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EBV-Positive Gastric Carcinomas in Poland

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Epstein-Barr virus plays a substantial role in numerous human neoplasms including gastric carcinoma, as proved recently. In our series EBV has been detected in five cases of forty (12.5%) gastric carcinomas. It may indicate that Poland is one of the countries with the highest EBV-induced gastric carcinoma incidence worldwide.

Introduction

Epstein-Barr virus (EBV), a member of *Herpesviridae* family and the well-known cause of world widely common infectious mononucleosis, is also proved to play a substantial role in several types of human cancers: Burkitt's lymphoma, nasopharyngeal carcinoma, B-cell lymphoma arising in immuno-compromised individuals, Hodgkin's disease (especially mixed cellularity type) and lymphoepithelioma-like carcinomas of the salivary gland, thymus, and lungs [2, 8, 12, 23].

The role of EBV in gastric carcinogenesis has been postulated recently [12, 25 - 28, 31]. The presence of EBV DNA in gastric carcinomas was documented in 1990 with polymerase chain reaction (PCR) [4]. Since then there has been a growing pile of evidences supporting the strong etiologic association between EBV and gastric carcinoma. First, *in situ* hybridization studies revealed that EBV gene product is uniformly present in all carcinoma cells in a proportion of cases [15]. Second, Southern blot hybridization of the EBV terminal repeat fragment disclosed that the EBV DNA in a carcinoma cell is monoclonal [16]. Third, it was shown that high titers of EBV-specific antibodies, especially of EBV viral-capsid antigen IgA and EBV early antigen R component IgG were present in serum many years before the diagnosis of EBV-positive gastric carcinoma [22].

However, the discovery of high concentration of the EBV-encoded small RNA (EBER) in EBV-associated gastric cancer cells, which is considered a unique marker of latent infection, seems to point out that EBV infection in gastric cancer may be considered as a latent state [12, 22].

The mechanisms by which EBV contributes to carcinogenesis of the gastric mucosa are unknown. The EBV gene expression pattern is reported to be similar to that of Burkitt's lymphoma: EBV-determined nuclear antigen 1 (EBNA1), latent membrane protein 2A (LMP2A), the BARF0 from the BamHI-A region and EBV-encoded small RNAs (EBERs) [12, 16, 26]. Some observations suggest that EBV infection occurs in the dysplastic phase of carcinogenesis because scattered EBV-positive cells are seen in dysplastic mucosa bordering the tumors but are absent in surrounding normal tissues usually including lymphocytes [13, 15, 32]. It is possible that EBV is required to maintain the malignant phenotype of EBV-associated gastric malignancies but none oncoprotein expression in EBV-positive carcinomas was found [15]. Some observations suggest that EBV is present in a clonal episomal form in the proliferating carcinoma cells [13, 24]. Recently several distinct or significantly frequent chromosomal aberrations in EBV-associated gastric carcinomas (loss of chromosome 4p, 11p and 18q) were described [33].

The way by which EBV enters gastric mucosa cells also remains mysterious. A recent observation points out that EBV enters the cells by receptor other than CD21 [31], but it still requires further study. The possibility of direct infection of various human epithelial cells *in vitro* by EBV was demonstrated [19].

Similar to infectious mononucleosis, EBV-associated gastric carcinoma occurs worldwide, but with variable incidence in different countries. It has been reported that association between EBV and gastric carcinogenesis varies from 4.3% in China, 5.2% in the United Kingdom, 6.2% in Italy, 6.7% in Hong Kong, 7% in Japan, 7 - 16% in the USA, 7.7% in France, 8.1% in Russia, 10.6% in Brazil, 11% in Taiwan to 17.9% in Germany [12, 26, 28].

In this study, we investigated the association between EBV and gastric carcinoma in patients from southern Poland. It is the first retrospective study in Poland of this kind. We have chosen the demonstration of EBV-encoded small RNA (EBER) by *in situ* hybridization in cells of advanced gastric carcinomas.

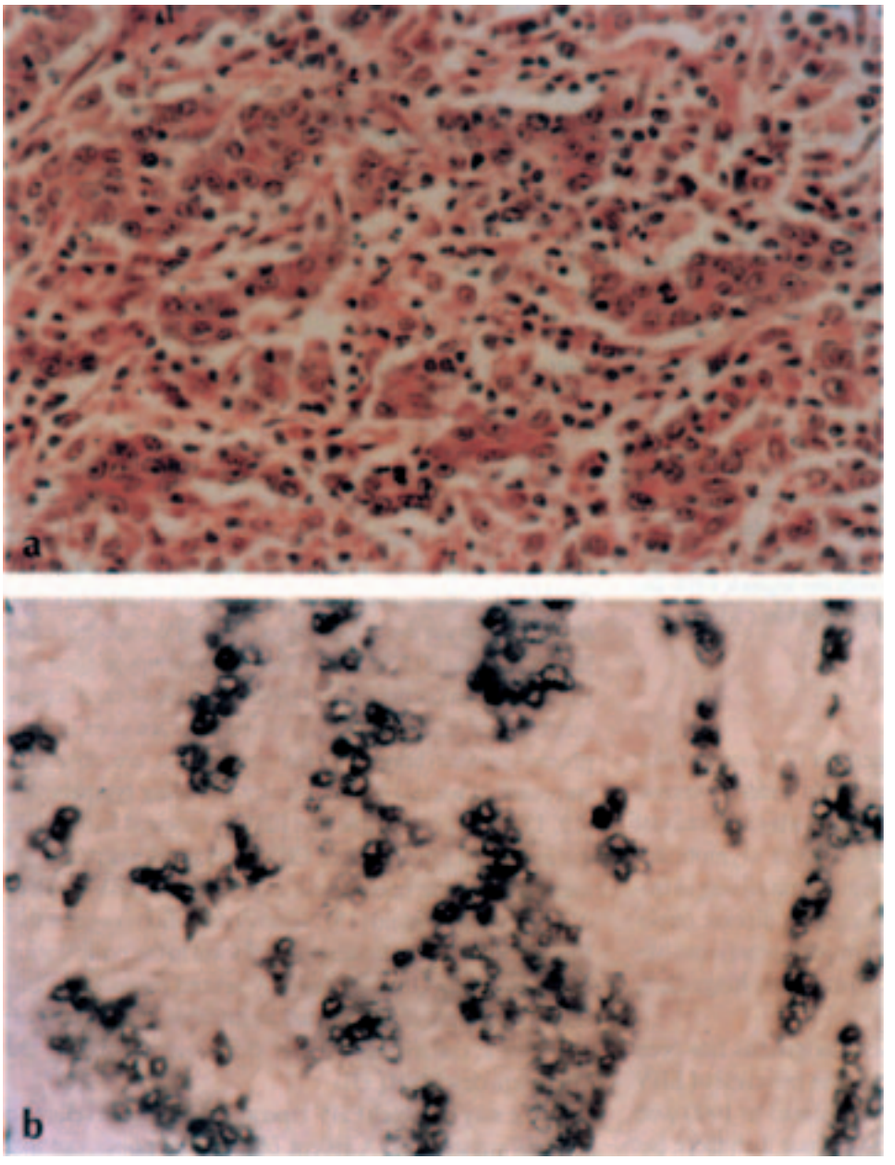

Fig. 1. a. Morphology of gastric adenocarcinoma with EBV infection: poorly differentiated carcinoma cells with large nuclei and prominent nucleoli occasionally forming tubular or cord-like pattern. HE. b. Dark blue staining of EBV-positive adenocarcinoma cells demonstrated with reaction of *in situ* hybridization.

Material and Methods

We studied 40 unselected gastric cancer patients who underwent surgery in the 1st Department of Surgery of Jagiellonian University, Collegium Medicum during the last three years: 30 men and 10 women aged from 35 to 81 years. Previously we found among them 21 cases with microsatellite instability whereas 19 were microsatellite stable [9]. The microsatellite status of the samples was categorized into two groups according to the current criteria of the National Cancer Institute Workshop report [3]: 1. with less than 30% loci with instability - low-frequency microsatellite instability (MSI-low), and 2. with 30% or more loci with instability - high-frequency microsatellite instability (MSI-high). Among the 21 gastric cancers with microsatellite instability 10 were classified as MSI-low and 11 as MSI-high.

Gastrectomy specimens were routinely sampled, and the carcinomas were classified according to the Lauren classification as intestinal, diffuse or mixed type [20]. Each carcinoma was also graded according to the Goseki classification [14] and staging was assessed according to TNM system recommended by the World Health Organization (WHO).

For all the tumor samples the process of *in situ* hybridization was carried out in a laboratory to detect DNA of Epstein-Barr virus in gastric carcinoma cells. The whole process was performed on Bench Mark Discovery Staining Module (Ventana Medical Systems Inc, Tucson, AZ). The necessary reagents i.e.: Alkaline Phosphatase Blue Detection Kit, INFORM EBV probes, INFORM Anti-FITC Linker Antibody, Protease I, buffers (EZ Prep buffer, Salt

Sodium Citrate buffer (SSC)) and other were purchased from Ventana Medical Systems, Inc.

Paraffin-embedded tissue sections were dewaxed using EZ Prep buffer, then digested with Protease I for 4 minutes. Probes were applied and denaturation was performed at 85˚C (10 minutes), then hybridization - at 37˚C (1 hour). The probes labeled with fluorescein contained a cocktail of oligonucleotides dissolved in formamide based diluent. After hybridization tissues were washed 3 times using 2x SSC buffer for 6, 2 and 6 minutes at 57˚C. Incubation with anti-fluorescein monoclonal antibody was performed for 20 minutes. Then Alkaline Blue Detection Kit was applied using standard Ventana Medical Systems Inc. protocol. The slides were counterstained with Nuclear Fast Red for 10 minutes. Slides were washed in warm tap water with detergent (to remove Liquid Coverslip (LCS)) and dehydrated in 70% ethanol (30sec.), 100% ethanol (2 times for 30sec.), acethone (1 minute), xylene (2 times for 3 minutes). Slides were coverslipped in permanent mounting medium (Consul-Mount, Shadon). In parallel with processing of the gastric carcinoma samples, positive and negative control slides obtained from Ventana were processed. The samples were interpreted as positive if the nuclei showed intense dark blue staining (Fig. 1).

The relationship between EBV status and age, sex, location, histological type, presence of cancer cells in lymphatic vessels of gastric mucosa, lymphocyte infiltration, grading, staging, survival time and microsatellite instability status were analyzed statistically. The statistical analysis of the results (chi square test, Fisher exact test, Kaplan-Meier survival diagram) was performed with Statistica 5.5 PL software (Statsoft, Tulusa, OK, USA). P-value of less than 0.05 was considered as statistically significant.

Results

Among forty gastric carcinomas five cases were EBV-positive and thirty-five cases were EBV-negative. Among the five positive cases, there was one woman and four men. The positive nuclear signal was usually uniformly strong in all carcinoma cells (Fig. 1). Only one EBV-positive gastric carcinoma was composed of foci of positive cells dispersed within the predominantly EBV-negative neoplastic infiltrate. Histologically, only one case among five EBV-positive was compatible with the "lymphoepithelioma-like" carcinoma pattern. Among the remaining gastric carcinomas, four had strong lymphocytic infiltration but mostly at the peripheral borders of the tumors, so they do not resemble the "lymphoepithelioma-like" pattern. All details are summarized in Table 1.

We found a statistically significant relation between Goseki grade and EBV presence. All EBV-positive carcinomas were Goseki grade III (4 cases) or Goseki grade IV (1 case). In contrast, we did not find a statistically significant correlation between the grade of gastric carcinomas expressed in a conventional three-tired system and EBV

TABLE 1

Clinical and pathological features of the examined gastric carcinomas

	EBV (+)	EBV (-)	p*
number of cases	5	35	
males	4	26	ns
females	1	9	
mean age (years) ± std dev.	55.00±14.19	64.94±10.02	ns
mean survival (days)	433.00	288.71	ns
location:			
whole stomach	0	6	
antrum	3	9	ns
body	0	6	
cardia	2	14	
Lauren:			
diffuse	4	15	ns
intestinal	0	13	
mixed	1	7	
Goseki:			
I	0	15	
II	0	1	0.04
III	4	7	
IV	1	12	
well differentiated	0	5	
moderately differentiated	0	4	ns
poorly differentiated	5	26	
lymphoid stroma:			
present	1	4	ns
absent	4	31	
ca cells in lymph vessels:			
present	2	17	ns
absent	3	18	
pT2	1	1	
pT3	3	31	ns
pT4	1	3	
pN0	1	6	
pN1	0	12	ns
pN2	2	12	
pN3	2	5	
pM0	5	17	
pM1	0	6	ns
pMx	0	12	
stage:			
II	1	7	
III A	1	9	ns
III B	2	13	
IV	1	6	
MSI-h	0	11	
MSI-l	3	7	ns
MSS	2	17	

*p <0.05 was considered statistically significant, chi-square or Fisher's exact test was used where appropriate

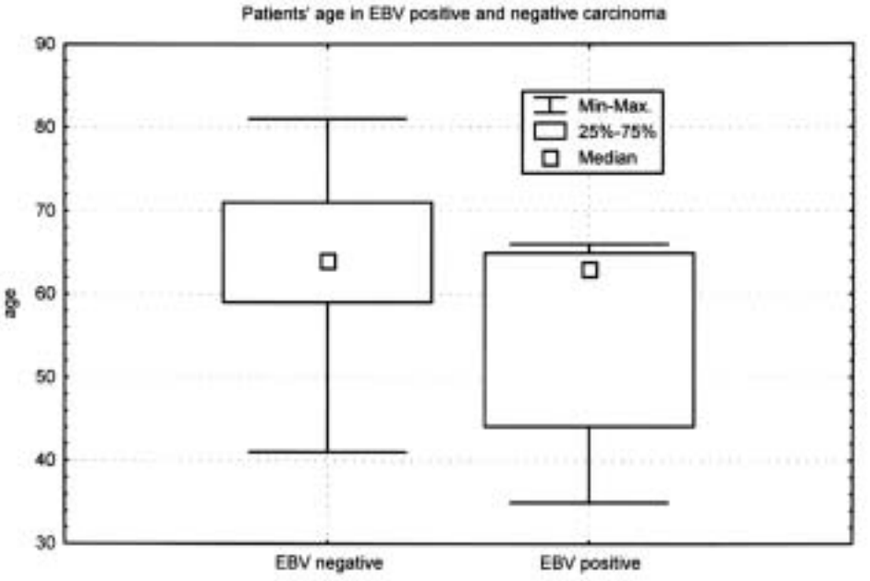

Fig. 2. Age of patients with and without EBV infection. Mean ages (see Table 1) insignificantly but definitely differ by about 10 years between the two groups, however medians are almost identical.

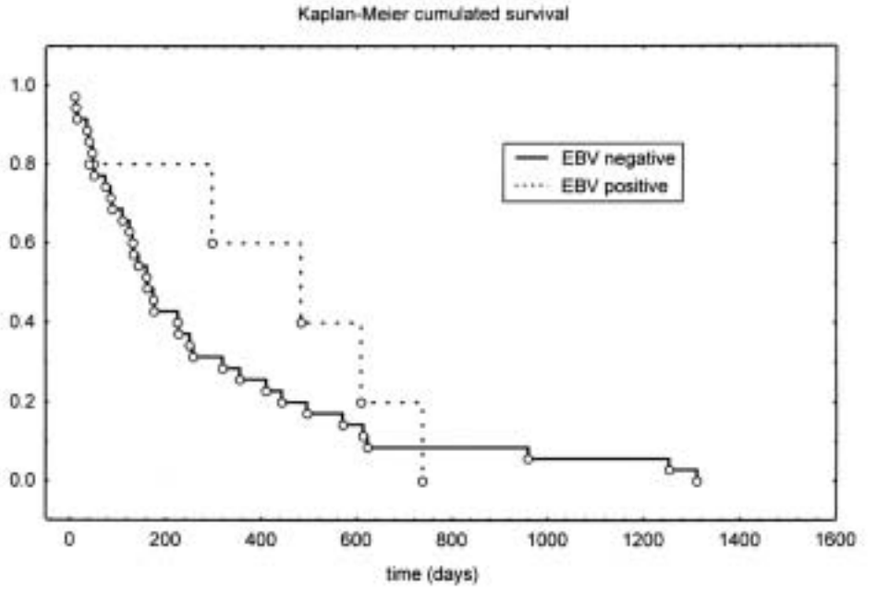

Fig. 3. Kaplan-Meier survival curves for patients with and without EBV infection.

presence in carcinoma cells. The statistical tendency of EBV presence only in the five uniformly poorly differentiated carcinomas should be strong, but it could not be proved because of very small size of the group.

There was no significant difference between sex and age distribution among EBV-positive and -negative cases, but there was a higher incidence of EBV-positive gastric carcinomas in males and we found an insignificant tendency of EBV infected patients being younger than the negative ones (Fig. 2). No significant correlation between EBV positivity and Lauren type, pTNM, median survival time after surgical operation (Fig. 3) and microsatellite instability was found.

Three of five positive cases were MSI-low, and two of them were microsatellite stable, but no statistically significant correlation between MSI status and EBV status was found.

Discussion

Gastric carcinoma is one of the leading malignant neoplasms in Poland especially among men. In a number of countries, the overall incidence of gastric carcinoma in the distal stomach is slowly decreasing, whereas the incidence in the proximal stomach is increasing slightly [11].

Epstein-Barr virus is the first virus for which a causal role in gastric carcinogenesis can be strongly suspected. It plays also an important role in the development of gastric stump carcinomas, usually independently on *Helicobacter pylori* infection [1, 30], but some authors' results suggest that previous *H. pylori* infection may be important [29]**.** In the present study EBV presence was demonstrated using method of *in situ* hybridization in 5/40 (12.5%). As reported in the literature in positive lesions EBV is found in almost all carcinoma cells, but usually not in surrounding lymphocytes, stromal cells, normal gastric mucosa and even intestinal metaplasia [12, 13, 16, 32]. This finding may indicate that EBV infection may be characteristic of the dysplastic phase of carcinogenesis [24]. Our results were similar, but not identical: in one case only several foci in the whole carcinomatous infiltration were EBV-positive. In contrast to some studies [6] in which EBV was detected in the tumor-infiltrating lymphocytes either in a few of them or in clusters we did not find EBV positivity in lymphocytes.

Japanese investigators did not find EBV in gastric carcinomas with microsatellite instability [7] and Taiwanese researchers did not find correlation of microsatellite instability and EBV presence [29]. However, we detected presence of the virus DNA in three gastric carcinomas with low microsatellite instability and two microsatellite stable tumors. This finding and other [20] may interfere with the hypothesis that microsatellite instability does not play a role in progression of EBV-associated gastric carcinomas and require further investigation. However, in contrast to Hong Kong researchers, in our series among gastric carcinomas with high microsatellite instability EBV positivity was not found [20]. A recent study demonstrated that MSI-positive EBV-negative gastric adenocarcinomas displayed as much methylation in CpG islands as EBV-positive gastric adenocarcinomas [18].

This study demonstrated that the high percentage of gastric cancer in Poland is associated with EBV infection. The incidence rate of 12.5% places our country among the highest incidence worldwide, but it requires further studies on a larger group of patients. However, the results suggest that in Central Europe (Germany - almost 18% [12, 26]) overall incidence is higher than in other parts of the world.

According to the literature, EBV positivity is observed in intramucosal carcinoma prior to invasive growth, perhaps at a very early step of carcinogenesis [12, 17, 23, 26]. However, in our series it is difficult to make suggestions about the time of infection because of the advanced stage of gastric carcinomas.

Worldwide observations show higher prevalence of EBV-positive gastric carcinoma in the proximal stomach and lower in the antrum for both sexes, whereas for lesions in middle stomach prevalence is higher for males than for females [5, 12, 17, 26, 28]. The cancers in our material, like many gastric carcinomas in Poland, diffusely infiltrated the stomach at the time of surgery, but the EBV-positive were located only in cardia or antrum.

The difference between mean ages of EBV-positive and EBV-negative gastric cancer patients observed in our series probably was a consequence of a too small size of the study group. Among the EBV-positive gastric carcinoma patients two were significantly younger (35 and 44 years) than the rest (63, 65 and 66 years), which significantly affected the mean age in this group. Medians of ages did not show significant difference (Fig. 2).

Usually in terms of a histological type, EBV infection occurred at higher frequencies among carcinomas with at least one of the following histological features: poor differentiation and/or marked lymphoid stroma, so they are called "lymphoepithelioma-like" [10]. In our series all EBV-positive gastric carcinomas were poorly differentiated, but a very small group of them (only five) made statistical test results virtually useless. However, Goseki grading which reflects at least some aspects of classic gastric carcinoma differentiation showed a significant difference between EBV infected and uninfected carcinomas.

Lymphoepithelioma-like carcinomas show a specific histology with dense lymphocyte infiltration, and poorly differentiated or undifferentiated carcinoma cells with large nuclei and prominent nucleoli occasionally forming tubular or cord-like structures. The tubular pattern in lymphoepithelioma-like carcinoma is usually observed in and near the mucosa, suggesting that tumor cells dedifferentiate during invasive infiltration [26, 29]. In our series the typical "lymphoepithelioma-like" carcinoma pattern was found only in 1/5 EBV-positive, but among EBV-negative neoplasms four cases had this feature.

In conclusion, our results are consistent with the results of previous studies suggesting that Epstein-Barr virus plays an important role in gastric carcinogenesis [12, 25, 26]. In our series among five detected EBV-positive gastric adenocarcinomas three showed low microsatellite instability and two were microsatellite stable. Furthermore, only one of them revealed typical "lymphoepithelioma-like" carcinoma pattern. We found EBV in 12.5% of examined gastric carcinomas, which may place Poland among countries with the highest incidence of EBV-induced gastric carcinomas worldwide.

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