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The role of the Epstein-Barr virus in the oncogenesis of EBV(+) gastric carcinomas

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Abstract Five hundred and thirteen cases of gastric carcinoma were investigated for the presence of viral RNA, and the clinico-pathological data, geno-type, BamHIF restriction fragment polymorphism (RFLP) and specific LMP-1 30 bp gene deletion were also examined. EBVs detected in lymphocytes in 20 normal gastric mucosa, 7 lymphoma cell lines (LCLs) maintained in severe combined immunodeficiency (SCID) mice and 18 non-Hodgkin's lymphomas were compared with those in the gastric carcinoma cases. Thirty-three cases (6.4%) were demonstrated to be positive for EBV by means of EBER-1 RNA in situ hybridization. Clinico-pathological data showed no statistically significant difference in histological grading, location of cancer and status of vessel and lymphatic invasion between the EBV-positive and -negative groups, although the former significantly predominated in the submucosal invasion group (submucosal vs mucosal P=0.021; submucosal vs advanced cancer P=0.033). Some of these data were different from corresponding data in earlier reports. In cases that were evaluated by molecular biology, type A, wild-type F and LMP-1 gene deletion predominated except one in 21 informative cases, one in 24 and two in 16, respectively. EBVs detected in lymphocytes in normal gastric mucosa, LCLs in SCID mice and non-Hodgkin's lymphoma were also predominantly affected by type A, wild-type F and LMP-1 gene deletion with few exceptions. The results indicate a lack of genetic differences among EBVs in gastric carcinoma, normal population, LCLs of SCID mice and non-Hodgkin lymphomas. Some EBV infections in gastric carcinomas may be transient, especially in the submucosal invasion group.

Key words Gastric carcinoma · Epstein-Barr virus · Oncogenesis

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Introduction

An apparently close relation between the Epstein-Barr virus (EBV) and the development of gastric carcinoma has been found. A particularly high incidence in specific types of lesions, such as the lympho-epithelioma-like gastric carcinoma (LEGC), has been reported [15, 21], and the presence of virus in all carcinoma cells and of monoclonality has been demonstrated by examination of EBV-terminal repeats and taken as evidence of a causal role [6, 10, 22]. However, EBV detection in some lesions has also been regarded as a passenger phenomenon on the basis of investigation of early cancers found together with LEGC [20]. While it has been demonstrated that EBV is present in about 10% of common type gastric carcinomas [14], leading to the speculation that it might be playing a part as an oncovirus in this situation [23], there is no definitive support for this hypothesis.

Analysis of the coding region of EBNA-2 allows division of EBV into types A and B [25]. In developed countries, type A is found almost exclusively in the peripheral blood of persons from Western countries, where only 3% of individuals carry type B EBV [3]. In contrast, both type A and type B are detected with a high incidence in normal individuals in equatorial Africa. Type A EBV is much more efficient at immortalizing B cells than is type B [11], and it has been proposed that type B EBV can only effectively transform B cells in immunodeficient populations [13]. It has been also reported that type A EBV detected in EBV-positive tumours demonstrates a

strict 30-bp sequence deletion within the 3′ end of the latent membrane protein-1 (LMP-1), resulting in the absence of ten amino acids (amino acids 343–352) from the cytoplasmic domain [5].

Transfectants of these isolates have been found to be aggressive when inoculated into severe combined immunodeficiency (SCID) mice, and the presence of this deletion may be associated with more aggressive behaviour in tumours [2].

EBV genomes from undifferentiated nasopharyngeal carcinoma (NPC) biopsies obtained from Southern Chinese patients have been shown to contain an additional BamHI restriction site in the BamHI F region ('f' variant) [9]. Since this variant is rarely detected in spontaneous lymphoblastoid cell lines from healthy Southern Chinese and North American donors, the f variant of EBV may be implicated in the pathogenesis of NPC [1].

A large series of gastric carcinoma cases was examined to investigate the role of EBV in gastric carcinogenesis. Comparison with EBER-1-positive reactive lymphocytes in normal gastric mucosa, EBV-positive lymphoma cell lines maintained in SCID mice and non-Hodgkin lymphomas as well as in the clarified model of EBV oncogenicity were made to allow us to draw conclusions.

Materials and methods

Five hundred and thirteen consecutive gastric carcinoma cases were selected from the patients' files of the Kitasato University Hospital and the Cancer Institute Hospital for the period from 1994 to 1995. All specimens were obtained by surgical resection, routinely fixed in 10% formalin and embedded in paraffin. Histopathological diagnosis was made by means of the modified classification described elsewhere [19].

Twenty cases in which lymphocytes in nonneoplastic gastric mucosa were sporadically positive for EBER-1 were selected from among EBV-negative gastric cancer cases. EBVs detected in these cases were thought to be consistent with those of the normal population.

Seven lymphoma cell lines (LCLs) established by injection of human lymphoma cells into SCID mice were kindly provided by Professor Shigeo Mori and Dr. Mami Shiota, Department of Pathology, Institute of Medical Science, Tokyo University. The presence of EBV and B lymphocyte surface markers and activation of full latent gene expression were proven and the cells had been maintained over ten generations in SCID mice.

Eighteen cases of EBV-associated non-Hodgkin lymphoma were selected on the basis of EBER-1 RNA in situ hybridization results from among 135 cases of nodal and extranodal non-Hodgkin lymphomas treated at the Kitasato University Hospital from 1989 to 1996. All specimens examined were 10% formalin-fixed and paraffin-embedded.

The EBV RNA ISH studies were performed using a digoxigenin-conjugated oligonucleotide probe complimentary to a portion of the EBER-1 gene. Sections 4 µm thick on slides coated with poly-L-lysine were routinely deparaffinized, dehydrated and predigested with 3 µg/ml proteinase K (Sigma, USA) and then hybridized for 2 h in a 37° C incubator. Anti-digoxigenin antibody–alkaline phosphatase (×100 dilution, Boehringer Mannheim, Germany) was employed with a NBT/BCIP kit (DAKO, Denmark) for detection of EBER-1 signals. Counterstaining was with 0.3% methyl-green solution. EBV-positive non-Hodgkin's lymphoma specimens were applied as positive controls.

Genomic DNA was extracted from microdissected carcinoma cells, normal gastric mucosa including EBER-1-positive lymphocytes and lymphoma cells on 10-µm sections cut from formalin-fixed and paraffin-embedded tissues using a standard protocol.

DNA aliquots (50 ng to 1 µg) were amplified in 50 µl of reaction mixture containing 1 µmol/l of each primer, 200 µmol/l dATP, dCTP, dGTP and dTTP, 10 mmol/l Tris HCl (pH 8.8), 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.1% Triton X-100 and 1 U of Taq polymerase (Takara, Japan), and overlaid with mineal oil, PCR amplification was performed in a Program Temp Control System PC800 (ASTEC, Japan). The primer combination used, cycle parameters for PCR amplification, and product sizes of the different regions of EBV are described in Table 1.

To perform an analysis of RFLP of the BamHIF, amplified PCR DNA was purified by phenol/chloroform extraction followed by ethanol precipitation. DNA pellets were resuspended in 50 µl of distilled water, and 5-µl aliquots were digested with 20 U BamHI restriction enzyme (Boehringer Mannheim Germany).

The amplified products and digested PCR products were electrophoresed in 2.0% soft agar gels (NuSieve 3:1, Takara) at 100 V for 40 min. After gels were denatured, neutralized and Southern blot transferred onto nylon membranes (Hybon N+, Amersham, UK), hybridization was performed using 10 pmol/ml of digoxygenin-labelled oligonucleotide probes against the respective samples with DIG Easy Hyb (Boehringer Mannheim) at 42–58° C for 12 h. After washing the membranes with 2×SSC, 0.1% SDS and 0.1×SSC, 0.1% SDS at 42° C to 58° C, luminescence was detected with X-ray films (Fuji RX-U, Japan) employing a Dig luminescent detection kit for nucleic acids (Boehringer Mannheim).

Statistically significant differences were analysed using the Mann-Whitney U-test and the Chi-quare test (Statview J4.5 software, Power Mackintosh 7600/120). The level of significance was set at $P<0.05$.

Table 1 Polymerase chain reaction (PCR) primer combinations and probes for Southern blot hybridization (*wt* wild type, *del* deleted type)

Targets	Primer and probe Sequence	PCR conditions	PCR products size (bp)
EBV geno-type (type A or B)	5′-AGGCTGCCCACCCTGAGGAT-3′ 5′-GCCACCTGGCAGCCCTAAAG-3′ Probe 5′-GTTGCCGCCAGGTGGCAGC-3′	94oC 60 s 560oC 60 s ×45 72oC 120 s	type A=168 type B=184
BamHIF RFLP	5′-CAACTGCCACAGACCCCATT-3′ 5′-GGCAATGGGACGTCTTGTAA-3′ Probe 5′-GCTAAGCCAGGATAATCAGG-3′	94oC 30 s 52oC 60 s ×40 72oC 90 s	Uncut=222 cut=126
LMP-1 gene deletion	5′-CGGAGGAGGTGGAAAACAAA-3′ 5′-GTGGGGGTCGTCATCATCTC-3′ Probe 5′-GGCGGGCCCTGGTCACCTCC-3′	94oC 60 s 61oC 60 s ×50 72oC 60 s	wt=161 del=131

Results

Thirty-three of 513 gastric carcinomas (6.4%) were proven to be positive for EBV by means of EBER-1 RNA ISH. When a case was positive, all carcinoma cells demonstrated EBER-1 positivity regardless of the histological type and grade of cancer invasion (Figs. 1, 2). Clinico-pathological data for the total and the EBV positive gastric carcinomas are summarized in Table 2. The age distribution was similar between EBV-positive and EBV-negative groups. There was a male predominance in the EBV-positive cases, but no statistically significant difference was noted, gastric carcinomas being generally more frequent in males. EBV-positive cases demonstrated no histological specificity, although well-differentiated adenocarcinoma cases were relatively few compared with moderately and poorly differentiated lesions. In the group with cancer invasion limited to the submucosa, the number of EBV-positive cases was significantly larger than for the other groups (P=0.021 and P=0.033). EBV-positive carcinomas did not occur in any particular locations. Vessel and lymphatic invasion did not differ between the EBV-positive and EBV-negative groups. No differences were noted with regard to lymph node metastasis.

Table 3 summarizes details for the 33 EBV-positive gastric carcinoma cases. There were two cases of LEGC, grouped together with the poorly differentiated adenocarcinomas. Twenty cases were type A and one was type B (Fig. 3); 23 were wild type F and one was an f variant (Fig. 4). Fourteen demonstrated a characteristic LMP-1 gene deletion and two were wild type LMP-1 (Fig. 5). In case 13, EBV polymorphism was B type and f variant, and the LMP-1 gene deletion was lacking. A detailed search in this case revealed no specific findings, such as an immunocompromised state.

Sixteen cases of 20 normal gastric mucosa with EBER-1 positive lymphocytes were informative. Fifteen cases were type A and one was type B. All cases were wild type F. Fourteen cases showed LMP-1 gene deletion but one case did not. In non-Hodgkin lymphomas and LCLs of SCID mice, 16 of 18 and 6 of 7 were type A, 16

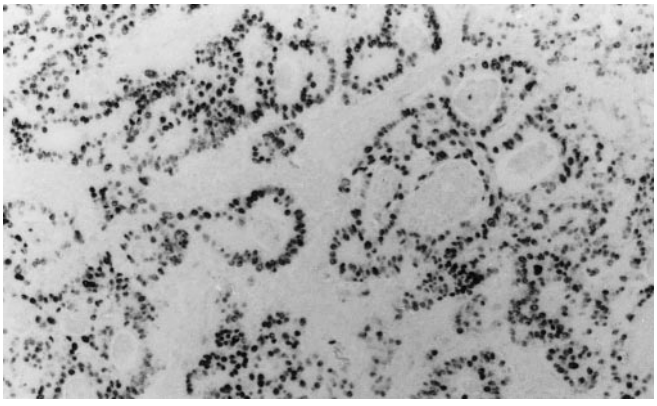


Fig. 1 EBER-1 in situ hybridization (ISH) in a well-differentiated adenocarcinoma. EBER-1 signals are located in the nuclei of cancer cells showing duct formation in association with dense small lymphocyte infiltration. ×200

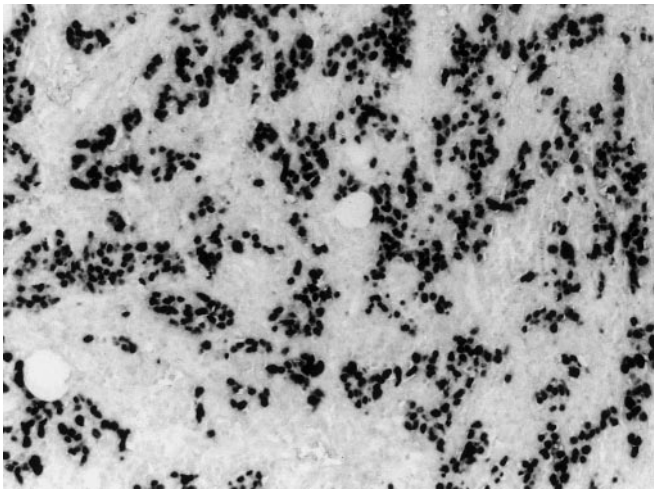


Fig. 2 EBER-1 ISH in poorly differentiated adenocarcinoma. EBER-1 signals are present in the nuclei of cancer cells, distributed diffusely with micro-tubular formation, surrounded by dense lymphoid stroma. ×200

Table 2 Clinico-pathological details of the gastric carcinomas (*M* male, *F* female, *well diff.* well differentiated, *Mod. diff.* moderately differentiated, *Poorly diff.* poorly differentiated, *m.m.* limited to within the mucosa, *s.m.* invasion of the mucosal muscle but not the proper muscle, *m.p.* invasion of the proper muscle but no penetration, *s.s.* invasion through the proper muscle but not reaching the serosa, *s.* invasion of the serosa)

	Total gastric carcinomas (513 cases)	EBV(+) gastric carcinomas (33 cases, 6.4%)	
Age	60.95	60.27	
M:F	367:146	28:5	
Histology			
Well diff.	132	5 (3.8%)	
Mod. diff.	113	9 (8.0%)	
Poorly. diff.	268	19 (7.1%)	
Depth			
m.m.	129	5 (3.9)	*
s.m.	111	13 (11.7%)	
m.p.	49	2 (4.1%)	**
s.s., s.	224	13 (5.8%)	
Location			
Cardia	66	7 (10.6%)	
Body	254	17 (6.7%)	
Antrum	193	9 (4.7%)	
Vessel invasion			
(+)	207	15 (7.2%)	
(–)	117	13 (11.1%)	
Lymphatic invasion			
(+)	179	11 (6.1%)	
(–)	145	17(11.7%)	

* P=0.021; ** P=0.033

Table 3 Summarized details of EBV-positive gastric carcinomas (*Mod.* moderately, *Poor.* poorly, *Well* well differentiated, *LEGC* lympho-epithelioma-like gastric carcinoma)

Case no.	Histological type	Cancer depth	EBV type	BamHIF RFLP	LMP-1 gene deletion
1	Mod.	m.	A	wt	+
2	Poor.	s.	A	wt	+
3	Mod.	s.m.	A	wt	+
4	Poor.	s.	A	ND	ND
5	Mod.	s.	ND	ND	ND
6	Mod.	s.m.	A	wt	ND
7	Poor.	s.	A	wt	ND
8	Poor.	s.m.	A	ND	+
9	Poor.	s.m.	A	wt	+
10	LEGC	m.p.	A	ND	ND
11	Mod.	s.m.	A	wt	+
12	Poor.	s.	A	wt	ND
13	Well	m.	B	f variant	wt
14	Poor.	s.m.	A	wt	+
15	Poor.	s.	ND	ND	+
16	Mod.	m.p.	ND	ND	ND
17	Mod.	s.m.	A	wt	+
18	Mod.	m.	ND	wt	ND
19	Well	s.m.	A	wt	+
20	Well	s.m.	ND	wt	ND
21	Mod.	s.m.	A	wt	+
22	Poor.	s.	A	wt	+
23	Well	m.p.	ND	wt	ND
24	Poor.	s.	ND	wt	ND
25	LEGC	s.	A	ND	ND
26	Poor.	s.m.	ND	wt	ND
27	Mod.	s.m.	A	wt	+
28	Well	m.	ND	ND	ND
29	Poor.	s.	A	wt	wt
30	Mod.	m.	A	wt	+
31	Poor.	s.m.	ND	ND	ND
32	Poor.	s.	ND	wt	ND
33	Poor.	s.	ND	wt	ND

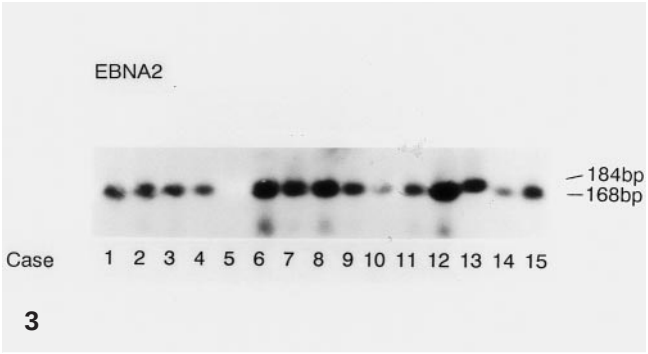
Table 4 EBV genotype, BamHIF RFLP and LMP-1 gene deletion of lymphocytes in normal gastric mucosa, lymphoma cell lines of SCID (severe combined immunodeficiency) mice and non-Hodgkin lymphomas

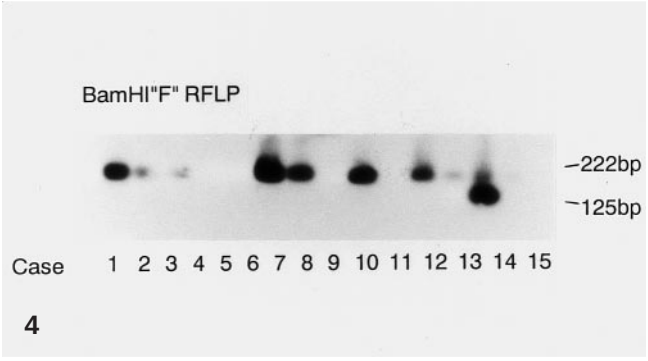
	EBV type A/B	BamHIF RFLP f/F	LMP1 gene deletion (+)/(–)
Lymphocytes in normal gastric mucosa	15/1	0/16	14 1
Lymphomas of SCID mouse	6/0	0/6	6/0
Non-Hodgkin lymphomas	16/0	0/16	16/0

and 6 were wild type F, and 16 and 6 showed LMP-1 gene deletion (Table 4).

Discussion

Investigation of a large series of Japanese gastric carcinomas revealed that in 6.2% of gastric carcinomas latent EBV infection was present [14]. The 6.4% of cases found to be positive in the present study by means of EBER-1 RNA ISH is thus in line with previous findings. Age, male-to-female ratio, histological grading of malignancy, location of cancer, vessel and lymphatic invasion, and lymph node metastasis were not linked with EBV positivity. The only associations of note were the high incidence in the submucosal invasion group. This differs from earlier reports [14] and may have been caused by





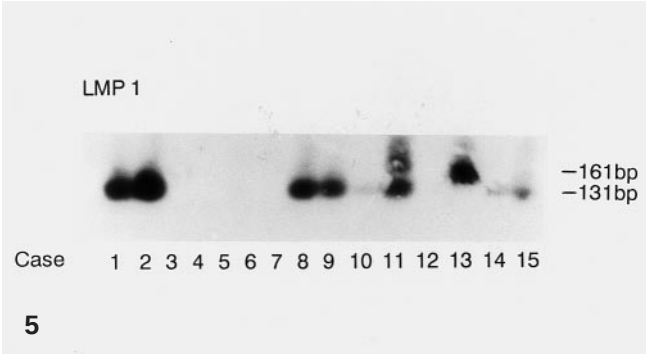


Fig. 3 Results of Southern blot hybridization of PCR products after amplification with primers for the EBNA-2 gene to determine the EBV genotype. In case 13, a single band 184 bp in length consistent with type B is found. All other cases evaluated show 168-bp products consistent with type A

Fig. 4 Results of Southern blot hybridization of PCR products after amplification with primers and digestion with the BamHI restriction enzyme for BamHIF RFLP. In case 13, a 126-bp band is seen, consistent with the f variant. All other cases evaluated demonstrate 222-bp uncut products consistent with the wild type

Fig. 5 Results of Southern blot hybridization of PCR products after amplification with primers for the LMP-1 gene; 131-bp products characteristic for 3′ end LMP-1 deletion are visible in all cases evaluated except case 13

the presence of transient EBV infection. It has been reported that EBV sometimes disappears completely in EBV-positive Burkitt lymphoma cell lines owing to loss of EBV plasmids [16]. Whereas this is not observed in lymphoblastic cell lines immortalized by EBV infection, EBV plasmids were found to be omitted in 2–4% of cells per generation in an EBV-positive Burkitt lymphoma cell line [16]. It is possible that EBV is maintained in tumour cells when it is needed for proliferation, but can be lost when this is not the case [8, 16]. In our experience, an established EBV-positive gastric carcinoma cell line lost EBV completely after a 2-year period of maintenance (data not shown). The other difference from the earlier report was that the described predominance of cancer localization in the middle to upper portion of the stomach [23] was not noted in the present study. We could not find any infectious route of EBV to gastric mucosa, as in earlier reports [15, 23]. It was proposed that EBV can infect gastric epithelium without any necessity for EBV receptor mediation. Thus, IgA antibody against EBV viral capsid antigen binds with EBV particles derived from B lymphocytes and is taken up by epithelial cells with the help of a secretory component [18]. Moreover, other researchers have reported that recombinant EBV could be infected in human gastric cell lines and proposed that this was mediated via a new receptor different from CD21 [24].

In the present study, most of the EBV evaluated in EBER-1-positive lymphocytes in normal gastric mucosa, being consistent with EBV in normal population, LCLs in SCID mouse and non-Hodgkin lymphomas, were of type A and wild type F and demonstrated the LMP-1 gene deletion. Results of the normal population are similar to those given in other reports from Japan [4, 17]. It might be suggested that the majority of EBVs in the normal population in Japan are type A and wild type F and have LMP-1 deletion, reflecting the results of LCLs in SCID mouse and non-Hodgkin lymphomas. In the case of gastric carcinomas the results were basically similar, but there was a exception. Thus, the EBV in case 13 was type B, f variant and wild type LMP-1. The present results for EBV genotype and BamHIF RFLP are in line with the predominance of type A and the low f variant incidence previously reported for Japan [4, 17].

Khanim et al. examined both normal donors and EBV-associated tumour patients, covering European, African, New Guinean, Chinese and Taiwanese populations, and found that EBV gene polymorphism in EBV isolates from healthy virus carriers occurred with similar frequencies in EBV-associated tumours from the same geographical regions [7]. The results of our study support this finding: EBV gene polymorphism in Japanese differs from that in Europeans, Africans and New Guineans. The extremely high incidence of LMP-1 gene deletion and predominance of type A is shared in common with the Chinese and Taiwanese [7], but the low incidence of f variant is different. A possible association with the f variant was suggested by the fact that the majority of NPC (68%) from Chinese had this variant, but it was also detected at a relatively high incidence (30%) in healthy Chinese donors [9]. No specific viral function has been attributed to BamHIF, although several open reading frames have been identified [7], so that this variation may be only a marker of a particular strain of EBV.

While Chen et al. described a greater oncogenic potential of the type A virus with the LMP-1 gene deletion in Brazilian Burkitt's lymphomas [2], this is clearly not the case for Japanese, Chinese and Taiwanese; there is a high incidence of this deletion even in healthy carriers. An investigation into the role of LMP-1 gene deletion in American patients with posttransplantation lymphoproliferative disorder (PT-LPD) revealed that EBV infection occurs with either wild or deleted LMP-1 type EBV and does not change thereafter [12]. It was concluded that the infection was an early event, with transformation induced regardless of the type. It seems likely that LMP-1 gene deletion is one of the factors in EBV oncogenesis, but this single mutation might only act in concert with other oncogenic factors.

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