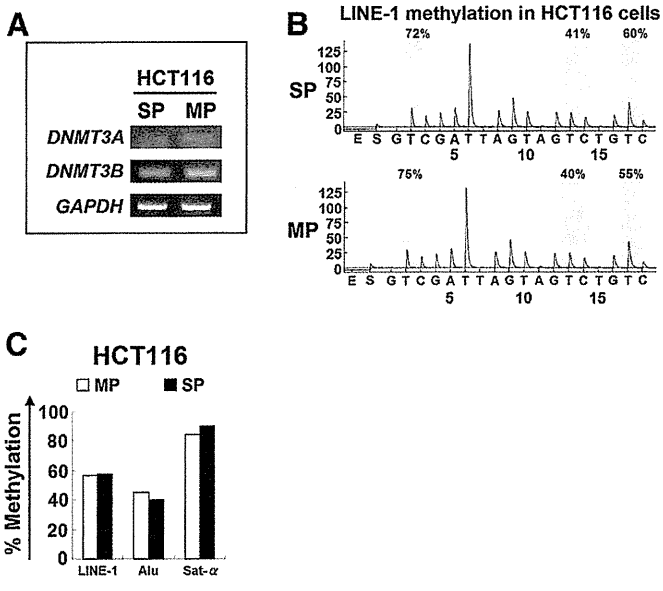

Fig. 5. Expression of DNMT3A and DNMT3B and methylation status of repetitive sequence in SP and MP cells. A. Expression of *DNMT3A* and *DNMT3B* mRNA in SP and MP cells derived from HCT116 cells. *DNMT3A* and *DNMT3B* mRNA expressions were evaluated in SP cells and MP cells derived from HCT116 cells. B. Quantitative pyrosequencing analysis of LINE-1 in SP and MP cells. Quantitative pyrosequencing analysis was performed using SP cells and MP cells derived from HCT116 cells. Representative pyrograms of SP cells and MP cells. Gray columns represent C-to-T polymorphic sites. C. Methylation status of LINE-1, Alu and Sat-alfa in SP cells and MP cells. SP cells and MP cells derived from HCT116 cells were analyzed. Data represent means. Open bars represent MP cells and closed bars represent SP cells.

Marzo et al., 1999; Mizuno et al., 2001; Girault et al., 2003; Saito et al., 2003; Etoh et al., 2004; Lin et al. (2007, 2010)) DNTM1 overexpression is correlated with poorer tumor differentiation in gastric carcinomas (Etoh et al., 2004), and it is correlated with poorer prognosis in hepatocellular and lung carcinomas. (Saito et al., 2003; Lin et al., 2010) Since CSCs/CICs are related to poorer prognosis and also poorer differentiation, these observations suggest that poorer differentiation and poorer prognosis might be caused by a high ratio of CSCs/CICs that is maintained by a high expression level of DNMT1.

We also investigated several functioning CSCs/CICs markers including ALDH1 enzymatic activity and the expression of CD44. ALDH1 enzyme identifies the cells that are resistant to alkylating agents, and gives these cells cytoprotective effects. ALDH1 members catalyze the final step in the conversion of retinol to retinoic acid that concerns with differentiation and self-renewal. (Kemper et al., 2010; Gires, 2011) This enzyme plays an important role in the maintenance of CSCs/CICs. CD44 also has a functional role in CSCs/CICs, such as survival, growth, differentiation and chemotherapy-resistance. (Kemper et al., 2010; Zeilstra et al., 2008) In addition, CD44 works as the adhesion molecule related in migration. (Cho et al., 2012) In this study, the cells that had high ALDH1 activity were detected only 0.2% in *DNMT1*$^{-/-}$ cells and CD44 expression greatly decreased in *DNMT1*$^{-/-}$ cells. Although we could not reveal the exact mechanisms of how DNMT1 controls the maintenance of CSCs/CICs, deletion of DNMT1 decreases CSCs/CICs and reduces the expressions of these functioning molecules. Therefore, we suppose that DNMT1 might be essential for initiating of the colon cancers. Actually, we observed that DNMT1 positive rates were significantly correlated with SOX2, that was reported to be as transcription factor in embryonic stem cells (Masui et al., 2007) and to highly express in CSCs/CICs of lung cancer (Nakatsugawa et al., 2011), positive rates in the immunohistochemical staining of primary colon cancer (data not shown). Although further analyses are required, this might be a clue that elucidates the role of DNMT1 in CSC/CICs.

Previous report described that there are no difference in genomic methylation status in both HCT116 cells and *DNMT1*$^{-/-}$ cells (Rhee et al., 2000), and we showed there are also no difference in genomic methylation status in SP and MP cells. Taken together, these results indicate that the methylation status of genome does not matter for the maintenance of CSCs/CICs.

In conclusion, we showed for the first time that *DNMT1* is essential for maintenance of human colon CSCs/CICs. Transient suppression of *DNMT1* is sufficient to exhaust CSCs/CICs. Our observations indicate the possibility that transient systemic or local gene suppression of *DNMT1* is an effective approach for eradicating CSCs/CICs, which will make disease more treatable by chemotherapy or radiotherapy.

Declaration of financial disclosure

The authors have no financial conflict of interest.

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Prognostic impact of the expression of ALDH1 and SOX2 in urothelial cancer of the upper urinary tract

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Aldehyde dehydrogenase 1 (ALDH1) and sex determining region-Y-related high mobility group box 2 (SOX2) have been identified as putative cancer stem-like cell/tumor-initiating cell markers in various cancer tissues. The aim of this study was to elucidate the prognostic impact of these putative cancer stem-like cell/tumor-initiating cell markers in upper urinary tract urothelial cell carcinoma. Immunohistochemical staining for ALDH1 and SOX2 was carried out on archival specimens from 125 patients with upper urinary tract urothelial cell carcinoma who underwent radical nephroureterectomy. The prognostic value of ALDH1 and SOX2 expression and other clinicopathological features was evaluated. On univariate analysis, tumor grade, pathological T stage, pathological N stage, lymphovascular invasion, ALDH1 expression and SOX2 expression were associated with a poor prognosis. On multivariate analysis, the independent factors of prognosis were tumor grade ($P=0.014$), pathological N stage ($P=0.005$) and ALDH1 expression ($P=0.002$). In subgroup analysis, those subgroups with no positive, one positive or two positive results in immunohistochemistry for ALDH1 and SOX2 expression had estimated 5-year cancer-specific survival rates of 80%, 49% and 22%, respectively ($P<0.001$). Neither ALDH1 nor SOX2 expression correlated with intravesical recurrence after radical nephroureterectomy. These findings suggest that cancer stem-like cells/tumor-initiating cells are linked to more aggressive behavior of upper urinary tract urothelial cell carcinoma, supporting the current cancer stem cell hypothesis. Thus, therapeutic targeting of cancer stem-like cells/tumor-initiating cells in upper urinary tract urothelial cell carcinoma is a future possibility.
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Keywords: ALDH1; cancer stem cell; carcinoma; prognosis; SOX2; upper urinary tract; urothelial cell

Upper urinary tract urothelial cell carcinomas are uncommon and account for only 5–10% of urothelial carcinomas.[1] Radical nephroureterectomy with excision of an ipsilateral bladder cuff is the standard therapy for patients with a normal contralateral kidney.[2] Upper urinary tract urothelial cell carcinomas that invade the muscle wall usually have a very poor prognosis, even if radical nephroureterectomy is performed appropriately.[1] The 5-year specific survival is <50% for pT2/ pT3 and <10% for pT4.[3,4] According to the most recent classifications, the primarily recognized prognostic factors are tumor stage and grade.[1] Gender, age and the initial location of the tumor within the upper urinary tract are no longer accepted as prognostic factors.[1] Lymphovascular invasion,[5–7] tumor necrosis,[8,9] tumor architecture[10] and concomitant carcinoma *in situ*[11,12] are associated with higher risks of recurrent disease and cancer-specific mortality. Molecular markers such as microsatellite instabilities,[13] E-cadherin, hypoxia-inducible factor-1α and a telomerase RNA component[14] have been shown to be useful for prognosis, although none of the markers has been externally validated.[1]

Cancer stem-like cells/tumor-initiating cells are a small population of cancer cells that have the properties of tumor-initiating ability, self-renewal and differentiation. Cancer stem-like cells/tumor-initiating cells are more resistant to chemotherapy and radio-

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therapy than non-cancer stem-like cell/tumor-initiating cell populations via various mechanisms,[15] suggesting that the existence of these cells is a prognostic factor in cancer patients. In this study, we investigated two cancer stem-like cell/tumor-initiating cell markers. Aldehyde dehydrogenase 1(ALDH1) is a cytosolic isoform of ALDH, and high levels of its activity are seen not only in hematopoietic stem/progenitor cells but also in solid cancers (eg, breast,[16,17] colorectal,[18] pancreas,[19] bladder[20] and prostate[21] cancers). Furthermore, expression of ALDH1 is a predictor of poor clinical outcome in the breast,[16,22] lung,[23] pancreatic[19] and bladder[20] cancers. Sex determining region-Y-related high mobility group box (SOX) 2 is a transcription factor that is involved in the maintenance of embryonic stem cell pluripotency and in multiple developmental processes. It is overexpressed in certain poorly differentiated subtypes of cancer (eg, lung,[24,25] breast,[26,27] and colorectal[28,29] cancers). SOX2 is not only a prognostic indicator in these cancers but also a candidate for cancer stem-like cell/tumor-initiating cell-targeting T-cell-based immunotherapy.[30]

The purpose of this study was therefore to evaluate the relationship between cancer stem-like cells/tumor-initiating cells and prognosis in upper urinary tract urothelial cell carcinoma by using the putative markers, ALDH1 and SOX2, with full clinicopathological data and follow-up. We also analyzed the association between cancer stem-like cell/tumor-initiating cell marker expression and recurrence, especially intravesical recurrence after radical nephroureterectomy.

Materials and methods

Patients

We reviewed the clinical pathology archives of 181 consecutive patients who underwent radical nephroureterectomy and were diagnosed as having upper urinary tract urothelial cell carcinomas at the Sapporo Medical University Hospital from June 1995 through May 2010. Patients with a previous history of bladder cancer and patients with concomitant bladder cancer were excluded. Finally, a total of 125 patients were enrolled in this study. Informed consent was obtained from the patients to use the surgical specimens remaining after pathological diagnosis for the investigational study, which was approved by the Institutional Review Board for Clinical Research at our university (No. 22–131). All hematoxylin- and eosin-stained slides were reviewed, and all of these specimens showed urothelial carcinoma. The median age at operation of the 89 male and 36 female patients was 69 years (range 32–88). Median follow-up was 69 months (range 6–192). All hematoxylin- and eosin-stained slides were reviewed, and clinical stage was assigned using the American Joint Committee on Cancer TNM Staging System for Renal Pelvis and Ureter Cancer (7th edition, 2010).[31] The patients' characteristics are shown in Table 1.

Table 1 Characteristics of the 125 patients

Characteristics	
Median age in years (range)	69 (32–88)
Median follow-up (months)	69
Sex	
Male	89 (71)
Female	36 (29)
Side	
Right	54 (43)
Left	71 (57)
Primary site (main)	
Renal pelvis	75 (60)
Ureter upper	11 (9)
Middle	10 (8)
Lower	29 (23)
Pathological stage	
Stage 0a	16 (13)
Stage 0is	2 (2)
Stage I	17 (14)
Stage II	21 (17)
Stage III	50 (40)
Stage IV	19 (15)
Chemotherapy	
Neoadjuvant	10 (8)
Adjuvant	6 (5)

Values are *N* (%) except where mentioned otherwise.

Immunohistochemistry and Scoring

Sections (4 μm) of the formalin-fixed, paraffin-embedded tumor specimens were immunostained after heat-induced epitope retrieval in citrate buffer (pH 6.0) using an autoclave with a monoclonal antibody against ALDH1 (dilution 1:1000; BD Transduction Laboratories, San Diego, CA, USA) and a polyclonal antibody against SOX2 (dilution 1:100; Invitrogen, Camarillo, CA, USA). Subsequent incubations with a secondary biotinylated antibody, avidin-conjugated peroxidase complex and chromogen were done on a Ventana NexES (Ventana Medical Systems, Tucson, AZ, USA). The slides were then counterstained with hematoxylin, rinsed, dehydrated through graded alcohols into nonaqueous solution, and cover-slipped with mounting media. Negative controls had the primary antibody replaced by buffer. All specimens were reviewed independently using light microscopy in at least five areas at ×400 magnification by investigators who were blinded to clinicopathological data (TT and YH). For ALDH1, tumors presenting at least one ALDH1-positive cancer cell were considered to be ALDH1 positive.[16,32] For SOX2, nuclear staining

was considered positive.[33] We previously reported that the SOX2-positive rates in lung cancer were 15%, 45% and 40% in <1%, 1–10% and >10% of tumors, respectively.[33] On the basis of these results, we used a 10% cutoff point for both negative and positive specimens. Breast and lung cancer tissues were used as positive controls for ALDH1 and SOX2, respectively.

Statistical Analysis

We tested the relationships between ALDH1/SOX2 and the other clinicopathological parameters, ie, the pathological T stage, pathological N stage, tumor grade and lymphovascular invasion by χ^2 tests. Cancer-specific survival, overall survival, recurrence-free survival and intravesical recurrence-free survival were assessed by the Kaplan–Meier method, and differences between two groups were compared using the log-rank test. For the test of intravesical recurrence-free survival, 16 patients with stage IV disease were excluded. The subgroups with two positive, one positive and no positive immunohistochemistry results for ALDH1 and SOX2 expression were analyzed. Univariate and multivariate regression analyses according to the Cox proportional hazards regression model, with cancer-specific survival as the dependent variable, were used to evaluate the expression of ALDH1 and SOX2 as potential independent prognostic factors. A value of $P<0.05$ was considered to indicate statistical significance. The calculations were performed using JMP™ software.

Results

Expression and Localization of ALDH1 and SOX2

Scattered ALDH1-positive cells were observed in 34 (27%) of the 125 cases (Figure 1b). The ALDH1

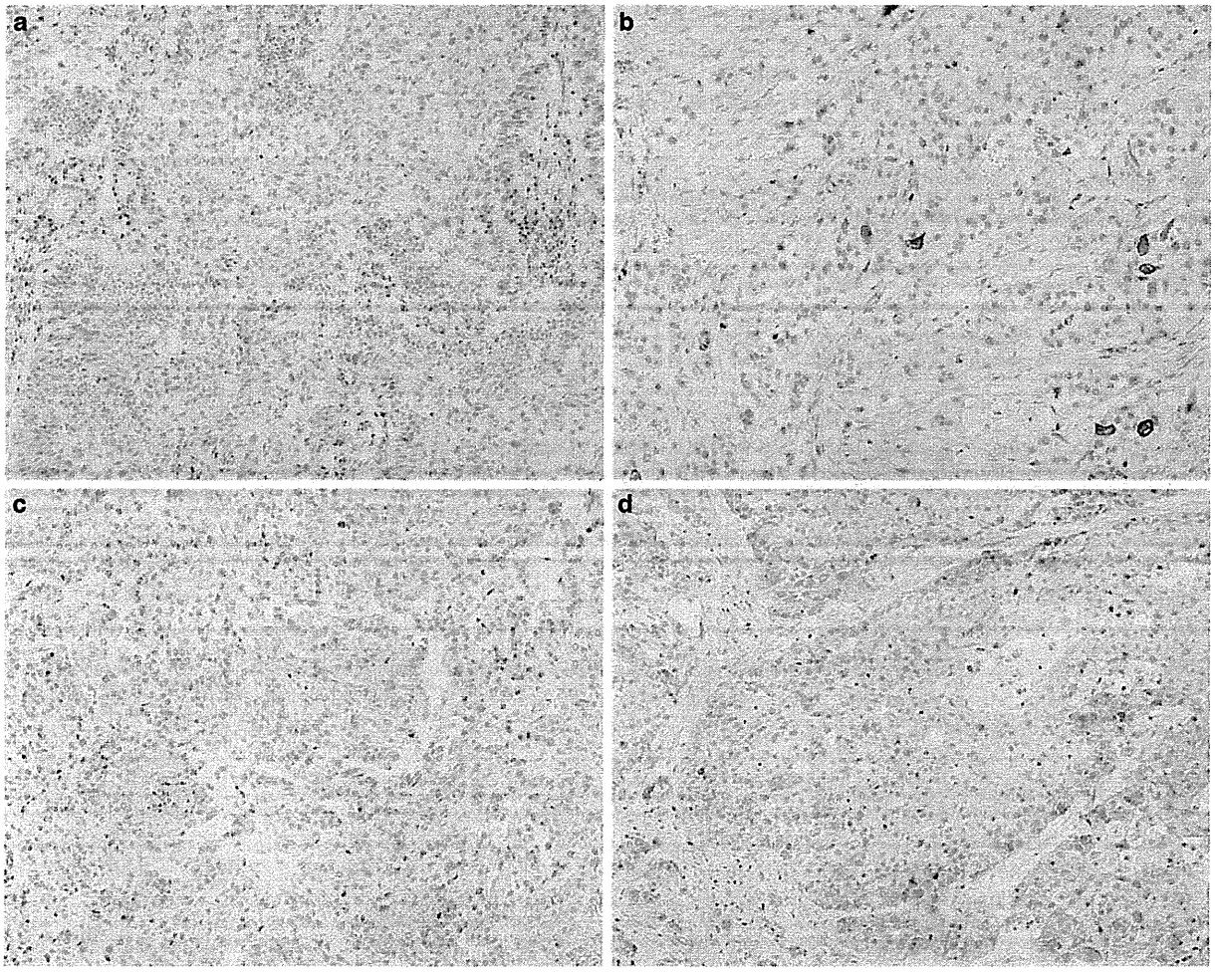

Figure 1 Representative immunohistochemical staining of aldehyde dehydrogenase 1 (ALDH1) and sex determining region-Y-related high mobility group box 2 (SOX2). (**a**) Negative ALDH1 expression in tumor cells, (**b**) positive ALDH1 expression in tumor cells, (**c**) negative SOX2 expression in tumor cells and (**d**) positive SOX2 expression in tumor cells.

Table 2 Frequency of positive expression of cancer stem-like cell/tumor-initiating cell (CSC/TIC) markers

CSC/TIC markers	n *(%)*
$ALDH1^{pos}SOX2^{pos}$	11 (9)
$ALDH1^{pos}SOX2^{neg}$	23 (18)
$ALDH1^{neg}SOX2^{pos}$	13 (11)
$ALDH1^{neg}SOX2^{neg}$	78 (62)

expression was strongly present in the cytoplasm. SOX2 expression was mainly positive in cells located in the peripheral regions of tumor nests, and diffuse cytoplasmic and nuclear staining was observed in 24 cases (19%) (Figure 1d). We examined the mRNA expression of ALDH1 and SOX2 by RT-PCR (Supplementary Information) and compared it with immunohistochemical expression of these genes in the same nine tissues. The concordance rates between the two methods were 78% for ALDH1 and 89% for SOX2 (Supplementary Figure S1). The rates of SOX2-positive cells were <1%, 1–10% and >10% in 19% ($n=24$), 62% ($n=77$) and 19% ($n=24$) of the cases, respectively. The percentages of ALDH1- and SOX2-positive cancer cells were counted and subjected to statistical analysis. The frequencies of the expression of cancer stem-like cell/tumor-initiating cell markers are shown in Table 2. In cases that were both ALDH1- and SOX2-positive, the tumor cells were ALDH1- or SOX2-positive or double-positive. Immunohistochemical staining of ALDH1 and SOX2 in a representative double-positive case is shown in Supplementary Figure S2.

Associations Between Expression of ALDH1 and SOX2 and Clinicopathological Variables (Table 3)

ALDH1 expression was linked to lymph node metastasis ($P=0.047$) and lymphovascular invasion ($P=0.038$). SOX2 expression was significantly associated with more advanced pathological T stage ($P=0.032$), more advanced pathological N stage ($P=0.019$), and as well as with a trend toward to higher tumor grade ($P=0.017$).

Association of ALDH1 and/or SOX2 with Survival and Recurrence

The 5-year cancer-specific survival rates of patients with ALDH1-negative and -positive tumors were 74% and 36%, respectively (Figure 2a). The 5-year cancer-specific survival rates of patients with SOX2-negative and -positive tumors were 72 and 46%, respectively (Figure 2b). There were significant differences in cancer-specific survival between patients with ALDH1-negative tumors and those with ALDH1-positive tumors ($P<0.001$, Figure 2a), and between patients with SOX2-negative tumors and those with SOX2-positive tumors ($P=0.003$, Figure 2b). Thus, both ALDH1 and SOX2 expression correlated with cancer-specific survival. The subgroups with no positive, one positive or two positive immunohistochemistry results for ALDH1 and SOX2 expression had estimated 5-year cancer-specific survival rates of 80%, 49%, and 22%, respectively ($P<0.001$, Figure 2c).

Table 3 ALDH1/SOX2 expression and pathological factors in patients with upper urinary tract urothelial cell carcinoma

Variable	*ALDH1*			*SOX2*		
	Positive (%)	*Negative (%)*	*P-value*	*Positive (%)*	*Negative (%)*	*P-value*
Pathological T stage						
pTa	2 (6)	0 (0)	0.184	1 (4)	1 (1)	0.032
pTis	3 (9)	13 (14)		1 (4)	15 (15)	
pT1	3 (9)	15 (16)		4 (17)	14 (14)	
pT2	6 (18)	17 (19)		1 (4)	22 (22)	
pT3	18 (52)	43 (48)		14 (58)	47 (46)	
pT4	2 (6)	3 (3)		3 (13)	2 (2)	
Pathological N stage						
pN0	27 (79)	85 (94)	0.047	18 (75)	94 (93)	0.019
pN1	4 (12)	2 (2)		2 (8)	4 (4)	
pN2	3 (9)	4 (4)		4 (17)	3 (3)	
Grade						
G1	0 (0)	3 (3)	0.083	1 (4)	2 (2)	0.017
G2	10 (29)	43 (47)		4 (17)	49 (48)	
G3	24 (71)	45 (50)		19 (79)	50 (50)	
Lymphovascular invasion						
Negative	17 (50)	64 (70)	0.038	13 (54)	68 (67)	0.242
Positive	17 (50)	27 (30)		11 (46)	33 (33)	

Kaplan–Meier plots and log-rank tests showed that the upper urinary tract urothelial cell carcinoma patients with ALDH1-positive tumor cells had significantly shorter overall survival, than those whose tumors were ALDH1-negative ($P<0.001$). The 5-year overall survival rates of patients with ALDH1-negative and -positive tumors were 63% and 31%, respectively. The 5-year overall survival rates of patients with SOX2-negative and -positive tumors were 62% and 36%, respectively. There was a significant difference in overall survival between the two groups ($P=0.019$).

The 5-year recurrence-free survival rates of patients with ALDH1-negative and -positive tumors were 43% and 24%, respectively (Figure 3a). There was a significant difference in recurrence-free survival between the two groups ($P=0.024$). In contrast, no difference was observed in recurrence-free survival between patients with SOX2-negative tumors and those with SOX2-positive tumors (Figure 3b). During the follow-up, 34 (32%) of 106 patients undergoing radical nephroureterectomy for stage ≤III disease had intravesical recurrence. Of the 34 patients, 13 (38%) had systemic recurrence and 8 (24%) died of UC. Neither ALDH1 nor SOX2 expression correlated with intravesical recurrence-free survival (Figures 3c and d).

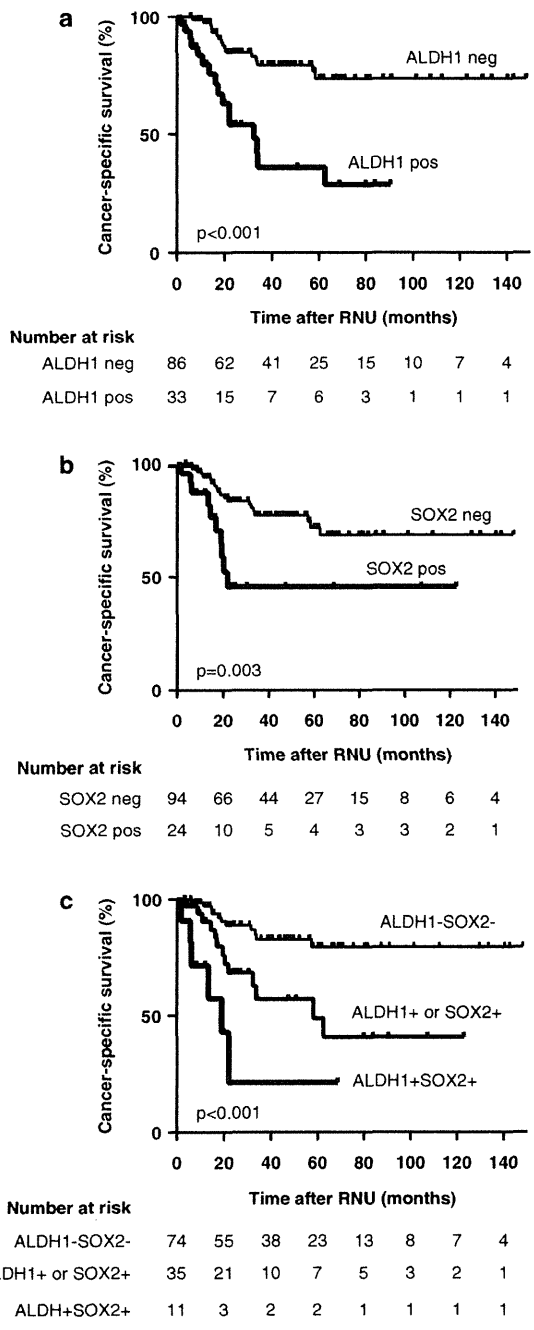

Figure 2 Kaplan–Meier curves for cancer-specific survival rates according to (**a**) aldehyde dehydrogenase 1 (ALDH1) expression status, (**b**) sex determining region-Y-related high mobility group box 2 (SOX2) expression status and (**c**) combined expression status of ALDH1 and SOX2.

In univariate analysis, the pathological T stage, pathological N stage, tumor grade, lymphovascular invasion, ALDH1 and SOX2 were associated with a poor prognosis (Table 4). In multivariate analysis, the independent factors of prognosis were the pathological N stage ($P=0.005$), tumor grade ($P=0.014$) and ALDH1 expression ($P=0.002$) (Table 4).

Discussion

To the best of our knowledge, this is the first study in which the relationships between expression of putative cancer stem-like cell/tumor-initiating cell markers and the most clinically relevant features of upper urinary tract urothelial cell carcinoma were evaluated. We demonstrated that expression of both ALDH1 and SOX2 correlated with cancer-specific survival. In contrast, expression of these markers was not associated with intravesical recurrence-free survival. These findings suggested that cancer stem-like cells/tumor-initiating cells were linked to more aggressive behavior of upper urinary tract urothelial cell carcinoma.

We demonstrated that ALDH1 was not only an independent factor for prognosis but also associated with recurrence-free survival, although there was no relationship between ALDH1 expression and intravesical recurrence-free survival. Brandt *et al*[34] found that ALDH1 was significantly upregulated in urothelial cancer stem-like cells compared with non-cancer stem-like cells, indicating a potential mode of chemoresistance in urothelial cancer stem-like cells. Su *et al*[20] reported that high ALDH1 expression was associated with poor prognosis for patients with bladder urothelial carcinoma and was an independent predictor for cancer-specific survival. Various studies have reported that immunohistochemically identified tumor ALDH1 expression is associated with a poor prognosis in breast,[16,22] lung,[23] and pancreatic[19] cancer patients. Conversely, ALDH1 has a favorable function in ovarian carcinoma and high expression of ALDH1 is a favorable prognostic factor in patients with ovarian cancer.[35] In a large study including 1420 patients with colorectal cancer of all stages, no significant correlation could be found between ALDH expression and survival,[36] whereas the ALDH1 expression pattern had a significant impact upon survival for G2 T3N0M0 colorectal cancer in another study.[37] Our findings suggest that upper urinary tract urothelial cell carcinoma contains ALDH1-positive cancer stem-like cells/tumor-initiating cells like bladder cancer, and that these cells are associated with survival or life-threatening disease, as 62% of the patients with intravesical recurrence were alive without any other recurrence.

Although the roles of SOX2 in cancer cells are still elusive, SOX2 is considered one of the candidate cancer stem-like cell/tumor-initiating cell antigens.[15] We previously demonstrated that SOX2-overexpressing lung adenocarcinoma cell lines showed higher rates of side population cells and higher tumorigenecity and that SOX2 mRNA knockdown of side population cells by gene-specific siRNA completely abrogated tumorigenecity *in vivo*.[33] In this study, we found that SOX2 was associated with cancer-specific survival in patients with upper urinary tract urothelial cell carcinoma. Although there has been no report showing the

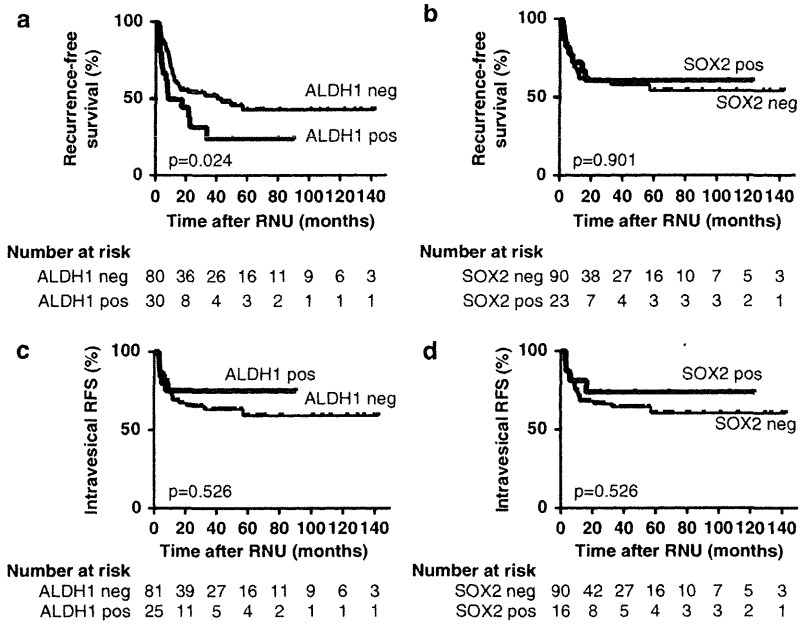

Figure 3 Kaplan–Meier curves for recurrence-free survival rates according to (**a**) aldehyde dehydrogenase 1 (ALDH1) expression status and (**b**) sex determining region-Y-related high mobility group box 2 (SOX2) expression status, and for intravesical recurrence-free survival rates according to (**c**) ALDH1 expression status and (**d**) SOX2 expression status.

Table 4 Prognostic factors for cancer-specific survival in univariate and multivariate analyses

Factor	*Univariate analysis*		*Multivariate analysis*	
	HR (95% CI)	*P-value*	*HR (95% CI)*	*P-value*
Pathological T stage	2.76 (1.69–4.89)	<0.001	1.68 (0.94–3.16)	0.082
Pathological N stage	2.75 (1.75–4.09)	<0.001	2.18 (1.29–3.60)	0.005
Grade	6.02 (2.53–17.7)	<0.001	3.36 (1.26–10.6)	0.014
Lymphovascular invasion	2.18 (1.52–3.25)	<0.001	1.22 (0.76–1.96)	0.433
ALDH1	1.97 (1.38–2.81)	<0.001	1.89 (1.28–2.79)	0.002
SOX2	1.78 (1.21–2.55)	0.005	1.30 (0.83–1.98)	0.256

relationship between SOX expression and prognosis in UC, Ben-Porath *et al*[38] reported enriched patterns of gene sets associated with embryotic stem cell identity, including SOX2, in the expression profiles of bladder carcinoma. They demonstrated that high-grade tumors showed an embryotic stem-like gene set enrichment pattern, and concluded that an embryotic stem-like signature was present in poorly differentiated cancers from distinct cells of origin. In the present study, SOX2 expression was significantly associated with tumor grade, pathological T stage and pathological N stage. This may explain why SOX2 expression was an independent factor for survival by univariate analysis but not by multivariate analysis. Several studies have reported that SOX2 is upregulated in various cancers other than urothelial carcinoma, including lung adenocarcinoma,[25] gastric carcinoma,[39] breast carcinoma,[27] head and neck squamous cell carcinomas,[40,41] hepatocellular carcinoma[42] and rectal cancer.[28] Meanwhile, another study on gastric cancer reported that SOX2 expression was related to better prognosis.[43] SOX2 expression is associated with a better outcome in squamous cell lung cancer.[44]

On the basis of the abilities for tumor initiation, self-renewal and differentiation, various putative cancer stem-like cell/tumor-initiating cell markers have been used.[45] As these markers (such as side population, CD44+/CD24-, CD133+, ALDH1, SOX2, Oct3/4, etc.) show distinct properties of cancer stem cells, tumor tissues can show heterogeneity when multiple markers are examined. These vary depending on the cancer, and not all tumor cells identified by certain markers are cancer stem-like cells/tumor-initiating cells.[46] In this study, 18%, 10% and 9% of the upper urinary tract urothelial cell carcinoma cases had $ALDH1^{pos}SOX2^{neg}$, $ALDH1^{neg}SOX2^{pos}$ and $ALDH1^{pos}SOX2^{pos}$ tumor

cells, respectively (Table 2). Furthermore, the number of upper urinary tract urothelial cell carcinoma cells immunohistochemically stained for both ALDH1 and SOX2, which are considered to have more characteristics of cancer stem-like cell/tumor-initiating cell, was limited in these cases (Supplementary Figure S2). These results are compatible with reported cancer stem-like cell/tumor-initiating cell frequencies, which ranged from 1 in 2500 to 1 in 36 000 in various cancers.[47]

There are several limitations to our study. First are the limitations inherent to any retrospective study. Second, radical nephroureterectomy was performed by various surgeons over a long time period. Third, immunohistochemistry has inherent limitations such as reproducibility and reliability. Finally, the roles of ALDH1 and SOX2 in upper urinary tract urothelial cell carcinoma require further investigation.

In summary, the current results demonstrate a direct link between the expression of cancer stem-like cell/tumor-initiating cell markers and patient survival in upper urinary tract urothelial cell carcinoma. Our data support the current cancer stem cell hypothesis for upper urinary tract urothelial cell carcinoma, which suggests that therapeutic targeting of cancer stem-like cells/tumor-initiating cells in upper urinary tract urothelial cell carcinoma is a future possibility.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Sorting nexin 5 of a new diagnostic marker of papillary thyroid carcinoma regulates Caspase-2

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Papillary thyroid carcinoma (PTC) is a well-differentiated endocrine malignant tumor that develops from thyroid follicular epithelium. The tumor represents the most common type of endocrine malignancy; however, its tumorigenesis is not fully elucidated. The aim of this study was to address the functional role of the sorting nexin (SNX) family in PTC because of recent experimental evidence suggesting that the SNX family members actively control endocytotic transportation as well as cell fate. Expression profiles of SNX family members of PTC showed a significant quantity of transcripts of SNX5. Further immunohistochemical analysis with an SNX5-specific monoclonal antibody established in this study consistently demonstrated the preferential expression of SNX5 in PTC (94.2%, 113/120 cases) as indicated by studies on 440 cases of various tumors. In contrast, other major carcinomas originating from the lung (2.6%, 1/38 cases), breast (5.1%, 2/39 cases), and intestine (4.2%, 1/24 cases) scarcely expressed SNX5. When we investigated models of murine thyroid tumors induced by the administration of carcinogens, high expression of Snx5 was also observed in well-differentiated thyroid tumors, further implying that the tumorigenesis of the thyroid gland was tightly associated with the abundance of SNX5/Snx5. Moreover epithelial cells expressing excess SNX5 showed high levels of Caspase-2 of an initiator caspase. Collectively these findings suggest that the evaluation of SNX5 expression would support pathological diagnosis of primary and secondary PTC. (*Cancer Sci* 2012; 103: 1356–1362)

The thyroid gland, controlling energy production and many metabolic pathways, is the most common site for the development of malignant tumors among a variety of endocrine organs.[1] The proportion of malignant thyroid tumors has steadily increased over the last three decades.[2–4] Most thyroid tumors originate from thyroid follicular epithelial cells known as thyrocytes and exhibit various histopathological subtypes, of which papillary thyroid carcinoma (PTC) comprises the predominant subtype, with a female:male ratio of about 3:1. While PTC generally has a favorable prognosis, the tumor can potentially metastasize to regional lymph nodes, the lung and other organs.[5–8] Pre-existing benign thyroid lesions and ionizing radiation are known risk factors, and gene alterations such as BRAF and RAS point mutations, and *RET/PTC* and *TRK* gene rearrangements have been reported in PTC.[9–13] Gene regulatory factors making critical contributions during the development of thyrocytes are of diagnostic value for PTC in the pathologic laboratory, including thyroid transcription factors including TTF-1 and TTF-2, a hematopoietically expressed homeobox (HHEX), and paired box gene-8 (PAX8).[14–17] However, the etiology of the tumor development has not been fully clarified.

Thyrocytes synthesize the thyroid hormones through a multiple intracellular process coordinated by thyroid-stimulating hormone. During the process, a transcytotic pathway of thyrocytes plays an important role as suggested by anatomic examinations and other studies.[18–20] Once an iodinated glycoprotein of thyroglobulin is synthesized, endosomes convey it into a luminal area surrounded by thyrocytes. Then the thyrocytes, if required, retrieve thyroglobulin molecules from the primary colloidal storage and liberate triiodothyronine (T3) and thyroxin (T4) through a lysosomal pathway using recycling endosomes.[21–23] Recent studies on the membrane-associated traffic system of endosomes have revealed a unique role of sorting nexin (SNX) retromer family members.[24–26] The SNX family has the capacity to bind phopsphatidylinositol phosphate of the lipid bilayer of endosomes through their signature moieties of the Phox-homology (PX) domain. Once such a membrane is recognized, various effector domains of SNX molecules characterize the subsequent process of the membrane compartment. Within various such domains, a Bin/Amphiphysin/Rvs167 (BAR) domain helps SNX molecules (called SNX-BAR) form a banana-shaped structure fitting the curvature of small vesicles, whose domain is also shared by non-SNX molecules involved in Alzheimer's disease and diabetes mellitus.[27–30] In addition to these functions, accumulating evidence reveals a more fundamental function of such SNX-BARs, regulating signal transduction and growth activities, to control epithelial cell integrity.[31–35] These facts led us to hypothesize a possible function of the SNX family in the tumor biology related to the development of PTC.

In this study we first demonstrated preferential expression of SNX5 of an SNX-BAR molecule in PTC as assessed by immunohistochemistry on 440 tumor cases. Murine models of PTC showing Snx5 overexpression in the tumors further supported importance of SNX5 in the pathogenesis of PTC. More interestingly Caspase-2 as an initiator caspase would be under the control of SNX5, implying that an SNX5-Caspase-2 axis might have a pivotal role of the development of PTC.

Materials and Methods

Tissues and cell culture. Thyroid cancer tissues were obtained from patients undergoing thyroidectomy in Sapporo Medical University Hospital and Muroran City General Hospital in Japan. All human materials analyzed in this study were obtained with informed consent and the approval of the institutional review board in each hospital. For primary culture of tumor cells, tissues were minced into small pieces and dispersed in PBS containing 0.7 mg/mL Blendzyme 3 (Roche, Basel, Switzerland) and 0.4 mg/mL DNase I (Sigma-Aldrich,

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St. Louis, MO, USA) as described previously.[36] After washing the cells three times with PBS, cells were cultured in RPMI1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum, 50 μg/mL streptomycin and 100 units/mL penicillin. Human embryo kidney (HEK) 293 cells and 8505c PTC cells were obtained from RIKEN Bioresource Center (Japan) and maintained in modified DMEM (Sigma-Aldrich) supplemented with the same reagents as described above. All cells were cultured at 37°C in a humidified atmosphere in 5% CO_2.

Reverse transcription-polymerase chain reaction analysis. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted to detect transcripts as previously reported.[36] Primer pairs were summarized in Table S1. The PCR cycling conditions were as follows: 95°C for 1 min; 60°C for 1 min; 72°C for 1 min with 25 cycles. Quantitative RT-PCR was performed as described in the manufacturer's protocol for Assays-on-Demand Gene Expression products (Applied Biosystems, Foster City, CA, USA). To compare the levels of transcripts, the ΔΔCT method was used to analyze triplicate specimens according to the manufacturer's instructions.

Antibodies, immunohistochemistry, and immunoblotting. A mouse anti-human SNX5 monoclonal antibody (clone 48C2; IgG2a subclass) was established per standard procedures by immunizing mice with recombinant SNX5 protein produced in bacterial BL21 cells containing the pET expression vector (Merck KGaA, Darmstadt, Germany) harboring human SNX5 cDNA, which was initially obtained from human epidermal HaCaT cells. A mouse anti-enhanced green fluorescent protein (EGFP) mAb (JL-8; Clontech, Mountain View, CA, USA) was used for detecting EGFP-tagged proteins in immunoblot analysis. A mouse anti-TTF-1 mAb (SPT24; Nichirei, Tokyo, Japan) and rabbit anti-thyroglobulin pAb (DAKO, Copenhagen, Denmark) were used for immunohistochemistry. For studying cells by immunoblotting, antibody sampler kits were used to analyze molecules regulating apoptosis, DNA repair or cell cycle (BD Biosciences, San Diego, CA, USA). For detecting Caspase-2, antibodies of mouse mAb (clone 35) were used provided with the sampler kit as well as rabbit pAb (poly6340) purchased from Biolegend (San Diego, CA, USA). Immunofluorescence, immunohistochemistry and immunoblotting were performed as previously described.[36,37] Immunofluorescent signals were detected under an immunofluorescence microscope (IX71; Olympus, Tokyo, Japan) or confocal laser microscope (R2100AG2; Bio-rad, Hercules, CA, USA). To obtain concordant results regarding the immunohistochemical expression of SNX5, the slides were examined on a multiheaded microscope by three investigators. The staining profile of SNX5 of tissue sections was graded in accordance with positive-staining areas as follows: less than 10% areas; (−), 10–50% areas; (+), over 50% areas; (++).

Animal models of thyroid tumors. Thyroid carcinomas were chemically induced in BALB/c female mice 6 weeks of age as described previously.[38] In brief, tumors were initiated with a single subcutaneous injection of *N*-bis(2-hydroxypropyl)-nitrosamine (DHPN; Toronto Research Chemicals, Toronto, Canada) at 2800 mg/kg body weight. One week later, drinking water containing 0.1% sulfadimethoxine (SDM; Sigma-Aldrich) was provided *ad libitum* for up to 12 weeks. Tissue specimens including normal thyroid gland and tumors around the trachea were obtained using forceps and scissors for microsurgery under a binocular wide-field dissecting microscope. All of the experiments using mice were performed in accordance with the institutional guidelines for the care and use of animals.

Cell transformation and cell proliferation assay. Expression vectors of pCMV-HA and pEGFP-C2 (Clontech) were used to transform HEK 293 cells. Transformation of cells was performed with LF2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For retrovirus-mediated gene transfer into 8505c cells, pLVSIN-CMV-puro vector harboring SNX5 cDNA was transfected into Lenti-X 293T cells using Lenti-X HTX Packaging System as described in the manufacturer's protocol (Takara, Tokyo, Japan). After transfection, cells were maintained in complete medium containing 1 μg/mL puromycin (Sigma-Aldrich). Growth activities of

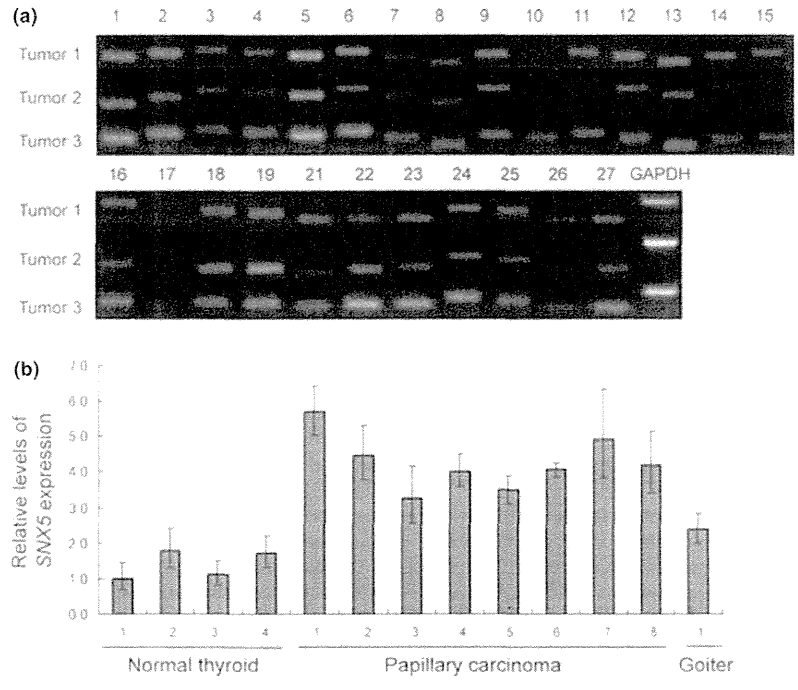

Fig. 1. Expression of sorting nexin (SNX) family members in papillary thyroid carcinoma (PTC). (a) The transcripts of SNXs assessed by reverse transcription-polymerase chain reaction (RT-PCR) in three cases of PTC. Numbers depicted correspond to the numbers of the members of the SNX family from SNX1 to SNX27. SNX20 is not assigned in humans. Glyceraldehyde 3-phosphate dehydrogenase is (GAPDH) depicted as a control. PCR cycles: 25 cycles. (b) The transcripts of SNX5 assessed by quantitative RT-PCR analysis in four normal thyroid tissues (derived from surgical specimens around tumors), eight cases of PTC and one case of nodular hyperplasia (goiter). Data are expressed as the fold change in each sample versus normal thyroid tissue number 1. Results show that the transcripts of SNX5 increased 3.2-fold in PTC ($P < 0.05$) and 1.8-fold in nodular hyperplasia ($P = 0.37$).

cells were investigated using a premix WST-1 cell proliferation assay system following the manufacturer's instructions (Takara).

Statistical analysis. Statistical significance was determined using the unpaired *t*-test and *P*-values of less than 0.05 were considered significant. Values were expressed as the mean ± standard deviation (SD).

Results

High expression of SNX5 in papillary thyroid carcinoma. Prior to starting this study, we conducted RT-PCR analysis to investigate which SNX family members were dominantly expressed in PTC. Examinations of tissue specimens from three PTC cases revealed that the transcripts of certain types of SNXs were indeed detected at various levels (Fig. 1a). Among those we examined, transcripts of SNX5 were most abundantly presented in the tumors. To a lesser extent, transcripts of SNX1, SNX2, SNX6, SNX9, SNX12, SNX13, SNX18, SNX19, SNX22, SNX23, and SNX24 were observed at moderate levels. We also performed quantitative RT-PCR analysis on normal and tumor tissue areas of PTC. The results demonstrated that the transcripts of SNX5 in the tumor lesions were 3.2-fold increased compared with those of normal thyroid areas (Fig. 1b).

To further determine the expression profile of SNX5 in tumor tissues, we established a mouse anti-human SNX5 monoclonal antibody (clone 48C2) specifically reacting to a part of the N-terminal domain of SNX5, but not other SNXs including SNX4, SNX6 (most similar to SNX5), and SNX8 (Fig. S1a,b). By using this mAb, we could certainly detect SNX5 in primary culture cells of PTC (Fig. S1c). We then performed immunohistochemistry with this mAb on tissue sections from a total of 267 cases of various thyroid tumors as summarized in Table 1. Indeed PTC, featuring papillary structure with empty-appearing nuclei, preferentially presented SNX5 (94.2%, 113/120 cases positive), like TTF-1 and thyroglobulin (Fig. 2). The sensitivity of SNX5 was 95.2% (100/105 cases; data not shown), very close to the values of TTF-1 and thyroglobulin (both indicating 100%, 105/105 cases). Interestingly, when we investigated the metastatic regions of PTC to the lymph nodes or lung, the expression of SNX5 was seemingly observed (Fig. 3b,c). Tumor tissues of composite-type carcinoma with PTC and poorly differentiated carcinoma demonstrated SNX5 expression only in the region of PTC (Fig. 3d). These findings suggested that investigation of the expression profile of SNX5 would be useful to define primary and secondary lesions of PTC. Normal thyrocytes very faintly expressed SNX5 (Figs 1b and 3a), implying that the amount of SNX5 would be enhanced during the tumorigenesis of PTC and other thyroid-origin tumors might show possible expression of SNX5 as well. In fact some other malignant thyroid tumors expressed SNX5. However, the positive rates were lower than those of PTC, ranging from 0% for medullary carcinoma (0/4 cases) and undifferentiated carcinoma (0/5 cases) to 41.2% for follicular carcinoma (7/17 cases). Benign tumors and tumor-like regions such as follicular adenoma and nodular hyperplasia (adenomatous goiter) also showed SNX5 expression with 28.6% (6/21 cases) and 67.9% (19/28 cases) positive rates, respectively. We further studied 173 cases of major malignant tumors not originated from thyroid as summarized in Table 2. As a result, we found very low positive rates of SNX5 in tumors that emerged in the lung (2.6%, 1/38 cases), breast (5.1%, 2/39 cases), colon (4.2%, 1/24 cases), liver (0%, 0/11 cases), kidney (0%, 0/21 cases), prostate (9.1%, 1/11 cases), ovary (14.3%, 2/14 cases), and uterus (6.7%, 1/15 cases). Our findings included other malignancies such as squamous cell carcinomas and lymphomas that occurred in a variety of organs, which showed negative expression of SNX5

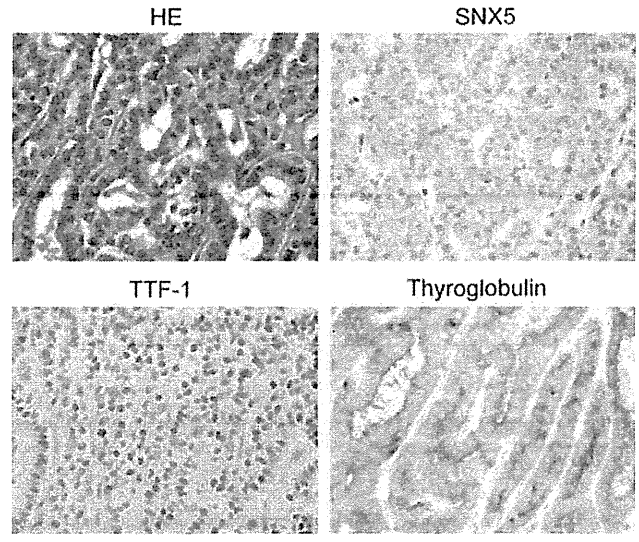

Fig. 2. Papillary thyroid carcinoma (PTC) preferentially presents sorting nexin 5 (SNX5). Immunohistochemical analysis of formalin-fixed paraffin-embedded (FFPE) tissue sections of PTC. Representative images of PTC are shown after hematoxylin and eosin (HE) staining of serial tissue sections, which were also used for immunohistochemical staining using anti-SNX5 mAb (48C2), anti-TTF-1 mAb (SPT24) and anti-thyroglobulin pAb. The nuclei of tumor cells express TTF-1 and the cytoplasm present thyroglobulin and SNX5 as well. Signals were visualized by ordinary procedures with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Original magnification: ×400.

Table 1. Expression of SNX5 in thyroid tumors

Organ	Tissue type	SNX5			Total
		++	+	−	
Thyroid gland	Papillary carcinoma	107 (89.2%)	6 (5.0%)	7 (5.8%)	120
	L/N metastasis	60 (85.7%)	5 (7.1%)	5 (7.1%)	70
	Follicular variant	0 (0%)	1 (50.0%)	1 (50.0%)	2
	Follicular carcinoma	5 (29.4%)	2 (11.8%)	10 (58.8%)	17
	Medullary carcinoma	0 (0%)	0 (0%)	4 (100%)	4
	Undifferentiated carcinoma	0 (0%)	0 (0%)	5 (100%)	5
	Follicular adenoma	4 (19.0%)	2 (9.5%)	15 (71.4%)	21
	Nodular hyperplasia	13 (46.4%)	6 (21.4%)	9 (32.1%)	28
					267

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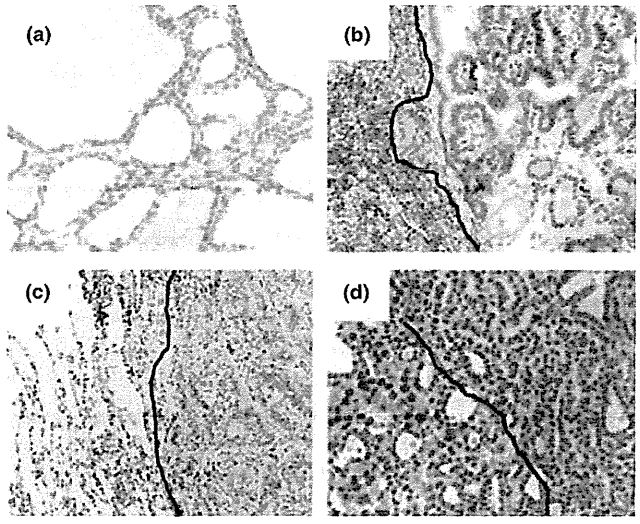

Fig. 3. Expression patterns of sorting nexin 5 (SNX5) in various tumors. Formalin-fixed paraffin-embedded (FFPE) tissue sections of various tumors were investigated and representative results are depicted. (a) Normal thyrocytes demonstrating very faint expression of SNX5. (b,c) Metastasis of papillary thyroid carcinoma (PTC) to a cervical lymph node and the lung as shown in (b) and (c), respectively. (d) Composite carcinoma of thyroid gland constituting poorly differentiated carcinoma demonstrating non-papillary structure with large nuclei in the left side and PTC in the right side. Note that only areas of PTC in (b–d) express SNX5. Solid lines separate the lesions of PTC. Original magnification: ×200.

(Table S2). Collectively these suggested the characteristic predominance of SNX5 in PTC, especially in the case of differential diagnosis from those of other tissue origin.[10] So far examination of the expression profile of SNX5 would be beneficial for thyroid tumors with papillary lesions, while we failed to find any significant relationship between expression profile of SNX5 and tumor stages in PTC cases in this study (data not shown).

High expression of Snx5 in chemically induced murine papillary thyroid carcinoma. Next we investigated murine models of PTC induced by the administration of chemical reagents.[38] After injection of DHPN as an initiator, mice were given free access to drinking water containing SDM as a promoter for consecutive weeks. PTC was eventually emerged 12 weeks after the administration of SDM (Fig. 4a). When the tumor lesions were examined by quantitative RT-PCR, the levels of transcripts of Snx5 of the tumors were found to be about two-fold those of normal thyroid tissue (Fig. 4b). This suggested that high expression of SNX5 might be a prerequisite for the tumorigenesis of PTC.

Induction of Caspase-2 by SNX5. Following these studies, we tried to determine the functional role of SNX5 in PTC cells. To do this we initially investigated HEK293 cells that expressed SNX5 at high levels (HEK293-SNX5 cells) and mock control cells (HEK293-control cells), because HEK293 cells are often used to study a fundamental role of a molecule in concern. When examined molecules regulating DNA repair, cell cycle and apoptosis, we unexpectedly found a novel role of SNX5 in the regulation of Caspase-2 (Fig. 5a,[39–41]). We next established and examined transformants of 8505c PTC cells that expressed SNX5 at high levels (8505c-SNX5 cells) and mock control cells (8505c-control cells). Like HEK293-SNX5 cells, 8505c-SNX5 cells showed upregulation of Caspase-2 (Fig. 5b). Immunohistochemical analysis on PTC tissue sections further demonstrated the presence of Caspase-2 (Fig. 5d), suggesting that SNX5 would induce Caspase-2 in PTC cells. Conversely, when we investigated 8505c transformed cells, overexpression of SNX5 conferred growth advantage (Fig. 5c). Therefore it was possible to consider that the action of Caspase-2 relating to apoptosis might probably be abrogated in the tumor cells.

Discussion

Here we report a unique role of SNX5 frequently presented in PTC. TTF-1, like Galectin-3 and other markers, is often used for the pathological diagnosis of PTC, although their expression is also noted in most other malignancies with papillary structures such as breast, intestine, and lung carcinomas.[42–44] Thyroglobulin is tissue-specific to the thyroid gland, but it should not be limited to PTC. As noted in this study, adenocarcinoma originated from tissues other than thyroid scarcely presented SNX5, which could be used to define primary and secondary PTC. Currently we do not know the mechanism of the upregulation of SNX5 in PTC, while our results indicated that SNX-BAR molecules, including SNX5, might have a cardinal role in the maintenance of thyrocyte function. This was expected from experimental evidence that SNX-BAR molecules such as SNX1, SNX2, SNX4, SNX5 and SNX6 operate to transfer small cargos between endosomes and the trans-Golgi network.[24–29] Regarding tumor biology, SNX2 is highly presented in tumor cells as a chimeric molecule with ABL1; however, the SNX5 gene is localized on chromosome 20p11, whose locus is believed unlikely to be altered in the majority of PTC.[45,46]

A further surprising finding of this study was that SNX5 could control Caspase-2. Caspases, a family of cysteine-dependent

Table 2. Expression of SNX5 in nonthyroid tumors

Organ	Tissue type	SNX5			Total
		++	+	–	
Lung	Adenocarcinoma	0 (0%)	1 (2.6%)	37 (97.4%)	38
Breast	Papillotubular carcinoma	1 (2.6%)	1 (2.6%)	37 (94.9%)	39
Colon	Tubular adenocarcinoma	0 (0%)	1 (4.2%)	23 (95.8%)	24
Liver	Hepatocellular carcinoma	0 (0%)	0 (0%)	11 (100%)	11
Kidney	Clear cell carcinoma	0 (0%)	0 (0%)	10 (100%)	10
	Urothelial carcinoma	0 (0%)	0 (0%)	11 (100%)	11
Prostate	Adenocarcinoma	0 (0%)	1 (9.1%)	10 (90.9%)	11
Ovary	Serous cystadenocarcinoma	0 (0%)	2 (25.0%)	6 (75.0%)	8
	Mucinous cystadenocarcinoma	0 (0%)	0 (0%)	6 (100%)	6
Uterus	Endometrioid adenocarcinoma	0 (0%)	1 (6.7%)	14 (93.3%)	15
					173

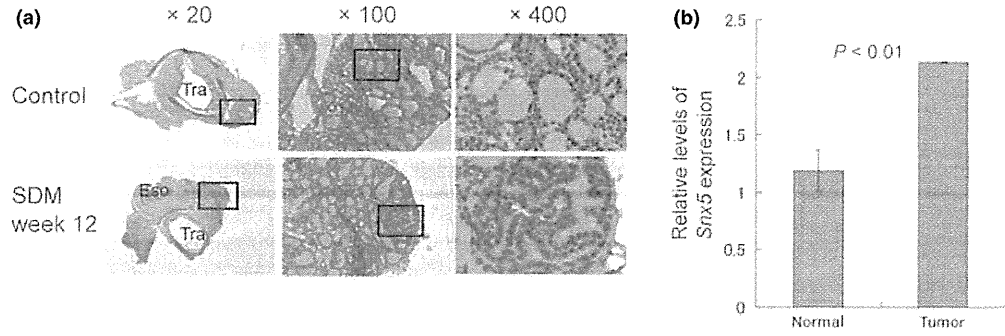

Fig. 4. Expression of sorting nexin 5 (Snx5) in murine papillary thyroid carcinoma (PTC) induced by specific carcinogens. (a) Frozen tissue sections of murine thyroid tumors stained with hematoxylin and eosin (HE). One week after injection of *N*-bis(2-hydroxypropyl)-nitrosamine (DHPN), sulfadimethoxine (SDM) was added to drinking water. Twelve weeks later, papillary structure resembling human PTC emerged in the tissues, compared with control mice with no chemicals. Boxed regions are magnified in high power views. Representative figures from each group of six mice are depicted. Eso, esophagus; Tra, trachea. Original magnifications: ×20, ×100, ×400. (b) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of Snx5 of tumors 12 weeks after administration of SDM. Data represent relative levels of Snx5 transcripts compared with the levels of 18s ribosomal RNA as an internal control. Values in each group of six mice are depicted as the mean ± standard deviation (SD).

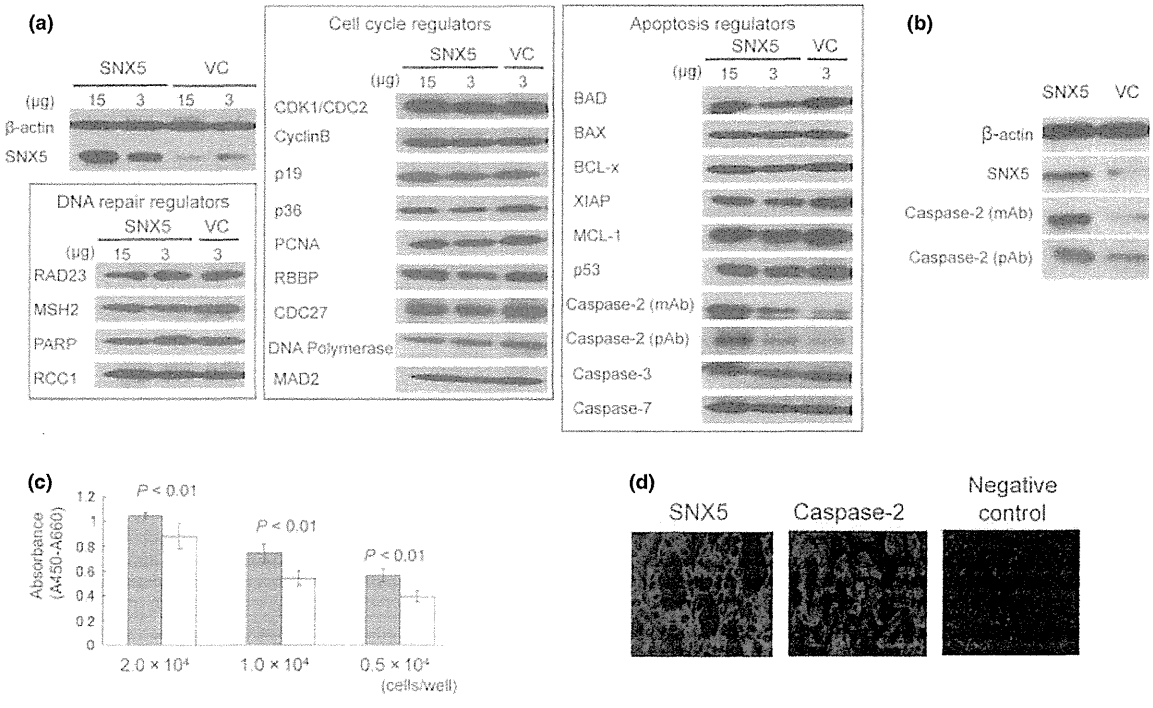

Fig. 5. Induction of Caspase-2 by sorting nexin 5 (SNX5) in papillary thyroid carcinoma (PTC). (a) Immunoblot analysis to explore molecules regulated by SNX5. HEK293 cells transiently introduced with pCMV-HA-SNX5 or mock vector were established as HEK293-SNX5 cells or HEK293-control cells, respectively, and analyzed. Left upper panel shows that the amounts of SNX5 at the protein level increase with the amounts of the pCMV-HA-SNX5 vector (depicted as SNX5) in contrast to the control (depicted as VC, vector control), where anti-SNX5 mAb (48C2) and anti-β-actin mAb were used to detect signals. In the right panel, examination of apoptosis regulators shows that Caspase-2 is increased in response to exogenous SNX5 in a dose-dependent manner, as observed by two different anti-Caspase-2 Abs of a mouse mAb (clone 35) and a rabbit pAb (poly6340). Molecules regulating DNA repair and cell cycle were also investigated as shown in the left lower and middle panels, respectively, where there are no significant differences at the protein level in the regulators. (b) Induction of Caspase-2 by SNX5 in 8505c PTC cells. The stable transformants of 8505c cells with pLVSIN-CMV-puro-SNX5 and mock vector were established after selection with puromycin as 8505c-SNX5 cells and 8505c-control cells, respectively. Immunoblot analysis of these cells with the same reagents and procedures of (a) demonstrates high expression of Caspase-2 in 8505c-SNX5 cells compared with 8505c-control cells. (c) Cell growth potentials of 8505c-SNX5 cells and 8505c-control cells established in (b). Three days after seeding different cell numbers per well using a 96-well flat-bottom plate, data were analyzed by WST-1 assay in triplicate. Values of arbitrary units of absorbance of 8505c-SNX5 cells and 8505c-control cells are shown in gray and open boxes, respectively. Data represent three independent experiments using three different transformants of 8505c-SNX5 cells and 8505c-control cells. (d) PTC simultaneously presents SNX5 and Caspase-2. Immunohistochemical studies were performed on frozen sections of PTC using mouse anti-SNX5 mAb (48C2) and anti-Caspase-2 mAb (clone 35). Images were obtained by immunofluorescence microscopy after staining of tissue sections with an Alexa 488-conjugated goat anti-mouse pAb (green). Mouse immunoglobulin G (IgG) was used as a negative control. Original magnification: ×200.

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aspartate-direct proteases, play critical roles in the initiation and execution of cell death.[47,48] Our results demonstrated that PTC and parts of well-differentiated tumors certainly expressed SNX5. In contrast, poorly differentiated thyroid carcinomas including undifferentiated carcinoma did not. These findings probably indicate some correlation of the expression profile of SNX5 and the malignant potential of thyroid tumors. It is reported that functional loss of SNX1 may affect alteration of a cell regulatory mechanism, eventually leading to malignant progression, implying its tumor suppressor activity in certain tumors.[31,35] Therefore, as an SNX-BAR, SNX5 might also have a tumor-suppressive function similar to SNX1. In particular, Caspase-2 controlled by SNX5 would have clinical relevance, comprising the slow growth potential of PTC.

While the manner of the action of SNX5 in the accumulation of Caspase-2 remains unknown, SNX-BAR molecules have been suggested to play multiple roles to preserve cellular integrity.[32–34] Considering this, together with our experimental results, excess amounts of SNX-BAR molecules can provoke unique functions in cells, where saturation for binding to the corresponding curvatures of endosomes eventually results in the emergence of a "free form" of SNX-BAR molecules from the endosome binding. In this regard SNX-BARs can act not only as transporters of vesicles, but also as possible sensors of the number or quality of the vesicles in cells. Thyrocytes are probably regulated by SNX-BARs, which might represent the number of intracellular loading units of endosomes with a traditional sorting function and eventually monitor cellular activities. It is well recognized that abnormalities of intra-cytoplasmic transfer of vacuoles such as endosomes occur in many tumor cells. SNX6, which has the ultimate function of metabolism of a p27kip1 tumor suppressor as indicated by cell-transformation experiments, is presented in PTC like SNX5.[49,50] So far further investigations will provide clues to fully illustrate the functional significance of the SNX5-Caspase-2 pathway in the tumorigenesis of PTC.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Establishment of an SNX5-specific mAb.

Table S1. RT-PCR primer sets used in this study.

Table S2. List of tumor tissues with negative expression of SNX5, that are not presented in Table 2.

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乳癌（第2版）

—基礎と臨床の最新研究動向—

III. 乳癌の分子生物学と発癌機序

分子生物学

乳がん幹細胞

安田和世　　廣橋良彦　　鳥越俊彦

III 乳癌の分子生物学と発癌機序

分子生物学

乳がん幹細胞

Breast cancer stem cells

安田和世　廣橋良彦　鳥越俊彦

Key words：乳がん，がん幹細胞，上皮間葉移行，EMT，シグナル伝達

1 がん幹細胞とは

がんは組織学的に多様な細胞から構成されているが，遺伝子に変異が生じた1個の体細胞に由来するがん細胞自体も組織の中では多様性をもっており，これが治療抵抗性の主な原因となっている．このような多様性を説明する概念として，正常組織に似た階層構造(ヒエラルキー)ががん細胞の社会にも存在し，その頂点に立つ根源細胞ががん幹細胞であると古くから提唱されていた．すなわち，がん幹細胞とは‘腫瘍内に存在し，自己再生能と様々な分化傾向を示すがん細胞を生み出す多分化能をもつ細胞’と考えられており[1]，ハチの社会に例えるならば‘女王バチ’に相当する．

がん幹細胞は正常幹細胞と同様の形質，すなわち非対称分裂，長寿命，高いストレス耐性，高い運動能を備えていると考えられているが，正常幹細胞との違いはその高い造腫瘍能にある．造腫瘍能は，NOD/SCIDマウスに代表されるような免疫不全動物への移植によって腫瘍を形成する能力で評価される．このような造腫瘍性の観点からはcancer initiating cellと呼ばれる．がん幹細胞が臨床的にも注目を浴びるのは，これらのがん幹細胞形質が抗がん剤や放射線治療への抵抗性と再発・転移の原因になっていると推測されるためである(**図1**)．そのため，現在世界中でがん幹細胞を標的とした治療薬の開発が行われている．

がん幹細胞はどのような細胞生物学的特徴をもっているのであろうか．がん幹細胞を選り分ける技術に限界があったためその実態は長い間不明であったが，セルソーターの発達により大きく進展した．なかでも，がん幹細胞研究のブレイクスルーとなったのは1997年のDickらによる急性骨髄性白血病(AML)幹細胞の発見である[2]．彼らは正常造血幹細胞が骨髄のCD34(+)CD38(−)細胞群に含まれていることを発見していたが，AML患者にはそれと同じ細胞表面マーカーをもつ少数の白血病細胞が存在し，それらが幹細胞様形質と高い造腫瘍能をもつleukemia initiating cellであることを報告した．その後，固形がんでも同様のcancer initiating cellの存在が報告され，2003年には乳がんでも報告された[3]．現在までに，脳腫瘍，肺癌，前立腺癌，骨肉腫などの様々な種類の固形がんにおいて，がん幹細胞の存在が報告されている[4]．

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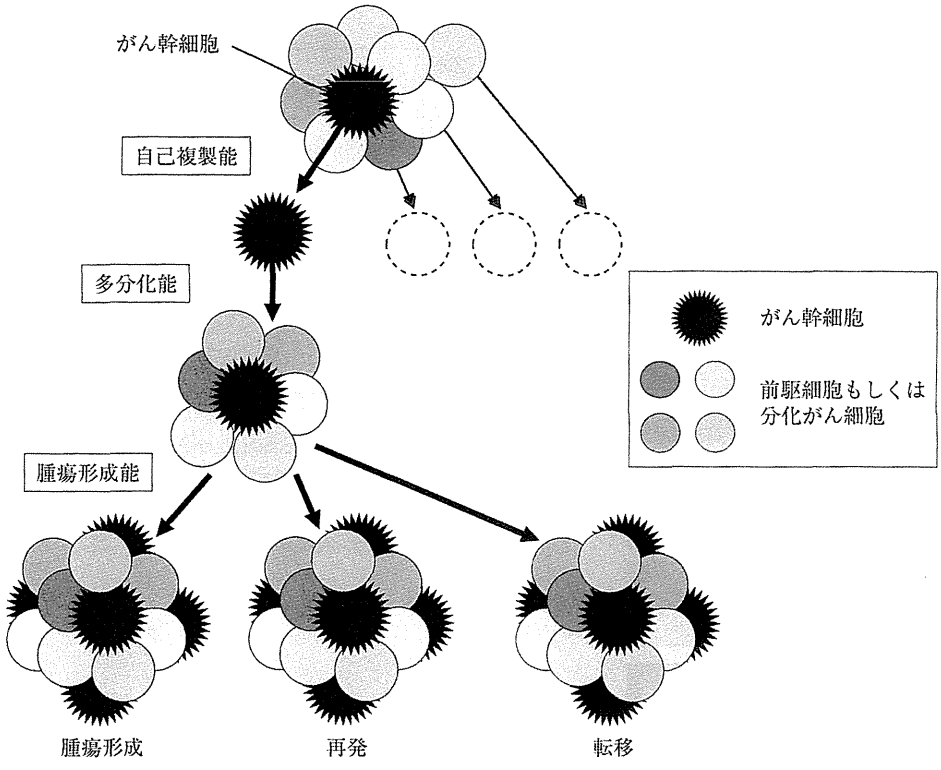

図1 がん幹細胞の概念

がん幹細胞は，自己複製能と多分化能をもち，がん細胞の多様性の根源をなすと考えられている．また，がん幹細胞は分化したがん細胞と比べて著しく高い造腫瘍能をもつ．がん幹細胞のストレス耐性能は治療抵抗性と関連し，高い遊走能は浸潤や転移の原因となる．

2 乳がん幹細胞のマーカーとがん幹細胞の同定

1）細胞表面マーカー

正常造血幹細胞の細胞表面マーカーによって白血病幹細胞が発見されたように，乳がん幹細胞のマーカーも正常乳腺幹細胞や他の正常組織幹細胞の研究をもとに探索された．Clarkeらは，乳がん患者の腫瘍組織内に11–35％存在するCD44(＋)CD24(－/low)細胞分画を免疫不全マウス(NOD/SCID)の乳腺脂肪組織に移植すると，1,000個の細胞で腫瘍を形成することを発見した[3]．更にその中でもEpCAM(＋)の細胞分画を選り分けると，わずか200個の移植で腫瘍を形成することを報告した．その後，CD44(＋)CD24(－)の乳がん細胞は高い浸潤能をもち，がんの転移とも関係することが確認されている[5]．こうして，EpCAM(＋)/CD44(＋)/CD24(－/low)の分画に乳がん幹細胞が濃縮されることが判明した．

2）ABC transporter

正常造血幹細胞の特徴として，ATP-binding cassette transporter(ABC transporter)の発現と活性が高いことが知られていた．ABC transporterはATP加水分解エネルギーを使用して細胞膜内外の物質輸送機能をもつ一群のタンパク質である．その一つであるABCG2(BCRP)は乳がん細胞の薬剤耐性にかかわる多剤耐性遺伝子産物としても知られている．ABCG2が蛍光色素Hoechst33342を細胞外に排出することを利用して，ABCG2活性の高い幹細胞様細胞を選別することができる．すなわち，細胞をHoechst33342色素で染色し，色素排出能の高い低染色性細胞を選別する方法である．この低