with a frequency of 8.2% in NMFH-1 cells (Fig. 2A), compared to the frequency of 0.4% in the control NMFH-1 cells treated with the ALDH inhibitor DEAB (Fig. 2B).

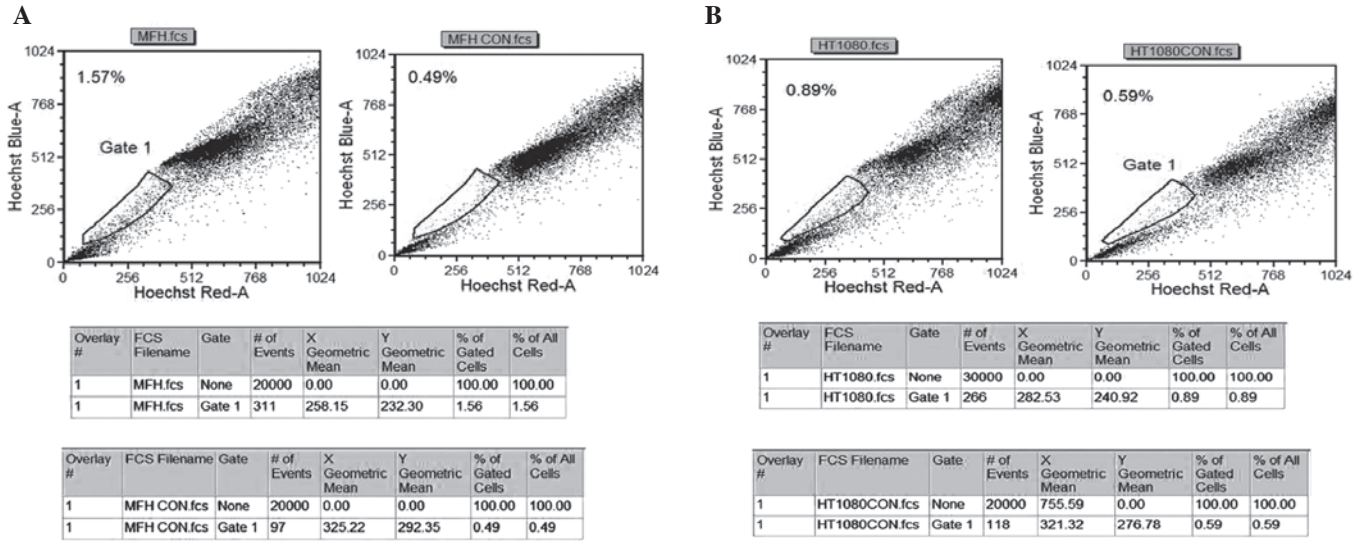
Spherical colony formation in NMFH-1 cells. The ability of NMFH-1 cells to generate spherical clones and self-renew was evaluated in a serum-starved culture assay. To investigate cell self-renewal, cultured spheres were dissociated into single cells and allowed to grow in monolayer culture and serum-starved anchorage-independent conditions with 1% methylcellulose medium: This process was repeated twice. The spherical colonies of NMFH-1 cells revealed expansion in the monolayer culture, which led to cell differentiation and self-renewal through the secondary formation of spherical colonies (Fig. 3).

Chemotherapy drug resistance between NMFH-1 and spherical colonies. CDDP and DXR inhibited the growth of NMFH-1 cells in a dose-dependent manner. The survival rates of adherent and spherical colonies subsequent to 48-h drug treatment are shown in Table II and Figs. 4-6. The difference in the growth inhibition rate between the adherent and spherical

Table II. Cell survival rates of NMFH-1 cells subsequent to 48 h treatment with CDDP, with or without 50 μM verapamil.

Treatment	Cell survival rate, %		χ^2	P-value
	Adherent colony	Spherical colony		
CDDP				
1 μM	83.91±3.98	98.97±0.40	446	0.0004
5 μM	64.38±11.15	87.80±12.10	337	0.0024
10 μM	50.17±13.57	83.76±14.05	279	0.0003
CDDP + verapamil				
1 μM	64.18±14.37	96.39±6.21	370	0.0001
5 μM	46.29±14.68	74.40±16.62	259	0.0016
10 μM	43.39±12.01	69.40±16.09	241	0.0001

Values are expressed as the mean ± standard deviation. [a]P<0.05. CDDP, cisplatin.



Overlay #	FCS Filename	Gate	# of Events	X Geometric Mean	Y Geometric Mean	% of Gated Cells	% of All Cells
1	MFH.fcs	None	20000	0.00	0.00	100.00	100.00
1	MFH.fcs	Gate 1	311	258.15	232.30	1.56	1.56

Overlay #	FCS Filename	Gate	# of Events	X Geometric Mean	Y Geometric Mean	% of Gated Cells	% of All Cells
1	MFH CON.fcs	None	20000	0.00	0.00	100.00	100.00
1	MFH CON.fcs	Gate 1	97	325.22	292.35	0.49	0.49

Overlay #	FCS Filename	Gate	# of Events	X Geometric Mean	Y Geometric Mean	% of Gated Cells	% of All Cells
1	HT1080.fcs	None	30000	0.00	0.00	100.00	100.00
1	HT1080.fcs	Gate 1	266	282.53	240.92	0.89	0.89

Overlay #	FCS Filename	Gate	# of Events	X Geometric Mean	Y Geometric Mean	% of Gated Cells	% of All Cells
1	HT1080CON.fcs	None	20000	755.59	0.00	100.00	100.00
1	HT1080CON.fcs	Gate 1	118	321.32	276.78	0.59	0.59

Figure 1. Detection of SP cells in human sarcoma cell lines. Proportion of the SP cells in the (A) malignant fibrous histiocytoma NMFH-1 cell line and (B) fibrosarcoma HT1080 cell line. SP, side population

colonies was statistically significant (P=0.0003), as 10 μM of CDDP inhibited cell growth by 50% in NMFH-1 cells in monolayer culture and 23% in sphere conditions, respectively. In addition, 10 μM DRX inhibited cell growth by 58% in adherent conditions and by 31% in spherical colony conditions. These results suggest that the spherical colonies are resistant to CDDP and DXR, which are the most commonly available chemotherapy drugs for sarcomas. The application of verapamil, an ABCG2 inhibitor, in combination with either CDDP or DXR resulted in increased cell growth inhibition compared with non-verapamil treatment. However, the growth inhibition rates were limited to only 26.8% for CDDP combined with verapamil and 31.1% for DXR combined with verapamil in adherent conditions. In spherical conditions, the growth inhibition rates were 19.4% for CDDP combined with verapamil and 25.5% for DXR combined with verapamil.

Expression of Nanog, Oct3/4, STAT3, SOX10 and ABCG2. The expression of the STAT3, Nanog, Oct3/4, Sox10 and ABCG2 genes, all of which are associated with the marker genes of pluripotent ES cells, was investigated by semi-quantitative RT-PCR analysis to determine whether these genes were expressed in adherent and sphere formation conditions. All five genes were expressed in spherical NMFH-1 cell colonies, and the spheres consistently demonstrated increased expression of these genes, with the exception of Nanog, compared with adherent cells (Fig. 7). ABCG2 encodes a membrane efflux transporter that is expressed in human hematopoietic stem cells (2), and the gene is also associated with chemotherapy resistance (18,25). The protein encoded by ABCG2 functions as a xenobiotic transporter, which may play a major role in multi-drug resistance. The ABCG2 protein is likely to act as a cellular defense mechanism in response to exposure to mitoxantrone and anthracycline (26). ABC transporters have the capacity to export numerous chemotherapy agents and are upregulated in CSCs derived from certain cancer cell lines (17,18). In particular, ABCG2 has been implicated in the high Hoechst 33342 dye efflux capacity

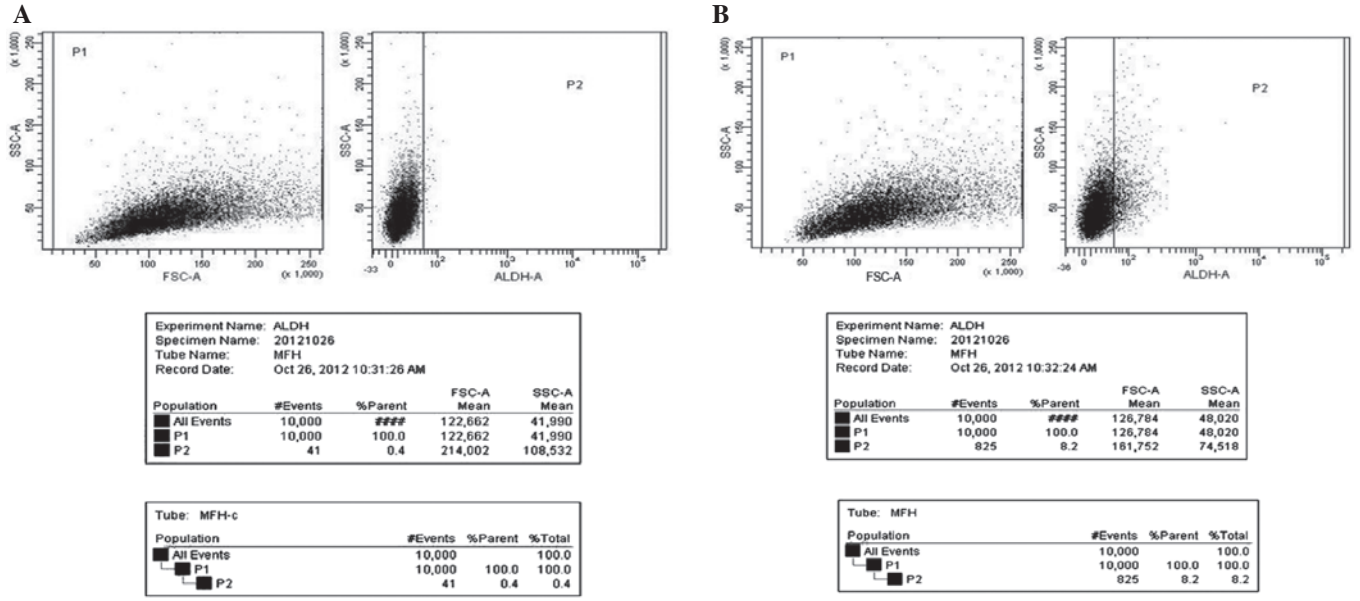


Figure 2. Cell populations expressing ALDH in the NMFH-1 cell line. (A) Fluorescence-activated cell sorting analysis of NMFH-1 cells, using the ALDH inhibitor 4-diethylaminobenzaldehyde as a control. (B) Aldefluor assay demonstrating that 8.2% of the human NMFH-1 cell population exhibits high ALDH1 activity. ALDH, aldehyde dehydrogenase.

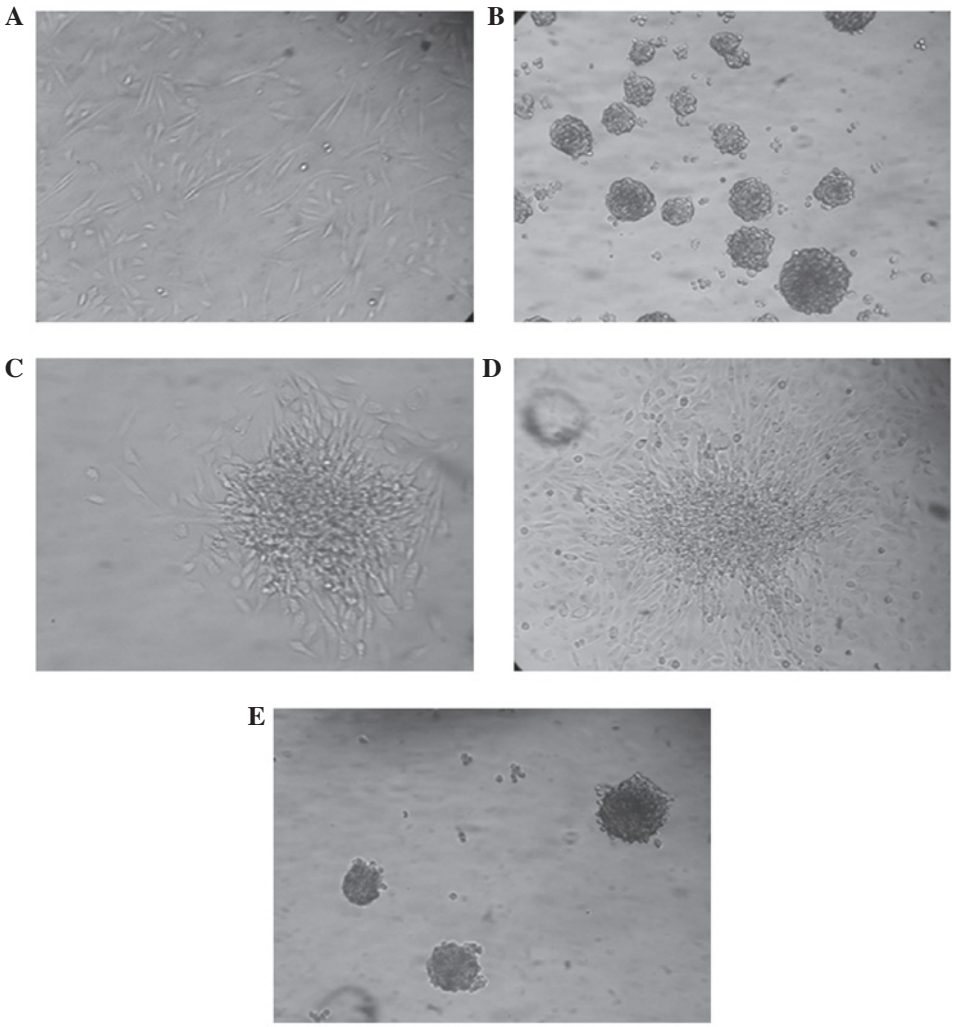


Figure 3. Spherical colony formation in NMFH-1 cells. Microscopic appearance of (A) NMFH-1 cells in the monolayer conditions and (B) spherical colony formation in anchorage-independent, serum-starved conditions subsequent to 12 days. (C and D) The spherical colonies were removed from the suspension culture and allowed to attach to a substratum. The adherent cells can be observed expanding from the sphere. (E) Secondary formation of spherical colonies was achieved by repeatly seeding into anchorage-independent, serum-starved conditions.

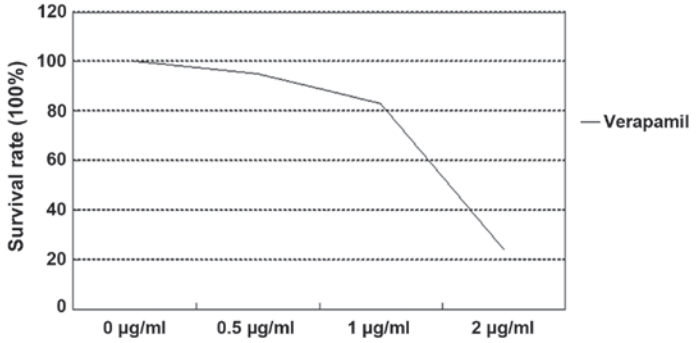


Figure 4. Resistance to chemotherapy drugs in spherical colonies of NMFH-1 cells. NMFH-1 cell growth was suppressed by verapamil in a dose-dependent manner. The cell growth inhibition rates was 1.2% at a concentration of 0.5 μg/ml (50 μM) verapamil.

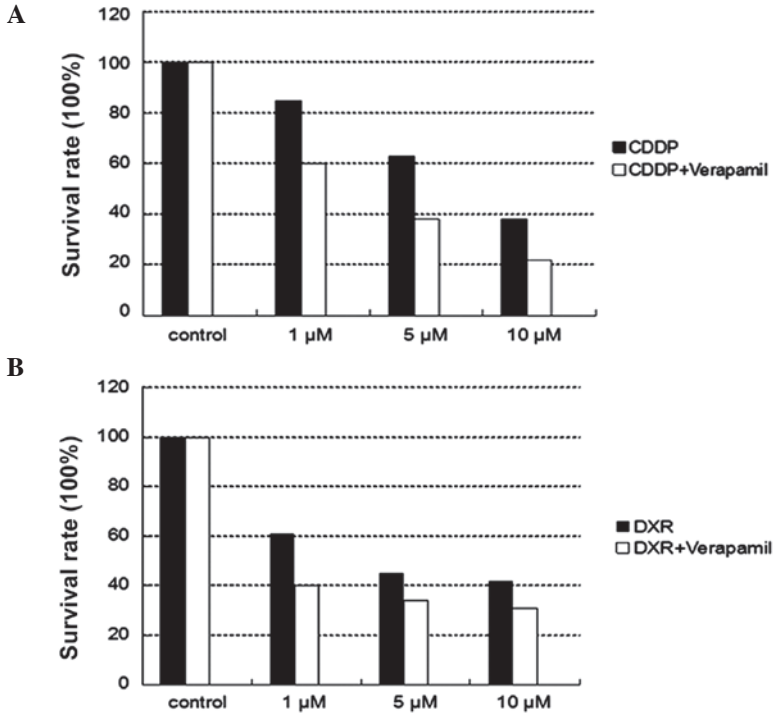


Figure 5. Cell growth of NMFH-1 cells was suppressed by (A) CDDP and (B) DXR in a dose-dependent manner. The growth inhibition rates exerted by 10 μM CDDP and 10 μM DXR were 50 and 58%, respectively. The application of verapamil, an inhibitor of adenosine triphosphate-binding cassette sub-family G member 2, in combination with the chemotherapy drugs increased the efficacy of the drugs. However, the effects were limited to a growth inhibition rate of 26.8% in CDDP-treated cells and 31.1% in DXR-treated cells. The data was obtained at a concentration of 50 μM verapamil in conjunction with 1 μM of CDDP and DXR. Similar results were obtained from treatment with 5 and 10 μM CDDP or DXR with verapmil. CDDP, cisplatin; DXR, doxorubicin.

that marks the SP cell phenotype. Therefore, to investigate abundance of ABC transporters associated with multi-drug resistance, RT-PCR was used to determine the relative mRNA expression of ABC transporters in NMFH-1 adherent and spherical cell colonies.

Expression of CD44 and CD133. The expression of the normal stem-associated cell proteins and candidate CSC markers CD133 and CD44 was examined by western blotting. The results revealed that the expression of the two proteins in the spherical colonies was significantly increased compared with the expression in the adherent NMFH-1 cells (Fig. 8), indicating that the spherical colonies possess certain stem cell-like properties. However, the expression of CD44 was considerably increased in the adherent and spherical colony formation conditions, which suggests that NMFH-1 cells may demonstrate a considerable migratory ability, as CD44 induces a metastatic phenotype in locally growing tumor cells (27,28). These results indicate that the spherical colony formation system may increase the expression of CD44 and CD133 in stem and progenitor cells.

Discussion

In general, there are two models of heterogeneity in cancer cells (1,29). The first model is that cancer cells of numerous phenotypes have the potential to proliferate extensively, and any one cell may have a low probability of exhibiting this potential in an assay of clonogenicity or tumorigenicity (1). The second model is that the majority of cancer cells have

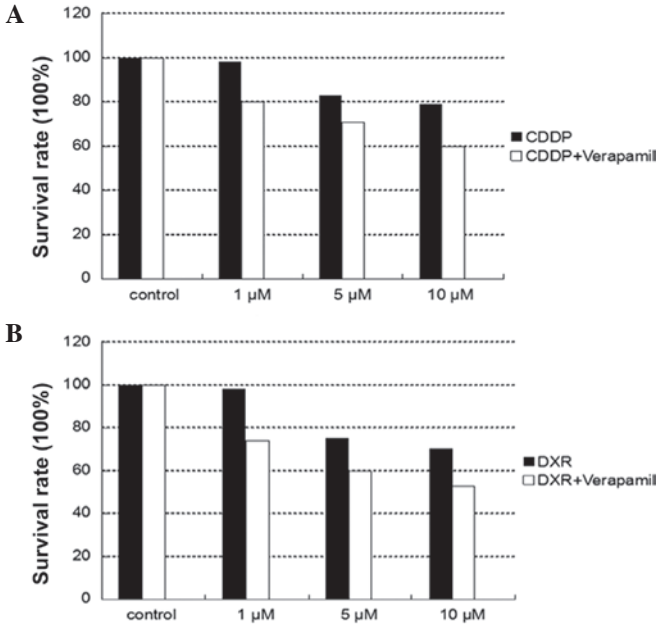


Figure 6. The growth of spherical colonies was suppressed by (A) CDDP and (B) DXR in a dose-dependent manner. The growth inhibition rates induced by 10 μM CDDP and 10 μM DXR were 23% and 31%, respectively. The application of verapamil in combination with drugs increased the efficacy of chemotherapeutic drugs. However, the effects were limited to 18.6% for CDDP and 22.3% for DXR. The data was obtained at a concentration of 50 μM verapamil with 1 μM CDDP or 1 μM DXR. Similar results were obtained from treatmetn with 10 μM CDDP or 10 μM DXR with verapmil. CDDP, cisplatin; DXR, doxorubicin.

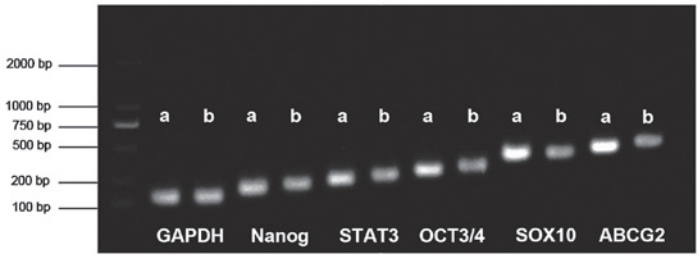


Figure 7. Expression of Nanog, OCT3/4, STAT3, SOX10 and ABCG2. Reverse transcription-polymerase chain reaction revealed strong expression of Nanog, STAT3, OCT3/4, SOX10 and ABCG2 in spherical NMFH-1 cell colonies, compared with the adherent cells. a, spherical colonies; b, adherent cells; STAT3, signal transducer and activator of transcription 3; SOX10, sex determining region Y-box 10; ABCG2, adenosine triphosphate-binding cassette sub-family G member 2.

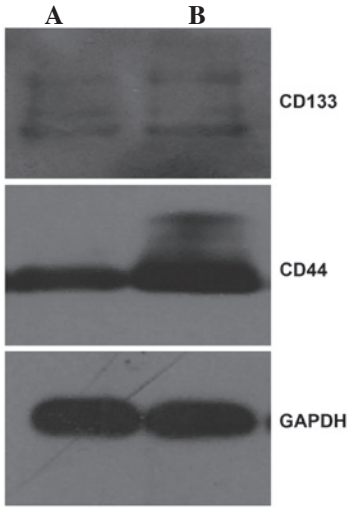


Figure 8. Expression of CD44 and CD133 in (A) adherent cells and (B) spherical colonies of NMFH-1 cells. Western blotting revealed strong expression of CD44 and CD133 in spherical colonies of NMFH-1 cells compared with the expression in adherent cells. CD, cluster of differentiation.

only limited proliferative potential, and only a rare subset of cancer cells consistently proliferates extensively in clonogenic assays and may form novel tumors on transplantation (29). This model predicts that a distinct subset of cells demonstrates an increased ability to form novel tumors, whereas the majority of cells do not possess this ability. Previous therapeutic failure to successfully treat the majority of cancers indicates that the second model of heterogeneity may be the more accurate model. The CSC hypothesis is consistent with the latter model that tumors contain a small subpopulation of cancer cells that share numerous stem cell-like properties, including proliferative potential and self-renewal, increased or decreased expression of stem cell-associated genes and cell surface markers. These rare stem cell-like tumor initiators are considered to be associated with initiating and maintaining the growth of tumors, and these cells may be responsible for local recurrence and distant metastasis.

In the present study, the novel myxofibrosarcoma cell line NMFH-1 was demonstrated to possess the abilities to form

spherical colonies and self-renewal in anchorage-independent, serum-starved culture conditions, which were previously developed to isolate cancer stem cells from hepatoma and certain bone sarcomas (6,7,20,23). The present study found that NMFH-1 adherent cells and spherical colonies expressed key marker genes of ES cells, consisting of Nanog, STAT3, Oct3/4 and SOX10. However, increased expression of STAT3, Oct3/4 and SOX10 was identified in spherical colonies compared with the adherent cultures. These genes play important roles in ES cells. Nanog maintains self-renewal in ES cells (30,31), STAT3 plays important roles in regulating cell growth, differentiation, apoptosis, angiogenesis and immune responses (32), SOX10 is involved in the regulation of embryonic development and the determination of cell fate (33), and Oct3/4 is a POU domain, octamer-binding transcription factor that is expressed in ES cells (34). In particular, previous studies have revealed that the increased or decreased expression of these stem cell-associated genes have been found in numerous primary cancers and CSCs (6,7,21,23,35). Therefore, certain key marker genes in ES cells may play a role in sarcoma oncogenesis.

To investigate the CSC properties of spherical colonies, the sensitivity of spherical colonies to chemotherapy agents was investigated. The spherical colonies exhibited general resistance to CDDP and DXR, and demonstrated increased survival ability compared with the adherent cells. In addition, verapamil, an ABCG2 inhibitor, in combination with either CDDP or DXR, demonstrated an increased inhibition of cell growth compared with non-verapamil treatment. It has been demonstrated that ABC transporters have the capacity to export numerous chemotherapy agents and are upregulated in CSCs derived from certain cancer cell lines (17,18). The ABCG2 protein functions as a xenobiotic transporter that may play a major role in multi-drug resistance (2). This protein is likely to act as a cellular defense mechanism in response to the exposure of cells to mitoxantrone and anthracycline (26), and is alternatively referred to as a breast cancer resistance protein (36). The present results indicate that verapamil may enhance the efficacy of chemotherapy agents by inhibiting ABCG2 from pumping chemotherapy drugs out of the cells. However, the ABCG2 inhibitor verapamil may only partially inhibit the growth adherent cells and spherical colonies. This may be due to cancer stem cells expressing other drug resistant proteins, including ABCB1 (37).

Furthermore, the present study demonstrated that the spherical colony culture system may enrich stem-like cells with the expression of the ABCG2 gene, according to the present results from RT-PCR. Previous studies have revealed that the ABCG2 gene is highly expressed in the placenta, normal stem cells and in certain tumor stem cells (2,38-40). ABCG2 levels were reduced when stem cells were induced to differentiate. In particular, ABCG2 has been implicated in the high Hoechst 33342 dye efflux capacity that is characteristic of the SP phenotype, and was subsequently identified and characterized as a novel stem cell marker (39). Thus, the spherical colonies overexpress ABCG2 and are more resistant to CDDP and DXR, indicating a possible contribution of these cells to cancer chemoresistance.

Finally, the expression of the candidate CSC markers CD44 and CD133 was examined in the spherical colonies. The lymphocyte homing receptor CD44 is expressed in numerous cells and is considered to be a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration (41). In particular, CD44 induces a metastatic phenotype in locally growing tumor cells (27,28). There is considerable evidence for the contribution of CD44 expression to the initiation and progression of numerous tumors and CSCs (42). CD133 is an important candidate CSC marker that localizes to membrane protrusions and is often expressed on adult stem cells, where it is hypothesized to maintaining stem cell properties by suppressing differentiation. CD133 has been used as a marker for hematopoietic stem cells (43) and neuronal stem cells (44). Previously, various studies have demonstrated that CD133 is associated with the initiation and progression of cancer stem and progenitor cells, and may be a valuable CSC marker in a variety of tumors, including epithelial cancers and solid sarcomas (10,11,45-50). Therefore, the increased expression of CD44 and CD133 in NMFH-1 spherical colonies may account for the increased survival and metastatic ability of these cells.

In conclusion, the present study revealed that the spherical colonies derived from NMFH-1 cells demonstrate stem-like properties in anchorage-independent conditions, and it was indicated that spherical colonies may contain CSC subpopulations. The current findings support the hypothesis that human NMFH-1 cells are heterogeneous, and that rare cells within the bulk of a tumor are responsible for the initiation and growth of NMFH-1 lesions. Due to the current inability to successfully treat the majority of tumors, the modulations of drug resistance in cancer chemotherapy may be promising. ABC transporter protein inhibitors, such as verapamil, may be valuable candidates. In addition, as CD44 plays an important role in tumor growth, progression and metastasis, the present interest in the use of CDs in tumor therapy should be increased.

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