GADD45A expression is correlated with patient prognosis in esophageal cancer

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Abstract. The prognosis of patients with esophageal cancer remains poor, and the tumor-node-metastasis classification system is not sufficient for predicting patient prognoses. Therefore, the identification of novel predictive markers for esophageal cancer is required. The present study investigated the clinicopathological significance of growth arrest and DNA damage-inducible 45α (GADD45A) and p53 in resectable esophageal squamous cell carcinoma (ESCC). The study consisted of 62 patients with esophageal cancer who underwent surgery between 2001 and 2007. The expression of the *GADD45A* gene product (GADD45A) and the p53 protein was analyzed by immunohistochemistry. The correlations among GADD45A expression, clinicopathological factors and prognosis were then analyzed in the patients with ESCC. GADD45A and p53 were expressed in 56.5% (35/62) and 48.4% (30/62) of patients, respectively. The expression of GADD45A did not show a marked correlation with that of p53. However, GADD45A expression correlated with pathological stage (stage 0-I vs. stages II-IV; P=0.014) and did not correlate with the tumor (T) or node (N) status. Furthermore, patients who were positive for GADD45A exhibited a significantly higher survival rate than those who were negative for GADD45A (log-rank test, P=0.009). Multivariate analysis indicated that T status, N status and GADD45A expression were significant variables predicting survival (hazard ratio, 2.486; 95% confidence interval, 1.168-5.290; P=0.018). Overall, GADD45A expression significantly affected the survival of patients with ESCC, and the reduced expression of GADD45A was correlated with a poor prognosis following curative surgery in these patients.

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Introduction

The prognosis of esophageal cancer patients remains poor, emphasizing the requirement for the development of novel treatment strategies. At present, the overall 5-year survival rate is <50%, despite the use of multimodality therapies. Numerous patients, even those with early-stage disease, develop local recurrence of tumors or distant metastasis within a short time period after surgery. To develop novel treatment strategies, it is important to understand the biological behavior of esophageal cancer. Recent studies have found that a number of genes and molecules show involvement in the origin and/or progression of esophageal cancer, including tumor protein p53 (1), deleted in esophageal cancer 1 (2), deleted in colorectal cancer (3), deleted in lung cancer 1 (4), cyclin D1 (5), adenomatous polyposis coli (6) and survivin (7). However, the exact mechanisms underlying esophageal squamous cell carcinoma (ESCC) development and progression remain unclear.

The p53 gene is required for the proper induction of the G_1 checkpoint and functions to upregulate growth arrest and DNA damage-inducible 45α (*GADD45A*) and WAF1/p21 expression (8). Additionally, GADD45A is a downstream mediator of p53 and is able to deactivate p53, thereby contributing to cell cycle regulation through binding with cyclin-dependent kinases and proliferating cell nuclear antigen (9,10). GADD45A stimulates DNA excision repair following cellular DNA damage (9). To the best of our knowledge, no studies have previously described the clinicopathological significance of GADD45A protein expression and its association with p53 protein in the progression of esophageal cancer.

Analysis of the immunoreactivity of p53 can be used as a measure of the loss of normal p53 function and the function of p53-related genes (11). These findings allow us to easily evaluate the status of the p53 gene by immunohistochemistry (11).

In the present study, the clinicopathological significance of GADD45A protein expression and its associations with the expression of its upstream mediator p53 were investigated in 62 patients with resectable ESCC.

Materials and methods

Tissue samples. Samples were obtained from 62 patients with ESCC who had undergone esophagectomy at the Department

of Gastroenterological Surgery, Nagoya City University Graduate School of Medical Science (Nagoya, Aichi, Japan) between 1997 and 2005, without pre-operative chemotherapy or radiation. The tumors were classified according to the guidelines for clinical and pathological studies on carcinoma of the esophagus (12). The samples were used after obtaining written consent from the patients.

Immunohistochemistry. Immunohistochemical staining was then performed on the formalin-fixed, paraffin-embedded ESCC tissues using rabbit polyclonal anti-GADD45A (cat no. sc-792; Santa Cruz Biotechnology, Dallas, TX, USA) or mouse monoclonal anti-p53 (cat no. M7001; Dako, Glostrup, Denmark) antibodies at 1:200 and 1:75 dilutions, respectively. Paraffin-embedded sections (4-μm) of tumors were deparaffinized, rehydrated, heat-treated by microwaving in 10 mM citrate buffer for 15 min for antigen retrieval and cooled to room temperature. The sections were then treated with 0.3% H_2O_2 in methanol for 30 min to neutralize endogenous peroxidases, blocked with non-specific goat serum for 10 min and incubated with anti-GADD45A or anti-p53 overnight at room temperature in a humid chamber. Immunoreactive protein was detected with a DAKO Envision system (horseradish peroxidase 3,3'-diaminobenzidine), and the sections were counterstained with hematoxylin. Immunostaining of GADD45A and p53 was subjectively assessed by two independent investigators, and discordant results were resolved by consultation with a third investigator. For the evaluation of GADD45A expression, immunostaining was considered positive only when unequivocally strong nuclear staining was present in >50% of the tumor cells, as analyzed using a Olympus BX51 light microscope (Olympus corporation, Tokyo, Japan). 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 (13). According to previous studies, the cutoff point for p53 positivity was set as positive staining of 20% of the cells (13).

Statistical analysis. The χ^2 test was used to compare the correlations between clinicopathological factors and the expression of GADD45A and p53. The cumulative survival rates were calculated according to the Kaplan-Meier method and compared by the Cox-Mantel test. Multivariate analysis of Cox's proportional hazard risk model was used to obtain the conditional risk of mortality due to ESCC. Statistical analysis was performed using the StatView software package (Abacus Concepts, Berkerly, CA, USA) Differences were considered statistically significant when $P<0.05$.

Results

Expression of GADD45A and p53. First, the expression of GADD45A and p53 proteins in ESCC tissues was investigated by immunohistochemistry. Representative images of GADD45A and p53 immunostaining are shown in Figs. 1 and 2. Typical ESCC cells showed diffuse nuclear staining for GADD45A and p53, while the cell membrane and cytoplasm were not stained. Immunostaining for GADD45A and p53 was positive in 27.7% (13/47) and 46.8% (22/47) of patients, respectively. GADD45A and p53 were expressed in 56.5% (35/62) and 48.4% (30/62) of esophageal cancer tissues, respectively (Table I). There were no correlations between the expression of GADD45A and that of p53 (data not shown). GADD45A expression was correlated significantly with pathological stage, but p53 expression did

Table I. Correlation of GADD45A immunohistochemistry results with clinicopathological factors, including patient and tumor characteristics, in esophageal cancer patients (n=62).

	Expression, n/total n		
Characteristic	GADD45A$^+$	p53$^+$	P-value
Age at surgery, years			0.993
<65	22/39	18/39	
>65	13/23	12/23	
Gender			0.952
Male	27/48	24/48	
Female	8/14	6/14	
Tumor status			0.354
T1	7/9	5/9	
T2	4/7	3/7	
T3	15/25	14/25	
T4	9/21	8/21	
Lymph node status			0.278
N0	7/9	6/9	
N1	5/10	6/10	
N2	11/23	9/23	
N3	5/11	4/11	
N4	7/9	4/9	
Pathological stage			0.130
0	2/2	2/2	
I	5/5	2/5	
II	2/6	3/6	
III	12/23	12/23	
IV	14/26	10/26	
0-I vs. II-IV			0.014
Histological differentiation			0.364
Well	14/25	10/25	
Moderate	18/27	14/27	
Poor	2/6	3/6	
Unknown	4	4	
Lymphatic invasion			0.696
Negative	5/8	5/8	
Positive	23/41	22/41	
Unknown	13	13	
Blood vessel invasion			0.634
Negative	11/21	13/21	
Positive	17/28	14/28	
Unknown	13	13	

GADD45A, growth arrest and DNA damage-inducible 45α; T, tumor; N, node.

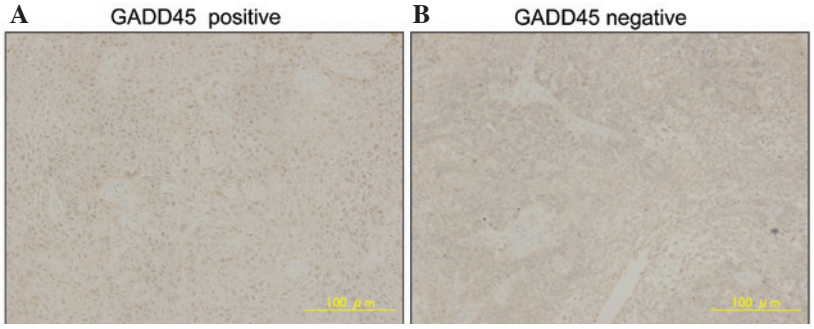

Figure 1. Representative immunostaining for GADD45A (x100 magnification). (A) Positive and (B) negative staining of GADD45A in tumor cells. GADD45A, growth arrest and DNA damage-inducible 45α.

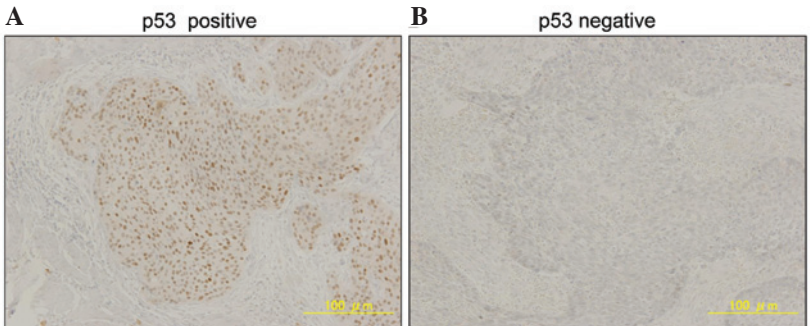

Figure 2. Representative immunostaining for p53 (x100 magnification). (A) Positive and (B) negative staining of p53 in the tumor cells.

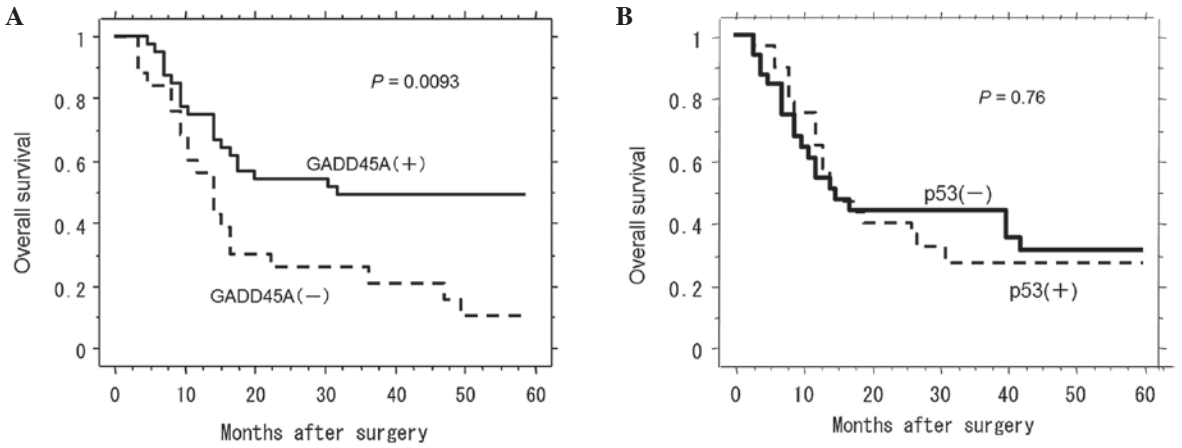

Figure 3. (A) Kaplan-Meier survival curve for esophageal cancer patients who were classified as showing either positive or negative GADD45A immunostaining. GADD45A status was found to be strongly associated (log-rank, P=0.0093) with patient survival. (B) Kaplan-Meier survival curves for esophageal cancer patients who were classified as showing either positive or negative p53 immunostaining. p53 status did not show a significant (log-rank, P=0.76) correlation with patient survival. GADD45A, growth arrest and DNA damage-inducible 45α.

not correlate with any factors of the tumor-node-metastasis classification (Table I) (12).

Survival curves and the expression of GADD45A and p53. GADD45A exhibited a significant effect on patient survival (Fig. 3A). Indeed, the patients with positive staining for GADD45A experienced a significantly longer survival time following surgery compared with the patients with negative results [27.0±1.3 months (n=35) vs. 12.0±1.2 months (n=27), respectively; P=0.0093 by Log-rank test; Fig. 3A]. However, there were no significant differences in survival following surgery between patients with negative staining and patients with positive staining for p53 [15.0±3.9 months (n=32) vs. 15.0±5.3 months (n=30), respectively; P=0.76 by Log-rank test; Fig. 3B].

Next, the study investigated the correlation between immunostaining for GADD45A and the survival time of patients with ESCC following surgery (median follow-up time, 22.3 months). Univariate analysis showed that, among the clinicopathological factors examined, the extent of the primary tumor (risk ratio, 8.849; P<0.0001), lymph node metastasis (risk ratio, 3.773; P<0.0001), lymphatic invasion (risk ratio, 4.975; P<0.0001), vein invasion (risk ratio, 2.906;

Table II. Univariate analysis, including immunostaining for GADD45A.

Parameter	Risk ratio	95% CI	P-value
Age at surgery, years			0.805
<65	1.000		
>65	1.669	0.871-3.194	
Gender			0.866
Male	1.000		
Female	0.956	0.097-.0435	
Primary tumor			<0.0001
T1	1.000		
T2-4	8.849	4.081-19.230	
Lymph node metastasis			<0.0001
N0-3	1.000		
N4	3.773	2.386-5.952	
Lymphatic invasion			<0.0001
Negative	1.000		
Positive	4.975	2.283-10.869	
Vein invasion			<0.0001
Negative	1.000		
Positive	2.906	1.724-4.901	
Immunostaining for GADD45A			0.013
Positive	1.000		
Negative	2.214	1.183-4.144	

CI, confidence interval; GADD45A, growth arrest and DNA damage-inducible 45α; T, tumor; N, node.

Table III. Multivariate analysis, including immunostaining for GADD45A.

Parameter	Risk ratio	95% CI	P-value
Primary tumor			0.019
T1	1.000		
T2-4	15.384	1.552-142.857	
Lymph node metastasis			0.004
N0-2	1.000		
N3-4	3.333	1.472-7.518	
Lymphatic invasion			0.305
Negative	1.000		
Positive	0.391	0.065-2.341	
Vein invasion			0.302
Negative	1.000		
Positive	1.524	0.684-3.401	
Immunostaining for GADD45A			0.018
Positive	1.000		
Negative	2.486	1.168-5.290	

CI, confidence interval; GADD45A, growth arrest and DNA damage-inducible 45α; T, tumor; N, node.

P<0.0001) and immunostaining for GADD45 (risk ratio, 2.214; P=0.013) were statistically significant prognostic factors. By contrast, immunostaining for p53 was not a prognostic factor in univariate analysis (Table II). Multivariate analysis revealed that GADD45 expression was an independent prognostic factor (risk ratio, 2.486; P=0.0181),

together with the extent of the primary tumor (risk ratio, 15.384; P=0.0194) and lymph node metastasis (risk ratio, 3.333; P=0.0038) (Table III).

Discussion

DNA repair is central to human genome integrity. A reduced capacity for DNA repair has been associated with certain types of genetic susceptibility to cancer. GADD45A is involved in DNA replication and repair, and interacts with proliferating cell nuclear antigen (9). DNA excision repair is stimulated *in vitro* and the passage of cells into the S phase is inhibited by GADD45. Additionally, GADD45A is involved in the maintenance of the p53-dependent cell cycle checkpoint and DNA repair. In normal cells, GADD45 is localized to the nucleus (14). Consistent with this, the current experiments showed that GADD45A was expressed in the nucleus in ESCC cells. As GADD45A plays a role in genomic stability (15), additional studies will be necessary to assess whether GADD45A contributes to the growth of esophageal cancers.

The present study analyzed the expression of GADD45A and p53 by immunohistochemistry. The mutant p53 protein is more stable than the wild-type protein and can be detected by immunohistology (16). The immunoreactivity of p53 can be used as a measure of the loss of normal p53 function (13). Therefore, the overexpression of p53 by immunohistochemistry may indicate mutant p53. While the present results suggested that p53 did not contribute to patient prognosis, controversy remains over whether p53 expression is a good prognostic marker in esophageal cancer (17,18). The present data suggested that the aberrant expression of p53 was not correlated with patient prognosis in esophageal cancer.

GADD45A is a downstream target of p53 signaling (19-21). However, as few studies have described the mechanisms mediating GADD45A expression in ESCC, it is not known whether p53 regulates GADD45A expression in this cancer type (22). Notably, the present study found that GADD45A, but not p53, was able to predict the prognosis of patients with ESCC. Thus, the data suggested that p53 is involved in the carcinogenesis of esophageal cancer and that GADD45A could be a useful biomarker for predicting prognosis in patients with ESCC. However, the expression of GADD45A was not markedly correlated with that of p53. Thus, the mechanism behind the regulation of GADD45A expression in ESCC remains unclear. Other factors, such as myc, phosphoinositide 3-kinase/AKT (23,24), activating transcription factor 2 (25) and Quercetin (26), which have been shown to regulate GADD45A expression, should be investigated in future studies.

A number of studies have shown that GADD45A functions to repair DNA damage under normal p53 signaling (27-29). On the other hand, more recent studies have demonstrated that there is no significant correlation between p53 status and basal *GADD45A* expression in tumor cells (13,14,30). The present study data also showed that GADD45A expression was not correlated with p53 status. Accordingly, the correlation between p53 status and GADD45A expression remains unclear and should be clarified in future studies.

Certain clinical studies have indicated that the expression of GADD45 is an indicator of a poor prognosis or malignant potential in cervical carcinomas (31), lung cancer (32) and gastric cancer (33). Moreover, the combined evaluation of thymidine phosphorylase and *GADD45* gene expression can predict therapeutic response in adenocarcinomas of the upper gastrointestinal tract (34). On the other hand, *GADD45* mRNA levels may not be useful for the prediction of the neoadjuvant chemotherapy success in individual cancer patients with locally advanced Barrett adenocarcinoma (35). The current study may be the first to demonstrate that GADD45A is an independent prognostic factor in esophageal cancer.

In the present study, it was found that decreased GADD45A expression in the cancer tissues accompanied the local progression of esophageal cancer (Fig. 1 and Table II). In addition, patients with lower GADD45A expression levels had a poorer prognosis (Fig. 2). Further studies are required to determine how deficiencies in DNA repair resulting from downregulation of GADD45A may promote tumor progression and lead to poor prognoses in patients with ESCC.

The *GADD45A* gene is localized to human chromosome 1 between p12 and p34 (36). A number of studies have suggested that the *GADD45A* locus (1p) may harbor tumor-suppressor genes for glioma (37), lung cancer (38) and gastric cancer (39). Therefore, loss of GADD45A may also contribute to the development of numerous other types of cancer. Further studies are required to determine whether there were chromosomal losses in the *GADD45A* locus in the esophageal tumor tissues examined in the present study. Additionally, determining whether *GADD45A* expression is mediated by other mechanisms, such as methylation of the promoter region, will be the focus of future studies.

In patients with esophageal cancer, numerous prognostic markers, including cyclin D1, E-cadherin and MDM2 proto-oncogene, E3 ubiquitin protein ligase, have been reported (40,41). Furthermore, we have also previously reported that survivin (7), pituitary tumor-transforming gene 1 (42), DNA fragmentation factor 45 (43) and drosha, ribonuclease type III (44) may be prognostic markers of ESCC. Thus, GADD45A represents an additional potential prognostic indicator for patients with ESCC.

Although the precise molecular mechanisms through which GADD45A is downregulated require clarification, the present study data clearly indicated that GADD45A may be a prognostic marker and molecular target for the development of effective therapeutic reagents for patients with esophageal cancer.

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