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Original article

Epstein-Barr virus infection in gliomas

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ABSTRACT

Purpose of the study: Epstein-Barr virus (EBV) has been involved in the development of some tumors, including Burkitt's lymphoma and Hodgkin's lymphoma. However, its potential role in glioma tumorigenesis remains debated. In this study, we investigated the EBV infection in gliomas from Tunisian patients.

Patients and methods: We conducted a retrospective study of 112 gliomas on archival material. The EBV DNA sequence was analyzed by polymerase chain reaction (PCR). Latent membrane protein 1 (LMP1) was detected by immunohistochemistry. *In situ* hybridization was used to detect EBV encoded small RNA (EBER). Clinicopathological features were recorded. Survival analysis was carried out using the Kaplan-Meier method and the Log-Rank test to compare EBV-positive and EBV-negative patients.

Results: Overall, there were twenty-four EBV-positive gliomas (21.4%). EBV DNA was identified in 24 cases. LMP1 and EBER were detected in four EBV DNA-positive cases. All EBV-positive cases were glioblastomas multiforme (GBM). Median overall survival and recurrence-free survival of EBV-negative patients were better than those of EBV-positive patients (Log Rank $p = 0.006$).

Conclusion: Altogether, these findings support the occurrence of EBV infection in Tunisian GBM. Furthermore, when compared to EBV-negative tumors, EBV infection seems to be associated with the worst patient prognosis. Advanced molecular studies are recommended to confirm these results and to shed further light on the potential role of EBV in these devastating tumors.

1. Introduction

Gliomas constitute a diversified group of malignant brain tumors, deriving from glial cells [1]. Based on the World Health Organization (WHO) criteria, gliomas comprise four histological grades (grade I-IV). Grade IV glioma or glioblastoma multiforme (GBM) constitutes the most frequent glial tumor in adults that is associated with the unfavorable prognosis and lower survival rates [1–4]. The etiology of these aggressive malignancies still remains unknown. Nevertheless, some factors have been involved, such as exposure to chemical agents or ionizing radiation and genetic predisposition in GBM patients [5]. More recently, the association of infectious agents with gliomas has been considered [6]. Recent studies reported associations with viral infections since they might be involved in oncomodulation of gliomas [7,8]. Several viruses have been detected in gliomas such as human cytomegalovirus (CMV), human papillomaviruses (HPV) 16 and 18, Epstein-Barr virus (EBV), Kaposi sarcoma-associated herpes virus, hepatitis B and C viruses, human adult T-cell leukemia virus type 1, John Cunningham virus, BK virus and simian virus 40 [6,8–16]. However, there were extensive controversies regarding the correlation between chronic viral infection and primary brain tumors [8,17]. Even if the majority of previous investigations had focused on CMV, more recent attentions have shifted to the involvement of EBV in gliomagenesis [18].

Albeit EBV infection is not generally related to cancer development, EBV is well established to be involved in some tumors such as Burkitt's lymphomas, Hodgkin's lymphomas, peripheral T-cell lymphomas, thymomas, nasopharyngeal and gastric carcinomas [9,19]. In the central nervous system (CNS), EBV is associated with some diseases such as acute cerebellar ataxia, acute encephalitis and meningitis [20].

The EBV, also called human herpesvirus 4 (HHV4), belongs to the herpes family and is present in almost 90% of the world's population [19,21]. Mainly transmitted *via* saliva and genital fluids,

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EBV infection can occur early in childhood and establishes lifelong latent infection, usually asymptomatic [9,22]. EBV has two different life cycles: an acute lytic cycle and a quiescent form. In latency, EBV can persist within B cells, which are the principal targets of EBV due to their expression of CR2, the major cellular receptor for this virus [9,19,22]. EBV may also persist in epithelial cells as well as some T and Natural Killer cells and remains invisible to the defense mechanisms [9]. There are three possible latency programs in these cells (Latency I, II or III). Each latency program leads to certain viral gene products, including the nuclear oncoproteins (EBNA1, -2, -3A, -3B, -3C, and -LP), the latent membrane proteins (LMP1, -2A and -2B) and multitude of EBV non-coding RNAs, such as EBERs and microRNAs (miRNAs) [9,23–26].

Although EBV is frequently present in primary CNS lymphoma diagnosed in immune-deficient subjects, the association of EBV infection with other brain tumors and mainly gliomas remains still debated [11,13,14,27–31]. To further explore the pathogenesis of gliomas, the study investigated the EBV infection in gliomas from Tunisian patients.

2. Materials and methods

2.1. Tissue samples

We performed a retrospective study of 112 specimens of gliomas diagnosed in the Pathology Department of the Farhet Hached University Hospital of Sousse (Tunisia) during 2010-2016. Approval for this study was obtained from the local Human Ethics Committee at the Farhet Hached Hospital and it conformed to the provisions of the Declaration of Helsinki.

The gliomas cases were classified into 12 pilocytic astrocytomas (grade I gliomas), 12 grade II gliomas, 6 grade III gliomas and 82 grade IV gliomas (glioblastoma multiforme (GBM)). Histological diagnosis review of all selected cases was performed by two pathologists (MM and MTY) using Hematoxylin and Eosin (HE) stained sections. All tissues had been routinely fixed in 10% buffered formalin and paraffin embedded. Representative tissue blocks were chosen to confirm that diagnostic tissue as originally reported was adequately represented in remaining tissue blocks.

Patient's clinicopathological data, including patient age at diagnosis, gender, tumor localization and the follow up were collected. Tumor recurrence and patient outcome were registered.

2.2. Epstein-Barr virus (EBV) infection

2.2.1. DNA extraction

DNA extraction was realized using the Qiagen QIAmp DNA FFPE tissue kit (Qiagen, Hilden, Germany), according to the manufacturer protocol. The presence of amplifiable DNA was verified through amplification of a 268-bp sequence of the human β-globin gene using a set of primers as described [32].

2.2.2. Polymerase chain reaction (PCR)

PCR was carried out using a primer set, allowing the amplification of a 175-bp fragment of the EBV BamM region (sense: 5′AACATGCTGTATGCCTCGCAGCG-3′; antisense: 5′AATTACTGGCGTGAATTGTGCCCA-3) [33]. The PCR reactions were performed using 200 ng of DNA template in a total volume of 25 μl as described previously [32]. The reaction mixtures contained 1 U of Taq polymerase (Promega), 1 × PCR buffer (10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl2, pH 8.3), 0.25 mM of each dNTP and 0.2 μM of each primer. The PCR conditions were as follows: 5 min at 95 °C, 35 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C) and a 10 min extension at 72 °C. PCR products were separated on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light [32].

2.2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed on sections of four microns thickness to assess the expression of the EBV latent membrane protein (LMP1) [32]. After tissue section rehydration, the antigen unmasking was carried out in a citrate buffer at 95 °C for 40 min. The activity of endogenous peroxidase was blocked by 3% hydrogen peroxide solution. Slides were then incubated with the LMP1 monoclonal antibody (clone MRQ-47, Cell Marque, USA) at room temperature for 30 min. Immunoreactivity was revealed by using the Envision + Dual Link System HRP kit (Dako, code K4063) and visualized by Diaminobenzidine. Finally, sections were counterstained with hematoxylin. All cases with LMP1-positive tumoral cells were considered EBV-positive.

2.2.4. In situ *hybridization*

In situ hybridization (ISH) was accomplished in 3 μm thick sections of paraffin-embedded tissues using digoxigenin conjugated oligonucleotide probes directed against EBV encoded small RNA (EBER; Dako Cytomation) and ISH kit (NCL-EBV-K; NovoCastra), according to the manufacturer's instructions. The slides were counterstained with hematoxylin, mounted in Faramount (Dako Cytomation) and examined under a light microscope. Positive staining was recognized by a nuclear blue-black color. All cases with EBER-positive tumoral cells were considered EBV-positive [32].

All PCR, IHC and ISH steps were conducted in parallel with positive and negative controls. Positive controls included nasopharyngeal carcinomas that were previously identified as EBV-positive [32]. Evaluation of IHC and ISH findings was independently assessed by two pathologists.

2.3. Statistical analysis

Statistics were analyzed by Chi-square test, using the Statistical Package for Social Science (SPSS) software Version 19.0 (IBM Corp., Armonk, NY, USA). For survival analysis, Kaplan–Meier method and Log-Rank test were used to assess survival time distributions according to EBV status. The probability value (p) < 0.05 was considered as statistically significant.

3. Results

Patient age ranged from 19 to 75 years with a mean age of 53 years. There were 68 male and 44 female. The tumors were diagnosed in the frontal lobe (25%), temporal lobe (17%), parietal lobe (12%), occipital lobe (2.7%) and mixed localizations involving more than one lobe (17.9%). In the remaining gliomas (25.4%), the tumor localization was unspecified in pathological reports.

The EBV infection status was assessed in all glioma samples included. Using EBV-specific PCR, EBV DNA was detected in 24 out of 112 glioma cases (21.4%, Fig. 1). Subsequent IHC showed that four of all gliomas (3.6%) and 16.7% of the EBV DNA-positive cases

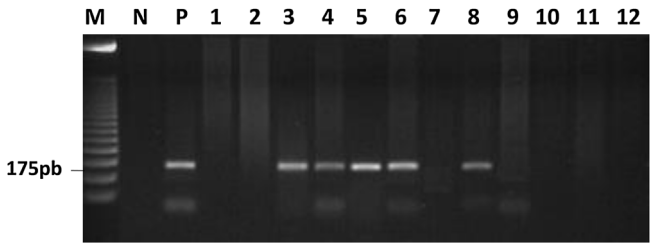

Fig. 1. Detection of EBV DNA (BamHI G region) by PCR in gliomas.
M: molecular weight marker with 50 bp (Promega). **P**: Positive control, **N**: negative control. **1-12**: glioma samples.

exhibited cytoplasmic staining of the EBV LMP1 in tumor cells. Finally, ISH revealed the presence of EBER in the nuclei of tumor cells in four of the EBV DNA-positive cases (16.7%). Representative examples of each assay were shown in Fig. 2.

Overall, the 24 EBV-positive cases corresponded exclusively to GBM, accounting for 29.3% of all GBM cases ($p = 0.0003$). No EBV was detected in the remaining gliomas (grades I-III).

Table 1 listed EBV infection and clinicopathological features of gliomas. EBV infection was more frequently reported in men (62.5%) than in women (37.5%). The median overall survival of EBV-negative cases was longer than that of EBV-positive gliomas (Table 1). In addition, the recurrence-free survival of patients with EBV-positive gliomas was significantly shorter compared with patients with EBV-negative tumors (Log-rank $p = 0.006$, Fig. 3). No other significant correlation was identified between EBV infection and gliomas features ($p > 0.05$ for all, Table 1).

4. Discussion

Over the past decades, the association of EBV has been well defined in B cell lymphomas and nasopharyngeal carcinomas [19,23,24,32]. The presence of EBV infection in gliomas has only recently been explored [10–13]. To the best of our knowledge, this is the first report investigating the EBV infection in gliomas in Africa and Arab world [18].

In this study, 112 Tunisian gliomas were screened for the presence of the EBV BamM region by conventional PCR. Twenty-four cases were associated with EBV infection. As well, previous studies have successfully detected EBV in glioma specimens and results varied considerably depending on geographical locations, populations and techniques used [10–13]. Recently, using multiplex droplet digital PCR, Lin et al. investigated the presence of *LMP1* sequence in 112 gliomas from archived tissues [12]. Only four GBM cases were associated with EBV (21.1%). No EBV was identified in all low-grade gliomas as well as in control specimens [12]. More recently, using RT-PCR and nucleotide sequencing, Strojnik et al. detected *EBNA* gene in three out of 33 GBM from Slovenian patients [10]. No EBV was found in all grade III gliomas [10]. Interestingly, herein, we identified no viral DNA in lower grades of glioma, (grade I-III), suggesting that EBV is preferentially associated with the most aggressive glial tumors. However, all these findings are in contrast to Fonseca et al. study [13]. Using fresh frozen gliomas from a Brazilian cancer center, these researchers screened 75 specimens of different histological subtypes to detect EBV DNA by PCR and direct sequencing [13]. EBV DNA was identified in 11 gliomas, including six low-grade gliomas, two grade III gliomas, one oligoastrocytoma, one ependymoma and only one GBM [13].

LMP1 and EBER are the major EBV oncoproteins actively involved in enhancing cell growth and anti-apoptotic effects [19,22]. In addition to conventional PCR method, we analyzed the presence of EBER by ISH and LMP1 by IHC in all glioma samples. Only four gliomas were positive for EBER and LMP1, all of them were GBM. Similarly, the recent study of Zavala-Vega et al. investigated EBV infection in GBM [11]. Using IHC and ISH on paraffin-embedded tissue samples, these researchers detected LMP-1 and EBER in six GBM, indicating EBV infection in 28.6% of Mexican patients [11]. Using a more sensitive and performed technique, the next generation sequencing (NGS), Cimino et al. identified viral sequences in five GBM (24% of cases) [15]. While, further examination of the EBV-positive tumors by ISH failed to detect EBER, suggesting that EBV might be transcriptionally inactive and more specific of a latent status [15].

Our findings suggested a potential prognostic role of EBV infection in gliomas. In fact, the median overall survival of EBV-negative patients was better than that of EBV-positive subjects. Furthermore, recurrence-free survival of EBV-positive patients was significantly shorter when compared with EBV-negative subjects. Interestingly, the previous study of Vidone et al. demonstrated for the first time the association of HPV with prognosis worsening in GBM patients upon age stratification [16].

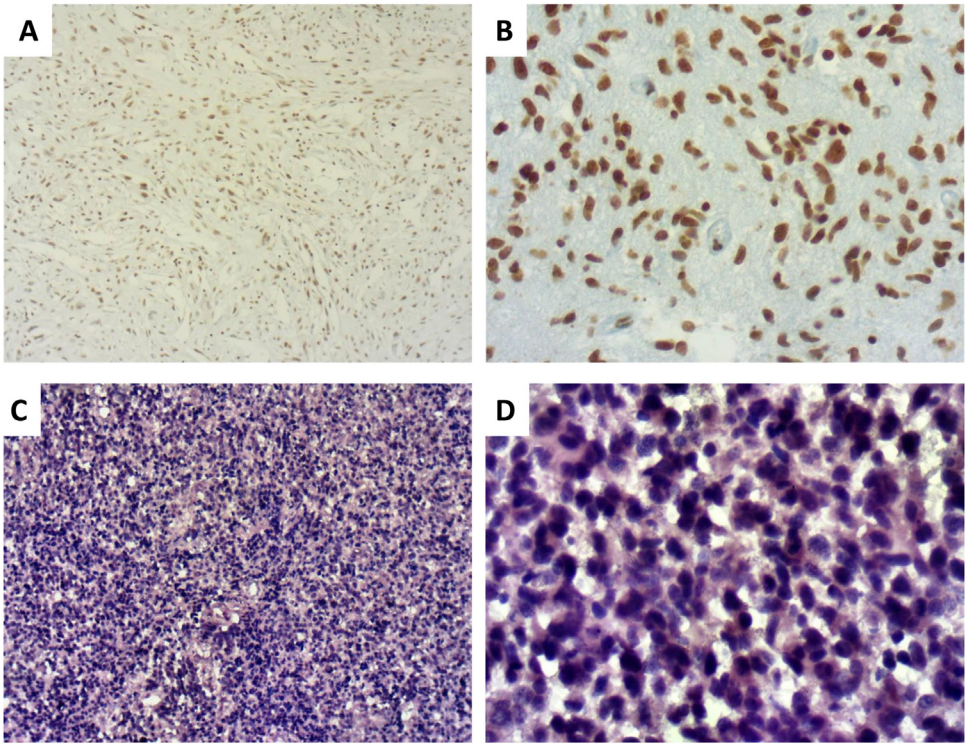

Fig. 2. EBV detection by immunohistochemistry and *in situ* hybridization in gliomas.
(**A-B**) LMP1 immunostaining in GBM (**A**: Mx200, **B**: Mx400). (**C-D**) EBER detection by *in situ* hybridization in GBM (**C**: Mx200, **D**: Mx400).

Table 1
Epstein-Barr virus (EBV) infection and clinicopathological features of gliomas.

	n (%)	EBV-positive (n = 24)	EBV-negative (n = 88)	*p*- Values
Age (years)				
19–35	18 (16.1%)	5 (20.8%)	13 (14.8%)	$p = 0.135$
36–55	71 (63.4%)	9 (37.5%)	62 (70.4%)	
>55 years	23 (20.5%)	10 (41.7%)	13 (14.8%)	
Gender				
Male	68 (60.7%)	15 (62.5%)	53 (60.2%)	$p = 0.231$
Female	44 (39.3%)	9 (37.5%)	35 (39.8%)	
Glioma grade				
I	12 (10.7%)	0	12 (13.6%)	**$p = 0.0003$**
II	12 (10.7%)	0	12 (13.6%)	
III	6 (5.4%)	0	6 (6.8%)	
IV	82 (73.2%)	24 (100%)	58 (65.9%)	
Tumor localization				
Frontal	28 (25%)	11 (45.8%)	17 (21.6%)	$p = 0.361$
Temporal	19 (17%)	3 (12.5%)	16 (18.2%)	
Parietal	14 (12.5%)	5 (20.8%)	9 (10.2%)	
Occipital	3 (2.7%)	2 (8.3%)	1 (1.1%)	
Mixed	20 (17.9%)	3 (12.5%)	17 (21.6%)	
Unspecified	28 (25%)	0	28 (31.8%)	
Tumor recurrence				
Presence	29 (25%)	7 (29.2%)	22 (25%)	$p = 0.311$
Absence	83 (75%)	17 (70.8%)	66 (75%)	
Median overall survival	67 (±14) months	19 (±3) months	89 (±19) months	**$p = 0.006$**

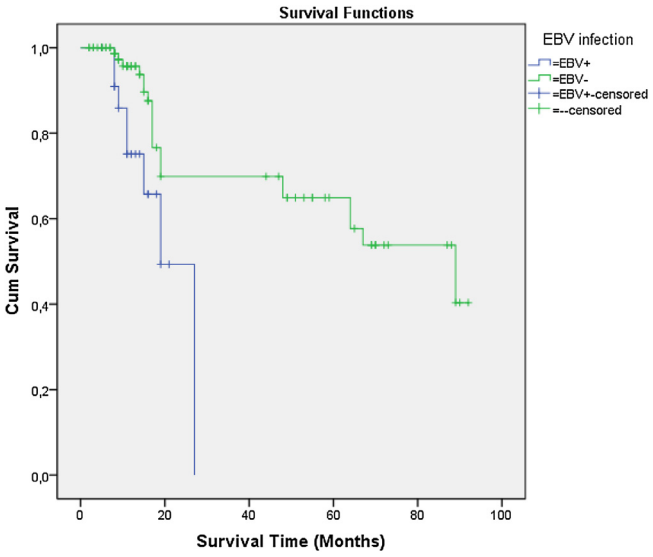

Fig. 3. Kaplan-Meier analysis of recurrence-free survival, according to EBV infection in Tunisian gliomas (Log Rank $P = 0.006$).

To further explore the involvement of EBV in glioma progression, some previous studies evaluated EBV serology in affected patients with controversial results [10,29,34]. Recently, no EBV was found in plasma of 45 patients with high-grade gliomas by Strojnik et al. [10]. However, earlier reports identified EBV antibodies in almost 90% of GBM patients and 89% of patients with glial tumors [34,36].

Nowadays, it is well known that EBV encodes for several miRNAs [23–26,35]. The expression of EBV miRNAs varies according to lymphoid and epithelial cells and viral latency programs. The BHRF1 miRNAs were overexpressed in cells undergoing type III EBV latency, including lymphobastoide cell lines as well as EBV-infected B-cell tumors. Additionally, BART miRNAs were identified mainly in epithelial cells displaying type II EBV latency, such as nasopharyngeal and gastric tumor cells [36–38]. In addition, some studies have reported the presence of EBV miRNAs in gliomas [39]. Herman et al. identified six EBV miRNAs, including miR-BART2-3p, miR-BART2-5p, miR-BART6-3p, miR-BART9, miR-BART15, and miR-BHRF1-3 in blood plasma of GBM subjects [39].

Nonetheless, some other studies have neglected any EBV presence in gliomas [14,17,27,40]. Using semi-quantitative PCR, Cosset et al. failed to detect EBV DNA in all 20 GBM biopsies and in the corresponding patient serum as well as in the remaining studied cases, including three low-grade gliomas, one oligodendroglioma, two meningiomas, one ependymoma, and one oligoastrocytoma [27]. Similarly, no evidence of DNA virus transcripts was described in low and high-grade gliomas by Khoury et al. [17]. Using real-time PCR analyses, Hashida et al. failed to detect as well *LMP1* gene in GBM from Japanese patients [14]. More recently, using NGS technique, Strong et al. neglected any major viruses could be associated with GBM [40]. Moreover, the authors considered that the detected EBV reads likely represented artifacts that were probably originated from infiltrating EBV-infected B-cells and/or from library or sequencing sample cross-contamination [40].

The observed discrepancies in EBV findings in gliomas could be caused partially by population or geographic differences, individual genetic variability, inherent heterogeneity of gliomas, differences in the actual viral genes probed, as well as methods sensitivity and precision [18,41,42]. In addition, differences in sampling, processing or preparation of specimens as well as difficulties with archived tissues may explain the discordant findings on EBV infection [18,41,42].

In conclusion, our findings support the EBV occurrence in only GBM specimens suggesting thereby their probable role in the oncogenesis and progression of these aggressive tumors. Furthermore, when compared to EBV-negative samples, EBV infection seems to be associated with the worst prognosis. The insight of the concept of EBV miRNAs in modulating the activity of cellular genes may ameliorate our understanding of the probable implication of EBV in gliomagenesis. Hence, more detailed and extensive molecular analyses are warranted to more explore the possible role of EBV in these malignancies, leading probably to new approaches for the management and treatment of these fatal tumors.

Author contributions

Study concept and design was done by SL and NM. Clinical data were provided by SM, HK and MM. Surgical pathological data were acquired by MTY and MM. The data were analyzed and interpreted by SL, NM and MM. Drafting of the manuscript and critical revisions of the manuscript for important intellectual content were done by all authors. Administrative, technical, or material support was provided by HK, BS and MM. The study was supervised by MM.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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