Declaration of Financial Disclosure

Hideo Takasu is an employee of Dainippon Sumitomo Pharma Co., Ltd.

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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

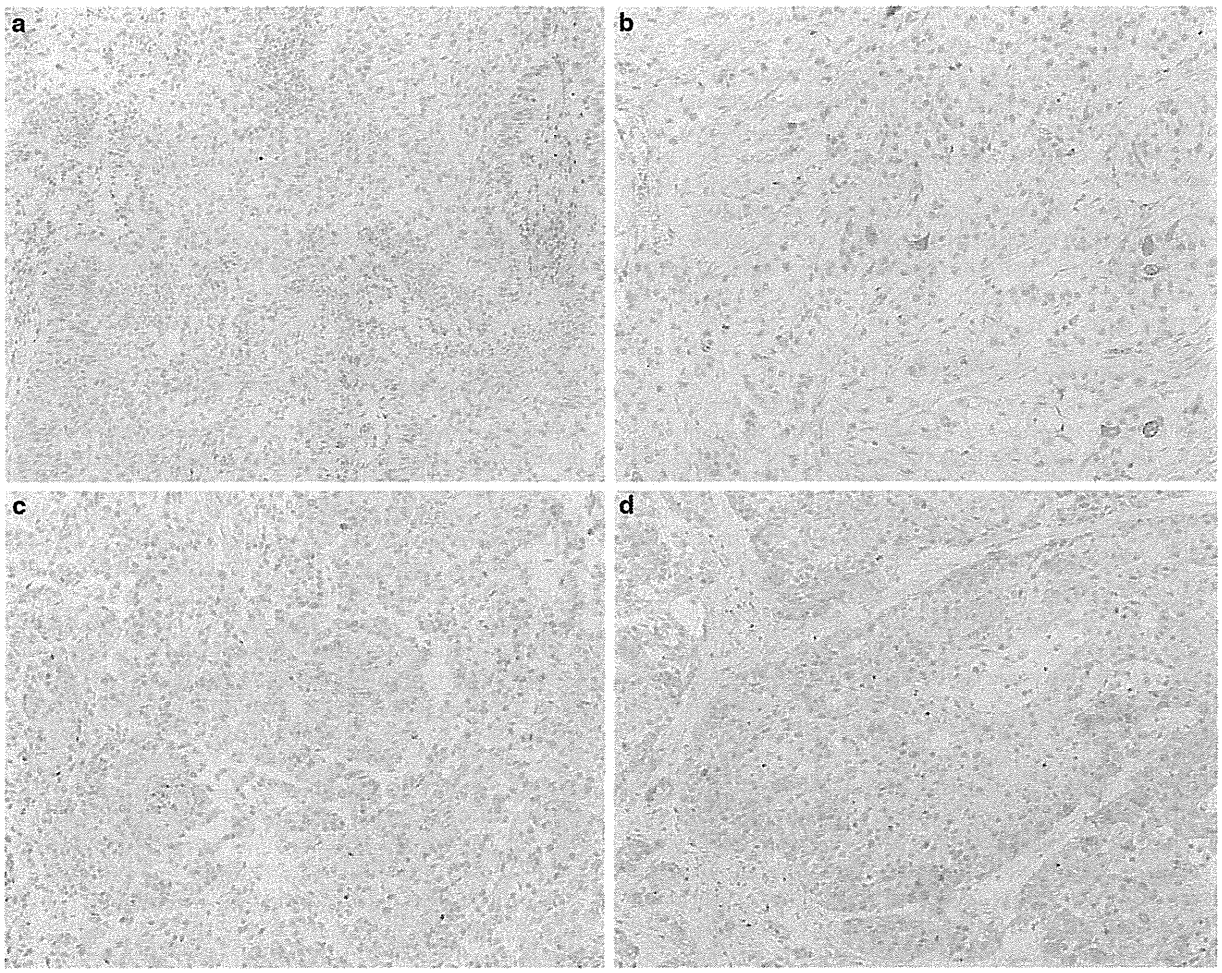

Figure 1 Representative immunohistochemical staining of aldehyde dehydrogenase 1 (ALDH1) and sex determining region-Y-related high mobility group box 2 (SOX2). (**a**) Negative ALDH1 expression in tumor cells. (**b**) positive ALDH1 expression in tumor cells, (**c**) negative SOX2 expression in tumor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

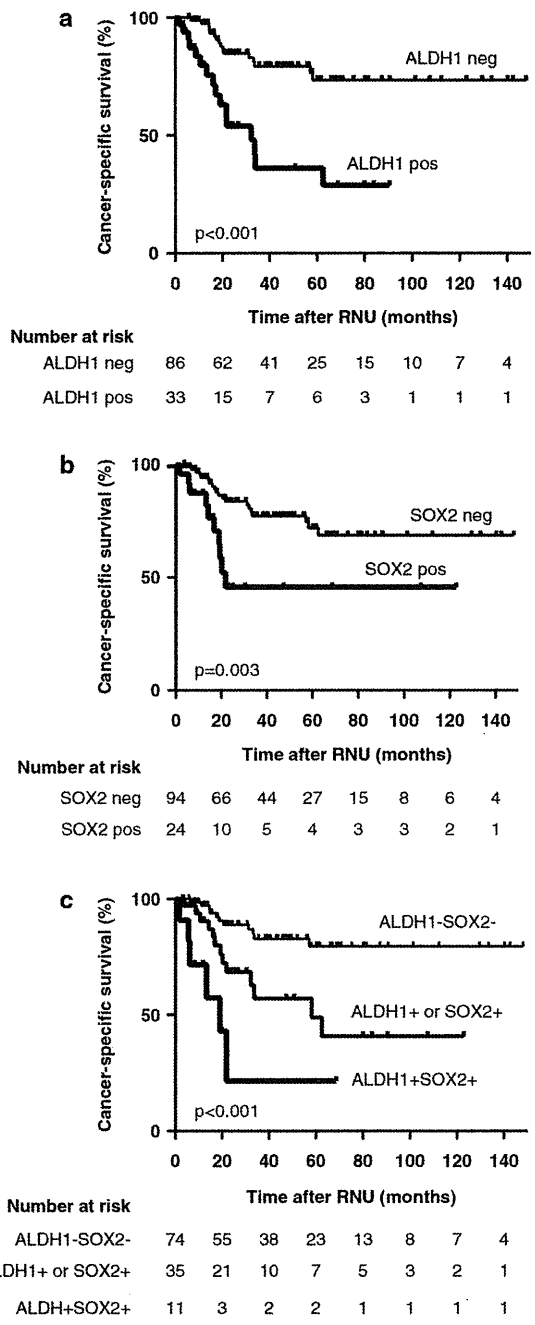

Figure 2 Kaplan–Meier curves for cancer-specific survival rates according to (**a**) aldehyde dehydrogenase 1 (ALDH1) expression status, (**b**) sex determining region-Y-related high mobility group box 2 (SOX2) expression status and (**c**) combined expression status of ALDH1 and SOX2.

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

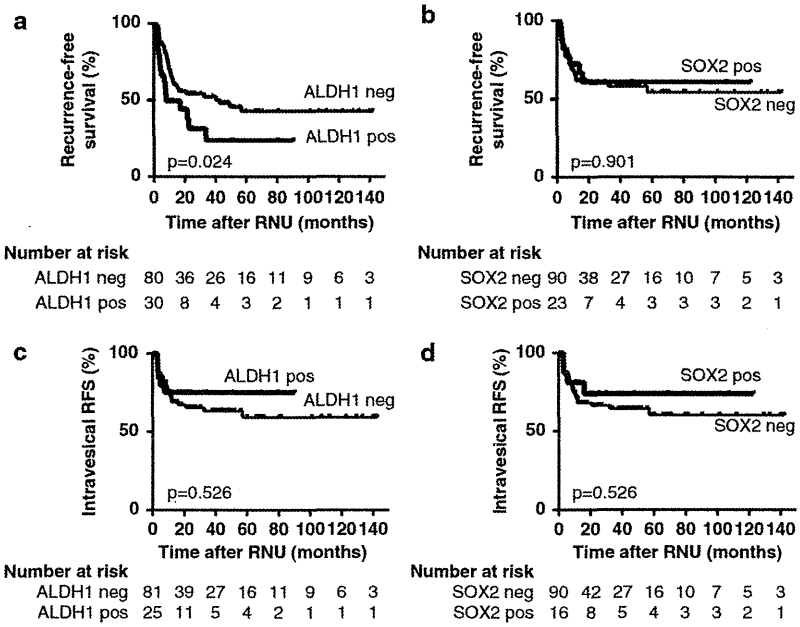

Figure 3 Kaplan–Meier curves for recurrence-free survival rates according to (**a**) aldehyde dehydrogenase 1 (ALDH1) expression status and (**b**) sex determining region-Y-related high mobility group box 2 (SOX2) expression status, and for intravesical recurrence-free survival rates according to (**c**)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

Nuclear, but not cytoplasmic, localization of survivin as a negative prognostic factor for survival in upper urinary tract urothelial carcinoma

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Abstract Survivin, a member of the inhibitor of apoptosis protein gene family, inhibits apoptosis and promotes mitosis. We determined whether nuclear or cytoplasmic localization of survivin could predict survival of patients with upper urinary tract urothelial carcinoma (UUTUC). Immunohistochemical staining for survivin was carried out on archival specimens from 125 consecutive patients with UUTUC who underwent radical nephroureterectomy. Nuclear and cytoplasmic staining of survivin was scored and compared with clinicopathologic features and cancer-specific survival (CSS). Nuclear expression of survivin was significantly correlated with tumor grade ($p<0.001$), lymphovascular invasion ($p=0.022$) and poor survival with an estimated 5-year CSS probability of 54 % for tumors with nuclear expression of survivin vs. 73 % for those without nuclear expression of survivin (hazard ratio=2.19; 95 % confidence interval=1.02–4.70; $p=0.043$). The 5-year cancer-specific survival rates of patients with cytoplasmic survivin-negative and -positive tumors were 66 and 67 %, respectively. There was no difference in survival between patients with cytoplasmic survivin-negative tumors and those with cytoplasmic survivin-positive tumors. Using univariate analysis, nuclear survivin expression, tumor grade, pathological T stage, pathological N stage, and lymphovascular invasion were the predictive variables for CSS. In contrast, cytoplasmic survivin expression had no prognostic relevance. These data suggest that nuclear accumulation of survivin represents biologic aggressiveness and that nuclear survivin is a negative prognostic marker in patients with resected UUTUC.

Keywords Survivin · Urothelial carcinoma · Upper urinary tract · Survival

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Introduction

Upper urinary tract urothelial carcinomas (UUTUC) are uncommon and account for only 5–10 % of urothelial carcinomas [1]. The primarily recognized prognostic factors are tumor stage and grade, whereas gender, age and the initial location of the tumor within the upper urinary tract are no longer accepted as prognostic factors [1]. Lymphovascular invasion [2–4], tumor necrosis [5, 6], tumor architecture [7], and concomitant carcinoma in situ [8, 9] are associated with higher risks of recurrent disease and cancer-specific mortality. Molecular markers such as microsatellite instabilities [10], E-cadherin, hypoxia-inducible factor-1α and a telomerase RNA component [11] have been shown to be useful for prognosis, although none of the markers has been externally validated.

Survivin, an inhibitor of apoptosis protein (IAP) family member that inhibits caspases and blocks cell death, is overexpressed in various human malignancies [12]. Survivin also plays a central role in cell division, and it is expressed in the nuclear or cytosolic pool in cancer cells [13]. Many studies have reported a correlation between survivin expression and an either unfavorable or favorable prognosis [14]. Recently,

nuclear expression of survivin has been reported to be associated with unfavorable outcomes in head and neck squamous cell carcinoma (SCC) [15–18], hepatocellular carcinoma [19, 20], esophageal SCC [21, 22], melanoma [23], glioblastoma [24], Merkel cell carcinoma [25], and bladder cancer [26–30]. Conversely, survivin nuclear positivity correlated with favorable prognoses in gastric [31], colorectal [32], and ovarian cancers [33, 34]. In non-small cell lung cancer, nuclear accumulation of survivin was a positive prognostic factor for survival in advanced disease [35], but a negative prognostic factor in patients with resected Stage I and II diseases [36].

To date, two studies have examined whether survivin expression has an impact on prognosis in patients with UUTUC. One study indicated no relationship between survivin expression and survival [37], whereas the other one showed that survivin expression was a poor prognostic factor [38]. In those studies, however, cells were considered survivin-positive when a distinct granular pattern was apparent within the cytoplasm of tumor cells. Therefore, we investigated the nuclear expression of survivin in patients with UUTUC and determined its prognostic relevance.

Materials and methods

Patients

We reviewed the clinical pathology archives of 181 consecutive patients who underwent radical nephroureterectomy and were diagnosed as having UUTUCs 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. 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 to 88). Median follow-up was 69 months (range 6 to 192). The clinical stage was assigned using the American Joint Committee on Cancer TNM Staging System for Renal Pelvis and Ureter Cancer (7th ed., 2010). Tumor grading was assessed according to the 1973 World Health Organization classification. The patients' characteristics are shown in Table 1.

Table 1 Characteristics of the 125 patients

Characteristic	*N*	(%)
Median age (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)
Tumor architecture		
Papillary	57	(46)
Sessile	66	(53)
Flat	2	(1)
Tumor grade (1973 WHO)		
G1	3	(2)
G2	53	(43)
G3	69	(55)
Pathological stage		
Stage 0a	16	(13)
Stage 0is	2	(1)
Stage I	17	(14)
Stage II	21	(17)
Stage III	50	(40)
Stage IV	19	(15)
Chemotherapy		
Neoadjuvant	10	(8)
Adjuvant	6	(5)

Immunohistochemistry and scoring

Sections (4 μm) of the formalin-fixed, paraffin-embedded tumor specimens were immunostained after heat-induced epitope retrieval in citrate buffer (pH6.0) using an autoclave with a polyclonal antibody against survivin (1:200, Novus Biologicals, Littleton, CO, USA). Subsequent incubations with a secondary biotinylated antibody, avidin-conjugated peroxidase complex, and chromogen were done on Ventana NexES (Ventana Medical Systems, Tucson, AZ, USA). The slides were then counterstained with hematoxylin, rinsed, dehydrated through graded alcohols into nonaqueous solution, and coverslipped with mounting media. Sections of colorectal adenocarcinoma were used as positive controls for survivin. Negative controls had the primary antibody replaced by buffer. All specimens were reviewed and scored independently using light microscopy in at least 5 areas at ×400 magnification by investigators who were blinded to clinicopathological data (TT and YH). Cases were scored positive when >10 % of the cells reacted

with the anti-survivin antibody, as proposed previously [26, 30].

Statistical analysis

We tested the relationships between nuclear or cytoplasmic survivin expression and the other clinicopathological parameters, i.e., the pathological T stage, pathological N stage, tumor grade and lymphovascular invasion by chi-square tests. Cancer-specific survival was assessed by the Kaplan–Meier method, and differences between two groups were compared using the log-rank test. 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 survivin expression as a potential independent prognostic factor. A value of $p<0.05$ was considered to indicate statistical significance. The calculations were performed using JMP™ software.

Results

Survivin expression in UUTUC and its associations with clinicopathological variables

Nuclear and cytoplasmic expression of survivin (Fig. 1) was found in 48 (38 %) and 30 (24 %) of the 125 cases, respectively. Coexistence of nuclear and cytoplasmic staining was observed in 15 cases (12 %). No normal urothelial cells were stained with the anti-survivin antibody either in the nucleus or in cytoplasm. Expression of nuclear and cytoplasmic survivin was positive in 20 (35 %) and 9 (11 %) of the 57 papillary tumors, respectively. In the 77 sessile tumors, expression of nuclear and cytoplasmic survivin was positive in 27 (41 %) and 19 (29 %), respectively. There was no relationship between tumor architecture and nuclear or cytoplasmic survivin expression. Nuclear survivin expression was significantly associated with higher tumor grade ($p<0.001$) and lymphovascular invasion ($p=0.022$), whereas no clinicopathological variables were linked to cytoplasmic survivin expression (Table 2).

Association of nuclear or cytoplasmic survivin expression with survival and recurrence

The 5-year cancer-specific survival rates of patients with nuclear survivin-positive and -negative tumors were 54 and 73 %, respectively (Fig. 2a). The 5-year cancer-specific survival rates of patients with cytoplasmic survivin-positive and -negative tumors were 67 and 66 %, respectively (Fig. 2b). There were significant differences in cancer-specific survival between patients with nuclear survivin-negative tumors and those with nuclear survivin-positive tumors (hazard ratio=2.19; 95 % confidence interval= 1.02–4.70; $p=0.043$) (Fig. 2a), but no significant differences between those with cytoplasmic survivin-negative tumors

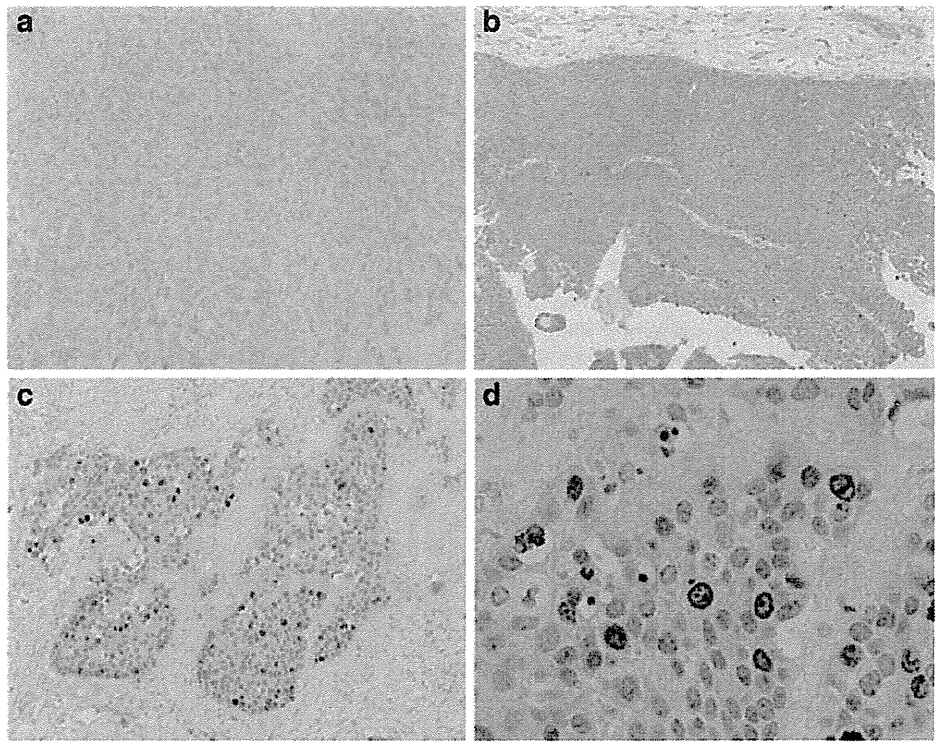

Fig. 1 Immunohistochemical staining for survivin in upper urinary tract urothelial carcinoma: **a** survivin-negative expression; **b** cytoplasmic survivin-positive expression; **c**–**d** nuclear survivin-positive expression

Table 2 Survivin staining patterns and pathological factors of patients with upper urinary tract urothelial carcinoma

Variable	Nuclear survivin expression			Cytoplasmic survivin expression		
	Positive (%)	Negative (%)	*p* value	Positive (%)	Negative (%)	*p* value
Pathological T stage						
pTis	1 (2)	1 (1)	0.058	2 (7)	0 (0)	0.222
pTa	5 (10)	11 (14)		4 (13)	12 (13)	
pT1	2 (4)	16 (21)		4 (13)	14 (15)	
pT2	9 (19)	14 (18)		4 (13)	19 (20)	
pT3	27 (56)	34 (44)		15 (50)	46 (48)	
pT4	4 (8)	1 (1)		1 (3)	4 (4)	
Pathological N stage						
pN0	42 (88)	70 (91)	0.107	26 (86)	87 (92)	0.656
pN1	1 (2)	5 (6)		2 (7)	3 (3)	
pN2	5 (10)	2 (3)		2 (7)	5 (5)	
Grade						
G1	0 (0)	3 (4)	<0.001	1 (3)	2 (2)	0.736
G2	11 (23)	42 (54)		11 (37)	42 (44)	
G3	37 (77)	32 (42)		18 (60)	51 (54)	
Lymphovascular invasion						
Negative	25 (52)	56 (73)	0.022	19 (63)	62 (65)	0.831
Positive	23 (48)	21 (27)		11 (37)	33 (35)	

and those with cytoplasmic survivin-positive tumors (hazard ratio=1.11; 95 % confidence interval=0.44–2.78; p=0.832) (Fig. 2b). A significant difference in CSS was observed between patients with nuclear survivin-positive tumors and those with survivin-negative tumors (hazard ratio=3.71; 95 % confidence interval=1.16–11.8; p=0.027) (Fig. 3), when the cutoff was set at 20 %.

In univariate analysis, the pathological T stage, pathological N stage, tumor grade, lymphovascular invasion, and nuclear survivin expression were associated with a poor prognosis (Table 3). In multivariate analysis, the independent factors of prognosis were the pathological T stage, pathological N stage and tumor grade (Table 3).

Discussion

Two studies have investigated the relationships between survivin expression and survival in patients with UUTUC. Nakanishi et al. [37] examined survivin expression in UUTUC using immunohistochemistry and its relationship with the prognosis, clinicopathologic parameters, bcl-2, p53 and proliferating cell nuclear antigen (PCNA) immunoreactivity. They found no correlation between survivin expression and prognosis, clinicopathologic findings, bcl-2, p53 or PCNA, and concluded the survivin cytoplasmic expression

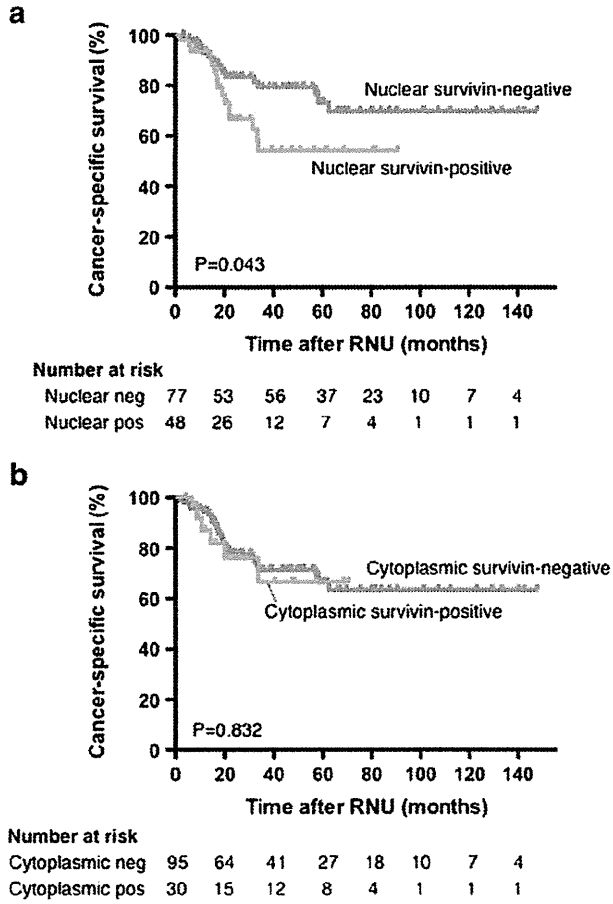

Fig. 2 Kaplan–Meier curves for cancer-specific survival rates according to **a** nuclear survivin expression status and **b** cytoplasmic survivin expression status. *RNU* radical nephroureterectomy

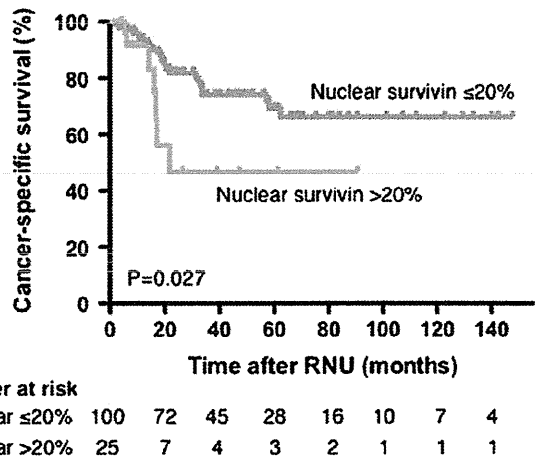

Fig. 3 Kaplan–Meier curves for recurrence-free survival rates according to nuclear survivin expression status (>20 % vs. ≤20 %). *RNU* radical nephroureterectomy

Table 3 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	1.92 (1.09–3.59)	0.023
Pathological N stage	2.75 (1.75–4.09)	<0.001	2.00 (1.24–3.12)	0.006
Grade	6.02 (2.53–17.7)	<0.001	3.72 (1.42–11.7)	0.006
Lymphovascular invasion	2.18 (1.52–3.25)	<0.001	1.30 (0.85–2.04)	0.236
Nuclear survivin	1.61 (1.01–2.44)	0.049	1.32 (0.81–2.02)	0.261
Cytoplasmic survivin	1.05 (0.64–1.59)	0.834	0.90 (0.54–1.39)	0.652

did not predict prognosis in UUTUC [37]. Jeong et al. [38] investigated the expression of apoptosis-related markers, including survivin, by using immunohistochemistry and the association with the clinical outcomes of patients with UUTUC. They demonstrated that survivin expression, the apoptosis index, pathological T stage, and pathological N stage were significantly associated with disease-specific survival in multivariate analysis [38]. Thus, the prognostic value of cytoplasmic expression of survivin in tumor cells was different in these studies and is still controversial, although we found that cytoplasmic survivin expression had no impact on survival in patients with UUTUC. One of the reasons is the difficulty and ambiguity of immunohistochemical scoring, especially when cells are weakly stained in the cytoplasm.

Recent reports [26–30] concerning survivin expression in urothelial carcinoma of the bladder indicated that nuclear expression of survivin correlated with clinical outcome and prognostic factors. Two of those studies compared the predictive value of nuclear versus cytoplasmic expression of survivin in bladder cancer cells. Yin et al. [29] evaluated the expression profile of the major apoptosis regulators, including caspases, IAPs (survivin, livin, XIAP, etc.), APAF1, SMAC, and BCL2 in non-muscle-invasive bladder cancer by immunohistochemistry. They demonstrated that survivin nuclear, but not cytoplasmic, expression was the only apoptotic marker that correlated significantly with tumor grade, stage, and patient outcome [29]. Another study, by Skagias et al. [30], analyzed tissues from 80 bladder cancers, including both non-muscle- and muscle-invasive diseases, by immunohistochemistry. They found correlations between nuclear survivin expression and increased grade, stage and the probability of tumor recurrence, but no relationship between cytoplasmic survivin expression and any clinicopathological parameter [30]. Several studies [13, 39] have reported that the nuclear pool of survivin is involved in promoting cell proliferation, whereas the cytoplasmic pool of survivin may participate in controlling cell survival but not cell proliferation. Furthermore, it is known that several splice variants of survivin have differential intracellular localization, e.g., survivin-ΔEx3 in the nucleus and survivin-2B in the cytoplasm [40, 41]. Nouraee et al. [42] examined the expression pattern of survivin and its major splice variants (survivin-ΔEx3 and survivin-2B) and their prognostic values by reverse transcriptase polymerase chain reaction. They demonstrated that the expression of survivin and survivin-ΔEx3 was preferentially elevated in tumors with high grades, whereas survivin-2B expression was lower in high-grade tumors [42]. Nuclear expression of survivin, including survivin itself and survivin-ΔEx3, is positively correlated with tumor cell proliferation [41], which may explain the reason why nuclear survivin correlates with survival and prognostic factors in various cancers.

To the best of our knowledge, this is the first study in which the relationships between nuclear expression of survivin and most clinically relevant features of UUTUC were evaluated. In the present study, we demonstrated that the UUTUC patients with nuclear survivin-positive tumor cells had significantly shorter OS, than those whose tumors were nuclear survivin-negative. In contrast, cytoplasmic survivin expression had neither prognostic relevance nor any association with other clinicopathological variables. Although nuclear survivin expression was not an independent prognostic factor in multivariate analysis, it was linked to tumor grade and lymphovascular invasion. Several investigators have found the prognostic impact of tumor grade on survival [43–45], and two large, independent, and multicenter studies [2, 4] showed that lymphovascular invasion was an independent prognostic factor in UUTUC and was associated with established features of biologically aggressive disease [46]. Therefore, nuclear accumulation of survivin represents biologic aggressiveness, which may have a prognostic impact on survival in patients with UUTUC. However, we did not find an independently increased risk of cancer-specific mortality with nuclear survivin expression.

This study has several potential limitations. First are the limitations inherent in any retrospective data collection. Second, radical nephroureterectomy was performed by various surgeons over a long time period. Third, immunohistochemistry has inherent limitations such as reproducibility and reliability. The data should be validated by molecular biology methods.

In summary, the current results demonstrate a negative association between the nuclear expression of survivin and patient survival in UUTUC. We conclude that nuclear survivin expression may be a superior biologic and prognostic marker for UUTUC.

Conflict of interest The authors declare no conflict of interest.

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Immunotherapeutic benefit of α-interferon (IFNα) in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients

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Survivin, a member of the inhibitor of apoptosis protein (IAP) family containing a single baculovirus IAP repeat domain, is highly expressed in cancerous tissues but not in normal counterparts. Our group identified an HLA-A24-restricted antigenic peptide, survivin-2B80–88 (AYACNTSTL), that is recognized by CD8 + CTLs and functions as an immunogenic molecule in patients with cancers of various histological origins such as colon, breast, lung, oral, and urogenital malignancies. Subsequent clinical trials with this epitope peptide alone resulted in clinical and immunological responses. However, these were not strong enough for routine clinical use as a therapeutic cancer vaccine, and our previous study of colon cancer patients indicated that treatment with a vaccination protocol of survivin-2B80–88 plus incomplete Freund's adjuvant (IFA) and α-interferon (IFNα) conferred overt clinical improvement and enhanced the immunological responses of patients. In the current study, we further investigated whether this vaccination protocol could efficiently provide not only improved immune responses but also better clinical outcomes for advanced pancreatic cancers. Tetramer and enzyme-linked immunosorbent spot analysis data indicated that more than 50% of the patients had positive clinical and immunological responses. In contrast, assessment of treatment with IFNα only to another group of cancer patients resulted in no obvious increase in the frequency of survivin-2B80-88 peptide-specific CTLs. Taken together, our data clearly indicate that a vaccination protocol of survivin-2B80-88 plus IFA and IFNα is very effective and useful in immunotherapy for this type of poor-prognosis neoplasm. This trial was registered with the UMIN Clinical Trials Registry, no. UMIN000000905. (*Cancer Sci* 2013; 104: 124–129)

Recent progress in human tumor immunology research has presented us with the possibility that immunotherapy could be established as an effective cancer therapy in the very near future.[1–6] Indeed, since the first discovery of a human tumor antigen in 1992,[7] many clinical trials for cancer vaccines have been carried out, and these studies have suggested that active immunization using HLA class I restricted tumor antigenic peptides and the whole or part of the tumor antigenic protein could work as activators of antigen-specific CTLs, at least in some cancer patients.[8–16] However, even in effective cases, vaccination with these molecules alone is not sufficient to evoke a potent and stable immune response and subsequent strong clinical effect. Thus, it is crucial to develop various methods for enhancing the immunological efficacy of tumor antigens.

We have studied how tumor antigenicity can be efficiently enhanced in cancer patients since 2003. In our studies, the HLA-A24-restricted peptide survivin-2B80-88 was given s.c. to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, and urinary bladder cancers, and lymphomas. Clinically, certain patients with colon, lung, and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed by computed tomography (CT).[8–12] These effects, however, were not strong enough for the clinical requirements as decided by the criteria for cancer chemotherapy. When assessed with the Response Evaluation Criteria in Solid Tumors, which requires more than 30% regression of tumors on CT, only one patient each of 15 with colon cancers and three with urinary bladder cancers had a positive clinical response, indicating that the therapeutic potential was obviously not strong enough for routine clinical use as a cancer treatment.

In a previous study,[8] to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we carried out and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA and IFNα resulted in clear clinical improvement and enhanced the immunological responses of patients. We also analyzed CTLs of these patients by single-cell sorting, and found that each CTL clone from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Pancreatic cancer is still one of most difficult malignant neoplasms to treat, so in the current study we investigated whether the most effective protocol for colon cancer patients, namely survivin-2B80-88 plus IFA and IFNα, could work similarly in pancreatic cancers as in colon cancers. Furthermore, we carried out frequency monitoring of survivin-2B80-88 peptide-specific CTL in cases of cancer patients treated with IFNα alone, and found no overt increase of these CTLs. Once the survivin-2B80-88 peptide was administered with IFNα, patients showed strong clinical and immunological responses as assessed by tetramer and enzyme-linked immunosorbent spot (ELISPOT) analyses. Taken together, our current data strongly suggest that vaccination using survivin-2B80-88 plus IFA and IFNα is actually very effective in patients with advanced pancreatic cancers from both the clinical and immunological points of view.

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Materials and Methods

Patients. Patient selection was done as reported in our previously published work. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University (Sapporo, Japan).[8–12] All patients gave informed consent before being enrolled. Patients who participated in this study were required to: (i) have histologically confirmed pancreatic cancer; (ii) be HLA-A*2402 positive; (iii) have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) be between 20 and 85 years old; (v) have unresectable advanced cancer or recurrent cancer; and (vi) have Eastern Cooperative Oncology Group performance status between 0 and 2. Exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the past 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection, or hematopoietic failure; (v) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, in the Sapporo Medical University Primary Hospital from December 2005 through to November 2010.

Peptide, IFA, and IFNα preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8–10,12] The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by HPLC analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) and stored at −80°C until just before use. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical (Osaka, Japan).

Patient treatment. In this clinical study, we used the protocol illustrated in Fig. 1, with the survivin-2B80-88 peptide plus IFA and IFNα. In this trial, the primary endpoint was safety. The second endpoint was investigation of the antitumor effects and clinical and immunological monitoring.

In this protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, IFNα at a dose of 3 000 000 IU was given s.c. twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination (Fig. 1).

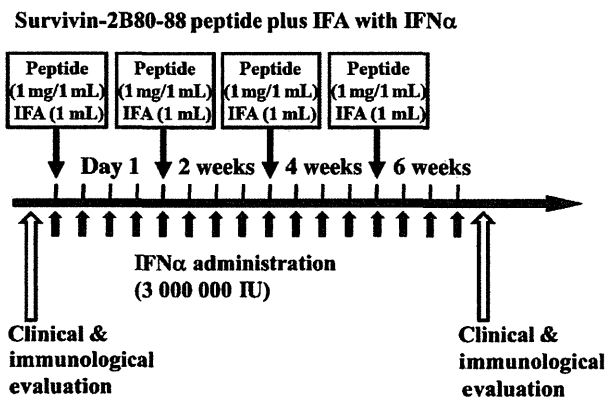

Fig. 1. Clinical protocol of study. Survivin-2B80-88 and incomplete Freund's adjuvant (IFA) were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, α-interferon (IFNα) was given twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination.

Toxicity evaluation. Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria.[8–10]

Clinical response evaluation. Physical examinations and hematological examinations were carried out before and after each vaccination.[8–10] A tumor marker (Ca19-9) was examined. Changes in the tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary pancreatic cancer tissues was done with anti-HLA class I heavy chain mAb EMR-8-5[13] (Funakoshi, Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response was defined as a $\geq$30% decrease from the baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for complete response, partial response, or PD.[8–10] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC, tetramer staining, and ELISPOT assay.** The samples for tetramer analysis and ELISPOT analysis were simultaneously obtained at the time of the hematological examination before and after each vaccination. These experiments were carried out as in our previous report. The PBMCs were isolated from blood samples by Ficoll–Conray density gradient centrifugation. Then they were frozen and stored at −80°C. As needed, frozen PBMCs were thawed and incubated in the presence of 30 μg/mL survivin-2B80-88 in AIM V (Life Technologies Corp, Grand Island, NY, USA) medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 2 days, 4 days, and 6 days after the addition of the peptide. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

The FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) and phycoerythrin (PE)-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from Medical and Biological Laboratories (MBL) Co., Ltd (Nagoya, Japan). For flow cytometric analysis, PBMCs, stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a PE-Cy5-conjugated anti-CD8 mAb (BD Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was carried out using FACSCalibur and CellQuest software (BD Biosciences). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.[8,10,12]

The ELISPOT plates were coated overnight in a sterile environment with an IFNγ capture antibody (BD Biosciences) at 4°C. The plates were then washed once and blocked with AIM V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBMCs (5×10^3 cells/well) that were stimulated *in vitro* as above were then added to each well along with HLA-A24-transfected T2 cells (T2-A24) (5×10^4 cells/well) that had been preincubated with or without survivin-2B80-88 (10 mg/mL) or

with an HIV peptide as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFNγ antibody and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Oberkochen, Germany). In this study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-postive spots as compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTLs. Survivin-2B80-88 peptide tetramer-positive CTLs were sorted and subsequently cloned to single cells using FACS (Aria II Special Order; BD Biosciences). The peptide-specific cytotoxicity of each of these CTLs was determined by pulsing T2A24 cells[8,17] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides, as previously described.

Results

Patient profiles, safety, and clinical responses. In the present protocol with the survivin-2B80-88 peptide plus IFA and IFNα, six patients were enrolled in the study (Table 1). None dropped out because of adverse events due to the vaccination. They consisted of three men and three women, whose age range was 50–80 years.

With respect to the safety, vaccination was well tolerated in all patients. Four patients had fever reaching nearly 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination except for induration at the injection site, which was conduced by IFA.

The clinical outcomes for the six patients treated with survivin-2B80-88 plus IFA and IFNα are summarized in Table 1. In some patients, particularly No. 1, the postvaccination Ca19-9 value was clearly decreased as compared with prevaccination, and was within the normal limit. Other patients (Nos. 2, 4, and 6) also had decreased or stable postvaccination levels of Ca19-9, although not as large. As for tumor size evaluated by CT, four patients (Nos. 1, 2, 4, and 6) were considered to have SD, but the other two patients (Nos. 3 and 5) had PD. Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced or stable Ca19-9 level.

Immune responses, single-cell cloning, and subsequent functional assessment of tetramer-positive CTLs. As in our previous study with colon cancer patients, we determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequencies before the first vaccination (prevaccination) and after the last vaccination (postvaccination) were assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with an HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10^4 CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

As summarized in Table 1, four of the six patients (Nos. 1, 2, 4, and 6) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and all four had SD by CT evaluation, suggesting that immune responses might appropriately reflect clinical responses with the current vaccination protocol.

As in our previous work, we also analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A*2402. As shown in Fig. 2, patient No. 1 (62 years old, female) had a reduced serum Ca19-9 level, and obvious immune responses as assessed by the survivin-2B80-88 ELISPOT and tetramer analyses (Fig. 3) after vaccination.

Subsequently, CD8 T cells of the tetramer-positive fraction were sorted by FACS, then cultured with 1, 3, and 10 cells/well for 7–10 days. Almost all growing T cells were survivin-2B peptide-specific T cells (data not shown), and we next assessed peptide-specific cytotoxicity by using these T cells. As Fig. 4 clearly shows, all T cells had very high peptide-specific cytotoxic potential. Taken together, these data clearly indicated that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα was capable of inducing a strong CTL response and for some pancreatic cancer patients might result in clinical effectiveness.

Assessment of treatment effect with IFNα alone. The above data strongly suggested that the current vaccination protocol

Table 1. Profiles of patients with advanced pancreatic cancer enrolled in the study and their clinical and immunological responses to vaccination with survivin-2B80-88 peptide, incomplete Freund's adjuvant and IFNα

Patient no.	Age/sex	Adverse effects	Tumor markers pre/post (CA19-9 U/mL)	CT eval.	Tetramer staining†		ELISPOT‡	
					Pre/post	% Increase	Pre/post	% Increase
1	62/F	Induration	136.5/31.4	SD	23/246	1069.6	27/294	1088.9
2	61/F	Induration Fever	63.6/60.6	SD	1/157	15700.0	25/71	284.0
3	56/M	Induration Fever Thrombopenia	171.4/978.8	PD	22/19	86.3	19/525	2763.2
4	80/F	Induration Fever	30.0/22.7	SD	9/1030	11444.4	1/101	10100.0
5	58/M	Induration Fever	436.0/2885.0	PD	3/0	0.0	34/20	58.8
6	50/M	Induration	4389.0/4295.0	SD	2/7	350.0	27/85	314.8

†Cytotoxic T-lymphocyte frequency of prevaccinated (pre) and postvaccinated (post) patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The numbers of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 × CD8 T cells are shown. ‡γ-Interferon (IFNγ) secretion of pre- and postvaccinated patients' CD8 T cells was assesed with enzyme-linked immunosorbent spot (ELISPOT) assay using T2-A24 cells pulsed with survivin-2B80-88 peptide. The numbers of spots in 5 × 10^3 CD8 T cells are shown. CT eval., evaluation by computed tomography; IFNα, α-interferon; PD, progressive disease; SD, stable disease.

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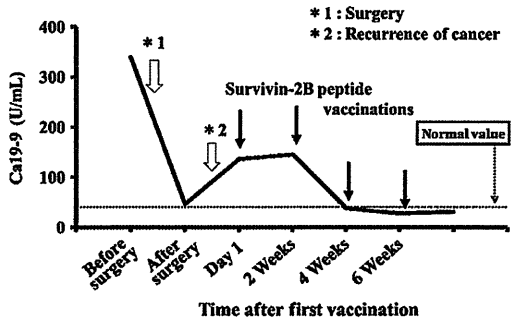

Fig. 2. Representative illustration of the clinical effect in patient No. 1 as assessed by the serum Ca19-9 level. Arrows indicate vaccinations with survivin-2B80-88 plus incomplete Freund's adjuvant with α-interferon (IFNα).

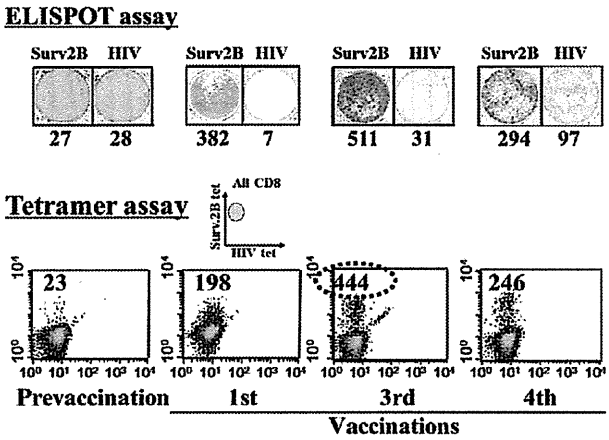

Fig. 3. Immunological analysis of CTL responses against HLA-A24 restricted survivin-2B80-88 peptide (surv2B) before and after vaccinations as assessed by enzyme-linked immunosorbent spot (ELISPOT) and tetramer (tet) analyses. Numbers in the ELISPOT assay indicate γ-interferon (IFNγ) secretion against survivin2B80-88 or HIV peptide pulsed T2-A24 cells in $10^4\times$ $CD8^+$ T cells. Numbers in tetramer analysis indicate survivin-2B80-88 peptide-specific $CD8^+$ T cells among $10^4\times$ $CD8^+$ T cells.

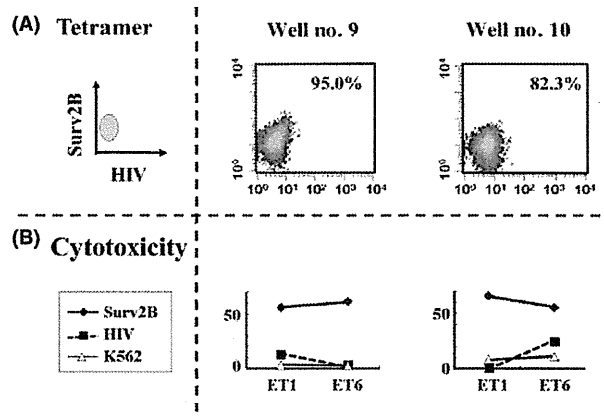

Fig. 4. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 CTL cells). Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Fig. 3 (circled) were sorted and cultured at 1, 3, and 10 cells /well for 7–10 days. Subsequently, clonal CTL cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer (Surv2B) (A) and against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide and against control K562 cells (B). ET, effector/target ratio.

with the survivin-2B80-88 peptide plus IFA and IFNα could work as a potential therapeutic regimen in pancreatic cancers. However, it remained to be clarified if IFNα alone without the peptide could function in a similar manner, at least to some extent, as this cytokine is considered to be the most potent for the activation and maturation of dendritic cells (DCs) as well as upregulation of HLA class I in tumor cells. To this end, we studied this profile in three patients with colon cancer, not pancreatic cancer, whose condition was similar to those in this study, that is, patients with unresectable advanced or recurrent cancer. This was done because patients with the latter cancer had highly advanced clinical cases, making this type of study impossible. As shown Table 2, all three patients showed no obvious increases, but rather reductions, in the frequency of survivin-2B peptide-specific T cells as assessed by tetramer analysis before and after two to four treatments with IFNα alone. Furthermore, this was also true for ELISPOT analysis. These data supported the idea that IFNα alone did not actively participate in the activation of survivin-2B peptide-specific T cells.

Discussion

Our group previously showed that the vaccination protocol of survivin-2B80-88 plus IFA and IFNα could work as a potent immunotherapeutic regimen in colon cancers.[8] In addition to colon cancer, survivin2B protein is expressed in most tumor cells of various tissue origins, such as those in the gastrointestinal and biliary tracts and pancreas, therefore, there is a possibility that the survivin2B peptide could work as a potential therapeutic tumor vaccine in cancer patients with these neoplasms.

In this present study, we assessed whether the vaccination protocol using survivin-2B80-88 plus IFA and IFNα could be effective in pancreatic cancer patients from immunological and clinical points of views. Consequently, our data strongly suggested that this protocol was very effective and useful in immunotherapy for advanced pancreatic cancers as in colon cancers. Actually it was shown that more than 50% of patients with pancreatic cancers showed positive clinical and immunological responses in tetramer and ELISPOT analyses. In some cases, the immunological response of survivin-2B80-88 peptide-specific CTLs was elucidated at the single-cell level. Taken together, the current data implied that our vaccination protocol was very useful in immunotherapy for pancreatic cancers.

As shown in Fig. 3, the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced from the third to the fourth vaccination. We speculate that there could be various reasons for this reduction. One might be immune escape by the downregulation of HLA expression, cytokines, or regulatory T cells. Another might be an activity of the stored samples, or differences between the environment of the peripheral circulation and the tumor. In other words, the peptide-specific CTL responses were reduced in immunological monitoring in the peripheral circulation, but maintained in the local cancer environment. In this case, the clinical responses, such as tumor marker (CA19-9) level and tumor size evaluated by CT, had been maintained also after that, even though the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced between the third and fourth vaccinations. Therefore, CA19-9 levels had been kept within normal limits and new cancer lesions had not appeared.

We evaluated immunological monitoring of this clinical protocol by tetramer staining and IFNγ ELISPOT assay. Tetramer staining recognizes the structure of the T cell receptor, and