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Gastric carcinoma: Monoclonal epithelial malignant cells expressing Epstein–Barr virus latent infection protein

(Epstein–Barr virus nuclear antigen 1/Epstein–Barr virus antibodies/Epstein–Barr virus cellular immunity)

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ABSTRACT **In 1000 primary gastric carcinomas, 70 (7.0%) contained Epstein–Barr virus (EBV) genomic sequences detected by PCR and Southern blots. The positive tumors comprised 8 of 9 (89%) undifferentiated lymphoepithelioma-like carcinomas, 27 of 476 (5.7%) poorly differentiated adenocarcinomas, and 35 of 515 (6.8%) moderately to well-differentiated adenocarcinomas. *In situ* EBV-encoded small RNA 1 hybridization and hematoxylin/eosin staining in adjacent sections showed that the EBV was present in every carcinoma cell but was not significantly present in lymphoid stroma and in normal mucosa. Two-color immunofluorescence and hematoxylin/eosin staining in parallel sections revealed that every keratin-positive epithelial malignant cell expressed EBV-determined nuclear antigen 1 (EBNA1) but did not significantly express CD45$^+$ infiltrating leukocytes. A single fused terminal fragment was detected in each of the EBNA1-expressing tumors, thereby suggesting that the EBV-carrying gastric carcinomas represent clonal proliferation of cells infected with EBV. The carcinoma cells had exclusively EBNA1 but not EBNA2, -3A, -3B, and -3C; leader protein; and latent membrane protein 1 because of methylation. The patients with EBV-carrying gastric carcinoma had elevated serum EBV-specific antibodies. The EBV-specific cellular immunity was not significantly reduced; however, the cytotoxic T-cell target antigens were not expressed. These findings strongly suggest a causal relation between a significant proportion of gastric carcinoma and EBV, and the virus-carrying carcinoma cells may evade immune surveillance.**

The human ubiquitous, transforming Epstein–Barr virus (EBV) is well documented to be causally associated with African Burkitt lymphoma, nasopharyngeal carcinoma, and B-cell lymphomas in immunodeficiency (1). EBV is also linked closely with Hodgkin disease (2, 3), thymic lymphoepithelioma-like carcinomas (4, 5), T-cell lymphomas in immunodeficiency (6, 7), and nasal T-cell lymphomas of the lethal midline granuloma-type (8). In these neoplastic cells, the EBV genome is present and the virus-encoded latent infection protein is expressed with elevated serum EBV antibodies. Lately, an association between EBV and certain cases of gastric carcinoma has been suggested, following the detection of the virus genomes in diseased tissues (9–14). To establish the causal role of EBV in primary gastric carcinoma, a common gastrointestinal cancer and the commonest of tumors in Japan, this paper concerns EBV-carrying tumor cell phenotype, clonality, EBV-encoded latent infection protein expression, and EBV-specific immune responses in a large number of patients with gastric carcinoma.

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MATERIALS AND METHODS

Subjects. One thousand consecutive, unselected cases of primary gastric carcinoma, resected from 626 male and 374 female Japanese patients 36–81 years old, were studied.

PCR. For PCR, DNA was extracted from frozen and formalin-fixed paraffin-embedded carcinoma tissues. An EBV-specific primer pair was directed for the amplification of 125 bp in *Bam*HI-W (15). The PCR products were subjected to Southern blot hybridization, using ^{32}P-labeled oligonucleotides as an internal probe. EBV PCR was also done in the *Bam*HI-K region, with a specific primer pair for the amplification of 269 bp, and was followed by Southern blotting (16). EBV genome-carrying Namalwa cells (17) were used as a positive control and the genome-free BJAB cells (18) were used as a negative control. The tumor specimens were also assessed for DNAs of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) (330-bp amplification in the coding region of HSV-1 DNA polymerase gene, which is identical to that of HSV-2) (19), human cytomegalovirus (CMV) (346 bp in the coding region of immediate early gene in *Hin*dIII-E) (20), varicella zoster virus (VZV) (642 bp in *Eco*RI-D) (21), and human herpes virus 6 (HHV-6) (776 bp in the *Sal* I fragment derived from Hashimoto strain) (22).

***In Situ* Hybridization.** *In situ* hybridization was carried out for EBV-encoded small RNA 1 (EBER1) in frozen and paraffin-embedded tissue sections with a digoxigenin-labeled EBER1 oligonucleotide probe (23). Hematoxylin/eosin staining was done in adjacent sections for the histological identification of EBV positivity.

Immunofluorescence. The anticomplement method was carried out for the detection of EBV-determined nuclear antigen 1 (EBNA1) (24). The indirect method was done for EBV early antigen (EA) and EBV capsid antigen (VCA) (25). The phenotype of cells expressing EBNA1 was defined by two-color immunofluorescence (6) using seropositive reference human serum (anti-EBNA1 of 1:1280) and fluorescein isothiocyanate-conjugated rabbit anti-human C3c (Dakopatts, Glostrup, Denmark) and a mixture of type I and type II keratin monoclonal antibodies (AE1 and AE3; ICN ImmunoBiologicals) for epithelial cells or CD45 monoclonal antibody (T29/33; Dakopatts) for leukocytes and tetramethylrhodamine isothiocyanate-labeled anti-mouse IgG (Dakopatts). Hematoxylin/eosin staining was also done in adjacent sections for histological identification of the site of immuno-

Abbreviations: EBV, Epstein–Barr virus; EBER1, EBV-encoded small RNA 1; EBNA, EBV-determined nuclear antigen; Lp, leader protein; LMP1, latent membrane protein 1; EA, EBV early antigen; VCA, EBV capsid antigen; HSV, herpes simplex virus; CMV, cytomegalovirus; VZV, varicella zoster virus; HHV, human herpes virus.

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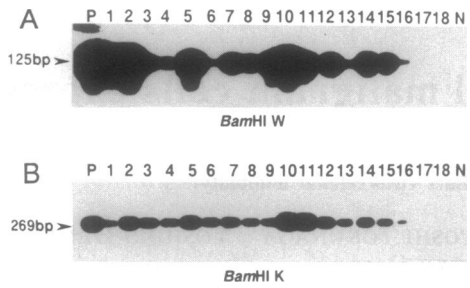

FIG. 1. Southern blot hybridization in primary gastric carcinomas. (*A*) EBV-specific 125-bp *Bam*HI-W. (*B*) EBV-specific 269-bp *Bam*HI-K PCR products. Lanes: 1–6, moderately to well-differentiated adenocarcinomas; 7–10, poorly differentiated adenocarcinomas; 11–16, undifferentiated lymphoepithelioma-like carcinomas; 17 and 18, EBV-negative gastric carcinomas; P, 10^5 Namalwa cells; N, 10^5 BJAB cells.

fluorescence. All stainings were carried out in frozen sections.

EBV Latent Infection Protein Expression. The expression of EBV-encoded latent infection proteins EBNA1, -2, -3A, -3B, and -3C; leader protein (Lp); and latent membrane protein 1 (LMP1) (26) was assessed by Western blotting (27) and immunofluorescence (28) in frozen tissues, with reference human serum containing antibodies to EBNA1, -2, -3A, -3B, and -3C; reference serum containing antibody to EBNA1 alone; and monoclonal antibodies to EBNA2 (PE2) (28), EBNA Lp (JF186) (29), and LMP1 (CS1-4) (30). Methylation of EBV latent infection protein genes was examined by Southern blotting in frozen tissues, with ^{32}P-labeled *Bam*HI-K for the EBNA1 coding region (31), *Bam*HI-Y for the EBNA2 coding region (32), *Xho* I in *Eco*RI-Dhet for the LMP1 coding region (31), and *Xho* I/*Eco*RI in *Eco*RI-Dhet for the LMP1 regulatory region (31) as probes after treatment of DNA isolated from carcinoma tissues with *Hpa* II and *Msp* I (33). Methylation was also assessed for EBNA1, -2, -3A, -3B, and -3C; Lp promoter regions in *Bam*HI-C (C promoter) and in *Bam*HI-W (W promoter); for EBNA1 F promoter region in *Bam*HI-F (F promoter); and EBNA1 F promoter regulatory elements in *Bam*HI-Q by Southern blotting, with *Bam*HI/*Eco*RI of *Bam*HI-C for C promoter, with *Bam*HI-W for W promoter, with *Bam*HI-F for F promoter, and with *Bam*HI-Q for F promoter regulatory elements as probes (33, 34) after treatment of cell DNA with *Hpa* II and *Msp* I.

Cell Clonality. To test the clonality of EBV-carrying gastric carcinomas, Southern blotting was done for the fused termini of EBV DNA in frozen tissues, with ^{32}P-labeled *Xho* I in *Eco*RI-Dhet as a probe (35). The method basically differentiates the latent form of EBV DNA from the lytic form and identifies the clonality of the latent EBV genome.

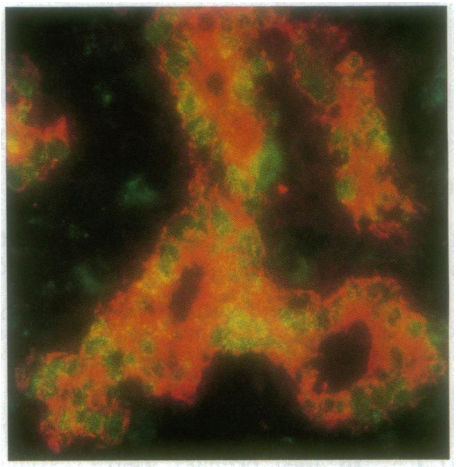

FIG. 3. Two-color immunofluorescence. Simultaneous detection of EBNA1 (yellow-green, stained with reference human serum containing anti-EBNA1) and keratin (red, stained with monoclonal antibodies) in a histological section of a well-differentiated gastric adenocarcinoma. (×600.)

EBV Antibodies. Serum samples were assayed for antibodies to VCA (IgG, IgM, and IgA) and EA (IgG and IgA) by the indirect immunofluorescence method and EBNA (IgG) by the anticomplement immunofluorescence method (36). Statistical significance was examined by Student's *t* test.

EBV Cellular Immunity. Cell-mediated immunity to EBV was assessed by T-cell-mediated regression of proliferating foci of exogenously EBV-infected autologous B lymphocytes (37). The strength of regression was expressed in terms of the minimum initial lymphocyte concentration required for a 50% incidence of regression of EBV-induced growth transformation, as calculated by the Reed–Muench formula. Statistical significance was examined by the Wilcoxon rank sum test.

RESULTS

Presence of EBV Genomes in Individual Neoplastic Cells of Gastric Carcinoma. When 1000 cases of primary gastric carcinoma were subjected to PCR Southern blots, 70 (7.0%) contained EBV genomic sequences of 125-bp *Bam*HI-W and 269-bp *Bam*HI-K (Fig. 1). Histologically, the EBV-positive tumors comprised 8 of 9 (89%) undifferentiated lymphoepithelioma-like carcinomas, 27 of 476 (5.7%) poorly differentiated adenocarcinomas, and 35 of 515 (6.8%) moderately to well-differentiated adenocarcinomas. *In situ* EBER1 hybridization and hematoxylin/eosin staining in parallel sections prepared from the same specimen showed that EBV genomes were present within every carcinoma cell in all 70 positive cases but not significantly in infiltrating lymphoid cells and in normal mucosa (Fig. 2). When metastatic lymph nodes were

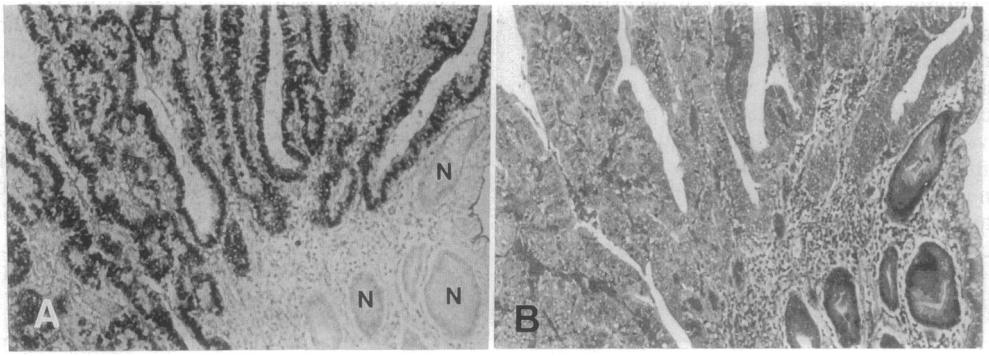

FIG. 2. (*A*) *In situ* EBER1 hybridization. (*B*) Hematoxylin/eosin staining in an adjacent pair of histological sections of a well-differentiated gastric adenocarcinoma. Every carcinoma cell but not lymphoid stroma and normal mucosa (N) is positive for EBER1. (×90.)

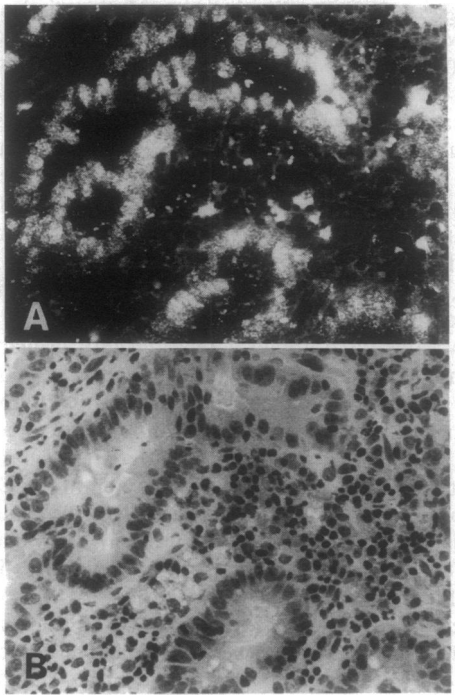

FIG. 4. (*A*) EBNA1 immunofluorescence. (*B*) Hematoxylin/eosin staining in an adjacent pair of sections of a well-differentiated gastric adenocarcinoma. Every carcinoma cell but not lymphoid stroma expresses EBNA1. Sections were stained with reference human serum containing anti-EBNA1. (×400.)

tested, all 15 lesions contained EBV genomes by PCR and *in situ* hybridization. EBV was not significantly detected in precancerous lesions and noncancerous portions. The 70 EBV-positive tumors were all negative for other HHVs: HSV-1, HSV-2, CMV, VZV, and HHV-6 DNAs (data not shown).

Detection of EBNA1 in Each Epithelial Malignant Cell in Gastric Carcinoma. Immunofluorescence was subsequently carried out to detect EBV-encoded latent infection protein expression. EBV-seropositive reference serum stained an abundance of EBNA1 in all 20 frozen specimens of either histological type (5 undifferentiated lymphoepithelioma-like carcinomas, 7 poorly differentiated adenocarcinomas, and 8 moderately to well-differentiated adenocarcinomas) of the 70 PCR-positive, *in situ* hybridization-positive gastric carcinomas. Two-color immunofluorescence of EBNA1 and keratin defined the phenotype of EBNA1-expressing cells as epithelial cells (Fig. 3). Immunofluorescence and hematoxylin/eosin staining in each adjacent pair of histological sections showed that EBNA1 was expressed in every malignant cell (Fig. 4). $CD45^+$ infiltrating leukocytes were not significantly stained (data not shown).

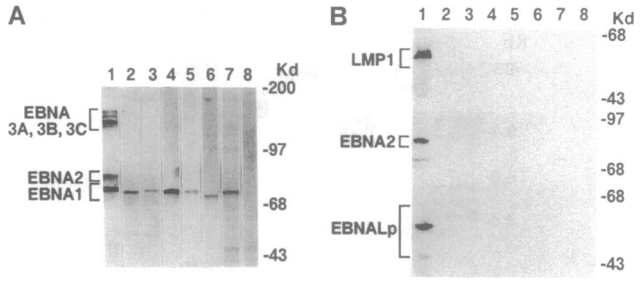

FIG. 5. Latent infection protein Western blotting in gastric carcinoma. All EBV-carrying gastrectomy specimens express EBNA1 but not EBNA2, -3A, -3B, and -3C; Lp; and LMP1. (*A*) Tested with reference human serum containing antibodies to EBNA1, -2, -3A, -3B, and -3C. (*B*) Tested with monoclonal antibodies to EBNA2, Lp, and LMP1. Lanes: 1, EBV-transformed B lymphocytes; 2 and 3, well-differentiated adenocarcinomas; 4 and 5, poorly differentiated adenocarcinomas; 6 and 7, undifferentiated lymphoepithelioma-like carcinomas; 8, EBV-negative gastric carcinoma.

Restricted Expression of EBV Latent Infection Proteins in Gastric Carcinoma. Western blotting further revealed that the 20 frozen EBV-carrying gastric carcinomas of either histological type were all positive for EBNA1 alone but not for other EBV-encoded latent infection proteins (EBNA2, -3A, -3B, -3C; Lp; and LMP1) (Fig. 5). Immunofluorescence also showed the same results (data not shown). When DNA extracted from the 20 EBV-carrying tumors was applied to *Msp* I and *Hpa* II and Southern hybridization was done, EBNA2 coding region, LMP1 coding sequence, and LMP1 regulatory sequence were all digested by methylation-insensitive *Msp* I but not by methylation-sensitive *Hpa* II (Fig. 6). Also, when C and W promoter regions for the synthesis of EBNA1, -2, -3A, -3B, and -3C; Lp; and F promoter region and F promoter regulatory elements for EBNA1 synthesis were assessed, they were well digested by *Msp* I but only partially digested by *Hpa* II (Fig. 6).

Monoclonality of EBV-Carrying Gastric Carcinoma. Southern blot hybridization of DNA extracted from the 20 frozen EBV-carrying gastric carcinomas of either histological type

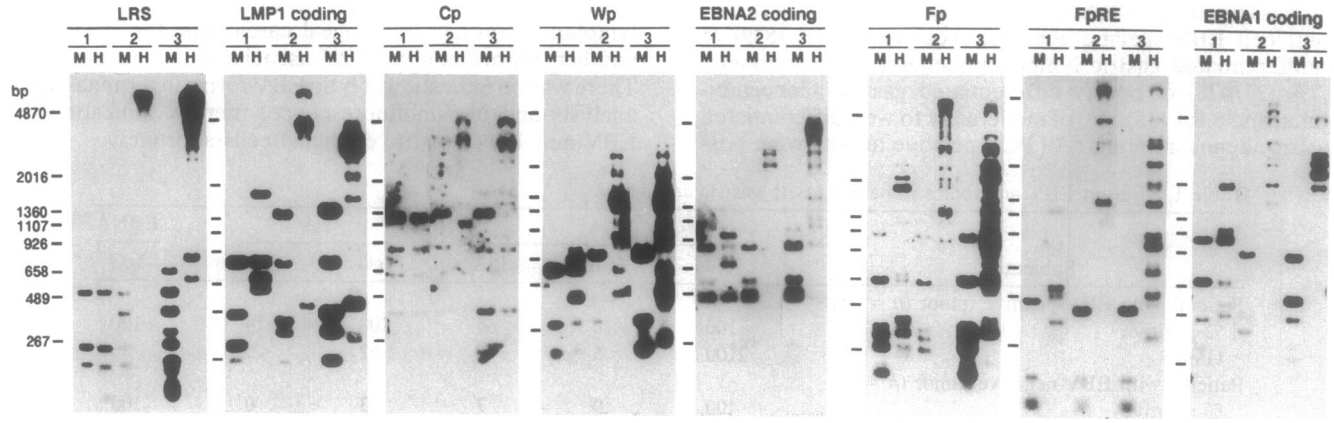

FIG. 6. Methylation status of EBV latent genes in gastric carcinoma. Cell DNA was applied to *Msp* I (lanes M) and *Hpa* II (lanes H) and Southern blotting followed. Lanes: 1, EBV-transformed B lymphocytes; 2, well-differentiated adenocarcinoma; 3, undifferentiated lymphoepithelioma-like carcinoma. LRS, LMP1 regulatory sequence; LMP1 coding, LMP1 coding region; Cp, C promoter region; Wp, W promoter region; EBNA2 coding, EBNA2 coding region; Fp, F promoter region; FpRE, F promoter regulatory elements; EBNA1 coding, EBNA1 coding region.

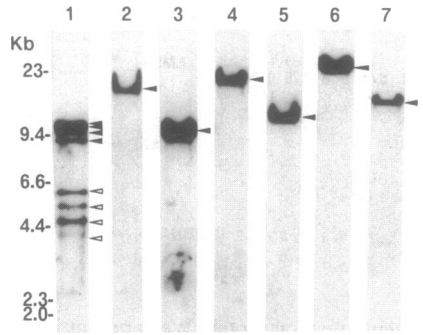

FIG. 7. Clonotypic EBV DNA analysis in gastric carcinoma. Lanes: 1, EBV-producer B95-8 cells; 2 and 3, well-differentiated adenocarcinomas; 4 and 5, poorly differentiated adenocarcinomas; 6 and 7, undifferentiated lymphoepithelioma-like carcinomas. ◀, Latent EBV DNA; ◁, productive EBV DNA.

for the fused termini of EBV genome revealed a single band in each case (Fig. 7). There was no ladder of smaller fragments on any blot (Fig. 7) nor was there EA and VCA immunofluorescence (data not shown).

Elevated EBV-Specific Antibodies in Patients with EBV-Carrying Gastric Carcinoma. Geometric mean titers of VCA IgG and EA IgG antibodies in 14 patients with EBV-carrying gastric carcinoma were all high (2100 and 62, respectively) when measured on admission, in comparison with those in 24 age-matched healthy counterparts (286 and <5) ($P < 0.01$) and those in 14 age-matched patients with EBV-negative gastric carcinoma (525 and <5) ($P < 0.01$) (Table 1). The difference in EBNA antibody titers was not significant; 56 in EBV-carrying carcinoma patients, 39 in healthy counterparts ($P > 0.05$), and 46 in EBV-negative tumor patients ($P > 0.05$) (Table 1).

Unaffected EBV-Specific Cellular Immunity in Patients with EBV-Carrying Gastric Carcinoma. EBV-specific cellular immunity, as expressed by the minimum initial lymphocyte concentration required for a 50% incidence of regression of EBV-induced growth transformation, was not significantly different among the three groups; 44.4×10^4 lymphocytes per ml in 10 patients with EBV-carrying gastric carcinoma, 23.3×10^4 lymphocytes per ml in 15 healthy counterparts ($P > 0.05$), and 42.1×10^4 lymphocytes per ml in 10 patients with EBV-negative gastric carcinoma ($P > 0.05$).

DISCUSSION

One thousand unselected primary gastric carcinoma cases were assessed by PCR Southern blots, and 70 tumors (7.0%) contained EBV-specific genomic sequences: 89% (8/9) of undifferentiated gastric lymphoepithelioma-like carcinoma, 5.7% (27/476) of poorly differentiated gastric adenocarcinoma, and 6.8% (35/515) of moderately to well-differentiated gastric adenocarcinoma. All PCR-positive tumors were positive for an *in situ* hybridization test. Metastatic lesions also had EBV. This large scale survey supports the previous findings (9–14) of the presence of EBV in diseased tissues of most cases of rare lymphoepithelioma-like carcinoma and certain cases of common adenocarcinoma of the stomach. The simultaneous viral and histological observations in each adjacent pair of sections clearly identified the site of EBV positivity as every neoplastic cell. EBV was not significantly present in normal mucosa and precancerous lesions. Since HSV-1, HSV-2, CMV, VZV, and HHV-6 DNAs were all negative, EBV is the only herpesvirus that is present in gastric carcinoma.

If EBV does have a causal relation with primary gastric carcinoma, the virus-encoded latent infection protein must be involved in the neoplastic cells. Indeed, immunofluorescence demonstrated the expression of an EBV oncoprotein, EBNA1, in all the PCR-positive, *in situ* hybridization-positive tumors tested. Simultaneous detection of EBNA1 in keratin-positive cells and hematoxylin/eosin stainings in each adjacent pair of sections of either histological type revealed that the EBV-specific protein is expressed in every epithelial malignant cell but not significantly in $CD45^+$ infiltrating leukocytes. It may therefore well be that the ubiquitous, potentially oncogenic EBV is specifically located and expressed in each gastric carcinoma cell in significant numbers of patients.

All EBV-carrying gastric carcinomas tested expressed EBNA1 alone, but not other latent infection proteins (EBNA2, -3A, -3B, and -3C; Lp; and LMP1) shown in Western blotting and immunofluorescence. This may be due to methylation of LMP1 coding and regulatory regions and also methylation of C and W promoters of EBNA genes, according to the *Msp* I and *Hpa* II digestion profiles. Although *Bam*HI-F is also methylated, EBNA1 expression may be due to methylation of the F promoter-negative regulatory element in *Bam*HI-Q (34). In fact, an active *Bam*HI-F promoter region (38, 39) has recently been found in EBV-carrying gastric carcinoma tissues (unpublished data). Thus, the characteristic EBNA1-restricted EBV expression in gastric carcinoma is the same as that in African Burkitt lymphoma, the most well known EBV-associated malignancy (40).

All EBV-carrying gastric carcinomas assessed had individual single clonotypes of EBV DNA, as determined by terminal repeat analysis. This suggests that each EBV-carrying gastric carcinoma of either histological type, not only rare lymphoepithelioma-like carcinoma (12, 14) but also common adenocarcinoma, is of monoclonal origin arising from a single EBV-infected cell, as are the cases in Burkitt lymphoma and nasopharyngeal carcinoma, the latter being another representative EBV-associated malignancy (35). There was no indication of lytic EBV forms in terminal repeat analysis and immunofluorescence, thereby indicating that EBV may infect gastric epithelial cells abortively.

Table 1. Serum EBV antibodies in patients with gastric carcinoma

Subjects	VCA IgG	VCA IgM	VCA IgA	EA IgG	EA IgA	EBNA IgG
Patients with EBV-positive tumor (n = 14)						
% positive	100	0	64	100	14	100
GMT	2100	<5	5	62	<5	56
Patients with EBV-negative tumor (n = 14)						
% positive	100	0	7	43	0	100
GMT	525	<5	<5	<5	<5	46
Healthy counterparts (n = 24)						
% positive	100	0	4	38	0	100
GMT	286	<5	<5	<5	<5	39

GMT, geometric mean titer.

High EBV antibody levels indicate high EBV activity in patients with the virus-carrying gastric carcinoma. EBV-specific T-cell-mediated immunity is normally retained in these patients as reflected by the normal EBNA antibody level; however, the gastric carcinoma cells expressing EBNA1 alone, an antigen that is not a target to T-cell cytotoxicity in contrast to other EBNAs and LMP1 (41, 42), may evade surveillance, similar to findings in African Burkitt lymphoma (43, 44).

Although the mechanism of EBV infection to normal gastric epithelial cells is unknown, our virologic and immunologic findings in a large number of patients may establish that a significant proportion of primary gastric carcinomas is a newly detected category of EBV-associated malignancies. Gastric carcinoma is a common gastrointestinal cancer and the commonest tumor in Japan, with nearly 95,000 patients per year being diagnosed (45). Thus, ≈7% of them (≈6700 cases) are EBV-carrying tumors.

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