HHS Public Access

Author manuscript

Eur J Gastroenterol Hepatol. Author manuscript; available in PMC 2021 November 01.

Published in final edited form as:

Eur J Gastroenterol Hepatol. 2019 November ; 31(11): 1328–1333. doi:10.1097/MEG.0000000000001521.

Clinicopathological characteristics of Epstein-Barr virus-positive gastric cancer in Latvia

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Abstract

Objective: Epstein-Barr virus (EBV)-associated gastric cancer (GC) has been proposed to be a distinct GC molecular subtype. The prognostic significance of EBV infection in GC remains unclear and needs further investigation. Our study aimed to analyze EBV-positive and EBV-negative GC patients regarding their personal and tumor-related characteristics, and compare their overall survival.

Methods: GC patients consecutively treated at the Riga East University Hospital during 2009–2016 were identified retrospectively. Tumor EBV status was determined by *in situ* hybridization

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Compliance with ethical standards

Consent to submit has been received explicitly from all co-authors, as well as from the responsible authorities.

At the time of enrolment all the participants have signed the informed consent. Our study was approved by the Ethics Committee of Riga East University Hospital Support Foundation and Riga East University Hospital.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of Interest

The authors declare that they have no competing interests.

for EBV-encoded RNA (EBER). Information about clinicopathological characteristics was obtained from patient questionnaires and/or hospital records. Overall survival was ascertained through July 30, 2017. Cox proportional hazard regression models adjusted for personal and tumor-related covariates compared survival between EBV-positive and EBV-negative patients.

Results: There were a total of 302 GC patients (61% males) with mean and standard deviation age 63.6 ± 11.5 years. EBER positivity was present in 8.6% of tumors. EBV-positive GC patients had better survival at 80 months (adjusted hazard ratio [HR] = 0.37, 95% confidence interval [CI] 0.19–0.72) compared to EBV-negative patients. Worse survival was observed for patients with stage III (HR = 2.76, CI 1.67–4.56) and stage IV (HR = 10.02, CI 5.72–17.57) compared to stage I GC, and overlapping and unspecified subsite (HR = 1.85; CI 1.14; 3.00) compared to distal tumors.

Conclusion: Tumor EBV positivity is a favorable prognostic factor in gastric cancer.

Keywords

Epstein-Barr virus; EBER-*in situ* hybridization; gastric cancer; survival

Introduction

Epstein-Barr virus (EBV) associated gastric cancer (GC) has been proposed to be a distinct molecular GC subtype. Presence of the EBV genome in GCs tumors was first reported in 1990 by Burke *et al.* [1] In 2014, The Cancer Genome Atlas provided a molecular classification defining EBV-positive gastric cancer as a separate GC subtype. [2] In a systematic review of observational studies, the worldwide crude prevalence of EBV in gastric adenocarcinoma was 8.29%, with lower prevalence in Asia (7.99%), intermediate in Europe (8.75%) and higher in the Americas (11.9%). [3]

Recently, a number of studies have investigated the association between EBV positivity and the prognosis of GC with conflicting results. Two large meta-analyses of 8,336 cases across 24 studies (with EBV prevalence varying from 2.02 % to 33.3 % and overall EBV positivity 9.3 %)[4] and of 4,599 cases (overall EBV positivity 8.2%)[5] found EBV positivity associated with favorable prognosis[4,5], and two additional studies with 566 (EBV positivity 7.2%)[6] and 192 (EBV positivity 33.3%)[7]patients showed better survival with EBV-positive tumors[6,7]. However, other reports have shown no correlation between EBV positivity and survival[8] or even poorer survival in EBV-positive patients[9].

Standard GC treatment guidelines do not differentiate between EBV-positive and EBV-negative tumors[10,11]. Tumor EBV status has been recognized as an emerging potential biomarker for personalized treatment strategies in GC, but is not currently recommended for clinical care[10]. Nevertheless, specific treatments for EBV-positive GC patients have been proposed, including proteosome inhibitors, pan-histone deacetylase inhibitors, antiviral drugs, EBV vaccines and various targeted and immunotherapy agents directed at PIK3/Akt/mTOR, PD-1, PD-L1, CTLA-4 and JAK2 [10,12]. The therapeutic effectiveness of these approaches is yet to be established. Thus, the prognostic significance of tumor EBV positivity in GC merits further investigation. In this study, we analysed EBV-positive and

EBV-negative Latvian GC patients concerning their personal and tumor-related factors, and to compare their overall survival.

Materials and Methods

Patient characteristics:

We retrospectively analysed data from consecutive GC patients treated at the Riga East University Hospital in 2009–2016 who were enrolled in the University of Latvia / Riga East University hospital biobank. At the time of enrolment all the participants have signed consent. Socio-demographic information was obtained by a standardized questionnaire. Personal characteristics obtained from the hospital database included sex, age, smoking status and body mass index (BMI). Age was dichotomized as ≤ 65 and > 65 years[13,14]. Smoking status was classified as never-smokers, current-smokers and former-smokers. BMI was classified as <18.5 kg/m^2 (underweight); 18.5 – 24.9 kg/m^2 (normal), and > 25 kg/m^2 (overweight). Self-reported history of other cancers was obtained from questionnaires. Data on patient survival from the date of diagnosis until the end of follow-up (July 30, 2017) were obtained from the hospital records database and The Centre of Disease Prevention and Control of Latvia. Our study was approved by the Ethics Committee of Riga East University Hospital Support Foundation and Riga East University Hospital.

Tumor characteristics:

Tumors were classified by AJCC stages I – IV (American Joint Committee on Cancer 7th edition); proximal (C16.0-C16.2 and C16.5-C16.6), distal (C16.3-C16.4) or overlapping/unspecified (C16.8-C16.9) location based on International Statistical Classification of Diseases and Related Health Problems, 10^{th} edition (ICD-10, 2017); and local recurrence as recorded in the hospital records database. All diagnoses of GC were confirmed histologically by an expert pathologist. We analyzed the following histopathological characteristics: Lauren's classification (intestinal-type, diffuse-type, mixed, indeterminate), grade (G1, G2, G3), and peritumoral atrophy and intestinal metaplasia. We chose not to add figures showing standard histopathologic grades. A grade (1, 2 or 3) was assigned to each cancer tissue using standard histopathologic methods that reflect cytologic differentiation features of the malignant cells (following the protocol [15]). With low grade (1) signifying that the cells are well differentiated and are thus more likely to grow slowly and remain localized, and high grade (3) signifying the cells are less differentiated and thus predicted to proliferate or spread.

Addressing the loss of the patients' data, we would like to note that none of the patients was lost during the follow up period. The focus of the study – survival in EBV positive and EBV negative gastric cancer patients had no missing data. This was achieved by gathering information from The Centre of Disease Prevention and Control of Latvia and strengthened the study's integrity. We also had registered all data in following categories: sex, age, other cancer in personal history, local recurrence of the tumor, atrophy and intestinal metaplasia in the adjacent tissues.

Missing data:

1. smoking status: 3 in EBV positive and 26 EBV negative group;
2. BMI: 3 in EBV positive and 22 EBV negative group;
3. stage: 0 in EBV positive and 10 EBV negative group;
4. tumor anatomical location: 11 in EBV positive and 64 EBV negative group;
5. Lauren's classification: 1 in EBV positive and 14 EBV negative group;
6. grade: 0 in EBV positive and 1 EBV negative group.

No participants that were excluded from the main analysis. Taking into account the bias missing data might cause we tried omitting categories which had missing values from the survival analysis.

Tissue cores were sampled from the paraffin embedded tumors and prepared as tissue microarrays (TMAs) in the Department of Pathology, University Medical Center Utrecht, Netherlands. TMAs were sent to the Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, North Carolina, USA for determination of EBV status by *in situ* hybridization of EBV-encoded small RNA (EBER) (Figure 1).

Formalin-fixed, paraffin-embedded tissues from gastric cancer resections were retrieved from the University of Latvia / Riga East University hospital biobank. By an experienced pathologist, two representative tumor regions were marked on a hematoxylin and eosin (H&E)-stained section of each tumor, avoiding areas of necrosis. From these tumor regions, a tissue cylinder with a diameter of 1.0 mm was punched out of the corresponding paraffin block ('donor block') and placed into the TMA paraffin block using a manual tissue arrayer (MTA-I, Beecher Instruments, Sun Prairie, USA), which was guided by the MTABooster® (Alphelys, Plaisir, France). The distribution and position of the cores was determined in advance with the TMA-designer Software (Alphelys-TMA Designer®, Version 1.6.8, Plaisir, France). *EBER in situ* hybridization was performed by an automated method using fluorescein-labeled EBER and oligo(d)T control probes on the Ventana Benchmark *in situ* hybridization system (Ventana Medical Systems, Tuscon, AZ, USA) as previously described (Ryan *et al.*, Lab Invest 2009 [16]). The oligo(d)T probe served as a control for RNA preservation in histological sections. A tumor was considered *EBER*-negative if *EBER* staining was undetected or was only expressed in benign-appearing lymphoid cells, and *EBER*-positive if the signal was localized to malignant epithelial cells.

Statistical analysis:

Chi-square tests were performed to compare EBV-positive and EBV-negative patients. Hazard ratios (HR) were derived from Cox proportional hazard regression models adjusted for personal (age, sex, BMI) and tumor-related (EBV status, tumor stage, topological localization) covariates. Cumulative survival curves were constructed by the Kaplan-Meier method. A two-sided p-value less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 22 software (IBM Corp. Released 2013. IBM Statistics for Windows, Version 22.0, Armonk, NY: IBM Corp.).

Results

Patients' characteristics:

There were 302 GC patients of which 61% were male. The mean age at diagnosis was 63.6 years (standard deviation [SD] 11.54; range 20 – 88). Slightly more patients were current or former smokers than never smokers. In almost half of the patients, BMI was within the normal range. There were 169 (56%) deaths during median follow-up of 34.3 months (range 0.27 – 156.2) (Table 1).

Tumor characteristics:

EBV positivity was present in 26 (8.6%) of the tumors. Male patients more often had EBV-positive GC (p = 0.01). A slight majority of all cancer cases (51%) were diagnosed at advanced stages, had histologically positive lymph nodes (51.3%, EBV-positive *vs* EBV-negative group p=0.15) and tumors located proximally (47.7%) in the stomach.

By Lauren classification, most cases were intestinal-type (49%), and poorly differentiated adenocarcinomas (73.2%). Patient and tumor characteristics (excluding sex) did not significantly differ between EBV-positive and EBV-negative GC patients (Table 1). The adjacent mucosa was atrophic for more than half of the tumors and exhibited intestinal metaplasia in one fourth, but these characteristics were not associated with tumor EBV status.

Statistical analysis:

In a multivariable Cox proportional hazard regression model adjusted for personal and tumor-related covariates, EBV-positive GC patients had better survival at 80 months (HR = 0.37; 95% confidence interval [CI] 0.19–0.72) compared to EBV-negative GC patients (Figure 2). Survival was not significantly associated with age, sex, BMI, stage II tumors and proximal tumor localization. Worse survival was observed for stages III and IV and for overlapping and unspecified tumor localisation (Table 2).

Discussion

In our series, EBV-positive GC patients had better survival compared to EBV-negative patients at median follow-up time of 34.3 months. These results are similar to several previous studies[4–7]. Reasons for this difference are uncertain, but may include enhanced cell-mediated cytotoxicity by tumor-infiltrating lymphocytes, more favorable mutation profile and/or greater sensitivity to chemotherapeutics[17].

We found tumor EBV positivity more frequently in male than female GC patients, similar to several other studies[3,5,6,18–21]. However, a few studies have found no difference in EBV frequency between sexes[22,23]. Males also have greater incidence of other EBV-associated malignancies, including nasopharyngeal carcinoma and Burkitt lymphoma, with somewhat less disparity post-menopause suggesting a potential protection by female sex hormones[24].

It has been reported that EBV-positive GC is more frequent in smokers[25,26]. Our study did not find any difference in smoking status between EBV-positive and EBV-negative

patients. In the case-case comparison study of 2,648 patients (184 EBV-positive) by Camargo *et al.* (2014), the unadjusted OR of EBV-positivity with smoking was 2.2 (CI 1.6 – 3.2), which was attenuated to 1.5 [1.0 – 2.3] by adjustment for possible confounders[25]. A smaller study of 205 patients by C. Koriyama *et al.* (2005) found prevalence of smokers in EBV-positive GC cases higher than among EBV-negative GC cases, but the difference was not significant ($p = 0.13$)[26].

Similar to several others[19,21] our study found no distinct histological features in EBV-positive GC. Previous reports regarding histological data are inconclusive. Some studies described higher EBV positivity in diffuse-type GC[5,22] while other studies showed predominance of intestinal-type[6] and poorer differentiation[1,5,27,28]. Van Beek *et al.* (2004) in a study with 566 patients found that EBV-positivity associated with intestinal-type histology ($p = 0.05$)[6]. Regarding differentiation, Abdirad *et al.* (2007) reported Japanese classification for 273 GC cases; solid poorly differentiated adenocarcinoma (por1) and non-solid poorly differentiated adenocarcinoma (por2) were the predominant histologic types in EBV-positive GC, but low numbers of cases in each group precluded formal statistical comparison with EBV-negative GC[22].

EBV associations with anatomical localisation have been inconclusive. Some reports have described predilection for the cardia[5] or proximal stomach[6], while other studies found fundus or body favored[12] and still other reports[22] like our study did not find any relation to localisation. Several reports have described significantly lower tumor-node-metastasis system-stage[1],[5,6] and less lymph node involvement[1,5,6,29] for EBV-positive GC. In our study, we did not observe these differences. We also attempted to characterise the background mucosa adjacent to tumors because EBV-positivity has been described in association with severe atrophic gastritis and a paucity of intestinal metaplasia[30]. However, we found no difference regarding surrounding lesions of atrophy and intestinal metaplasia in EBV-positive and -negative GCs, in agreement with another report[9].

In our study, survival was not significantly associated with age, sex, BMI, stage II tumors and proximal tumor localization. Worse survival was observed for stages III and IV and for overlapping and unspecified tumor localisation.

Some of the hypothesis why EBV positive GC patients have better survival are:

Greater number of gene mutations and the production of neoantigenes:

Cancers with a greater number of gene mutations provoke a stronger antitumor immune response. The thinking behind this hypothesis relates to the production of neoantigens—fragments of proteins expressed on the surface of cancer cells that are encoded by mutated genes. Neoantigens are unique to cancer cells because they are derived from a mutant gene, which may encode a mutant protein that differs from that expressed by normal cells. Therefore, neoantigens have the potential to be recognized as foreign by the cells of the immune system that patrol the body. A greater number of neoantigens mean increased stimulation of immune cells and a stronger immune response [31] and correlate with patient response to both CTLA-4 and PD-1 inhibition. According to data 19% of gastric intestinal type adenocarcinomas have high mutation burden (defined as >20 mutations/Mb) [32].

The Cancer Genome Atlas classification distinguished EBV positive GC subtype based on molecular changes (some of them are: (1) higher prevalence of DNA hypermethylation, (2) strong predilection for *PIK3CA* mutation, (3) frequent *ARID1A* (*55%*) and *BCOR* (*23%*) mutations, recurrent *JAK2* and *ERBB2* amplifications and only rare *TP53* mutations, (4) prominent pattern of nucleotide A to C transversions base changes and (5) highly transcribed EBV viral mRNAs and miRNAs). Mutation rates were below 11.4 mutations per megabase (Mb) [2].

High percentage of the tumor infiltrating lymphocytes:

EBV positive GC subtype is described to have high percentage of the tumor infiltrating lymphocytes [33] and the amount of lymphocytes is significantly associated with improved survival [34,35].

Morphological evidence of an activated cytotoxic T-cell infiltrate in EBV-positive gastric carcinoma preventing lymph node metastases:

Additionally, van Beek et al. have suggested that local triggering of cellular immune responses in EBV-positive GC prevents lymph node metastasis formation [36].

In summary, based on our findings which showed no substantial differences clinically (besides male sex in EBV positive group) and morphologically between EBV-positive and EBV-negative GC patients as well as evidence in the published papers, we suggest that reasons contributing to the survival difference could be: greater number of gene mutations and the production of neoantigenes, increased primary tumor inflammation and decreased secondary spread.

Our study is the first report presenting Northern European' data regarding the association of EBV with GC and survival analysis of EBV-positive and -negative patients. We used standardized collection of patient data, biomaterial, and histological (including EBV status) analysis. We also included a range of covariates in the Cox regression model and our study is one of the largest single centre ones. Thus, the observed difference in survival between EBV-positive and -negative GC patients is meaningful. The novelty of the study is the analysis of the clinical and pathological characteristics in EBV positive and EBV negative gastric cancer groups in a high gastric cancer incidence country, with homogenous Caucasian population, similar diet patterns (all patients were carnivores) and evenly distributed characteristics (with exception of male sex) between both groups. As well as the multifactorial regression model used to analyse survival data.

Unfortunately, we did not have data on some important risk factors for developing gastric cancer, including salt intake and *H. pylori* infection. However, these characteristics would not be expected to confound an association between tumor EBV-status and mortality.

In conclusion, our study supports other data that EBV-positive GC has better survival. Tumor EBV status should be considered as a prognostic factor in design and analysis of clinical trials. Furthermore, EBV-positive GC may be amenable to targeted therapy and immunotherapy to improve patient outcomes in the future.

Source of funding:

Project No. LZP-2018/1-0135 'Research on implementation of a set of measures for prevention of gastric cancer mortality by eradication of *H. pylori* and timely recognition of precancerous lesions' of the Latvian Council of Research. This study was supported in part by the Intramural Research Program, US National Cancer Institute.

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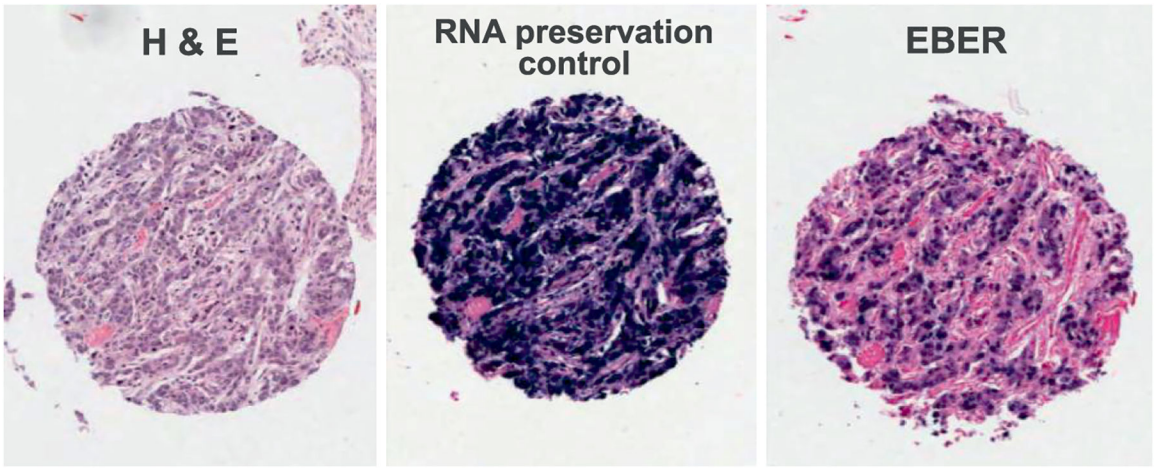

Figure 1.

Representative photomicrographs of an EBV-positive gastric cancer tumor stained with hematoxylin and eosin (left panel), RNA preservation control (middle panel), and EBER-ISH (right panel)

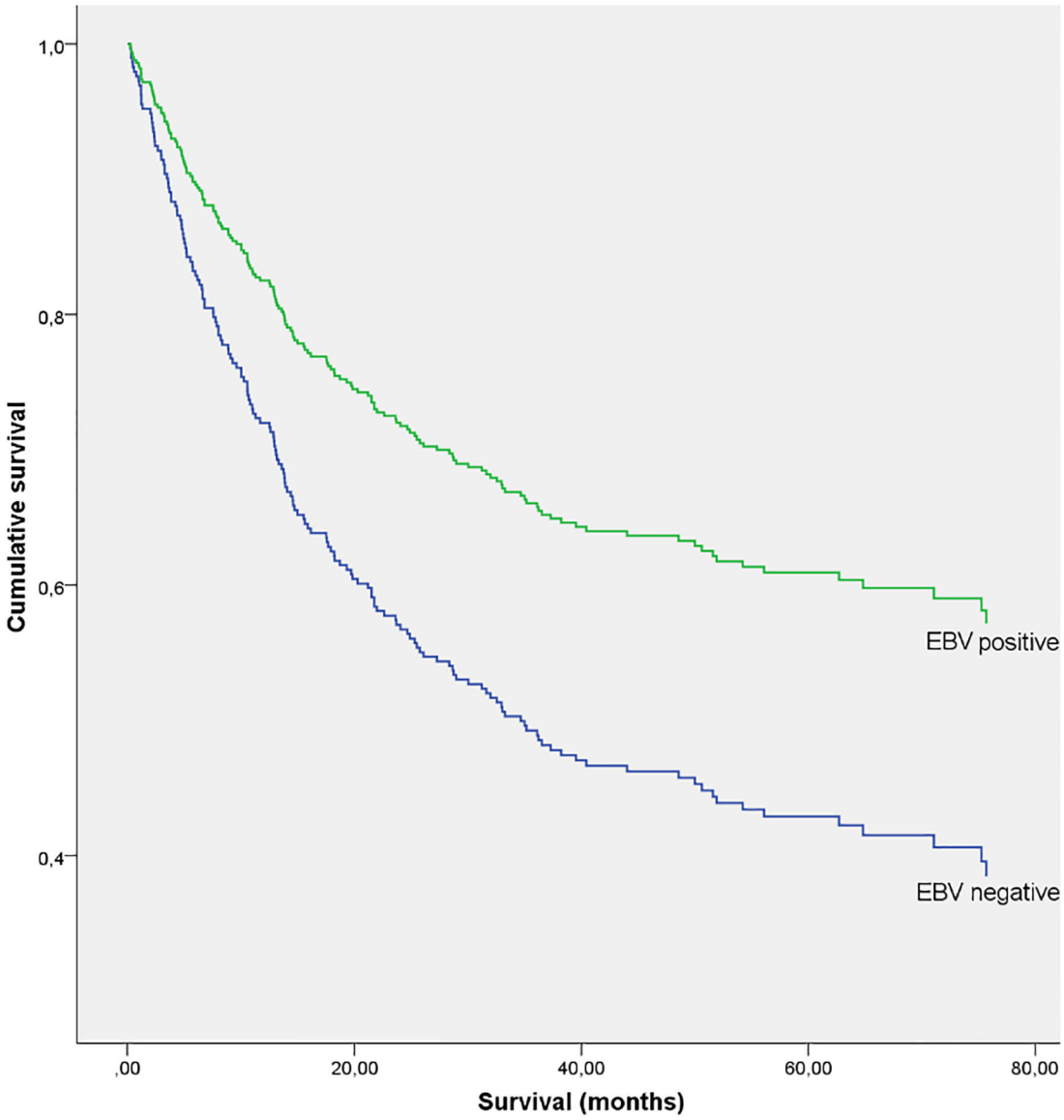

Figure 2.
Kaplan-Meier curve of cumulative survival (all stages combined)

Table 1.

Socio-demographic and tumour-related characteristics of EBV- positive and EBV-negative GC patients

	Total %	EBV-positive gastric carcinomas (n=26)	EBV-negative gastric carcinomas (n=276)	*p* value (refers to all the group)
		N	N	
Sex				
female	39.4	4	115	
male	60.6	22	161	0.01
Age				
Mean ± SD	63.6 ±11.54	63.8 ± 11.9	62.1 ± 11.5	0.86
Age ≤ 65 years	53.0	14	146	
Age > 65 years	47.0	12	130	0.54
Smoking status [a]				
never	43.4	8	123	
current	25.8	8	70	
former	21.2	7	57	0.60
BMI [b]				
< 18.5	3.6	1	10	
18.5 – 24.9	45.7	12	126	
≥ 25	42.4	10	118	0.92
Previous history of other cancers				
no	94.7	26	260	
yes	5.3	0	16	0.37
Stage [c]				
0	0.3	0	1	
I	22.2	4	63	
II	23.2	8	62	
III	36.1	7	102	
IV	14.9	7	38	0.33
Tumour location				
Proximal	47.7	13	131	
Distal	18.9	2	55	
Overlapping/unspecified	33.4	11	90	0.27
Local recurrence				
no	95.4	25	263	0.84
Lauren's classification [d]				
Intestinal	49.0	14	134	
Diffuse	31.5	7	88	
Mixed	14.5	4	40	0.97

	Total %	EBV-positive gastric carcinomas (n=26)	EBV-negative gastric carcinomas (n=276)	*p* value (refers to all the group)
		N	N	
Grade [e]				
G1 and G2	26.4	8	72	
G3	73.2	18	203	0.51
Intestinal metaplasia in adjacent tissues				
no	74.2	19	205	
yes	25.8	7	71	0.52
Atrophy in adjacent tissues				
no	42.7	11	118	
yes	57.3	15	158	0.56

EBV, Epstein-Barr virus.

[a.] smoking status: unknown – 9.6% of all patients, 3 patients in EBV positive and 26 EBV negative group;

[b.] BMI: unknown – 8.3% of all patients, 3 patients in EBV positive and 22 EBV negative group;

[c.] stage: unknown – 3.3% of all patients, 0 in EBV positive and 10 EBV negative group;

[d.] Lauren's classification: indeterminate – 5% of all patients, 1 in EBV positive and 14 EBV negative group;

[e.] grade: unknown - 0.4% of all patients, 0 in EBV positive and 1 EBV negative group.

Table 2.

Associations of personal and tumour-related factors with survival

Variable	Adjusted hazard ratio*	95% CI	P value
EBV- positive status	0.37	0.19; 0.72	< 0.01
Age > 65 years	0.87	0.60; 1.12	0.21
Female sex	0.87	0.62; 1.22	0.42
BMI – underweight[a]	1.92	0.92; 4.02	0.08
BMI – overweight[a]	0.72	0.51; 1.01	0.06
Tumour stage – II[b]	1.15	0.63; 2.12	0.64
Tumour stage – III[b]	2.76	1.67; 4.56	< 0.01
Tumour stage – IV[b]	10.02	5.72; 17.57	< 0.01
Tumour anatomical location - proximal	1.39	0.87; 2.21	0.17
Tumour anatomical location – overlapping and unspecified[b]	1.85	1.14; 3.00	0.01

CI, confidence interval; EBV, Epstein-Barr virus.

[a] normal weight as referent

[b] stage I as referent

[c] distal location as referent