Clinicopathological Significance of Abnormalities in Gadd45 Expression and Its Relationship to p53 in Human Pancreatic Cancer

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ABSTRACT

Purpose: **The *growth arrest and DNA damage-inducible 45* gene (*GADD45a*) is one of the downstream mediators of the *p53* gene that stimulates DNA excision repair. The present study was designed to assess the clinicopathological significance of *GADD45a* and p53 in resectable invasive ductal carcinomas (IDCs) of the pancreas.**

Experimental Design: **This study included 72 pancreatic IDC patients who received surgery between 1982 and 2001. Point mutations in exons 1 and 4 of *GADD45a* and the expression of the *GADD45a* gene product (Gadd45) and p53 protein were analyzed by direct DNA sequencing and immunohistochemistry.**

Results: **Point mutations were found in exon 4 of *GADD45a* in eight cases (13.6%). Gadd45 and p53 were expressed in 54.2% (39 of 72) and 47.2% (34 of 72) of the patients. The expression of Gadd45 did not necessarily correlate with that of p53. However, Gadd45 expression correlated significantly with the grade of the pT factor of the tumors. Coexpression analysis of Gadd45 and p53 indicated that in patients with p53(+) IDC, the Gadd45(+) group had a significantly lower survival rate than the Gadd45(−) group. Furthermore, Gadd45 expression had no effect on the efficacy of the adjuvant chemotherapy. Multivariate analysis indicated that pTNM (tumor-node-metastasis) stage, grade, and adjuvant chemotherapy were significant variables for survival. Furthermore, in the p53(−) group, there were no significant variables. In contrast, in the p53(+) group, pTNM stage, histological grade, and Gadd45 expression were significant variables.**

Conclusions: **The frequency of *GADD45a* mutation is appreciable in human pancreatic IDC, and the expression of Gadd45, combined with that of p53, significantly affects the survival of patients with resectable IDCs of the pancreas.**

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INTRODUCTION

Pancreatic cancer is one of the most common causes of cancer death in developed countries (1). IDC[2] of the pancreas is highly aggressive and malignant, with an extremely poor prognosis. One of the reasons for this malignant potential is that the biological characteristics of this tumor may be quite different from those of other carcinomas.

We have studied the genetic etiology of pancreatic IDC, especially its association with the efficacy of chemotherapy. Our previous studies showed that mutations in the Ki-*ras* gene and overexpression of various apoptosis-associated genes such as *CDKN1A* (*Waf1, p21*), B-cell lymphoma/leukemia-2 (*Bcl-2*), and *Bax* and that of various growth factors such as epidermal growth factor, epidermal growth factor receptor, and transforming growth factor β were associated with the progression of pancreatic IDC (2–8). An analysis of their interrelationships with p53-associated genes might be beneficial in evaluating the efficacy of adjuvant therapy for pancreatic IDC (2, 3, 8).

Depending on the nature of the DNA insult, checkpoints delay the transitions from G_1 to S or G_2 to M and may inhibit DNA replication during S phase. Presumably, these delays allow time for DNA repair before entry into the S and M phase, respectively (9–11). The *p53* gene induces G_1 arrest and/or apoptosis through either its downstream mediators such as Bax or WAF/1 or its upstream mediators such as mouse double min 2 (MDM2). In addition, the *p53* gene is required for the G_1 checkpoint and functions to up-regulate expression of the *growth arrest and DNA damage-inducible 45* gene (*GADD45a*) and WAF/1-p21 (12, 13). *GADD45a* is also one of the downstream mediators of p53 and deactivates p53 that contributes to cell cycle regulation through binding with both cyclin-dependent kinases and proliferating cell nuclear antigen (14, 15). *GADD45a* stimulates DNA excision repair when cellular DNA is damaged (14). To our knowledge, there is only one report on the mutation of *GADD45a*, which reported that no mutations were seen in human breast cancer (16) and in various human tumor cell lines (17), and the clinicopathological significance of expression of GADD45a protein (Gadd45) and its relationship with p53 protein in the progression of various malignancies is unclear.

The immunoreactivity of the p53 protein indicates the loss of normal function of p53 and its associated genes. These findings allow us to easily evaluate the status of the *p53* gene by immunohistochemistry.

The present study aims to clarify the clinicopathological significance of *GADD45a* abnormalities (mutation and protein

[2] The abbreviations used are: IDC, invasive ductal carcinoma; ACT, adjuvant chemotherapy; TNM, tumor-node-metastasis; Ab, antibody.

expression) and their relationship to the upstream mediator p53 in 72 patients with resectable IDC of the pancreas.

MATERIALS AND METHODS

Patients. Seventy-two patients (38 females and 34 males; age range, 35–80 years; mean age, 65.4 years) with pancreatic IDC underwent pancreatectomies between 1982 and 2001 at the First Department of Surgery, Shimane Medical University. The patients' profiles are summarized in Table 1. A standard or pylorus-preserving pancreatoduodenectomy was performed in 38 patients, a distal pancreatectomy was performed in 21 patients, and a total pancreatectomy was performed in 13 patients. The tumors were staged according to the International Union Against Cancer classification (TNM classification; Ref. 18). None of the patients received any type of treatment before their surgical procedures. Forty-one of the patients received ACT after their surgery, and most patients were given 5-fluorouracil or its derivatives, alone or with cyclophosphamide, and some received intensive regimens including Adriamycin and cisplatin.

Extraction of Genomic DNA. In a 1.5-ml Eppendorf tube, six slices of 5-μm sections from formalin-fixed and paraffin-embedded specimens were used to extract the genomic DNA as described previously (19). One ml of xylene was added, and the tube was centrifuged at 12,000 rpm at room temperature for 3 min, three times, in a refrigerated centrifuge MR-15A (Tomy Seiko Co., Ltd., Tokyo, Japan). The supernatant was discarded, and 1 ml of ethanol was added. After centrifuging at 12,000 rpm at room temperature for 3 min, three times, the suspension was discarded, and the precipitate was dried under a vacuum at 55°C for 10 min. Three hundred μl of lysis buffer containing proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 50–100 μg/ml were added, and the mixture was then shaken for 36 h at 37°C in Thermomix D-3508 (B. Braun Melsungen AG, Melsungen, West Germany). During this time, 50 μl of proteinase K solution at 1 mg/ml [diluted with Tris-EDTA buffer (pH 8.0)] were added three times every 12 h. An equal volume of phenol, chloroform, and isoamyl alcohol at a proportion of 25:24:1 was added and mixed at 20–40 rpm by Rotator RT-50 (TAITEC Co., Tokyo, Japan) for 30–60 min at room temperature. After centrifuging at 12,000 rpm at 4°C for 5 min, the supernatant was transferred to a new tube. This step was performed three to five times, until the supernatant became clear. An equal volume of chloroform and isoamyl alcohol at 24:1 was added and mixed at 20–40 rpm by a rotator at room temperature for 20 min. After centrifuging at 12,000 rpm at 4°C for 5 min, the supernatant was transferred to a new tube, and 0.1 of the above volume of 3 M sodium acetate (pH 7.4) and 2.5× the above volume of −20°C ethanol were added and mixed. After precipitation at −80°C for 30 min, the sample was centrifuged at 12,000 rpm at 4°C for 10 min, the supernatant was discarded, and 80% ethanol (1.5 ml) was added. After centrifuging at 12,000 rpm at 4°C for 10 min, the supernatant was discarded, and the precipitate was dried under a vacuum for 10 min at 55°C. The extracted DNA was dissolved in 449.1 μl of TE buffer solution (pH 8.0), and then 0.9 μl of 10 mg/ml RNase A (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to a total of 500 μl (the final working solution contained RNase A at 20 μg/ml). After incubating at 37°C for 60 min, the genomic DNA was purified again as described above.

Table 1 Gadd45 expression and clinicopathological characteristics

Feature	No. of patients	No. expressing (%)	
		Gadd45(+)	p53(+)
Overall	72	39/72 (54%)	34/72 (47%)
Age (yrs)			
<65	28	15/28 (54%)	9/28 (32%)
≥65	44	24/44 (55%)	25/44 (57%)
Gender			
Male	34	17/34 (50%)	15/34 (44%)
Female	38	22/38 (58%)	19/38 (50%)
Grade			
1 (well differentiated)	35	20/35 (57%)	17/35 (49%)
2 (moderately differentiated)	32	16/32 (50%)	14/32 (44%)
3 (poorly differentiated)	5	3/5 (60%)	3/5 (60%)
4 (undifferentiated)	0	0	0
UICC pTNM stage (1997)			
I	10	5/10 (50%)	6/10 (60%)
II	4	1/4 (25%)	0/4 (0%)
III	36	17/36 (47%)	14/36 (39%)
IV	22	16/22 (73%)	14/22 (64%)
pT (primary tumor)[a]			
1	5[b]	1/5 (20%)	4/5 (80%)
2	25	11/25 (44%)	12/25 (48%)
3	23	12/23 (52%)	6/23 (26%)
4	19[b]	15/19 (79%)	12/19 (63%)
Nodal involvement			
−	21	10/21 (48%)	10/21 (48%)
+	51	29/51 (57%)	24/51 (47%)

[a] 1, limited to pancreas, <2.0 cm; 2, limited to pancreas, >2.0 cm; 3, extended to peripancreatic structures including the duodenum, bile duct, mesentery, mesocolon, omentum, and peritoneum; 4, extended to adjacent structures including the stomach, spleen, colon, portal vein, celiac artery, and the superior mesenteric and common hepatic arteries and veins.

[b] $P < 0.05$ by χ^2 test.

PCR and DNA Sequencing. The *GADD45a* gene fragment was amplified by PCR. Exon 1 and exon 4 were 265 and 397 bp, respectively. The PCR primers used were 5′-GCCTGTGAGTGAGTGCAGAA-3′ (sense) and 5′-GGAGTT GCCCTGTGCAAACT-3′ (antisense) for exon 1 and 5′-GAACCCAACTACCTTGAAGA-3′ (sense) and 5′-CCCCTTGGCATCAGTTTCTG-3′ (antisense) for exon 4. The sequencing primers were 5′-TAGCCGTGGCAGGAGCAG-3′ (sense) for exon 1 and 5′-TGTCT CCATGTCACATAGCC-3′ (sense) for exon 4.

The PCR was run in 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 2 min with a Thermal cycler (Perkin-Elmer, Norwalk, CT). We used the ABI Prism 310 Genetic Analyzer (Perkin-Elmer Corp., Foster City, CA) for direct DNA sequencing.

Abs. The anti-Gadd45 polyclonal Ab (C-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA) is an affinity-purified rabbit polyclonal Ab raised against a peptide corresponding to the sequence mapping at the COOH-terminal 146–165 amino acids of human Gadd45 and cross-reacts with murine Gadd45. It was used at a dilution of 1:400.

The anti-p53 monoclonal Ab (Ab-6, DO-1) was purchased from Oncogene Science (Uniondale, NY). DO-1 reacts with a stable determinant of p53 and recognizes an epitope between amino acids 37 and 45 (Ser-Leu). DO-1 was produced by

hybridoma cells, which were derived from myeloma cells fused with splenocytes, and then hyperimmunized with recombinant human wild-type-p53 (20). DO-1 has been reported to partially cross-react with denatured mutant-type-p53. DO-1 was diluted to a working concentration of 2 μg/ml for these experiments.

Immunohistochemistry. All specimens were fixed in formalin and embedded in paraffin. Sections 4-μm thick were deparaffinized in xylene for 5 min, three times, and hydrated in 100%, 95%, and 45% ethanol and, finally, in PBS. After antigen retrieval by microwave oven, the endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 in methanol for 15 min, and nonspecific binding was blocked with 10% normal serum for 10 min. The sections were incubated with primary Ab at room temperature for 2 h. After rinsing twice in PBS, the specimens were incubated with a biotinylated secondary Ab (Nichirei Corp., Tokyo, Japan) at 37°C for 15 min, washed twice in PBS, and then incubated with peroxidase-labeled avidin-biotin (Nichirei) for 10 min at room temperature. After rinsing twice in PBS, the immunoreaction of the specimens was visualized with a 0.05% 3,3′-diaminobenzidine (Nichirei) solution for 6–10 min at room temperature. After rinsing in distilled water, the specimens were counterstained with methyl green, dehydrated, and mounted.

Evaluation of Immunostaining. For the evaluation of Gadd45 expression, immunostaining was taken to be positive only when unequivocally strong nuclear staining and cytoplasmic staining were present in >50% of the tumor cells. Those cases with only faint staining were regarded as negative. For evaluation of p53 expression, immunostaining was scored as positive only when the nucleus of the tumor cells was stained (21). According to previous reports, 20% positive was regarded as the cutoff point for p53 positivity (22–24).

Statistical Analysis. The χ^2 test was used to compare the correlations between the clinicopathological factors and the expressions of Gadd45 and p53. The postsurgical status of all patients was surveyed on March 31, 2001. The cumulative survival rates were calculated according to the Kaplan-Meier methods and compared by the Cox-Mantel test. A multivariate analysis of Cox's proportional hazard risk model was used to obtain the conditional risk of death due to IDC of the pancreas. Five patients (two died of bleeding, one died of acute myocardial infarction within 1 month of surgery, and the remaining two died of other diseases) were excluded from the survival statistics. They were included only in the frequencies of Gadd45 and p53 abnormalities. Statistically significant differences were defined as $P < 0.05$.

RESULTS

***GADD45a* Point Mutation.** We investigated *GADD45a* gene mutations in exon 1 for 61 cases and in exon 4 for 59 cases. No mutations were found in exon 1. However, in exon 4, point mutations were seen in 13.6% (8 of 59) of the cases. The point mutations occurred between codons 141 and 159. A representative mutation is shown in Fig. 1 (58-year-old male patient with poorly differentiated adenocarcinoma). GenBank data (Version L24498.1, GI: 403127) were used for the normal control sequence of *GADD45a*. The mutation profiles in eight cases were summarized in Table 2. In eight cases with *GADD45a* mutation,

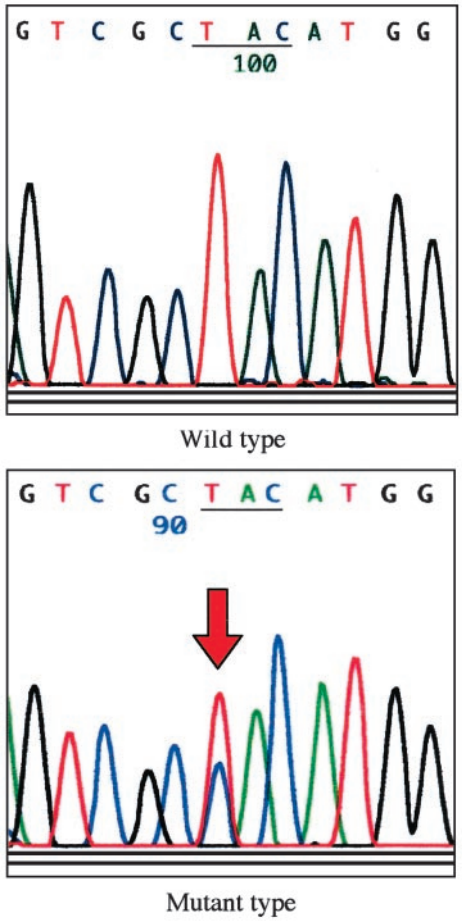

Fig. 1 A representative analysis of a point mutation of *GADD45a* at exon 4. The *arrow* indicates a point mutation from TAC to CAC.

Gadd45 expression was seen in five cases, and p53 expression was seen in one case. There were no correlations between the *GADD45a* mutations and clinicopathological factors including age, gender, histological grade, clinical stage, pT, pN, M, and vascular invasion.

Expression of Gadd45 and p53. Representative immunostaining for Gadd45 and p53 is shown in Fig. 2. Gadd45 and p53 were expressed in 54.2% (39 of 72) and 47.2% (34 of 72) of pancreatic IDCs (Table 1), respectively. There were no correlations between their expressions. Gadd45 expression correlated significantly with histological grade and pT, but p53 expression did not correlate with any factors of the TNM classification (Table 3).

Survival Curves and Gadd45 and p53 Expression. Gadd45 and p53 expression did not have any significant effect on the patient survival (Fig. 3). However, their coexpression showed that in the patients with p53(+) IDC, the Gadd45(+) group had a significantly lower survival rate than the Gadd45(−) group (Fig. 4).

ACT seemed to improve patient survival, but this difference did not reach statistical significance. Neither Gadd45 nor p53 expression showed any effect on the efficacy of ACT (Fig. 5).

Table 2 Mutation of *GADD45a* in exon 4

No.	Codon	Mutation of codon	Amino acid change	Mutant status	Gadd45 expression	p53 expression
1	159	GTG » GCG	Val » Ala	Transversion	−	−
2	157	GTT » GCT	Val » Ala	Transition	+	+
3	147	TGC » TAC	Cys » Tyr	Transition	+	−
4	150	AGT » ATT	Ser » Ile	Transversion	+	−
5	146	TTT » TGA	Phe » Nonsense	Transversion	−	−
6	150	AGT » GGT	Ser » Gly	Transition	−	−
7	141	AGT » ACT	Ser » Thr	Transversion	+	−
8	152	TAC » CAC	Tyr » His	Transition	+	−

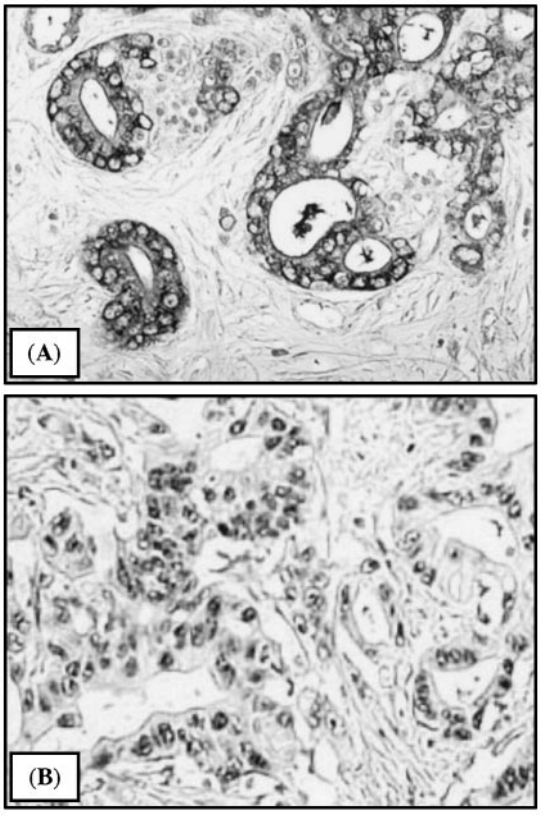

Fig. 2 Immunostaining of Gadd45 and p53. *A*, Gadd45(+) immunostaining. The nuclear membrane and cytoplasm are positively stained. *B*, p53(+) immunostaining. The nucleus is positively stained.

Multivariate Analysis. Multivariate analysis demonstrated that pTNM stage, histological grade, and ACT were significant variables for survival but that Gadd45 expression and p53 expression were not significant variables for survival. When the patients were classified into two groups, the p53(+) group and the p53(−) group, there were no significant variables in the p53(−) group. However, pTNM stage, histological grade, and Gadd45 expression were significant variables in the p53(+) group (Table 4).

DISCUSSION

The *GADD45a* gene is localized to the short arm of human chromosome 1 between p12 and p34 (25). This gene was first isolated and cloned from Chinese hamster cells, and then human *GADD45a* was also cloned (26–29). To our knowledge, only one report on the mutation of *GADD45a* has been published (16), and Blaszyk *et al.* (16) reported that in human breast cancers, there were no mutations over the entire coding region of the *GADD45a* gene. The present study may be the first report on point mutations in the *GADD45a* gene in human malignant cells. We analyzed the mutations in exons 1 and 4 of *GADD45a* by direct DNA sequencing. In exon 4, point mutations were found in 13.6% (8 of 59) of the cases. Whereas the point mutations do not appear to contribute to the patients' prognosis, the frequency is appreciably high and may be specific to pancreatic cancer. Considering the fact that Gadd45 pays a role in genomic stability (30), additional comprehensive studies will be necessary to assess whether these mutations do indeed contribute to the emergence of pancreatic cancers. In the present study, only one of eight cases with *GADD45a* mutation showed p53 expression. On the other hand, it was reported that unequal segregation of chromosomes occurred in several *GADD45a*−/− cell lineages, and it may contribute to the aneuploidy (30). Because the present study did not analyze loss of heterozygosity, it was unclear whether all of the mutations were haploid or not; in other words, it was unclear whether there is any evidence for loss of heterozygosity or not. Accordingly, there is a possibility that some of the wild-type allele may be due to normal stromal cell DNA.

In the present study, when the patients were grouped by p53 expression, the expression of Gadd45 had a significant influence on the survival of the patients. In the p53(+) group, the survival rate of the Gadd45(+) subgroup was significantly lower than that of the Gadd45(−) subgroup. It has also been confirmed that the immunoreactivity of the p53 protein is an appropriate indicator of altered p53 function (31, 32), although

Table 3 Correlation between the expression of Gadd45 and p53 and the clinicopathological characteristics

Variable	Correlation coefficient (*P*)	
	Gadd45	p53
Age (yrs)	0.061 (0.6148)	0.169 (0.1559)
Gender	−0.079 (0.5103)	−0.059 (0.6247)
Grade	−0.034 (0.7786)	0.008 (0.9503)
pT	−0.304 (0.0091)	−0.029 (0.8101)
pN	0.084 (0.4826)	−0.005 (0.9662)
M	−0.087 (0.4678)	0.081 (0.4990)

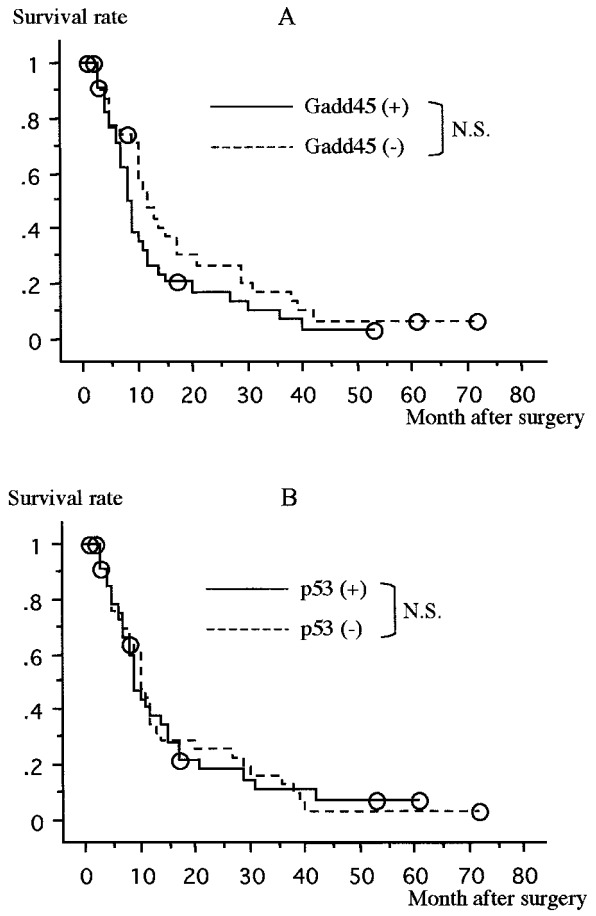

Fig. 3 Survival curves grouped by Gadd45 (*A*) and p53 (*B*) expression. *A*, the mean survival was 12.7 months for the Gadd45(+) group (n = 39) and 17.1 months for the Gadd45(−) group (n = 33; P = 0.1216). *B*, the mean survival was 14.4 months for the p53(+) group (n = 34) and 15.3 months for p53(−) group (n = 38; P = 0.9743).

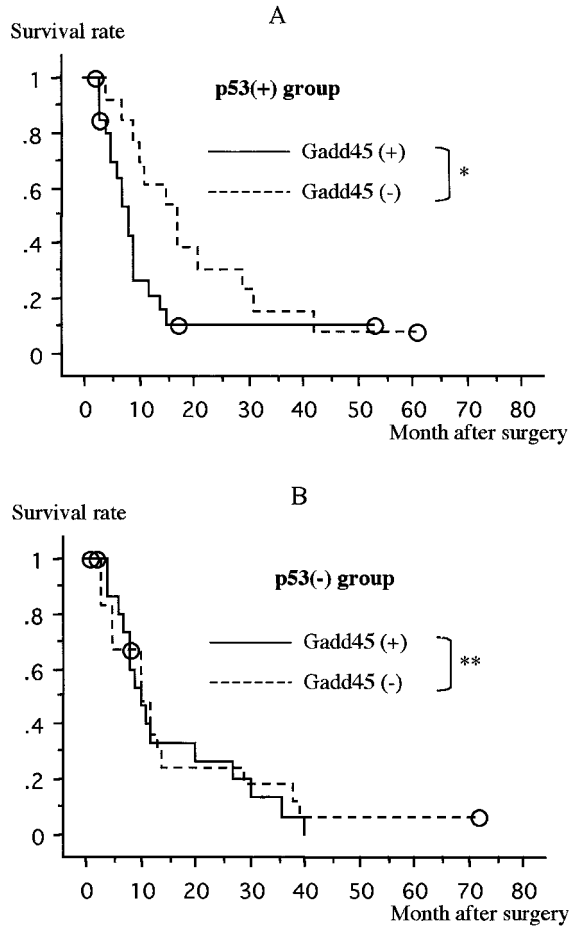

Fig. 4 Coexpression of Gadd45 and p53 and survival curves after pancreatectomy. *A*, in the p53(+) group, the mean survival was 8.3 months for the Gadd45(+) group (n = 21) and 19.6 months for the Gadd45(−) group (n = 13; ∗, P = 0.049). *B*, in the p53(−) group, survival was 15.5 months for the Gadd45(+) group (n = 18) and 15.0 months for Gadd45(−) group (n = 20; ∗∗, P = 0.7204).

p53 expression does not always reflect the status of p53 mutations at the gene level. In other words, the absence of p53 expression is not synonymous with normal p53 function. Even when p53 is not immunostained, there still might be a null status, deletion, frameshift mutation, or nonsense mutation in the *p53* gene or, alternatively, MDM2 overexpression (33–35). The *GADD45a* gene is one of the downstream mediators of p53 that adjusts the cell cycle upon stimulation by p53 (36). *GADD45a* induces G_1-S or G_2-M arrest with p53 (12, 37, 38). Accordingly, it has been believed that *GADD45a* works normally to repair DNA damage under a normal p53 status. On the other hand, two recent publications indicated that whereas no significant correlation was found between p53 status and basal levels of *GADD45a*, several tumor cell lines have augmented *GADD45a* expression (39, 40). However, the role of *GADD45a* overexpression in abnormal status of the p53 system is unclear. It was reported, for instance, that expression of *GADD45a* mRNA and its protein was increased in the WI-L2-NS lymphoid cell line, which showed a very high constitutive level of mutated p53 protein (41). The present results suggest that if overexpression of Gadd45 reflects an overfunction of *GADD45a*, the suppressive effects of *GADD45a* on tumor growth might be abrogated in human pancreatic IDC with p53 abnormalities. These results are contradictory with the report that *GADD45a* overexpression reduced colony formation in several cell lines (42). However, it was also reported that high levels of *GADD45a* did not inhibit cellular growth of the WI-L2-NS cell line that had no mutations in the *GADD45a* gene. Accordingly, these results (including our results) indicate that tumor cells can sometimes abrogate the growth-inhibitory function of the *GADD45a* gene, especially in abnormal status of p53. Accordingly, correlations among p53 status, *GADD45a*, and its own expression are still unclear and should be clarified in future studies. Some clinical studies have reported that Gadd45 expression is an indicator of poor prognosis or malignant potential. Sengupta *et al.* (43) reported that in epithelial ovarian cancers, Gadd45 expression was significantly correlated with WAF/1-p21 expression and that Gadd45 expression was a significant prognostic factor on a univariate analysis. Santucci *et al.* (44)

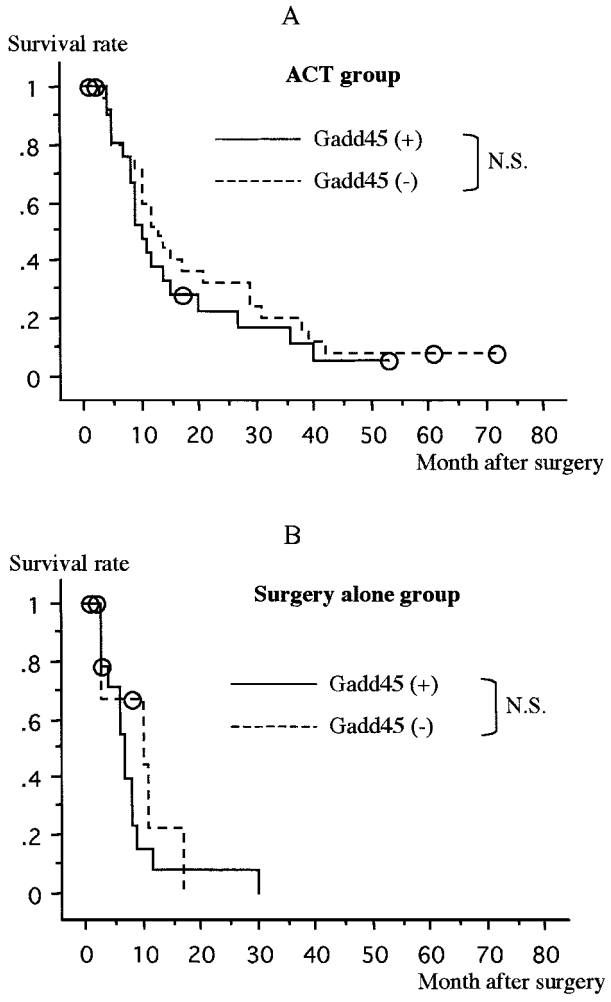

Fig. 5 Gadd45 expression and survival curve after pancreatectomy for patients with (*A*) or without (*B*) ACT. *A*, in the ACT group, the mean survival was 15.5 months for the Gadd45(+) group ($n = 22$) and 18.6 months for the Gadd45(−) group ($n = 27$; $P = 0.4223$). *B*, in the surgery alone group, survival was 8.3 months in the Gadd45(+) group ($n = 17$) and 9.4 months in the Gadd45(−) group ($n = 6$; $P = 0.4371$).

reported that lack of *GADD45a* induction after radiotherapy was correlated with good clinical response in cervical carcinomas. Song *et al.* (45) reported that in lung cancer cell lines, *GADD45a* expression was increased more than 10-fold. These findings suggest that the expression of Gadd45 is associated with an accelerated progression to malignancy and with resistance to adjuvant therapy. In patients with p53(−) IDC, the expression of Gadd45 did not show any significant effect on survival. Accordingly, when p53 and *GADD45a* function normally, the malignant potential of other cell growth-related genes such as *Waf1*, *Bax*, or *transforming growth factor* β might affect the tumor.

In conclusion, not only is the frequency of *GADD45a* mutations appreciable in human pancreatic IDC, but deregulated Gadd45 expression is also a useful indicator of poor prognosis when combined with p53 expression.

Table 4 Multivariate analysis by Cox's proportional hazard risk model[a]

Variables	Conditional risk ratio (95% confidence limit)	P (χ^2)
Overall patients		
pTNM	1.673 (1.210–2.313)	0.0018
Histological grade	2.203 (1.343–3.614)	0.018
ACT	0.518 (0.273–0.983)	0.0442
Gadd45 expression	1.387 (0.784–2.454)	0.2606
Gender	1.121 (0.603–2.084)	0.7182
Age	0.999 (0.970–1.029)	0.9494
p53 expression	1.014 (0.598–1.722)	0.9575
p53 (+) group		
pTNM	2.528 (1.460–4.377)	0.0009
Histological grade	2.839 (1.270–6.345)	0.0110
Gadd45 expression	3.611 (1.317–9.901)	0.0126
ACT	0.566 (0.214–1.502)	0.2533
Age	1.028 (0.972–1.088)	0.3309
Gender	1.252 (0.419–3.735)	0.6872
p53 (−) group		
ACT	0.440 (0.170–1.139)	0.0906
Gender	1.715 (0.661–4.640)	0.2595
pTNM	1.285 (0.786–2.102)	0.3178
Histological grade	1.545 (0.637–3.749)	0.3357
Age	0.988 (0.947–1.030)	0.5653
Gadd45 expression	0.862 (0.383–1.940)	0.7189

[a] Dependent variable, month; censoring variable, death due to pancreatic cancer.

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