Genetic Analysis of Epstein–Barr Virus Latent Membrane Protein 1 and Immunohistochemical Expression of Transforming Growth Factor (TGF)-β1, TGF-βRII, p21, p16, E2F1, Thymidylate Synthase, and NF-κB in Epstein–Barr Virus Encoded RNA-positive Gastric Adenocarcinoma

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Background: Although clinicopathologic differences have been described between Epstein-Barr virus (EBV)-positive and negative gastric adenocarcinomas, the pathogenetic basis for these differences remains unclear. In this study, efforts were made to confirm that expression of EBV-latent membrane protein (LMP1) and immunohistochemical characteristics of EBV-positive gastric adenocarcinomas.

Methods: We investigated genomic deletion, and RNA & protein expression of the EBV-LMP1, as well as immunohistochemical protein expression of transforming growth factor (TGF)-β1, TGF-βRII, p21, p16, E2F1, thymidylate synthase, and NF-κB in relation to EBV positive gastric adenocarcinoma.

Results: A total of 38 Epstein-Barr Virus Encoded RNA-positive and 80 negative gastric carcinomas were examined. A 30 bp DNA deletion in the EBV-LMP1 gene, initiating at codon 342, was detected in 94.4% of EBV-positive cases. By RT-PCR and western blotting, EBV-LMP1 mRNA and protein expressions were absent in all cases, regardless of DNA deletion. No significant differences in TGF-β1, TGF-βRII, p21, p16, E2F1, or thymidylate synthase expression were identified. However, the decreased expression of p16 was found in 84.2% of EBV-positive carcinomas, relative to only 57.5% of EBV-negative tumors (p=0.024).

Conclusion: EBV-LMP1 DNA deletion, mRNA and protein losses are highly prevalent in EBV-positive gastric adenocarcinoma among Korean patients, along with decreased p16 expression.

Key Words: Stomach neoplasms; Epstein-Barr virus; EBV-Associated Membrane Antigen; Protein p16

Gastric carcinoma is the second most frequent cause of cancer-related death in Korea, and it is a very common cancer worldwide. Most gastric adenocarcinomas are associated with intestinalized chronic gastritis caused by Helicobacter pylori infection; however, 5-10% of the cases are related to Epstein-Barr virus (EBV) infection. Gastric carcinoma with lymphoid-rich stroma (GCLRS) (also known as lymphoepithelioma-like carcinoma) is particularly associated with EBV infection.1,2 Viral-associated tumorigenesis may be important not only for viral-targeted therapies, but also in relation to early detection and prevention in the future. In pursuit of this, EBV-associated gastric cancer must be differentiated from ordinary gastric adenocarcinoma and its pathogenic mechanisms unraveled.

In most cases of GCLRS, EBV expression has been exclusively detected in the tumor cells, implicating a potential causative role for EBV within the epithelial cell itself.3-5 Studies on apoptosis,6-9 microsatellite instability (MSI) and p16 expression in EBV-positive gastric carcinomas10,11 have been reported. However, the pathogenesis of EBV-positive gastric carcinoma remains far from clear.

The EBV products may interact with a variety of anti-apoptotic molecules, cytokines, and signal transducers or exhibit these properties directly to promote malignant transformation.10 In the present study of Korean patients, therefore, efforts were made to confirm an major transforming factor in EBV-associated malignancy, that is whether EBV-positive gastric carcinomas express EBV-latent membrane protein 1 (LMP1), as has been implicated in other EBV-related tumors such as lymphoma and nasopharyngeal carcinoma.10 Recently, absence of EBV-LMP1 expression in conjunction with a 30 bp gene deletion and evidence of
lytic infection in EBV-associated gastric adenocarcinoma have been described. Little is known about these aspects of EBV biology in Korean patients. Further, the immunohistochemical staining for EBV-LMP1 needs to be clarified. Accordingly, we have investigated genomic deletion, and mRNA and protein expression of the EBV-LMP1 in Korean gastric carcinoma patients. In addition, we investigated the immunohistochemical expression of transforming growth factor (TGF)-β1, TGF-β receptor II, p21, and NF-κB which have been linked to EBV-LMP1 related tumorigenesis. And we also evaluated the expression of other cell cycle regulators such as p16 and E2F1 together with thymidylate synthase (TS) which have also been linked to EBV infection.

**MATERIALS AND METHODS**

**Materials**

We retrospectively reviewed the slides from 1,700 patients who were diagnosed as gastric adenocarcinoma and who underwent gastric resection at Wonju Christian Hospital, Yonsei University Wonju College of Medicine from 1989 to 2002. GCLRS was admitted when there was no proliferation of connective tissue in tumor stroma and lymphocytic infiltration was present in more than 30% of tumor area. As controls, 80 cases of ordinary gastric adenocarcinoma without lymphoid-rich stroma were chosen by systematic sampling of every 5th gastric adenocarcinoma resection among all the gastrectomies, that were consecutively registered at Wonju Christian Hospital over the past 4 years. GCLRS was excluded from the sampling of controls. 18 fresh samples among the Epetein-Barr virus encoded RNA (EBER) positive gastric carcinoma were available.

After reviewing the pathology reports and clinical charts of the patients, we determined the stage of each adenocarcinoma according to the standards of the American Joint Committee on Cancer (AJCC). 60 to 80 mg of fresh tissue was incubated with 95 mL of TE buffer, 10 mL of 10% SDS and 50 μL of proteinase K (10 mg/mL) in a 55°C water bath for one day. On the next day, 1 mL phenol was added, following which the sample was shaken for 10 min and then centrifuged at 13,000 rpm and 4°C for 5 min. The supernatant was transferred to a new tube, an equal amount of phenol/chloroform/isoamylalcohol (25:24:1) was added, and the sample was shaken again. It was then centrifuged for 5 min and the supernatant transferred to a new tube. An equal amount of chloroform/isoamylalcohol (24:1) was added and the sample was then shaken and centrifuged. 10 μL of 5 M sodium acetate (pH 5.2) and two volumes of 100% ethanol were added to the supernatant and this was centrifuged at 4°C and 13,000 rpm for 30 min. After the DNA pellet was washed with 70% ethanol, 300 μL of sterile water was added and incubated at 37°C for one day.

**In situ hybridization with EBER**

Tissue microarray blocks were sectioned at 5 μm thickness and dried at 30°C; each slide was treated with 10 μg/mL of proteinase K for 20–30 min at room temperature. 20 μL of EBER oligonucleotide probe hybridization solution (Novocastra, Newcastle, UK) was placed on the slide and incubated in a humidified chamber at a constant temperature of 37°C for 2 h. To suppress nonspecific background stain, the slides were treated with a blocking solution. Alkaline phosphatase-conjugated antibody to fluorescein isothiocyanate was added and incubated for 30 min. To demonstrate alkaline phosphatase activity, the slide was treated with a dilute solution of levamisol, an enzyme substrate (5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium), in the dark overnight. Mayer’s hematoxylin counterstaining was then performed. Dark brown nuclear staining was scored as positive under the light microscope.

**DNA preparation from frozen tissue**

Total RNA was isolated from fresh samples using an RNeasy Mini kit (Qiagen, CA, USA), followed by cDNA synthesis using a 1st strand cDNA synthesis kit (Roche, Germany). 200 ng of total RNA was incubated for 10 min at 25°C and then for 1 h at 42°C in the total volume of 20 μL, with 1X reaction buffer, 5 mM MgCl₂, 1mM NTP mix, 3.2 μg of random hexamer, 50 U of RNase inhibitor and 20 U of AMV reverse transcriptase, and
then it was heated for 5 min at 99°C, and finally stored at -20°C.

Polymerase chain reaction (PCR) & sequencing

EBV-LMP1 DNA and mRNA were assessed by PCR. An EBV-LMP1 positive lymphoma cell line (SNU-1183) was used as an external positive control and BJAB, an EBV-negative B lymphoma cell line was used as a negative control; these were kindly provided by Dr. W.K. Lee (Myongji University, Yongin, Korea) and Dr. J. C. Lee (Chonbuk National University, Chonju, Korea), respectively. Beta-actin was used as a constitutive internal control for RNA yield and quantification. The PCR reaction mixture consisted of 400 ng of the template, 200 μM of each dNTP, 1.5 mM MgCl₂, 2.5 U of Taq polymerase (Promega, USA, catalog #: M2865), and 20 pmole of each primer. The PCR primer sequences were: 5′-AGCGACTCTGCTGGAAATGAT-3′ (sense), and 5′-TGATTAGCTAAGGCATTCC-3′ (anti-sense).11 Cycling conditions included heating at 94°C for 5 min, followed by a total of 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Electrophoresis, purification and sequencing of the PCR products were then performed.

Western blotting

Protein levels of EBV-LMP1 were assessed by Western blotting. Protein extracts (30 μg) from archival frozen tissues were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated for 1 h at room temperature in 5% nonfat dry milk containing TBS-Tween. The blots were probed overnight with a mouse anti-human LMP antibody (Zymed) and rabbit anti-beta-actin antibody (Cell biosciences).23 Of 1,700 reviewed cases of gastric adenocarcinoma, 38 cases of GCLRS (2.2%) were identified. By light microscopy, the mucosal tumor component was typically a tubular adenocarcinoma, showing abortive tubules with an anastomosing pattern, whereas tumor in the deeper layers of the gastric wall often revealed a more diffuse invasive growth pattern showing a mixture of poorly differentiated adenocarcinoma and numerous lymphoid cells (Fig. 1). In all the cases of gastric adenocarcinoma with lymphoid-rich stroma, the tumor cells showed positive EBER in situ hybridization reactions and thus were classified as EBV-positive gastric adenocarcinoma (Fig. 2). There were no positive EBER signals detected in lymphocytes, stroma, or the non-neoplastic mucosal mucosa. EBV-positive gastric carcinomas were distributed in the antrum (28.9%), the body (63.2%), and the cardia (7.9%), and 47.4% of the tumors were stage I, 15.8% were stage II, 31.6% were stage III and 5.2% were stage IV according to the standards.

Statistical analysis

Data are expressed as the score and means ± standard deviation. Comparison of the scores between the two groups (EBV-positive versus negative gastric carcinoma) was performed using the Student’s t-test. The correlation of each parameter score was verified by bivariate correlation analysis and was expressed as a correlation coefficient (γ). In the statistical analysis, p values less than 0.05 were considered statistically significant. The statistical analysis was performed using the SPSS software, version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Of 1,700 reviewed cases of gastric adenocarcinoma, 38 cases of GCLRS (2.2%) were identified. By light microscopy, the mucosal tumor component was typically a tubular adenocarcinoma, showing abortive tubules with an anastomosing pattern, whereas tumor in the deeper layers of the gastric wall often revealed a more diffuse invasive growth pattern showing a mixture of poorly differentiated adenocarcinoma and numerous lymphoid cells (Fig. 1). In all the cases of gastric adenocarcinoma with lymphoid-rich stroma, the tumor cells showed positive EBER in situ hybridization reactions and thus were classified as EBV-positive gastric adenocarcinoma (Fig. 2). There were no positive EBER signals detected in lymphocytes, stroma, or the non-neoplastic gastric mucosa. EBV-positive gastric carcinomas were distributed in the antrum (28.9%), the body (63.2%), and the cardia (7.9%), and 47.4% of the tumors were stage I, 15.8% were stage II, 31.6% were stage III and 5.2% were stage IV according to the standards.
Immunohistochemical staining for EBV-LMP revealed a strong positivity in the section of control (Fig. 3A) and diffuse but weak cytoplasmic and nuclear positivity in the tumor cells and lymphoid stroma of EBV-positive gastric adenocarcinoma (Fig. 3B). Non-neoplastic intestinal metaplasia in EBV-positive tumor and some of EBV-negative gastric adenocarcinoma also showed diffuse weak EBV-LMP staining (Fig. 3C, D). EBV-LMP1 DNA PCR detected the 286 bp product in 94.4% of tumors, while the 316 bp product was amplified in only 5.6%. A 30 bp DNA deletion starting from codon 342 was confirmed by sequencing (Fig. 4). In all cases including absence of deletion, no RT-PCR expression of EBV-LMP1 mRNA was detected, with appropriate internal beta-actin positive controls to assure RNA quality. Western blotting for LMP1 was investigated with appropriate external and internal beta-actin positive controls and revealed no protein expression in all cases (Fig. 5).

Immunohistochemical results for p16, p21, TGF-1, TGF-RII, thymidylate synthase and E2F1 are shown in Fig. 6A, B and Table 1. NF-κB was detected only in the cytoplasm of the non-tumorous mononuclear cells in the vicinity of the tumors, but this was absent in tumor cells in all cases. Statistical analysis revealed no significant differences for any of these parameters stratified by tumor stage. Correlation analysis of each parameter is shown in Table 1. Expression of TGF-β1 correlated with the expression of TGF-βRII & E2F1 and visa versa, demonstrating that these 3 molecules were closely correlated with one another. The cell cycle regulator proteins (p16 and p21) and the thymidylate synthase protein showed significant correlation each other,
but not with other parameters.

A statistically significant difference was demonstrated only for loss of p16 expression (p=0.024) between the EBER-positive group and the EBER-negative gastric adenocarcinoma group (Fig. 7, Table 2).

**DISCUSSION**

To further our understanding of EBV-related gastric tumorigenesis in comparison to ordinary gastric adenocarcinoma, we have demonstrated a 30-bp deletion and loss of mRNA and pro-
tein expression of the EBV-LMP1 viral gene in EBV-associated gastric adenocarcinoma among Korean patients. Loss of p16 expression in the EBV-positive cases was also observed. Other molecules described in EBV pathogenesis, including TGF-β1, TGF-βRII, and p21 revealed no differences between EBV-associated gastric adenocarcinoma and ordinary gastric adenocarcinoma. Interestingly, the absence of NF-κB expression was identified in not only the EBV-positive group but also in the EBV-negative group. These findings indicate that EBV-LMP1 loss is virtually universal in EBV-positive gastric adenocarcinoma among Korean patients.

EBV-positive lymphomas demonstrate EBV genome in most tumor cells, whereas it is present in only a very small portion of normal cells. Because of this dichotomy, EBV-targeted therapies to prevent expression of viral oncogene and to enhance the immune response against the virally encoded antigens have been proposed. This approach might also be applied to the treatment of EBV-positive gastric carcinoma by developing new therapies that target EBV molecular tumorigenesis in this distinct form of gastric carcinoma. If true, it would then become paramount to evaluate all gastric cancers for EBV involvement in order to direct therapy.

Histologically, EBV-positive gastric carcinomas show a characteristic lymphoid-rich stroma; however, in the mucosal layer, the tumors characteristically manifest as tubular adenocarcinomas with an anastomosing gland pattern and scarce lymphoid cells, similar to ordinary gastric adenocarcinoma. Therefore, small mucosal biopsy samples may yield false negative results in the morphologic identification of EBV-related gastric adenocarcinoma. Fortunately, EBER in situ hybridization is a very useful method to confirm EBV infection, especially in small biopsies where routine histology can be misleading.

**Fig. 5.** Western blotting confirms absence of EBV-LMP1 protein in EBER-positive gastric adenocarcinomas; lane 1 to 6: EBER-positive gastric carcinoma, lane (-): negative control (B cell lymphoma cell line BJAB), lane (+): positive control (cell line SNU-1183).

**Fig. 6.** Photomicrographs show the immunohistochemical expression of TGF-β (A), TGF-βRII (B), thymidylate synthase (C), E2F1 (D, arrow: positive cells), p16 (E&F), p21 (G), and NF-κB (H). The reduced p16 expression in EBV-positive gastric carcinoma (F) compared to the EBV-negative gastric adenocarcinoma (E) is distinct. The immunohistochemical staining for NF-κB reveals the positive reaction in the mononuclear inflammatory cells infiltrating the lymphoid-rich stroma of the tumor, and not in the nuclei of the carcinoma cells (H).

**Fig. 7.** The immunohistochemical expression of p16 demonstrates the statistically significant difference between the EBV-positive group and the EBV-negative group, but the other parameters are not different between the two groups. *p=0.024 (<0.05)
EBV-LMPs are transmembrane molecules that are expressed during latent EBV infection, together with the EBV nuclear antigens. Two transmembrane EBV-LMP proteins (LMP1 and LMP2) play important roles in the initiation and maintenance of malignant transformation in EBV-infected lymphocytes and fibroblasts. These proteins are believed to play similar roles to the EBV oncopogenes. EBV infection has been described as having four types of latency patterns, according to the expression of latent genes. Type 0 is the pattern detected in healthy individuals, and type I, II, and III are associated with malignancy. Of them, EBV-positive gastric carcinoma shows a type I pattern with the production of BARF-1 and absence of LMP1. Evidence of lytic infection has also been reported.

Studies on the expression of EBV-LMP in EBV-positive gastric carcinoma among Koreans are rare. In this study, immunohistochemical staining for EBV-LMP revealed a diffuse weak positivity in the cytoplasm and nuclei of the tumor cells as well as lymphoid cells (Fig. 1). These immunohistochemical results conflict with previous reports describing EBV-LMP1 immunohistochemical expression only in the lymphoid cells of EBV-positive gastric carcinoma, using the same primary antibody as in the present study. To more fully evaluate and clarify this discrepancy, we analyzed EBV-LMP1 mRNA expression by RT-PCR and western blotting, using fresh tumor tissue. We found complete absence of mRNA and protein expression in all cases. Further, a 30 bp deletion in EBV-LMP1 DNA was confirmed, in agreement with previous reports. Thus, based on both our DNA, mRNA and western blotting results, the immunohistochemical staining we observed in both tumor and lymphoid cell cytoplasm and nuclei is almost certainly an artifact. The use of immunohistochemical staining for EBV-LMP1 is therefore, not recommended as a diagnostic test for EBV-related gastric carcinoma.

Loss of mRNA expression is part of the mechanism for LMP1 absence in EBV-related gastric adenocarcinoma, because even tumors without gene deletion lacked mRNA expression. In a previous report by Salamon and colleagues, the absence of the EBV-LMP1 in EBV-positive gastric carcinoma was associated with the methylation of the episomal DNA of the EBV. Moreover, hypermethylation of EBV-LMP1 resulting in the absence of LMP1 expression has been described in various EBV-associated tumors. Although expression of EBV-LMP1 was not detected in EBV-positive gastric carcinomas, most of the tumor cells were EBER-positive whereas the normal mucosa was negative. This suggests that the EBV may act through alternate tumorigenesis mechanisms than those involving EBV-LMP1 or that loss of expression of this molecule may promote EBV-associated neoplasia.

In regard to the role of TGF-β1 in EBV-positive gastric carcinoma, it has been reported that an EBV-infected gastric cancer cell line was more resistant to TGF-β1-mediated growth inhibition and apoptosis than a TGF-β1-susceptible gastric cancer cell line. The mechanism whereby tumor cells acquire resistance to TGF-β1 via EBV infection also has been discussed. It has been reported that EBV-LMP1 represses TGF-β signaling through the NF-κB pathway at the transcriptional level by competing for a limited pool of transcriptional co-activators. However, in our study, none of the tumors demonstrated NF-κB nuclear expression. It was only expressed in the cytoplasm of the adjacent lymphoid cells in EBV-positive gastric carcinomas. This indicates that NF-κB does not appear to be frequently activated in gastric carcinoma from Korean patients.

An in vitro study of cell lines showed the role of EBV-LMP1 in inhibiting the TGF-β signaling on the human cell cycle inhibitor p21 WAF/Cip1 and Smad 7 gene promoters. In the present study, no differences were detected in the expression of the TGF-β and p21 WAF/Cip1, relative to EBV infection. While correlation between the TGF-β1 expression and the TGF-βRII expression was detected, no statistically significant differences between the EBV-positive group and the EBV-negative group were demonstrated. Based on these results, it is difficult to explain EBV suppression of the growth inhibitory role of TGF-β1 in EBV-positive gastric carcinoma.

E2F-1 plays a central role not only in regulating the transition from G1 phase to S phase of the cell cycle, but it also plays a role in thymidylate synthase gene expression. Mauser et al. described that EBV immediate-early protein BZLF1 mediates the switch between latent and lytic form of EBV infection and induces expression of E2F1 in EBV-associated gastric adenocarcinoma cells. In our study, E2F1 showed weak nuclear positivity in tumor cells. Further, thymidylate synthase expression was correlated with E2F1 expression. However, in comparison with the ordinary gastric adenocarcinoma, no significant difference related to EBV infection was detected. In addition, no correlation between thymidylate synthase expression and EBV infection was detected.

In the EBV-positive group, a significant difference was detected in the expression of p16 compared with the EBV-negative group. P16 is an inhibitor of the G1S transition in the cell cycle. Expression levels of p16 were significantly lower in EBV-positive gastric carcinoma, which is in agreement with the previous reports that were concerned with aberrant methylation of the
p16 gene promoter. Further study of the methylation mechanisms and role of p16 loss will provide direct implications for insight in the pathogenesis of EBV-related gastric carcinoma.

In summary, diagnostic EBV testing in gastric adenocarcinoma is best accomplished by EBER in situ hybridization. Our results show that it is not feasible by EBV-LMP immunohistochemistry, due to false-positive non-specific reactions, as confirmed by negative western blotting, RT-PCR and DNA deletion results. Tumor morphology on superficial mucosal biopsy samples is also unreliable due to the similarity of EBV-positive tumors at the mucosal surface to ordinary gastric adenocarcinoma. This study confirms EBV-LMP1 deletion and expression loss in Korean patients with EBV-positive adenocarcinomas, in combination with decreased p16 expression. Further elucidation of these pathways will hopefully lead to viral-targeted therapy of EBV gastric adenocarcinoma.

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