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Epstein-Barr virus in gastric cancer and association with 30 bp del-latent membrane protein 1 polymorphism

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SUMMARY

OBJECTIVE: This study aimed to determine the frequencies of Epstein-Barr virus, types 1 and 2 infection, and 30 bp del-latent membrane protein 1 viral polymorphism in gastric adenocarcinomas, as well as to investigate the association between Epstein-Barr virus infection and tumor location, type, and the patient's sex.

METHODS: Samples were collected from 38 patients treated at a university hospital in Rio de Janeiro, Brazil. Epstein-Barr virus detection and genotyping were performed by polymerase chain reaction, followed by polyacrylamide gel electrophoresis and staining by the silver nitrate method.

RESULTS: Overall, 68.4% of patients had Epstein-Barr virus-positive tumors. Of these, 65.4% presented infection by Epstein-Barr virus type 1, 23.1% by Epstein-Barr virus type 2, and 11.5% had coinfection with types 1 and 2. The 30 bp del-latent membrane protein 1 polymorphism was found in 42.3% of Epstein-Barr virus-positive tumors, 23.1% had the wild-type virus, and 23.1% had the wild-type and the polymorphism concomitantly. In 11.5% of Epstein-Barr virus-positive tumors, it was impossible to determine whether there was polymorphism or not. Tumor location in the antrum (22 of 38) and diffuse type (27 of 38) were predominant. There was no significant difference in Epstein-Barr virus infection or the 30 bp del-latent membrane protein 1 polymorphism between men and women.

CONCLUSION: Epstein-Barr virus infection was found in 68.4% of tumors investigated in this study. To the best of our knowledge, this is the first article showing the coinfection of Epstein-Barr virus types 1 and 2 in gastric carcinoma in Brazil.

KEYWORDS: Stomach neoplasms. Herpesvirus 4, human. Coinfection.

INTRODUCTION

Gastric cancer (GC) is the fifth most common and fourth most lethal malignant tumor worldwide[1]. For 2020, there were an estimated 1,089,103 new cases and 768,793 deaths for GC globally[1]. In Brazil, there were an estimated 21,480 new cases in 2022[2]. In 2020, 13,850 died because of the disease in the country[2]. Several risk factors are associated with GC, including chronic *Helicobacter pylori* infection, family history, diet, alcohol consumption, smoking, and infection by Epstein-Barr virus (EBV)[3]. Nearly 10% of gastric carcinomas are associated with EBV, and the virus infects more than 90% of the global population[4]. EBV is classified into two major types: 1 and 2 (or types A and B) based on differences in viral nuclear antigen (EBNA) genes, especially *EBNA2*, *EBNA3A*, *-3B*, and *-3C*[5]. Type 1 is the most prevalent worldwide. EBV-1 can convert human B-lymphocytes into lymphoblastoid cell lines more efficiently than EBV-2[5]. Several of the EBV-encoded latent proteins are involved in cellular transformation[6]. The latent membrane protein 1 (LMP1), encoded by the *BNLF1* gene, is an essential EBV protein. It can induce phenotypic changes in B-cells and epithelial cells[7,8]. The 30 base pairs (bp) deletion in the third exon of

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BNLF1 (called the 30 bp del-LMP1) causes a loss of ten amino acids. Some studies associate the 30 bp del-LMP1 polymorphism with the oncogenesis of various tumors, including nasopharyngeal carcinoma and GC[8].

This study aimed to determine the frequency of EBV types (1 and 2) and the prevalence of the viral 30 bp del-LMP1 polymorphism in gastric adenocarcinomas in 38 patients treated at a university hospital in Rio de Janeiro, Brazil. Moreover, the study investigated the association between EBV type and the 30 bp del-LMP1 polymorphism with the tumor location, patients' sex, and histological tumor type.

METHODS

This was a hospital-based study. Samples were collected from 38 patients (26 men and 12 women) diagnosed with primary gastric adenocarcinoma at the Hospital Universitário Clementino Fraga Filho, Federal University of Rio de Janeiro, Brazil. The mean age of patients was 63.2±10.8 years. For men, the mean age was 65.9±9.96 years, and the mean age for women was 57.4±9.66 years. After diagnosis, patients underwent surgical resection, performing total or partial gastrectomy. The histological type of tumors was classified according to the Laurén classification. Sample collection occurred between April 2013 and August 2019. The Institutional Research Ethics Committee approved the study (#23511719.0.0000.5257).

DNA extraction: Genomic DNA was extracted from fresh tumor tissue using the phenol:chloroform method, according to previously described by Maccormick et al.[9].

Detection of Epstein-Barr virus DNA: Two regions of the EBV genome were selected for polymorphism analysis by polymerase chain reaction (PCR): the U2 region encoding *EBNA-2* (to recognize type 1 or 2) and a sequence at the exon 3 of the *BNLF1* gene (to detect 30 bp del-LMP1 variant). To analyze *EBNA-2*, the method described by Kunimoto et al.[10] was adapted for multiplex PCR, using two pairs of primers. For type l: forward, 5′-ACAACCACTCATGATGCCAC-3′ and reverse, 5′-ACCGTGGTTCTGGACTATCT-3′. For type 2: forward, 5′-GGTAGCCTTAGGACATACTC-3′ and reverse, 5′-TGGAGGGAGTCCTGTACTAT-3′[10]. PCR conditions were: initial denaturation of 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The final extension was performed at 72°C for 5 min. The products generated were fragments of 240 bp (type 1) or 233 bp (type 2)[10] visualized as DNA bands by electrophoresis in 10% polyacrylamide gel, followed by silver nitrate staining according to previously described by Silva et al.[11]. For the *BNLF1* fragment, the primers used were: forward, 5′-TGGAGGGAGAGTCAGTCAGGC-3′ and reverse, 5′-ATTGACGGAAGAGGTTGAAAAC-3′[12]. Amplification conditions were: initial denaturation of 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min. The final extension was performed at 72°C for 5 min. The products generated were fragments of 254 bp (wild) or 224 bp (deleted), visualized by gel electrophoresis and silver nitrate staining[11].

RESULTS

Demographic and clinical data of patients were collected from medical records and reports.

Table 1 shows the association between patients' characteristics and the presence or absence of tumoral EBV.

Table 2 includes only patients with EBV-positive tumors (n=26), associating the characteristics of patients with the EBV type and 30 bp del-LMP1 polymorphism.

Of the 38 patients included in the study, 23 had died by January 31, 2022. The overall mean survival of patients with EBV-positive tumors was 800.96 days. Furthermore, patients with EBV-negative tumors had an overall mean survival of 1,160.83 days, meaning a difference of 359.87 days.

Table 1. Characteristics of patients with gastric carcinoma and tumoral EBV infection.

Characteristics	Total	EBV-positive (%)	EBV-negative (%)
Number of patients	38	26 (68.4)	12 (31.6)
Sex			
Male	26	17 (65.4)	9 (34.6)
Female	12	9 (75)	3 (25)
Mean age (years)	63.2	62.4	64.9
Tumor location			
Cardia	5	3 (60)	2 (40)
Cardia/body/fundus	1	0 (0)	1 (100)
Antrum	22	16 (72.7)	6 (27.3)
Antrum/body	1	1 (100)	0 (0)
Body	7	4 (57.1)	3 (42.9)
Fundus	1	1 (100)	0 (0)
Cardia/body/fundus/antrum	1	1 (100)	0 (0)
Tumor type[§]			
Intestinal	10	6 (60)	4 (40)
Diffuse	27	19 (70.4)	8 (29.6)
Intestinal+diffuse	1	1 (100)	0 (0)

EBV: Epstein-Barr virus. [§]Tumor type according to the Laurén classification.

Table 2. Characteristics of patients with gastric carcinoma and genotype of EBV-positive tumors.

Characteristics	EBV type (%)			30 bp deletion (%)			
	EBV 1	EBV 2	EBV 1/2	W	D	W/D	ND
Sex							
Male (n=17)	10 (58.8)	5 (29.4)	2 (11.8)	5 (29.4)	8 (47)	2 (11.8)	2 (11.8)
Female (n=9)	7 (77.8)	1 (11.1)	1 (11.1)	1 (11.1)	3 (33.3)	4 (44.4)	1 (11.1)
Age (years)							
≥65 (n=13)	8 (61.5)	4 (30.8)	1 (7.7)	5 (38.5)	5 (38.5)	1 (7.7)	2 (15.4)
<65 (n=13)	9 (69.2)	2 (15.4)	2 (15.4)	2 (15.4)	6 (46.1)	4 (30.8)	1 (7.7)
Tumor type[§]							
Intestinal (n=6)	4 (66.7)	1 (16.7)	1 (16.7)	1 (16.7)	4 (66.7)	1 (16.7)	0 (0)
Diffuse (n=19)	12 (63.2)	5 (26.3)	2 (10.5)	6 (31.6)	7 (36.8)	3 (15.8)	3 (15.8)
Int+Diff (n=1)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Tumor location							
Cardia (n=3)	3 (100)	0 (0)	0 (0)	1 (33.3)	1 (33.3)	0 (0)	1 (33.3)
Noncardia (n=23)	14 (60.9)	6 (26.1)	3 (13)	6 (26.1)	10 (43.5)	5 (21.7)	2 (8.7)

EBV: Epstein-Barr virus (types 1, 2, or 1/2); 30 bp deletion: the 30 bp del-LMP1 polymorphism of the gene that encodes EBV protein LMP1 (latent membrane protein 1); W: wild type; D: 30 bp deletion; W/D: wild type+30 bp deletion; ND: not detectable. §Tumor type according to the Laurén classification. Int+Diff: Intestinal+Diffuse.

Among deceased patients, the mean survival was 264.06 days for those with EBV-positive tumors and 424.43 days for patients with EBV-negative tumors, a difference of 160.37 days. Figure 1 shows the survival curve of patients according to the Kaplan-Meier method.

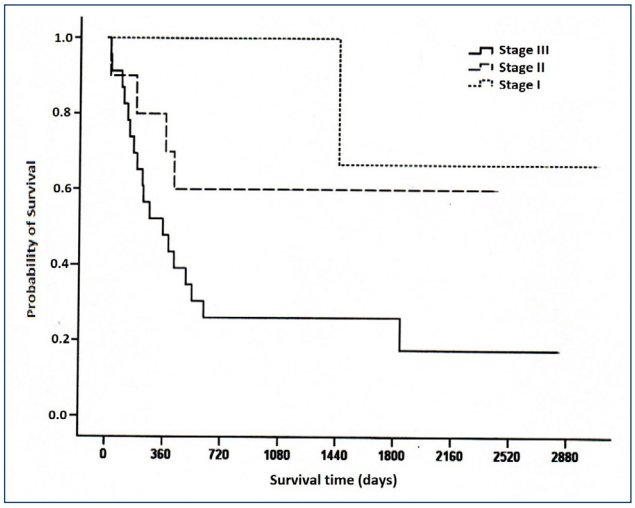

Figure 1. Kaplan-Meier curve correlating tumor staging with survival of gastric cancer patients.

DISCUSSION

Several studies have investigated the frequency of EBV-associated GCs. An investigation from "The Cancer Genome Atlas" network, with 295 tumors from North America, Europe, and Asia, found EBV in 9% of samples[4]. A study from the "Asian Cancer Research Group" with 300 GCs from Asian patients detected the virus in 6.5% of them[4]. In Brazil, research conducted in São Paulo showed 10.5% of EBV-positive among 286 tumors[13]. In contrast, a study from Amazonas, northern Brazil, using the PCR technique found EBV in 80% of the biopsies from 10 patients with GC[14]. Noteworthy is that, in the research from Amazonas, tumor biopsies were pulverized, ensuring the availability of a substantial amount of material for DNA extraction[14]. In our investigation, EBV was detected in 68% of the evaluated GC tumors. The disagreement between this study and some others may be partly explained by the use of different methodologies. Some studies used 5-µm-thick sections of paraffinized tumor tissue to detect viral DNA. On the contrary, we used tumor fragments of at least 1 cm in diameter, substantially increasing the amount of material available to assess the presence of EBV DNA. This difference in tissue collection was possibly an important factor in detecting EBV in 68% of samples. Our research showed that among EBV-positive tumors, 65.4% were of type 1, and 23.1% were of type 2. In 11.5% of the cases, there was coinfection of types 1 and 2. As far as we know, the phenomenon of EBV 1 and 2 coinfections has not yet been reported in GCs among Brazilian patients. The investigated

population had peculiar characteristics, presumably resulting from the Brazilian people's miscegenation. The LMP1 protein has a relevant contribution to cellular proliferation and survival that occur in EBV-associated malignancies[15]. EBV-positive GC tends to have a distinct clinicopathological phenotype compared to EBV-negative tumors. Some studies have indicated that EBV-positive GC was more prevalent in younger patients compared to EBV-negative tumors. Moreover, the disease is more commonly associated with males[16,17], as well as with Caucasians and Hispanics[18]. According to scientific literature, EBV-positive tumors preferentially occur in proximal portions of the stomach, more frequently in the cardia and gastric body, and are associated with diffuse histology[18]. In our study, the mean survival of patients with EBV-positive tumors was considerably shorter than the survival of those with EBV-negative tumors. Regarding overall mean survival, the difference was of 359.87 days. However, this result contradicts what is shown in the scientific literature, which shows a better prognosis for patients with EBV-positive tumors. Nonetheless, the poorer prognosis presented for EBV-positive patients found in our investigation may perhaps be explained by the fact that, in this sample, there were 27 tumors of the diffuse type against only 10 of the intestinal type and one with both. In the literature, it has been reported that diffuse tumors have a worse prognosis than intestinal ones[19,20].

CONCLUSION

The frequency of EBV-positive tumors in this study was 68.4%. The 30 bp del-LMP1 polymorphism was found in 42.3% of EBV-positive tumors. There was no significant difference in the frequency of EBV infection or the 30 bp del-LMP1 polymorphism between men and women. Tumor location in the gastric antrum and diffuse histological type were predominant. As far as we know, this is the first study to show the coinfection of EBV types 1 and 2 in gastric carcinoma in Brazil.

AUTHORS' CONTRIBUTIONS

ERCS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **MSMS:** Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **NHSC:** Investigation, Methodology, Validation, Writing – review & editing. **MFDG:** Conceptualization, Writing – original draft, Writing – review & editing. **ÁLVLB:** Methodology, Validation, Visualization. **WMVS:** Formal Analysis, Writing – original draft. **MGCC:** Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft. **GPBN:** Conceptualization, Data curation, Formal Analysis, Writing – original draft.

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