Epstein-Barr virus in gastric carcinoma and adjacent normal gastric and duodenal mucosa

S Shousha, Y A Luqmani

Abstract

Aim—To look for Epstein-Barr virus (EBV) in a series of gastric cancers of common and rare histological types.

Methods—Formalin sections from 19 cases of gastric carcinomas of different types were studied using in situ hybridisation with fluorescein conjugated EBER oligonucleotides and the avidin-biotin complex immunoperoxidase technique, using the monoclonal antibody Dako-EBV CS 1-4.

Results—The only positive tumour was a lymphoepithelioma-like gastric carcinoma. The remaining 18 cases, which included 11 consecutive cases of usual adenocarcinomas, three early gastric cancers, two adenosquamous carcinomas, and a case each of signet ring carcinoma and neuroepithelioma, were all negative. However, scattered EBV positive cells were seen in the normal gastric or duodenal mucosa bordering the tumours in seven out of 11 cases.

Conclusions—Lymphoepithelioma-like gastric carcinoma seems to be the main, if not the only, EBV positive gastric cancer. The presence of EBV positive normal gastric and duodenal cells suggests that these cells may act as a reservoir for the virus in some people—a possibility that deserves wider investigation.

(J Clin Pathol 1994;47:695-698)

Epstein-Barr virus (EBV) has recently been demonstrated in cases of poorly differentiated gastric carcinoma with heavy lymphocytic infiltration.¹⁻⁵ The positive tumours were mainly present in the fundal region of the stomach, and the patients were mainly of Asian origin. The viral genome was identified using the polymerase chain reaction (PCR),¹⁻³ in situ hybridisation,³⁻⁵ or Southern blot techniques.⁵ In one of these studies EBV was detected by PCR, but not by in situ hybridisation, in the normal gastric mucosa of only one out of seven patients with EBV positive tumours.³ In two other case studies the normal mucosa was EBV negative using in situ hybridisation⁴ and Southern blotting.⁵

Shibata and Weiss⁶ studied 138 cases of gastric adenocarcinomas of the usual type and found evidence of EBV in 22 (16%), using PCR and in situ hybridisation. Most of the positive tumours were situated in the gastric antrum in male patients. In two of these

patients the EBV genome was also detected in adjacent dysplastic epithelium, but not in the normal mucosa or in mucosa showing intestinal metaplasia.⁶ These findings could not be confirmed by Leoncini *et al*⁷ who studied 65 consecutive cases of gastric carcinoma and could only find four (6%) PCR-EBV positive cases, of which only three were positive by in situ hybridisation. All positive cases had a prominent lymphoid infiltration, but the degree of tumour differentiation was poor in two and moderate in the other two. These authors also failed to demonstrate EBV in the adjacent dysplastic, metaplastic, or normal gastric mucosa by PCR or in situ hybridisation.⁷

The present study was designed to try to resolve this controversy by investigating a consecutive series of gastric adenocarcinomas, and to extend the field of investigation by examining other uncommon histological varieties of gastric carcinoma which have not been studied so far. Adjacent non-neoplastic gastric and duodenal mucosa was also examined whenever available.

Methods

Nineteen cases of gastric carcinoma treated by partial gastrectomy were studied. These included one case of lymphoepithelioma-like gastric carcinoma, two cases of adenosquamous carcinomas, three cases of early gastric carcinoma, one case of gastric neuroepithelioma, one case of signet ring carcinoma and 11 consecutive cases of gastric adenocarcinoma of the usual type. All the archival haematoxylin and eosin stained sections of each case were reviewed and a representative section of the tumour was selected. Some of these sections also contained adjacent non-neoplastic mucosa. New consecutive 5 µm thick sections were cut from the corresponding paraffin wax blocks and mounted on either ordinary glass slides, for immunohistology, or on slides coated with aminopropyl triethoxy silane for in situ hybridisation.

For in situ hybridisation, sections were dewaxed in xylene, rehydrated in graded alcohol, followed by washing for two minutes in phosphate-buffered saline (PBS), and for five minutes in PBS containing 0.1% Triton X-100. Sections were then treated with 50 µg/ml proteinase K in 0.1 M TRIS, 0.05 M EDTA, pH 8, for 30 minutes at 37°C, and subsequently washed in 0.1 M glycine. Postfixation in 4% paraformaldehyde in PBS for five

Department of
Histopathology,
Charing Cross and
Westminster Medical
School, London
S Shousha

Department of
Medical Oncology
Y A Luqmani

Correspondence to:
Dr S Shousha, Department
of Histopathology, Charing
Cross Hospital, Fulham
Palace Road,
London W6 8RF

Accepted for publication
14 January 1994

minutes was followed by washing in PBS for five minutes. Hybridisation was carried out using a mixture of fluorescein conjugated EBER oligonucleotides (Dako Ltd, High Wycombe, UK) following the manufacturer's protocol except that hybridisation was performed for 16 instead of two hours at 37°C. Sections were covered with siliconised cover-slips; these were sealed using rubber solution. A Vectastatin kit was used for detection using alkaline phosphatase, following an amplification step involving sequential incubation (one hour each) with anti-fluorescein isothiocyanate (FITC) antibody and an anti-mouse immunoglobulin alkaline phosphatase conjugate. Sections were then lightly counterstained with haematoxylin before mounting in Glycergel.

As a negative control, sections were treated in the same way except for omitting the application of the specific probe. Sections of a known EBV positive case of Hodgkin's disease were used as positive controls.

For immunohistology, sections were dewaxed and rehydrated in graded alcohol. Endogenous peroxidase was blocked by incubating the slides for 30 minutes at room temperature in 3% hydrogen peroxide in methanol. Slides were rinsed in tap water, then three times for five minutes each, in 0.1M TRIS-buffered saline (TBS) (pH 7.6) before incubating the sections in 10% normal rabbit serum in TBS for 30 minutes to block non-specific reactions. Excess serum was decanted and sections were then incubated with the monoclonal antibody Dako-EBV CS 1-4 (Dako) diluted 1 in 50 in TBS for 30 minutes at room temperature. Sections were then rinsed in TBS as before and incubated for 30 minutes with biotinylated rabbit anti-mouse immunoglobulin (Dako) diluted 1 in 250 in TBS. After three rinses in TBS sections were incubated with streptavidin-biotin complexed to horseradish peroxidase (Dako) for 40 minutes. Sections were then rinsed in TBS and

peroxidase activity was visualised by incubation for six minutes in 0.5% diaminobenzidine (Sigma UK) and 0.01% hydrogen peroxide. Sections were counterstained with Harris's haematoxylin and mounted with Ralmount (BDH UK). Negative controls were treated as above except the specific antibody was omitted. Sections of a known case of EBV positive Hodgkin's disease were used as a positive control in each run.

Results

The patients included 14 men and five women. They varied in age between 38–89 years with a mean and a median of 64 years. The tumours were situated in the proximal part of the stomach in 11 cases, in the distal part in six, and midway in two cases. The tumour sections examined contained adjacent normal gastric tissue in nine cases, duodenal tissue in three, and oesophageal tissue in four cases.

Of all the 19 tumours examined, *in situ* hybridisation showed EBV positivity only in the lymphoepithelioma-like gastric carcinoma. The patient was a 65 year old woman who presented with a five month history of epigastric discomfort. Five years earlier she had had a prolonged illness that started suddenly, characterised by a persistent "terrible taste" in her mouth, depression, and intermittent blurred vision. Her husband died three months before her admission from "repeated gastrointestinal bleeding", about which no further information is available. The ulcerated tumour was sited along the lesser curve of the fundal region, and measured 8 × 7.5 cm across. It had infiltrated the full thickness of the stomach wall.

Microscopically, the tumour consisted mostly of sheets of malignant cells with large vesicular nuclei and prominent nucleoli. Neoplastic glandular structures were present in some parts. A heavy lymphocytic infiltrate was seen overlying the malignant cells and heavily infiltrating the stroma in between. Extensive metastasis were present in 16 lymph nodes dissected from around the stomach, lesser and greater omentum, and gastrosplenic area. Positive EBV *in situ* hybridisation was seen as a diffuse cytoplasmic and nuclear red staining in most tumour cells (fig 1) and in lymphatic tumour emboli in the overlying non-neoplastic mucosa (fig 2). The overlying and adjacent gastric and oesophageal mucosae were negative in this case, as well as some parts of the tumour.

Immunohistological assessment showed diffuse, faint, cytoplasmic and focal dark brown nuclear staining. *In situ* hybridisation of further newly cut sections from the other archival paraffin wax blocks from this case confirmed the presence of EBV in the primary tumour and lymph node metastasis, and the absence of virus in normal gastric and oesophageal mucosae. The patient died 11 months after surgery.

All the remaining 18 tumours were negative. However, scattered positive cells were seen in the adjacent normal gastric or duodenal

Figure 1 EBV positive lymphoepithelioma-like gastric carcinoma (*in situ* hybridisation).

Figure 2 EBV positive, red stained, malignant cells in a lymphatic. The surrounding normal gastric mucosa is EBV negative (in situ hybridisation).

(A)

(B)

Figure 3 EBV positive, red stained, normal duodenal (A) and gastric cells (B) (in situ hybridisation).

mucosa in seven out of 11 cases (three usual type adenocarcinoma, two superficial, one signet ring and one adenosquamous carcinoma). Positive normal gastric cells were seen in five out of nine cases (three of six proximal and two of three distal), and normal duodenal mucosa in all three cases examined; one case had both normal duodenal and gastric positive cells. No positive cells were seen in normal oesophageal mucosa examined in four cases, and no EBV positive lymphocytes were identified in any of the cases. Positivity in normal gastric and duodenal cells was seen by both in situ hybridisation and immunoperoxidase techniques (fig 3). The positive cells were mainly seen in the lower parts of the gastric and duodenal crypts. They were more abundant in the duodenal mucosa overlying prominent Brunner's glands. Positive cells were occasionally seen in the latter glands. In the corresponding haematoxylin and eosin stained sections, most of the positive cells looked larger than the surrounding cells and had large central nuclei.

Discussion

Our findings suggest that EBV is absent in the rare histological types of gastric carcinoma that include adenosquamous, neuroectodermal, signet ring, and early superficial carcinomas. It is probably also absent in the common type of adenocarcinoma, as our consecutive 11 cases were all negative. It seems that the only EBV positive gastric tumours are the lymphoepithelioma-like neoplasms, as was originally suggested in the earlier reports.

The clinical history of the positive case reported here included some unusual features that are difficult to interpret and evaluate, but we report them in case similar findings are encountered in other patients with similar tumours. The sure finding is the short clinical history related to epigastric pain and the apparent rapid progression of the tumour as manifested by its large size and extensive lymph node dissemination. In this respect similar extensive lymph node metastases were seen in some,¹⁴⁵ but not all²³⁷ previously reported cases.

The demonstration of EBV positive cells in a few normal gastric and duodenal epithelial cells in some cases is intriguing. In all previous reports where normal gastric mucosa adjacent to gastric carcinoma was examined, the normal mucosa was reported as EBV negative,³⁵⁻⁷ while dysplastic gastric mucosa was reported as positive by one group⁶ and negative by another.⁷ None of the previous authors commented on the duodenal mucosa. However, Shibata *et al*, in their earlier report, mentioned that PCR, but not in situ hybridisation, detected "lesser amounts" of EBV in two stomach sections without tumour from a patient with EBV positive gastric carcinoma.³ These authors used radiolabelled isotopes for their in situ hybridisation which can sometimes be difficult to interpret when the signal is weak. The normal positive cells in our study were detected only by careful searching and

were relatively more abundant in the duodenum, which previous authors do not seem to have examined. The in situ hybridisation probe and the antibody used for immunohistology recognise different component parts of the EBV genome but both produced positive signals in cells at the same sites in consecutive sections, making it unlikely that the observed positivity was the result of a non-specific or a cross-reaction. EBV can infect oropharyngeal epithelial cells^{8,9} and is capable of infecting and replicating in human epithelial systems.¹⁰ There have also been reports about the presence of EBV in bleeding colonic ulcers¹¹ and in oesophageal ulcers in three patients with AIDS.¹² Thus it may be that the virus can also infect gastrointestinal cells. This would explain the existence of EBV positive gastric carcinomas, which the accumulating evidence seems to have proved beyond any doubt. Determination of the prevalence of these EBV positive cells in various normal and diseased conditions is outside the scope of this study, but is currently being investigated.

This study was supported by grants from Gunnar Nilsson Research Trust Fund and Dako Ltd, UK.

- 1 Burke AP, Yen TSB, Shekitka KM, Sobin LH. Lymphoepithelial carcinoma of the stomach with Epstein-Barr virus demonstrated by polymerase chain reaction. *Mod Pathol* 1990;3:377-80.

- 2 Min K-W, Holmquist S, Peiper SC, O'Leary TJ. Poorly differentiated adenocarcinoma with lymphoid stroma (lymphoepithelioma-like carcinomas) of the stomach. Report of three cases with Epstein-Barr virus genome demonstrated by the polymerase chain reaction. *Am J Clin Pathol* 1991;96:219-27.
- 3 Shibata D, Tokunaga M, Uemura Y, Sato E, Tanaka S, Weiss LM. Association of Epstein-Barr virus with undifferentiated gastric carcinomas with intense lymphoid infiltration. Lymphoepithelioma-like carcinoma. *Am J Pathol* 1991;139:469-74.
- 4 Niedobitek G, Herbst H, Young LS, Rowe M, Dienemann D, Germer C, et al. Epstein-Barr virus and carcinomas. Expression of the viral genome in an undifferentiated gastric carcinoma. *Diagn Mol Pathol* 1992;1:103-8.
- 5 Pittaluga S, Loke SL, So KC, Cheung KN, Ma L. Clonal Epstein-Barr virus in lymphoepithelioma-like carcinoma of the stomach: demonstration of viral genome by in situ hybridization and Southern blot analysis. *Mod Pathol* 1992;5:661-4.
- 6 Shibata D, Weiss LM. Epstein-Barr virus-associated gastric adenocarcinoma. *Am J Pathol* 1992;140:769-74.
- 7 Leoncini L, Vindigni C, Megha T, Funto I, Pacenti L, Musaro M, et al. Epstein-Barr virus and gastric cancer: data and unanswered questions. *Int J Cancer* 1993;53:898-901.
- 8 Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. *N Engl J Med* 1984;310:1225-30.
- 9 Greenspan JS, Greenspan D, Lennette ET, Abrams DI, Conant MA, Petersen V, et al. Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. *N Engl J Med* 1985;313:1564-71.
- 10 Li QX, Young LS, Niedobitek G, Dawson CW, Birkenbach M, Wang F, et al. Epstein-Barr virus infection and replication in a human epithelial cell system. *Nature* 1992;356:347-50.
- 11 Okano M, Thiele GM, Davis JR, Nauseef WM, Mitros F, Purtilo DT. Adenovirus type-2 in a patient with lethal hemorrhagic colonic ulcers and chronic active Epstein-Barr virus infection. *Ann Int Med* 1988;108:693-9.
- 12 Kitchen VS, Helbert M, Francis ND, Logan RPH, Lewis FA, Boylston AW, et al. Epstein-Barr virus associated oesophageal ulcers in AIDS. *Gut* 1990;31:1223-5.