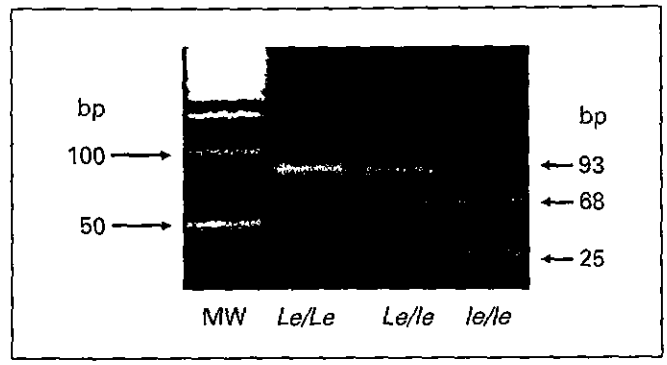

Fig. 2. PCR-RFLP for detection of the T59G mutation. Cleavage at a restriction enzyme in the PCR products from *le* alleles separates the 93-bp fragment into 2 fragments of 68 and 25 bp. MW = Molecular weight marker.

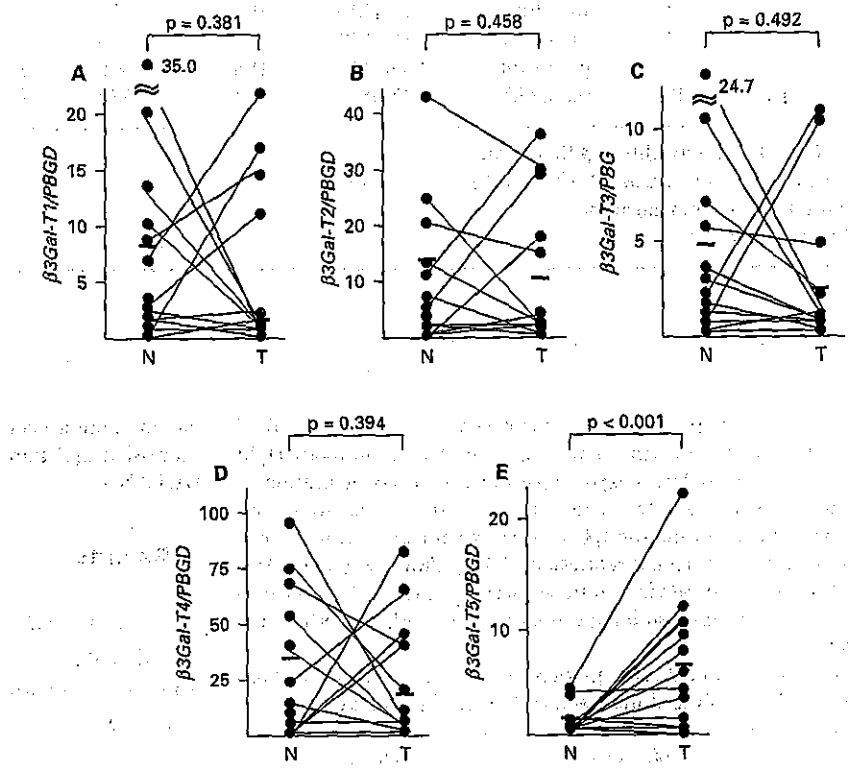

Fig. 3. Quantitative analysis of *β3Gal-T* mRNA expression in pancreas tissues by the LightCycler method. Each solid circle represents the ratio of *β3Gal-T/PBGD*. Lines connect values for samples of cancerous and adjacent noncancerous tissues from the same patients. The bar across each group represents the average value of each group. N = Noncancerous tissue; T = cancerous tissue. **A** *β3Gal-T1;* **B** *β3Gal-T2;* **C** *β3Gal-T3;* **D** *β3Gal-T4;* **E** *β3Gal-T5.*

Table 3. Statistical analysis of *β3Gal-T* gene expression levels in noncancerous and cancerous tissues

Glycosyltransferase	Amount of transcript mean ± SD		Statistical significance
	noncancerous	cancerous	
β3GalT1	7.2±10.9	2.4±5.0	0.381
β3GalT2	16.1±24.1	11.3±14.1	0.458
β3GalT3	4.4±7.6	2.4±4.5	0.492
β3GalT4	32.1±33.8	15.1±16.9	0.394
β3GalT5	0.8±2.3	6.8±6.4	<0.01

who were homozygous for the *Le* allele (table 2). A schematic representation of the *Le* genotype is shown in figure 2.

Gene Expression Levels of β3Gal-T1, T2, T3, T4, *and* β3Gal-T5

Quantitative RT-PCR analysis was performed to compare the difference in each *β3Gal-T* gene expression between cancerous and adjacent noncancerous tissues of each case. Although the levels of *β3Gal-T1, T2, T3,* and *β3Gal-T4* gene expression showed variable values in non-

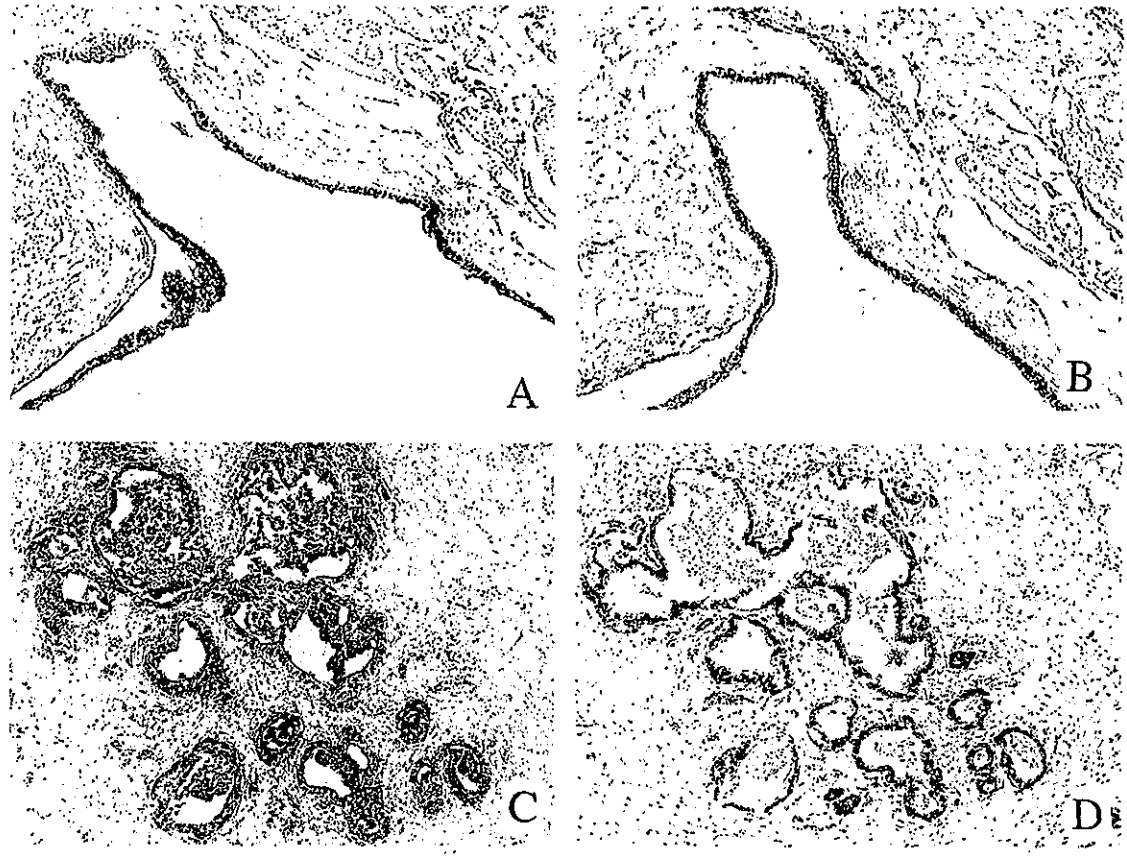

Fig. 4. Representative patterns of immunostaining for CA 19-9 and DUPAN-2 in noncancerous (**A**, **B**) and cancerous (**C**, **D**) tissues. CA 19-9 (**A**) and DUPAN-2 (**B**) were expressed slightly as the apical types in noncancerous tissues. Magnification: × 100. CA 19-9 (**C**) and DUPAN-2 (**D**) were stained as the apical and cytoplasmic types. Immunoreactive intensities of both antigens in cancerous tissues correlated with each other. Magnification: × 100. All tissues were derived from patient 9 in table 2.

cancerous and adjacent cancerous tissues in each case, *β3Gal-T5* gene expression in cancerous tissues exhibited statistically significant values for upregulation in cancerous tissues (fig. 3). The mean of the *β3Gal-T5* transcript amount exhibited statistically significant values for upregulation in cancerous tissues, 6.8 ± 6.4, as compared with noncancerous tissues, 0.8 ± 2.3 ($p < 0.01$; table 3). In contrast, there was no significant difference in the averages of gene expressions of *β3Gal-T1, T2, T3,* and *β3Gal-T4* between cancerous and noncancerous tissues (table 3).

Immunohistological Expression of CA 19-9 and DUPAN-2

To investigate the correlation between *β3Gal-T1, T2, T3, T4,* and *β3Gal-T5* gene transcripts and the expression of CA 19-9 or of its precursor, DUPAN-2, we next stained all tissues with both CA 19-9 and DUPAN-2. Both antigens were slightly or weakly stained on the apical surface of the cells and in the supranuclear regions of the cytoplasm in normal pancreatic ducts (fig. 4A, B). On the other hand, in cancerous tissues, both antigens were distributed over the entire surface and throughout the cytoplasm showing valuable staining levels in atypical or malignant cells (fig. 4C, D). Cancerous tissue derived from the *Le*-negative patient showed no CA 19-9 immunoreactivity, but high and diffuse DUPAN-2 staining, as described in another study (data not shown) [32].

Although there was no relation between gene expression levels of *β3Gal-T1, T2, T3, T4,* and *β3Gal-T5* in each cancerous tissue and its serum value of CA 19-9, a good correlation was observed between *β3Gal-T5* gene expression level and both CA 19-9 and DUPAN-2 immunoreactivities except for the *Le*-negative patient ($p < 0.01$; fig. 5).

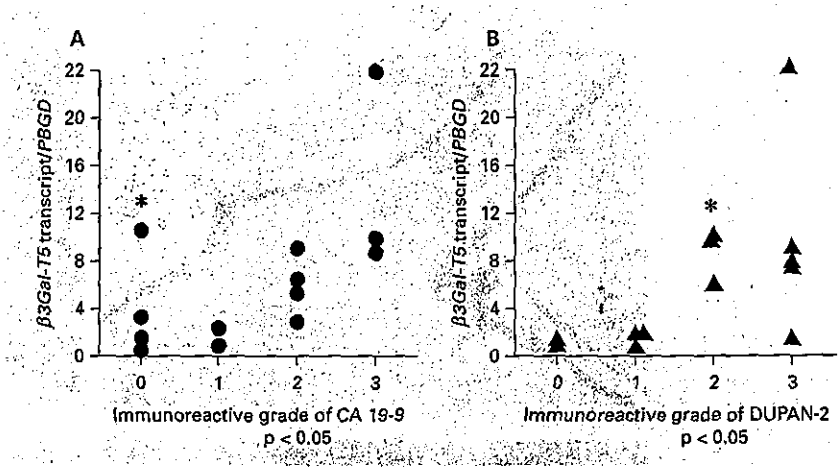

Fig. 5. Correlation between the gene expression of *β3Gal-T5* and immunoreactivity in cancerous tissues. Individual *β3Gal-T5* gene expression and CA 19-9 (**A**) or DUPAN-2 (**B**) immunohistochemical value. Statistic significance (p value) between *β3Gal-T5* levels and CA 19-9 grades was calculated, excluding the *Le*-negative patient. * = *Le*-negative patient.

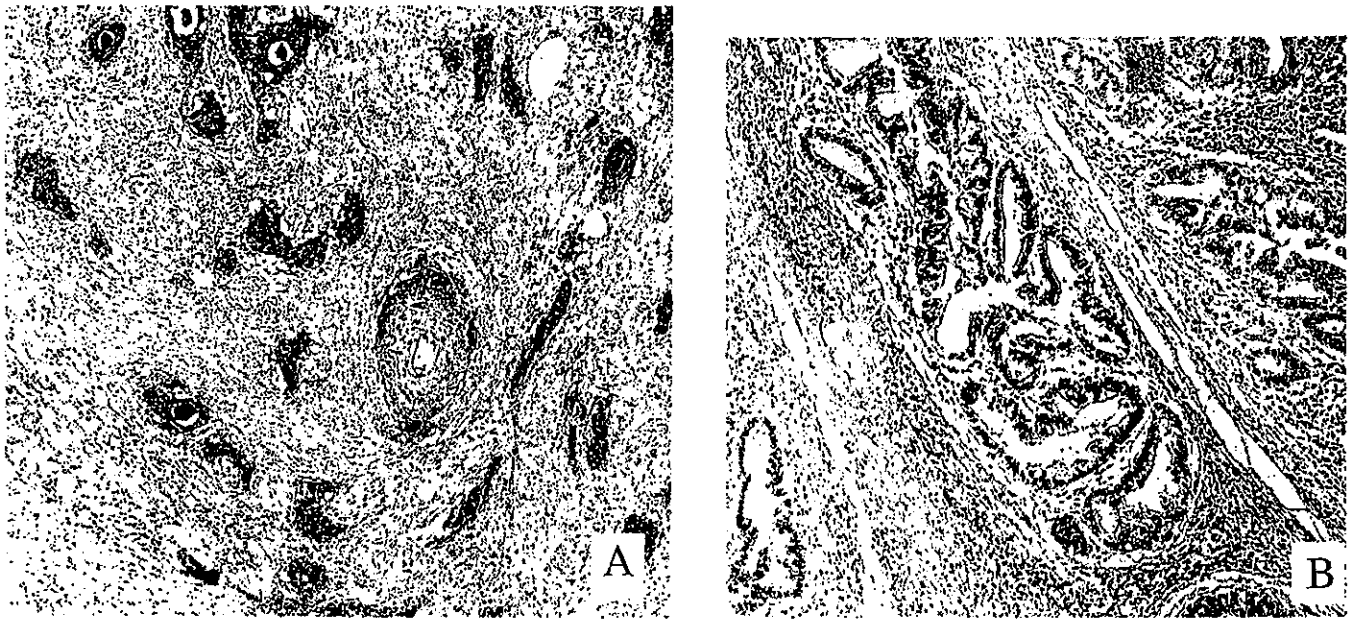

Fig. 6. Representative patterns of immunostainig for CA 19-9 in cancerous tissue with high and low gene expression. The tissues showing high (**A**) and low (**B**) *β3Gal-T5* gene expression were derived from patient 6 and 7 in table 2, respectively.

Cancerous tissue with high immunoreactivity of CA 19-9 antigen was likely to indicate high levels of *β3Gal-T5* gene transcript (fig. 6A). In contrast, low expression of the antigen was found in the cancerous tissue with low gene expression of *β3Gal-T5* (fig. 6B). There was no correlation between immunoreactivity levels of both antigens and other *β3Gal-T* gene expression levels in cancerous tissues.

Discussion

Pancreatic cancer is a formidable disease with an extremely poor prognosis; fewer than 20% of affected patients survive the first year, and only 3% are alive 5 years after the diagnosis [33]. Curative resection is possible in selected patients (10–15%), with the expectation of extended 5-year survival rates ranging from 6 to 20% in

some series [34]. Conventional diagnostic and therapeutic strategies, however, still remain to be insufficient to improve survival, which might be ascribed to the incomplete recognition of biological tumor characteristics of this disease. Therefore, understanding the biological basis of this disease could pave the way for improving diagnostic and therapeutic approaches.

The formation of tumor-associated carbohydrate antigen in cancerous tissues is attributed to regulated expression of the glycosyltransferase genes involved in the synthesis of the antigens [5]. Those genetic features of glycosyltransferase fate the formation or structure of the carbohydrate chain [2]. Various researches discuss aberrant glycosylations of the carbohydrate structures in various cancerous tissues in terms of the regulated gene expressions of glycosyltransferases because alteration of some glycosyltransferases predominantly influences the synthesis of these carbohydrate determinants [15, 35, 36]. There is increasing evidence that glycosyltransferases are coded by families of multiple related genes whose expression is carefully regulated in different tissues and in different physiological or pathological states [37–39]. Gene alterations, such as *K-ras* and *p53* gene mutations, have been well studied and believed to be the most notable molecular abnormalities in pancreatic cancer [23, 40]. However, characterization of glycosyltransferase expression related to carbohydrate determinants in this disease has not yet been well described.

In the present study, we performed a quantitative analysis of 5 *β3Gal-T* genes in human pancreatic tissues by real-time PCR, which enables us to easily perform continuous and more precise quantitative measurement, and examined the correlation between those gene expression levels and immunoreactivity of CA 19-9 and/or its precursor DUPAN-2. Our observation indicates that only *β3Gal-T5* gene expression augmented in the cancerous tissues as compared with noncancerous tissues, and that only *β3Gal-T5* transcript among the members of *β3Gal-T* genes correlated with CA 19-9 or DUPAN-2 expression, while the expression of other member transcripts, i.e. *β3Gal-T1, T2, T3,* and *β3Gal-T4,* did not. DUPAN-2 is considered to convert easily to CA 19-9 without any regulated expression of the *Le* gene, because the *Le* gene was found to be ubiquitously and abundantly expressed [41]. This result denies the possibility that the Le enzyme plays a key role in the overexpression of CA 19-9 antigen, as we described previously [16].

It is quite plausible that a large proportion of cancer cells coexpressed both antigens, leading to parallel immunostaining of these 2 antigens, except for the *Le*-negative patient in the present study, which is consistent with another study [42]. Indeed, the *Le*-negative patient showed no CA 19-9 immunoreactivity regardless of whether the tissue was noncancerous or cancerous, due to the lack of the Le enzyme. Although there was only 1 *Le*-negative case, *β3Gal-T5* gene transcription correlated well with DUPAN-2 immunoreactivity in a similar way to other cases. Immunoreactivity of these 2 antigens in tumor cells seems to reflect the expression of the *β3Gal-T5* gene. Therefore, the parallel induction of these 2 antigens and *β3Gal-T5* gene expressions in pancreatic cancer accordingly might support the concept of a direct relationship between the β3Gal-T5 enzyme and the terminal product, CA 19-9. Additionally, physiological and kinetic characterization of β3Gal-T5 incorporating donor Gal into the GlcNAc-based acceptor to produce CA 19-9 might efficiently be helpful to evaluate β3Gal-T5 involvement in the formation of CA 19-9, i.e., the acceptor substrate specificity using purified β3GalTs from human pancreatic cancer tissue instead of cancer cell lines might also be an additional option to provide evidence that β3GalTs are related to the formation of the type 1 chain or CA 19-9.

In summary, we quantified the gene expressions of *β3Gal-T* genes in pancreatic cancer tissues and analyzed the expression of CA19-9 or DUPAN-2 immunohistochemistry. Our present results indicate that the mRNA content of *β3Gal-T5* correlates with the levels of the immunohistochemical expression of CA 19-9 in pancreatic cancer cells. Based on these results, we concluded that β3Gal-T5 is the most feasible candidate for the synthesis of CA 19-9.

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Expression of Uridine Diphosphate N-Acetyl-α-*D*-Galactosamine: Polypeptide N-Acetylgalactosaminyl Transferase 3 in Adenocarcinoma of the Pancreas

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Key Words
Pancreatic carcinoma · GalNAc-T3 · Tumor differentiation

Abstract
Uridine diphosphate (UDP) N-acetyl-α-*D*-galactosamine:polypeptide N-acetylgalactosaminyl transferase 3 (GalNAc-T3), one of the enzymes that catalyze the initial glycosylation of mucin type O-linked proteins, was shown to associate with the differentiation of cancer cell lines and the prognosis of some kinds of cancers. In the present study, the association of GalNAc-T3 expression with clinicopathologic features of pancreatic adenocarcinoma and patients' survival was examined. The level of expression of GalNAc-T3 was analyzed immunohistochemically in paraffin-embedded tumor samples from surgically resected specimens of 59 patients with pancreatic ductal adenocarcinoma. The correlations of GalNAc-T3 expression with clinicopathologic features and prognosis were studied. Thirty-five tumors showed high-intensity GalNAc-T3 staining, whereas 24 showed low-intensity staining. A close association was observed between GalNAc-T3 staining intensity and histologic differentiation and the stage of the tumors. The low-intensity group showed a high rate of the poorly differentiated subtype ($p < 0.001$) and T4 of the pTNM staging system ($p < 0.05$). These findings indicate that the expression of GalNAc-T3 is associated with the differentiation and aggressiveness of ductal adenocarcinoma of the pancreas.

Introduction

Ductal adenocarcinoma of the pancreas is one of the main causes of cancer death with poor prognosis in Western countries and Japan [1, 2]. Surgical resection offers the only curative treatment; however, the actuarial 5-year overall survival rate was only 10–30%, even though tumors of 2 cm or less in diameter were curatively resected [3–6]. Reasons for the high mortality rate are

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(1) difficulty in diagnosing at an early stage because the tumors usually progress asymptomatically, and (2) aggressive biological behavior of the tumor [4, 5].

Multiple subsets of genes have changed for activation or inactivation during the development and progression of pancreatic carcinoma [7–11]. Frequent genetic alterations in pancreatic cancer reported to date were K-ras oncogene [7, 8], tumor suppressor genes such as p16 [9] and p53 [10], and growth factors such as epidermal growth factor [7, 8] and insulin-like growth factor I [11]. However, the exact mechanisms for progression of pancreatic cancer have not been elucidated to date. Therefore, further progress in the research of the progression of pancreatic cancer is needed.

It has been reported that the expression of cell surface carbohydrate antigens (CAs), such as CA 19-9 and Sialyl-Tn, alter in gastrointestinal cancers including pancreatic cancer during malignant transformation and tumor progression [12]. They may determine the metastatic behavior of tumor cells affecting adhesion, motility, and immunogenicity [12]. Glycoprotein O-glycans, which are GalNAc-Ser or Thr O-linked oligosaccharides, are found in secretions and on the cell surfaces of cancer cells [13–17]. The structures of O-glycans are often changed in an unusual or abnormal way in cancers, and this change may contribute to the phenotype and biological features of cancer cells [12].

Uridine diphosphate (UDP) N-acetyl-α-*D*-galactosamine:polypeptide N-acetylgalactosaminyl transferases (GalNAc transferases) are families of enzymes that catalyze the initial glycosylation of mucin type O-linked proteins by transferring the monosaccharide N-acetylgalactosamine (GalNAc) from the nucleotide sugar UDP GalNAc to the hydroxyl group of serine and threonine amino acid residues. Thirteen human isozymes of it have been identified so far [18–26]. Among these, mRNA expression of GalNAc-T3 is highly tissue specific and is detected in organs with secretary epithelial cells such as the pancreas [22]. It has been suggested that the GalNAc-T3 expression is significant in the differentiation or progression of pancreatic carcinoma [22].

However, the association between GalNAc-T3 expression in pancreatic carcinomas and their clinicopathologic features, such as histologic differentiation, invasiveness, and metastasis of the tumor, and patients' prognoses has not been clarified yet. In the present study, the expression of GalNac-T3 in pancreatic adenocarcinoma with clinicopathologic data of the patients was evaluated, which might allow a better prognostic stratification in the clinical field.

Patients and Methods

Patients

Surgical specimens were obtained from 59 patients who had undergone surgery for primary ductal adenocarcinoma of the pancreas. All patients had curative resections of primary tumors at the Department of Surgery and Clinical Oncology, Osaka University Graduate School of Medicine during the period from January 1992 to June 2001. The patients included 34 males and 25 females with ages ranging from 48 to 79 (mean 65.8) years. The stage of the disease was classified according to the pTNM staging system [27]. Resected specimens were examined macroscopically to determine the location and size of tumor. Then tissue samples were fixed in 10% neutral buffered formalin, and routinely processed through graded series of ethanol solutions for paraffin embedding. Four-micrometer-thick histologic sections were cut and stained with hematoxylin and eosin to determine the following categories: histologic diagnosis, differentiation of tumor cells, and the existence of metastasis to the lymph nodes. Twenty-nine cases had well-differentiated adenocarcinoma, 24 moderately differentiated adenocarcinoma, and 6 poorly differentiated adenocarcinoma.

After surgery, we followed patients with measurement of serum carcinoembryonic antigen and CA 19-9 levels, ultrasonography and computed tomography at about 3- to 6-month intervals. The patients were followed until March 31, 2002: the follow-up period for survivors ranged from 10.9 to 119.0 (median 33.2) months after surgery.

Immunohistochemical Analysis

The preparation and specificity of polyclonal anti-GalNAc-T3 antibody has been described previously [23, 24, 28]. Antibody was diluted in phosphate-buffered saline with 2% bovine serum albumin. A final dilution of 1:3,000 was used for immunohistochemistry. Immunohistochemistry was performed using paraffin-embedded tissue sections with the immunoperoxidase procedure (avidin-biotin-complex method). Antigen retrieval was performed by heating the deparaffinized rehydrated sections in 10-m*M* citrate buffer (pH 6.0) at 98°C for 5 min by using microwave as described previously [29]. Sections were counterstained lightly using Mayer's hematoxylin. Specimens of breast carcinoma known to have GalNAc-T3 were stained in parallel as a positive control [23, 24]. For negative controls, nonimmunized rabbit IgG (Vector Laboratories, Burlingame, Calif., USA) was used as the primary antibody.

Stained sections were evaluated in a blinded manner without prior knowledge of the clinicopathologic features of the patients. Staining intensity in the cytoplasm of tumor cells was categorized as follows: weaker (low intensity), or equal to stronger (high intensity) than that in noncancerous pancreatic ductal cells, which was determined as the positive control. When the staining intensity of tumor cells varied in different areas of the same specimen, the predominant pattern was chosen as the expression level.

Statistical Analysis

Statistical analyses were performed using the JMP software (SAS Institute Inc., Cary, N.C., USA). The χ^2 test and Fisher's exact probability test were used to analyze the correlation between GalNAc-T3 expression at immunohistochemistry and clinicopathologic features. Kaplan-Meyer methods with log-rank test were used to calculate the overall survival rate and differences in survival curves [30]. p values of less than 0.05 were considered statistically significant.

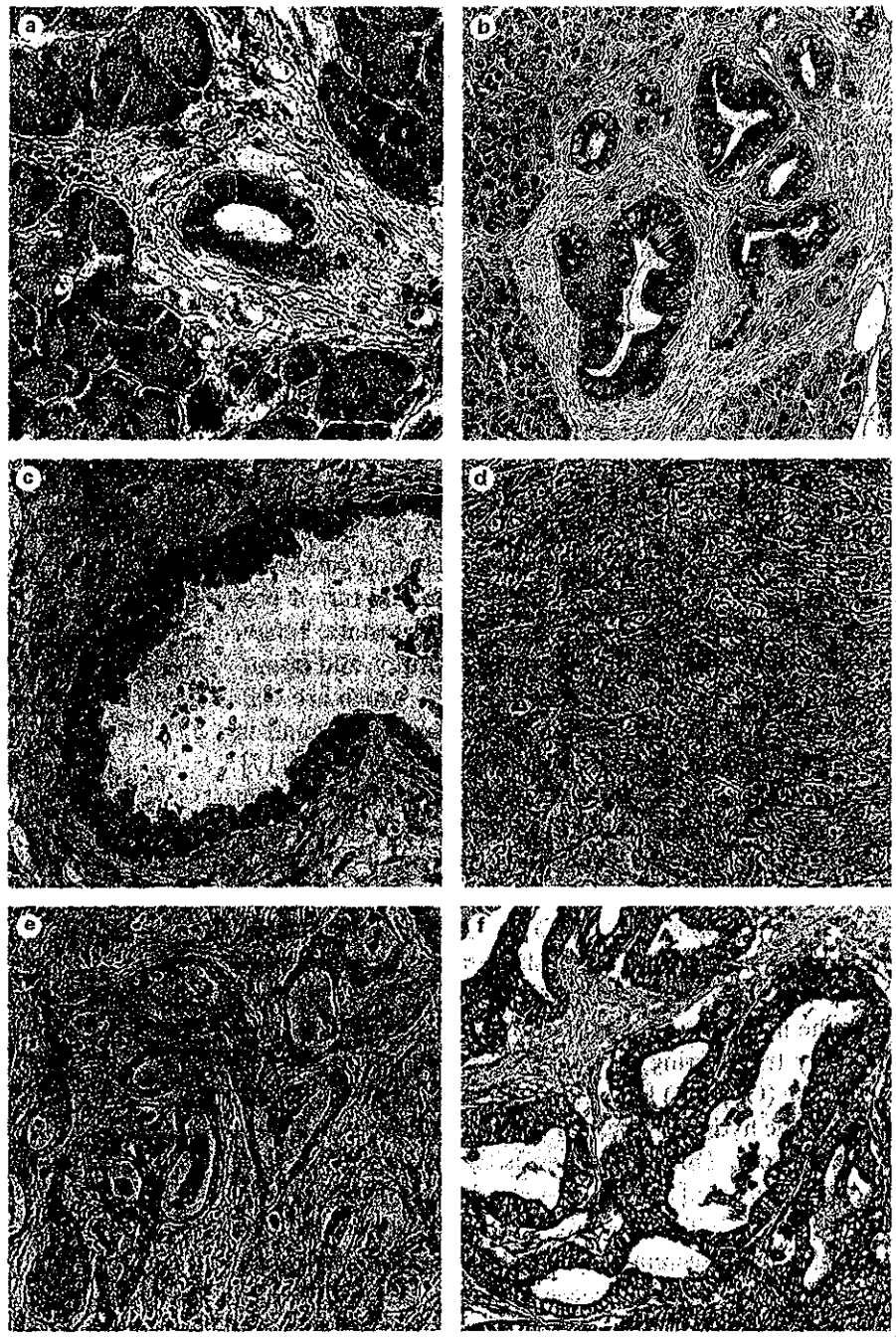

Fig. 1. GalNAc-T3 immunostaining. **a** Normal ducts of the pancreas are moderately stained with GalNAc-T3 (magnification, ×100). **b** Hyperplastic ducts of the pancreas with high intensity (×40). **c** Dysplastic ducts of the pancreas with high intensity (×40). **d** Poorly differentiated adenocarcinoma of the pancreas showing low intensity staining (×40). **e** Moderately differentiated adenocarcinoma of the pancreas with high intensity (×40). **f** Well-differentiated adenocarcinoma of the pancreas with high intensity (×40).

Results

GalNAc-T3 Expression

GalNAc-T3 staining of both normal pancreatic duct and ductal adenocarcionoma of the pancreas were observed predominantly in the cytoplasmic perinuclear regions. Normal pancreatic duct cells were stained moderately positive (fig. 1a). In addition, inflammatory pancreatic ducts and hyperplastic or dysplastic pancreatic epithelium were stained similar to normal pancreatic ducts (fig. 1b, c). The cancerous portion in 24 cases showed a weaker signal compared with the noncancerous ducts in the same specimen; therefore, these cases were regarded as the low-intensity group (fig. 1d). Thirty-five

Table 1. Relationship between GalNAc-T3 expression and clinicopathologic factors of patients with pancreatic adenocarcinoma

Factors	Total cases	GalNAc-T3 expression		p value
		low intensitiy	high intensity	
Age, years				
≦60	15	6 (40.0)	9 (60.0)	NS
>60	44	18 (40.1)	26 (59.1)	
Gender				
Male	33	16 (48.5)	17 (51.5)	NS
Female	26	8 (30.8)	18 (69.2)	
Location of tumor				
Head	47	20 (42.6)	27 (57.4)	NS
Body/tail	12	4 (33.3)	8 (66.7)	
T (pTNM)				
T1	3	1 (33.3)	2 (66.7)	<0.05[a]
T2	3	1 (33.3)	2 (66.7)	
T3	30	9 (30.0)	21 (70.0)	
T4	23	13 (56.5)	10 (43.5)	
Differentiation				
Well	29	5 (17.2)	24 (82.8)	<0.0001
Moderately	24	13 (54.2)	11 (45.8)	
Poorly	6	6 (100)	0 (0)	
Lymph node metastasis				
Absent	23	9 (39.1)	14 (60.9)	NS
Present	36	15 (41.7)	21 (58.3)	
Stage (pTNM)				
I	1	0 (0)	1 (100)	<0.05[b]
II	15	4 (26.7)	11 (73.3)	
III	20	7 (35.0)	13 (65.0)	
IV	23	13 (56.5)	10 (43.5)	

Figures in parentheses indicate percentages.
[a] T1 + T2 + T3 vs. T4.
[b] I + II + III vs. IV.

cases showed similar or higher signal, and were regarded as the high-intensity group (fig. 1e, f). Four cases showed a heterogeneous staining pattern with high intensity in the main part of the tumor, whereas low-intensity staining in some part of the tumor. These cases were included in the high-intensity group.

Relationship between GalNAc-T3 Staining Status and Clinicopathologic Features of Pancreatic Adenocarcinomas

The correlations between GalNAc-T3 expression and clinicopathologic features in ductal adenocarcinomas of the pancreas are summarized in table 1. GalNAc-T3 staining status significantly affected the differentiation of the tumor and T factor of the TNM classification ($p < 0.001$ and $p < 0.05$, respectively). The rate of high-intensity staining was 24/29 (82.8%) in well-differentiated adenocarcinoma, 11/24 (45.8%) in moderately differentiated adenocarcinoma, and 0/6 (0%) in poorly differentiated adenocarcinoma. Thirteen out of 24 cases (54.2%) with low GalNAc-T3 staining intensity were classified as T4 of the TNM classification, compared with 10 of 35 (28.6%) of the high-expression group.

Uni- and Multivariate Analyses for Prognostic Factors in Pancreatic Adenocarcinoma

The 5-year overall survival rates of all the patients were 31.8%. GalNAc-T3 expression did not show a prognostic significance for the overall survival of patients with adenocarcinoma of the pancreas (table 2). Uni- and multivariate analyses revealed differentiation of the tumor and the presence of lymph node metastasis as independent prognostic factors (tables 2, 3).

Table 2. Univariate analysis of clinicopathologic factors for overall survival of patients with pancreatic adenocarcinoma

Factors	Patients	1-year overall survival rate %	3-year overall survival rate %	5-year overall survival rate %	p value
Age, years					
≦60	15	79.4	64.2	64.2	NS
>60	44	80.2	55.9	22.4	
Gender					
Male	33	84.1	63.8	19.8	NS
Female	26	82.7	64.4	33.3	
Location of tumor					
Head	47	74.6	61.2	33.2	NS
Body/tail	12	100	48.5	0	
Differentiation					
Well/moderately	53	85.7	61.7	29.2	<0.05
Poorly	6	33.3	33.3	33.3	
Lymph node metastasis					
Absent	23	86.4	66.5	43.6	<0.05
Present	36	75.4	52.0	10.8	
T (pTNM)					
T1	3	100	66.7	66.7	NS
T2	3	100	66.7	66.7	
T3	30	85.1	61.1	36.4	
T4	23	67.4	55.3	17.3	
GalNAc-T3 expression					
Low intensity	24	72.2	67.0	33.5	NS
High intensity	35	84.6	53.9	30.4	

Table 3. Multivariate analysis of clinicopathologic factors for overall survival of patients with pancreatic adenocarcinoma

Factors	Relative risk	95% CI	χ^2 value	p value
Lymph node metastasis				
Absent	3.35	1.19–2.93	7.86	<0.005
Present				
Differentiation				
Well/moderately	5.65	1.24–4.13	6.25	<0.05
Poorly				

Discussion

A close relationship between the alterations of O-glycans in cancer phenotype such as differentiation, invasiveness, and adhesiveness of the tumor cells has been reported [12]; however, the biosynthesis of O-glycans is a multifaceted process, and the precise mechanism affecting the transformation of cancer cells has not been clarified yet. MUC1 is one of the main mucin in the pancreas, and the frequent alterations of O-glycans of MUC1 changes the biological characteristics of tumor cells [12]. Thus, studies of the expression of glycosyltransferases, which catalyze the first step to O-glycans, in pancreas cancer with data of clinical characteristics may reveal the effect of O-glycosylation on abnormal biological features of cancer such as cancerous transformation, differentiation, and progression.

The expression of GalNAc-T3 is highly tissue specific and is detected in organs with secretary epithelial cells [16], and it can be correlated with the degree of differentiation in the adenocarcinoma cell line including pancreatic cancer cells [22]. Therefore, it could be hypothesized that

an aberrant expression of GalNAc-T3 in human pancreas cancer has a functional role in the differentiation and progression. It might be helpful for predicting the progressive potential of tumor cells, and a more effective treatment approach. Consistent with the hypothesis described above, our results demonstrate that there is a significant positive relationship between high expression of GalNAc-T3 and well histological differentiation in surgically resected pancreatic adenocarcinomas; this result is coherent with previous studies of pancreatic adenocarcinoma cell lines [22], colorectal cancers [24], and non-small-cell lung cancers [28].

Our immunohistochemical analysis shows 24 cases out of 29 well-differentiated adenocarcinomas, and none of the 6 poorly differentiated adenocarcinomas were of high intensity. Studies of specimens from the breast and colorectum showed that GalNAc-T3 is expressed to a significant degree in breast carcinoma and normal colorectal epithelium, but not in normal mammary epithelium [23]. Combined with this report, our results may imply that GalNAc-T3 is expressed in active and mature glandular tissue such as from the pancreas and colorectum, but not in silent glandular tissue such as from the breast, and that the GalNac-T3 expression in pancreatic adenocarcinomas is influenced by the tumor differentiation. This result is contrary to a previous report about the expression of GalNAc-T3 in colorectal carcinoma [24], where GalNAc-T3 expression was decreased in most tumors compared with normal colorectal mucosa and tumor tissue contained less carbohydrate than normal mucosa. In our present study, more than 80% of the cases with well-differentiated adenocarcinoma of the pancreas showed equal to higher GalNAc-T3 expression compared with normal counterparts. In the pancreas, tumors and tumor cell lines overexpress MUC1 [31], being associated with increased expression of carbohydrate SLe^a and SLe^x in the tumorigenesis [32]. Therefore, differences in the results from colorectal carcinomas and pancreatic carcinomas may be due to the dissimilar character of both carcinomas, and the amount of carbohydrate would be different. The expression of GalNAc-T3 may differ in different organs and further investigation for other types of carcinomas is needed.

A significant correlation was observed between GalNAc-T3 expression and the T factor of the pTNM staging system. Pancreatic adenocarcinomas with low intensity showed higher rates of T4 compared with the high-intensity group, which implies that the low-intensity group with poorer differentiation compared with the high-intensity group has a more progressive and invasive behavior. Our preliminary findings with pancreatic cancer cells transfected with *GalNAc-T3* showed the suppressed growth of the transfected cells compared with cells transfected with the vector alone, suggesting a reverse association of GalNAc-T3 expression with cell proliferation probably according to the differentiation of pancreatic ductal cancer cells. However, there were no differences in overall survival between the two groups, which might be due to relatively small numbers of patients in the present study, and a poor prognosis for patients with pancreatic adenocarcinoma regardless of the tumor stage.

In summary, the present findings show that low GalNAc-T3 expression in pancreatic adenocarcinoma is associated with a poorer histologic phenotype and a high incidence of T4 of the pTNM staging system, namely, progressive and invasive characteristics. Further investigation with more clinical samples could help clarify the significance of GalNAc-T3 expression in the outcome of ductal carcinoma of the pancreas.

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Increased Expression of Valosin-Containing Protein (p97) is Associated With Lymph Node Metastasis and Prognosis of Pancreatic Ductal Adenocarcinoma

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Background: Valosin-containing protein (VCP, also known as p97) exhibits antiapoptotic function and metastasis by activation of nuclear factor kappa-B signaling pathway. Our previous study showed that VCP expression level correlated with prognosis of hepatocellular and gastric carcinoma. In the present study, association of VCP expression with lymph node metastasis and prognosis of pancreatic ductal adenocarcinoma (PDAC) was examined.

Methods: VCP expression in 83 patients (46 males and 37 females) of ages ranging from 43 to 80 (median, 66) years who had undergone curative surgery for primary PDAC was analyzed by immunohistochemistry, in which staining intensity in tumor cells was categorized as weaker or equal to (low expression) or stronger (high expression) than that in noncancerous ductal tissue.

Results: Thirty-two tumors (38.6%) and 51 tumors (61.4%) were classified as low-VCP-expressing and high-VCP-expressing tumors, respectively. VCP expression correlated significantly with lymph node metastasis ($P < .01$) but not with various clinicopathologic factors, including age, gender, and histologic differentiation. Multivariate analysis revealed VCP expression as an independent prognosticator for both disease-free and overall survival, along with histologic differentiation, T stage of pathologic tumor-node-metastasis (pTNM) classification, and lymph node metastasis. Furthermore, VCP expression was a prognosticator for disease-free and overall survival in each relatively early stage (I or II) and advanced stage (III) group of pTNM classification.

Conclusions: Our results indicate the potential usefulness of VCP expression as a marker of metastasis and overall prognosis of PDAC.

Key Words: Pancreatic adenocarcinoma—Prognosis—Valosin-containing protein—Lymph node metastasis.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common causes of cancer deaths; the incidence of PDAC ranks fifth as a cause of cancer mortality in Western countries.[1,2] Despite recent advances in diagnostic and therapeutic modalities, the prognosis of PDAC is poor.[3,4] Although only surgical resection offers patients an opportunity to live longer and possibly be cured, only 15% to 20% of patients with PDAC have a resectable tumor at the time of diagnosis.[5,6] In fact, complete removal of macroscopically detectable cancer tissues does not prevent early tumor recurrence.[2,6] Such recurrence probably arises from growth of occult cancer cells that had already invaded or metastasized out of the surgical region by the time of surgery.[7,8] Therefore, understanding the biologic basis of tumor aggressiveness and the metastatic potential of PDAC is important and could allow for better therapeutic approaches.

Previous studies suggested that multiple subsets of genes are either activated or inactivated during develop-

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ment and progression of PDAC.[9-11] Frequent genetic alterations reported to occur in PDAC include K-ras oncogene,[9] tumor suppressor genes such as p53,[10] and growth factors such as epidermal growth factor.[11] However, the exact underlying mechanisms of the progression of pancreatic cancer are not yet understood.

Recently, we identified the gene encoding valosin-containing protein (VCP, also known as p97) associated with metastasis of murine osteosarcoma cell line by using the mRNA subtraction technique.[12] VCP, a member of the superfamily of ATPases associated with various cellular activities, is involved in the ubiquitin-dependent proteasome degradation pathway of inhibitor $\kappa B\alpha$ ($I\kappa B\alpha$), an inhibitor of $NF\kappa B$.[13] Murine osteosarcoma cells transfected with VCP gene showed constant activation of $NF\kappa B$, rapid degradation of p-$I\kappa B\alpha$, decreased apoptosis rates after $TNF\alpha$ stimulation, and increased metastatic potential.[12] Although persistent activation of $NF\kappa B$ has been reported in some cases of PDAC and with PDAC cell lines,[14] little is known about the role of VCP in human malignant tumors, including PDAC. Indeed, our previous study showed that VCP expression level correlated with the recurrence rate and prognosis of hepatocellular carcinoma, in which hematogenous metastasis is considered to be the principal pattern of cancer spread.[15]

In the present study, we examined the expression of VCP in patients with curatively resected PDAC by immunohistochemical analysis to clarify its correlation with clinicopathologic factors and postoperative survival.

PATIENTS AND METHODS

Patients and Tissue Samples

Surgical specimens were obtained from 83 patients who had undergone curative resection for primary PDAC at the Department of Surgery and Clinical Oncology, Osaka University Graduate School of Medicine, and Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Disease, during the period of June 1982 to December 2001. Curative resection was defined as the complete resection of tumorous lesions, with microscopic negative surgical margin. The patients included 46 males and 37 females of ages ranging from 43 to 80 (median, 66) years. The stage of the disease was classified according to the pathologic tumor-node-metastasis (pTNM) staging system.[16]

Resected specimens were examined macroscopically to determine the location and size of tumor. Then tissue samples were fixed in 10% formalin and routinely processed for paraffin embedding. Histologic sections were cut at 4-μm thickness and stained with hematoxylin and eosin and reviewed by two investigators (YT and YH) to determine histologic differentiation and existence of metastasis to the lymph nodes. Forty-four cases were well-differentiated adenocarcinoma, 32 were moderately differentiated adenocarcinoma, and seven were poorly differentiated adenocarcinoma.

After surgery, we followed-up with measurement of serum carcinoembryonic antigen and carbohydrate antigen 19-9 levels, ultrasonography, and computed tomography at about 3- to 6-month intervals. Adjuvant chemotherapy was administered to 24 patients. Chemotherapeutic protocols were as follows: mitomycin C injection via portal vein in 2 patients; 5-fluorouracil via hepatic artery alone in 5, via portal vein alone in 2, and via combined hepatic artery and portal vein in 11; and oral medication in 5. Radiotherapy was administered to 18 patients. Five patients received combined chemotherapy and radiotherapy. In total, 37 patients received adjuvant therapy and 46 patients did not. The patients were followed-up until April 2003; the follow-up period for survivors ranged from 17.1 to 119.0 (median, 40.4) months after surgery.

Immunohistochemical Analysis

Immunohistochemistry was performed with paraffin-embedded tissue sections by means of the immunoperoxidase procedure (avidin-biotin-complex method). In brief, antigen retrieval was performed by heating the deparaffinized rehydrated sections in 10 mM citrate buffer for 5 minutes. Mouse monoclonal anti-VCP (p97) antibody (PROGEN Biotechnik, Heidelberg, Germany) was used as the primary antibody at a final dilution of 1:3000. Sections were lightly counterstained with methyl green. For negative controls, nonimmunized mouse IgG (Vector Laboratories, Burlingame, CA) was used as the primary antibody. Stained sections were evaluated in a blinded manner by two investigators (SY and YT) without prior knowledge of the clinicopathologic features of patients. Staining intensity in the cytoplasm of tumor cells was categorized as follows: weaker or equal to (low expression) or stronger (high expression) than that in noncancerous pancreatic ductal cells, which served as the positive control. When the staining intensity of tumor cells varied in different areas of the same specimen, the predominant pattern was chosen as the expression level.

The strong correlation of VCP expression between mRNA level, as determined by reverse transcription polymerase chain reaction (RT-PCR) or in situ hybridization (ISH), and protein level, as determined by immunohistochemistry, has been described previously.[15,17]

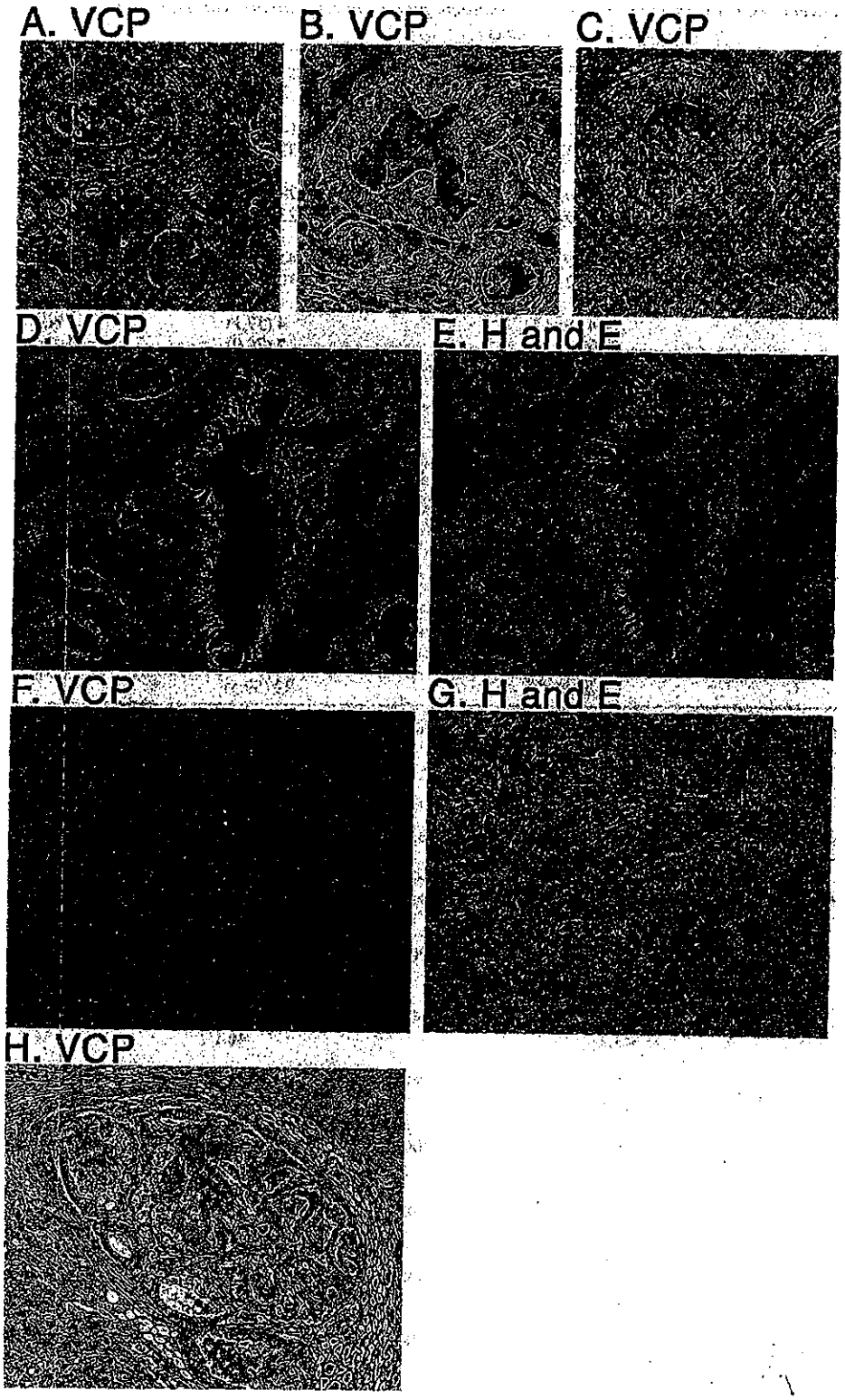

FIG. 1. (A) Positive control of valosin-containing protein (VCP) staining of normal pancreatic ductal cells (magnification, ×400). (B) Well-differentiated adenocarcinoma of the pancreas with high VCP expression. Tumor cells show strong VCP staining (magnification, ×400). (C) Well-differentiated adenocarcinoma of the pancreas with low VCP expression. Tumor cells are faintly stained with VCP (magnification, ×400). (D) Well-differentiated adenocarcinoma of the pancreas with strong VCP staining. (E) Hematoxylin and eosin staining of the same section (magnification, ×33). (F) Poorly differentiated adenocarcinoma of the pancreas with faint VCP staining and (G) hematoxylin and eosin staining of the same section (magnification, ×33). (H) Lymph node with metastatic pancreatic adenocarcinoma with strong VCP expression (magnification, ×33).

TABLE 1. *Relationship between VCP expression and clinicopathologic factors of 83 patients with pancreatic ductal adenocarcinoma*

Factors	Total no. of patients	Patients with low VCP expression (%)	Patients with high VCP expression (%)	*P* value
Age (y)				
≤60	24	10 (41.7)	14 (58.3)	NS
>60	59	22 (37.3)	37 (62.7)	
Gender				
Male	46	18 (40.0)	28 (60.0)	NS
Female	37	14 (37.3)	23 (62.7)	
Tumor location				
Head	66	25 (37.9)	41 (62.1)	NS
Body/tail	17	7 (41.2)	10 (58.8)	
T (pTNM)				
T1	7	3 (42.9)	4 (57.1)	NS
T2	10	3 (30.0)	7 (70.0)	
T3	40	17 (42.5)	23 (57.5)	
T4	26	9 (34.6)	17 (65.4)	
Histologic differentiation				
Poorly	7	1 (14.3)	6 (85.7)	NS
Moderately	32	11 (34.4)	21 (65.6)	
Well	44	20 (45.5)	24 (54.5)	
Lymph node metastasis				
Absent	35	20 (57.1)	15 (42.9)	<.01
Present	48	12 (25.0)	36 (75.0)	
Stage (pTNM)				
I	9	6 (66.7)	3 (33.3)	NS
II	48	17 (35.4)	31 (64.6)	
III	26	9 (34.6)	17 (65.4)	
Adjuvant therapy				
Not performed	46	17 (37.0)	29 (63.0)	NS
Performed	37	15 (40.5)	22 (59.5)	

VCP, valosin-containing protein; NS, not significant; pTNM, pathologic tumor-node-metastasis.

TABLE 2. *Univariate analysis of clinicopathologic factors for disease-free and overall survival of patients with pancreatic ductal adenocarcinoma*

Factors	No. of patients	5-y disease-free survival rate %	*P* value	5-y overall survival rate %	*P* value
Age (y)					
≤60	24	45.5	NS	48.4	NS
>60	59	27.5		31.4	
Gender					
Male	46	23.0	NS	27.8	NS
Female	37	43.2		46.1	
Tumor location					
Head	66	35.7	NS	38.7	NS
Body/tail	17	18.8		20.8	
Histologic differentiation					
Poorly	7	14.3	<.0001	14.3	<.001
Well/moderately	76	34.0		37.6	
Lymph node metastasis					
Absent	35	50.7	<.001	53.6	<.01
Present	48	17.4		19.1	
T (pTNM)					
T1–T2	17	57.8	<.05	54.5	<.05
T3–T4	66	23.4		28.8	
VCP expression					
Low expression	32	48.3	<.001	59.0	<.001
High expression	51	22.0		21.3	
Adjuvant therapy					
Not performed	46	30.6	NS	35.2	NS
Performed	37	33.8		36.4	

NS, not significant; pTNM, pathologic tumor-node-metastasis; VCP, valosin-containing protein.

Statistical Analysis

Statistical analyses were performed with JMP software (SAS Institute, Cary, NC). χ^2 and Fisher's exact probability tests were used to analyze the correlation between VCP expression and immunohistochemistry and clinicopathologic features. Kaplan-Meier methods with log-rank test were used to calculate overall survival rate and differences in survival curves.[18] Cox's proportional hazards regression model with stepwise analysis was used to analyze the independent prognostic factors.[19] P values of $<.05$ were considered statistically significant.

RESULTS

Expression of VCP in PDAC

Immunohistochemical assays were performed on tissues from 83 patients with PDAC and matched nontumor tissues from the same section. Positive control sections of normal ductal cells of the pancreas were stained moderately, irrespective of the presence of pancreatitis (Fig. 1A). In both normal pancreatic ducts and PDAC, VCP staining was observed in the cytoplasm (Figs. 1A and 1B). Twenty-seven cases showed low VCP expression in cancer cells in every area of the tumor, whereas four cases showed high expression at the peripheral zone of the tumor but low expression in the larger central area of the tumor. Overall, 32 cases (38.6%) were classified in the low-expression group (Figs. 1C, 1F, and 1G). The remaining 51 (61.4%) showed constant high expression in the tumor and were classified in the high-expression group (Figs. 1B, 1D, and 1E). Metastatic lymph nodes with PDAC were examined immunohistochemically, and 10 of 11 lymph nodes were strongly stained with VCP monoclonal antibody (Fig. 1H).

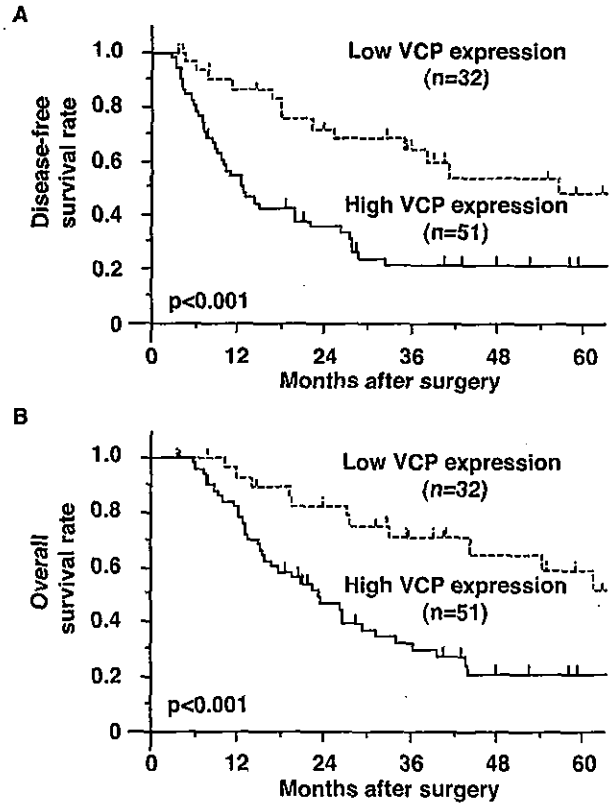

FIG. 2. Disease-free (A) and overall (B) survival of patients with low and high valosin-containing-protein (VCP)-expressing pancreatic ductal adenocarcinomas. Significant difference was observed between the two groups.

Relationship Between VCP Staining Status and Clinicopathologic Features

Table 1 shows the relationship between various clinicopathologic features and VCP expression. There were no significant differences between low- and high-VCP expression groups with regard to age, gender, location of primary tumor, T factor and stage of pTNM classification, histologic differentiation, and adjuvant therapies, with the exception of presence of lymph node metastasis ($P < .01$).

TABLE 3. *Multivariate analysis of clinicopathologic factors for disease-free and overall survival of patients with pancreatic ductal adenocarcinoma*

Factors	Relative risk	95% CI	χ^2 value	P value
Disease-free survival				
VCP expression				
Low expression	2.28	1.09–2.15	6.36	<.05
High expression				
Lymph node metastasis				
Absent	2.27	1.10–2.11	6.63	<.01
Present				
Histologic differentiation				
Well/moderately	5.57	1.43–3.61	9.65	<.01
Poorly				
T (pTNM)				
T1–T2	2.14	1.00–2.30	3.92	<.05
T3–T4				
Overall survival				
VCP expression				
Low expression	2.42	1.11–2.26	6.65	<.01
High expression				
Lymph node metastasis				
Absent	2.42	1.12–2.21	7.02	<.01
Present				
Histologic differentiation				
Well/moderately	6.43	1.49–4.04	10.3	<.01
Poorly				
T(pTNM)				
T1–T2	2.32	1.05–2.39	4.90	<.05
T3–T4				

CI, confidence interval; pTNM, pathologic tumor-node-metastasis; VCP, valosin-containing protein.

Univariate and Multivariate Analyses of Prognostic Factors in PDAC

Five-year disease-free and overall survival rates were 31.9% and 35.0%, respectively. Forty-seven patients died with a tumor and 51 had recurrence of a tumor: in the liver in 21, lymph node in 24, peritoneum in 2, and other organs in 4.

The prognostic significance of VCP expression was analyzed for disease-free and overall survival rates. Patients with low VCP expression had better 5-year survival rates than those with high expression (disease-free: 48.3% vs. 22.0%; overall: 59.0% vs. 21.3%; $P < .001$ and $P < .01$, respectively) (Table 2, Fig. 2). Univariate analysis revealed that VCP expression level, presence of lymph node metastasis, histologic differentiation, and T stage of pTNM staging system were significant prognosticators for both disease-free and overall survival (Table 2).

Multivariate analysis with factors proven to be significant in the univariate analysis revealed that VCP expression level, presence of lymph node metastasis, histologic differentiation, and T stage of pTNM staging system were independent prognostic factors for both disease-free and overall survival (Table 3). Indeed, there was a significant difference in disease-free survival and overall survival rates between patients with low- and high-VCP expression when the patients were divided into the relatively early (stage I or II) and advanced (stage III) groups of pTNM classification (Fig. 3).

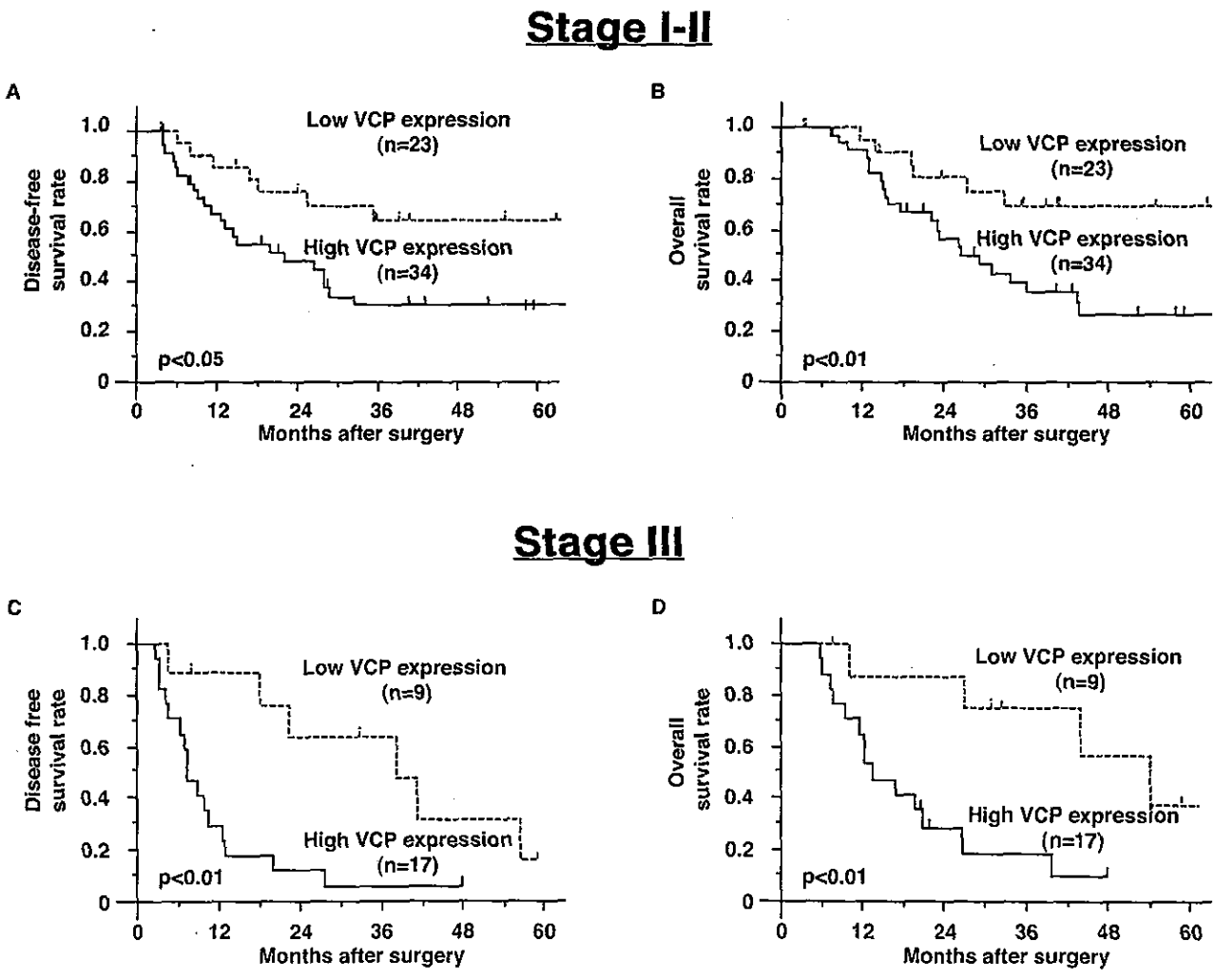

FIG. 3. Disease-free and overall survival of patients with low and high valosin-containing-protein (VCP)-expressing pancreatic ductal adenocarcinomas classified as early stage (I or II; A, disease-free, B, overall) or advanced stage (III; C, disease-free, D, overall) in pathologic tumor-node-metastasis (pTNM) staging. Significant difference was observed between the two groups for both stages.

DISCUSSION

Prediction of the clinical course of patients with PDAC on the basis of pathobiological differences of tumors at surgery could provide important information for clinicians.[5,6] Several clinicopathologic variables, such as tumor size, histologic differentiation, lymph node metastasis, and extent of portal system involvement and retroperitoneal invasion, have been recognized as prognostic indicators.[20] We undertook the present work in an effort to determine whether increased VCP expression is a valid biologic indicator for aggressiveness of PDAC. The prognostic value of VCP cannot be realized until analysis of patients with malignant tumors suggests a possible relationship between their expression and clinical outcome, as determined by disease recurrence or, consequently, patient survival.

Our results clearly demonstrated that the expression of VCP correlated significantly with lymph node metastasis of PDAC. Furthermore, the majority of lymph node metastases originating from PDAC exhibited high expression of VCP. The immune response against tumor takes place at the draining lymph nodes, where dendritic cells activate naive T lymphocytes, which in turn attack cancer cells through the secretion of various cytokines such as TNF.[21] VCP-expressing cancer cells might be resistant to such immunologic attacks via the antiapoptotic NFκB signaling pathway, eventually allowing their survival in lymph nodes.

Univariate and multivariate analyses revealed that the VCP expression level is an independent prognosticator for PDAC. In fact, VCP expression level proved to be a prognosticator for PDAC in patients at both the relatively early stage (I or II) and advanced stage (III) of pTNM classification; 5-year overall survival rates for patients with low and high VCP expression were 70.0% and 27.7% in the early stage group and 37.5% and 8.9% in the advanced stage group, respectively. A combination of VCP expression level and pTNM staging would be more useful for stratifying patients at high or low risk for tumor recurrence. Because the present study involved patients receiving different types of treatment, the prognostic value of VCP expression is less meaningful than for patients treated in the same chemoradiation protocol.

Recent studies showed that gemcitabine-based chemotherapy improved the prognosis of PDAC.[22,23] Immunostaining of surgical specimens of PDAC for VCP could be a valuable guide in clinical decision-making about appropriate adjuvant therapies. For patients with low-VCP-expressing PDAC at an early stage, a favorable outcome could be expected without adjuvant therapies, but patients with high-VCP-expressing and/or advanced-stage PDAC should be treated intensively with adjuvant therapies.

In conclusion, we identified VCP as a new biological marker of aggressive PDAC and noted that the expression of VCP correlated significantly with lymph node metastasis and prognosis of PDAC. Immunohistochemical analysis of VCP could be a useful marker in predicting the postoperative prognosis of PDAC. These findings set the stage for future studies about the exact role of VCP in PDAC.

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