Original

**Detection of Epstein-Barr Virus Infection in Gastric
Carcinoma Using In Situ Polymerase Chain
Reaction and Analysis of Epstein-Barr
Virus-Associated Gastric Carcinoma**

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Abstract: We performed two independent evaluations of in situ polymerase chain reaction (in situ PCR) and Epstein-Barr virus encoded small RNA in situ hybridization (EBER ISH) for detection of Epstein-Barr virus infection in gastric carcinoma. In situ PCR is a novel technique with a high sensitivity which is a powerful aid for morphological diagnosis. Paraffin sections of gastric carcinomas from 96 cases were analyzed. Twenty-two of 96 cases (23%) were positive by in situ PCR, and 14 cases (15%) were positive by EBER ISH. Therefore there were 8 cases of EBV-DNA-positive gastric carcinoma without expression of EBER. To determine the significance of the expression of EBER in EBV-infection positive gastric carcinoma, we performed a clinicopathological study. There were two pathologic features in the gastric carcinoma with EBER. There was a significantly higher incidence of undifferentiated type and advanced cancer in EBER-positive gastric carcinomas. Therefore the expression of EBER contributes to the clinical pathologic feature of Epstein-Barr virus-associated gastric carcinoma.

Key words: epstein-barr virus associated gastric carcinoma (EBVaGC), in situ polymerase chain reaction (in situ PCR), EBV-encoded small RNA (EBER), in situ hybridization (ISH)

Introduction

Epstein-Barr virus (EBV), a ubiquitous human herpes virus, was the first virus to be identified in a human neoplastic cell¹⁾. EBV has one unique feature among the herpes viruses; it can infect B lymphocytes and immortalize cells^{2,3)}. EBV is well known to be the causative agent of infectious mononucleosis and it is also associated with a number of malignancies including Burkitt's lymphoma and nasopharyngeal carcinoma. Recently, it has been reported that EBV is associated with Hodgkin's disease⁴⁾, various types of lymphomas⁵⁾, salivary gland carcinoma⁶⁾, thymic carcinoma⁷⁾ and testicular tumor⁸⁾. Epstein-Barr virus-associated gastric carcinoma (EBVaGC) is the most common EBV-related neoplasm in

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Japan. EBVaGC is observed in about 10% of gastric cancer patients in Japan, and according to this, EBVaGC occurs in about five thousand patients per year.

Analysis of EBVaGC is generally by EBV-encoded small RNA in situ hybridization (EBER ISH). EBER is specific to EBV latently infected cells and is found in high copy numbers. Furthermore EBER ISH has a comparatively high sensitivity, but EBER is not expressed in the lytic state. Moreover, EBER is undetectable by ISH when there is only a small copy number^{9,10)}. EBV-DNA-positive nasopharyngeal carcinoma with no EBER expression has been reported¹¹⁾. Using DNA ISH, one study suggested that EBV infection without EBER was present in non-carcinomatous gastric epithelium cells¹²⁾. Clearly there remains a need for a means of detecting EBV infection-positive gastric carcinoma, which is not affected by the infection type or the number of copies of the virus. We tried to detect viral genomic DNA using an in situ polymerase chain reaction technique (in situ PCR). In situ PCR is a detection technique which has a high sensitivity and specificity. Since this method is performed directly on a slide, morphological diagnosis is possible.

We examined 96 gastric carcinoma specimens by in situ PCR and EBER ISH to determine the presence of EBV-positive gastric carcinoma without EBER. We also performed a clinicopathological study to elucidate the significance of the expression of EBER in EBV infection-positive gastric carcinoma.

Materials and Methods

Clinical specimens and cell lines

Formalin-fixed, paraffin-embedded gastric carcinoma tissues were observed by EBER ISH and in situ PCR. The gastric carcinoma specimens from 96 cases used in this study were from the files of our department.

To evaluate the sensitivity of in situ PCR for detection of EBV DNA, Raji cells (an EBV-immortalized Burkitt's lymphoma cell line) were used as an EBV-positive control. ML-1 cells were used as the negative control.

EBER in situ hybridization

Fixation of tissues was performed by paraformaldehyde (PFA) at 4°C for 24 hours and 70% ethyl alcohol (EA) at 4°C for 4 days after for preservation of RNA. The 6- μ m sections were cut from paraffin-embedded blocks. After deparaffinization, protease digestion was performed by Pepsin 0.01N-HCL (2 μ g/ml) (Sigma, St. Louis, USA) at 37°C for 30 minutes. Hybridization for 24 hours was performed using a biotin-labeled EBER Probe (Research Genetics, Huntsville, USA: 750126). We used biotin-thyramidin (First Chemistry Medicine, Tokyo Japan: TSA-Indirect ISH NEL-730) in order to detect probe with a high sensitivity. After hybridization, the positive signals were visualized by diaminobenzidine plus Ni · Co (DAB-Co, Ni).

In situ polymerase chain reaction for Epstein-Barr virus DNA

Fixation was performed as in the EBER ISH technique. The 8- μ m sections were cut from paraffin-embedded blocks. The in situ PCR was performed as previously described¹³⁻¹⁷⁾. In this protocol, it is important to avoid damage to the tissue sections on the glass slide, especially to sections of undifferentiated carcinoma. The main determinant of tissue damage appeared to be protease digestion and PCR. Our experience suggests that pepsin is more

suitable than proteinase K for limiting tissue damage.

Paraffin sections 8- μ m thick were dewaxed and rehydrated in xylene through a graded ethanol series (70, 90, and 100%). Then, we performed protease digestion on the slides by pepsin 0.01 N-HCL (2 μ g/ml) (Sigma) at 37°C for 30 minutes, washed them in PBS, and air-dried them. A slide seal for in situ PCR (TaKaRa, Kyoto, Japan) of 50 μ l was stuck on a slide, the PCR labeling solution was applied to the tissue sections, and it was equipped with a cover film. PCR primers were EBV Bam HI-W Primer TC60: 5'-CCAGAGGTAAGTGGACTT-3' and TC61: 5'-GACCGGTGCCTTCTTAGG-3' (Research Genetics Huntsville USA). The Bam HI-W fragment contained a long region of DNA. PCR labeling solution was set to a total of 50 μ l, containing 5 μ l of 10 $\times$ PCR buffer, 4 μ l of 2.5 mM-dNTP mixture, 2 μ l of 5U/ μ l Taq DNA polymerase, 1 μ l of Biotin-16-dUTP, 6 μ l of 25 mM-MgCl₂, 1 μ l of each primer (50 pmol/ μ l) and 30 μ l of sterile water. The reaction was performed in a TaKaRa Thermal Cycler MP. TP-3000, with 10 cycles of denaturation for 1 min at 90°C, annealing for 2 min at 55°C, and primer extension for 1 min at 72°C. PCR products were visualized by DAB.

Classification and statistical analysis

Various clinical pathologic publications followed the General Rules for the Gastric Cancer Study¹⁸⁾. Differences were analyzed by the χ^2 test or Fisher's exact test, and a P value of <0.05 was considered significant.

Results

Detection of EBV infection in gastric carcinoma

To detect EBV infection in gastric carcinoma, two independent evaluations of in situ PCR and EBER ISH were performed using samples from 96 cases of gastric carcinoma. Twenty-two of 96 cases (23%) showed positive signals in the nuclei of carcinoma cells by in situ PCR (Table 1), and 14 of 96 cases (15%) showed positive signals in the nuclei of carcinoma cells by EBER ISH (Table 1). Seventy-four cases showed no response either in situ PCR or EBER ISH (Table 1). All of the EBER-positive cases were also positive by in situ PCR (Table 1). Therefore 8 of 82 cases were positive by in situ PCR but negative by EBER ISH.

The results of in situ PCR of Raji cells used as a positive control are shown in Fig. 1. The positive signal was diffuse in cells (Fig. 1). There was no signal in ML-1 cells which are negative for EBV (Fig. 2). The case shown in Fig. 3 was positive by both EBER ISH and in situ PCR. Positive signals were seen in the nucleus of a cancer cell by EBER ISH (Fig. 3a), and in situ PCR gave clearer positive signals (Fig. 3b). The case shown in Fig. 4 was EBER ISH-negative (Fig. 4a) and in situ PCR-positive (Fig. 4b). Again positive signals by in situ PCR were in the nuclei of cancer cells (Fig. 4b). Similar results were obtained in at least two independently performed experiments. These results were in agreement with in situ PCR hybridization (Fig. 5).

Positive signals on in situ PCR were detected in almost all the carcinoma cells that were observed by EBER ISH. There were no positive signals in a non-carcinomatous gastric epithelium sample including intestinal metaplasia by in situ PCR. In several cases, EBV-positive signals by in situ PCR were observed in lymphocytes in tissue sections, but morphology confirmed the exclusion of such a non-specific reaction.

Table 1. Macroscopic findings of the Gastric Carcinoma with EBV infection

		Group A	Group B	Non EBVaGC
EBER ISH in situ PCR		negative positive	positive positive	negative negative
numbers		8	14	74
Age (mean $\pm$ SD)		68.63 $\pm$ 13.95	69.85 $\pm$ 7.33	65.57 $\pm$ 12.26
Sex	Male	5	12	56
	Female	3	2	18
Tumor location	U	4	6	17
	M	1	4	24
	L	3	3	31
	Remnant stomach	0	1	2
Macroscopic types				
	early 0	1	3	38
	advanced 1	1	0	4
	2	3	2	16
	3	3	6	18
	4	0	1	3
	5	0	2	0
Peritoneal metastasis	P0	8	10	66
	P1	0	4	8
Liver metastasis	H0	8	10	73
	H1	0	4	1

Fig. 1. In situ PCR of Raji cells as a positive control for EBV.
Positive signals are diffuse within cells ($\times 200$).

Fig. 2. In situ PCR of ML-1 cell as a negative control for EBV.
There is no signal ($\times 200$).

Clinical pathologic findings of EBV infection-positive gastric carcinoma

Samples were divided into two groups; group A, which was positive by in situ PCR only, and group B, which was positive by EBER ISH. The cases negative for EBV by both in

Fig. 3. A case of EBV-DNA-positive gastric carcinoma with expression of EBER (histological typing reveals a poorly differentiated adenocarcinoma, non-solid type).
 (a) Positive signals are seen in the nucleus of a cancer cell by EBER ISH ($\times 200$).
 (b) Results of in situ PCR of the case shown in Fig. 3a.
 Clearer positive signal is observed in the nucleus of a cancer cell ($\times 200$).

Fig. 4. A case of EBV-DNA-positive gastric carcinoma without expression of EBER (histological typing is tubular adenocarcinoma, moderately differentiated type).
 (a) There is no signal in the nuclei of a cancer cell by EBER ISH ($\times 100$).
 (b) In situ PCR of the case shown in (a). Positive signal by in situ PCR is seen in the nucleus of a cancer cell ($\times 100$).

situ PCR and EBER ISH were designated as non-EBVaGC.

There was no significant difference in mean age or sex between groups A and B (Table 1). Both group A and B tumors were mostly distributed in the upper part of the stomach, although group non-EBVaGC was predominantly in the lower part (Table 1). Both groups A and B had many cases of type 3 advanced cancer, when it was divided into the elevated type and depression type, there was no significant difference between EBV-positive gastric carcinoma and non-EBVaGC (Table 1). When the depth of tumor invasion was examined, advanced cancer was significantly more predominant in group B ($p < 0.05$) (Table 2). In histological typing, 10 of 14 cases (71.2%) were undifferentiated carcinoma in group B while differentiated carcinoma accounted for 6 of 8 cases (75%) in group A ($p < 0.05$) (Table 2). There were no differences between the groups in nodal involvement, peritoneal

Fig. 5. In situ PCR hybridization of the case that was positive on in situ PCR ($\times 400$)
(histological typing reveals a poorly differentiated adenocarcinoma, non-solid type).

metastasis, liver metastasis, lymphatic invasion, venous invasion, pattern of tumor infiltration into the surrounding tissue (INF), or cancer-stroma relationship (Table 2).

Discussion

In this study, we demonstrated that levels of EBER expression were associated with different clinicopathological features of EBVaGC. EBER-positive gastric cancers were significantly more likely to be advanced cancer with greater tumor invasion than non-EBVaGC. Moreover, there was a tendency for undifferentiated carcinoma compared to gastric cancer to show only EBV-DNA expression.

The relation between EBV and gastric carcinoma was originally reported in lymphoepithelioma-like undifferentiated adenocarcinoma by Burke *et al.* in 1990¹⁹⁾. The features of such stomach cancer, which has extensive lymphocyte infiltration have been reported as carcinoma with lymphoid stroma²⁰⁾. EBV-associated gastric carcinoma accounts for about 10% of ordinary gastric carcinoma^{21, 23)}. Some authors analyzed the pathologic features of EBVaGC by using EBER ISH²³⁻²⁷⁾. According to these reports, the pathologic features of EBVaGC are male predominance, occurrence primarily in the upper part of the stomach, moderately or poorly differentiated histological type, and a tendency for lymphocyte infiltration. By macroscopic typing of advanced cancer, elevated lesions with ulcer and scarce infiltration tendency were observed^{23, 26, 27)}. The frequency of EBVaGC is the same from early gastric cancer to advanced gastric cancer²⁷⁾. EBV in EBVaGC is monoclonal or oligoclonal in the intramucosal or early invasive stage, but invariably monoclonal in advanced carcinoma^{24, 25)}. In our series, the distinctive pathologic features of EBVaGC were depth of tumor invasion and histological type. The depth of tumor invasion was significantly greater in the EBER ISH-positive group ($p < 0.05$), but there was no significant difference in the group that showed positivity by in situ PCR only. On histology, differentiated carcinoma was predominant in the group that was positive by in situ PCR only, and undifferentiated carcinoma was predominant in the EBER ISH-positive group. EBER-positive gastric carcinoma is significantly more common in undifferentiated advanced cancer than in gastric carcinoma positive for EBV-DNA without EBER.

Table 2. Histological features of the Gastric carcinoma with EBV infection

		Group A (n=8)	Group B (n=14)	Non EBVaGC (n=74)
EBER ISH in situ PCR		negative positive	positive positive	negative negative
Histological typing				
differentiated type	pap	1	0	4
	tub1	2	1	14
	tub2	3	3	16
undifferentiated type	por1	0	3	5
	por2	2	6	23
	sig	0	0	8
	muc	0	1	4
differentiated/undifferentiated		6/2*	4/10*	34/40
Depth of invasion				
early	m	1	1	8
	sm	1	0	19
advanced	mp	0	3	9
	ss	6	2	9
	se	0	6	27
	si	0	2	2
early/advanced		2/6	1/13*	27/47*
Nodal involvement	positive	4	9	38
	negative	4	5	36
lymphatic invasion	positive	6	12	53
	negative	2	2	21
venous invasion	positive	6	11	45
	negative	2	3	29
INF [†]	α	2	1	13
	β	4	5	27
	γ	2	8	34
Stroma ^{††}	med	2	4	14
	int	4	2	31
	sci	2	8	29

*p<0.05

[†] pattern of tumor infiltration into the surrounding tissue^{††} cancer-stroma relationship

Therefore, it is considered that the gastric carcinoma cell that expresses EBER has more malignant potential than the gastric carcinoma cell which contains only EBV-DNA.

EBER are two small non-polyadenylated RNAs, EBER1 and EBER2, which are found at high concentrations (up to 10⁷ copies per cell) in EBV latently infected cells. The EBER directly inhibits the activation of purified PKR (double stranded RNA-activated protein kinase)²⁸⁾. The inhibition of PKR activity is related to cell immortalization, by maintaining protein synthesis and it restricts the anti-viral effects of interferon²⁹⁾. Moreover, PKR is a

tumor suppressor gene, suggesting that the restriction of PKR causes cell carcinogenesis³⁰⁾. Thus, the EBER may directly contribute to carcinogenesis. The results of our series support this.

Why would gastric carcinoma cells containing EBV-DNA without EBER exist? In an ordinary EBV latent infected cell, there are several possible explanations; EBV may be mutated, there may be a certain factor which affects expression of EBER in an infected cell, or only EBER which is a small molecule was lost in process of PCR.

To detect the EBV genome in gastric carcinoma, we performed in situ polymerase chain reaction. EBV-positive gastric carcinoma without EBER was present at higher levels than anticipated. We showed that EBER expression is an important factor in the clinicopathological features of Epstein-Barr virus associated gastric carcinoma. In recent years, there have been reports of specific gene therapy for EBV-associated neoplasms using an EBV-driven lytic system³¹⁾. The clinical entity of EBVaGC is low at present, but it is a possibility that the prophylaxis or conservative treatment in the far advanced stages of EBVaGC differ from non-EBVaGC. This means that identification of an oncogenic virus in pathological diagnosis may be reflected in medical treatment. Many clinical analyses including studies using clinical samples are regarded as important for fundamental research. Genetic analysis using paraffin sections may be utilized in not only detection but also in the study of the systems of oncogenes, cytokines, re-activation, apoptosis, and proliferation.

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[Received February 17, 2000: Accepted February 24, 2000]