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Analysis of multiple markers for cancer stem-like cells in human thyroid carcinoma cell lines

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Abstract. Cancer stem-like cells (CSCs) play important roles in cancer initiation and progression. CSCs have been isolated using several markers, but those for thyroid CSCs remain to be confirmed. We therefore conducted a comprehensive search for thyroid CSC markers. Expression of nine cell surface markers (CD13, CD15, CD24, CD44, CD90, CD117, CD133, CD166, and CD326) and aldehyde dehydrogenase (ALDH) activity, which are CSC markers in various solid cancers, and the ability to form spheres *in vitro* and tumors *in vivo* were investigated using eight thyroid cancer cell lines (FRO, KTC1/2/3, TPC1, WRO, ACT1, and 8505C). Among these, four cell lines (FRO, KTC3, ACT1, and 8505C) possessed the both abilities; however, common markers indicative of CSCs were not observed. The pattern of ability to form spheres was completely matched to that of tumor formation, suggesting that our sphere assay is valuable for assessment of tumor-forming ability. Next, the cells were sorted using these markers and subjected to the sphere assay. In three cell lines (FRO, KTC3, and ACT1), ALDH^{pos} cells showed higher sphere forming ability than ALDH^{neg} cells but not in other cells. CD326^{hi} also appeared to be a candidate marker only in FRO cells. However, these subpopulations did not follow a classical hierarchical model because ALDH^{neg} and CD326^{low} fractions also generated ALDH^{pos} and CD326^{hi} cells, respectively. These data suggest that ALDH activity is probably a major candidate marker to enrich thyroid CSCs but not universal; other markers such as CD326 that regulate different CSC properties may exist.

Key words: Thyroid cancer, Cancer stem cells, ALDH, CD326

THYROID cancer is the most common endocrine malignancy, and its incidence is increasing globally. There are four main types of thyroid cancer derived from thyroid follicular epithelial cells: papillary (PTC), follicular (FTC), poorly differentiated (PDTC), and anaplastic thyroid cancer (ATC). Although the overall prognosis of PTC and FTC is excellent, PDTC is moderately aggressive and ATC is one of the most malignant human cancers with significantly poorer outcomes [1].

There is accumulating evidence that solid tumors contain a distinct subpopulation of cancer stem-like cells (CSCs), or tumor-initiating cells, that play impor-

tant roles in cancer initiation, progression, recurrence, and metastasis. Therefore, the identification and analysis of CSCs are fundamental for cancer study. The “gold standard” to identify CSCs is to isolate cells using biomarkers (e.g. cell surface marker such as CD133) and inject them into immunodeficient mice to see whether they develop tumors [2].

Regarding biomarkers, recent studies have reported several CSC markers in solid tumors (e.g. CD44⁺/CD24⁻ for breast cancer [3], CD133⁺ for brain tumor [4] and colon cancer [5], CD44⁺ for gastric cancer [6]). In some types of cancers, two or more different markers have independently been identified in the same type of cancer: for example, CD44⁺/CD24⁻ [3], aldehyde dehydrogenase (ALDH) 1 activity [7], and CD133 [8] for breast CSCs; CD133 [5] and CD44⁺/CD24⁺ [9] for colon CSCs. However, their association and/or combination have not been fully investigated.

In thyroid cancer, we and other groups separately

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reported side population (SP) [10] and CD133⁺ [11, 12] as CSC markers using thyroid cancer cell lines. However, the identity of the cell line, ARO cells, that had been used in functional characterizations in all above-mentioned studies became questionable. Schweppe *et al.* [13] found quite a few misidentifications and redundancies (presumably due to cross-contamination) in thyroid cancer cell lines using DNA profiling, and the ARO cells are likely identical to the colon cancer cell line, HT-29. Therefore, it is necessary to reconfirm the validity of these markers.

Recently, Todaro *et al.* [14] have demonstrated that a small subpopulation with high ALDH activity showed cancer stem-like features in primary thyroid cancer cells. However, they did not analyze other markers such as CD133, and there is no other study confirming this finding so far. In the present study, therefore, we carried out a comprehensive assessment of CSC markers which have been frequently identified in many types of solid cancers using thyroid cancer cell lines.

Materials and Methods

Cell culture

FRO, KTC2, KTC3, ACT1, and 8505C cell lines were originally established from human ATC; KTC1 and TPC1 were from human PTC; WRO was from human FTC. FRO, TPC1, and WRO were obtained from Dr. James Fagin (currently Memorial Sloan-Kettering Cancer Center, NY, USA [13]). KTC1, KTC2, and KTC3 were kindly provided by Dr. Junichi Kurebayashi (Kawasaki Medical School, Kawasaki, Japan [15, 16]). ACT1 was obtained from Dr. Naoyoshi Onoda (Osaka City University, Osaka, Japan; originally established by Dr. Seiji Ohata of Tokushima University [17]). 8505C was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. All cells were grown in RPMI1640 (FRO, KTC2, KTC3, TPC1, and WRO) or DMEM (KTC1, ACT1, and 8505C) supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin (PS) at 37°C in a humidified atmosphere with 5% CO₂. For sphere formation, cells were seeded on HydroCell dish/plate (CellSeed) and incubated in serum-free DMEM/F12 (1:1) supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, and B27 without vitamin A (Life technologies) (sphere medium). We used Promega's short tandem repeat (STR) profiling (10 loci) service (Promega Japan) to confirm the identities of the cell lines.

In vivo tumor xenograft

Cells were suspended in 50 µL growth medium and mixed with an equal volume of growth factor-reduced matrigel (Life technologies). The mixed cell suspension was injected s.c. into 6-week-old male BALB/c nu/nu mice (CLEA Japan). Tumor sizes were measured with calipers, and tumor volumes were calculated according to the formula: $a^2 \times b \times 0.4$, where a is the smallest tumor diameter and b is the diameter perpendicular to a . Animal care and experimental procedures described in this study were done in accordance with the Guidelines for Animal Experiments in Nagasaki University with the approval of the Institutional Animal Care and Use Committee (#1001150813-2). All efforts including euthanasia were made to reduce suffering.

RT-PCR analysis for CD44v

Total RNA was extracted from cells using ISOGEN reagent (NIPPON GENE) according to the manufacturer's instruction and then reverse transcribed using a SuperScript III first-strand synthesis supermix kit (Life technologies) with random hexamers. The following PCR amplifications were performed using KOD FX polymerase (TOYOBO) and primers as previously described [18]. PCR products were electrophoresed onto 2% agarose gel containing ethidium bromide and visualized under UV light.

Flow cytometry and fluorescence-activated cell sorting (FACS)

After trypsinization, cell suspension was prepared in FACS buffer (PBS/2% FBS/1 mM EDTA). The cells were stained at room temperature (RT) for 30 min with the following antibodies: anti-CD24-FITC, anti-CD44-PE, and anti-CD166-PE (BD Biosciences); anti-CD133/1-PE (Miltenyi Biotech); anti-CD13-FITC, anti-CD15 (SSEA-1)-PE, anti-CD90-PE, anti-CD117 (c-KIT)-PE, and anti-CD326 (EpCAM)-PE (eBiosciences). To discriminate dead cells, the cells were then incubated with 7-AAD (BD Biosciences) at RT for 5 min. To test for ALDH activity, an ALDEFLUOR assay kit (Aldagen) was used according to the manufacturer's protocol; labeling was performed for 30 min. Diethylamino-benzaldehyde (DEAB), an inhibitor of ALDH activity, was used as a negative control. The labeled cells were analyzed/sorted using a FACS Vantage SE with a ClonCyt option (BD Biosciences). Data analysis was done with FlowJo software (Tree Star).

Statistical Analysis

Difference between groups was examined for statistical significance with unpaired *t*-test or one-way ANOVA followed by Turkey's *post-hoc* test. *P*-value not exceeding 0.05 was considered statistically significant. Data were analyzed with PRISM software (GraphPad Software).

Results

The ability of tumor/sphere formation of thyroid cancer cell lines

We prepared eight thyroid cancer cell lines and first performed STR profiling to confirm the identities of these lines. Our FRO cells appeared to be identical to the WRO82-1 cells in Dr. Haugen's laboratory [13]; however, the data from our WRO cells did not match with their results. The data of the other cell lines were all matched. Details are provided in Table 1.

We then assessed the ability of tumor formation in nude mice. Matrigel was used to accelerate implantation. FRO, KTC3, ACT1, and 8505C cells formed tumors; size and growth rate of tumors varied among cell lines and the number of cells injected (Fig. 1A). In contrast, KTC1, KTC2, TPC1, and WRO did not have the ability of xenograft formation in nude mice. These data suggest that FRO, KTC3, ACT1, and 8505C contain a reasonable number of CSCs or tumor-initiating cells.

Next, we evaluated the ability of sphere formation. We used serum-free medium with some growth factors/additives and low-attachment surface plates to optimize the condition. As shown in Fig. 1B, FRO, KTC3, ACT1, and 8505C, which have *in vivo* tumorigenic potential, showed sphere formation, whereas KTC1, KTC2, TPC1, and WRO did not at all. The pattern of being able to form spheres was completely matched to that

of tumor formation in this setting, suggesting that our sphere assay is valuable to assess tumorigenic potential.

Expression profile of candidate CSC markers

We analyzed expression profiles of possible CSC markers in the eight thyroid cancer cell lines by flow cytometry. Based on previous studies investigating CSC markers in solid tumors [3-7, 9, 19-27], we chose the following: CD13, CD15 (SSEA-1), CD24, CD44, CD90, CD117 (C-KIT), CD133, CD166, CD326 (EpCAM), and ALDH activity. Regarding cell detachment, we compared trypsin and Cellstripper (Cellgro Mediatech), which is a non-enzymatic cell dissociation solution used to preserve cell surface antigens. No obvious difference was found in their expression patterns (data not shown). Therefore, we used trypsinization for cell detachment for all subsequent experiments. The results are shown in Fig. 2. The expression of some markers varied among different cell lines, while that of others was constant; high CD44, no CD133, and moderate CD166 expression was found in all the cell lines. Unfortunately, no correlation was observed between the expression pattern and tumorigenicity in nude mice/sphere-forming ability.

Regarding CD44, it has been recently demonstrated that an alternative splicing variant form of CD44 (CD44v) plays an important role in the protection of CSCs against reactive oxygen species (ROS), suggesting that the expression of CD44v may be increased in CSCs [28]. We next examined its expression by RT-PCR. As shown in Fig 2B, CD44v (mainly CD44v8-10) was abundantly expressed in FRO and ACT1 cells, whereas CD44s (standard isoform) was far dominant in the other cell lines. Again, there was no correlation between the expression pattern of CD44v and tumorigenic potential.

Table 1 Short tandem repeat (STR) profiling data of the thyroid cancer cell lines in this study

Locus	FRO	WRO	TPC1	KTC1	KTC2	KTC3	ACT1	8505C
TH01	6	7, 9, 3	9	9	6	6, 9	6, 9	6, 9
D21S11	29, 31	29	30	29	30	30	30, 31	28, 32, 2
D5S818	11, 12	12	8, 10	11, 12	12	12	11, 13	10, 11
D13S317	13	9, 10	11, 12	11	8, 12	8	10, 12	13
D7S820	11	8, 12	11	11	11	11	10, 11	10
D16S539	11, 12	11, 12	9	12	10, 11	10, 12	9, 11	12
CSF1PO	11	10	11, 12	10, 12	12, 13	10	11, 12	12, 13
AMEL	X	X	X	X, Y	X	X	X	X
vWA	17, 18	16	14, 18	14, 17	17, 18	18	17, 18	17, 19
TPOX	8, 11	8	11	11	8	9	8	10, 11

Fig. 1 Tumor/sphere formation of thyroid cancer cell lines
 A, Indicated number of depicted thyroid cancer cells were injected into nude mice as described in Materials and methods. Tumor volumes were measured every week. Data are presented as the mean and SD of 10 tumors. B, Indicated number of depicted thyroid cancer cell lines were seeded in each well of a 96-well HydroCell plate, and incubated in sphere medium for 4 weeks. The number of spheres with a diameter ≥ 100 μ m was scored and plotted. Each bar represents the mean of 12 wells.

Fig. 2 Expression profile of candidate CSC markers in thyroid cancer cell lines

A, 1×10^6 indicated cells were plated in each 10 cm dish 24 h before analysis. The cells were detached, labeled with indicated fluorochrome-conjugated antibodies or ALDEFLUOR reagent, and then analyzed by flow cytometry. In each histogram, expression of indicated cell surface marker is shown as shaded area. In ALDEFLUOR assay, samples incubated in the presence of DEAB was first analyzed to set a region for high ALDH cells (open region), and then test samples were measured. Similar results were obtained in at least two separate experiments. X-axis and Y-axis in all plots are in log and linear scale, respectively. B, Expression of CD44 variants in thyroid cancer cell lines. Total RNA was extracted from indicated cell lines and subjected to RT-PCR. M: DNA size marker. NC: negative control (RT-). CD44s: CD44 standard isoform. CD44v8-10: CD44 variant containing exons v8-10.

Sphere-forming ability of subpopulations sorted by FACS

As our sphere assay appeared to be a promising way to assess tumorigenic ability, we next compared sphere-forming ability of two subpopulations (marker-high and marker-low or -negative) using two cell lines, FRO and KTC3, harboring relatively higher sphere-forming ability. In FRO cells, there were no differ-

ences between the two subpopulations sorted using CD13, CD15, CD44, and CD166 markers (Fig. 3). By contrast, CD326^{hi} and ALDH^{pos} cells demonstrated significantly higher sphere-forming ability than CD326^{low} and ALDH^{neg} cells, respectively (Fig. 3). In KTC3 cells, only ALDH^{pos} cells showed moderate but significant difference compared with ALDH^{neg} cells (Fig. 3). Other markers including CD326 did not affect the ability of

Fig. 3 Sphere-forming ability of subpopulations sorted by FACS

Cells gated for the expression of indicated CSC markers were directly sorted into a 96-well HydroCell plate and incubated for 4 weeks. In each well, 20 (FRO), 300 (KTC3), 300 (ACT1), or 300 (8505C) cells were sorted. X-axis and Y-axis in all plots are in log and linear scale, respectively. The number of spheres with a diameter $\geq 100 \mu\text{m}$ in a well was counted and plotted. Each bar represents the mean of at least 8 wells. * $p < 0.05$ vs the other.

sphere-formation (Fig. 3). We then additionally compared the sphere forming ability between ALDH^{pos} and ALDH^{neg} subpopulations in ACT1 and 8505C cells. As shown in Fig. 3, there was significant difference in ACT1 cells, but this was not the case in 8505C cells. Taken together, these data suggest that ALDH activity seems to be a potential CSC marker but not universal.

ALDH activity and CD326 expression in FRO cells

To investigate a relationship between two markers, ALDH activity and CD326 expression, in FRO cells, we performed ALDEFLUOR assay followed by CD326 staining. As shown in Fig. 4, CD326^{hi} cells were comprised of ALDH^{pos} and ALDH^{neg} cells. On the other hand, $\text{CD326}^{\text{low}}$ cells were all ALDH^{neg} . We then sorted each subpopulation and assessed its sphere forming ability. As expected, $\text{CD326}^{\text{low}}/\text{ALDH}^{\text{neg}}$ and $\text{CD326}^{\text{hi}}/\text{ALDH}^{\text{pos}}$ cells demonstrated poor and higher sphere forming ability, respectively (Fig. 4). Interestingly, $\text{CD326}^{\text{hi}}/\text{ALDH}^{\text{neg}}$ cells also showed high sphere forming ability to a similar extent as the $\text{CD326}^{\text{hi}}/\text{ALDH}^{\text{pos}}$

Fig. 4 Dual ALDH and CD326 staining

After ALDEFLUOR assay, FRO cells were further incubated with anti-CD326-PE antibody and then subjected to FACS. The indicated subpopulations (left dot plot) were directly sorted into a 96-well HydroCell plate and incubated for 4 weeks. In each well, 20 cells were sorted. Both X- and Y-axes in the dot plot are in log scale. The number of spheres with a diameter $\geq 100 \mu\text{m}$ in a well was counted and plotted. Each bar depicts the mean of at least 16 wells. * $p < 0.05$ vs $\text{CD326}^{\text{low}}/\text{ALDH}^{\text{neg}}$.

Fig. 5 Ability of asymmetric division

Cells were sorted from the indicated gate, cultured for 2 weeks, and then re-analyzed by flow cytometry. X-axis and Y-axis in all plots are in log and linear scale, respectively. Similar results were obtained in at least two separate experiments.

cells. Since the $CD326^{hi}$ population contained both $ALDH^{pos}$ and $ALDH^{neg}$ fractions and these fractions included tumorigenic cells, CD326 may be a better marker than ALDH activity to obtain tumor-initiating cells as much as possible or exclude cells without tumor-forming ability more strictly in this type of cells.

Self-renewal and differentiation of the subpopulations

Next, to evaluate the ability of self-renewal and differentiation, which means generating phenotypically same and different populations and are characteristics of CSC, we sorted the subpopulations of $ALDH^{pos}$ and $ALDH^{neg}$ (and $CD326^{hi}$ and $CD326^{low}$ in only FRO cells), further cultured for 2 weeks, and re-analyzed the expression of those markers by flow cytometry. As expected, marker-high cells generated both marker-high and marker-low (or -negative) cells (Fig. 5), suggesting that the highly sphere-forming populations possessed both self-renewal and differentiation capacities. However, marker-low or -negative cells also generated both types of cells (Fig. 5). These data suggest that the change of phenotype is not uni-directional as for the expression of these markers.

Discussion

In the present study, we used 8 thyroid cancer cell lines for assessment of tumorigenic ability in nude

mice and *in vitro* sphere forming ability. Both abilities were observed in the 4 lines (FRO, KTC3, ACT1, and 8505C), but the other 4 lines (KTC1, KTC2, TPC1, and WRO) did not show either ability, indicating that our sphere assay is strongly associated with nude mouse xenograft experiment in regard to evaluating the ability of tumor formation. This provides strong advantages, because the *in vitro* sphere assay is more cost and time efficient, and also gives higher reproducibility.

Primary cancer cells have advantages to study CSCs in many aspects because they are pathologically natural and thus may reflect characteristics of *in vivo* tumor. However, there are also some disadvantages in using primary cells. The cells must be extensively digested mechanically and enzymatically, and the effect/damage of the treatment may vary with tumor type and type of marker, from patient to patient, and even within a single tumor tissue depending on the amount of connective tissue, place (central or peripheral), etc. Therefore, the cells obtained through the treatment might not be representative of whole tumor cells. Of course, cell lines show differences in many properties including gene expression profile compared with *in vivo* primary tumors [29], presumably due to selection and serum-supplemented condition for faster growing. However, cell lines still retain characteristics of primary tumors and valuable for cancer study [9, 29]. In addition, they are easily used repeatedly to confirm reproducibility

and are not contaminated with stromal cells. Regarding CSC marker between primary cancer and cancer cell line, many common markers in same types of cancer have been reported: *e.g.* CD44⁺/CD24⁻ and ALDH activity in primary breast cancer cells [3, 7] and breast cancer cell lines [30-32]; CD133 in primary brain cancer cells [4, 33] and glioma cell lines [34, 35]; CD133 in primary liver cancer and cell lines [36, 37]; CD44 in primary gastric cancer and cell lines [6, 38]. Therefore, we used thyroid cancer cell lines and our established sphere assay to perform comprehensive search for thyroid CSC markers.

Among the tumor-forming 4 lines (FRO, KTC3, ACT1, and 8505C), ALDH activity measured by the ALDEFLUOR assay significantly discriminated sphere forming ability in 3 lines (FRO, KTC3, and ACT1), suggesting that the ALDH activity is probably the most promising candidate of thyroid CSC marker. However, the ALDH activity was not a marker in the remaining line, 8505C cells, in our study. Indeed, in the report by Todaro *et al.*, ALDH^{high} cells did not develop tumors in mice in 8/32 (25%) cases [14]. These data suggest that ALDH activity is not a common CSC marker among thyroid cancers, and there may be other CSC markers. In fact, in FRO cells, CD326^{hi} cells, which were not completely overlapped with the ALDH^{pos} fraction, also showed higher sphere forming ability, implicating that at least two different types of tumor initiating cells exist in this type of cancer cells. It has been demonstrated that CSC marker varied depending on types of breast cancer cell line as well as primary breast cancer cases [30]. Moreover, even in a single tumor, multiple distinct populations with CSC properties were found in hepatocellular carcinoma [39] and glioblastoma [40]. These reports are consistent with our findings.

The cells with higher sphere forming capability (ALDH^{pos} in FRO, KTC3, and ACT1; CD326^{hi} in FRO) displayed both self-renewal and differentiation (generating both same and different “heterogeneous” populations), which is a hallmark of CSCs. However, the other fractions (ALDH^{neg} or CD326^{low}) also gave rise to both two populations. We cannot rule out the possibility that these progenies are from cells with other type of unknown CSC marker. Akunuru *et al.* [41] have shown that multiple distinct CSC subpopula-

tions that were interconvertible each other were found in lung cancer cells. In squamous cell carcinoma cell lines, there are two distinct CSC populations (CSCs with epithelial characteristics and with features of epithelial-mesenchymal transition), and they are also migratory each other [42]. Furthermore, Iliopoulos *et al.* [43] have shown that IL6 can convert non-CSCs to CSC in breast and prostate cancer cell lines as well as from cells derived from human breast tumors. In melanoma cells, a distinct subpopulation of slow-cycling JARID1B-positive cells is required for tumor maintenance [44]. However, interestingly, these cells do not follow a hierarchical CSC model, as they can be derived from JARID1B-negative cells. In thyroid cancer, therefore, the state of CSCs might be also flexible in certain circumstances.

The present study indicates that markers and their expression mechanisms of thyroid CSCs may be complicated. Compared with other organs' cancer, studies of thyroid CSCs are still limited, and further researches are definitely required. For instance, not only a single marker but several/different markers should be considered in each cancer. In addition, even in a single tumor, it would be necessary to examine the CSC properties in different portions using *in situ* techniques or cell separation from different locations. In conclusion, our results suggest that ALDH activity is a major marker to enrich thyroid CSCs but not universal. There may be different CSC markers depending on individual cases. Further characterization is needed to understand the whole view of thyroid CSCs. This may lead to the establishment of a novel tailored therapy for various types of thyroid cancer.

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Disclosure

The authors declare that they have no competing interests.

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