# Epstein-Barr Virus-Positive Gastric Carcinoma Has a Distinct Protein Expression Profile in Comparison with Epstein-Barr Virus-Negative Carcinoma

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## ABSTRACT

***Purpose:*** **EBV has been detected in 2–16% of gastric carcinomas. However, there is little information available about the gene expression profile of EBV-positive gastric carcinomas.**

***Experimental Design:*** **EBV infection was examined using EBV-encoded small RNAs (EBERs) *in situ* hybridization, and 63 (5.6%) of 1127 consecutive gastric carcinomas were found to be EBV-positive. The expressions of 27 tumor-associated proteins were evaluated immunohistochemically in 63 EBV-positive gastric carcinomas and 287 EBV-negative carcinomas using the tissue array method. In addition, the genotype of EBV was investigated by PCR amplification of *LMP1* (latent membrane protein 1), Epstein-Barr nuclear antigen 2 (EBNA2), and *EBNA3B* genes.**

***Results:*** **EBV-positive gastric carcinomas are characterized by the presence of lymphoid stroma, proximal location, and predominance in males. In comparison with EBV-negative carcinomas, EBV-positive carcinomas showed frequent loss of expression of p16, smad4, FHIT, and KAI-1 (kangai 1; $P < 0.05$), but retained the expression of APC (adenomatous polyposis coli), DCC (deleted in colorectal cancer), and some DNA repair proteins ($P < 0.05$). There was negative association between EBV infection and the expression of MUC1, MUC2, MUC5AC, p53, CEA, C-erbB2, and smad7. Using hierarchical cluster analysis, we divided EBV-positive gastric carcinomas into two clusters. Those patients with cluster 1 (42 cases) carcinomas had a better prognosis than those with cluster 2 (12 cases; $P = 0.0002$) or those with EBV-negative carcinomas (280 cases; $P = 0.0251$). Fifty-one (92.7%) of 55 EBV-positive carcinomas demonstrated the 30-bp deletion in *LMP1* gene, and 53 (96.4%) of 55 cases were type 1 for *EBNA2* and *EBNA3B* genes.**

***Conclusion:*** **EBV-positive gastric carcinomas have a distinct protein expression profile as well as distinct clinicopathological features, as compared with EBV-negative carcinomas. The subclassification of EBV-positive carcinomas, by hierarchical cluster analysis, is significantly associated with patient survival.**

## INTRODUCTION

The EBV is a ubiquitous human herpes virus implicated in the etiology of many human malignancies, including lymphomas such as Burkitt's lymphoma and Hodgkin's lymphoma (1). In addition, EBV has been detected in a range of tumors of nonlymphoid lineages, including carcinomas of the nasopharynx and stomach, and smooth muscle cell-derived neoplasms in immunosuppressed individuals. Studies of conventional gastric adenocarcinomas have revealed the presence of EBV in 2–16% of cases worldwide, but the absolute number of EBV-associated adenocarcinomas of the stomach is considerable because of the high incidence of gastric carcinoma (2–4). However, little is known regarding the molecular characteristics of EBV-positive gastric carcinomas. Recent studies suggest that hypermethylation of CpG islands is a mechanism of tumor suppressor gene silencing in EBV-positive gastric carcinomas (5), but allelic loss at some markers such as 5q [*APC* (adenomatous polyposis coli)], 17p (*p53*), and 18q (*smad4*) is inversely correlated with EBV positivity (6). The *p53* tumor suppressor pathway (7) and microsatellite instability, a hallmark of a defective DNA mismatch-repair system (8), are not encountered in EBV-positive gastric carcinomas. By comparative genomic hybridization, loss of chromosomes 4p, 11p, and 18q was found to be significantly more frequent in EBV-carrying gastric carcinomas than in non-EBV-carrying gastric carcinomas (9). These results suggest that EBV-positive gastric carcinomas have a different expression status from many cancer-related genes, especially tumor suppressor genes, and different gene expression status is associated with distinct morphological and clinical features in EBV-positive gastric carcinomas.

EBV *LMP1* (latent membrane protein 1) is considered to be a potential viral oncogene, and in human B cells, *LMP1* is essential for B-lymphocyte growth transformation and likely plays an important role in the initiation and maintenance of the immortalized state (10, 11). Recently, a specific *LMP1* genotype that contains a 30-bp deletion in the 3′ COOH-terminal region of the *LMP1* gene was detected in EBV-associated gastric carcinomas (12). Apart from the *LMP1* genotype, other viral gene polymorphisms have also proved to be valuable tools in characterizing EBV strains. Two broad EBV types, 1 and 2, can

Received 7/28/03; revised 12/1/03; accepted 12/8/03.

**Grant support:** Grant FG03-11-02 from the 21C Frontier Functional Human Genome Project from the Ministry of Science & Technology of Korea.

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be distinguished by sequence divergence in the *EBNA2* (Epstein-Barr nuclear antigen), *-3A*, *-3B*, and *-3C* loci (13). In this study, using the tissue array method, we investigated the expression status of 27 known tumor-associated proteins in 63 EBV-positive gastric carcinomas and 287 EBV-negative nonselected gastric carcinomas. Using hierarchical cluster analysis, we were able to subdivide the EBV-positive gastric carcinomas into two groups, with each group showing different clinical outcome. In addition to gene expression profile, the genotype of EBV was investigated by PCR amplification of *LMP1, EBNA2,* and *EBNA3B* genes.

## MATERIALS AND METHODS

**Specimens.** The files of 1127 surgically resected gastric cancer cases examined at the Department of Pathology, Seoul National University College of Medicine over a period of 2 years (January 1995 to December 1996) were examined to evaluate their EBV status by *in situ* hybridization. To compare EBV-positive and negative carcinomas, we selected 287 consecutive EBV-negative gastric carcinoma cases treated over a period of 6 months (January 1995 to June 1995), which samples had previously been characterized for the expression status of 20 tumor-associated proteins (14). The age, sex, tumor location, gross type according to Borrmann's classification, tumor size, lymphatic invasion, and pTNM (tumor-node-metastasis) stage (15) were evaluated by a review of the medical charts and pathological records. Glass slides were reviewed to determine the histological type according to WHO classifications (16). The clinical outcome of the patients was followed from the date of surgery to either the date of death or December 1, 2000, resulting in a follow-up period ranging from 1 to 72 months (mean, 54 months). Those cases lost to follow-up and those ending in death from any cause other than gastric cancer were regarded as censored data during the analysis of survival rates.

**Tissue Array Methods.** Twenty-one array blocks containing a total of 1127 cases were conducted, as described previously (Superbiochips Laboratories, Seoul, Korea; Refs. 14, 17). Because it had previously been proven that there is excellent agreement between the staining results obtained from different intratumoral areas of gastric carcinomas (17), a core was sampled from each case. An adequate case was defined as a tumor occupying more than 10% of the core area. Each block contained an internal control consisting of nonneoplastic gastric mucosa from body, antrum, and intestinal metaplasia. Four μm were cut from each tissue array block, deparaffinized, and dehydrated.

**EBER *in Situ* Hybridization.** Sections were digested with proteinase K and were hybridized for 2 h at 37°C with a fluorescein-conjugated EBV oligonucleotide probe for EBV-encoded small RNAs (EBERs; Novocastra, Newcastle upon Tyne, United Kingdom). We performed EBER *in situ* hybridization on 21 array slides. Hybridization products were detected using an alkaline phosphatase-conjugated antibody to FITC [affinity-isolated rabbit F(ab′)]. 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium was used as an enzyme substrate to demonstrate alkaline phosphatase activity. The slides were counterstained with Mayer's hematoxylin, and positive staining was observed under light microscopy as black granules at the site of hybridization. Only those cases with signals within tumor cell nuclei were considered to be positive. Sixty-three cases (5.6%) of a total of 1127 consecutive cases of gastric carcinomas were found to be EBV-positive.

**Immunohistochemistry.** Immunohistochemical staining against tumor-associated gene products was performed using a streptavidin peroxidase procedure after an antigen retrieval process using microwaves or autoclaves. Immunostaining was performed on seven array slides with 287 EBV-negative carcinomas and 63 EBV-positive carcinomas. Twenty-seven antibodies among various commercially available antibodies were selected, after a test procedure using a human control slide for immunohistochemistry (Superbiochips Laboratories, Seoul, Korea). Table 1 and Fig. 1 list the antibodies used in this study. For statistical analysis of this large scaled data (14, 18), the results of immunostaining were considered to be positive if 10% or more of the neoplastic cells were stained. MUC1, MUC2, MUC5AC, and MUC6 protein expression status was considered to be positive when more than 20% of the cancer cells showed cytoplasmic staining (17).

**PCR Studies for the 30-bp Deletion in *LMP1* Gene and for EBV 1 and 2 Typing.** The DNA of cancerous tissue from 55 of 63 patients with EBV-positive gastric cancers was obtained from formalin-fixed, paraffin-embedded surgical blocks. The DNA was extracted by proteinase K digestion and the phenol/chloroform procedures. The extracted DNA was amplified by PCR with two 20-base oligonucleotide primers flanking the site of the characteristic 30-bp deletion of *LMP1*, as previously published (19): 5′-CGGAAGAGGTTGAAAACAAA-3′ and 5′-GTGGGGGTCGTCATCATCTC-3′. The amplified product of the 3′ end of the EBV *LMP1* gene is 161 bp, whereas a product containing the characteristic deletion is 131 bp. Two 20-base oligonucleotide primers flanking a region of the *EBNA2* and *EBNA3B* differing between type 1 and type 2 EBV were used, as reported previously (12): 5′-AGGCTGCCCACCCTGAGGAT-3′ and 5′-GCCACCTGGCAGCCCTAAAG-3′ for *EBNA2*, and 5′-CCCTTGCGGATGCAGCCAAT-3′ and 5′-GGCTGATATGGAATGTGCCC-3′ for *EBNA3B*. The expected amplification products of *EBNA2* are 168 bp for type 1 and 184 bp for type 2, and the expected amplification products of *EBNA3B* are 125 bp for type 1 and 149 bp for type 2 (Fig. 2).

**Statistical Analyses.** Either the $\chi^2$ test or Fisher's exact test (2-sided) was performed to determine the correlation between the EBV status of the gastric carcinomas and the clinicopathological parameters, and between the EBV status and the protein expression status. Survival curves were estimated using the Kaplan-Meier product-limit method, and the significance of the differences between the survival curves was determined using the log-rank test. The results were considered to be statistically significant at a $P$ of less than 0.05. Hierarchical cluster analysis and classifications were based on 54 cases of EBV-positive gastric carcinomas, which cases were available for immunostaining against all 27 antibodies. All statistical analyses were conducted using the SPSS 11.0 statistical software program (SPSS, Chicago, IL).

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*Table 1* Antibodies used for immunohistochemical study

| Antibody | Retrieval methods | Dilution | Source | Nonneoplastic mucosa | Altered expression in cancer |
|---|---|---|---|---|---|
| p16 | Autoclave | 1:50 | Pharmingen (San Diego, CA) | Nucleus | Loss |
| smad4 | Microwave | 1:50 | Santa Cruz (Santa Cruz, CA) | Nucleus | Loss |
| FHIT | Microwave | 1:250 | Zymed (South San Francisco, CA) | Nucleus | Loss |
| KAI1 | Microwave | 1:200 | Santa Cruz (Santa Cruz, CA) | Cytoplasmic | Loss |
| MGMT | Microwave | 1:50 | Chemicon (Temecula, CA) | Nucleus | Loss |
| p53 | Microwave | 1:100 | DAKO (Carpinteria, CA) | Negative | Nucleus |
| APC | Microwave | 1:400 | Abcam (Cambridge, UK) | Cytoplasmic ± nucleus | Loss |
| DCC | Microwave | 1:50 | BD Biosciences (Franklin Lakes, NJ) | Cytoplasmic | Loss |
| E-cadherin | Microwave | 1:200 | Transduction (Lexington, KY) | Membranous | Loss |
| Rb | Microwave | 1:50 | Pharmingen (San Diego, CA) | Nucleus | Loss |
| VHL | Microwave | Prediluted | Neo Markers (Fremont, CA) | Cytoplasmic | Loss |
| PTEN | Microwave | 1:50 | A.G. Scientific (San Diego, CA) | Cytoplasmic | Loss |
| rad21 | Microwave | 1:100 | IMGENEX (San Diego, CA) | Nucleus | Loss |
| rad9 | Microwave | 1:100 | IMGENEX (San Diego, CA) | Nucleus | Loss |
| Ku-70 | Microwave | 1:1000 | Santa Cruz (Santa Cruz, CA) | Nucleus | Loss |
| MUC1 | Microwave | 1:100 | Novocastra (Newcastle, UK) | Negative | Cytoplasmic |
| MUC2 | Microwave | 1:100 | Novocastra (Newcastle, UK) | Cytoplasm[a] | Cytoplasmic |
| MUC5AC | Microwave | 1:100 | Novocastra (Newcastle, UK) | Cytoplasm[b] | Cytoplasmic |
| MUC6 | Microwave | 1:100 | Novocastra (Newcastle, UK) | Cytoplasm[b] | Cytoplasmic |
| CEA | Microwave | 1:50 | DAKO (Carpinteria, CA) | Negative | Cytoplasmic |
| C-erbB2 | Microwave | 1:75 | DAKO (Carpinteria, CA) | Negative | Membranous |
| smad7 | Microwave | 1:100 | Santa Cruz (Santa Cruz, CA) | Negative | Cytoplasmic |
| bcl-2 | Microwave | 1:100 | DAKO (Glostrup, Denmark) | Negative | Nucleus |
| CD44 | Microwave | 1:40 | Novocastra (Newcastle, UK) | Negative | Membranous |
| β-Catenin | Microwave | 1:200 | Transduction (Lexington, KY) | Membranous | Nucleus |
| Cox-2 | Microwave | 1:400 | Cayman (Ann Arbor, Michigan) | Negative | Cytoplasmic |
| p63 | Microwave | 1:100 | Santa Cruz (Santa Cruz, CA) | Negative | Nucleus |

[a] Cytoplasmic staining in intestinal metaplasia and negative in gastric proper gland.
[b] MUC5AC was positive in superficial glands and MUC6 was positive in deep glands of gastric mucosa.

## RESULTS

**Clinicopathological Features of EBV-Positive Carcinomas.** In the EBER-positive cases, the EBER signals were specifically localized in the nuclei of nearly all of the tumor cells. The EBV-positive gastric carcinomas tended to have lymphoid stroma ($P < 0.001$; Table 2). They were mostly of the poorly differentiated type and proximal location, and were more prevalent in male patients ($P < 0.05$). However, there was no difference in pTNM stage between the EBV-positive and EBV-negative carcinomas. During the follow-up period, 117 (34.1%) of the 343 patients died (16 from the EBV-positive group and 101 from the EBV-negative group). The survival rate of the patients with EBV-positive carcinomas (5-year survival rate, 74.1 ± 5.6%) was better than that of the patients with EBV-negative carcinomas (5-year survival rate, 65.4 ± 2.9%), but this was not statistically significant ($P = 0.143$). By means of Kaplan-Meier survival curves stratified according to disease progression (advanced and early carcinomas), the EBV status was found to be correlated with patient survival in the case of advanced carcinoma, with marginal statistical significance ($P = 0.053$).

**Gene Expression Profile of EBV-Positive Carcinomas.** Table 3 shows that there is a distinct pattern of protein expression in the case of EBV-positive gastric carcinomas. EBV-positive carcinomas had a more frequent loss of expression of p16 ($P < 0.001$), smad4 ($P < 0.001$), FHIT ($P < 0.001$), KAI-1 (kangai 1; $P = 0.002$), MGMT ($O^6$-methylguanine DNA-methyltransferase; $P = 0.06$), and E-cadherin ($P = 0.089$) than did EBV-negative carcinomas. However, the losses of expression of some tumor suppressor proteins, including APC ($P = 0.002$) and DCC (deleted in colorectal cancer; $P = 0.025$), were not frequently encountered in EBV-positive carcinomas. Most EBV-positive carcinomas showed the preservation of DNA repair gene products, such as rad21 (0 of 62, 0%), rad9 (1 of 61, 1.6%), and Ku-70 (2 of 63, 3.2%), and only a few cases of EBV-positive carcinomas expressed MUC1 (5 of 63, 7.9%) or MUC2 proteins (3 of 63, 4.8%). There was an inverse correlation between EBV infection and the overexpression of p53 ($P < 0.001$), CEA (carcinoembryonic antigen; $P = 0.004$), C-erbB2 ($P = 0.007$), and smad7 ($P = 0.003$). No significant association was found between the EBV status and the expression status of PTEN (phosphatase and tensin homolog deleted on chromosome 10), rb, VHL (von Hippel Lindau), MUC6, bcl-2, CD44, β-catenin, Cox-2, or p63 proteins in gastric carcinomas.

**Hierarchical Cluster Analysis of EBV-Positive Carcinoma.** Fifty-four EBV-positive gastric carcinomas were analyzed by hierarchical clustering according to the protein expression profile, and the tumors were separated into two main branches (Fig. 3). The left branch, named cluster 1, consisted of 42 EBV-positive carcinomas and was characterized by lower lymph node metastasis and lower pTNM stage (Table 4). To investigate whether the two groups might represent clinically distinct subgroups of patients, univariate survival analysis was performed. The right branch, cluster 2, consisting of 12 cases, was associated with poor prognosis in comparison with cluster 1 ($P = 0.0002$). The survival rate of patients with EBV-positive

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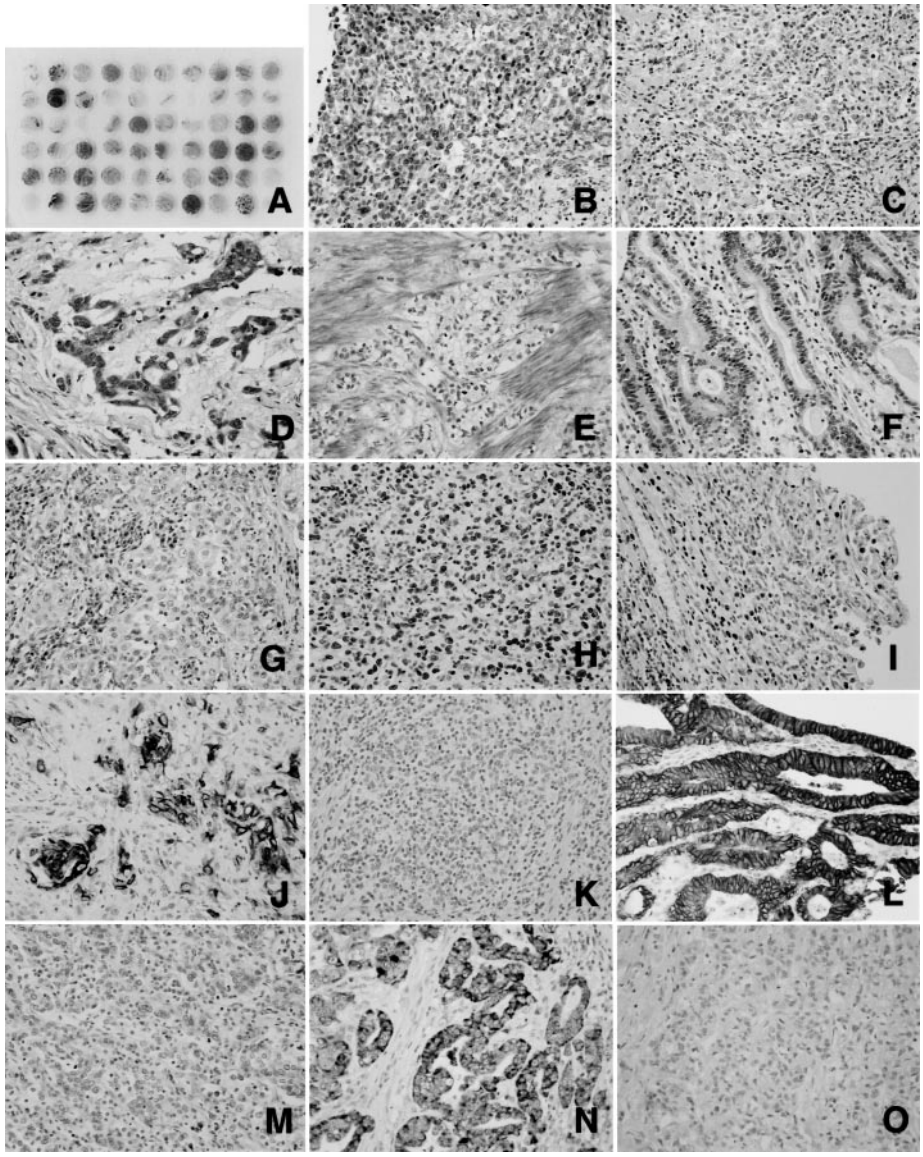


*Fig. 1* Immunohistochemical analyses in EBV-positive gastric carcinomas. *A*, overview of tissue array slide (EBV *in situ* hybridization, ×1). *B*, p16-positive tumor sample (×400). *C*, p16-negative (×400). *D*, DCC (deleted in colorectal cancer)-positive (×400). *E*, DCC-negative (×400). *F*, MGMT ($O^6$-methylguanine DNA-methyltransferase)-positive (×400). *G*, MGMT-negative (×400). *H*, Ku-70-positive (×400). *I*, Ku-70-negative (×400). *J*, MUC1-positive (×400). *K*, MUC1-negative (×400). *L*, C-erbB2-positive (×400). *M*, C-erbB2-negative (×400). *N*, Cox-2-positive (×400). *O*, Cox-2-negative (×400).

carcinomas was found to be better than that of patients with EBV-negative carcinomas, but cluster 2 of the EBV-positive carcinomas was associated with the worst outcome (Fig. 4).

**Genotypes of Epstein-Barr Virus.** Fifty-three (96.4%) of 55 EBV-positive gastric carcinomas were classified as type 1 for *EBNA2* and *EBNA3B*, whereas 2 (3.6%) of 55 showed mixed infection status of type 1 and type 2. The 30-bp *LMP1* deletion variant was detected in 51 (92.7%) of 55 EBV-positive carcinomas. By univariate survival analysis, type 1 for *EBNA2* and *EBNA3B* and the 30-bp deletion of *LMP1* were associated with poor prognosis but without statistical significance ($P = 0.3913$ and 0.7742, respectively).

## DISCUSSION

In this study, 63 (5.6%) of 1127 consecutive gastric carcinomas were EBV-positive, and this rate was similar to the findings of previous reports in the United Kingdom (3), Italy (20), and Japan (21). Between January 1 and June 30, 1995, the EBV-positive rate was 5.6% (17 of 304), and between July 1, 1995, and December 31, 1996, the EBV-positive rate was also 5.6% (46 of 823). The former 17 EBV-positive gastric carcinomas had a similar protein expression profile to that of the total of 63 EBV-positive carcinomas (data not shown). The clinicopathological characteristics of these 63 EBV-positive gastric carcinomas, such as rich lymphoid stroma, proximal location, and predominance in males, were also in agreement with the results of other investigations (22, 23).

The role of EBV in carcinogenesis of the stomach is not completely understood. The latency type of EBV in gastric adenocarcinomas is distinct from the known EBV latency types, *e.g.*, in Burkitt's lymphomas and nasopharyngeal carcinomas. Recently, it has been suggested that EBV-positive carcinomas

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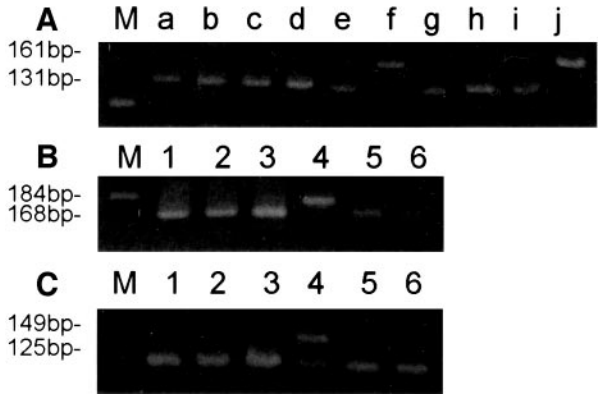


*Fig. 2* PCR results. *A*, the deletion in *LMP1* (latent membrane protein 1) gene. *Cases f* and *j*, the wild type of *LMP1* gene; *Cases a–e, g–i*, the 30-bp deletions in *LMP1* (*M*, marker). *B*, the genotype for *Epstein-Barr nuclear antigen 2 (EBNA2*). *Case 4*, type 2 for *EBNA2* gene; *Cases 1, 2, 3, 5*, and *6*, type 1. *C*, the genotype for *EBNA3B. Case 4*, the mixed type 1 and 2 infection status.

*Table 2* Comparison of EBV-positive and EBV-negative gastric carcinomas according to the clinicopathological findings

| | EBV-negative carcinomas ($n$ = 287) | EBV-positive carcinomas ($n$ = 63) | $P$ |
|---|---|---|---|
| Age (years) | 54.8 ± 13.1 | 53.4 ± 12.7 | >0.05 |
| Sex | | | <0.001 |
| Male | 190 | 57 | |
| Female | 97 | 6 | |
| WHO classification | | | <0.001 |
| Well and moderately differentiated | 110 | 13 | |
| Poorly differentiated | 112 | 48 | |
| Mucinous | 17 | 0 | |
| Signet ring | 48 | 2 | |
| pTNM stage | | | >0.05 |
| I | 126 | 27 | |
| II | 53 | 19 | |
| III | 64 | 14 | |
| IV | 44 | 3 | |
| Lymphoid stroma | | | <0.001 |
| Absent | 280 | 35 | |
| Present | 7 | 28 | |

have distinct molecular characteristics, as well as clinicopathological characteristics (5–8). The loss of p16 protein is commonly associated with epigenetic mechanisms and is more common in EBV-infected tumors arising in the body of the stomach (24, 25). In EBV-positive gastric carcinomas, bcl-2 expression was found to be significantly higher than that in EBV-negative carcinomas, whereas no significant difference in bcl-x expression was observed (26). By means of an analysis with 27 commercially available antibodies, we showed that EBV-positive carcinomas had a distinct expression profile in comparison with EBV-negative carcinomas.

In this study, EBV-positive gastric carcinomas had a more frequent loss of expression of p16, smad4, FHIT, and KAI-1 than did EBV-negative carcinomas. These proteins have been reported to be important in gastric carcinogenesis and tumor progression (14). The losses of expression of MGMT and PTEN (phosphatase and tensin homolog deleted on chromosome 10), the promoter of which was reported to be frequently hypermethylated in EBV-positive gastric carcinomas (5), were more frequent in EBV-positive carcinomas, but without statistical significance. The expression loss of APC, one of the tumor suppressor proteins associated with colorectal carcinogenesis (27), was found in 4 of 61 EBV-positive carcinomas, but a previous study demonstrated that the rate of promoter hypermethylation of *APC* gene was about 95% in EBV-positive carcinomas (5). Another mechanism regulating gene expression, in addition to aberrant methylation, may be at work in EBV-positive carcinomas. We previously demonstrated the negative association between microsatellite instability, which is the result of mismatch repair failure and the presence of EBV in the tumor (8). In this report, negative associations were also found be-

*Table 3* Aberrant expression of various proteins in EBV-positive and EBV-negative gastric carcinoma

| | EBV positive | EBV negative | $P$ |
|---|---|---|---|
| Tumor suppressor proteins with frequent loss in EBV-positive carcinomas | | | |
| p16 (loss) | 46/60 (76.7%) | 84/271 (31.0%) | <0.001 |
| smad4 (loss) | 19/63 (30.2%) | 31/277 (11.2%) | <0.001 |
| FHIT (loss) | 49/62 (79.0%) | 128/268 (47.8%) | <0.001 |
| KAI-1 (loss) | 24/63 (38.1%) | 55/278 (19.8%) | 0.002 |
| Tumor suppressor proteins with infrequent loss in EBV-positive carcinomas | | | |
| p53 (positivity) | 3/63 (4.8%) | 97/283 (34.3%) | <0.001 |
| APC (loss) | 4/61 (6.6%) | 67/274 (24.5%) | 0.002 |
| DCC (loss) | 6/61 (9.8%) | 61/270 (22.6%) | 0.025 |
| Tumor suppressor proteins without significant correlation between EBV-positive and -negative carcinomas | | | |
| MGMT (loss) | 14/63 (22.2%) | 36/278 (12.9%) | 0.060 |
| E-cadherin (loss) | 37/63 (58.7%) | 125/267 (46.8%) | 0.089 |
| PTEN (loss) | 15/61 (24.6%) | 56/273 (20.5%) | 0.482 |
| rb (loss) | 1/63 (1.6%) | 9/278 (3.2%) | 0.696 |
| VHL (loss) | 7/63 (11.1%) | 25/279 (9.0%) | 0.597 |
| DNA repair proteins with infrequent loss in EBV-positive carcinomas | | | |
| rad21 (loss) | 0/62 (0%) | 24/267 (9.0%) | 0.012 |
| rad9 (loss) | 1/61 (1.6%) | 26/266 (9.8%) | 0.035 |
| Ku-70 (loss) | 2/63 (3.2%) | 39/273 (14.3%) | 0.015 |
| Mucin phenotype | | | |
| MUC1 (positivity) | 5/63 (7.9%) | 70/281 (24.9%) | 0.003 |
| MUC2 (positivity) | 3/63 (4.8%) | 74/274 (27.0%) | <0.001 |
| MUC5AC (positivity) | 15/63 (23.8%) | 110/283 (38.9%) | 0.024 |
| MUC6 (positivity) | 6/63 (9.5%) | 37/282 (13.1%) | 0.435 |
| Proteins with infrequent overexpression in EBV-positive carcinomas | | | |
| CEA (positivity) | 22/63 (34.9%) | 155/281 (55.2%) | 0.004 |
| C-erbB2 (positivity) | 1/63 (1.6%) | 38/281 (13.5%) | 0.007 |
| smad7 (positivity) | 9/63 (14.3%) | 93/282 (33.0%) | 0.003 |
| Proteins without correlation between EBV-positive and -negative carcinomas | | | |
| bcl-2 (positivity) | 3/63 (4.8%) | 33/280 (11.8%) | 0.100 |
| CD44 (positivity) | 9/63 (14.3%) | 51/272 (18.8%) | 0.405 |
| β-catenin (positivity) | 12/63 (19.0%) | 56/269 (20.8%) | 0.994 |
| Cox-2 (loss) | 5/62 (8.1%) | 31/272 (11.4%) | 0.445 |
| p63 (positivity) | 1/61 (1.6%) | 8/274 (2.9%) | 1.000 |

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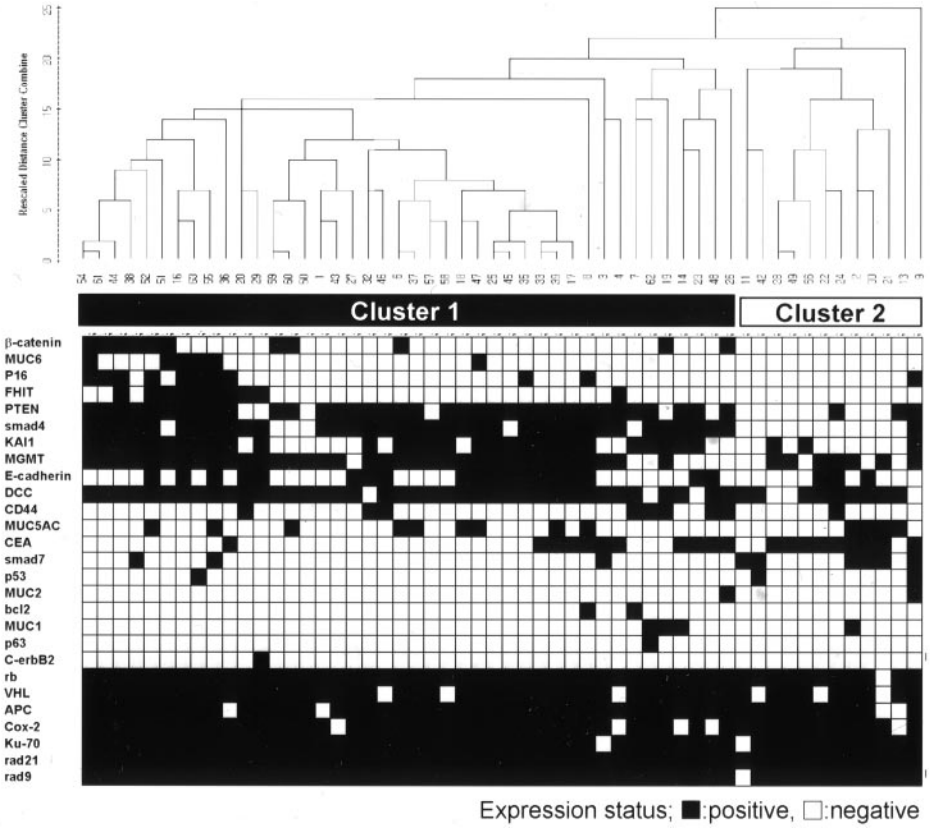


*Fig. 3* Hierarchical cluster analysis of EBV-positive carcinoma. EBV-positive gastric carcinomas were separated into two main branches, *Cluster 1* (42 cases) and *Cluster 2* (12 cases).

*Table 4* Clinicopathological characteristics in cluster 1 and cluster 2 of EBV-positive gastric carcinomas

| | Cluster 1 | Cluster 2 | *P* |
|---|---|---|---|
| Tumor (T) size (mean ± SD) | 5.0 ± 2.5 | 8.0 ± 2.7 | 0.001 |
| pT stage | | | 0.056 |
| pT1 | 11 | 0 | |
| pT2 | 26 | 8 | |
| pT3 | 5 | 4 | |
| Lymph node (N) metastasis | | | 0.022 |
| Absent | 19 | 1 | |
| Present | 23 | 11 | |
| pTNM[a] stage | | | 0.026 |
| I | 21 | 1 | |
| II–IV | 21 | 11 | |

[a] TNM, tumor-node-metastasis.

tween EBV infection and the aberration of other DNA repair proteins.

Previous studies suggested that the outcome of patients with EBV-positive gastric carcinomas was better than that of patients with EBV-negative carcinomas, but in most of these reports, the results were not statistically significant (7). Recently, it was reported that EBER expression was related to poor prognosis in intestinal-type carcinoma (28). In this study, however, when stratified according to Lauren's classification, EBV status was not associated with patient survival rate, either in the intestinal type or in the diffuse type (data not shown). For the hierarchical cluster analysis according to protein expression profile, the EBV-positive gastric carcinomas were subdivided into two groups. We observed differences in pTNM stage and clinical outcome for tumors classified as cluster 1 *versus* cluster 2. The survival rate of cluster 1 was significantly better than that

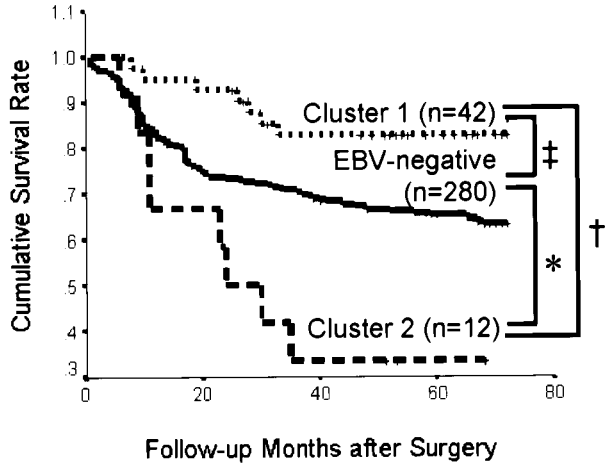


*Fig. 4* Survival curves using the Kaplan-Meier method. The survival rate of patients with cluster 1 of the EBV-positive carcinomas was found to be better than that of patients with EBV-negative carcinomas (‡, $P = 0.0251$), but cluster 2 of the EBV-positive carcinomas was associated with the worst outcome (*, $P = 0.0215$, †, $P = 0.0002$).

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of EBV-negative carcinomas, but cluster 2 was associated with the worst outcome. Therefore, overall EBV status in gastric carcinomas had no effect on patient prognosis. Different downstream pathways or different genetic events beyond EBV infection could result in different clinical outcomes in cluster 1 and cluster 2, and this needs further investigation.

In this study, most of EBV-positive gastric carcinomas were found to be type 1 for *EBNA2* and *EBNA3B* and the 30-bp deletion variants of *LMP1*, and these results were consistent with a previous report (12). These patterns are distinct from the viruses detected from saliva of normal population, of which only about one-half is infected with viral type 1.[4] The COOH-terminal 30-bp *LMP1* deletion variant has been identified in nasopharyngeal carcinoma and various EBV-associated lymphoid tumors. It has been suggested that 30-bp deletion variants of *LMP1* induces a more aggressive transformation of epithelial cells, and a 30-bp deletion variant was associated with clinical aggressiveness of EBV-associated Hodgkin's disease (29, 30). However, 30-bp deletion variants of *LMP1* were not associated with tumor progression or lymph node metastasis in this study (data not shown). In addition, the genotype of EBV was not associated with the subclassification by hierarchical cluster analysis (data not shown). LMP1 and EBNA2, which are known to play a major role in EBV-induced oncogenesis, have not been routinely detected by immunohistochemistry in EBV-positive gastric carcinomas (12), and it can be suggested that LMP1 is not important in the tumor progression of EBV-positive gastric carcinoma.

In conclusion, EBV-positive gastric carcinomas have a distinct protein expression profile, as well as distinct clinicopathological features. In comparison with EBV-negative carcinomas, EBV-positive carcinomas showed frequent loss of expression of some tumor suppressor genes, but retained the expression of APC, DCC, and some DNA repair proteins. There were negative associations between EBV infection and the expression of MUC1, MUC2, MUC5AC, p53, CEA, C-erbB2, and smad7. By means of hierarchical cluster analysis, EBV-positive carcinoma can be subclassified into two groups, which have different clinical outcomes. The genotype of EBV-associated gastric carcinomas, was mostly of type 1 for *EBNA2* and *EBNA3B* and for the 30-bp deletion variants of *LMP1*.

## ACKNOWLEDGMENTS

We thank S. P. Kim., S. I. Choi., and Superbiochip Laboratories (Seoul, Korea) for their technical assistance.

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