Role and Prognostic Significance of Proapoptotic Proteins in Epstein-Barr Virus-infected Gastric Carcinomas

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Abstract. *Background: The purposes of the present study were to evaluate the role and the prognostic values of proapoptotic proteins involved in the death receptors (Fas and TRAIL receptors) and mitochondrial pathways (Bax) in Epstein-Barr virus (EBV)-infected gastric carcinomas. Materials and Methods: Fifty-five EBV-infected gastric carcinomas were identified by in situ hybridization for EBV-encoded small RNAs. Immunohistochemistry was peformed for Fas, Fas-ligand, FADD, TRAIL, DR4, DR5 and Bax. Apoptotic indices (AIs) were determined using TUNEL assay and assessed. Results: No remarkable differences in protein expressions were observed between EBV-infected gastric carcinomas and conventional gastric carcinomas. Bax positivity tended to be associated with higher AI ($p=0.068$), whereas Fas and FADD positivitives were related to lower AI ($p=0.006$ and 0.059, respectively). Proteins involved in TRAIL pathways showed no statistical significant relationship with AI. TNM stage and Fas and FADD expressions were related to overall survival ($p<0.05$), but TNM stage was the only independent prognostic factor. Conclusion: Apoptosis in EBV-infected gastric carcinomas probably occurs via the mitochondrial pathway through Bax, rather than via the death receptor pathways. Fas and FADD expressions, and pathological tumor stage (TNM stage) may be the prognostic factors.*

The Epstein-Barr virus (EBV) is a ubiquitous human herpes virus implicated in the etiology of many human lymphoid (1-3) and epithelial malignancies (2, 3). Almost sixteen years have passed since EBV was first detected in three cases of gastric carcinoma (4). EBV-infected gastric carcinoma has been described in different populations from low-incidence areas, such as, Western Europe and the United States, to high-risk countries, like Korea and Japan (3). The worldwide occurrence of EBV-infected gastric carcinoma is estimated at more than 50, 000 cases/year (5, 6). Today, it is well known that 2-16% of gastric carcinomas throughout the world reveal monoclonal proliferations of EBV-infected carcinoma cells (5-7), and 5.6% (8) or 13% (9) of Korean gastric carcinomas have been reported to be infected with EBV. Moreover, although EBV has been detected in only a small proportion of gastric carcinomas, gastric carcinoma is the most common malignancy in Korea (10). Consequently, gastric carcinoma represents the most significant EBV-infected malignancy in Korea. However, the mechanism by which EBV contributes to the carcinogenesis of the gastric mucosa remains unknown.

Carcinogenesis is a complex process involving multiple genetic changes. Overall tumor cell growth is known to be the result of the breakdown of the dynamic balance between cell proliferation and apoptosis. Recently, we suggested that nuclear factor-kappa B, which is an antiapoptotic protein, might be crucial in the oncogenesis of EBV-positive gastric carcinomas (11). However, no study has been performed on the expression profiling of proapototic proteins in EBV-infected gastric carcinomas despite the fact that apoptosis is related to cell transformation into cancer and resistance to cancer therapy. But, a few studies have been performed on these proteins in gastric carcinomas of unknown EBV status (12-16). Generally, two major apoptotic pathways have been well documented: that *via* the death receptors and that *via* the mitochondria (17). Mechanisms *via* the death receptors play an important role in induction of apoptosis. The best characterized death receptors are TNFR1 (tumor necrosis factor receptor 1), Fas (Apo-1, CD95) and TRAIL (TNF-related apoptosis inducing ligand)

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Key Words: Epstein-Barr virus, stomach neoplasms, human herpesvirus 4, immunohistochemistry, prognosis, apoptosis, Fas, FADD, Bax.

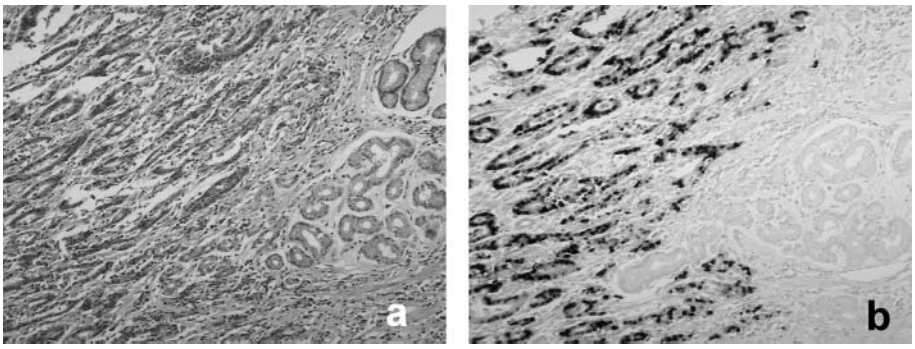

Figure 1. *Epstein-Barr virus infected gastric carcinoma. a H&E. b EBER-in situ hybridization. Most cancer cell nuclei (left 2/3 portion) reveal strong EBER signals (black granules), while normal epithelial cells (right 1/3 portion of the figure) produce no signal.*

receptors (DR4 and DR5) (17, 18). Through the apoptosis pathway, Fas-ligand and TRAIL molecules bind to their respective death receptors, *i.e.*, Fas, DR4 and DR5 (17, 18). Activation of death receptors leads to a signal transduction cascade initiated by Fas-associated death domain (FADD) and caspase-8. The activation of caspase 8 (initiator of apoptosis) activates other caspases, which induces the consequent activation of caspase 3 (executor), and eventually ends in apoptosis (17).

As for the mitochondrial pathway, it involves bcl-2 family members, including the apoptosis-promoting Bax. Many members of the bcl-2 family are resident proteins of the mitochondrial membranes and influence mitochondrial permeability transfer. Moreover, Bax release leads to the activation of caspase 3 and thus apoptosis (17).

In the present study, we focused on the Fas-ligand and TRAIL pathways, and BAX. The purposes of the present study were to evaluate the roles of these proapoptotic proteins and to assess their prognostic values in EBV-positive gastric carcinoma.

Materials and Methods

Specimens and tissue array technique. Initially, 821 consecutive surgically resected gastric carcinomas were collected from the files of the Department of Pathology, Seoul National University College of Medicine, dating from January 1995 to June 1996. A tissue array method was used in which each tissue array paraffin block contained 60 cores comprising 56 neoplasms and 4 normal tissues (a 2.0 mm-diameter column of a tissue core). Each tissue core was punched out from each original paraffin block of carcinoma samples to construct a new tissue array paraffin block. Using *in situ* hybridization for EBV-encoded small RNAs (EBER) on 16 array slides, EBER signals were found in 47 (5.7%) of these 821 cases. Eight cases of EBV-infected gastric cancer, as described in our previous report (19), were added and thus the new tissue array block was composed of a total of 55 cases of EBV-positive gastric cancer.

Age, sex, tumor size and location, lymphoid stroma, tumor differentiation, Lauren's classification, and pathological tumor stage (TNM stage; T: depth of tumor invasion, N: lymph node metastasis, M: distant metastasis) according to the AJCC system were assessed (20). Patient overall survival times were calculated from the date of operation to the date of death or the last follow-up. The follow-up period was 6-139 months (median: 51 months).

Table I. *Profiles of antibodies used for immunohistochemistry, and the staining results of 55 cases of Epstein-Barr virus-infected gastric carcinoma.*

Antibody	Clone	Dilution	source	No. of positive cases (n=55)
Fas	.	1:20	Oncogene	32 (58.2%)
Fas-ligand	G247-4	1:50	Pharmingen	26 (47.2%)
FADD	S-18	1:500	Santa Cruz	18 (32.7%)
TRAIL	H-257	1:100	Santa Cruz	37 (67.3%)
DR4	C-20	1:100	Santa Cruz	27 (49.1%)
DR5	C-20	1:50	Santa Cruz	13 (23.6%)
Bax		1:1000	Pharmingen	35 (63.6%)

EBER-in situ hybridization. Three-micrometer thick sections from each tissue array block were deparaffinized and dehydrated. Sections were then digested with proteinase K and hybridized for 2 hours at 37˚C with a fluorescein-conjugated oligonucleotide probe for EBER (Novocastra, Newcastle upon Tyne, UK). Hybridization products were detected using an alkaline phosphatase-conjugated antibody to fluorescein isothiocyanate (affinity-isolated rabbit F(ab')). 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium was used as an enzyme substrate to demonstrate alkaline phosphatase activity. Slides were counterstained with Mayer's hematoxylin and positive staining was observed under a light microscope as black granules at the site of hybridization (Figure 1). A few infiltrating lymphocytes also stained for EBER, but only cases with signals within tumor cell nuclei were considered positive.

Immunohistochemistry on tissue array slides. After a microwave antigen retrieval process, immunohistochemistry was performed using seven antibodies as detailed in Table I. The previously

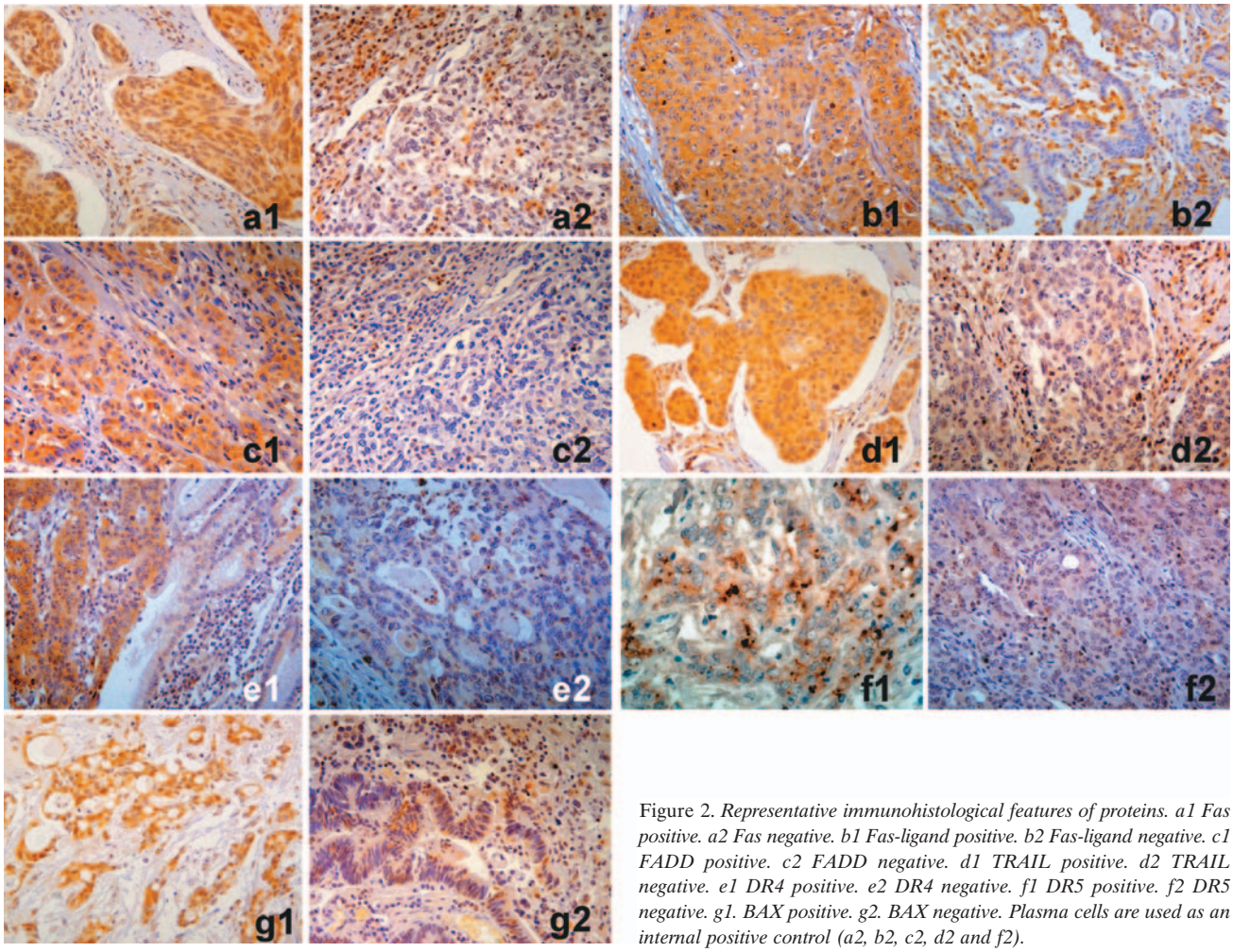

Figure 2. *Representative immunohistological features of proteins. a1 Fas positive. a2 Fas negative. b1 Fas-ligand positive. b2 Fas-ligand negative. c1 FADD positive. c2 FADD negative. d1 TRAIL positive. d2 TRAIL negative. e1 DR4 positive. e2 DR4 negative. f1 DR5 positive. f2 DR5 negative. g1. BAX positive. g2. BAX negative. Plasma cells are used as an internal positive control (a2, b2, c2, d2 and f2).*

demonstrated positive control tissue slide and the sample tissue array slides were processed at the same time. For Fas, Fas-ligand, FADD, TRAIL, DR4, DR5 and Bax antibodies, positive staining was defined as cytoplasmic staining with moderate to strong intensity in 10% or more of the tumor cells (Figure 2). In the case of DR5 antibody, Golgi pattern (punctuate cytoplasmic) staining in ≥1% of the tumor cells was defined as positive staining (Figure 2, f1).

TUNEL assay. Apoptosis was detected on tissue array paraffin sections by the labeling of fragmented DNA using Apoptag® peroxides (kit S7101, Chemicon International, Temecula, CA, USA). Tissue sections were deparaffinized, pretreated with proteinase K, rinsed and quenched in 1% H_2O_2. Samples were then incubated with terminal deoxynucleotidyl transferase (TdT), in the presence of nucleotides labeled with digoxigenin. (TdT catalyzes a template-independent addition of digoxigenin-labeled nucleotide triphosphates to the 3'-OH ends of double- or single-stranded DNA.) The slides were then incubated with stop-wash buffer, incubated with anti-digoxigenin peroxidase, and washed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) to induce a color reaction. Sections were counterstained with Mayer's hematoxylin. Slides that were treated in the same way, but which were not exposed to TdT, served as negative control.

Tumor cells showing homogeneous dense nuclear staining with a nuclear outline and perinuclear clearing, were counted as TUNEL-positive tumor cells (Figure 3). Whole tumor cells in each tissue core were assessed and the number of tumor cells in each tissue core were found to range from 245 to 2, 870 (mean: 859, median: 595). Apoptotic index (AI) was defined as the percentage of tumor cells that were positive for TUNEL staining.

Statistics. All statistical analyses were conducted using the SPSS Ver. 10.0 (SPSS, Chicago, IL, USA). The chi-square test or Fisher's exact test (2-sided) was used, as appropriate. One-way ANOVA was used to analyze continuous variables. Cumulative survival rates were obtained using the Kaplan-Meier analysis method and differences in survival were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. P values of <0.05 were considered to be statistically significant.

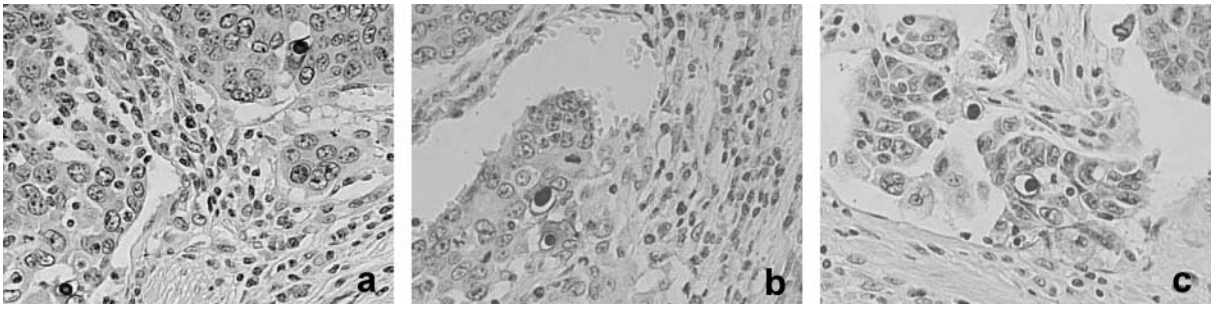

Figure 3. *TUNEL staining for cell apoptosis in three representative cases (a, b and c).*

Table II. *Clinicopathological profile of the 55 patients with Epstein-Barr virus-infected gastric carcinoma.*

Clinicopathological features		Total cases (n=55)	Living patients (n=39)	Dead patients (n=16)	*P-value
Gender	Male	47	34	13	NS
	Female	8	5	3	
Age (years)	Range (Median)	28-73 (57)	29-73 (54)	28-73 (62)	NS
Tumor size (cm)	Range (Mean)	0.6-14.0 (5.7)	0.6-14.0 (5.3)	2.5-11.0 (6.6)	NS
Tumor location	Low 1/3	3 (5%)	2 (5%)	1 (6%)	NS
	Middle 1/3	31 (56%)	24 (62%)	7 (44%)	
	Upper 1/3	15 (27%)	10 (26%)	5(31%)	
	Whole	6 (11%)	3 (8%)	3 (19%)	
Lauren classification	Intestinal	14 (25%)	12 (31%)	2 (13%)	NS
	Diffuse	36 (65%)	23 (59%)	13 (81%)	
	Mixed	5 (9%)	4 (10%)	1 (6%)	
Lymphoid stroma	Present	25 (45%)	17 (44%)	8 (50%)	NS
	Absent	30 (55%)	22 (56%)	8 (50%)	
Tumor stage (TNM stage according to AJCC system)	IA	11 (20%)	11 (28%)	0	<0.05
	IB	11 (20%)	9 (23%)	2 (13%)	
	II	15 (27%)	7 (18%)	8 (50%)	
	IIIA	11 (20%)	9 (23%)	2 (13%)	
	IIIB	3 (5%)	2 (5%)	1 (6%)	
	IV	4 (7%)	1 (3%)	3 (19%)	
Follow-up (months)	Range (Median)	6-139 (51.0)	25-139 (53.0)	6-87 (17.5)	NS

*P-value for living and dead patient data was calculated using the Kaplan-Meier log-rank test in SPSS 10.0. NS, not significant.

Results

Cilnicopathological features. The clinicopathological features are summarized in Table II. EBV-infected gastric carcinomas tended to be prominent in male patients, in the diffuse type according to the Lauren's classification, and in patients with lymphoid stroma. The follow-up period ranged from 6 to 139 months, with a median of 51 months, and 16 patients (29%) succumbed.

Apoptosis. Immunohistochemical staining data for apoptosis-related proteins are summarized in Table I and a representative immunohistochemical staining pattern is depicted in Figure 2. The mean apoptotic index (AI) with

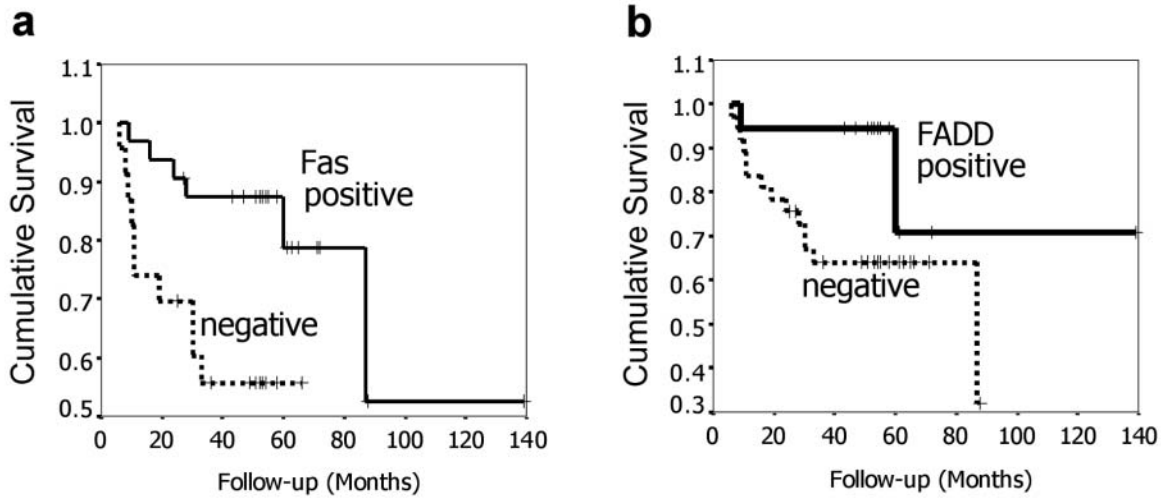

Figure 4. *Kaplan-Meier survival plots. Positivities for Fas (a) and FADD (b) are found to be positively correlated with patient's survival ($p<0.05$).*

the TUNEL assay was 0.416±0.472 (median: 0.250; range: 0-1.84). Bax positivity tended to be associated with higher AI ($p=0.068$), while Fas ($p=0.006$) and FADD positivities ($p=0.059$) were related to lower AI. Relationships between proteins involved in TRAIL pathways (TRAIL, DR4 or DR5) and AI were not significant.

Survival analyses. Univariate analysis using the Kaplan-Meier model showed that TNM stage ($p=0.0002$), Fas positivity ($p=0.0101$) and FADD positivity ($p=0.0485$) were all associated with longer overall survival (Table II and Figure 4). However, according to multivariate analysis (Cox-Regression hazard model), TNM stage was the only independent prognostic factor (Table III).

Table III. *Multivariate analyses of the prognostic factors for overall survival (Cox proportional hazards model) in the 55 patients with Epstein-Barr virus-infected gastric carcinomas.*

Prognostic factors	Hazard ratio (95% confidence interval)	*P*-value
Pathological TNM stage (AJCC system)		0.049
IA *vs.* IV	0.000 (0.000 ~ 8.25±2.92)	
IB *vs.* IV	0.061 (0.009 ~ 0.407)	
II *vs.* IV	0.229 (0.054 ~ 0.970)	
IIIA *vs.* IV	0.071 (0.011 ~ 0.470)	
IIIB *vs.* IV	0.156 (0.015 ~ 1.581)	

Discussion

Curiously, the TNFR1 pathway among three major death receptors does not appear to affect cell death in EBV-infected cells, or rather promotes cell survival (21, 22). Meanwhile, Fas-ligand and TRAIL pathways are regulated and lead to apoptosis, when EBV-negative human stomach cancer AGS cells (American Type Culture Collection) were incubated with *Helicobacter* (23). At this point, it is imperative to know whether Fas and TRAIL receptor pathways in EBV-infected gastric carcinomas induce apoptosis or not. In the present study, proteins involved in Fas or TRAIL pathways (TRAIL, DR4 or DR5) were not correlated with high apoptotic indices, while Bax positivity tended to be associated with a higher apoptotic index. Therefore, apoptosis in EBV-infected gastric carcinomas does not occur *via* the death receptor pathways (Fas or TRAIL receptors pathways). Instead, apoptosis in EBV-infected gastric carcinomas seems to occur *via* the mitochondrial pathway (Bax pathway), although further study of the mitochondrial pathway is required, such as the investigation of mitochondrial transmembrane potential (24) and mitochondrial apoptosis-related proteins like Smac/DIABLO and Htr/A2 (25, 26). The outcome of the present study, *i.e.* that apoptosis occurs *via* a mitochondrial pathway, agrees with results obtained in an EBV-negative, Korean human gastric carcinoma cell line, in which apoptosis was found to occur *via* the mitochondrial apoptotic pathway (24). However, our findings differ from those obtained in an EBV-negative American gastric carcinoma cell line, in which apoptosis was found to involve Fas-ligand and TRAIL pathways when cells were incubated with *Helicobacter* (23).

In the present study, Fas and FADD positivities were found to be related to a higher apoptotic index. Therefore, Fas and FADD appear to act as antiapoptotic factors in the

present study. This opposite function of these proteins which are ordinarily associated with the death receptor pathway is not so paradoxical, as the fact that the TNFR1 (one of the death receptors) pathway promotes cell survival is already known, even though the exact mechanism of its opposite function is unknown (2, 22). Furthermore, in the present study, the TRAIL pathway was found not to be related to the apoptotic index and thus did not function as a proapoptotic factor. The intricacy of the apoptotic network could explain this apparent conflict in protein function, as the apoptosis pathways are composed of the signaling networks controlled by many molecular links, which can disturb the functions of proapoptotic factors. For example, ligation of the TRAIL receptors, DR4 and DR5, can result in the activation of nuclear factor-kappa B, an antiapoptotic factor (27, 18), and TNFR1 and Fas can also activate nuclear factor-kappa B (18). Moreover, it has been reported that nuclear factor-kappa B is more frequently expressed in EBV-infected gastric carcinomas than in EBV-negative gastric carcinomas ($p<0.05$) (11).

Fas and FADD deserve to be considered prognostic biological markers in EBV-infected gastric cancer, although in the present study, they showed significance only upon univariate analysis. In fact, TNM stage was found to be the only independent prognostic factor with multivariate analysis. This is not a surprising result, considering two things. First, FADD was found to be significantly related to tumor stage ($p=0.030$), while Fas was found to be only marginal associated with tumor stage ($p=0.069$) (data not shown). Second, mutually interacting factors like these proapoptotic proteins lose their prognostic impacts statistically when they are counted simultaneously in multivariate analysis. In the present study, Fas and FADD were positive correlated with each other ($p<0.01$) (data not shown). Meanwhile, Bax was not a prognostic factor in EBV-positive gastric carcinomas despite its proapoptotic function. This finding suggests that a cell survival factor, such as nuclear factor-kappa B could be more important than an apoptotic factor in a patient's prognosis (11).

In the present study, no remarkable difference was observed between EBV-infected gastric carcinomas and conventional gastric carcinomas of unknown EBV status, with respect to the immunoexpressions of the proapoptotic proteins involved in the Fas or Bax pathway. In previous literature, Fas positivity was observed in 60% of conventional gastric carcinoma cases (12, 13); Fas-ligand positivity in 54.0% (15), 63.5% (13), and 69.1% (12); FADD positivity in 36.4% (12), and Bax positivity in 57.9% (16). To the best of our knowledge, no report is available on the immunoexpressions of proteins involved in the TRAIL pathway. In particular, golgi pattern staining shown by DR5 in the present study was intriguing, but we cannot refer to literature due to absence of any reports on DR5 immunostaining. Meanwhile, the mean apoptotic index found in the present study tended to be lower than those previously reported in EBV-infected gastric carcinomas, 0.97 ± 0.41 (28), and lower than in gastric carcinomas of unknown EBV status, 1.00 ± 0.80 (29) - 2.09 ± 1.0202 (30). We provide an indirect explanation for this discrepancy. These previous reports show that the apoptotic index in EBV-infected gastric carcinoma is lower than in EBV-negative gastric carcinomas ($p<0.05$) (28), and apoptotic index in advanced carcinoma is lower than in early carcinoma (14). Thus, it is possible that the EBV-infected carcinoma cases used in the present study fall into the category of the more advanced stage than those used in the published studies, which provided no detail on stage.

We conclude that apoptosis in EBV-positive gastric carcinomas may occur *via* the mitochondrial pathway (Bax pathway), rather than *via* the death receptor pathways, and that Fas and FADD immunoexpressions may be prognostic factors, in addition to the pathological tumor stage (TNM stage), which according to our results was the only independent prognostic factor of overall survival.

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