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The usefulness of three-dimensional cell culture in induction of cancer stem cells from esophageal squamous cell carcinoma cell lines

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ABSTRACT

In recent years, research on resistance to chemotherapy and radiotherapy in cancer treatment has come under the spotlight, and researchers have also begun investigating the relationship between resistance and cancer stem cells. Cancer stem cells are assumed to be present in esophageal cancer, but experimental methods for identification and culture of these cells have not yet been established. To solve this problem, we created spheroids using a NanoCulture® Plate (NCP) for 3-dimensional (3-D) cell culture, which was designed as a means for experimentally reproducing the 3-D structures found in the body. We investigated the potential for induction of cancer stem cells from esophageal cancer cells.

Using flow cytometry we analyzed the expression of surface antigen markers CD44, CD133, CD338 (ABCG2), CD318 (CDCP1), and CD326 (EpCAM), which are known cancer stem cell markers. None of these surface antigen markers showed enhanced expression in 3-D cultured cells. We then analyzed aldehyde dehydrogenase (ALDH) enzymatic activity using the ALDEFLUOR reagent, which can identify immature cells such as stem cells and precursor cells. 3-D-cultured cells were strongly positive for ALDH enzyme activity. We also analyzed the expression of the stem cell-related genes Sox-2, Nanog, Oct3/4, and Lin28 using RT-PCR. Expression of Sox-2, Nanog, and Lin28 was enhanced. Analysis of expression of the hypoxic surface antigen marker carbonic anhydrase-9 (CA-9), which is an indicator of cancer stem cell induction and maintenance, revealed that CA-9 expression was enhanced, suggesting that hypoxia had been induced. Comparison of cancer drug resistance using cisplatin and doxorubicin in 3-D-cultured esophageal cancer cells showed that cancer drug resistance had increased. These results indicate that 3-D culture of esophageal squamous cell carcinoma lines is a useful method for inducing cancer stem cells.

1. Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth leading cause of cancer death, but is one of the least studied of all cancers [1,2]. Prognosis for this cancer remains poor, even with multimodal treatment, due to high resistance to chemotherapy and radiotherapy.

Cancer stem cells have recently been recognized as a contributing factor to the resistance to multimodal treatment for cancer therapy [3–5]. According to the definition proposed by Reya et al. at the 2006 meeting of the American Association for Cancer Research, cancer stem cells are considered to have four characteristics: tumorigenicity, multipotency, self-renewal, and drug resistance [6,7]. These cells have been identified using intracellular metabolic activity and cell surface CD antigens, and have been studied using cells isolated from cultured cell lines and clinical samples. The following findings have been garnered from a range of cancers (hematological malignancy, colon cancer, brain tumors, breast cancer, etc.). (1) CD44, CD133, CD338 (ABCG2), CD318 (CDCP1), and CD326 (EpCAM) are candidates for surface CD antigens in cancer stem cells [8–12]. (2) The enzymatic activity of aldehyde dehydrogenase (ALDH) is elevated in cancer stem cells and precursor cells, and by measuring this enzymatic activity, it is possible to identify cells with the multipotency characteristic of cancer stem cells [13–18]. (3) Sox-2, Nanog, Oct3/4, and Lin28 have been identified as stem cell-related genes, and cells that strongly express these genes have acquired resistance to chemotherapy [19–22]. (4) Cancer stem cells are induced and maintained in hypoxic conditions [11,23]. However, with research on esophageal cancer having failed so far to identify cancer stem cells, there is a

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need to develop new methods for inducing esophageal cancer stem cells.

Tumors in the body have a 3-dimensional (3-D) structure, and tumor cells form a microenvironment through interactions with their surroundings in three dimensions. Cancer drug resistance studies in other cancers have reported the benefits of using spheroids formed in 3-D cell culture, which experimentally reproduces the 3-D structure of cancerous tissue in the body, instead of using conventional 2-dimensional (2-D) cell culture on a flat plate [24–26].

In this study, we investigated the usefulness of 3-D cell culture in inducing esophageal cancer stem cells by culturing esophageal cancer cell lines in 2-D and 3-D culture and comparing cell surface antigen markers, expression of strongly analyzed ALDH-positive cells, gene expression, and sensitivity to anti-cancer drugs.

2. Materials and methods

2.1. Tumors and cell culture methods

The human esophageal squamous cell carcinoma lines TE2 and TTn (supplied by Professor Hisahiro Matsubara (Fig 1A), Department of Frontier Surgery, Graduate School of Medicine, Chiba University) [27–29] were cultured in a 75-cm^2 flask (Greiner Bio-One, Frickenhausen, Germany) at 37 °C in 5% CO_2 in RPMI-1640 cell culture medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Gibco; Grand Island, NY) and the antibiotics penicillin (5000 units/mL) and streptomycin (5000 μg/mL) (Gibco).

2.2. 3-D cell culture using NanoCulture® Plates (NCP)

3-D culture was done for 2 weeks in RPMI-1640 medium supplemented with 6.7% FCS using a 24-well NCP inoculated with 5×10^4 cells per well. The spheroids formed in 3-D culture were harvested by pipetting, re-inoculated onto a flat culture plate, and cultured for 2 days.

2.3. Analysis of cancer stem cell surface CD antigens

A FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was used to analyze cell surface antigen markers. Fluorescence-labeled antibodies CD44 [5], CD133 [6], CD326 [8] (eBioscience, San Diego, CA), CD318 [7], and CD338 [9] (Biolegend, San Diego, CA) were used for surface markers.

2.4. ALDEFLUOR assay

The ALDEFLUOR® kit (StemCell Technologies, Inc., Vancouver, BC, Canada) was used for ALDEFLUOR analysis. Cells were incubated for 60 min at 37 °C in a buffer containing 100 U/mL BODIPY-aminoacetaldehyde (BAAA), washed twice, and analyzed with flow cytometry. When analyzing the differences in ALDH enzymatic activity, we confirmed that the activity of ALDH-positive cells was reduced by diethylaminobenzaldehyde, which inhibits ALDH activity.

2.5. Analysis of gene expression

The expression of Sox-2, Nanog, Oct3/4, and Lin28 was analyzed with real-time PCR after extraction of mRNA. Primers with the following sequences were used.

Sox2-forward: 5′-ATGGACAGTTACGCGCACAT-3′, Sox2-reverse: 5′-GACTTGACCA

CCGAACCCAT-3′, Nanog-forward: 5′-GACTTGACCACCGAACCCAT-3′, Nanog-reverse: 5′-CTGGATGTTCTGGGTCTGGT-3′, Oct3/4-forward: 5′-GACAACAATGA GAACCTTCA-3′, Oct3/4-reverse: 5′-GA CAACAATGAGAACCTTCA-3′, LIN28-forward: 5′-AAAGGAGACAGGT GCTAC-3′, LIN28-reverse: 5′-ATATGGCTGATGCTCTGG-3′, GAPDH forward: 5′-AGCCACATCGCTCAGACACC-3′, GAPDH reverse: 5′-GT ACTCAG CGGCCAGCATCG-3′. mRNA extraction, PCR, and electrophoresis were done using a Total RNA Minikit® (ATP Biotech Inc.

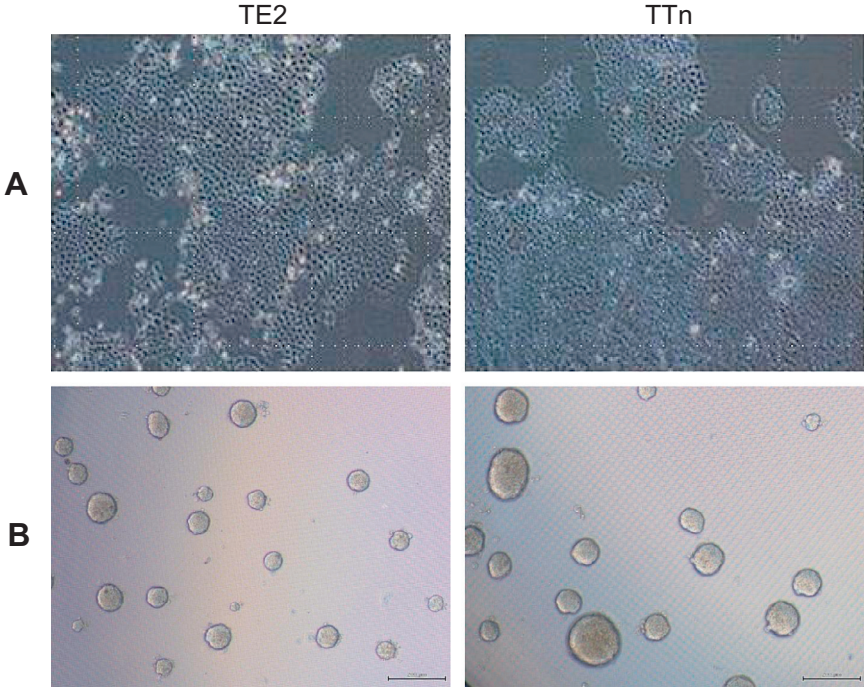

Fig. 1. Morphological changes in 2-D and 3-D cell culture of the esophageal squamous cell carcinoma lines TE2 and TTn. (A) 2-D culture. (B) 3-D culture. Spheroids formed in 3-D culture.

Taipei, Taiwan), High capacity RNA-to-cDNA kit® (Applied Biosystems, Foster City, CA) [30], and 1.5% agarose gel AmpliTaq Gold® PCR Master Mix® (Applied Biosystems), in accordance with the protocols for each product. PCR was done using a VeritiTM Thermal Cycler (Applied Biosystems), with DNA denatured at 98 °C for 1 min, and then amplified with 35 cycles of 98 °C for 10 s, 55 °C for 30 s (60 °C for Nanog), and 72 °C for 1 min.

2.6. Determination of carbonic anhydrase-9 (CA-9)

2-D-cultured cells and 3-D-cultured cells were each adjusted to 1×10^5 cells/100 μL/tube, and 2 μL anti-CA-9 (Abcam, Cambridge, UK) was added to each tube and mixed. The antibody reaction proceeded at 4 °C for 60 min. Then, after washing twice in FACS buffer and resuspending at 1.5 mL/tube, 15 μL PE-α-mouse IgG antibody was added and mixed as secondary antibody (100-fold dilution) and incubated at 4 °C for 45 min. After reaction and sufficient washing in FACS buffer, 1 μL propidium iodide (Sigma–Aldrich) was added and mixed to remove dead cells. Expression of CA-9 was analyzed with flow cytometry.

2.7. Cancer drug sensitivity test

To test cancer drug sensitivity, the cells were inoculated into a 96-well flat plate at 4×10^4 cells/well, and the concentration of drug needed for 50% cell growth inhibition (IC50 value) was investigated. Two-fold dilution series were created fromcisplatin (250 ng/mL) and doxorubicin (500 ng/mL), and cells were cultured at 37 °C in 5% CO_2 for 7 days. Viable cells were counted after staining with Alamar Blue (AbD Serotec, Oxford, UK).

2.8. Statistical analysis

The Student's *t*-test was used to test the difference in mean values for IC50 in the cancer drug sensitivity test, and a two-tailed $p < 0.05$ was considered to indicate a significant difference (IBM SPSS statistics19).

3. Results

3.1. Spheroids were created in 3-D cultures of the esophageal squamous cell carcinoma lines TE2 and TTn

Culture of cancer cells using NCP does not require the establishment of any particularly complex culture conditions. It is possible to form spheroids of colon and breast cancer cells inoculated onto plates and cultured for 7 days at 5% CO_2 and 37 °C [26], although spheroid formation is not possible with some tumors, such as scirrhous gastric cancer cell lines (e.g., HSC58, HSC44PE). 3-D culture of the esophageal squamous cell carcinoma lines TE2 and TTn was successful in forming spheroids after 7 days of culture under the same conditions as above (Fig. 1B). To obtain spheroids large enough to guarantee enough cells for each analysis, culture was done for 14 days.

3.2. Analysis of cancer stem cell surface antigen markers

The expression of the known cancer stem cell surface antigen markers CD44, CD133, CD338, CD318, and CD326 is a feature shared by cell groups in other cancers that exhibit the multipotency and drug-resistance characteristics of cancer stem cells. When comparing the expression of these markers in esophageal squamous cell carcinoma cells cultured in 2-D and 3-D culture, we found that CD44, CD133, and CD338 expression was weaker in 3-D culture, and CD318 and CD326 expression did not differ (Fig. 2).

3.3. The proportion of strongly ALDH-positive cells increased in 3-D culture

We therefore investigated ALDH enzymatic activity in 3-D culture of esophageal cancer cells. Cells showing ALDH enzymatic activity can be detected as strongly FL-1-positive cells with flow cytometry because BAAA taken into the cell is converted into BAA (BODIPY-aminoacetate). As shown in Fig. 3A, 21.8% of TE2 cells and 20.7% of TTn cells were confirmed as ALDH positive after 2-D culture, whereas after 3-D culture, the values for strongly ALDH-positive esophageal cancer cells increased to 79.3% (TE2) and 47.8% (TTn). These results indicate that 3-D culture increases the number of cells with stemness properties, suggesting that there was a greater level of induction of stem-like cells.

3.4. Expression of the cancer stem cell-related genes Sox-2 and Nanog was enhanced

Sox-2, Nanog, Oct3/4, and Lin28 have been identified as genes that induce or express the stemness property, and elevated expression of these genes has also been reported in cancer stem cells. Our comparison study using RT-PCR to analyze the expression of these genes in 2-D-cultured and 3-D-cultured esophageal cancer cells found that 3-D-cultured cells exhibited enhanced expression of Sox-2 and Nanog (Fig. 3B). The enhanced expression of Sox-2, Nanog, and Lin28 in 3-D culture was confirmed with real-time PCR analysis (Fig. 3C).

3.5. Expression of the hypoxic marker CA-9 was enhanced in 3-D culture

Expression of high levels of CA-9 indicates that cancer cells are in hypoxic conditions, and hypoxia has been found to maintain and induce cancer stem cells [11,23]. In this study, we therefore examined CA-9 expression to investigate the hypoxic condition of spheroids formed by 3-D culture. The experiment showed that CA-9 expression was conspicuously enhanced in 3-D-cultured cells, suggesting that cells become more strongly hypoxic in the 3-D culture environment (Fig. 3D).

3.6. Cancer drug sensitivity tests showed that resistance to cisplatin and doxorubicin was enhanced after 3-D cell culture

The acquisition of cancer drug resistance is one of the key elements in experimentally confirming the presence of cells with stemness properties. In this experiment, we investigated the growth inhibition effect of different concentrations of cisplatin and doxorubicin, which are widely used clinically, in 2-D-cultured and 3-D-cultured cells. As shown in Fig. 4, the 3-D-cultured esophageal squamous cell carcinoma cell line TTn showed lower sensitivity to these cancer drugs than did the 2-D-cultured cells. The IC50 values for cisplatin were 11.7 ng/mL in 2-D culture and 23.1 ng/mL in 3-D culture, whereas the IC50 values for doxorubicin were 30.2 ng/mL in 2-D culture and 43.6 ng/mL in 3-D culture. The difference between the IC50 values was tested using the Student's *t*-test, which confirmed that cancer drug resistance was significantly elevated after 3-D culture (cisplatin: $p = 0.0003$; doxorubicin: $p = 0.015$).

4. Discussion

3-D cell culture is considered to be a useful way of experimentally reproducing the 3-D structure of cancerous tissue found in the body. 3-D culture was originally devised as a method for experimentally reproducing the interactions between the tumor and its

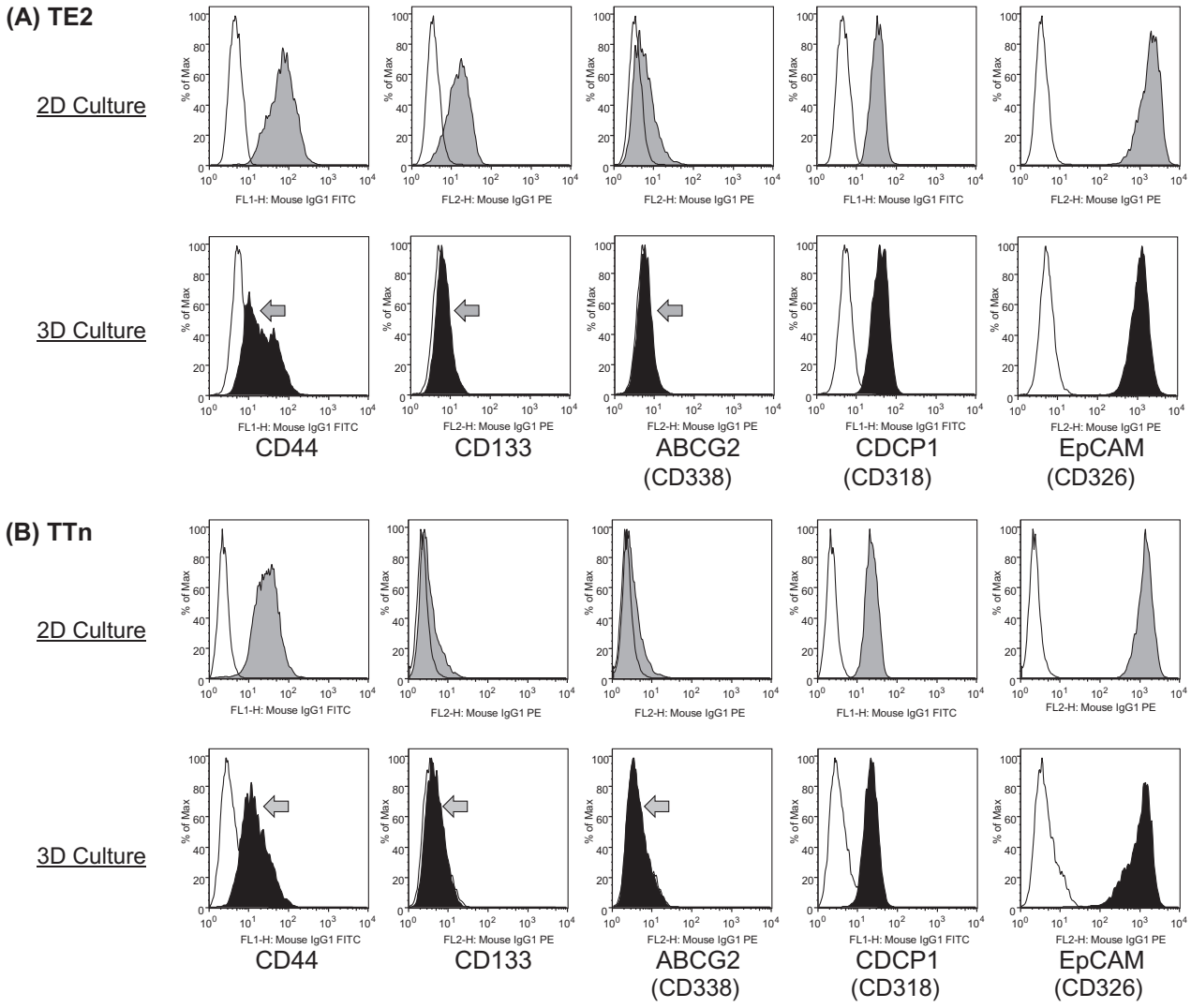

Fig. 2. Luminance of cell surface antigen markers in 2-D and 3-D cell culture of esophageal squamous cell carcinoma lines. (A) is the result of staining after (i) 2-D culture and (ii) 3-D culture of the TE2 cell line. (B) is the TTn cell line.

microenvironment. In 1944, Holtfreter discussed a method for culture of spherical cell reaggregates, and in 1970, Sutherland et al. reported the creation of spheroids in soft agar [31,32]. Subsequently, a 3-D culture method was proposed using a scaffold created from collagen and other extracellular matrix materials [25], and the arrival of nanofibers and other new materials involved in cell adhesion has prompted expectation of further developments [33,34]. NCP are 3-D culture plates that allow spheroids to be cultured in culture medium. They are created by imprinting onto the plate base a nanoscale pattern that mimics an extracellular matrix material such as collagen. Cells cultured in 3-D culture using NCP are easier to harvest than cells formed as spheroids in soft agar. Because it was possible to create spheroids by culturing the esophageal squamous cell carcinoma lines TE2 and TTn using NCP, in this study, we investigated the cancer stem cell-like characteristics minimally present in 2-D cultured cells, and further conducted a cancer drug sensitivity test to investigate the validation of NCP 3-D cell culture for cancer stem cell induction.

One way of verifying the induction of cancer stem cells is to analyze surface antigen markers with flow cytometry. Because CD44, CD133, and CD338 are expected to be confirmed as cancer stem cell markers in histopathologically equivalent cervical cancers and other esophageal squamous cell carcinoma lines, and CD318 is thought to be a surface marker of presumed immature cancer stem cells, we compared the expression of each of these markers. We also examined CD326 as a marker for mesenchymal cells, because it is thought that cancer stem cells undergo epithelial-to-mesenchymal transition during metastasis [35]. In this experiment, expression of CD44, CD133, and CD338 was weaker in 3-D-cultured cells than in 2-D-cultured cells, but expression of CD318 and CD326 did not differ. These results indicate that the cancer stem cell markers already reported have low significance in esophageal squamous cell carcinoma lines, and the results highlight the need to continue searching for other CD antigens.

ALDH is an intracellular enzyme that oxidizes intracellular aldehyde to carboxylic acid. It is abundantly expressed in immature cells such as precursor cells, and through retinol metabolism, it contributes to the initial phase of stem cell induction byoxidation of retinol to retinoic acid [10]. It is thought that ALDH is a shared stem cell marker, because strongly ALDH-positive cells have been identified in mouse and human blood stem cells and stem cell precursors, and numerous studies have also found strong expression of ALDH in cancer stem cells [14–18]. Our experiment found that the expression

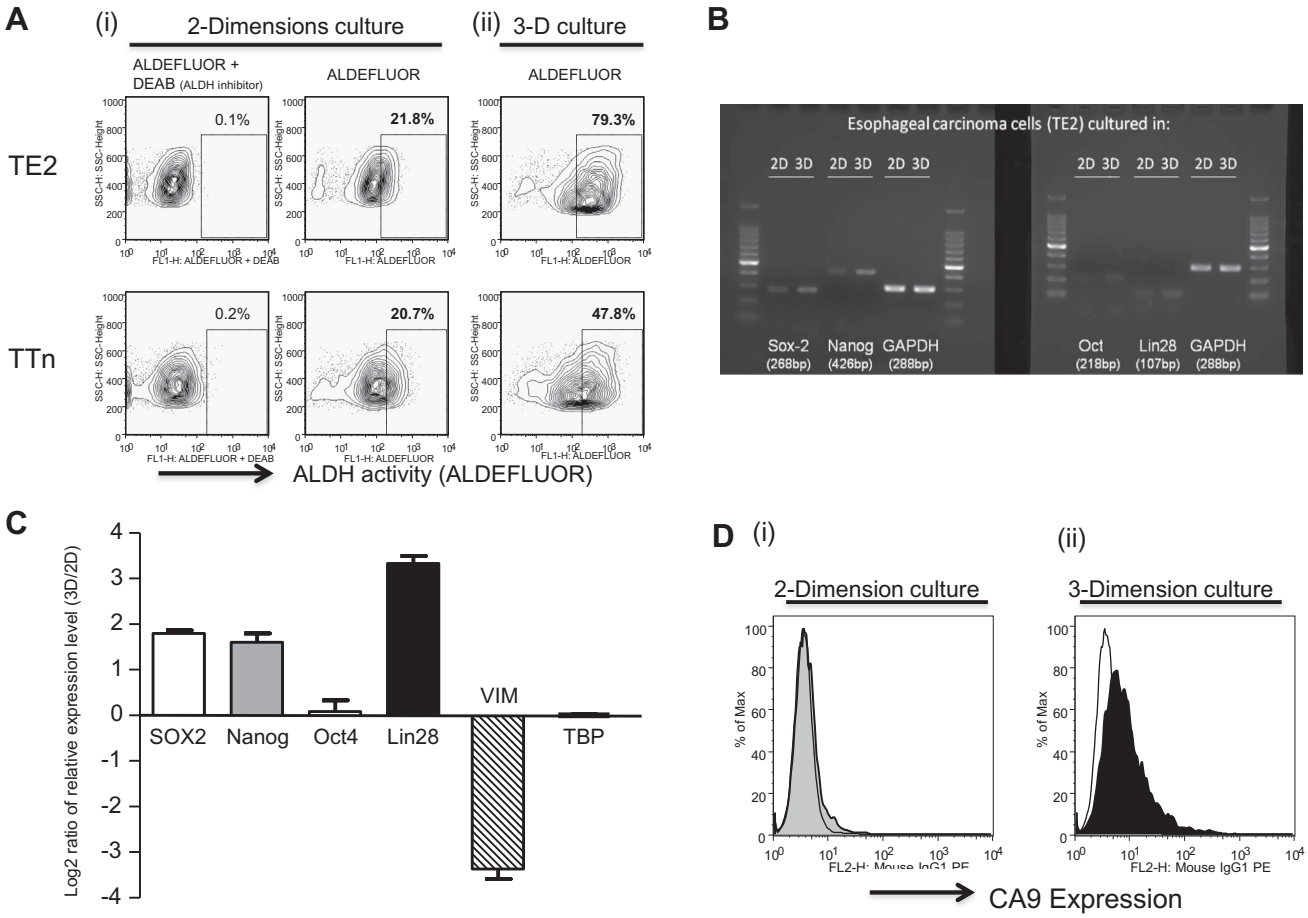

Fig. 3. (A) Determination of ALDH enzymatic activity with ALDEFLUOR. The upper row is TE2, and the lower row is TTn. (i) Conditions in the 2-D cell culture were set using an ALDH inhibitor. (ii) The ALDH positive rate in 3-D cell culture was investigated. (B) Analysis of cancer stem cell-related the genes in esophageal squamous cell carcinoma line TE2 (RT- PCR). 2-D: 2-D cultured cells. 3-D: 3-D cultured cells. (C) Real-Time PCR (Log2 ratio of relative expression level: 3-D/2-D). (D) Comparison of CA-9 expression in the esophageal squamous cell carcinoma line TE2 in 2-D and 3-D culture.

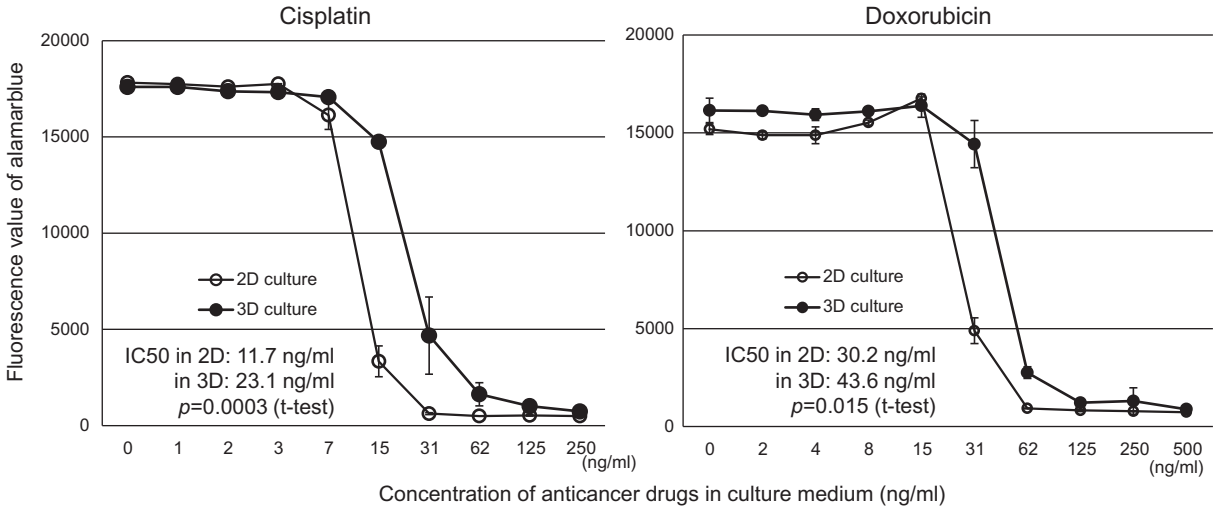

Fig. 4. Cancer drug sensitivity test after 2-D and 3-D culture of esophageal squamous cell carcinoma lines. In the dose-response curve, the IC_{50} values of cisplatin and doxorubicin increased in 3-D culture.

of strongly ALDH-positive cells was greater after 3-D culture than after 2-D culture, suggesting that 3-D culture had resulted in induction of immature cells. It is possible that cancer growth could be stopped through treatments targeting immature, strongly ALDH-positive cells before differentiation into the various mature cells involved in building cancerous tissue.

The stem cell-related genes Sox-2, Nanog, Oct3/4, and Lin28 contribute to cancer drug resistance and differentiation potential in cancer cells [19–22]. Our result suggested that cells with the cancer drug resistance and multipotency characteristics of stem cells could be induced by 3-D culture of esophageal squamous cell carcinoma lines.

CA-9 is a tumor-related protein that belongs to the carbonic anhydrase gene family and contributes to invasion and adhesion of malignant tumors. Cells in tumors that express high levels of CA-9 are thought to exist under hypoxia [23]. We similarly found enhanced expression of CA-9 in esophageal cancer cells after 3-D culture. This suggests that cells in 3-D culture were exposed to stronger hypoxic conditions than those in 2-D culture, and that these culture conditions are more conducive to induction of cancer stem cells.

The results of the cancer drug sensitivity test of esophageal squamous cell carcinoma lines to cisplatin and doxorubicin after 2-D and 3-D culture showed that the IC50 values for each drug were significantly higher after 3-D culture, indicating that characteristics of cancer stem cells had been induced, thus increasing cancer drug resistance. 3-D culture using esophageal squamous cell carcinoma lines shows potential for efficiently inducing esophageal cancer stem cells, and this may be due in part to the hypoxic conditions of this culture method. In terms of future prospects for the clinical application of 3-D culture to the treatment of esophageal cancer, this method shows potential for use in screening new cancer drugs and molecularly targeted drugs, which are currently in the research and development phase. In the field of basic research, 3-D culture of esophageal squamous cell carcinoma lines may also be applicable to the identification and characterization of esophageal cancer stem cells, and shows promise as a useful method for cancer stem cell induction.

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