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Expression of CD44 Variants and Its Association with Survival in Pancreatic Cancer

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Since the CD44 variant 6(v6) molecule has been noted as a marker for tumor metastasis and prognosis in several tumors, we examined whether or not v6 is a useful marker for evaluating the prognosis of pancreatic cancer patients. In addition, we attempted to assess the clinicopathological implications for pancreatic cancer of the variant 2 (v2) isoform using a recently developed monoclonal antibody against a v2 epitope. The expression of CD44 variants was evaluated immunohistochemically in paraffin-embedded pancreatic cancer tissues from 42 patients who were confirmed surgically and histologically to have received curative resection. An indirect immunoperoxidase method was used with monoclonal antibodies against epitopes of the standard (CD44s) portion, v6 and v2. Protein expression data were evaluated statistically for any correlations with the length of survival or with histological parameters. The expression of CD44v6 and v2 was observed only in tumor cells, if at all. On the other hand, expression of total CD44 (including CD44v, as well as CD44s) was observed in both tumors and adjacent normal sites. Tumor tissue from 21 (50%) and 16 (38%) patients showed positive immunoreactivity with mAb 2F10 (anti-CD44v6) and mAb M23.6.1 (anti-CD44v2), respectively. The expression of CD44v6 and v2 was correlated with decreased overall survival (P=0.0160 and P=0.0125, respectively). A significant correlation was obtained between CD44v2 peptide expression and vessel invasion (P=0.026). These results suggest that CD44v2 and CD44v6 may be useful markers for poor prognosis in curatively resected primary pancreatic cancer.

Key words: CD44 variants — Pancreatic cancer — Metastasis — Prognosis — Immunohistochemistry

CD44 is a heavily glycosylated cell surface molecule which is involved in cell-cell and cell-matrix interactions[1,2] and mediates several functions, such as extracellular matrix cell adhesion,[3,4] lymphocyte homing,[5,6] T cell activation[7] and tumor metastasis.[7,8] The CD44 family of polymorphic transmembrane glycoproteins is encoded by a complex gene[7,9–12] that occupies a stretch of 60–80 kD assigned to the human chromosomal locus 11p13. Its human form is composed of at least 21 exons, 10 of which are constitutively expressed on almost all cell types to produce a heavily glycosylated 85–90 kD isoform known as standard form CD44s (Fig. 1). The remaining exons can be alternatively spliced in various combinations, and their products are incorporated into the polypeptide backbone encoded by the standard form exons. This results in a large array of protein isoforms (CD44v) which are differentially expressed in various tissues and at various stages in development.[13]

Recently, it was demonstrated that the expression of one variant isoform of CD44 distinguished metastatic from non-metastatic pancreatic carcinoma cell lines in the rat.[8] Evidence that CD44v itself has a role in metastasis came from the demonstration that transfection with cDNA encoding this isoform converted non-metastatic carcinoma rat cells into metastatic cells.[8] Although the functions of CD44v isoforms in humans remain unclear, it is thought that CD44v isoforms play an important role in the growth and metastasis of several kinds of tumors.[14–18] It has been demonstrated that the disturbed activity of the *CD44* gene in malignant tumors results in the accumulation of immature mRNA transcripts containing introns[10,19] from the *CD44* gene. Moreover, in a recent study,[20] direct comparison of Southern blots with western blots from matched tumor cell line extracts indicated that most of the diverse mRNA isoforms are not detectably translated into proteins. These findings, together with data from reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis, show that the overexpression at the protein level involves only a minority of aberrant RNA transcripts.

Recent clinicopathological studies have revealed that the expression of individual variant exons is associated with poorer prognosis in several malignancies; for example, that of exon 14 (v9) is increased in gastric adenocarcinoma,[21] that of exon 11 (v6) in colon, breast,[22,23] and pancreas[24,25] cancers, and that of exon 7 (v2) in breast

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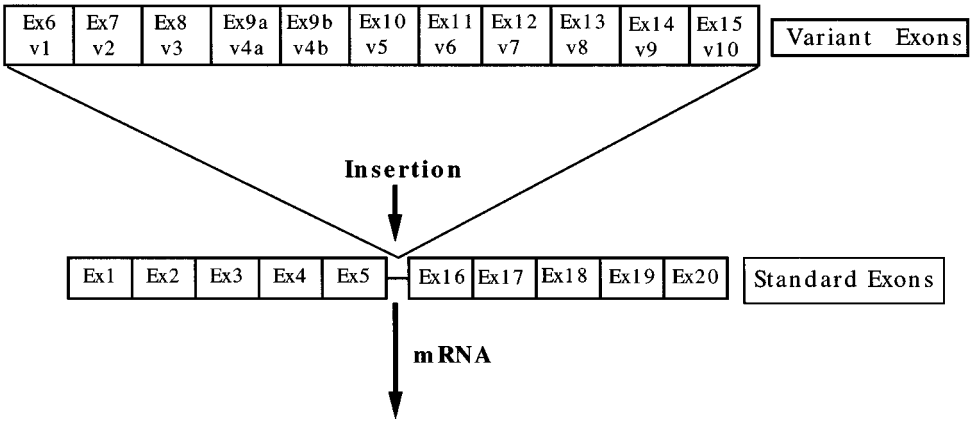

Fig. 1. Map of the CD44 gene products. CD44 variant exons are alternatively spliced into the mRNA strand corresponding to the standard form. Therefore, positive staining with mAb 2C5 against the epitope of CD44 standard portion indicates the protein expression of all CD44 isoforms, including CD44v as well as CD44s. Monoclonal antibodies, mAb M23.6.1 and mAb 2F10 recognize the peptides encoded by the variant exon 2 and variant exon 6, respectively.

cancer.[26] On the other hand, several reports have provided data indicating that CD44s expression is associated with longer survival in neuroblastoma and decreased expression of CD44v6 is correlated with decreased survival in laryngeal squamous carcinoma.[27] It is difficult to interpret this large body of information and to compare the various studies. However, it seems reasonable to say that there is clear organ specificity in the expression of the *CD44* gene.

Based on this background, we studied the correlation between the expression of CD44 variants and survival in patients with pancreatic adenocarcinoma, which has the worst prognosis of all gastrointestinal cancers.[28–30] We analyzed the correlations between clinicopathological indices and immunohistochemical staining for surgical specimens curatively resected from primary pancreatic adenocarcinoma using several monoclonal antibodies: anti-CD44s, anti-CD44v6 (exon 11) antibody and a recently developed anti-CD44v2 (exon 7) antibody whose specificity for the CD44v2 epitope has been confirmed by Borgya *et al*.[31]

MATERIALS AND METHODS

Tissue specimens Samples of formalin-fixed, paraffin-embedded tumor tissue from 42 invasive pancreatic adenocarcinomas surgically resected between 1990 and 1995 at the National Cancer Center Hospital, Tokyo, were studied. All patients were confirmed to have received curative resection after intensive histological exploration, and no patients had distant metastases at the time of operation. The median age of the patients was 58.9 years (range: 40 to 76). Twenty-seven patients were male and 15 were female. Patient distribution by stage according to UICC classification (UICC, 1987) was: stage I, 5 patients; stage II, 3 patients; stage III, 27 patients; and stage IV, 7 patients. In the present study, we did not include patients with metastases to distant organs. However, some patients had metastases to lymph nodes far from the pancreas and these were removed because our surgical team performed with curative intent. Such metastases are defined as one of the categories of distant metastases (LYM) in the UICC classification. Consequently, our cases with curative resection included stage IV cases who belonged to the M1 category with LYM factor.

Clinicopathological indices Staining results in primary tumors were compared with clinicopathological features, including tumor-node-metastasis (TNM) staging, grading, tumor localization, tumor diameter, vessel invasion, lymphatic invasion, nodal status and sex. Since histology often varied within the same tumor, the histological grading of the cancer was based on the dominant pattern.

Immunohistochemistry Three micrometer sections were cut on silane-coated glass slides (Muto Pure Chemicals, Tokyo), deparaffinized in xylene, dehydrated through a graded series of ethanol, and washed in running water. These sections were then treated with Serotec Target Unmasking Fluid (STUF; SEROTEC, Oxford, UK) for 10 min in a 600 W microwave oven and left to cool for 15 min. Sections were rinsed in 2 changes of deionized water and phosphate-buffered saline (PBS), and preincubated with 20% normal rabbit serum in Tris-buffered saline (TBS) at 37°C for 60 min. After having been washed with TBS, separate sections were incubated with the primary

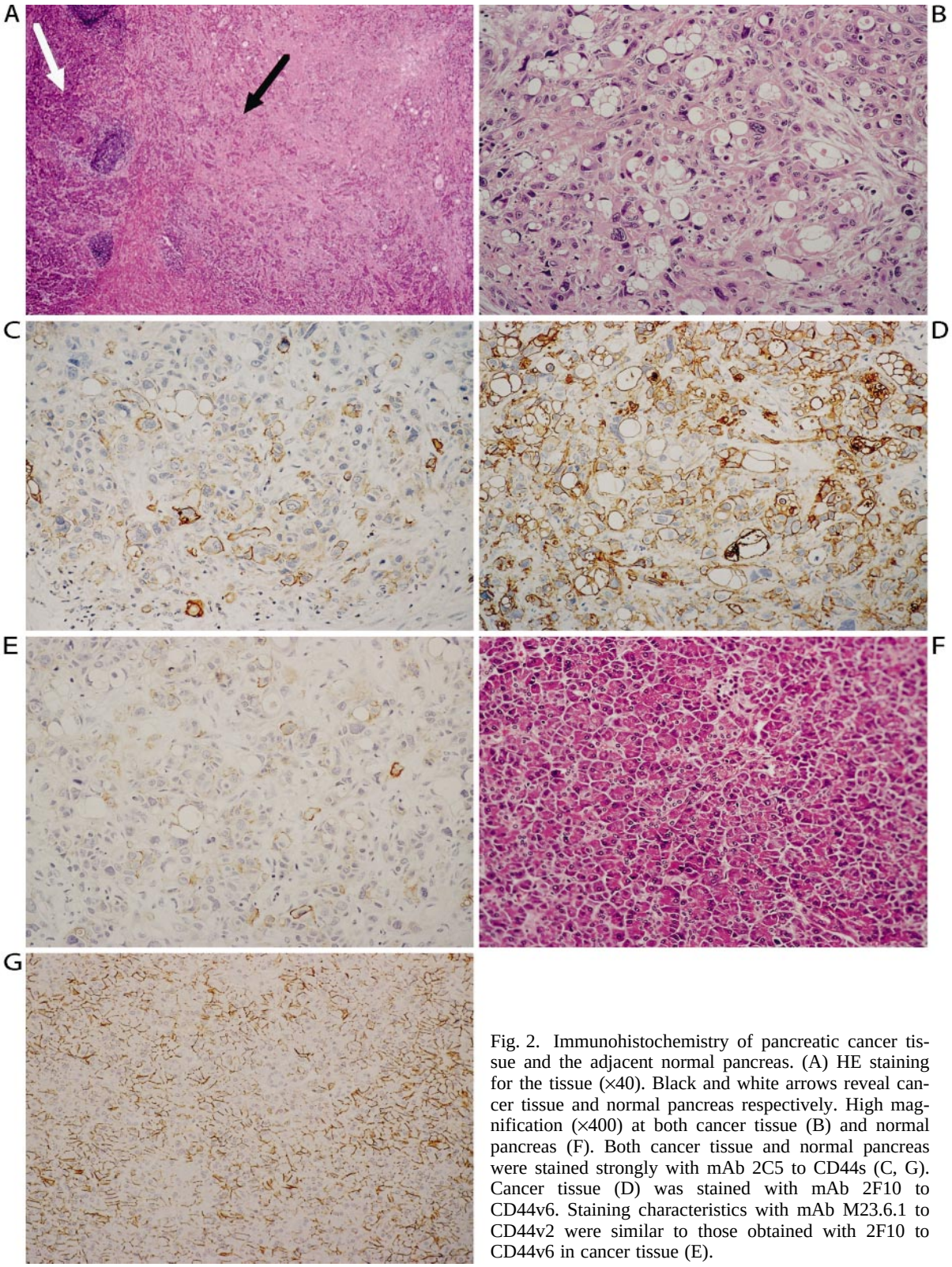

Fig. 2. Immunohistochemistry of pancreatic cancer tissue and the adjacent normal pancreas. (A) HE staining for the tissue (×40). Black and white arrows reveal cancer tissue and normal pancreas respectively. High magnification (×400) at both cancer tissue (B) and normal pancreas (F). Both cancer tissue and normal pancreas were stained strongly with mAb 2C5 to CD44s (C, G). Cancer tissue (D) was stained with mAb 2F10 to CD44v6. Staining characteristics with mAb M23.6.1 to CD44v2 were similar to those obtained with 2F10 to CD44v6 in cancer tissue (E).

Table I. CD44 Variant Expressions of 42 Patients with Primary Pancreatic Cancer Correlated with Clinicopathological Indices

Index	CD44v6 expression		*P* value	CD44v2 expression		*P* value
	Positive	Negative		Positive	Negative	
Sex						
Male	13	14		10	17	
Female	8	7	0.75	6	9	0.85
Histological grading[a)]						
Well/Mod	16	18		12	22	
Poor/Undif	5	3	0.43	4	4	0.44
Tumor site						
Head	15	11		12	14	
Body/Tail	6	10	0.20	4	12	0.17
Tumor diameter						
≤2	1	2		1	2	
>2	20	19	0.55	15	24	0.86
Vessel invasion						
No	5	9		2	12	
Yes	16	12	0.19	14	14	0.026
Lymphatic invasion						
No	6	2		5	3	
Yes	15	19	0.12	11	23	0.11
Lymph node metastasis						
No	4	4		3	5	
Yes	17	17	1.00	13	21	0.97
TNM stage[b)]						
Stage I	2	3		1	4	
Stage II/III/IV	19	18	0.63	15	22	0.37
Vital status						
Alive	4	12		2	14	
Dead	17	9	0.011	14	12	0.007

a) Histological grading and *b*) TNM staging are classified according to TNM Classification of Malignant Tumor (UICC, 1987): Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated; Undif, undifferentiated.

monoclonal antibodies 2C5 (3.3 μg/ml, R&D Systems, Arbington, UK), 2F10 (10 μg/ml, R&D Systems) and M23.6.1 (20 μg/ml), which recognize epitopes of the CD44s portion, v6 portion and v2 portion, respectively, in 1% normal rabbit serum diluted with TBS at 4°C overnight in a wet box and then at 37°C for 10 min the next morning. After the primary antibody treatment, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol at 4°C for 10 min, and the sections were incubated with a 1:400 dilution of biotinylated anti-mouse IgG (DAKO, Santa Barbara, CA). This was followed by incubation with horseradish peroxidase-conjugated avidin-biotin complex (ABComplex; DAKO) at room temperature for 60 min. Immunostaining was visualized with 3,3′-diaminobenzidine (Sigma, St. Louis, MO) for 20 min, and the reaction was stopped by washing with water. Finally, the sections were counterstained with Mayer's hematoxylin. Between all antibody incubations, the sections were washed three times for 5 min with TBS on a shaking platform.

Negative controls included sections treated with 1% normal rabbit serum alone in place of the primary antibody. Staining was defined as positive if more than 5% of tumor cells were stained clearly and the accompanying control, without primary antibody, was negative.

Statistical analysis The correlation between CD44 peptide expression and clinicopathological indices was analyzed by using Fisher's exact test. We defined overall survival as survival from the date of surgery until December 26th, 1996. The primary determinant in this study was survival time, as measured from the date of surgery until the time of the last follow-up visit or death. Data on survival were excluded if the patient died from other causes. Survival curves for 40 patients (excluding the complica-

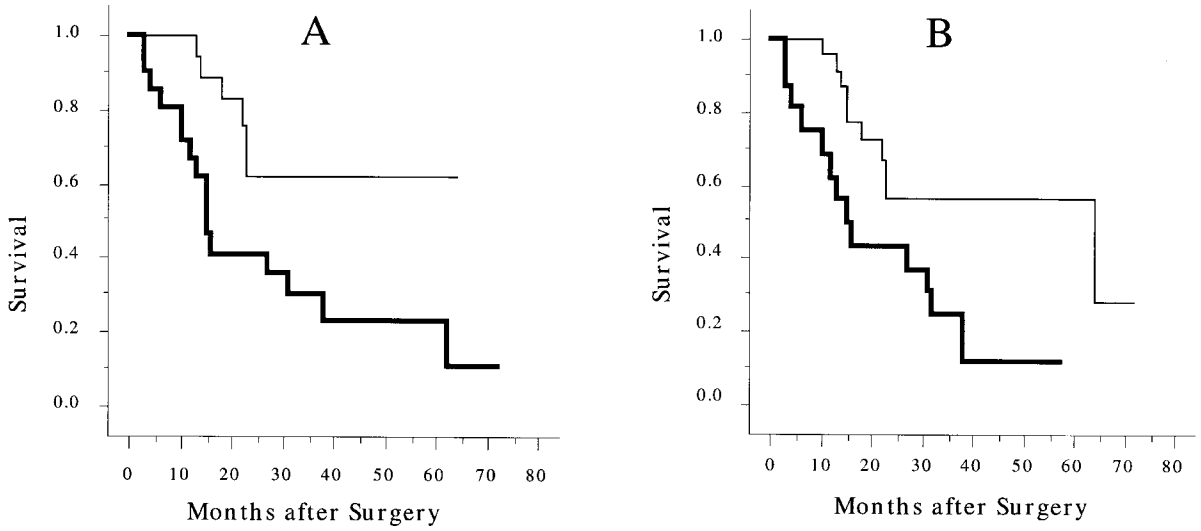

Fig. 3. Overall survival probability of pancreatic cancer patients categorized according to CD44 isoform expressions. (A) v6 epitope, CD44v6-positive patients showed a significantly poorer prognosis than CD44v6-negative patients (P=0.0160). (B) Similarly, CD44v2-positive patients had a significantly poorer prognosis than CD44v2-negative patients (P=0.0125).

tion of death) were constructed according to the Kaplan-Meier method[32] and differences between the curves were calculated. The P value was calculated by use of the logrank test and the Breslow-Gehan-Wilcoxon test. A P value of less than 0.05 was considered significant.

RESULTS

Immunohistochemical staining Forty-two pancreatic cancer tissue sections were stained with three monoclonal antibodies directed against the total CD44 (CD44v as well as CD44s), CD44v6 (exon 11) and CD44v2 (exon 7). Moreover, adjacent normal epithelia in cancer tissue were examined for reactivity with the same antibodies. As shown in Fig. 1, CD44 variant exons are alternatively spliced into the mRNA strand corresponding to the standard form. Therefore, positive staining with 2C5 antibody indicates the protein expression of all CD44 isoforms, including CD44v as well as CD44s. Total CD44 expression was observed in all cancer tissues and all normal tissues (Fig. 2, C and G). In contrast, CD44v6 and CD44v2 were not detected in normal tissues. Among 42 primary pancreatic cancer tissues, 21 (50%) showed staining with mAb 2F10 (CD44v6) and 16 (38%) with M23.6.1 (CD44v2) (Fig. 2, D and E). All CD44v2-positive samples were also CD44v6-positive, but CD44v6-positive were not always CD44v2-positive. The intensity of CD44v2 staining was generally weaker than that of CD44v6 staining. Table I summarizes the CD44s and CD44 variant staining status of the 42 patients.

Correlation between CD44 variant expression in primary pancreatic cancer and clinicopathological indices Table I gives the relevant clinical characteristics of the 42 patients whose tumors were analyzed immunohistochemically. For CD44v6 expression, no significant correlations were obtained for any clinicopathological indices. In contrast, a significant correlation was observed between CD44v2 expression and vessel invasion (P=0.026). However, evaluation of other prognostic factors revealed no significant correlations. In particular, neither the TNM stage nor the tumor diameter was correlated with CD44 variant expression.

Expression of CD44 variants and prognosis The relationship between CD44v6 and CD44v2 expression and survival rate was examined in 40 curatively resected primary pancreatic cancer patients (excluding the complication of death) during a follow-up of 3–72 months (mean 25 months). Kaplan-Meier curves (Fig. 3) showed that patients with CD44v6-positive primary tumors had a shorter survival time than those with CD44v6-negative tumors (logrank test: P=0.0160; Breslow-Gehan-Wilcoxon test: P=0.0069). The 5-year survival rate was 62% among patients with CD44v6 negativity and 25% among patients with CD44v6 positivity. Also, for CD44v2 staining, a similar association was obtained between CD44v2 expression and survival time (logrank test: P=0.0125; Breslow-Gehan-Wilcoxon test: P=0.0128). The 5-year survival rate was 57% among CD44v2-negative patients and 11% among CD44v2-positive patients. The average survival of CD44v6-positive and CD44v6-negative

patients was 22.0 months (3–72 months) and 31.3 months (4–64 months), respectively. Also, the average survival of CD44v2-positive and CD44v2-negative patients was 20.9 months (3–57 months) and 30.0 months (4–72 months), respectively. At the conclusion of the study, 19% of patients with CD44v6-positive tumors were alive, as compared to 57% of patients with CD44v6-negative tumors ($P=0.011$, Table I). In addition, 13% of patients with CD44v2-positive tumors were alive, in contrast to 54% of patients with CD44v2-negative tumors ($P=0.007$, Table I).

DISCUSSION

Many studies have indicated that the expression of CD44 variants is associated with tumor metastasis and progression in several types of tumors, such as gastric cancer,[21] colonic cancer,[15–17, 22] breast cancer,[23, 26] and non-Hodgkin's lymphoma.[33] Here, we demonstrated immunohistochemically the association between CD44 variant expression and clinicopathological indices in curatively resected primary pancreatic cancer using previously characterized monoclonal antibodies against total CD44 and CD44v6 and a new monoclonal antibody against an epitope encoded by exon 7 (CD44v2) of the human *CD44* gene. From our previous study using western blot analysis, patients with CD44v6 positivity were not always CD44v2 positive and it is considered that the CD44 molecule detected by v2 antibody includes all the other variants.[20]

In the clinicopathological evaluation, a significant association was found between the expression of CD44v2 and vessel invasion. However, 14 of 26 (54%) tumors negative for CD44v2 expression exhibited vascular invasion versus 14 of 16 (88%) tumors positive for CD44v2 expression. Since the rate of vascular invasion of CD44v2-negative tumors was high, there is need for caution in interpreting the pathological significance of CD44v2-containing CD44 proteins in pancreatic cancer. Also, the expression of CD44v2 was not associated with liver metastasis (data not shown).

Concerning prognosis, we found here that expression of both CD44v6 and CD44v2 is significantly associated with increased mortality. Patients with CD44v6-negative primary tumors had a longer survival time than those with CD44v6-positive tumors. The projected 5-year survival rate was 62% among patients with CD44v6 negativity and 25% among patients with CD44v6 positivity. A similar association was also obtained between CD44v2 expression and survival time. The projected 5-year survival rate was 57% among CD44v2-negative patients and 11% among CD44v2-positive patients. Compared to well-known survival rates of pancreatic cancer, cases used in this study showed an extremely high survival rates. However, the 5-year survival rate of all surgically resected cases is 25% in our institution (unpublished data), and this rate is comparable to those reported previously.[27, 34, 35] In the present study, to evaluate the significance of the expression of CD44v2 and v6 as prognostic markers, we thought it was important to use specimens from patients who had the same background. Therefore, we evaluated only cases which were confirmed to have received surgically and histologically curative resections, as shown by intensive histological examination.

There are now many reports concerning the relationship between the expression of the *CD44* gene and survival time. The present data for the CD44 variants in pancreatic cancer are consistent with the results obtained for other adenocarcinomas, such as colonic,[22] breast[23] and stomach cancer[21] and non-Hodgkin's lymphoma.[33] On the other hand, several studies have provided data indicating that CD44s expression is associated with longer survival in neuroblastoma[36] and CD44v6 expression is consistent with a longer survival in laryngeal squamous cell carcinoma.[27] Furthermore, Sugino *et al*. noted that initially there is a marked increase in the expression of the *CD44* gene in early bladder carcinoma, relative to normal urothelium, but this diminishes as the tumors acquire a more aggressive phenotype.[37] Furthermore, there are distinct differences in the patterns of expression of the *CD44* gene in normal cells and tissues depending on location and cell lineage; for example, abundant expression of CD44 variants is seen in normal skin and in squamous epithelium.[13] In normal colonic mucosa, very weak expression of CD44v was found in the basal layers of epithelium (at the base of crypts), but none was detected in surface epithelium.[38] From these findings, we see that the expression pattern of the *CD44* gene is complex, with CD44 variant expression occurring in a tissue- and location-specific manner. Regarding the role of CD44v2 or v6 in functions such as cell-cell adhesion and tumor metastasis, it is so far unknown whether such functions are modified by the conformational changes caused by the insertions in the CD44 variants or by a direct effect of the variants. Consequently, it is unknown whether CD44 variant expression abnormalities are causal or are only incidental effects in the pathogenesis of pancreatic cancer. However, as for other adenocarcinomas, the present findings strongly indicate that CD44v2 and CD44v6 are useful markers for clinical prognosis in curatively resected primary pancreatic cancer.

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