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Rapid Communication

Association of Epstein-Barr Virus with Undifferentiated Gastric Carcinomas with Intense Lymphoid Infiltration

Lymphoepithelioma-like Carcinoma

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Some undifferentiated gastric carcinomas with intense lymphoid infiltration have a striking resemblance to nasopharyngeal lymphoepithelioma. The authors identified eight such cases (seven patients from Japan and one from the United States) of undifferentiated gastric carcinoma (lymphoepithelioma-like carcinoma [LELC]) and examined them for Epstein-Barr (EBV) viral sequences using polymerase chain reaction (PCR) and* in situ *hybridization (ISH) techniques. EBV was detected in seven of the eight cases by PCR, including a lymph-node metastasis. ISH that was performed in six of these cases showed EBV genomes to be uniformly present in the carcinoma cells and not present in the reactive lymphoid infiltrate or normal gastric mucosa. PCR of a polymorphic EBV locus (lymphocyte-determined membrane antigen) showed that a single genotype was present in each gastric LELC, consistent with a clonal process. These findings suggest that some undifferentiated gastric carcinomas are EBV-related and that focal EBV infection occurs before transformation. (Am J Pathol 1991, 139:469–474)

The nasopharynx is the site of a distinctive type of undifferentiated carcinoma associated with a prominent lymphoid stroma. This unique carcinoma has been designated "lymphoepithelioma" or nasopharyngeal carcinoma (NPC). The Epstein-Barr virus (EBV) may play an oncogenic role in NPC since its DNA is present in virtually all NPC specimens.[1,2]

Histologically similar undifferentiated carcinomas with intense lymphoid infiltration (lymphoepithelioma-like carcinomas [LELC]) occur at other sites, although generally they are rare lesions. The similarities between NPC and LELC at other sites has prompted the search for EBV in these LELC. EBV DNA has been demonstrated in LELC of the salivary gland[3] and thymus,[4–6] and most often in Asians. EBV DNA was not demonstrated in a survey of LELC from the oropharynx, skin, or cervix, although one of four LELC of the lung contained EBV sequences.[7]

To our knowledge, there has been only one published report of a gastric tumor with a morphologic appearance identical to undifferentiated NPC. In that study by Burke et al in 1990,[8] the gastric LELC was found to be associated with EBV; however, the cellular location of the EBV sequences was not documented as the EBV was only detected by polymerase chain reaction (PCR). With the exception of this case report, no other examples of gastric carcinomas associated with EBV have been documented. The demonstration of EBV in gastric carcinoma cells would imply, similar to Burkitt's lymphoma and NPC, that EBV may play an important role in its oncogenesis. For these reasons, eight gastric LELCs were examined by PCR and *in situ* hybridization (ISH) for EBV sequences.

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Methods

Specimens

The gastrectomy specimens were selected on the basis of histology. Eight formalin-fixed, paraffin-embedded gastrectomy specimens of undifferentiated gastric carcinoma with intense lymphoid infiltration resembling NPC were analyzed. Only one such case was found in a retrospective search of more than 200 cases from the combined surgical pathology files of the Los Angeles County–University of Southern California, Stanford, and City of Hope, Medical Centers. Seven additional cases were obtained from three hospitals in the Kagoshima region of Japan. In addition, ten gastrectomy specimens with gastric adenocarcinoma without intense lymphoid infiltration from Kagoshima were examined.

PCR

Formalin-fixed, paraffin-embedded tissues were cut into 10 μm thick sections and extracted as previously described.[9] The PCR[10] was performed as previously described[11] with primers (SL1, SL3) and a probe (SL2) specific for an 80-base pair region of the EBV EBNA 1 gene. As a positive amplification control, primers (PC03, PC04)[12] for the beta-globin genomic sequence were also simultaneously present during the amplification. All EBV positive and negative samples showed amplification of the normal genomic sequence. Positive controls consisting of Raji DNA, and negative controls consisting of human genomic DNA as well as an assay with no added sample were done with each experiment. In separate experiments[11] this PCR assay detected EBV in EBV-positive cell lines (Jijoye, EB1, EB2, EB3, Daudi) but not in EBV-negative cell lines (Ramos, SiHa, HeLa).

The relative amounts of EBV DNA were further characterized by subjecting serial dilutions of the DNA extracted from the formalin-fixed specimens to 50 PCR cycles.[11]

The EBNA-positive formalin-fixed specimens were amplified with a second set of primers (SL18, SL19), which immediately flank the portion of the EBV lymphocyte-determined membrane antigen (LYDMA) gene composed of variable numbers of tandem 33-base pair repeats as previously described.[11] The PCR products are Southern blotted after electrophoresis through a 3% NuSieve (FMC, Rockland, ME), 0.5% agarose gel, using a 32P labeled probe (SL20) homologous to the tandem repeats.

Immunohistochemistry

The normal and tumor tissues were stained for keratin using a keratin mixture containing the four monoclonal antibodies AE1 (Hybritech, San Diego, CA), CAM5.2 (Becton Dickinson, Mountain View, CA), UCD3 (Triton Biosciences, Alameda, CA), and EAB-902 (Enzo Biochemicals, New York, NY) using the ABC method.[13]

In situ *Hybridization*

The *in situ* DNA hybridization studies were done using a ^{35}S-labeled Bam HI-W fragment of the EBV genome as previously described.[14] Previous negative hybridization studies with heterologous cytomegalovirus and papillomavirus probes showed the specificity of the Bam HI-W fragment for EBV sequences.[14] In each experiment, a fixed NPC specimen served as a positive control and EBV-negative lymphoid tissue served as a negative control. In addition, five squamous cell carcinomas from the head and neck region and ten adenocarcinomas from a variety of sites were analyzed and were EBV-negative.

Results

The demographic and gross characteristics of the gastric LELC from the eight patients are presented in Table 1.

Table 1. *Lymphoepithelioma-like Carcinoma of the Stomach*

Case	Age/Sex	Site	Stage*	Size (cm)	LN status	Survival (mo)†
A	74/M	Cardia	II	6 × 5	2/38	8
B	66/F	Cardia	IB	5.5 × 4	negative	12
C	78/M	Body	IB	4.5 × 4.5	negative	27
D	71/F	Body	IB	1.4 × 1.6	negative	68
E	64/M	Cardia	IC	4 × 4	negative	7
F	57/M	Body	IB	2.5 × 2	negative	4
G	75/F	Antrum	IC	2 × 2	negative	64
H	62/M‡	Cardia	IB	5 × 2	negative	2

* TNM.[25]
† All alive.
‡ Caucasian immigrant in the United States from the Ukraine, other patients were in Japan.

The average age of the patients was 68.4 years with a male to female predominance. All of the patients are alive after a median follow-up of 10 months.

The tumors from all eight patients showed diagnostic features of LELC characterized by diffuse infiltrating nests of undifferentiated carcinoma cells surrounded by a prominent lymphoid stroma and reactive follicles (Figure 1A). Occasional neoplastic glands were present. The lymphoid stroma consisted primarily of small mature lymphocytes, scattered, transformed lymphoid cells, and varying amounts of plasma cells. The uninvolved gastric mucosa was normal although small lymphoid follicles were typically present in the lamina propria. The epithelial nature of the carcinoma cells was confirmed with the demonstration of keratin-positivity by immunohistochemistry (Figure 1B).

EBV sequences were detected in seven of the eight fixed tissues of gastric LELC by PCR (Table 2), including a lymph-node metastasis (case A). In contrast, only one of ten specimens with gastric adenocarcinoma without intense lymphoid infiltration was EBV-positive (P = 0.0029, Fisher's exact test, two-sided). There were no histologic differences between the EBV negative and positive gastric LELC, although the EBV positive cases were present in the cardia and body of the stomach and the EBV negative case was present in the antrum. EBV could not be detected from sections with normal uninvolved lymph node or gastric mucosa except in case B, suggesting that the EBV DNA was specifically associated with the carcinoma cells. Dilution analysis of the extracted DNA showed that large amounts of EBV were associated with the LELC tissues and lesser amounts were present in the two EBV positive sections uninvolved with tumor.

PCR of a polymorphic region of the LYDMA gene comprised of variable numbers of tandem 33-base pair repeats (VNTR) showed that predominantly a single LYDMA VNTR genotype was present in each gastric LELC (Figure 2). The LYDMA VNTR PCR products in the normal tissues of case B were different and smaller than the genotype of the tumor tissue.

In situ hybridization for EBV DNA sequences of the tumor and normal tissues were done (Table 2). EBV sequences were localized to the epithelial component of the tumors in all six cases attempted (Figure 3). The signals were moderate to strong and of uniform intensity from tumor cell to tumor cell. Surrounding lymphocytes in tumor and normal tissues were negative for EBV ge-

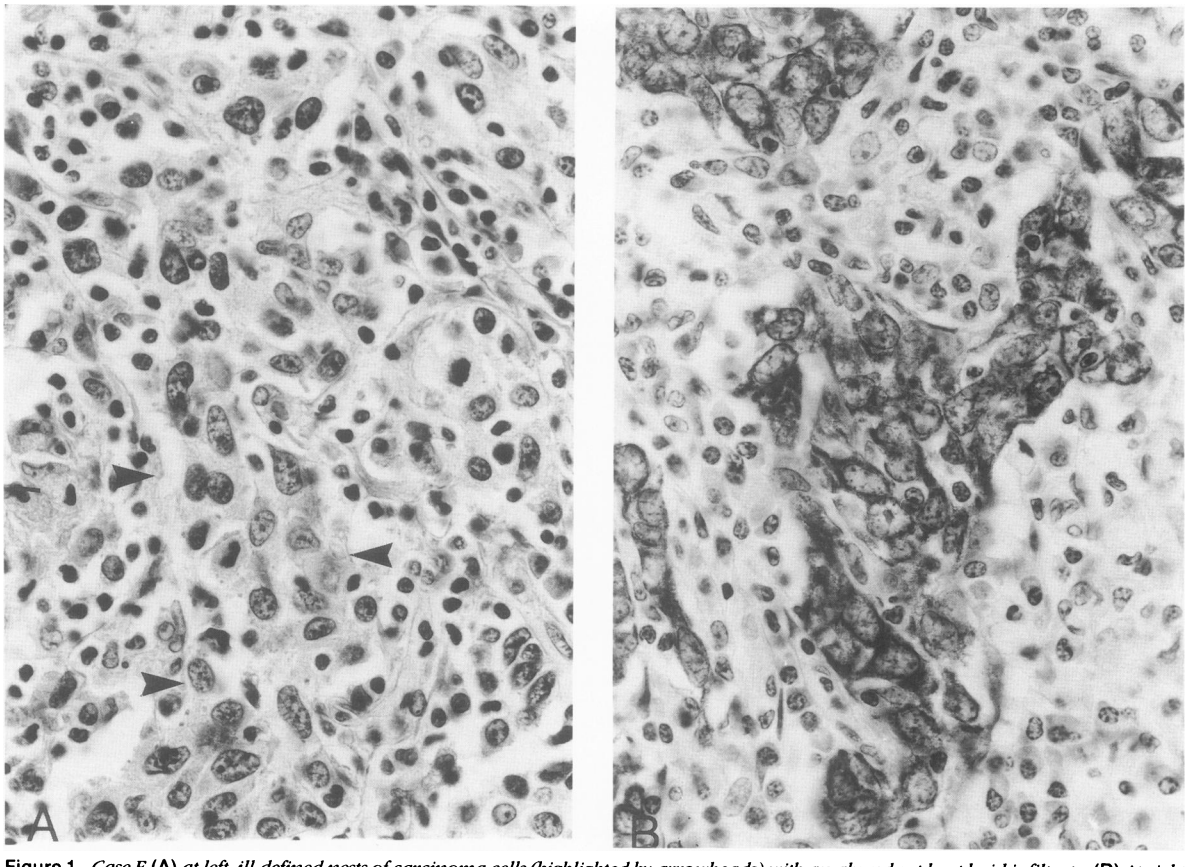

Figure 1. *Case E* **(A)** *at left, ill-defined nests of carcinoma cells (highlighted by arrowheads) with an abundant lymphoid infiltrate.* **(B)** *At right, immunoperoxidase study for keratin labels the carcinoma cells.*

Table 2. *PCR and ISH Studies*

Case	EBV–PCR Assays					ISH
	Primary	Normal mucosa	Normal LN	LN metastasis	LYDMA PCR	Tumor cells
A	+ + +	ND	–	+ + +	single	+
B	+ + +	+*	+ +*	NM	single†	+
C	+ + +	–	–	NM	single	+
D	+ + +	–	ND	NM	single	ND
E	+ + +	–	–	NM	single	+
F	+ + +	–	–	NM	single	+
G	–	–	–	NM	ND	–
H	+ + +	–	ND	NM	single	+

* ISH was negative for EBV sequences.
† The LYDMA PCR band for case B in the normal mucosa and normal lymph node were single, of equal size, but of a different size than the LYDMA band generated from the primary tumor (Figure 2).
ND = no data.
NM = no lymph node metastasis.
– = negative, and + = positive.
For PCR assays: relative amounts of EBV present, + = only first dilution EBV positive, + + = at least second 10-fold dilution EBV positive, + + + = EBV positivity greater than or equal to genomic dilution.

nomes. The normal gastric mucosa was also EBV negative. EBV could not be detected by ISH in the normal gastric mucosa and normal lymph node of case B, which were EBV PCR positive.

Discussion

The gastric LELC in this study are similar to the gastric carcinomas with intense lymphoid infiltration described by Watanabe et al,[15] but generally lacked glandular differentiation and thus were more histologically similar to the EBV-related undifferentiated carcinomas of the nasopharynx. These gastric LELC appear to be an uncommon variant of gastric carcinoma. Only one such case was found in the combined surgical pathology files of the

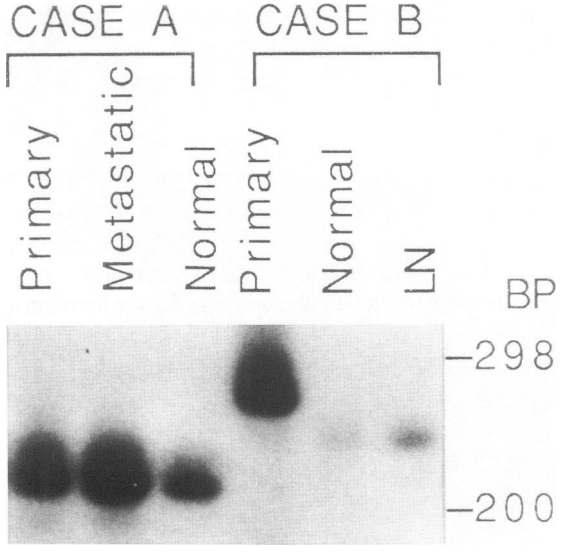

Figure 2. *Southern blot analysis of the LYDMA VNTR PCR products. In case A, the same-sized LYDMA PCR product is amplified from all the sections with tumor—the primary tumor, lymph-node metastasis and normal gastric mucosa with a small focus of tumor. In contrast, different-sized LYDMA PCR products are present in case B. A single-sized LYDMA PCR product was amplified from the primary tumor whereas a single-sized but smaller LYDMA PCR product was amplified from tumor free normal gastric mucosa and lymph node, indicating that the EBV-related proliferations in the tumor and the nontumor tissues are different.*

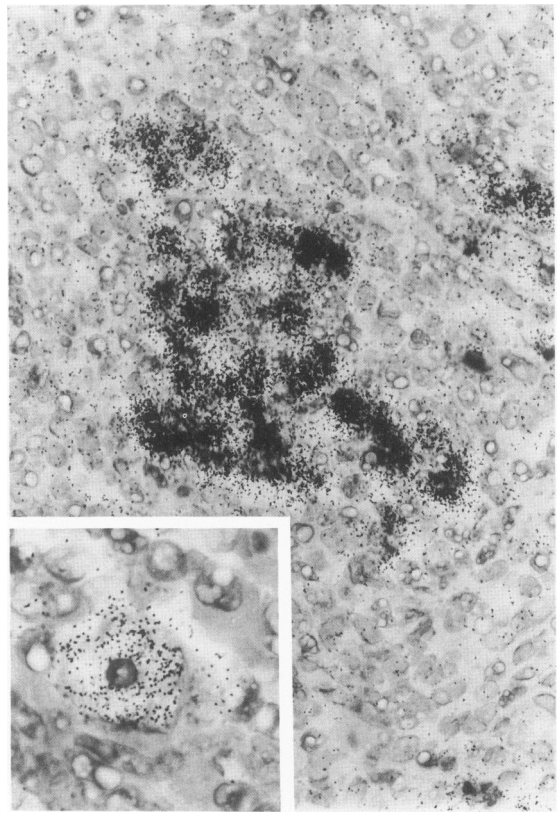

Figure 3. In situ *hybridization study for EBV using an ^{35}S-labeled probe. Intense labeling of the LELC from case C is present. The insert shows a solitary metastatic carcinoma cell within a lymph-node sinus from case A with strong nuclear positivity (note prominent nucleolus) in contrast to adjacent negative staining lymphoid and endothelial cells.*

City of Hope, Stanford, and Los Angeles County–University of Southern California, Medical Centers. Seven cases from Japan, which has a much higher incidence of gastric cancer than the United States,[16] were identified and analyzed. The rarity of gastric LELC precluded examination of fresh specimens and therefore only formalin-fixed, paraffin-embedded tissues were studied by PCR and ISH techniques. Using these techniques, large amounts of EBV sequences could be specifically located in tumor tissues and cells in the majority (87%) of gastric LELC. In contrast, only one of ten Japanese gastrectomy specimens with adenocarcinoma without intense lymphoid infiltration was EBV-positive ($P = 0.0029$). The presence of EBV in gastric cancer without extensive lymphoid infiltration is currently under further investigation.

The demographics of the patients with gastric LELC were similar to other EBV-related neoplasms. There was a male predominance (2.5:1), and the single example of an EBV negative gastric LELC was from a female. This male predominance seen in this study is also present in EBV-related Burkitt's lymphoma and NPC.[17] Like most gastric carcinomas,[16] the gastric LELC occurred in the elderly (average age, 68.4 years). Although follow-up is limited, the patients with EBV positive or negative gastric LELC appeared to have a high survival rate, similar to the higher survival rates noted for gastric carcinoma with lymphoid stroma.[15]

EBV infection of the tumor cells may occur before or after transformation. The ISH studies suggest that every tumor cell is uniformly EBV infected and support the model that EBV infection occurs early in oncogenesis with a subsequent clonal expansion of EBV containing tumor cells. The finding of EBV both in the primary carcinoma and its lymph-node metastasis (case A) and with an identical LYDMA VNTR genotype (see below) also supports this hypothesis.

The general lack of EBV detection by PCR or ISH in normal gastric mucosa suggests that if EBV infection of normal gastric epithelium is an early event in oncogenesis, the initial infection may be focal. EBV is shed from the nasopharynx of most normal EBV seropositive individuals.[18] Although speculative, the gastric mucosa may be infected from the chronic exposure to swallowed EBV virions. Alternatively, the gastric mucosa may be infected by EBV-infected lymphocytes in the lamina propria. Expression of the EBV surface receptor (C3d receptor CR2 or CD21)[19,20] appears important for infection, and such a receptor is present on oral epithelium, presumably allowing the entry of EBV into these cells.[21] The lack of EBV detection in the normal gastric epithelium suggests that this receptor is not normally present on gastric epithelial cells, and the CD21 antibody B2 does not react with normal gastric epithelium (Weiss, unpublished observations, 1991). Chronic gastric atrophy and intestinal metaplasia may be precursors of gastric carcinoma[22] and these precursor states may in some instances also induce the expression of the EBV receptor or allow latency. This would permit EBV infection of the gastric epithelium to precede transformation. Dietary factors related to gastric cancer such as nitrite, nitrate, salt, carbohydrate, or fresh vegetable consumption and the refrigeration of food[16] may also influence EBV receptor expression.

It would be difficult to demonstrate the clonal expansion of EBV infected gastric carcinoma cells. The usual method employing Southern-blot hybridization with a terminal tandem repeat probe[23] requires fresh tissue, and using PCR to amplify this VNTR is impractical since it is much greater than 1,000 base pairs. EBV remains episomal in most tissues and its integration site is not well characterized.[24] Alternatively, the number of EBV species in a given tissue can provide an approximation of clonality. The LYDMA gene of EBV is polymorphic with a region consisting of variable numbers (between 3 and 7) of tandem 33 base pair repeats (VNTR), and is more suitable for PCR. The size of this LYDMA VNTR is characteristic for a given EBV isolate and variable between EBV isolates, although different isolates may have the identical LYDMA VNTR genotype. PCR primers homologous to sequences immediately adjacent to the VNTR can amplify this polymorphism. In this study, a single predominate band consistent with a clonal proliferation was observed in the gastric LELC, although polyclonal proliferations could also generate a single band.

Tissues without neoplastic cells were generally EBV negative. The exception was the normal lymph node and gastric mucosa from case B which were EBV PCR positive but EBV negative by ISH. Immunohistochemical analysis (not shown) did not show occult metastatic tumor cells in these tissues. PCR of the LYDMA VNTR showed that there were at least two different EBV isolates in this patient (Figure 2). One isolate with a larger LYDMA VNTR genotype was present in the neoplastic epithelial cells of the tumor tissue whereas another isolate with a smaller LYDMA VNTR genotype was absent from the tumor tissue but present in the lymphoid tissues of the normal lymph node and mucosa. It is likely that the EBV detected by PCR in the normal lymph node and mucosa represented low levels of reactivation (below the sensitivity of ISH) of a second latent EBV infection in lymphoid cells secondary to immunosuppression by the gastric cancer. This phenomenon does not seem to commonly occur since EBV sequences were not detected in the normal lymph nodes or mucosa of six other cancer cases.

In summary, this study demonstrates that some undifferentiated gastric carcinomas with intense lymphoid infiltration, which resemble NPC, are EBV-related and provide the first direct evidence that gastric epithelial cells

can be infected with EBV. The exact role of EBV in the oncogenesis of these tumors is unknown and will require further investigation.

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