

EPSTEIN-BARR-VIRUS-ASSOCIATED GASTRIC CANCER IN RUSSIA

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The present investigation was carried out to estimate the prevalence of EBV-associated cases among gastric carcinoma (GC) patients of Russia and the Republics of the former Soviet Union (FSU). With this aim, formalin-fixed paraffin-embedded blocks from 206 gastric carcinomas obtained from patients of the Cancer Research Center, Moscow, were investigated by EBV-encoded RNA-1 (EBER-1) *in situ* hybridization applied to paraffin sections. As a result, 18 GC cases (8.7%) revealed uniform EBER-1 expression restricted to the carcinoma cells. Hybridized signals not detected in non-neoplastic gastric epithelium. EBV involvement was significantly more frequent among males, especially in tumors of less differentiated types (moderately differentiated tubular adenocarcinomas and poorly differentiated solid adenocarcinomas) and located in the upper stomach (cardia and middle). Most EBV-positive GCs were characterized by strong lymphoid-compartment involvement. Our findings concerning the distribution of EBV-positive GCs by sex, site and histological type are similar to those in Japan, however, EBV-positive rate of GC cases in Russia is higher than in Japan and lower than in USA. *Int. J. Cancer* 73:786–789, 1997.

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Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that is known to produce lifelong infection in most humans. It is well documented as being causally associated with infectious mononucleosis (IM), African Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC) and B-cell lymphoma in immunodeficiency (Epstein and Achong, 1986). Moreover, the implication of EBV in the pathogenesis of a large variety of other human diseases, including malignant ones, is widely discussed nowadays (for review, see Grasser *et al.*, 1995). Since it is able to infect at least 2 types of cells (lymphoid and epithelial), EBV is associated with malignancies of either cellular origin.

In addition to its role in nasopharyngeal carcinoma (NPC), traditionally exemplified as an epithelial-cell-derived EBV-associated tumor (De Thé *et al.*, 1969; Zur Hausen *et al.*, 1970) EBV has been implicated in the pathogenesis of other epithelial-cell tumors, such as those of the salivary and parotid glands (SG,PG) (Tsai *et al.*, 1996; Gallo *et al.*, 1994) and gastric carcinomas (GC) (Shibata and Weiss, 1992). These types of human malignancy mostly represent undifferentiated lympho-epithelioma-like tumors morphologically similar to NPC. The presence of the EBV genome in carcinoma cells (but not in lymphoid ones infiltrating the tumors), as well as monoclonal proliferation of EBV-infected tumor cells and a wide spectrum anti-EBV antibodies found at high levels in serum, are characteristic features of EBV-associated tumors of epithelial origin.

Direct evidence of EBV involvement in the development of GC has been proved mostly by the uniform presence of EBV in carcinoma cells by EBER-1 ISH and monoclonal EBV episomes confirmed by Southern-blot analysis (Tokunaga *et al.*, 1993a,b; Imai *et al.*, 1994; Shin *et al.*, 1996). According to reported data, the incidence of EBV-associated GCs was 6.7% in Japan (Tokunaga *et al.*, 1993a) and 16% in USA (Shibata and Weiss, 1992) of the total number of primary gastric tumor specimens examined. EBV-associated GC was shown to be characterized by predominance in males and preferential location in the upper and middle part of the stomach (Uemura *et al.*, 1994). Although EBV-positive cases were observed for all histological types, high levels of EBV involvement were described for GCs with extensive lymphoid stroma and

less differentiation for tumor cells (Uemura *et al.*, 1994; Shibata, 1996). Since GC is the second commonest tumor in Russia (Dvoirin *et al.*, 1996), the present investigation was undertaken to determine the incidence of EBV-associated cases among GC patients of the former Soviet Union (FSU) and to clarify the clinicopathological features of these tumors.

MATERIAL AND METHODS

Subjects

The biopsy specimens were collected from unselected primary GC patients observed in the Cancer Research Center, Moscow, from 1992 to 1994. These patients were inhabitants of different regions of the FSU. In all, formalin-fixed, paraffin-embedded blocks from 206 cases were used for the investigation, including 101 male cases, 83 female ones and 22 cases for which the gender was unknown. For each case, information on age, primary site of cancer, histological type and depth of invasion were collected. Paraffin blocks taken from the main tumors were subjected to hematoxylin-and-eosin (H + E) staining and *in situ* hybridization (ISH).

Histology

Histological examination was performed as described (Tokunaga *et al.*, 1993a,b), using the classification scheme of the Japanese Research Society for Gastric Cancer (1981). Briefly, histologic patterns were classified as follows: well- and moderately differentiated tubular adenocarcinoma (tub1 and tub2 respectively), solid and non-solid poorly differentiated adenocarcinoma (por1 and por2), signet-ring-cell carcinoma (sig), and mucinous carcinoma (muc). In addition, poorly or undifferentiated lympho-epithelioma-like carcinoma (not included in our study) was detected. Sites of tumors were classified as cardia, middle portion or antrum. The depth of invasion was determined as mucosa, submucosa, muscular layer or serosa.

In situ hybridization

To study EBV presence in GC biopsy specimens, the method of *in situ* hybridization on paraffin-embedded sections was applied. As a probe for EBV latent mRNA, EBER-1, a complementary digoxigenine-labeled 30-base oligomer, was used as described (Chang *et al.*, 1992). Briefly, after standard procedures of deparaffinization, re-hydration and predigestion with protease, hybridization with a digoxigenin-labeled probe (0.5 ng/μl) was performed overnight at 37°C. After washing by 0.5× standard saline citrate, hybridization was detected by an anti-digoxigenin-antibody-alkaline-phosphatase conjugate (Boeringer, Mannheim, Germany), as suggested by the manufacturer.

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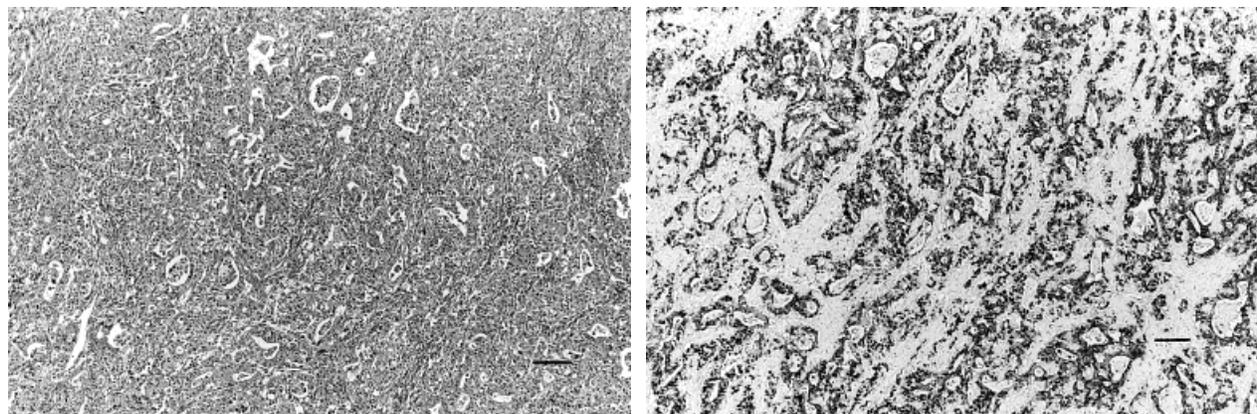


FIGURE 1 – Moderately differentiated tubular adenocarcinoma with occasional tubule formation and moderate lymphocytic infiltration (a) with EBER-positive signals in all carcinoma cells (b). Scale bar, 25 μ m.

TABLE I – DISTRIBUTION OF GASTRIC-CANCER CASES AND EBV-POSITIVE CASES BY GENDER IN RUSSIA

Gender	Study cases	EBV-positive	Percent positive
Male	101	15	14.9
Female	83	2	2.4
Unknown	22	1	4.5
Total	206	18	8.7

TABLE II – DISTRIBUTION OF EBV-POSITIVE GASTRIC-CANCER CASES BY SITE OF TUMOR

Site	Male	Female	Total
Cardia	6/37 (16.2%)	1/11 (9.1%)	7/48 (14.6%)
Middle	8/39 (20.5%)	1/41 (2.4%)	9/80 (11.3%)
Antrum	1/22 (4.5%)	0/28 (0.0%)	1/50 (2.0%)
Unknown	0/3 (0.0%)	0/3 (0.0%)	1/7 (14.3%)
Total	15/101 (14.7%)	2/83 (2.4%)	18/206 (8.7%)

For 22 cases, gender is not known.

Statistical analysis

Distribution of the EBV-associated GC cases by gender, tumor site, histological type, age, and depth of invasion was performed by linear logistic regression using the general models for binary outcomes algorithm from the EPICURE package of statistical programs for analysis of epidemiological data (Preston *et al.*, 1991).

RESULTS

Prevalence of EBV-positive cases

Out of 206 GC cases tested by *in situ* hybridization for the presence of EBV-encoded small RNA 1 (EBER-1), a positive signal of hybridization was detected in 18 (8.7%). Almost all carcinoma cells showed intensive EBER-1 ISH signals restricted to the nuclei (Fig. 1a,b). At the same time, normal gastric mucosa with or without atrophic gastritis, intestinal metaplasia of several degrees, hyperplastic alveolar epithelium, cystic glandular hyperplasia, and intestinal epithelium were completely negative for EBV ISH (Fig. 2a,b).

Factors that may influence EBV involvement

Age. There was no clear evidence of the relationship between age patients' (range, 31 to 81 years) and the occurrence of EBV-associated GCs.

Gender: In contrast, EBV-associated cases of GC clearly demonstrated gender dependence (Table I). EBV involvement was significantly more prevalent among tumors of males (15 from 101 cases, or 14.9%) than females (2 from 83, or 2.4%), corresponding to an unadjusted odds ratio (OR) of 7.06 for males vs. females (95% confidence intervals (CI) 1.91 and 45.7, $p = 0.0019$).

Tumor site. The analysis of tumor site showed virtually no EBV involvement in GCs localized in the antrum (Table II). The frequency of EBV-positive tumors in the cardia (14.6%) and in the middle stomach (11.3%) was significantly higher in comparison with the antrum involvement in 2% GC cases (OR 0.16, 95% CI 0.01 and 0.90).

Histological type. Analysis of the relationship between histological type of the GCs and EBV involvement revealed that most EBV-associated cases belong to less differentiated tumors (Table

TABLE III – DISTRIBUTION OF EBV-POSITIVE GASTRIC-CANCER CASES BY HISTOLOGICAL TYPE

Type	Male	Female	Total
tub1	1/19 (5.3%)	0/11 (0%)	1/30 (3.3%)
tub2	7/33 (21.2%)	1/24 (4.2%)	8/57 (14%)
por1	5/23 (21.7%)	1/19 (5.3%)	6/42 (14.3%)
por2	2/20 (10.0%)	0/19 (0%)	2/39 (5.1%)
sig	0/3 (0%)	0/4 (0%)	0/7 (0%)
muc	0/3 (0%)	0/6 (0%)	0/9 (0%)
Total	15/101 (14.9%)	2/83 (2.74%)	18/206 (8.7%)

tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; por1, solid poorly differentiated adenocarcinoma; por2, non-solid poorly differentiated adenocarcinoma (scirrhous type); sig, signet-ring-cell carcinoma; muc, mucinous carcinoma. For 22 cases, gender is not known.

III). EBV prevalence was not related to the division of the tumors into intestinal and diffuse. However, the appearance of EBV-related cases was more frequent among moderately differentiated, tubular (tub2) adenocarcinomas than among well-differentiated (tub1) adenocarcinomas (14.0% vs. 3.3%) and in solid-type, poorly differentiated adenocarcinomas (por1) in comparison with the non-solid (por2) type (12.5% vs. 4.5%). There were no EBV-positive cases among signet-ring-cell and mucinous carcinomas. Lympho-epithelioma-like tumors were absent in this study. Interestingly, EBV-positive tumors were found to be characterized by slight-to-moderate lymphocyte infiltration in and around the tumor nests.

Depth of invasion. There was no clear evidence of the relationship between EBV and depth of tumor invasion. Although submucosa was invaded in 25% and serosa in 10% (as compared with no tumor invasion into mucosa and muscular layer), there was no significant difference in homogeneity by depth of invasion.

DISCUSSION

To study the prevalence of EBV-associated cases among GC patients of the Cancer Research Center, Moscow, gastrectomy

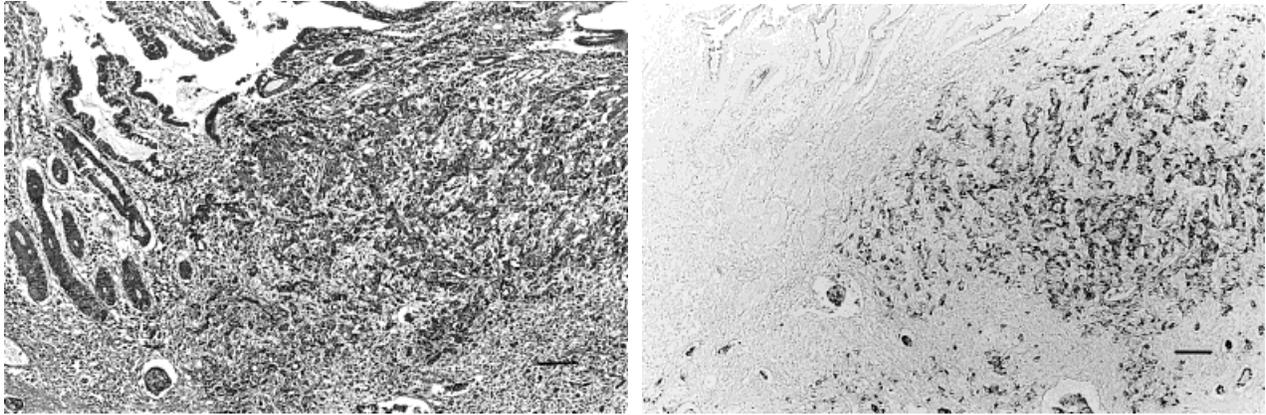


FIGURE 2 – Intramucosal lace pattern with intestinal metaplasia in the surrounding mucosa (a) showing EBV-positive signals only in carcinoma cells (b). Scale bar, 25 μ m.

specimens collected from respective patients were examined by *in situ* EBV-encoded small RNA-1 hybridization applied to paraffin sections. The results obtained indicated that the frequency of EBV-positive signal of hybridization was 8.7%, signals being detected in 18 of 206 biopsy specimens. This rate is somewhere between the relatively low positive rate in a high gastric-cancer-incidence area (Japan) and the high positive rate in a low-incidence area (USA): 6.7% and 16% respectively (Tokunaga *et al.*, 1993a; Shibata and Weiss, 1992). It is important to note, however, that the GC incidence rate (world standardized) even in the Russian Federation varies significantly: from 56.0 to 18.9 among men in Kostroma province and Dagestan respectively, and from 23.7 to 6.6 among women in Vologda province and Dagestan respectively (Dvoirin *et al.*, 1996). The rates for Moscow are 28.0 for men and 14.3 for women. These figures may indicate that the prevalence of EBV-associated GCs in the above-mentioned geographic regions of Russia is also different, and should be estimated for each region separately.

Statistical analysis of the data obtained revealed a clear gender difference, a lack of EBV involvement in antrum, and no very clear pattern with respect to age. These results are very similar to data on EBV-related GC in Japan, characterized by male predominance, site of tumor, histological type, age, and depth of invasion (Tokunaga *et al.*, 1993a,b).

Our findings revealed the presence of viral transcripts (EBER-1 ISH) in virtually all tumor cells, in line with reported data (Tokunaga *et al.*, 1993a). These results together with the monoclonality of EBV episomes as detected by Southern-blot analysis, also the elevated IgG antibodies against EBV capsid antigen in EBV-positive GC (Imai *et al.*, 1994), provide direct evidence of EBV involvement in a relatively small proportion of GCs. From the above observations, it could be assumed that EBV infection must take place early in the neoplastic process, providing infected tumor cells with a strong selective advantage and thus possibly being of pathogenetic significance for the virus-associated GC. The absence

of CD21 expression on gastric-carcinoma cells (Niedobitek *et al.*, 1992) suggests that gastric epithelial cells are infected by the virus through the mechanism of cell fusion between EBV-infected lymphocytes and epithelial cells (Bayliss and Wolf, 1980). The same mechanism has been assumed as a basis for EBV-associated carcinogenesis of nasopharyngeal carcinoma (Lenoir and De Thé, 1978). However, the use of monoclonal antibodies to CD21 has shown the presence of EBV receptor on human pharyngeal epithelia (Young *et al.*, 1986).

To elucidate the role of EBV in the pathogenesis of gastric carcinoma, several investigations have focused on the study of EBV-latent-protein expression in tumor cells of epithelial origin. As demonstrated by Sugiura *et al.* (1996), EBV-carrying gastric-carcinoma cells uniformly expressed EBNA-1 and LMP2A in approximately 40% cases, but did not express other members of the EBV-latent-protein family. Thus, the spectrum of EBV latency in GC cells according to the above authors resembles those in Burkitt's lymphoma (1st type of latency) rather than in NPC (2nd type of latency). Expression of LMP1 in 3 out of 12 EBV-positive patients has been found, however, by immunohistochemistry (Shin *et al.*, 1996). This finding suggests that the expression pattern in EBV-associated GC is similar to NPC-type latency, in line with the epithelial origin of GC. Further investigations should be carried out to clarify the role and significance of EBV in development of this malignancy.

In summary, our results, together with the findings of other investigators, support the notion that whereas EBV may be an important contributing factor in the development of GC, other factors not yet identified may also be involved in the genesis of gastric carcinoma.

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