

Epstein-Barr Virus Nuclear Antigen-2 Detection and Typing in Immunocompromised Children Correlated With Lymphoproliferative Disorder Biopsy Findings

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Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis, plays a significant role as a cofactor in the process of tumorigenesis, and has consistently been associated with a variety of malignancies especially in immunocompromised patients. Forty-four children and adolescents (21 liver transplant patients, 7 heart transplant, 5 AIDS, 3 autoimmune hepatitis, 2 nephritic syndromes, 2 medullar aplasia, 2 primary immunodeficiency disorder patients, 1 thrombocytopenic purpura and 1 systemic lupus erythematosus) presenting with chronic active EBV infection (VCA-IgM persistently positive; VCA-IgG > 20 AU/mL and positive IgG – EBNA) had peripheral blood samples obtained during clinically characterized EBV reactivation episodes. DNA samples were amplified in order to detect and type EBV on the basis of the EBNA-2 sequence (EBNA2 protein is essential for EBV-driven immortalization of B lymphocytes). Although we have found a predominance of type 1 EBNA-2 virus (33/44; 75%), 10 patients (22.73%) carried type 2 EBNA-2, and one liver transplant patient (2.27%) a mixture of the two types, the higher proportion of type 2 EBV, as well as the finding of one patient bearing the two types is in agreement with other reports held on lymphoproliferative disorder (LPD) patients, which analyzed tumor biopsies. We conclude that EBNA-2 detection and typing can be performed in peripheral blood samples, and the high prevalence of type 2 in our casuistic indicates that this population is actually at risk of developing LPD, and should be monitored.

Key-Words: EBVA, EBNA-2, infectious mononucleosis, lymphoproliferative disorder, EBV genotyping, EBV-PCR.

Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis, plays a significant role as a cofactor in the process of tumorigenesis, and has consistently been associated with a variety of malignancies including endemic Burkitt's lymphoma, nasopharyngeal carcinoma, certain B and T-cell lymphomas, approximately 50% of Hodgkin's disease, different types of gastric carcinomas, and other lymphoproliferative diseases [1]. Immunosuppressive drugs such as cyclosporine A and tacrolimus seem to act synergistically with EBV as tumor inducing factors [2].

EBV infection of primary B cells results in the establishment of a latent infection, in which only 11 of potentially more than 80 viral proteins are expressed. These gene products include EBV nuclear antigens EBNA 1, 2, 3A, 3B, 3C and 5, latent proteins (LMP) 1, 2A and 2B, and small non coding RNAs (EBER 1 and 2). The Epstein-Barr virus EBNA-2 protein is a transcriptional activator that regulates viral latent genes and cellular gene expression, and is also essential for EBV-driven immortalization of B lymphocytes [3,4]. EBNA-2 is critically required for B-cell transformation, as the P3HR1-derived EBV strain, which lacks EBNA-2, is deficient in this capacity, and the transforming potential is reconstituted with EBNA-2 expression [5,6].

EBV type 1 and type 2 (formerly called type A and B), appear to be mainly caused by divergence within the EBNA-

2 protein, because EBV type 2, when compared to type 1, has a lower transforming efficiency and a poorer initial outgrowth [7,8]. Both EBV types occur worldwide, with different geographical distributions. In general, EBV type 1 is the predominant type in lymphatic cells of immunocompetent, infected individuals. However, in certain T-cell deficient hosts, especially those with acquired immunodeficiency syndrome, there are increased infection rates with EBV type 2 [9,10].

Persistent infection with more than one EBV type or strain is not unusual, particularly in immunocompromised patients. Intertypic recombinants have been identified in the oropharynx and lymphocytes of immunodeficient patients, and less commonly from healthy people. Scientific evidence suggests that some immune impairment promotes co-infection with multiple strains of EBV, and fosters the development of intertypic recombinant viruses [10]. Human immunodeficiency virus type 1-infected patients develop a number of lymphoproliferative disorders ranging from non neoplastic lymphadenopathies to B-cell lymphomas [11]. Different point mutations in the EBNA-2 gene were described in some EBV type-1 isolates from immunodeficiency virus-infected patients, but not from immunodeficiency virus-negative subjects [12].

Although a large amount of *in vitro* scientific evidence concerning the differences of EBNA-2 type 1 and type 2 is available, little is known about whether both strains really differ in their biological activities *in vivo*. Aitken et al. [13] found EBNA-2 sequence heterogeneity in EBV type-1 isolates from African and New Guinean endemic Burkitt's lymphoma cases, suggesting that variant EBV type-1 sub-strains may be associated with these tumors.

Human EBV establishes latency and promotes the long-term survival of its host B cell by targeting the molecular machinery controlling cell fate decisions. EBNA-2 is a key determinant in the EBV-driven B-cell growth transformation

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process. By activating the array of viral and cellular target genes, EBNA-2 initiates a cascade of events which ultimately cause cell cycle entry and the proliferation of the infected B cell [14]. The mechanism of transcriptional activation by EBNA-2 also involves phosphorylation of the C-terminal domain (CTD) of RNA polymerase II [15]. It was also established that somatic hypermutation and class-switch recombination in germinal centers critically depend on activation-induced cytidine deaminase (AID). Dereglulation of AID may lead to the aberrant activation or persistence of both genetic processes, thus contributing to the pathogenesis of B-cell lymphomas by mistargeted mutagenesis or recombination [16]. The EBNA-2 also up-regulates *bfl-1*, an anti-apoptotic gene which confers protection from apoptosis under conditions of growth factor deprivation [17].

Taken into account that conventional diagnostic tools are rarely useful in evaluating EBV-related disorders in immunodeficient patients, polymerase chain reaction (PCR) offers the possibility of rapid and accurate EBV detection and typing in patients at risk of developing lymphoproliferative disorders, allowing a better understanding of the different pathogenic potentials of the two main viral types [18]. Another interesting point is to determine whether peripheral blood leukocytes could be used as the primary source of DNA instead of biopsy specimens, which would allow pediatricians to monitor immunocompromised patients with chronic-active infections by means of a less invasive procedure, as soon as EBV detection takes place. Therefore, we aimed at detecting and typing the EBV EBNA-2 gene in a group of immunocompromised children and adolescents by analyzing peripheral blood samples.

Material and Methods

This study was approved by the Ethics Committee of the "Faculdade de Medicina da Universidade de São Paulo", Brazil. After informed consent, we analyzed peripheral blood samples of 44 immunocompromised patients presenting with chronic-active EBV infection. These patients, 24 boys and 20 girls, from 5 to 14 years old, were assisted at the Children's Institute: 21 with liver transplants, 7 with heart transplants, 5 with AIDS, 3 with autoimmune hepatitis, 2 with nephritic syndromes, 2 with medullar aplasia, 2 with primary immunodeficiency disorders, 1 with thrombocytopenic purpura and 1 with systemic lupus erythematosus. All 44 cases were diagnosed as bearing chronic-active EBV infection on the basis of serological results: VCA-IgM persistently positive; VCA-IgG > 20 AU/mL and positive for IgG – EBNA. IgG antibodies to VCA were determined by capture ELISA (Diasorin, Italy); VCA-IgG positive > 20 AU/mL and VCA-IgM positive (qualitative test); IgG to EBNA (nuclear antigen) was determined by ELISA (Sanofi Diagnostics Pasteur, France), samples being classified as acute EBV infection (positive for IgM and IgG to VCA but negative to IgG-EBNA), post-acute EBV infection or carrier state (positive for IgG to VCA and IgG – EBNA).

DNA Extraction from Whole Blood

Two milliliters of whole blood (EDTA, Becton Dickinson) was drawn from patients after informed consent of parents. DNA extraction was performed according to a previously described salting-out protocol [19]. DNA concentration was estimated by UV spectrophotometry at 260 nm (DU-70, Beckman).

Amplification and Detection

Before EBV specific amplifications, all DNA samples were tested with primers to amplify a fragment of the human beta-actin gene (sense 5'-GTGGGGCGCCCCAGGCACC-3' and anti-sense 5'-CTCCTTATTGTCACGCACGATTTTC-3') in order to ensure DNA integrity and absence of amplification inhibitors. PCR assays were performed in 50 µL of total volume containing approximately 100 ng genomic DNA, 100 mM Tris-HCl, 500 mM KCl; 2.5 units Taq DNA polymerase (Amersham, Biotech), 200 µM dNTP (Invitrogen, USA), 1.5 mM MgCl₂; 0.4 µM each primer. In the first round of amplification, primers E2p1 and E2p2 aimed at amplifying a fragment of 596bp covering almost the entire EBNA2 gene extent (E2p1: 5'-AGGGATGCCTGGACACAAGA-3' and E2p2: 5'-TGGTGCTGCTGGTG GTGGCAAT-3'). In the second round of amplification, the pair of primers Ap1 and Ap2 aimed at amplifying a 497 bp fragment characterizing the EBV type 1 EBNA2 gene product, while primers Bp1 and Bp2 amplified a 150 bp fragment specific to the EBV type 2 EBNA2 gene product (Ap1: 5'-TCTTGATAGGGA TCCGCTAGGATA-3'; Ap2: 5'-ACCGTGGTTCTGGACTATCTGGATC-3'; Bp1: 5'-CATGGTAGCCTTA GGACATA -3'; Bp2: 5'-AGACTTAGTTGATGCCCTAG-3') [18]. All amplifications (beta-actin and EBV specific) were carried out as follows: after an initial denaturation step of 5 min. at 95°C, 40 cycles of amplification were performed in either the first, or the second round of amplifications (MJ Research, PT-150 minicycler). Cycles consisted of 1 min. at 95°C, 1 min. at 55°C and 1 min. at 72°C, followed by a final extension step of 5 min. at 72°C. Afterwards, 10 mL of amplification products were analyzed using horizontal electrophoresis (Horizon H-58, Life Technologies, USA), in 2% ethidium bromide-stained agarose gel (Sigma, USA). In each experiment, a negative control containing sterile water instead of genomic DNA, or DNA from a non infected individual was tested, as well as a positive control (EBV DNA from viral cultures).

Results

All 44 DNA samples were successfully amplified by the human beta-actin primers thus confirming integrity of DNA samples, and the absence of PCR inhibitors. The first round of amplification was followed by the second PCR with EBNA-2 type 1 and type 2 specific primers (Figure 1). From a total of 44 samples, there were 33 type 1 EBNA-2 virus (33/44 or 75.0%), 10 type 2 (10/44 or 22.73%), and one DNA sample belonging to a liver transplant recipient patient which amplified type 1 and type 2 at the same time (01/44 or 2.27%). Table 1 summarizes PCR detection and typing and the patients' underlying disease.

Figure 1. Electrophoresis of the nested-PCR products. **A-** First round (external primers E2p1 and E2p2). M - molecular weight marker - 100 bp (Amersham); lanes 1A, 3A, 5A and 7A – negative controls (sterile water instead of DNA); lane 2A- patient 1, lane 4A- patient 2; lane 6A- positive control (EBV-DNA). **B-** Second round (EBV type 1 and 2 specific primers, Ap1-2 and Bp1-2, respectively). M - molecular weight marker - 100 bp (Amersham); lane 1B - negative control (sterile water instead of DNA); lanes 2B and 3B – Patient 1; lanes 4B and 5B – Patient 2; lane 6B - positive control (EBV type 1); lane 7A- positive control (EBV type 2).

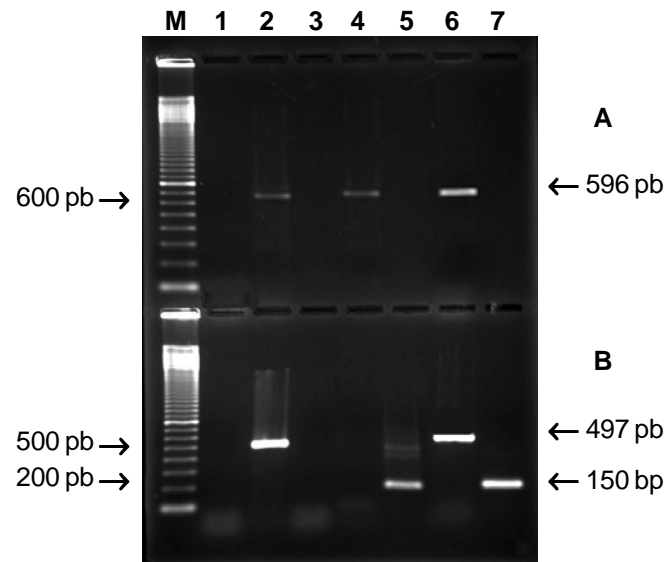


Table 1. Description of patients according to the underlying disease and the EBNA-2 typing by PCR.

Disease	N	Type 1	Type 2	Type 1 and 2
Liver transplantation	21	15	5	1
Heart transplantation	7	6	1	0
AIDS	5	2	3	0
Autoimmune hepatitis	3	3	0	0
Nephritic syndrome	2	2	0	0
Medullar aplasia	2	2	0	0
Primary immunodeficiency	2	2	0	0
Thrombocytopenic purpura	1	1	0	0
SLE	1	0	1	0
Total	44	33	10	1

Discussion

In Brazil there are three reports on the detection and typing of EBV in pediatric patients. Araújo et al. [20] found type 1 EBV in the majority of Brazilian Burkitt's lymphoma cases: 20/30 type 1, 7/30 type 2 and 3 samples were not amplifiable. More recently [21], the same group studied the phenotype and the expression of EBV gene products in 90 classical Hodgkin and non Hodgkin's disease pediatric cases by immunohistochemistry, and *in situ* hybridization. They found that EBV infection was present in 86.7% of patients, but exclusively in classical Hodgkin's disease biopsies, although associated with all EBV subtypes. Hassan et al. [22] compared an EBV-RNA (EBER) *in situ* hybridization assay with a nested-

PCR that aimed at typing the EBNA-2 gene in 41 children presenting with B-cell non Hodgkin's lymphoma, including 35 Burkitt's lymphomas. They successfully detected EBV genomes in 68% of biopsies, and type 1 and type 2 EBV accounted for 80 and 20% of the samples, respectively. PCR and *in situ* hybridization were concordant in 95% of results.

We studied 44 children and adolescents presenting with severe underlying diseases and receiving immunosuppressive therapy. However, we did not investigate any patient with overt lymphoproliferative disorders. Nevertheless, our findings corroborate those of Hassan et al. [22] as we found very similar distribution of type 1 and 2 EBNA-2: 75% of type 1; 22.73% of type 2 and 2.27% of simultaneous infection (type 1 and type 2).

Norin et al. [23] studied the incidence of lymphoproliferative disorders (LPD), in a series of 500 liver transplantations. They found that the rate varied from 1 to 10% depending on the type of organ transplanted, and the immunosuppressive regimens used. They concluded that post transplant LPD constitute a significant cause of complication. In the present study, there was a predominance of type 1 EBNA-2 EBV among the 21 liver transplant patients studied (15/21 or 71.43%), with type 2 found in 5/21 patients (23.81%), and both types in one patient (1/21 or 4.76%). Whether EBV typing will be useful in preventing, and promoting early diagnosis of LPD in these patients has yet to be verified.

EBV causes a wide spectrum of diseases in the setting of immunodeficiency including primary or secondary (acquired) immunodeficiencies, which have been increasingly reported [24]. The major clinical phenotype is the EBV genome-positive LPD, which ranges from benign lymphoproliferation to malignant lymphoma with chromosome alterations. Severe or fatal infectious mononucleosis may develop in some patients with immunodeficiency disorders such as X-linked diseases. Canioni et al. [25] evaluated distinctive features of LPD in a group of 18 primary immunodeficiency patients and compared the findings with those of 10 post transplant children. They concluded that LPD is much more pleomorphic in primary immunodeficiency patients compared to post transplant recipients, even if some LPD are similar in both groups. A low T-cell count and abnormal T-cell function indicated bad prognosis in both groups. Polymorphic LPD were most frequent (n=19), whereas lymphomas were rare (n=7), and pseudo-tumoral lymphoid hyperplasia (n=2) was only observed in the group of primary immunodeficiency patients. Several reports have demonstrated that the responses of B-cells to the Epstein-Barr virus are highly heterogeneous in common variable immunodeficiency. Kondo et al. [26] have studied patients with selected primary immunodeficiency disorders (Bloom's syndrome, Wiskott-Aldrich Syndrome, IgA deficiency). In Bloom's Syndrome, a mild B-cell function abnormality was found, while in the Wiskott-Aldrich's patients responses were variable. In a patient with IgA deficiency, peripheral blood mononuclear cells responded adequately to EBV in terms of proliferation, but poorly in IgA production, suggesting an abnormality only in the IgA production mechanism. Unfortunately, none of the mentioned studies have determined EBV type 1 and type 2 prevalence. In our casuistic, we have included only two patients with primary autoimmune deficiency, and in both cases the classical EBNA-2 type 1 was found. EBNA-2 and LMP1 gene analysis of 48 LPD from HIV-1 patients evidenced EBNA-2 transcripts in all tumors, 50% of samples were co-infected with both EBV type 1 and 2 strains and/or a multitude of type 1 variants, with predominance of type 1 in large cell lymphomas, and type 2 in Burkitt-like tumors [11]. In our study, the five AIDS patients included did not present with LPD, but were undeniably at-risk patients. Although we observed a predominance of type 2 virus (2 type 1, and 3 type 2 EBNA-2), further studies with more AIDS

patients are needed to confirm this finding. Furthermore, we did not find any AIDS patient presenting with both EBV types. Kanegane et al. [27] have associated cases of acute idiopathic thrombocytopenic purpura with primary EBV infections. More recently, Yenicesu et al. [28] conducted a retrospective study based on the medical notes of pediatric patients with idiopathic thrombocytopenic purpura in whom EBV, CMV and rubella virus serology were performed, and viral infection was detected in 13.3% of cases. These studies did not analyze the EBV infection at the molecular level. In our study group, only one patient with idiopathic thrombocytopenic purpura was included, and had a type 1 EBV virus.

EBV infection can cause a number of renal disorders ranging from microscopic hematuria, to acute renal failure. Membranous nephropathy is an uncommon and usually secondary cause of nephritic syndrome in children, and has been reported after chronic EBV infections with persistent antigenemia. Araya et al. [29] reported two pediatric cases of secondary membranous nephropathy associated with acute and chronic EBV infection. The association of EBV with renal histological changes consistent with membranous nephropathy has been previously suggested, but not directly described. In the study performed by Araya et al. [29] EBV infections were not detected and typed by PCR, so that they cannot state what type of EBV was at the origin of infections. In our study, the two patients with nephritic syndrome analyzed had type 1 EBNA-2 virus. In addition, to examine the possible involvement of EBV in the etiology of renal cell carcinoma (RCC), 9 RCC, 2 nephroblastoma (Wilm's tumor), and 2 RCC cell lines were subjected to mRNA in situ hybridization and indirect immunofluorescence staining. It was found that EBV infected all the RCC and nephroblastoma patients irrespective of the histological or clinical stage. The results also suggest that EBV expression may be involved in the pathogenesis of RCC and nephroblastoma [30].

To investigate the possibility that Epstein-Barr virus might play a role in systemic lupus erythematosus (SLE), EBV was searched for by PCR and by culture isolation in oropharyngeal lavage fluids of 15 SLE patients, of 13 acute infectious mononucleosis patients, and of 28 healthy individuals. EBV type 1 DNA was demonstrated by PCR in the oropharyngeal secretions of 8 SLE patients, and the virus was isolated from 6 DNA-positive specimens. Moreover, 50% of the SLE patients, and 100% of the infectious mononucleosis patients, but none of the EBV-seropositive normal individuals, produced IgG antibodies raised against synthetic EBNA-2 peptides used in the study. The authors concluded that EBV may establish a persistent infection, at least in a certain number of SLE patients [31]. We have analyzed only one DNA sample from a SLE patient undergoing prolonged immunosuppressive therapy, and experiencing an EBV reactivation episode at the time of blood sampling. The nested-PCR to type the EBNA-2 gene found a type 2 virus in this patient. Again, it is premature to affirm the predominance of a certain subtype of EBV based on one SLE case, but it is important to emphasize that in the

totality of patients included in the present study, it was possible to detect and type EBV using DNA extracted from peripheral blood samples drained during clinically characterized reactivation episodes.

Our nested-PCR technique has successfully detected and typed all 44 samples included in the present study probably because all patients were correctly diagnosed as having chronic-active EBV infection, and the samples were drawn from patients during reactivation episodes. Kunimoto et al. [32] have used a one-step amplification targeting the gene encoding the EBNA-2 nuclear antigen. EBV was detected in mouth washings of 21 (23%) out of 91 seropositive healthy adults. Twenty samples (22%) contained type 1, and only one (1%) type 2. Seventy-nine patients with various types of tonsillitis were also studied. EBV was detected in mouth washings of 37 patients (47%): 34 (43%) were type 1, and 3 (4%) were type 2. Double infection was not seen in either group. They concluded that EBV type 1 was the dominant type in Japan, and that the type 2 variant was quite rare. More recently, Peh et al. [33] aimed at comparing the performances of a one-step and a nested-PCR to detect the EBNA-2 gene. They studied 38 paraffin-embedded tissue lymphomas and 14 nasopharyngeal carcinomas, along with 12 reactive lymph nodes and tonsils as normal controls. All cases showed presence of type 1 (called type A) virus, consistently detected by nested-PCR, but not with the one-step amplification. They did not find any type 2 (called type B) samples, or mixed infection in the casuistic. They concluded that the nested-PCR technique used had successfully increased the sensitivity of EBV detection and typing, and type 1 EBV was the prevalent strain associated with human diseases in Malaysia.

In our casuistic we found a predominance of type 1 EBNA-2 virus among the 44 samples studied, corresponding to 75.0% of the total. However, 22.73% of samples carried type 2 EBNA-2 virus, and 2.27% a mixture of the two types. The fact that the casuistic was composed of immunocompromised children and adolescents could account for the higher proportion of type 2 EBV with respect to studies conducted in healthy populations. When these results are compared with those of LPD bearing patients, the prevalence of type 1, type 2, and both types, are similar, indicating that our population is actually at risk of developing LPD.

Strategies for the prophylaxis and treatment of these potentially life-threatening complications of EBV infection have advanced dramatically. They include immunological-based approaches targeted at EBV-infected cells, as well as improvement in the treatment of the underlying and predisposing diseases [34]. EBV detection and typing might also contribute to these strategies at a diagnostic and prognostic level.

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