Frequent Expression of Thymidine Phosphorylase in Epstein-Barr Virus-associated Gastric Carcinoma of Diffuse Type

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Abstract. *The aim of the present study was to elucidate the etiological roles of Epstein-Barr virus (EBV) in the development of EBV-associated GC (EBV-GC), EBV-GCs and non EBV-GCs were compared with regard to the expression of thymidine phosphorylase (TP), which is known to have angiogenic activity in various tumor tissues. Patients and Methods: TP expression was examined by immunohistochemistry assay among 156 gastric carcinoma cases (21 EBV-GC cases and 135 non EBV-GC cases). Results: The frequency of tumors with TP expression was nearly twice as high in EBV-GCs (71%) than in non EBV-GCs (37%) (p=0.005). However, such an association was only observed in Lauren's diffuse-type tumors. Conclusion: Our finding suggests that the mechanism involved in TP expression of gastric carcinoma appears to be different in intestinal- and diffuse-type tumors.*

In the early 1990s, the technique of *in situ* hybridization (ISH) of Epstein-Barr virus-encoded small RNA (EBER) became available, and revealed that about 10% of gastric carcinomas had involvement of Epstein-Barr virus (EBV) (1, 2). Although EBV is suspected to play etiological roles in some gastric carcinomas, its mechanism is as yet unclear. The expression pattern of latency-associated EBV gene products in EBV-associated gastric carcinomas (EBV-GCs) is similar to tumors of latency I, and the following six EBV genes are expressed: *EBER1, EBER2, EBNA1, LMP2A, BARF0,* and *BARF1* (3, 4). However, *LMP1*, an EBV oncogene, is scarcely expressed in EBV-GCs.

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There are a number of studies comparing the expression of oncoproteins, suppressor-gene products, and other host-cell proteins in EBV-GC and non EBV-GC (5-14). Recently, Wu *et al*. reported that the frequency of the high-producer allele in the tumor necrosis factor alpha (*TNF-α*) gene was significantly higher among EBV-GC cases compared with controls (15). It is also known that TNF-α and other cytokines, such as interferon gamma (IFN-γ) and interleukin 1-alpha (IL1-α), up-regulate the expression of thymidine phosphorylase (TP), an angiogenic factor (16 17). Tumor-associated macrophages particularly play an important role in promoting angiogenesis by producing these cytokines (18, 19). TP is also identical to platelet-derived endothelial cell growth factor (PD-ECGF), an enzyme involved in pyrimidine nucleoside metabolism (20-22), and TP expression is an important prognostic factor of gastric carcinomas (23-25) and for other various tumor sites (26-33).

In the present study, we examined the relationship between TP and EBER expression in gastric carcinomas.

Patients and Methods

Study materials. The study was carried out on 156 gastric carcinoma cases, 119 from The Department of Surgical Oncology, Digestive Surgery, Kagoshima University Faculty of Medicine, diagnosed during the period 1986-1999, and 37 from Nampu Hospital in Kagoshima, diagnosed during 1989-1998. The studies of TP expression and EBER presence in gastric carcinomas were independently conducted using immunostaining (24, 25) and ISH assays (10), respectively. The data sets used in these studies were collated and a single data set was created. TP immunostaining and EBER-ISH assay in gastric carcinomas were conducted where either examination had not been conducted.

This study was approved by the Ethics Committee of Kagoshima University Graduate School of Medical and Dental Sciences.

Immunohistochemistry assay to detect TP. Tissue specimens of the carcinomas and normal tissues from patients were stained immunohistochemically using monoclonal antibody against a

Table I. *Univariate analysis of thymidine phosphorylase expression according to various clinicopathological features and EBER expression.*

	Total	TP+	%	OR[a]	95% CI[a]	*P*-value[a]
Gender						0.884
Female	49	20	41%	1	reference	
Male	107	45	42%	1.1	0.5-2.1	
Age (years)						*P* for trend=0.391
≤59	42	16	38%	1	reference	
60-69	58	23	40%	1.1	0.5-2.4	
≥70	56	26	46%	1.4	0.6-3.2	
Histology						0.448
Intestinal	99	39	39%	1	reference	
Diffuse	57	26	46%	1.3	0.7-2.5	
Tumor site						*P* for heterogeneity=0.149
Upper third	50	27	54%	1	reference	
Middle third	32	11	34%	0.9	0.3-2.3	
Lower third	37	14	38%	1.9	0.8-4.6	
Tumor size (mm)						*P* for trend=0.024
<30	46	11	24%	1	reference	
30-54	54	27	50%	3.2	1.3-7.5	
≥55	55	26	47%	2.9	1.2-6.7	
Lymph node invasion						0.001
Negative	91	29	32%	1	reference	
Positive	51	31	61%	3.3	1.6-6.8	
Lymph vessel invasion						0.168
Negative	75	27	36%	1	reference	
Positive	81	38	47%	1.6	0.8-3.0	
Venous invasion						0.089
Negative	118	45	38%	1	reference	
Positive	37	20	54%	1.9	0.9-4.0	
Depth						*P* for trend=0.013
Intramucosal	155	2	22%	1	reference	
Submucosal	26	15	58%	4.9	1.8-13	
Muscularis propia	48	27	56%	4.6	2.0-11	
Serosa	27	11	41%	2.5	0.9-6.7	
Stage						*P* for trend<0.001
I	73	18	25%	1	reference	
II	26	15	58%	4.2	1.6-11	
III	21	11	52%	3.4	1.2-9.2	
IV	18	12	67%	6.1	2.0-19	
EBER						0.005
Negative	135	50	37%	1	reference	
Positive	21	15	71%	4.3	1.5-12	

TP: Thymidine phosphorylase; OR: odds ratio; CI: confidence interval; EBER: Epstein-Barr virus-encoded small RNA. [a]ORs and their corresponding 95% CIs and *p*-values were obtained by logistic regression models.

glutathione-*S*-transferase fusion protein that contained a 244-amino acid sequence (residues 7-250) of the amino terminus of PD-ECGF/TP as described previously (24, 25). In brief, paraffin-embedded tissue samples were cut into 3 μm-thick sections, deparaffinized with xylene and dehydrated with 98% ethanol. Endogenous peroxidase was blocked by immersion in absolute methanol with 0.3% hydrogen peroxide at room temperature for 20 min. The blocked sections were incubated overnight at 4˚C with monoclonal antibody against TP diluted 1000-fold with phosphate-buffered saline (PBS). The following morning, the slides were incubated for 30 min at room temperature with biotinylated antimouse immunoglobulins (Ig) G diluted 1:100 with PBS. The sections were washed 3 times in PBS and then incubated for 30 min with streptavidin-horseradish-peroxidase complex diluted 1:100 with PBS. After PBS washing, the sections were incubated with 0.5 mg/ml diaminobenzidine and 0.03% (v/v) hydrogen peroxide in PBS, and counterstained with hematoxylin prior to mounting. For evaluation of TP expression, specimens were considered as TP positive when >5% of the carcinoma cells were stained according to the previous studies (24-27). The evaluation of TP expression was performed without knowledge of the patients' clinicopathologic factors including EBER status.

In situ hybridization assay to detect EBER. ISH assay of paraffin-embedded tissue samples obtained from the main tumor was conducted using a digoxigenin-labeled EBER-1 oligonucleotide probe as described before (34). A case was considered to be EBER positive based on a positive signal under microscopy. Paraffin sections from a known EBER-positive

Table II. *Univariate analysis of thymidine phosphorylase expression according to various clinicopathological features and EBER expression by Lauren's histological type.*

	Total	TP+	%	ORa	95% CI[a]	*P*-value[a]
Intestinal-type tumors						
Tumor size (mm)						0.001
<30	38	7	18%	1	reference	
≥30	61	32	52%	4.9	1.9-13	
Lymph node invasion						0.002
Negative	67	19	28%	1	reference	
Positive	27	17	63%	4.3	1.7-11	
Depth						<0.001
Intramucosal	44	7	16%	1	reference	
Submucosal+	55	32	58%	7.4	2.8-19	
Stage						<0.001
I	58	13	22%	1	reference	
II-IV	35	22	63	5.9	2.3-15	
EBER						0.745
Negative	90	35	39%	1	reference	
Positive	9	4	44%	1.3	0.3-5.0	
Diffuse-type tumors						
Tumor size (mm)						0.742
<30	8	4	50%	1	reference	
≥30	48	21	44%	0.8	0.2-3.5	
Lymph node invasion						0.250
Negative	24	10	42%	1	reference	
Positive	24	14	58%	2.0	0.6-6.2	
Depth						0.991
Intramucosal	11	5	45%	1	reference	
Submucosal+	46	21	46%	1.0	0.3-3.8	
Stage						0.209
I	15	5	33%	1	reference	
II-IV	30	16	53%	2.3	0.6-8.3	
EBER						0.005
Negative	45	15	33%	1	reference	
Positive	12	11	92%	22	2.6-187	

TP: Thymidine phosphorylase; OR: odds ratio; CI: confidence interval; EBER: Epstein-Barr virus-encoded small RNA. [a]ORs and their corresponding 95% CIs and *p*-values were obtained by logistic regression models.

gastric tumor were used as positive control, and a sense probe for EBER-1 was used as negative control in every assay. EBV-GCs had the uniform presence of EBER in tumor cells but not in the surrounding normal epithelial cells.

Histology. Histologically, all cases were classified as intestinal or diffuse-type gastric carcinomas according to Lauren (35). Intestinal- and diffuse-types of Lauren classification correspond, in principle, to well-differentiated and poorly differentiated types of Nakamura and Sugano (see 36), respectively. Note here, however, that Lauren's classification was created by the studies of advanced tumors while the classification of Nakamura and Sugano was obtained from the analysis of early carcinomas. If more than two histological types were found in the same patients, the case was classified according to the predominant histological type.

The location of tumor defined as the predominant site was divided into the following three sites: cardia or upper third part, middle part, and antrum or lower third part according to the guidelines of the Japanese Research Society for Gastric Cancer (37). The depth of invasion was classified as mucosal, submucosal, muscularis propria and subserosal involvement. A tumor invading beyond the submucosa is considered to be advanced cancer (37).

Statistical analysis. Logistic regression analysis was conducted to examine the association of EBER status (positive or negative) with TP expression (positive or negative) using age, sex, tumor location (cardia, middle, or antrum), and depth of invasion as covariates. Maximum likelihood estimates of odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated. *P*-value for trend of age was calculated using age as a continuous variable in a logistic model. All the *p*-values presented were two-sided.

Results

We examined the association between EBER presence and TP expression in 156 gastric carcinomas (21 EBV-GC and 135 non EBV-GC cases). The average age at diagnosis was 65 years

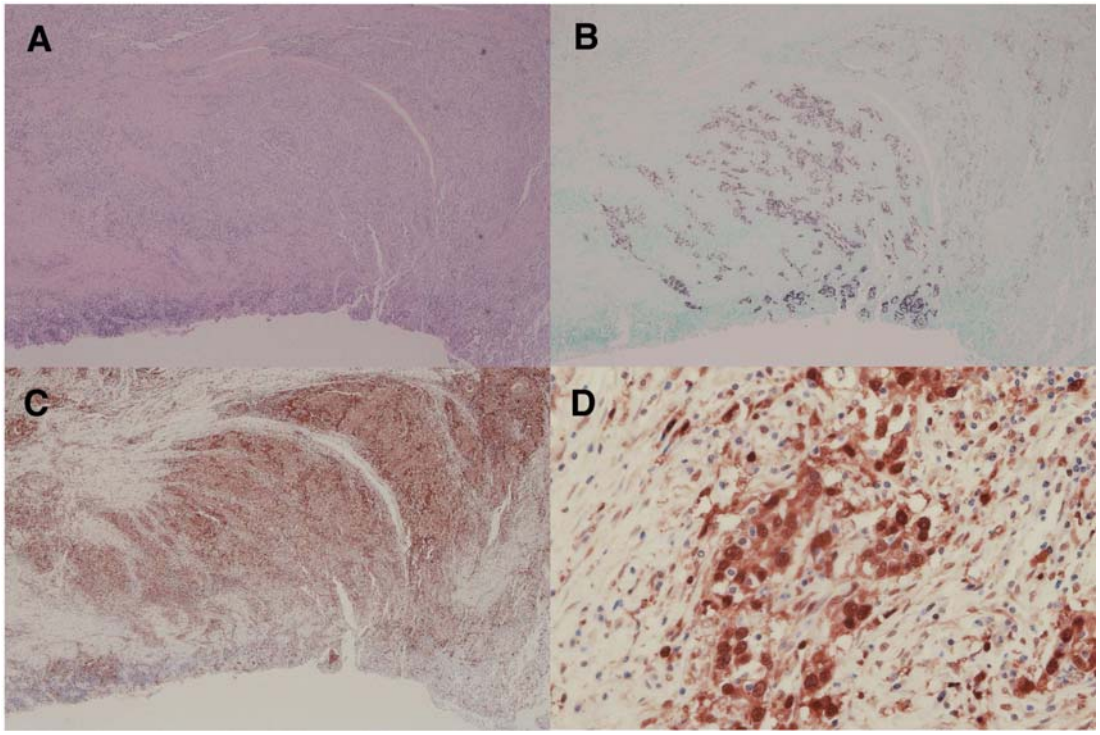

Figure 1. *Poorly differentiated adenocarcinoma. A: Hematoxylin-eosin staining (×40); B: EBER-1 in situ hybridization (×40), EBER-1-positive; C: thymidine phosphorylase (TP) immunostaining (×40), TP-positive; D: TP immunostaining (×400). Strong TP immunostaining can be observed in both cytoplasm and nuclei of carcinoma cells.*

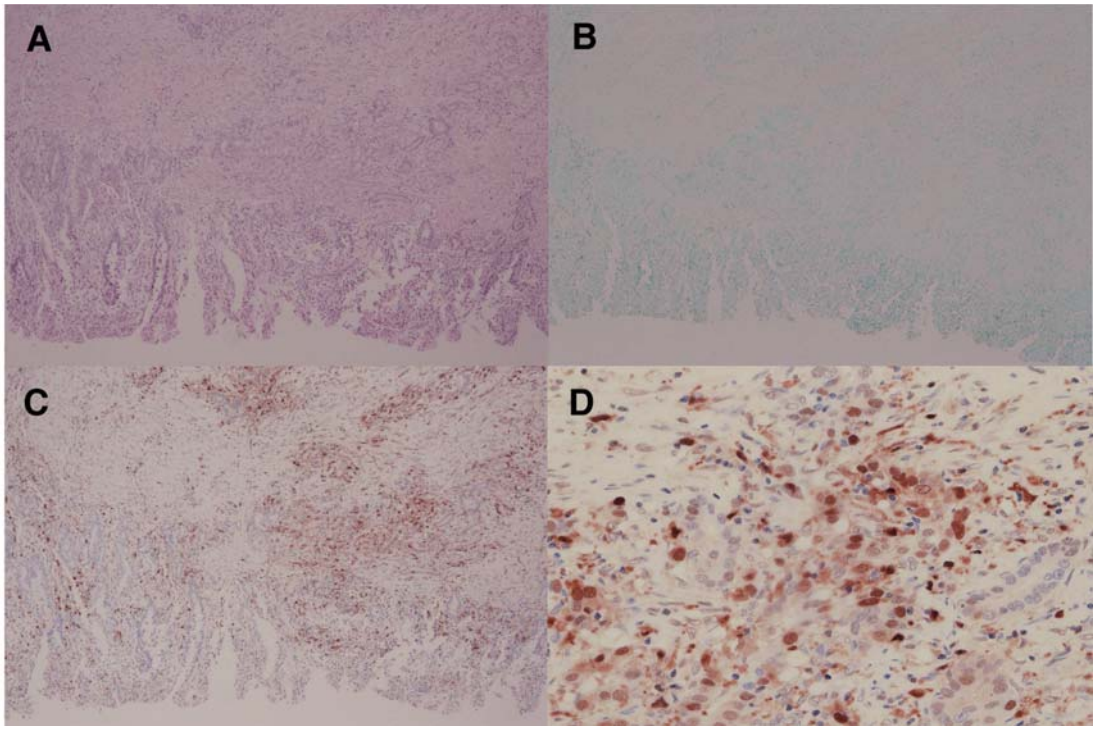

Figure 2. *Moderately differentiated adenocarcinoma lesion of the same patient as shown in Figure 1. A: Hematoxylin-eosin staining (×40); B: EBER in situ hybridization (×40), EBER-1-negative; C: TP immunostaining (×40), TP-positive; D: TP immunostaining (×400), TP-positive. TP immunostaining in the nuclei of carcinoma cells is weak.*

among both men (range 38-87 years) and women (range 32-86 years). TP was expressed in 65 (42%) of 156 cases examined. In univariate analysis, the proportion of TP-positive tumors was related to larger tumors, lymph node invasion, tumor depth, and advanced clinical stage (Table I). These associations were observed only in intestinal-type tumors (Table II).

The frequency of TP expression was nearly twice as high in EBV-GCs than in non EBV-GCs: 71% (15/21) in EBV-GCs and 37% (50/135) in non EBV-GCs. However, the association between EBER expression and TP expression was virtually limited to diffuse-type tumors (Table II). Moreover, the EBER expression was higher in diffuse-type GCs than in intestinal-type GCs, and the difference was statistically significant in a logistic analysis (p=0.035). After excluding EBV-GCs from the analysis for diffuse-type, ORs were as follows: tumor size (30 mm+): 0.6, lymph node invasion: 1.8, tumor depth (submucosal+): 1.0, and clinical stage (II-IV): 1.7. None of these associations were statistically significant. Thus, weaker associations between TP expression and prognostic factors, observed in diffuse-type of GCs, cannot be explained by the high proportion of EBV-GC cases.

When we limited the analysis to EBV-GC cases alone, TP expression also tended to be associated with larger tumors, lymph node invasion, tumor depth, and advanced clinical stage, with ORs nearly equal to or even higher than those for non EBV-GCs (data not shown). However, these associations were not statistically significant because of the small sample size of EBV-GC.

In the present study, we found an interesting case with both intestinal and diffuse tumor types. Figures 1 and 2 show EBER and TP expression patterns in the different histological types from the same patient. Figure 1 shows an EBER-positive diffuse-type (poorly differentiated, non-solid type) carcinoma with strong TP expression in both cytoplasm and nuclei of carcinoma cells. Figure 2 is an EBER-negative intestinal-type (moderately differentiated tubular type) carcinoma in the same patient, showing weak TP expression in the nuclei of carcinoma cells. Since the predominant tumor of this case was differentiated tubular type, the case was classified as intestinal-type in the analysis.

Discussion

The present study showed that TP expression was related to EBER expression in diffuse-type gastric carcinomas but not in intestinal-type tumors. The difference between the two histologic types of Lauren's classification was apparent even in a single case, where an EBER-negative intestinal-type lesion and EBER-positive diffuse-type lesion coexisted. TP staining was more evident in the diffuse-type lesions than in the intestinal-type lesions, particularly in the nucleus of carcinoma cells.

TP expression in diffuse-type tumors showed no association with tumor size, lymph node invasion, tumor depth, or clinical stage, although TP expression in intestinal-type gastric carcinomas was strongly related to those factors. Shimaoka *et al*. also reported that TP-positive differentiated adenocarcinomas invaded more deeply than the TP-negative ones, whereas that was not the case in undifferentiated adenocarcinomas (25). These observations suggest that the mechanisms of TP expression and/or its role in diffuse-type GCs may be different from those in intestinal-type GCs. As shown in the results, however, this difference cannot be explained by EBER presence, although EBV-GC was significantly related to diffuse-type tumors.

TP metabolites in the cytoplasm are suspected to be responsible for angiogenic activity of TP (22, 38). Although TP staining was observed not only in the cytoplasm but also in the nucleus in several studies (29, 39-41), as well as in the present study, its biological significance is yet to be elucidated. Fox *et al*. pointed out the possibility that TP in the nucleus may modulate the cellular thymidine pool for DNA synthesis, while TP in the cytoplasm may have other effects (40).

It has been reported that TNF-α and IFN-γ, which are known to up-regulate TP expression, were highly produced among patients with infectious mononucleosis, EBV-positive T lymphoproliferative diseases, and other EBV-related lymphomas (42-45), probably by EBV-LMP1 and/or EBNA2 (44-46). Lay *et al*. (45) suggested that EBV selectively up-regulate TNF-α expression over IFN-γ and IL1-α. Regarding EBV-GC, Ohtani *et al*. (47) examined mRNA expression levels of IFN-γ in stromal cells obtained from EBV-GCs and non EBV-GCs but there was no statistically significant difference. On the other hand, Wu *et al*. (15) found the TNF-α-producing allele of the *TNF-α* gene more frequently among EBV-GC cases than controls. According to these observations, TP overexpression in EBV-GCs might mainly be induced by TNF-α, although there is no direct evidence to prove it. Further studies are warranted to clarify which viral product(s) are involved in TNF-α production in EBV-GCs since LMP1 and EBNA2 are scarcely expressed in EBV-GCs.

In EBV-GC, infiltrating mononuclear cells showed stronger expression of Ki-67, a cell proliferation marker, than in non EBV-GC (48). In addition, there are studies showing the correlation between TP and Ki-67 expression in carcinoma (49) and adenoma (50) although the mechanism is yet unknown. van Triest *et al*. (51) reported that vascular endothelial growth factor was related to both TP immunostaining and Ki-67 index in colorectal carcinomas, suggesting a correlation between TP expression and Ki-67 index. In EBV-GC, *p16* and E-cadherin genes are frequently down-regulated (8, 11-14) probably through hypermethylation of their promoter regions (11, 13). The down-regulation of *p16* in EBV-GC may result in rapid cell-cycling and increased DNA synthesis, which increases the Ki-67 index and may induce TP expression.

In conclusion, the present study showed that the EBV genome expression affects TP expression in diffuse-type GCs but not intestinal-type GCs. Our findings suggest that EBV may be at work in relatively late stages of diffuse-type tumor development. Together with the observation that TP expression is related to prognostic factors only in intestinal-type tumors, the mechanisms involved in TP expression of gastric tumors appear to be different in intestinal- and diffuse-type tumors.

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