

Identification of rhodamine 123-positive stem cell subpopulations in canine hepatocellular carcinoma cells

HARUMICHI ITOH¹, SHIMPEI NISHIKAWA¹, TOMOYA HARAGUCHI¹, YU ARIKAWA¹,
MASATO HIYAMA², TOSHIE ISERI³, YOSHIKI ITOH³, MUNEKAZU NAKAICHI³,
YASUHO TAURA², KENJI TANI² and KAZUHITO ITAMOTO¹

Departments of ¹Small Animal Clinical Science, ²Veterinary Surgery and ³Veterinary Radiology,
Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi City, Yamaguchi 753-8511, Japan

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Abstract. The majority of cases of chemotherapy for hepatocellular carcinoma (HCC) are not effective in human or veterinary medicine due to resistance against anticancer agents. In human medicine, hepatocellular carcinoma stem cells (HCSCs) were recently identified as cytokeratin 19 (CK19)-, cluster of differentiation (CD)-44-, and CD133-positive. However, there are few previous reports regarding canine HCSC (cHCSC). Additionally, to the best of our knowledge, the chemoresistance against anticancer agents of these cHCSCs has not been investigated. In the present study staining of cHCSCs was performed with rhodamine 123, a low-toxicity fluorescent dye for mitochondria, by flow cytometry. There were two subpopulations in the HCC cell line defined by their higher (Rho^{Hi}) and lower (Rho^{Lo}) fluorescence intensity of rhodamine 123. The Rho^{Hi} subpopulation demonstrated a higher Nanog gene expression, sphere-forming ability, and chemoresistance against gemcitabine. However, there was no significant difference between Rho^{Hi} and Rho^{Lo} regarding the proliferation rate and chemoresistance against mitoxantrone and doxorubicin. The present results indicate that the expression of rhodamine 123 identifies different stem cell subpopulations in a canine HCC cell line.

Introduction

Canine hepatocellular carcinoma (cHCC) accounts for 50% of primary liver tumors (1). cHCC is classified clinically as massive, nodular, or diffuse; with ~60% of cHCC patients

categorized as massive, 30% as nodular and 10% as diffuse (2). In addition, nodular and diffuse cHCC exhibit a higher metastatic rate than massive tumors. As a result of the low metastatic rate, patients with massive cHCC demonstrate a good prognosis when treated surgically. However, nodular and diffuse cHCC have a worse prognosis compared with massive cHCC (3). For unresectable massive cHCC, and for a part of nodular and diffuse cHCC cases, transcatheter arterial embolization or transcatheter arterial chemoembolization have been employed experimentally as minimally invasive treatments, and partial therapeutic effects have been reported (4). However, no curative surgical treatment has been established for nodular and diffuse cHCC to date.

Chemotherapy has generally been considered to have a limited therapeutic effect in primary liver tumors. The presence of P-glycoprotein in the tumors is suggested as a part of therapy resistance mechanism (5). A previous study reported that chemotherapy using mitoxantrone (MTX) resulted in a partial response in a dog with cHCC (6). Although a response has been reported in a dog following the empirical use of gemcitabine (GEM) (7), a retrospective study of 18 cHCC dogs with GEM (4 massive, 10 nodular and 4 diffuse) concluded that the effect of the therapy was worse than surgical treatment and that the single use of GEM did not improve the survival rate in dogs with cHCC (8). Considering these issues, effective chemotherapy for cHCC has not yet been established.

A previous study indicated that cancer tissue is not comprised of a single type of cells, but that cancer stem cells (CSCs) maintain tumor function and morphology (9). CSCs are defined by their ability to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor (10). Therefore, a novel therapeutic strategy targeted at CSCs has recently been investigated. Similarly, a side population of hepatocellular carcinoma stem cells (HCSCs) has been reported (11) to be marked by cluster of differentiation (CD) 133-positive (12,13), CD90-positive (14), and CD44-positive (15) cells. However, there are a small number of reports for canine HCSCs (cHCSCs), such as CD90- and CD44-positive (16,17).

Rhodamine 123 is a low-toxic fluorescent dye for staining mitochondria and is used to determine mitochondrial activity by flow cytometry (18). In human medicine, rhodamine 123

Correspondence to: Professor Shimpei Nishikawa, Department of Small Animal Clinical Science, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi City, Yamaguchi 753-8511, Japan
E-mail: sn2007@yamaguchi-u.ac.jp

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is considered one of the markers that identify hematopoietic stem cells (HPCs) (19) and renal carcinoma stem cells (20). However, to the best of our knowledge, no previous studies have reported that rhodamine 123 characterizes cHSCs.

The aim of the current study was to determine cHSCs in a cHCC cell line using rhodamine 123 and flow cytometry. Additionally, the various biological characteristics and chemoresistance were compared between subpopulations of stem cells with higher (Rho^{Hi}) and lower (Rho^{Lo}) rhodamine expression.

Materials and methods

Cell line and culture. The CHCC cell line (AZACH) was purchased from Cosmo Bio Co., Ltd., (Tokyo, Japan) and maintained in Eagle's minimum essential medium (EMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with Sigma-Aldrich 5% fetal bovine serum (FBS; Merck KGaA, Darmstadt, Germany), amino acid supplement (GlutaMAX; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and antibiotic-antimycotic agents (PSM; 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, final concentrations, all from Nacalai Tesque, Inc., Kyoto, Japan). They were subsequently cultured for two days at 37°C in an atmosphere containing 5% CO₂.

Flow cytometry and cell sorting. The cells were enzymatically dissociated using Accutase solution (Innovative Cell Technologies, Inc., San Diego, CA, USA) after washing with phosphate-buffered saline (PBS). The cells were resuspended with Dulbecco's PBS supplemented with 1% FBS and 1 mM EDTA-3Na (Wako Pure Chemical Industries, Ltd.). Cells were stained with a viability probe (Zombie NIR; BioLegend, Inc., San Diego, CA, USA) to stain the dead cells. In addition, wells were incubated with 0.1 µg/ml rhodamine 123 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at 37°C for 30 min and washed twice with PBS. Subsequent to washing, the labeled cells were analyzed using flow cytometry (Accuri C6; BD Biosciences, Franklin Lakes, NJ, USA) and sorting was performed using a cell sorter (SH800; Sony Biotechnology, Inc., Tokyo, Japan). A negative control was run using DPBS without rhodamine 123. Data were analyzed using FlowJo software (version 10.1; Tree Star, Inc., Ashland, OR, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the Rho^{Hi} and Rho^{Lo} subpopulations incubated for 24 h after sorting using a commercially available kit (miRNeasy Mini kit; Qiagen, Tokyo, Japan). RT to single-strand cDNA was performed using a commercially available kit (ReverTra Ace qPCR RT Master Mix with gDNA Remover; Toyobo Co., Ltd., Osaka Japan) according to the manufacturer's instructions. For qPCR, the Nanog primers (forward, TGGAACAATCCGCTCCACAA and reverse, GATGGACTCCAGATCACCCATAGAA) and templates were mixed with the SYBR Premix Ex TaqII (Takara Bio, Inc., Otsu, Japan). DNA was amplified by 45 cycles of denaturation for 5 sec at 95°C and annealing for 30 sec at 60°C using the Thermal Cycler Dice Real-Time

System II (Takara Bio, Inc.). Data generated from each PCR reaction were analyzed using the Thermal Cycler Dice Real-Time System version 2.10B (Takara Bio, Inc.). The relative quantity of mRNA was normalized to that of hypoxanthine phosphoribosyltransferase 1 (forward, GGAGCATAATCCAAAGATGGTCAA and reverse, TCAGGTTTATAGCCAACACTTCGAG). The data analysis was performed using the 2^{-ΔΔC_q} method (21).

Cell proliferation assay. Cell proliferation was analyzed using WST-8 and a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Briefly, sorted Rho^{Hi} and Rho^{Lo} subpopulations were seeded into 96-well plates at 3x10³ cells/well. Subsequently, 100 µl fresh medium containing 10 µl CCK-8 solution was added to each well after 12, 24, 48 and 72 h. The absorbance at a wavelength of 450 nm of each well was measured on an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) followed by incubation at 37°C for 1 h. A total of six replicates were prepared for each group.

Sphere formation assay. Sorted cells were seeded in ultra-low attachment plates (Corning Inc., Corning, NY, USA) at a density of 1x10⁶ cells/dish and cultured with EMEM supplemented with 5% FBS, amino acid supplement (GlutaMAX) and antibiotic-antimycotic agents (PSM; 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, final concentrations) for 3 days. Spheres were counted from 6 sites using a fluorescence microscope (BZ-9000; Keyence Corporation, Osaka, Japan) for quantitative analysis of sphere formation.

Chemoresistance assay. The cytotoxic effect in each subpopulation was determined by WST-8 (CCK-8) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at a density of 3x10³ cells/well with 0.1, 0.5, 1, 5, 10 and 50 µM of doxorubicin (DOX), MTX and GEM (Wako Pure Chemical Industries, Ltd.). Following incubation for 24 h at 37°C in an atmosphere containing 5% CO₂, 100 µl fresh medium containing 10 µl CCK-8 solution was added to each well, followed by incubation at 37°C for 1 h. The absorbance at a wavelength of 450 nm of each well was measured on an Epoch microplate spectrophotometer. A total of six replicates were prepared for each group.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). The results were expressed as means ± standard error. Comparisons between two groups were performed using the independent t-test. Multiple comparisons were performed with one-way ANOVA and P<0.05 was considered to indicate a statistically significant difference.

Results

Rhodamine 123 staining and sorting of cHCC. Flow cytometry of cultured cHCC excluding dead cells confirmed that rhodamine was expressed. Cells labeled by rhodamine

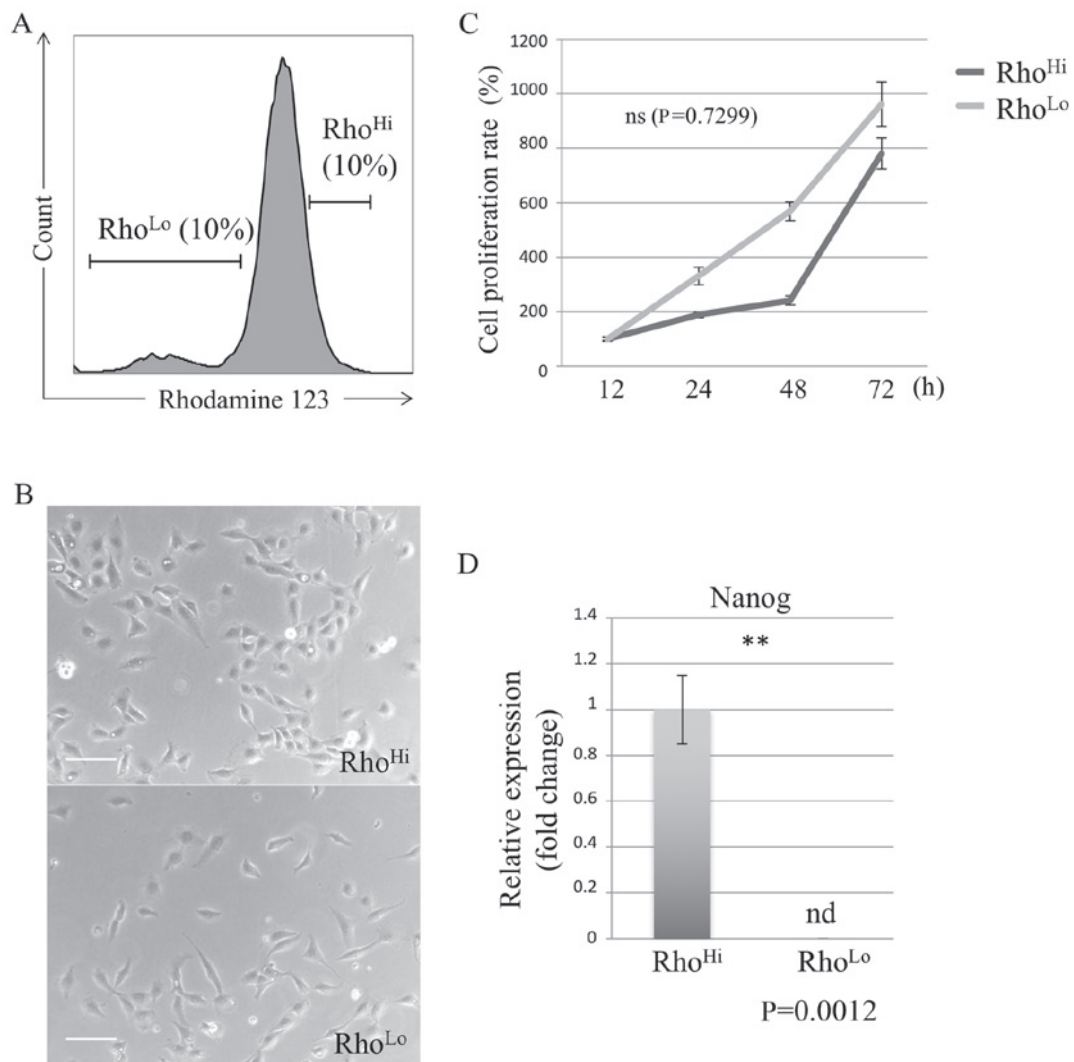


Figure 1. Detection of Rho^{Hi} and Rho^{Lo} subpopulations of cHCC and evaluation of proliferation rates and Nanog expression levels. (A) Flow cytometric analysis of cHCC. The difference in the expression (~10%) of rhodamine was considered to characterize cells as Rho^{Hi} and Rho^{Lo}. (B) The sorted Rho^{Hi} and Rho^{Lo} cells exhibited similar morphologies following incubation for 24 h (scale bar, 100 μ m). (C) No difference in the cell proliferation rates was identified between the Rho^{Hi} and Rho^{Lo} subpopulations. (D) Rho^{Hi} exhibited higher Nanog expression levels than Rho^{Lo}. Values are expressed as means \pm standard error (n=5). ** $P<0.01$ Rho^{Hi} vs. Rho^{Lo}. Ns, not significant; nd, not detected; cHCC, canine hepatocellular carcinoma.

were sorted by their expression of rhodamine into Rho^{Hi} and Rho^{Lo} (Fig. 1A). Subsequent to sorting, the Rho^{Hi} and Rho^{Lo} subpopulations were incubated for 24 h, and the two subpopulations exhibited a similar morphology (Fig. 1B).

Comparison of proliferation rate. To assess the difference in the proliferation potential between the Rho^{Hi} and Rho^{Lo} subpopulations, the proliferation rate was measured in each subpopulation using the WST assay. As a result, the proliferation potential of the Rho^{Hi} and Rho^{Lo} subpopulations of cHCC was not identified to be significantly different (Fig. 1C).

Comparison of Nanog expression. The gene expression of Nanog, a common type of stem cell marker, was evaluated using RT-qPCR. The gene expression level of Nanog in Rho^{Hi} was identified to be higher than that in Rho^{Lo} (Fig. 1D).

Comparison of sphere formation. A sphere formation assay was performed using ultra-low attachment dishes and demonstrated

the formation of tumor spheres in cultures of Rho^{Hi} and Rho^{Lo} subpopulations (Fig. 2A). However, the Rho^{Hi} subpopulation exhibited a significantly higher number of sphere formations per visual field than the Rho^{Lo} subpopulation (Fig. 2B).

Comparison of chemoresistance ability. Chemoresistance against MTX, GEM, and DOX was determined for the Rho^{Hi} and Rho^{Lo} subpopulations. Rho^{Hi} exhibited a higher survival rate than Rho^{Lo} when GEM was administered (Fig. 3B). However, no significant difference in the chemoresistance potential was identified between the Rho^{Hi} and Rho^{Lo} subpopulations with MTX or DOX administration.

Discussion

In the current study Rho^{Hi} demonstrated higher Nanog expression level and sphere formation ability than Rho^{Lo}. In addition, Rho^{Hi} exhibited greater chemoresistance potential to GEM when compared with Rho^{Lo}. However, no

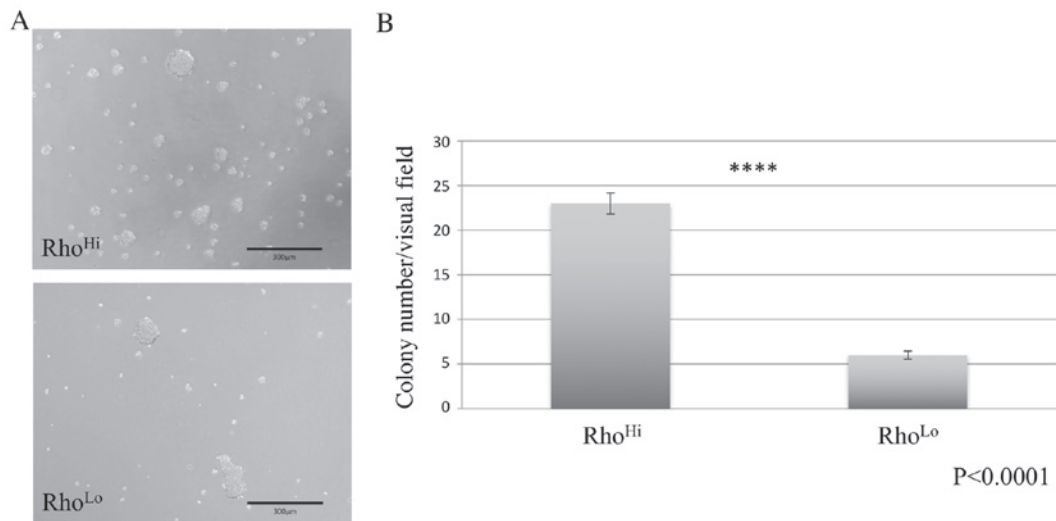


Figure 2. Sphere formation ability of Rho^{Hi} and Rho^{Lo} subpopulations. (A) Three days after cultivation of Rho^{Hi} and Rho^{Lo} subpopulations in ultra-low attachment dishes, the two subpopulations exhibited spherical colonies (scale bar, 300 μm). (B) Quantitative analysis of sphere formation revealed a higher sphere formation ability in the Rho^{Hi} subpopulation. ****P<0.0001 as indicated between Rho^{Hi} and Rho^{Lo}.

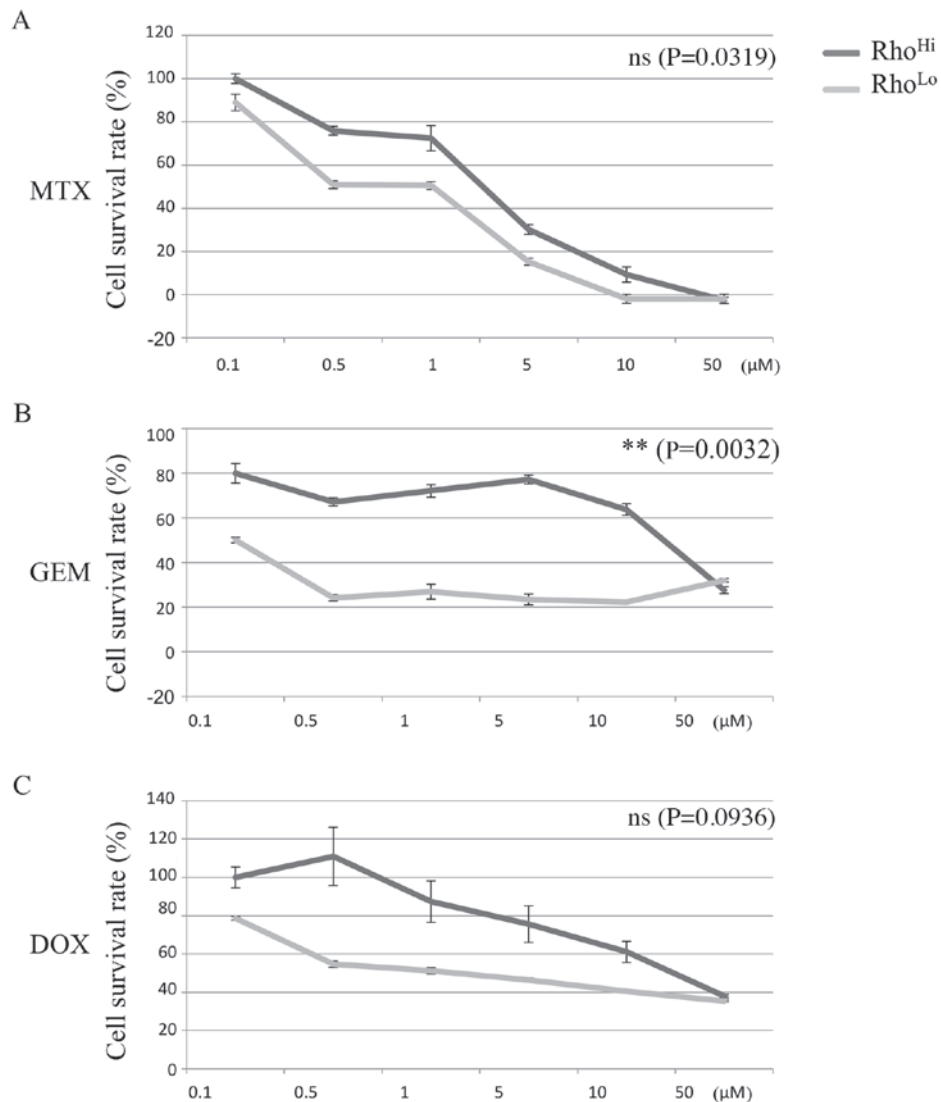


Figure 3. Chemoresistance against MTX, GEM, and DOX was determined for the Rho^{Hi} and Rho^{Lo} subpopulations. (A) No significant difference regarding the chemoresistance potential was shown between the Rho^{Hi} and Rho^{Lo} subpopulations with MTX treatment. (B) Rho^{Hi} exhibited a higher survival rate than Rho^{Lo} following GEM treatment. (C) No significant difference regarding the chemoresistance potential was identified between the Rho^{Hi} and Rho^{Lo} subpopulations with DOX treatment. **P<0.01 as indicated between Rho^{Hi} and Rho^{Lo}. ns, not significant; MTX, mitoxantrone; GEM, gemcitabine; DOX, doxorubicin.

significant difference between Rho^{Hi} and Rho^{Lo} regarding the proliferation rate or chemoresistance against MTX and DOX was identified.

Rhodamine 123 is absorbed easily by living cells, becoming concentrated in the mitochondria (22). Mitochondria are categorized as intracellular organelles and functionally supply adenosine triphosphate as a result of cell respiration and metabolism. Previous studies propose that reactive oxygen species produced in mitochondrial respiration are correlated with aging and the formation of malignancies (23,24). Furthermore, recent studies have revealed rhodamine to be a marker of stem cells. However, identifying which stem cells display either higher or lower expression levels of rhodamine is complicated. Regarding adult stem cells, a few reports have identified HPC as having a low expression of rhodamine 123 (19,25). Conversely, in cancer cells, a previous study revealed that subpopulations with a higher expression of rhodamine exhibited higher proliferation, sphere formation, radio-resistance and tumor differentiation potential than those with a lower expression of rhodamine (26).

To the best of our knowledge, there is only one report regarding HCSCs identified by rhodamine expression in humans (27). In the present study, primary HCCs were obtained from clinical patients; primary HCC were compared with cells cultured with DOX and 5-fluorouracil, which were regarded as HCSCs. HCSCs exhibited more stem cell markers, sphere formation, and tumor differentiation and a lower level of rhodamine expression than primary cells (27). The present study demonstrated that the expression of rhodamine was a poor stem cell marker; however, it did not examine chemoresistance or sphere-forming ability in the Rho^{Hi} and Rho^{Lo} subpopulations (24). Additionally, cells from the clinical patients cultured with DOX and 5-fluorouracil were regarded as HCSCs. Thus, these were cells selected for their chemoresistance and ability to proliferate well in dishes (24). As a result, Rho^{Lo} cells may have been exhibited as being poorly differentiated.

Treatment for cHCC is limited, and poor prognoses have been reported for cHCC with metastasis or diffusion through an entire hepatic lobe, although massive cHCC has a good prognosis when it is treated surgically. Thus, more effective treatments are required for nodular and diffuse cHCC. A recent study in human medicine have proposed a novel strategy for the treatment of cancer targeted at CSCs, and notable results have been presented regarding CSCs from basic and clinical trials (28). Veterinary medicine follows human medicine, and CSCs have been reported from various types of solid tumor and hematological malignancies (29-32). To the best of our knowledge, the current study is the first to describe CSCs identified by rhodamine in a canine cancer cell line, and it may lead to basic studies regarding cHCSC-targeting treatment. Further studies are required to reveal the mechanism by which mitochondrial activity affects the stem cell characteristics and chemoresistance of cHCC.

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