Epstein-Barr virus in gastric adenocarcinomas: association with ethnicity and CDKN2A promoter methylation

Q N Vo, J Gerauds, M L Gulley, D A Boudreau, J C Bravo, B G Schneider

Aims: It has been shown previously (by immunohistochemistry) that gastric adenocarcinomas harbouring Epstein-Barr virus (EBV) frequently lose p16 protein. This study aimed to examine the mechanisms of inactivation of the CDKN2A gene and correlate the results with clinicopathological features.

Methods: Methylation specific polymerase chain reaction was used to detect CDKN2A promoter methylation in gastric adenocarcinomas from American patients. In addition, immunohistochemistry was used to detect the loss of the p16 protein and in situ hybridisation was used to detect the presence of EBV. The tumours were also analysed for the presence of microsatellite instability.

Results: Eleven (10%) of 107 tumours harboured EBV in the malignant cells. In gastric cancers without EBV, 32% exhibited CDKN2A promoter methylation and 26% had p16 protein loss. In contrast, 91% of the tumours containing EBV had CDKN2A promoter methylation (p = 0.0003) and 90% showed p16 protein loss (p = 0.0001). The presence of EBV was also associated with male sex (p = 0.03) and was more common in tumours from Texas Hispanics than from non-Hispanic whites or African-Americans (p = 0.01). EBV was not associated with microsatellite instability, histological subtype, stage, or grade of the tumour, or age or survival time of the patient.

Conclusions: The presence of EBV in gastric adenocarcinomas is strongly associated with CDKN2A inactivation by promoter methylation. In addition, these findings suggest that there are ethnic differences in tumour virology and pathogenesis.

Gastric cancer is distinctive in its association with two different infectious agents that may contribute to the development of the disease. The first of these associations to be identified was that with Helicobacter pylori, a bacterium that colonises the stomach and serves as a risk factor for gastric carcinoma of the intestinal subtype. The second infectious agent found to be associated with a subset of gastric cancers is the Epstein-Barr virus (EBV). This virus was initially recognised for its association with Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma. More recently, it was found to be present in gastric adenocarcinomas, especially lymphoepithelioma-like (LEL) variants, which morphologically resemble nasopharyngeal carcinomas, but also in gastric cancers not morphologically distinguishable from non-EBV containing gastric adenocarcinomas.

The virus is present in 3–16% of gastric adenocarcinomas, in 67–100% of LEL carcinomas, and in 29–35% of gastric remnant adenocarcinomas (for a review, see Takada). Monoclonality in the size of the fused termini of the viral genome indicates that the virus was present in the original cell from which the tumour arose, and identification of EBV in premalignant lesions further implicates the virus as an early acting contributor to the process of carcinogenesis. When gastric epithelial cells were infected with EBV containing a selectable marker, clones arose that had higher proliferation rates and a higher saturation density than control cells, and which formed colonies in soft agar. Clearly, EBV is able to drive gastric epithelial cells in vitro towards a malignant phenotype, but the mechanisms by which the virus accomplishes this remain obscure.

"Epstein-Barr virus is able to drive gastric epithelial cells in vitro towards a malignant phenotype, but the mechanisms by which the virus accomplishes this remain obscure"

In a previous report, we found that EBV associated gastric adenocarcinomas showed p16 loss, as measured by immunohistochemistry (IHC), more frequently than did non-EBV associated tumours. To gain insight into the mechanisms of this loss, we performed methylation specific polymerase chain
reaction (MS-PCR) and microsatellite analysis on a larger set of gastric cancers. Here, we report the striking association of EBV with promoter methylation of the CDKN2A gene in gastric cancers.

METHODS

Specimens
Formalin fixed, paraffin wax embedded gastrectomy specimens (n = 122) from patients with gastric adenocarcinoma were obtained from hospitals in San Antonio, Texas (n = 67); New Orleans, Louisiana (n = 50); and Rochester, Minnesota (n = 5). Specimens were serially acquired, but cases with small amounts of tumour in the section were omitted from the study, as were cases with finely dispersed tumour cells. Methylation analysis data were obtained for 118 of the 122 cases, and EBV analysis data were obtained for 107 cases. The ethnicity, age, and sex of the patient and anatomical site of the tumour were obtained from medical records or tumour registries. The ethnic groups represented were white (persons of European ancestry; n = 48, 36 men, 12 women), Texas Hispanics (persons of predominantly Mexican ancestry; n = 47, 35 men, 12 women), and African–Americans (n = 25, 16 men, nine women); the ethnic origins of two patients were unknown. Staging was performed using standard TNM criteria. Tumours were graded and classified as intestinal, diffuse, or mixed subtype by an experienced surgical pathologist (JCB) according to the criteria of Laurén. Survival data were available for 100 patients from Texas and Louisiana. All specimens and patient information were obtained with the approval of the institutional review boards of the Louisiana State University Health Sciences Center and/or the University of Texas Health Science Center at San Antonio. This set of cases overlapped but is not identical to that used in our previous study of EBV in gastric cancer.

Harvesting of DNA from formalin fixed, paraffin wax embedded gastric tumour samples
Paraffin wax embedded sections of gastric adenocarcinomas were prepared for microdissection as described previously. Tumour cells and benign cells were harvested by laser capture microdissection (LCM) with the PixCell I instrument (Arcturus Engineering, Mountain View, California, USA) using a 30 µm laser spot with 50 to 60 mW power and a 50 to 100 msec pulse width. Cell preparations of 2000 to 3000 pulses were harvested and digested in 40 µl of 3M NaOH at 75°C for 15 minutes. Then, the solution. DNA was precipitated with ethanol using glycogen as a co-precipitant and resuspended in 25 µl of freshly prepared, pH 5.0, 4.8M sodium bisulfite and 14 µl of 10M hydroquinone and incubating for four hours at 55°C. The samples were concentrated with centrifugal filter devices (Centriprep YM-30, Millipore Corporation, Bedford, Massachusetts, USA). Desulfonation was performed by adding 4.5 µl of 3M NaOH to each sample, incubating at 37°C for 15 minutes, and then adding 28 µl of 5M ammonium acetate to neutralise the solution. DNA was precipitated with ethanol using glycogen as a co-precipitant and resuspended in 25 µl of water.

Methylation specific polymerase chain reaction
The sodium bisulfite modification technique relies on the capacity of bisulfite to convert unmethylated cytosine residues, but not 5-methylcytosine, to uracil by deamination. Primer sequences, as described by Herman et al., discriminated methylated from unmethylated sequences of the CDKN2A promoter based upon the chemically induced differences induced by bisulfite modification. Forward primers were labelled with γ-32P ATP using T4 polynucleotide kinase. PCR reactions contained 100mM of each primer, 200µM of each dinucleotide triphosphate, and 1.5mM MgCl2. Annealing temperatures for primers were 60°C for the primers specific for unmethylated DNA and 63°C for the primers specific for methylated DNA. After 95°C for three minutes, amplifications were carried out for 40 cycles consisting of 95°C for 45 seconds, 60°C/63°C for 45 seconds, and 72°C for one minute. PCR products were electrophoresed on 6% polyacrylamide gels for two hours at 45 W. Gels were dried under vacuum at 80°C and analysed by autoradiography on Kodak X-omat AR film.

Immunohistochemistry
Unstained sections (5 µm) were cut on to capillary gap slides, and heated at 60°C for 20 minutes. The anti-p16 monoclonal antibodies used were: Ab7 (Lab Vision Corporation, Fremont, California, USA) and clone PMG175-405 (PharMingen, San Diego, California, USA). Normal mouse IgG was used as a negative control. Sections of all cases were incubated with Ab7 at 1 µg/ml at 4°C overnight, after antigen retrieval in 0.1M EDTA, pH 8.0 (20 minutes at 95–100°C). A subset of cases was also reacted with the PMG175–405 monoclonal antibody, as described previously. The Vectastain Elite ABC kit from Vector (Burlingame, California, USA) was used for detecting bound antibodies, according to the manufacturer’s recommendations. Diaminobenzidine with a haematoxylin counterstain was used for colour development. Normal colonic mucosa and a p16 positive lung cancer xenograft were used as external positive controls. In addition, non-neoplastic stromal cells served as internal positive controls for p16 in every tumour section. A mesothelioma and pancreatic adenocarcinoma, both of which were p16 negative, were used as external negative controls. Sections were examined for evidence of nuclear staining above any cytoplasmic background; cytoplasmic staining itself was disregarded. If there was nuclear staining in a diffuse or mosaic distribution throughout the tumour, it was considered positive for p16. If the neoplastic nuclei failed to stain whereas admixed non-neoplastic cells reacted positively, the lesion was scored as negative. In a subset of carcinomas, loss of p16 reactivity was not diffuse, but limited to well defined areas within the lesion. This staining pattern was also considered abnormal and may reflect a relatively late event in the development of the tumour.

In situ hybridisation
In situ hybridisation for EBV encoded RNA 1 (EBER1) and U6 was performed on paraffin wax embedded sections, as described previously, using digoxigenin labelled riboprobes complementary to EBER1 transcripts. U6 transcripts served as a control for RNA preservation because U6 is a ubiquitous cellular RNA similar in size, copy number, and nuclear localisation to EBER1. Dr R Ambinder provided probe templates for
EBV status was determined using the log rank test. All analyses were done with SAS (SAS Institute Inc, Cary, North Carolina, USA).

RESULTS

To determine the mechanism of p16 inactivation in gastric cancers, we performed MS-PCR for the promoter region of the CDKN2A gene and obtained results from 118 gastric adenocarcinomas. Of these, 44 (37%) showed methylation of the CDKN2A promoter (fig 1). Details of clinicopathological associations with methylation are described elsewhere (QN Vo et al, unpublished data, 2002).

Figure 1 Lanes marked “U” contain PCR products amplified with primers specific for unmethylated sequences, and lanes marked “M” contain PCR products amplified with primers specific for methylated sequences. Analysis of five gastric adenocarcinomas by methylation specific PCR shows that tumours 1 and 5 have bands indicating the presence of methylation in the CDKN2A promoter. Tumours 2, 3, and 4 have bands indicating only the unmethylated form of the promoter. Tumour 1 is an Epstein-Barr virus associated tumour.

EBER1 (RA386) and U6 (RA390). Positive controls included EBV related Hodgkin’s disease cases, and negative controls included normal epithelium and other non-malignant structures in each case. Rare small lymphocytes stained with the EBER1 probe in some tumour sections, but only tumour specific EBER1 staining was considered to be a positive result.

Statistical analysis

The relation between EBV or methylation status and categorical variables such as sex, site, and grade was analysed using the χ² test or Fisher’s exact test. The time to event was used to calculate the relative hazard of death given EBV status. A Kaplan-Meier curve was constructed, and the association with EBV status was determined using the log rank test. All analyses were done with SAS (SAS Institute Inc, Cary, North Carolina, USA).

DISCUSSION

In this set of gastric adenocarcinomas, 10% were EBV related, as defined by in situ hybridisation to EBER transcripts, a method that is considered the gold standard for defining EBV associated tumours. This result was similar to the percentage reported by us (12%) in a previous study on a smaller set of
mostly North American cases, and similar to the percentage reported in a study on Japanese Americans (10%). Similar percentages have also been reported by other groups in patients from areas with a higher incidence of gastric cancer, and somewhat higher values (16%) were reported in another set of American gastric cancers.

The current cohort of patients with gastric cancer is more ethnically diverse than in our previous study. Our current study included non-Hispanic white, Hispanic, and African–American populations, mostly from South Texas and Louisiana. Remarkably, nine of the 11 patients with EBV positive tumours were found to be Hispanic men. In south Texas, Hispanic men have an increased incidence of gastric cancer, over twice as high as that of non-Hispanic white men.

It is possible that EBV contributes to this increased risk in this population. However, a study of 135 consecutive gastric cancers in Mexico found only 8% of cases to be EBV related, a proportion consistent with results reported in North American and European patients. A population based study of south Texas ethnic groups would be useful to confirm and characterise the interesting variation in host–virus interactions that our data suggest.

Geographical variations in EBV associated tumours have long been noted. Burkitt’s lymphoma, the first tumour known to be associated with EBV, is found primarily in equatorial Africa and in New Guinea. Hodgkin’s disease is more frequently associated with EBV in Hispanic Americans. EBV associated nasopharyngeal carcinoma has its highest incidence in Southeast Asia. The reasons for such geographical or ethnic variations remain obscure because EBV infection is carried by over 90% of adults worldwide.

“The ability of the virus to control the expression of viral genes by promoter methylation provides a selective advantage that allows the virus to persist in the host for many years.”

Previously, we found in gastric cancers a significant association between EBV and p16 loss, as determined by IHC. This association was confirmed in our current set of tumours (p = 0.0001). Furthermore, the presence of EBV was highly associated with methylation of the CDKN2A promoter (p = 0.0003). In all cases but three, tumours were both negative by immunostaining and methylation positive. In one case, IHC could not be performed, but the tumour showed methylation. In another case, a tumour with methylation was p16 positive by IHC. Such an event could occur if methylation was present in only one allele of the gene. In the third case, no methylation was present, although p16 protein was not detected by IHC. Such results could be explained by gene inactivation by mutation or homozygous deletion.

### Table 2 Clinical and biological features in association with EBV

<table>
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<th></th>
<th>EBV positive</th>
<th>EBV negative</th>
<th>p Value</th>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
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<tr>
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<td>11</td>
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<tr>
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<tr>
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<td>Methylated</td>
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<tr>
<td>Negative</td>
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<td>Mean (SEM) age</td>
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<td>49</td>
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<tr>
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<tr>
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<tr>
<td>1</td>
<td>2</td>
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<tr>
<td>M (n=108)</td>
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<tr>
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<tr>
<td>Poor</td>
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<tr>
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<tr>
<td>Mixed</td>
<td>1</td>
<td>5</td>
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</table>

Unless otherwise noted, values are number of patients. EBV, Epstein–Barr virus; IHC, immunohistochemistry; MS-L, low frequency microsatellite instability; MS-H, high frequency microsatellite instability; MSS, microsatellite stable; MS-PCR, methylation specific polymerase chain reaction; NS, not significant.
Suppression of p16 expression associated with EBV infection or EBV proteins has been observed in other systems. In mouse embryo fibroblasts, EBV latent membrane protein 1 (LMP1) acts to prevent senescence by inhibiting the induction of p16. Consistent with the capacity of EBV to inactivate p16, we and others have previously reported frequent p16 loss (as determined by IHC) in nasopharyngeal carcinomas.

As with infection with *H pylori*, EBV infection persists over decades, evading attempts by the immune system to eradicate it. Normal individuals live in symbiosis with the virus by mounting an effective defence against EBV driven cell proliferation through the response of CD8 positive T cells. The T cells of individuals with EBV associated gastric cancer are able to cause regression of proliferating foci of exogenously infected autologous B cells. In the presence of a vigorous immune response, the virus apparently evades immune surveillance by maintaining latency in a quiescent state, in which few viral genes are expressed, and this control is achieved largely by promoter methylation.

EBV appears to be distinctive among large DNA viruses in showing a lower CpG content than would be expected by chance. This could be explained by its use of methylation in suppressing the expression of viral proteins. Because of the spontaneous deamination of methylated cytosines, CpG6s tend to be reduced in the viral genome.

In nasopharyngeal carcinomas, lymphomas, and normal B cells, methylation in the viral CpG rich BamH1 C and W promoters controls expression of the EBV encoded nuclear antigen (EBNA) proteins. In vitro studies of Burkitt’s lymphoma cell lines and nasopharyngeal carcinoma cells have shown that the expression of LMP1 is associated with hypomethylation of the LMP1 regulatory sequences (for a review, see Robertson). It seems that the ability of the virus to control the expression of viral genes by promoter methylation provides a selective advantage that allows the virus to persist in the host for many years.

Extrapolations regarding patterns of methylation in other EBV associated diseases must be performed with caution because the mechanism by which EBV contributes to gastric cancer may be distinct from the mechanisms occurring in other EBV related systems. Under in vitro conditions, the expression of LMP1 alone can transform fibroblasts. However, in gastric epithelial cells, we and others have found weak or no expression of LMP1 in EBV associated gastric cancers. Imai et al showed that in EBV associated gastric cancers inactivation of LMP1, in addition to EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and Lp, was accomplished by the methylation of LMP1 regulatory sequences and the C and W promoter regions.

Sugiuira et al have described the latency pattern of EBV in gastric cancer as more closely resembling the latency I pattern in Burkitt’s lymphoma, rather than the latency II pattern of most nasopharyngeal carcinomas. That study reported the finding in gastric cancers of transcripts for EBNA1 (initiated from the Q promoter), LMP1 in nasopharyngeal carcinomas and the BamHI-A regions of the viral genome, but no transcripts of LMP1, LMP2B, or EBNA2. Similarly, zur Hausen et al have described a transcription pattern that includes BARF1, BARF0, LMP2A, and Q/K driven EBNA1 but not LMP1, EBNA2, or ZEBRA transcripts in EBV associated gastric cancers.

Those authors described a distinctive pattern of expression for gastric cancer that resembles that of Burkitt’s lymphoma except for the additional transcription of BARF1 and LMP2A in gastric cancer.

zur Hausen et al have proposed that BARF1 may substitute for LMP1 in its oncogenic potential in gastric cancer. BARF1 can transform fibroblasts and immortalise kidney epithelial cells in culture. The overexpression of BARF1 can activate the c-myc proto-oncogene in Louckes B cells. BARF1 has a region of homology to c-fms, a tyrosine kinase proto-oncogene, which encodes the colony stimulating factor 1 receptor. BARF1 may help the virus to evade the immune system by acting as an antagonist to colony stimulating factor 1, which is a macrophage/monocyte growth and differentiation factor (reviewed in Hamilton).

“Neither of the promoters of the tumour suppressor genes hMLH1 and hMSH2 appeared to be methylated in our set of tumours”

It is possible that CDKN2A promoter methylation in EBV associated gastric cancers is a cellular change secondary to the acceleration of cell proliferation caused by the presence of a viral oncogene such as BARF1. An alternative possibility is that CDKN2A promoter methylation is more directly related to the activities of the virus, and that the ability of the virus to achieve methylation of its own genes for evasion of immune surveillance may be accompanied by the ability to influence the methylation of cellular tumour suppressor genes. If this last hypothesis is true, it is noteworthy that there is apparently some specificity of the cellular tumour suppressor genes that become methylated because neither of the promoters of the tumour suppressor genes hMLH1 and hMSH2 appeared to be methylated in our set of tumours. This we infer from the fact that no EBV positive cases were MSI-H, a defect that results from inactivation of the DNA mismatch repair system, usually caused, in gastric cancers, by hMLH1 or hMSH2 promoter methylation. A leukaemia model demonstrates how an oncogene can cause methylation of a promoter of a specific

**Figure 2**  (A) Immunohistochemical staining using an antibody against p16 in this Epstein-Barr virus (EBV) associated gastric cancer reveals that tumour cell nuclei (arrowhead) have no detectable p16 protein, in contrast to the p16 positive inflammatory and stromal cells adjacent to the tumour (arrow) and to the p16 positive staining tumour nuclei in the EBV negative carcinoma (B). Haematoxylin stained.
tumour suppressor gene. In this model system, a leukaemia promoting chimaeric oncogene, PML-RAR, is able to recruit DNA methyltransferases to the promoter of the tumour suppressor gene RARβ2, thus reducing its expression by hypermethylation of the promoter. Whether the effect of EBV upon CDKN2A promoter methylation is direct or indirect, the increased proportion of promoter methylation detected in EBV associated tumours compared with non-EBV associated tumours is thought provoking. In summary, we have identified a distinct pattern of epigenetic alteration in EBV associated gastric cancers. Such distinctiveness might become relevant to the event that treatments based on the reversal of aberrant methylation become available.

ACKNOWLEDGEMENTS
We thank Drs D Bradburn, D Bostwick, W Hinchen, and E Averyt for help in obtaining specimens, and P Eagan for skillful assistance with in situ hybridisation. We thank Dr R Ambrider for the EBER1 and U6 probes and templates. We wish to acknowledge the assistance of Ms J Johnson and the staff of tumour registries of Touro Infirmary, St Luke's Baptist Hospital, and the Audie Murphy Memorial Veterans Administration Hospital, especially M Roberts, A Burt, and B Kirk. This work was supported by the Health Excellence Fund of the Board of Regents of the State of Louisiana. Q Vo was supported in part by the Scott Graduate Scholars Program.

Take home messages

- The presence of Epstein-Barr virus (EBV) in gastric adenocarcinomas was strongly associated with CDKN2A inactivation by promoter methylation. This might be useful if treatments based on the reversal of aberrant methylation become available.
- The presence of EBV was associated with male sex and was more common in tumours from Texas Hispanics than from non-Hispanic whites or African-Americans.
- EBV was not associated with microsatellite instability, histological subtype, stage, or grade of the tumour, or age or survival time of the patient.

REFERENCES
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doi: 10.1136/jcp.55.9.669

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