Isolation of cancer stem like cells in primary endometrial cancer using cell surface markers CD133 and CXCR4

細胞表面マーカーCD133，CXCR４を用いたヒト子宮内膜癌からの癌幹細胞様細胞の単離

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Contents

1. Abstract

Endometrial cancer is the most common gynecologic malignant tumor of the female reproductive system in developed countries, besides, incidence rates of endometrial cancer is increasing per year. Although surgery, radiation, and chemotherapy were established for the remedy, the novel therapies are urgently necessary, especially for recurrent diseases that have acquired radio- or chemo-resistance as a big challenge for us. It has been reported that a small subpopulation of cancer cells has a great advantage of cell proliferation, proposing the concept of cancer stem cells (CSCs) in malignant tumors that are responsible for tumor formation and progression. CSCs in endometrial cancer, side-population (SP) cells has been identified because the SP phenotype is associated with a high expression level of the ATP-binding cassette transporter protein ABCG2/Bcrp1. However, in order to develop the molecular therapy target, cell surface markers are more convenient than SP fraction. In this research, the identification of CSCs in endometrial cancer was focused on the cell surface markers and stem cell markers. The tissues were got from human endometrial cancer which had obtained from endometrial patients during surgical procedures.

Isolated endometrial cancer cells strongly expressed mRNA of stemness-related genes, such as *c-Myc, Sox-2, Nanog, Oct 4A, ABCG2, BMI-1, CK-18,* and *Nestin* . The cells expressed many kinds of surface markers, such as CD24， CD133，CD47， CD29， CD44, CXCR4, SSEA3 and SSEA4. CD24, CD133, CXCR4 and CD47 accounted for 22.1±3.4 %, 25.6±5.4 %, and 12±1.3%, 10.2±2.9%, respectively. CD44, and CD29 constituted 94.3 ±2.7 %, and 93.1±3.7%, respectively. SSEA-3 and SSEA-4 did only 0.6± 0.1% and 0.43±0.1%, respectively.

I focused on CD24, CD133, CXCR4 because these cell surface markers expressed patients independent manner. The mRNA levels of the stemness genes were compared with the difference of the expression of cell surface marker. The expression of stemness genes were higher in CXCR4+ cells than in CXCR4- cells, as well as in CD133+ and in CD133- cells. However, there was no difference in the presence of CD24 between these cells.

Following sorting, the percentage of CD133+CXCR4+ cells was 9.3% in isolated endometrial cells. They grew faster, exhibited higher expression of stemness-related genes, produced more spheres, and had higher clonogenic ability and tumorigenicity than other subpopulations. CD133+CXCR4+ cells were also more resistant to anti-cancer drugs than other subpopulations. These findings indicate that CD133+CXCR4+ cells may be CSC like cells in primary endometrial cancer.

The cell surface markers CD133 and CXCR4 may be useful for the development of drugs against CSC molecular targets.

Key words: Cancer stem cells (CSCs), endometrial cancer, CD133, CXCR4

2. Introduction

Endometrial cancer is one of the most common malignancies in the world. United state of American cancer statistics indicate that the incidence of endometrial cancer is increasing yearly, with 49,560 and 54,870 new cases diagnosed in 2013 and in 2015, respectively [1, 2]. There are several pathological types of endometrial cancer, including endometrioid carcinoma, mucinous adenocarcinoma, papillary serous adenocarcinoma, clear cell adenocarcinoma, undifferentiated carcinoma, and mixed carcinoma. Even if a types differ in endometrial cancer, conventional treatments, such as operation and chemotherapy, have been already established, and unfortunately, sometimes recurrence will occur. Previous studies have promoted the cancer stem cell (CSC) theory as a prominent explanation for tumor heterogeneity, and have suggested that CSCs have certain abilities including self-renewal, invasion, anti-tumor drug resistance, and tumor recurrence [3, 4]. According to the CSC hypothesis, tumors are organized in a hierarchy of heterogeneous cell populations, and only a small subpopulation of the cells within them are located as CSC. CSC have the ability to self-renewal, can give rise to different progeny, and utilize common signaling pathways [5], and to sustain tumor formation and growth [6]. The CSC hypothesis not only provides a mechanism for therapeutic methods, but also explains the mechanism of resistance for chemotherapy or radiation. That is, the theory has had a significant influence on our understanding of cancer metastasis, biology, and progression and has provided a molecular target for anti-cancer therapeutic methods.

The validity of the CSC theory has been demonstrated in leukemic cells; CSCs have been shown to account for <1% of total tumor cells [4, 7,

8].First, the CSC in leukemic cells expressed surface markers or other specific markers and was able to use to identify a population of CSC from total tumor cells. And then, CSCs have been discovered in various kinds of tumors including breast [9], prostate [10], pancreas [11], melanoma tumors and so on [12]. However, to identify and isolate CSCs from solid tumors was very challenging because most solid tumors are unlike the hematopoietic, the normal tissue developmental gradation has not been identified and therefore, it is more difficult to select candidate markers [13]. The minority subpopulation was isolated from a solid human breast cancer tumor. These cells were identified by cell surface markers of CD44+CD24-/low Lineage- ; the cells are tumorigenic; when low numbers of CD44+CD24- cells were injected into immune deficient mice, tumors formed at very high frequency, while alternate phenotypes failed to form tumors. And now, it was reported a number of cell surface markers, including CD24, CD44, CD49, CD90, CD133, ALDH1, EpCAM, ESA and SP fraction were common to several types of cancer [14].

Gargett C E et al. identified the epithelial stem/progenitor cells in the human endometrium [15]. The human endometrium is a highly dynamic tissue that undergoes cycles of growth, differentiation, shedding, and regeneration throughout the reproductive life of women and contains rare populations of epithelial and stromal colony-forming cells [16]. Friel A M et al. showed that SP cells isolated from the endometrial cancer cell lines AN3CA and Ishikawa were chemoresistant and had high proliferative activity and tumorigenicity [17]; while Hubbard S A et al. demonstrated that the small population of clonogenic cells isolated from endometrial cancer patient tissues possessed self-renewal, differentiation, and tumorigenic abilities [18].

CD133 (human prominin-1) is a membrane glycoprotein with a putative function in plasma membrane organization. It is the first marker used to identify and isolate CSCs from the in endometrial cancer. Rutella S et al. and Nakamura M et al. analyzed tumor samples for CD133 expression. They isolated CD133+ cells and assessed their phenotypic characteristics, self-renewal capacity, ability to maintain CD133 expression and form sphere-like structures in long-term cultures, sensitivity to chemotherapeutic agents, gene expression profiles, and the ability to initiate tumors in NOD/SCID mice [19, 20].

CXC motif chemokine receptor 4 (CXCR4) is a stromal cell-derived factor-1 receptor secreted by bone marrow, liver, lung, and neural cells [21]. A previous study detected CXCR4 expression in malignant tumor cells and showed activation of CXCR4 causes signaling through numerous pathways, leading to enhanced survival, increased proliferation, drug resistance, degradation of extracellular matrix, and angiogenesis [22].

In this research, based on the previous study [23, 24], I isolated cancer stem cells from primary endometrial cancer using by cell surface markers CD133 and CXCR4, and it suggested that the obtained endometrial cancer stem cells are tumorigenic and display stem cell-like properties.

3. Materials and Methods

3.1 Human primary endometrial cancer tissues

All endometrial cancer tissues were collected in accordance with the guidelines of the Research Ethics Committee of the University of Toyama. The tumor samples were obtained during surgical resection after obtaining informed consent from the of endometrial cancer patients. The sampled tumor tissue was divided in three. One section was fixed with 4% paraformaldehyde for pathology, one was washed and dissociated into 1-2mm^3 fragments, then transplanted subcutaneously into nude mouse (6-8 weeks), and one was used to isolate cancer cells.

3.2 The isolated cells from human endometrial cancer tissues

The small tissue fragments were washed with phosphate buffered saline (PBS, WAKO, Tokyo, Japan), digested twice with 0.4% collagenase(Sigma-Aldrich, ST Louis, USA), for 15 min in a 37 °C water bath, and then filtered with gauze. Cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, ST Louis, USA) supplemented with 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan), 1% L-glutamine (Nacalai Co., Kyoto, Japan), and 20% fetal bovine serum (Biosera, Sydeny, Australia) in a cell culture incubator at 37 °C under 5% CO_2

3.3 Flow cytometric analysis

EC cells at 70%-85% confluence were dissociated with trypsin (0.25%, Sigma-Aldrich, ST louis, USA), incubated in PBS, then blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich, ST louis, USA) for 30 min. The cells (1×10^6) were stained with antibodies against CD24 (anti CD24-FITC; BD Pharmingen, Franklin Lakes, New Jersey, USA), CD133 (anti CD133-FITC; Miltenyi Biotec, Gladbach, Germany), CD47 (anti CD47-FITC; Santa Cruz Biotechnology, Dallas, Texas, USA), CD29 (anti CD29-

PE; BD Pharmingen, Franklin Lakes, New Jersey, USA), CD44 (anti CD44-PE; BD Pharmingen, Franklin Lakes, New Jersey, USA), CXCR4 (anti CXCR4-PE; Beckman Coulter, Brea, California, USA), SSEA3 (anti SSEA3-FITC; BD Pharmingen, Franklin Lakes, New Jersey, USA), and SSEA4 (anti SSEA4-PE; BD Pharmingen, Franklin Lakes, New Jersey, USA), then washed twice with PBS and analyzed using the FACS Canto II system (BD Biosciences, Franklin Lakes, New Jersey, USA).

3.4 Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's protocol. Isolated RNA (1 μg) was reverse transcribed with the ReverTra Ace qPCR RT Kit Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was amplified using the Taq DNA polymerase kit (Qiagen, Dusseldorf, Germany) with specific primers for *c-Myc, Sox-2, Nanog, Oct 4 A*, *ABCG2*, *BMI-1*, *CK-18, Nestin* and *ß-actin* (Table 2) under the following conditions: initial denaturation (95 ºC, 4 min); 35 cycles of denaturation (94 ºC, 1 min), annealing (*c-Myc, Sox-2, Nanog, ABCG2, CK-18, Nestin* and *ß-actin* with 56 ºC, *Bmi-1* with 50 ºC, *Oct 4 A* with 60 ºC, respectively, for 1 min), extension (72 ºC, 1 min); and a final extension (72 ºC, 10 min) with GeneAmp PCR System(Applied Biosystems, Foster City, California, USA). Primers and PCR conditions are detailed in Table 1. PCR products were separated by electrophoresis on 2% agarose gels (Wako, Osaka, Japan), visualized with ethidium bromide (Wako, Osaka, Japan), and analyzed using the Image Reader LAS-3000 software (Fujifilm, Tokyo, Japan).

Table 1

Genes	Primer	Size (bp)	Annealing temperature(°C)	Cycle number
c-Myc	F:GATTCTCTGCTCTCCTCGACGGAG	273	56	35
	R:GCGCTGCGTAGTTGTGCTGATGTG			
Sox-2	F:AGTCTCCAAGCGACGAAAAA	410	56	35
	R:GGAAAGTTGGGATCGAACAA			
Nanog	F:CAGAAGGCCTCAGCACCTAC	216	56	35
	R:CTGTTCCAGGCCTGATTGTT			
Oct 4A	F:GAAGCTGGAGAAGGAGAAGCTG	224	60	35
	R:CAAGGGCCGCAGCTTACACATGTTC			
ABCG2	F:TCAGGTAGGCAATTGTGAGG	210	56	35
	R:CTTCAGCATTCCACGATATGG			
BMI-1	F:AATCAAGGAGGAGGTGA	370	50	35
	R:CAAACAAGAAGAGGTGGA			
CK-18	F:TGGTCACCACACAGTCTGCT	357	56	35
	R:CCAAGGCATCACCAAGATTA			
Nestin	F:ACAGCGGAATTCCTGGAG	410	56	35
	R:CTGAGGACCACGACTCTCTA			
ß-actin	F:CGGGACCTGACTGACTAC	252	56	35
	R:GAAGGAAGGCTGGAAGAG			

Table 1 PCR primer and PCR control condition details

3.5 Magnetic-activated cell sorting (MACS)

CD133+CXCR4+ EC cells were separated by Magnetic-activated cell sorting technology (MACS: Magnetic-Activated Cell Sorting, Miltenyi Biotec, Bergisch, Gladbach, Germany). Cultured EC cells at 70%-85% confluence were trypsinized and resuspended in cold (2-8℃) MACS buffer

(PBS, pH 7.2, 0.5% BSA, and 2 mM EDTA). Cells were stained with the CD133 FITC-conjugated primary antibody (Miltenyi Biotec, Bergisch, Gladbach, Germany) according to the manufacture's recommendations: 10 µL of the CD133 FITC-conjugated antibody were added per 10^7 total cells in 100 µL of buffer, mixed well, and then incubated in the dark at 2-8℃. Cells were washed with buffer and centrifuged at 300×g for 10 minutes, twice. The cells were then resuspended and 20 µL anti-FITC Multisort microbeads (Miltenyi Biotec, Bergisch, Gladbach, Germany) and 80 µL buffer were added per 10^7 total cells, mixed well and incubated 15min at 2-8°C. The cells were then washed, centrifuged at 300×g for 10min, and applied to a column placed in the magnetic field of the MACS Separator (Miltenyi Biotec, Bergisch, Gladbach, Germany). The unlabeled cells which passed through the column, were collected; the total effluent is the unlabeled cell fraction. Next, buffer washes were performed three times: the column was removed from the separator and the buffer was pipetted onto the column. The magnetically labeled cells were immediately flushed out by firmly pushing the plunger into the column. For the second magnetic labeling and separation, 20 µL of MultiSort Release Reagent (Miltenyi Biotec, Bergisch, Gladbach, Germany) were added per 1 mL of cell suspension, mixed well, and incubated for 10 min in the dark at 2-8 °C. The released fraction was then washed by adding 1-2 mL of buffer per 10^7 cells and centrifuged at 300×*g* for 10 min. The supernatant was discarded, a total of 10^7 cells were resuspended cells per 50 µL buffer, and 30 µL of MultiSort Stop Reagent (Miltenyi Biotec, Bergisch, Gladbach, Germany) were added per 10^7 total cells and mixed well. Next, the MACS Micro Beads were added to magnetically label the cells for the second marker, the CXCR4 PE conjugated antibody (Beckman coulter, Brea,California, USA), and the

reaction volume was adjusted to 100 μL. The reaction was mixed well and incubated according to the manufacturer's recommendations. Magnetic separation was performed as described above.

3.6 Immunofluorescence

EC cells (2.5x 10^4) were suspended in 50 μL PBS, dripped onto glass slides, and then centrifuged at 800 rpm for 7 min using a cytospin (Thermo scientific, Massachusetts, Texas, USA). Slides were air-dried overnight, fixed in acetone for 15 min at -20 °C, and blocked in Block Ace（DS Pharm Biomedical, Tokyo, Japan）for 15 min. The cells were then incubated with primary antibodies (Santa Cruz Biotechnology, Texas, USA) against c-Myc (1:200), Klf-4 (1:200), Oct3/4 (1:200), Nanog (1:200), and Sox-2 (1:50) overnight at 4 °C. The slides were washed three times with PBS, then incubated with an Alexa Flour-conjugated secondary antibody in the dark at 37 °C for 1 h. The slides were counterstained with 4,6-diamidino-2-phenylidole (DAPI) (Cosmo Bio. Tokyo Japan) to demonstrate the presence of nuclei, negative groups were stained without a primary antibody. Stained slides were examined using a Leica fluorescent microscope (LEITZ DMRD, Wetzlar, Germany) with cellSens standard software (OLYMPUS, Tokyo, Japan) and images were obtained with a digital camera (DP73; OLYMPUS, Tokyo, Japan).

3.7 Cell proliferation assay

After sorting, unsorted cells (parent cells), CD133+CXCR4+ cells and CD133-CXCR4- cells were seeded into 24-well plates at a concentration of 5×10^3 per well. The cells were cultured in DMEM (Sigma-Aldrich, ST Louis, USA) supplemented with 1% penicillin-streptomycin(Nacalai Tesque, Kyoto, Japan) , 1% L-glutamine(Nacalai Co., Kyoto, Japan), and 20% fetal bovine serum (Biosera, Sydney, Australia) in a cell culture incubator at 37 °C under

5% CO_2. Medium was changed every 24 h and the cells of each subgroups were counted at every two day from day 2 to day 12.

3.8 Sphere formation assay

Following cell sorting, parent cells or CD133+CXCR4+ cells or CD133-CXCR4- cells were seeded at a density of $1.0x10^3$ cells in 24-well ultra-low attachment dishes (Corning, New York, USA) containing serum-free medium composed of DMEM/F12 (Sigma-Aldrich. St. Louis, USA) with 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan), 1% L-glutamine (Nacalai Co., Kyoto, Japan), respectively, then cultured at 37 °C under 5% CO_2 for 7 days.

3.9 Soft agar colony formation assay

EC cells (2×10^4) from each group, that is unsorted cells, CD133+CXCR4+ EC cells and CD133-CXCR4-EC cells, were added to 3 mL 0.4% Noble Agar (BD, New Jersey, USA), mixed well, then seeded onto 60mm dishes containing 5 mL 0.5% Bacto Agar (BD, New Jersey, USA). The cells were cultured at 37 °C under 5% CO_2 in an incubator for 14 days then examined using a microscope (Nikon, ECLIPSE, TE2000-V, Tokyo, Japan). Colonies with a diameter greater than 500 μm were counted and analyzed.

3.10 Chemosensitivity assay

Chemosensitivity was evaluated using the Cell Counting Kit-8 (Dojindo , Tokyo, Japan) assay according to the manufacturer's protocol; 5×10^3 cells per well were seeded in 96-well plates, pre-incubated for 24 h, then treated with different concentrations of cisplatin (Sigma-Aldrich, Missouri, USA) and paclitaxel (Sigma-Aldrich, Missouri, USA). Following 24 h incubation, 10 μL of CCK-8 solution was added to each well and the cells were further incubated for 2 h at 37 °C under 5% CO_2 in an incubator. Cell viability was determined by measuring absorbance at 450 nm with a

Multi-Mode Microplate Reader (Filter Max F5, Molecular Devices, USA).

3.11 Xenograft tumor formation assay

Male, 6- to 8- week-old nude mice were purchased from Japan SLC (Tokyo, Japan) and housed under pathogen-free conditions. Tumorigenicity experiments were performed according to the guidelines provided by the Experimental Animal Center, Toyama University. To determine tumorigenicity, various amounts (1×10^4, 1×10^3, and 1×10^2) of CD133+CXCR4+ cells, and CD133-CXCR4- cells were diluted in PBS. Next, the CD133+CXCR4+ cells were subcutaneously injected into the left flanks of nude mice and CD133-CXCR4- cells were subcutaneously injected into the right flanks. Tumor formation was evaluated 12 weeks post injection.

3.12 Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical analysis was carried out using the statistical software Statistical Product and Service Solutions. Student's *t*-test was used to compare the means between the different groups. Values are presented as the mean with standard error. All data were statistically analyzed using SPSS, version 21 (SPSS, Inc, Chicago, Illinois, USA). A P value <0.05 was considered as significant.

4. Results

4.1 Expression of stem cell related genes, stem cell surface markers and chemokines in isolated endometrial cancer cells.

The characterization of stemness in isolated cells from endometrial tissues was determined by measuring mRNA expression using semi-quantitative RT-PCR. Several stemness genes, including *c-Myc, Sox-2, Nanog, Oct 4 A, ABCG2, BMI-1, CK-18* and *Nestin* were expressed in the isolated cells (Figure 1 A).

Next, the presence of CD24, CXCR4, CD133, CD44, CD49, CD29, SSEA-3, and SSEA-4 in isolated cells was examined by flow cytometry; CD24, CXCR4, and CD133 accounted for 22.1±3.4 %, 25.6±5.4 %, and 12 ±1.3 %, respectively. CD44, CD47, and CD29 accounted for 94.3±2.7 %, 10.2±2.9%, and 93.1±3.7%, respectively. However, SSEA-3 and SSEA-4 constituted only 0.6±0.1% and 0.43±0.1%, respectively (Figure 1B-1, 1B-2).

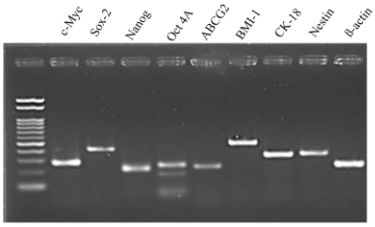
4.2 Subgroup of isolated cells after sorting by MACS.

The mRNA levels of the stemness genes were higher in CXCR4+ cells than CXCR4- cells, as well as CD133+ and CD133- cells. However, there was no difference in the presentation of CD24 between these cells (Figure 1C)

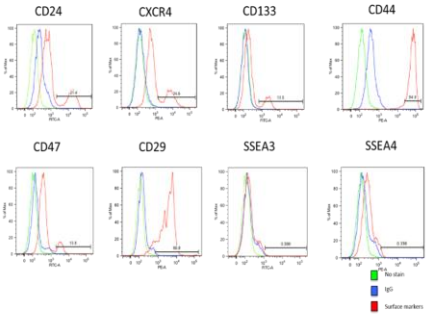
The proportion of CD133+CXCR4+ cells was 9.3%, CD133+CXCR4- cells was 1.9%, CD133-CXCR4+ cells was 27.8%, and CD133-CXCR4- cells 61.0 % in isolated cells (Figure 1D).

Figure 1

A



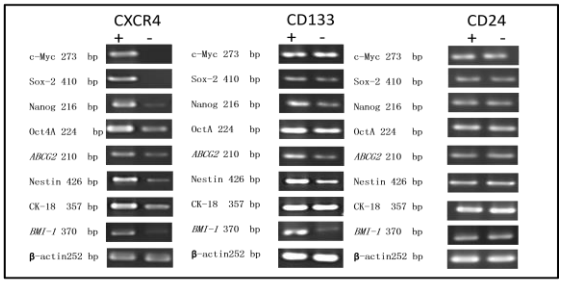
B-1



B-2

FACS%	CD24	CXCR4	CD133	CD44	CD47	CD29	SSEA3	SSEA4
Average	22.1±3.4	25.6±5.4	12±1.7	94.3±2.7	10.2±2.9	93.1±3.7	0.6±0.1	0.43±0.1

C



D

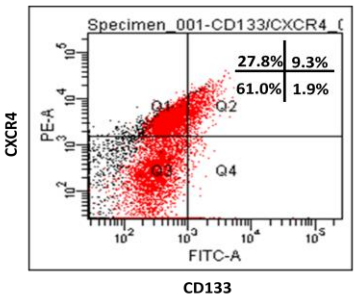


Figure1 The expression of stemness genes and surface markers in isolated cells from endometrial cancer tissues. (A) EC cells expressed stemness related genes including *c-Myc, Sox-2, Nanog, Oct 4 A, ABCG2, BMI-1, CK-18, Nestin* and *ß-actin*. *ß-actin* is the negative control. (B-1, B-2) The expression of CD24, CD133, CD47, CD29, CD44, CXCR4, SSEA3 and SSEA4 were analyzed by flow cytometry. (C) The mRNA expression of comparison and analysis between CD24, CD133, and CXCR4 positive and negative subpopulation by RT-PCR. (D) The CD133+CXCR4+ cells ration is 9.3%.

4.3 The expression of mRNA compared with CD133+CXCR4+ and CD133-CXCR4- cells.

The cells were examined the expression of genes thought to play key roles in stem cell biology, *such as c-Myc, Sox-2, Nanog, Oct 4 A, ABCG2, BMI-1, CK-18,* and *Nestin*. The mRNA expression was showed that *c-Myc, Sox-2, Nanog, ABCG2, BMI-1,* and *Nestin* was higher in the CD133+CXCR4+ cells and lower in the CD133-CXCR4- cells (Figure 2 A). Immunocytochemical staining further demonstrated that c-Myc, KLF4, OCT3/4, Nanog, and SOX2 levels were higher in CD133+CXCR4+ cells compared to parent cells and CD133-CXCR4- cells (Figure 2B).

Figure 2A

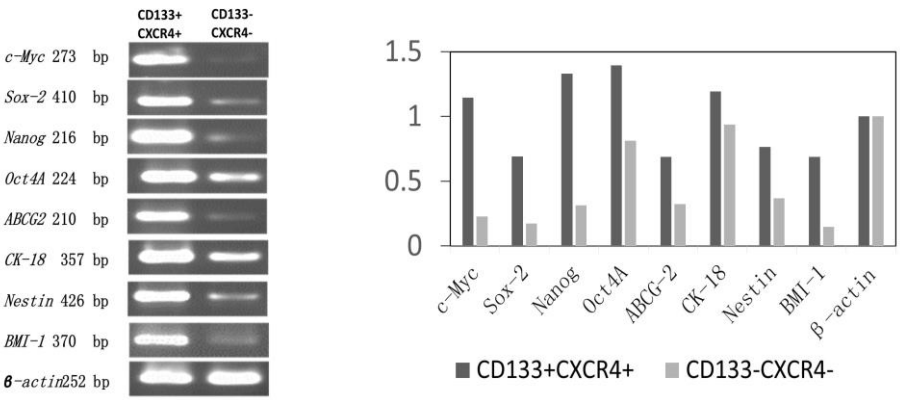
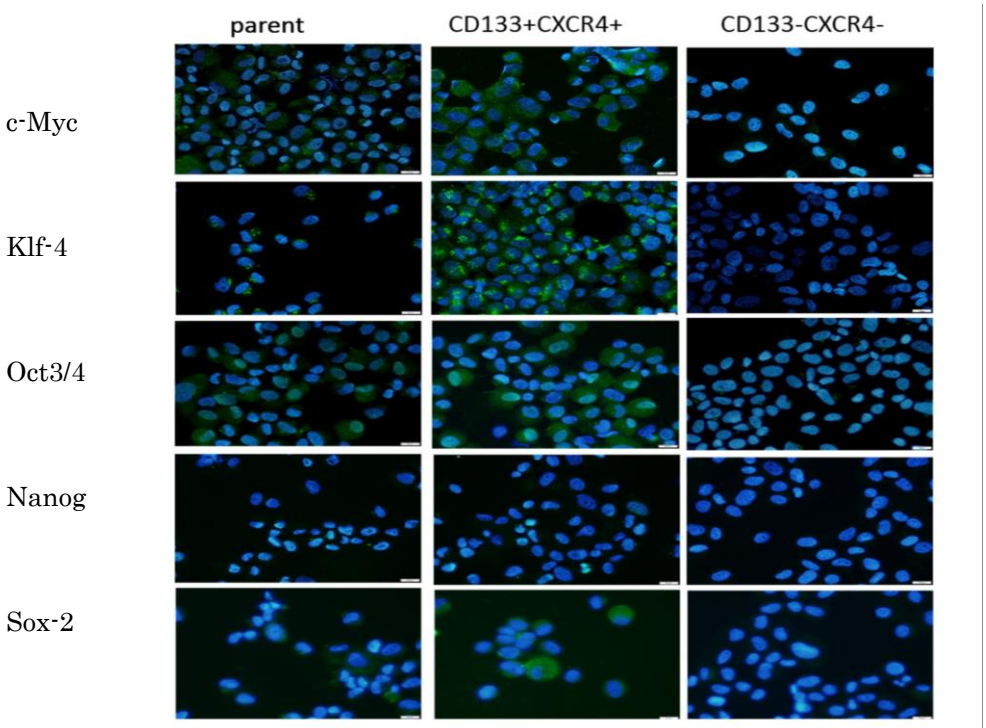


Figure 2 The expression of comparison and analysis between CD133+CXCR4+ and CD133-CXCR4- cells. (A) The cells expressed higher stemness genes such *c-Myc, Sox-2, Nanog, Oct 4 A,ABCG2, BMI-1, CK-18,* and *Nestin* in CD133+CXCR4+ cells than CD133-CXCR4- cells. ß-actin was used as parameter.

Figure 2B



(B) The immunocytochemical stain of the subgroups. Nuclei were stain with DAPI. (× 20)

4.4 CD133+CXCR4+ cells have increased proliferative and clonogenic capacity

The proliferative capacity was compared among each subgroup in *vitro*. CD133+CXCR4+ cells were cultured in normal medium for 12 days. The growth curve showed that CD133+CXCR4+ cells grew faster than parent cells and CD133-CXCR4- cells (Figure 3 A). Besides, analysis of sphere formation was examined (Figure 3 B). The CD133+CXCR4+ cells formed the most spheres compared with other subgroups ($p<0.05$). Then separated the formed spheres into three groups which included large, middle and small (L>100 μm, M 50-100 μm, S 20-50 μm) types. The result revealed that the CD133+CXCR4+ cells formed spheres were not only more than EC cells and CD133-CXCR4- cells, but also had the largest size (Figure 3 C).

As is shown in Figure 3D, the colony-formation capacity was tested in dishes with soft agar. CD133+CXCR4+ cells formed an average of 2345 colonies with larger sizes, however, CD133-CXCR4- cells and parent cells showed an average of 354 and 1432 colonies with smaller sizes, respectively. The colony-forming ability showed the significant difference ($p<0.05$).

Figure 3

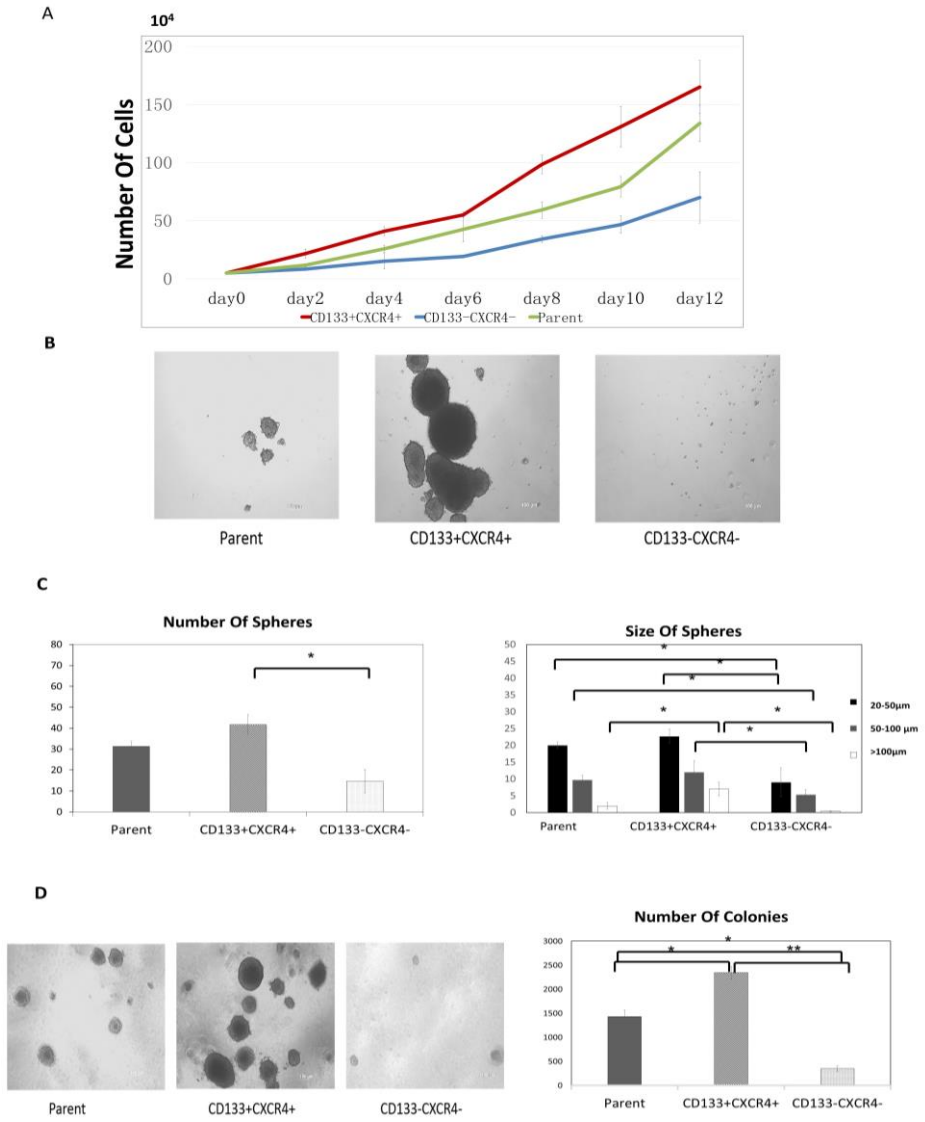


Figure 3 Characteristics of parent cells, CD133+CXCR4+ cells and CD133-CXCR4- cells. (A) The growth curve of cells that counted day 2 to day 12 after sorting cell. (B) The photograph of spheres after sorting, cultured for 7 days. (C) The number and size of spheres. Spheres

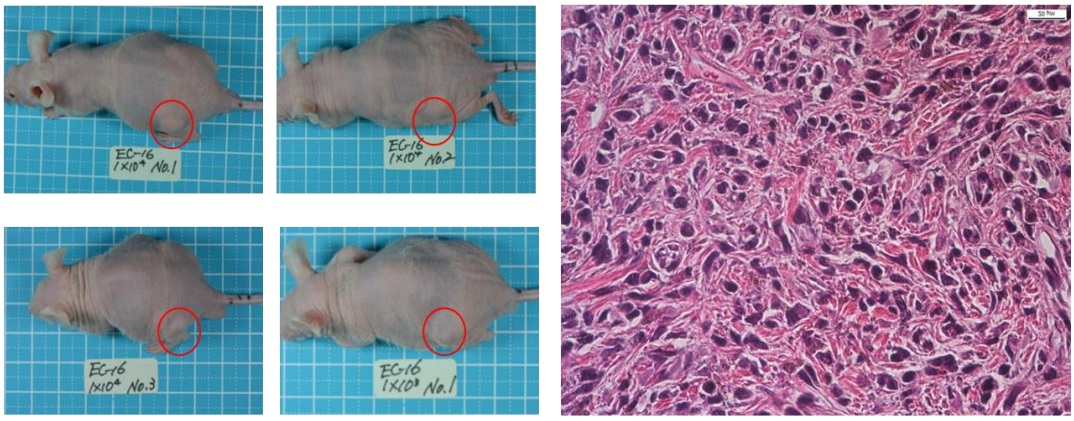
were observed in all subgroups, but whether the numbers or the size of the spheres, the CD133+CXCR4+ cells were the most and largest (* $p<0.05$). (D) Colony-forming assay demonstrated the difference in the each subgroups (* $p<0.05$, * * $p<0.01$).

4.5 CD133+CXCR4+ cells have tumorigenic potentials

The tumorigenic potential was evaluated with CD133+CXCR4+ cells and CD133-CXCR4- cells using node mice. 1×10^4 CD133+CXCR4+ cells initiated subcutaneous tumors in nude mice (3/3), 1×10^3 CD133+CXCR4+ cells also formed tumor (1/3). Carcinoma cells of immature morphologies were identified in the graft (Figure 4). Meanwhile, CD133-CXCR4- cells completely failed to form tumors in nude mice (Figure 4 A and B).

Figure 4

A



B

Number of cells	CD133+CXCR4+	CD133-CXCR4-
1.0×10^4	3/3	0/3
1.0×10^3	1/3	0/3

Figure 4 Tumorigenicity ability of sorted cells in *vivo*. (A) Photograph of tumor formation in nude mice after subcutaneous injection (1.0×10^4 cells and 1.0×10^3 cells). Histological analyses showing hematoxylin and eosin staining. It was shown that the cells possessed the bigger and irregular nucleus and sometimes multiple nuclei. (B) Table of rate of tumor formation. It was compared between CD133+CXCR4+ cells and CD133-CXCR4- cells.

4.6 CD133+CXCR4+ cells show enhanced anti-drugs resistance

Whether the CD133+CXCR4+ cell had the stronger chemoresistance was also tested, the anti-drugs resistance assay was performed using CD133+CXCR4+ cell, CD133-CXCR4- cells and parent cells, which were treated by cisplatin and paclitaxel with different concentrations. Figure 5 A and B showed CD133+CXCR4+ cells were significantly more resistant to cisplatin and paclitaxel compared to the other subpopulations at different concentrations.

Figure 5

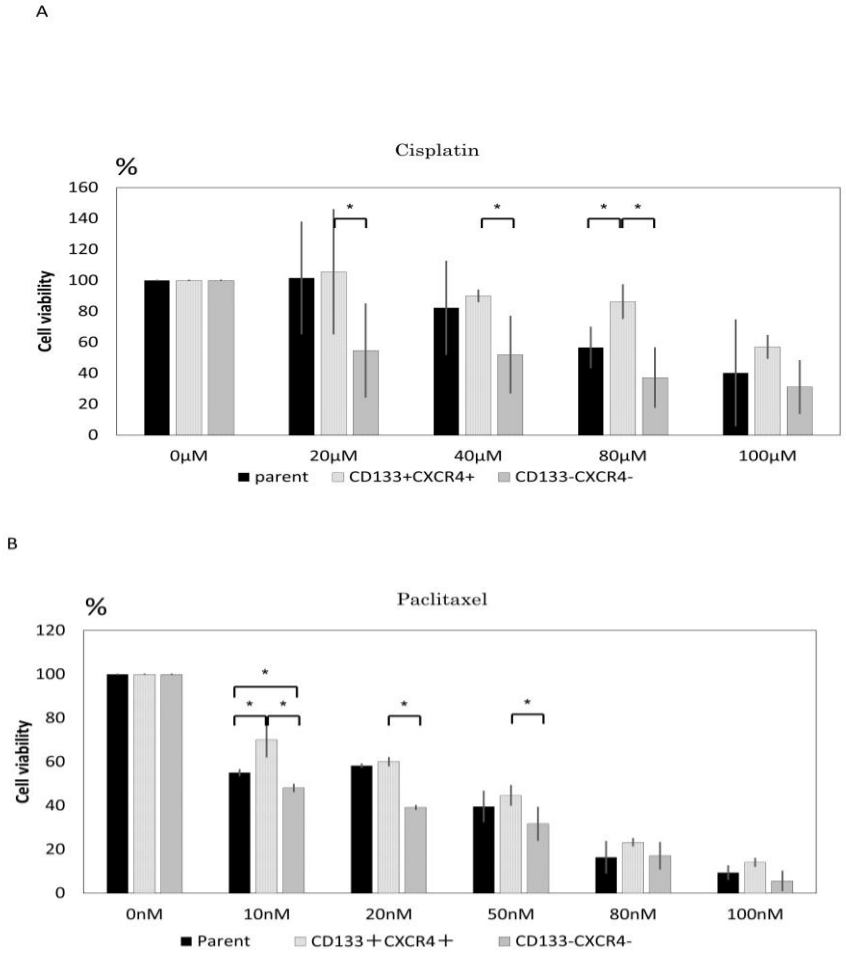


Figure 5 Drug resistance of the each subpopulation. (A and B) Cisplatin and paclitaxel were added into the sorted cells at different concentration to evaluate the cell viability (* $p<0.05$).

4.7 Clinical data from patients

The information was obtained from 20 patients in accordance with the approved of the Research Ethics Committee of Toyama University Hospital (Table 2). The age, stage, grade, and therapies were recorded. I collected 20 samples from the patients, but only 5 primary cells were isolated successfully. The isolated cells were from the stage Ⅲ to Ⅳ tissues.

Table 2

Diagnosis Pathology	ID	Previous Clinical Back ground				
		At surgery Age	Diagnosis			comments
			FIGO2008	Grade in adeno-carcinoma	Therapy	Isolated cells
Endometrioid carcinoma	1	69		G1	death	
	2	77	Ⅳb	G3	TC	◎
	3	52	Ⅳb	G2-3	TC,RT,AP,TC	relapse ◎
	4	85	Ⅳb	G2	TC	○
	5	68	Ⅲc	G2	TC	
	6	62	Ⅲc	G3	TC	○
	7	63	Ⅱc	G1	TC	
	8	70	Ⅱb	G2	TC	
	9	91	Ⅱb	G1	TC	
	10	63	Ⅰb	G1		◎
	11	70	Ⅰb		TC	
	12	55	Ⅰb	G2		
	13	59	Ⅰb	G1	TC	
	14	62	Ⅰa	G3	TC	
	15	48	Ⅰa	G2	TC	
	16	59	Ⅰa	G1		
	17	46		G1		
Serous adenocarcinoma	1	67	Ⅲc		TC, AP,TC	death
	2	72	Ⅲa		TC,AP,TC	death
	3	68	Ⅰa		TC	

FFIGO2008:Diagnostic criteria of The Japan Society of Obstetrics and Gynecology
◎: growth well
○: growth slowly

Table 2 The clinical characteristics of patients.

5. Discussion

It was widely accepted that CSCs play an important roles in cancer development and progression [4, 8, 10]. There were some reports about CSCs in endometrial cancers [23, 24]. However, most of studies carried out using cell lines, Ishikawa and AN3CA [20]. In this study, CSCs in primary endometrial cancer tissues were examined.

Isolated cells from primary endometrial cancer tissues expressed a number of mRNA of stemness related genes, such as *c-Myc, Sox-2, Nanog, Oct 4A, ABCG2, BMI-1, CK-18,* and *Nestin.* The other hand, the cells expressed a number of cell surface markers, such as CD24, CD29, CD44, CD47, CD133, CXCR4. Several cell surface markers, such as CD24, CD44, and CD133, are common in certain solid tumors including breast, brain, colorectal, and endometrial cancers [13]. I investigated relationship between the expression of stemness genes and the cell surface markers. CD133+ and CXCR4+ EC cells exhibited stronger expression of stem-related genes, such *as c-Myc, Sox-2, Nanog, Oct 4 A, ABCG2, BMI-1, CK-18,* and *Nestin* rather than CD133- or CXCR4- EC cells. While there was hardly any difference between CD24+ and CD24- EC cells (Figure 1C). Therefore, I focused on the CD133 and CXCR4 markers.

CD133 is an 866-amino-acid single-chain transmembrane glycoprotein with a molecular weight of 120 kDa. Previous studies have indicated that CD133 expression is risk factor for endometrial cancer [25]. In this study, I detected CD133 expression was 12% of EC cells. Nakamura M et al. [20] reported that CD133+ endometrial cancer cell lines possess increased proliferative and tumorigenic potentials and are resistant to cisplatin- and paclitaxel- induced cytotoxicity. Vincent Z et al. [26] also identified CD133+ as a reliable marker for CSC characterization in the Colo205

colon adenocarcinoma cell line. Moreover CD133+ endometrial cancer cell lines have been shown to exhibit higher expression of MT1-MMP, through which their increased invasive ability is mediated [27].

CXCR4 has been shown to be expressed in all types of human tumors, including endometrial cancer. The paper reported that CXCR4 were significantly up-regulated in of endometrial cancer as compared to atypical, simple hyperplasia and normal endometrium [24]. Teicher B. A et al [28] showed that CXCL12/CXCR4 axis is involved in tumor progression, angiogenesis, metastasis, and survival, CXCR4 were thought to be caused some key signaling pathways and promoted chemotaxis, survival proliferation, and transcription gene expression.

Above it, the both cell surface marker of CD133 and CXCR4 is very key marker to candidate of CSCs. CD133+CXCR4+ EC cells in my results existed less than 10% of the total population, which is consistent with previous findings that CSCs constitute a small population of cancer cells in malignant tumors. CD133+ cells account for 10.1% and 20.1% in the Ishikawa and MFE280 EC cell lines, respectively [20]. Over the past two decades a number of researchers have sought to identify appropriate markers for CSCs, most of which were single markers However, much of the research has demonstrated the importance of combination markers. Hermann P C et al. [29] have demonstrated that CD133+ and CXCR4+ constitute two distinct subpopulations in pancreatic cancer with a migratory and invasive phenotype. Other studies have shown that patients with high ratios of CD133+CXCR4+ exhibit a significantly reduced two-year survival rate compared with patients with low CD133+CXCR4+ cell ratios [30]. A recent study has reported that the use of a novel class of CXCR4 antagonists [31], alone or in combination with

chemotherapeutic agents and/or CD133 targeting agents, might reduce anti-drug ability and the development of tumor formation [32].

CD133+CXCR4+ EC cells grew faster than CD133-CXCR4- EC cells under normal culture conditions. CD133+CXCR4+ EC cells formed more spheres and colonies than the CD133-CXCR4-EC cells and parental cells. Sphere formation has been observed in stem cells from various normal and cancer tissues, indicating that sphere formation might constitute an ordinary characteristic of stemness [33, 34, 35, 36]. Spheres have also been shown to have higher tumorigenic ability than parental cancer cells in xenografts experiment [37].

Boyer L.A et al. [38] have suggested that *OCT4, SOX2*, and *NANOG* contribute to pluripotency and self-renewal by activating their own genes, which encode components of key signaling pathways. *BMI-1* is a polycomb gene associated with maintenance of self-renewal ability, which has been implicated in various cancers [39, 40, 41].

In addition, it has been reported the downregulation of *ABCG-2* expression inhibits the self-renewal capacity of cells and significantly enhances the efficacy of chemotherapy-induced apoptosis in colon adenocarcinoma cells and CD133-positive colorectal carcinoma cells [42]. *Nestin*, an intermediate filament protein and a stem cell marker, is expressed in several tumors. Bokhari et al. found that of the EC cancer lines, AN3CA and KLE cells exhibited a significantly higher number of CD133+ cells and higher *Nestin* expression levels than Ishikawa cells [43], while CK18 expression varied in different cancer types.

Zhang B, et al. [44] demonstrated that *CK18* expression is correlated with clinical stage, lymph node metastasis, number of positive lymph nodes, and recurrence and metastasis in non-small cell lung cancer. They also found that patients with high CK18 expression have poorer overall

survival (OS) and Disease-free survival (DFS) than patients with low *CK18* expression.

In the present study, CD133+CXCR4+ EC cells did not only exhibited higher expression of the stemness genes compared to CD133-CXCR4- EC cells, but also express stronger the c-Myc, KLF-4, OCT3/4, NANOG, and SOX2 by immunofluorescence staining.

Moreover, the results showed that $1.0x10^3$ CD133+CXCR4+ EC cells formed tumors xeno-transplanted into nude mice, while CD133-CXCR4- EC cells did not.

Studies performed with several cancer lines have revealed that CD133+ cells are more resistant to anti-tumor drugs and radiotherapy. The CD133+ human fibrosarcoma cell line exhibits significant resistance to both cisplatin and doxorubicin, drugs currently used in the clinical setting for the treatment of fibrosarcoma[45]. Cioffi M, et al [32]. evaluated the sensitivity of sorted CD133+CXCR4+ ovarian cells to cisplatin, which is a drug commonly used for the treatment of ovarian cancer, and found that CD133+CXCR4+ cells expressed the highest level of ABCG-2, a surface marker transporter involved in resistance to chemotherapy. Consistent with those findings, my results show that sorted CD133+CXCR4+ EC cells were more resistant to cisplatin and paclitaxel, drugs routinely used for the treatment of endometrial cancer.

It is very difficult to isolate the primary cells from the tumor tissue. In this study, 5 lines were successfully grew out of 20 patients' specimens. Although patient EC16 received chemotherapy and radiotherapy, she still relapsed. EC16 cell grew fast and eugenic observed by microscope. So EC 16 cells were used for this research. Many reports have shown that CSCs isolated from cell lines could be successfully used for in *vitro* and in *vivo*, it can not be considered as accurate surrogates for clinical cancers. Accompany with the passage, the cell line may have differentiation ability

and occur the gene mutation. To overcome the deficiencies of cell lines, primary cells were used.

All these data indicate that CD133+CXCR4+ EC cells possess greater proliferation, clonogenic, tumorigenic, and chemo-resistance abilities.

The CSCs like cells are able to isolate from primary endometrial cancer tissues. The results suggested that CD133+CXCR4+ EC cells from isolated cells have characterization of cancer stem cells, and it may be cancer stem like cells. Combination of the surface marker of CD133 and CXCR4 is useful method to detect the cancer stem cell, and constitute excellent novel molecular targets for endometrial cancer therapy.

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