ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

The prognostic role of EBER in pediatric cancer

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Abstract
The Epstein–Barr virus (EBV) infection is an endemic disease, over 90% of the population being exposed to it by adulthood. EBV is implicated in the etiology of a significant number of neoplasms, which acquire particular features in terms of course and prognosis. Incidence rates are much higher in children. To establish the link between EBV and neoplasms, EBER (Epstein–Barr virus non-encoded RNAs) needs to be highlighted in tumor tissue. The role of EBV in patient response to oncological treatment remains controversial.

Keywords: EBER, cancer, children, chemotherapy.

Introduction

The incidence of pediatric cancer is continuously growing and contributes, from an epidemiological standpoint, to the high incidence of the disease worldwide. In 2012, GLOBOCAN estimated to 14.1 millions the number of annual new cases, to 8.2 millions the number of annual cancer-related deaths and to 32.6 millions the number of cancer patients for whom less than five years have passed since diagnosis/treatment [1].

The growing incidence of pediatric cancer depends on the age of onset of the disease, some studies estimating the growth rate to be much lower. However, an extremely important difference between pediatric and adult cancer is made by prognosis. An Italian study assessing data from 31 cancer registries for 2003–2008 revealed that the mortality rate in that timeframe was three times lower than in the 1970's [2].

The multifactorial etiology of cancer poses significant problems in terms of screening, early diagnosis or effective treatment. The continuous phenotypic change of cancer cells in their effort to adapt to the aggression of their host's immune system or of the oncological treatments administered represents an extremely important cause of treatment failure.

The Epstein–Barr virus (EBV) is representative for a virus group that is extremely widespread in the general population, over 90% of the individuals developing antibodies before reaching adulthood. The initial infection is asymptomatic in most of the cases, which allows for a lifelong persistent latent infection to settle in the host's body. Expression of a true arsenal of proteins with extremely diverse roles, it ensures cell proliferation and the survival of the virus. In the latency period, expressed proteins ensure the proliferation of cells responsible for preserving the viral reservoir, keeping it "invisible" to the immune system and infecting new cells or hosts [3]. The factors associated with EBV reactivation or dramatic clinical presentations, which may culminate in patient death, remain unknown. EBV has the versatile capacity of infecting a wide variety of cell types: T-cells, B-cells, natural killer (NK), epithelial cells and muscle cells [4].

EBV is considered by *World Health Organization* (WHO) to be a class I carcinogenic agent and it is associated in terms of etiologic factors with nasopharyngeal cancer, Hodgkin lymphoma, non-Hodgkin lymphoma – Burkitt lymphoma. The tumor spectrum in which its involvement is identifiable is constantly changing, the latest sites referring to stomach and breast cancer [5, 6]. It is one of the largest viruses with double-stranded linear DNA genomes, encoding over 100 genes, most of whom are dormant during the latency period. During the latent infection stage, some of them encode important proteins such as EBV nuclear antigen (EBNA) 1, 2, 3A, 3B, 3C and EBNA-leader protein (EBNA-LP), latent membrane proteins (LMP) 1, 2A, 2B [7]. The EBV genome also contains two small EBER (Epstein–Barr virus non-encoded RNAs), EBER-1 and EBER-2 [7]. EBER is the sole gene expressed in all types or latency stages [8].

EBV may be considered the causative agent of a malignancy provided that the presence of viral genes or gene products in the tumor tissue is demonstrable. Given its constant presence, EBER is an ideal candidate in proving the viral etiology of EBV for some malignancies. Demonstrating the viral etiology of a malignancy may have a practical relevance through the potential screening of the oncological treatment administered: the viremia level or other proteins expressed by the viral infection may become therapeutic targets. The presence of EBER in tumor tissue is determined through *in situ* hybridization, the gold standard in place. Among all EBV genes, EBER is the most intensely expressed in EBV-induced tumors [9].

The pro-tumor role of EBER has not been fully clarified

yet. It appears that, in gastric cancer, EBV stimulates the insulin-like growth factor (IGF-1), an autocrine factor, which accelerates tumor growth [10]. On the other hand, the presence of EBV though LMP-1 increases cancer cell resistance to Fluorouracil (5FU)- and Taxotere-based chemotherapy [10]. Another mechanism consists in stimulating IL-10 secretion, thus favoring cell multiplication [11]. It appears that EBER inhibits apoptosis by blocking the protein kinase phosphorylation and it confers resistance to the pro-apoptotic effects of IFN-γ [11, 12]. Moreover, the detachment of EBV-infected cells in the EBER interstitial environment stimulates the production of type 1 IFN and of proinflammatory cytokines, responsible for the presence of B-type clinical symptoms in lymphoproliferative disorders [11].

Working hypothesis. Goals

In patients with EBV-induced neoplasms, the natural history and the prognosis of the disease are different. The immunosuppression induced by the tumor itself or by the treatment administered may be accountable for the viral reactivation of EBV. This can be objectivized by determining the anti-viral capsid antigen immunoglobulin G titers (anti-VCA IgG) and viral DNA in the blood. The pre-therapeutic anti-VCA IgG titer may be considered an independent tumor prognostic factor, as in the case of nasopharyngeal carcinoma [13]. The sensitivity and specificity values of EBV DNA appear to be higher than those of anti-VCA Ig in cancer diagnosis [14].

EBV DNA serum is a marker of viral replication correlated with viral load. Published studies also revealed that the EBV DNA level in cancer patients and their response to treatment are directly proportional [15]. Still, EBV DNA cannot replace EBER in identifying the viral etiology of a neoplasm [16].

The goals of this study were to assess possible correlations between the anti-VCA IgG titer (serological marker for EBV infection intensity) and the presence of EBER in tumor tissue and also to evaluate the prognostic role of EBER in the response treatment of pediatric neoplasms.

Materials and Methods

A retrospective study including 20 children monitored either by the "Prof. Dr. Ion Chiricuţă" Oncological Institute of Cluj-Napoca or by the Pediatric Emergency Hospital of Cluj-Napoca (Romania) was conducted. Inclusion criteria: age <18 years, histopathologically confirmed malignancy, chemotherapeutic treatment, available paraffin block, positive serous status with known anti-VCA IgG serum level. Exclusion criteria: absence of histopathological confirmation of the tumor type, biological or clinical status not allowing for the administration of oncological treatment, non-compliant patient, incomplete clinical or paraclinical data and chemotherapy in a medical center other than those indicated above. The study group included 12 boys and eight girls.

Paraffin blocks were retrieved from the archives of the "Prof. Dr. Ion Chiricuţă" Oncological Institute or of the Pediatric Emergency Hospital (Cluj-Napoca). Glass slides were prepared from these blocks. They were first reread to confirm initial diagnostic and they subsequently followed the preparation stages imposed by immunohistochemistry (IHC).

Clinical and paraclinical data from the patients' records were also assessed – following classic prognostic factors in connection with the EBER status: treatment response, tumor dimension, "bulky" stage, changes in hematological and biochemical parameters during chemotherapy.

Detection of EBV by *in situ* hybridization

Section selection

Four to 6 μm sections were cut from the paraffin blocks and stained with Hematoxylin and Eosin (HE), in compliance with standard protocol. The glass slides of interest were selected among the examined sections (criteria: maximal density of cancer cells or of malignant lymphoid cells).

Section preparation for hybridization

New 5 μm sections were cut from the paraffin blocks from which the sections examined previously had been obtained. The sections were spread on glass slides in compliance with the usual procedure. The deparaffination of the sections was performed by successively immersing them in three Xylene baths of three minutes each, followed by two minute-baths in 99% and 95% ethylic alcohol respectively. The glass slides were subsequently immersed in double-distilled water for one minute.

Pre-treatment and protease digestion

Proteinase K was applied on the sections – 100 mL of 15 μg/mL solution (500 μg of lyophilized Proteinase K to which 1 mL of 50 mM Tris/HCl buffer with a pH 7.6 was added). The glass slides were incubated for 30 minutes at a temperature of 37^0C. After digestion, the glass slides were immersed in a double-distilled water bath for three minutes.

Section dehydration

Section dehydration was achieved by three minute-immersions in two ethylic alcohol baths (95% and 99%, respectively).

Hybridization

An oligonucleotide probe was used for the detection of Epstein–Barr viral RNA, conjugated with Fluorescein (Novocastra Fluorescein-conjugated probes for *in situ* hybridization Epstein–Barr Virus Probe ISH Kit), out of which 20 μL of probe were added per section. The process lasted for 20 minutes and unfolded at a temperature of 37^0C. Digestion was blocked by incubating the glass slides for 10 minutes with 100 μL of blocking solution.

Detection

The anti-FITC/AP antibody (Anti-Fluorescein Isothiocynanate/Alkaline Phosphatase) was added to the sections, for binding purposes, for 30 minutes. The glass slides were placed in a Tris-buffered saline (TBS) solution for three minutes and in Alkaline Phosphatase substrate buffer for five minutes. In order to prove Alkaline Phosphatase activity, the glass slides were incubated in the dark, overnight, at room temperature, with the mixture obtained from 5-Bromo-4-chloro-3-indolyl phosphate and Nitro blue

tetrazolium, in Dimethylformamide, to which Levamisole was added in compliance with the proportions indicated by the producer. The following day, the glass slides were washed for five minutes, counterstained with Mayer's Hematoxylin and mounted (aqueous mounting medium).

Statistical analysis

Chi-square or Fisher Exact Test was used to evaluate correlations between quantitative variables, following standard application criteria for each test. Normality of continuous data was tested using the Shapiro–Wilk test. Based on normality testing results, between-group differences in continuous data were assessed using either the Student *t*-test or the Mann–Whitney *U*-test. Receiver operating characteristic (ROC) analysis, including curve construction, area under curve (AUC) with a 95% confidence interval (95% CI) and cut-off determination was performed. Sensitivity and specificity were also determined for the identified cut-off value. A significance threshold of $p \leq 0.05$ was selected for all tests. SPSS 13.0 (Chicago, IL, USA) and MedCalc 8.3.1.1 software applications were used for data analysis.

Results

The mean age of the patients enrolled in the study group was of 12.4 years, the youngest subject being two years and the oldest being 17 years.

The types of neoplasms developed by these patients were: Hodgkin lymphoma – eight patients, non-Hodgkin lymphoma – eight patients, nasopharyngeal carcinomas – four patients.

Several aspects related to the neoplastic tumor the patients had developed were assessed in correlation with the EBER status.

Histological type

The classification according to tumor type and EBER status, as well as the microscopic details of the neoplasms studied are presented in Table 1 and Figures 1–6.

Table 1 – ***EBER status versus histological type***

Histological type	EBER-positive	EBER-negative
Hodgkin lymphoma	4	4
Non-Hodgkin lymphoma	3	5
Nasopharyngeal carcinoma	3	1

EBV infection intensity

In a previous cohort study (previous published), which included 35 patients, the regression curve revealed an anti-VCA IgG cut-off value of 213.44 IU/mL, predictive of whether or not complete remission will be achieved in children with EBV-induced neoplasm at the end of treatment with classic chemotherapy agents.

Comparing the EBER status against the EBV infection intensity – highlighted using the anti-VCA IgG titer level – results in the following contingency table (Table 2).

Table 2 – ***EBER status contingent on the anti-VCA IgG titer***

EBER status	IgG <213.44 IU/mL	IgG >213.44 IU/mL
EBER-negative	5	5
EBER-positive	4	6

The statistical analysis did not reveal any significant correlation between the anti-VCA IgG titer and the EBER status (p=0.653).

Treatment response

In the study group, complete remission was achieved for 70% of the EBER-negative patients and for 60% of the EBER-positive patients (Table 3), the difference being statistically insignificant (p=0.639).

Table 3 – ***Treatment response versus EBER status***

EBER status	CR	< CR
EBER-negative	7	3
EBER-positive	6	4

CR: Complete remission.

Initial tumor dimension

The initial dimension of the tumor does not appear to have influenced the EBER-positive status in any way, the differences obtained being statistically insignificant (p=0.497).

Variations in hematological and biochemical parameters during treatment, contingent on the EBER status

We assessed the connection between the EBER status (positive or negative) and the initial values, as well as their variations (nadir) during chemotherapy, so as to verify whether or not the presence of EBV induces additional toxicity. Blood count was used to assess the level of leukocytes, neutrophils, lymphocytes, monocytes, thrombocytes and hemoglobin level. The following parameters were taken into account from biochemical analyses: aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and lactate dehydrogenase (LDH) (Table 4).

Table 4 – ***ASAT values contingent on the EBER status: statistical significance***

	ASAT	C2	C3	C4	C5	C6	ALAT	C2	C3	C4	C5	C6
Mann–Whitney *U*-test	21.000	9.500	11.500	10.000	14.500	9.000	34.500	32.000	28.500	15.000	10.000	6.500
Wilcoxon test	66.000	64.500	66.500	55.000	50.500	30.000	79.500	87.000	83.500	60.000	46.000	12.500
Z-test	-1.724	-2.495	-2.540	-1.667	-.811	-.640	-.530	-.293	-1.348	-1.000	-1.466	-.300
Asymptotic	.085	.013	.011	.096	.418	.522	.596	.769	.178	.317	.143	.764
Exact Sig. [2*(1-tailed Sig.)]	.094	.010	.009	.112	.435	.610	.605	.813	.182	.364	.171	.786

Out of the elements taken into account in the study, the EBER-positive status only had a statistically significant impact on the ASAT level, during the second and third chemotherapy cycles. A tendency towards statistical significance was noted for the forth cycle, also suggesting that EBER presence significantly increases cholestasis in the context of chemotherapy.

Disease-free survival rate *versus* EBER status

Disease-free survival (lack of relapse) does not seem to be influenced by the EBER status, the differences being statistically insignificant (Tables 5 and 6; Figure 7).

Table 5 – ***Disease free survival rate versus EBER status***

Group	No. of patients	Occurrence	Censored *N*	%
EBER-negative	10	0	10	100
EBER-positive	10	2	8	80
Total	20	2	18	90

Table 6 – ***Disease free survival versus EBER status: statistical significance***

	Chi-square	df	Sig.
Log-rank (Mantel–Cox)	2.110	1	.146

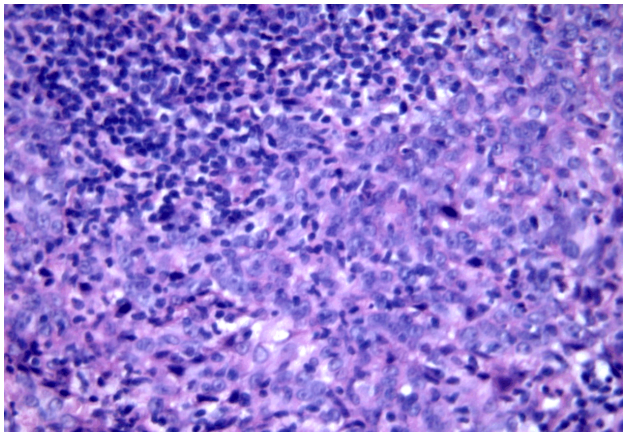

Figure 1 – ***Lateral cervical lymph node metastasis of nasopharyngeal carcinoma. HE staining, ×400.***

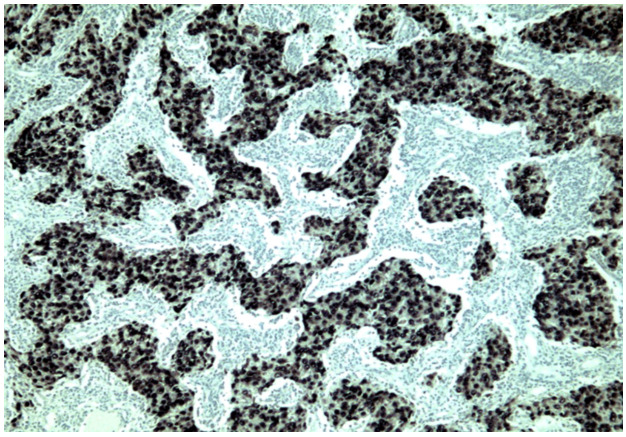

Figure 2 – ***EBER positive for the case presented on previous figure: nasopharyngeal carcinoma. IHC for EBER, ×200.***

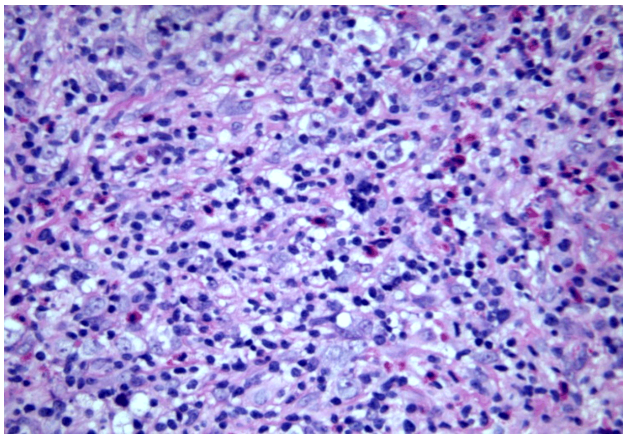

Figure 3 – ***Nodular sclerosis Hodgkin lymphoma. HE staining, ×400.***

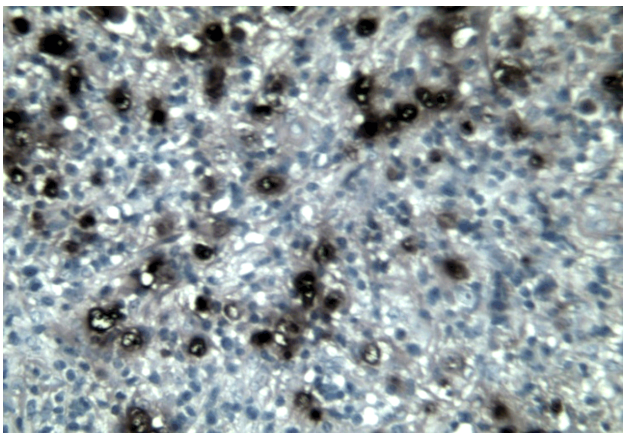

Figure 4 – ***Nodular sclerosis Hodgkin lymphoma, EBER-positive. IHC for EBER, ×1000.***

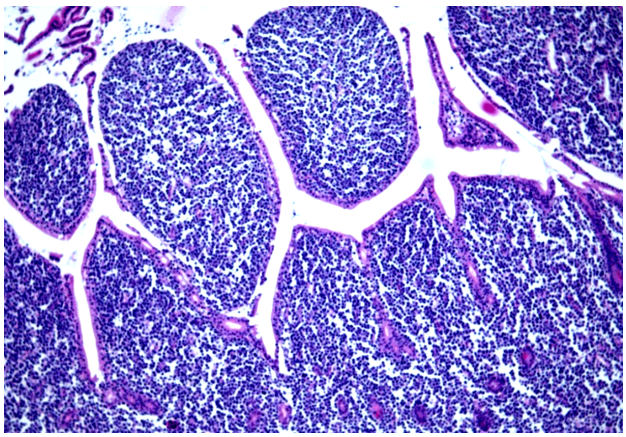

Figure 5 – ***Burkitt lymphoma. HE staining, ×100.***

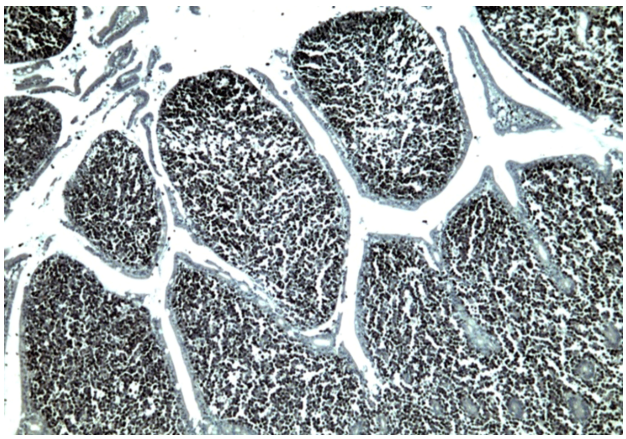

Figure 6 – ***EBER positive Burkitt lymphoma. IHC for EBER, ×100.***

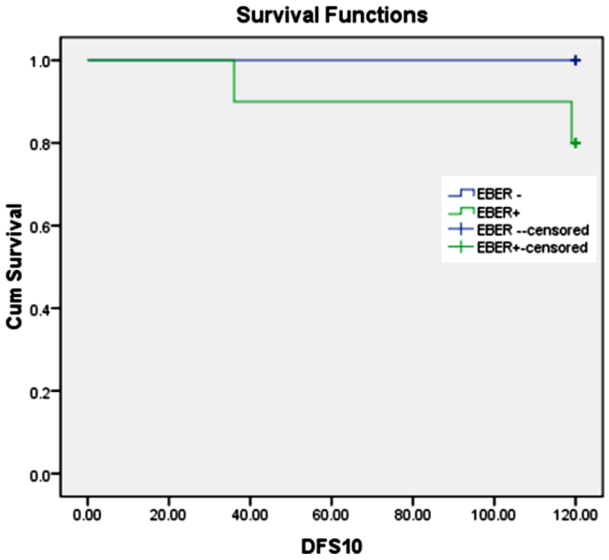

Figure 7 – ***Ten-year disease-free survival (DFS10) rates contingent on the EBER status.***

Discussion

Fifty percent of the children with Hodgkin lymphoma in the study group where EBER-positive, the rate being of 37.5% in those with non-Hodgkin lymphoma and of 75% in those diagnosed with nasopharyngeal carcinoma. In specialized literature, data on EBV positivity in children with neoplasms significantly vary. On the one hand, this discrepancy reflects the geographical differences recorded in connection with the incidence of the EBV infection. A Chinese study reveals an incidence rate of 73.5% whereas an Iraqi study reports an EBER-positive rate of less than 5% in children with lymphoma [17, 18]. It appears that EBV is most frequently associated with the mixed cellularity subtype (71%) and with nodular sclerosis (54.2%) [19].

The number of new cancer cases that can be attributed to EBV infection varies according to the regional socio-economic level, with a high number of cases in areas with limited development [20]. Khan & Hashim estimated that the global death burden, for the period of 1990–2010 attributed to EBV-related neoplasm worldwide was of 142 979, which is translated into 1.8% of all cancer deaths. More concerning is that for the mentioned period the authors reported an increasing trend in the number of cases [21].

The EBV infection is strongly linked to the development of nasopharyngeal carcinoma. In virtually all cases, EBV DNA can be identified in geographic areas with high and intermediate-incidence and in approximately 80% of cases in low-incidence areas (limited available data) [20]. The virus infects epithelial cells from the oropharynx and the associated B-cells, in the mucosa, by using the CD21 complement receptor [22].

Evidence regarding the implication of EBV in carcinogenesis of nasopharyngeal carcinoma is brought by the identification of a clonal EBV genome in the tumor cells [23, 24]. These facts argue in favor of the presence of the infection prior to the development of the neoplastic process [22]. However, the exact role of the EBV infection in the development of nasopharyngeal carcinoma is still debated. In most cases, the virus establishes a non-lithic or minimally lithic relationship with the host cell, favored by a low level of viral gene expression [21]. In the case of nasopharyngeal carcinoma, the virus assumes a type II latency behavior [characterized by expression of EBV-encoded RNAs (EBERs), BamHI-A rightward transcripts (BARTs), Qp promoter-induced EBV nuclear antigen-1 (EBNA-1) and a variable expression of LMP-1, LMP2A and LMP2B] [21, 23]. In undifferentiated nasopharyngeal carcinomas, the most common early chromosomal abnormalities involve losses of genetic material in 3p and 9p. This is translated into inactivation of RASSF1A, p16, p14ARF, CDKN2A [23, 25]. Evidence suggests that p16 hypermethylation significantly increases the risk of development of nasopharyngeal carcinomas [26, 27]. The silencing of p16 and RASSF1A creates a favorable context for the progression of the EBV infection, which in turn will promote tumor progression [23].

In the case of Hodgkin's lymphoma, EBV shows a similar latency state [23]. The detection of EBV gene products in tumor cells is highly variable and dependent of several factors (age, area and histological subtype) [20]. Although the data is limited, it estimated that the overall prevalence of EBV infection in HL is around 40% [20].

The tumor cells of classic Hodgkin's lymphoma [Hodgkin and Reed–Sternberg (HRS) cells] are considered transformed B-cell with a germinal center or post germinal center origin [23, 27]. The infection does not adversely affect the integrity and viability of the cell, but favors viral replication. LMP-1 mimics the presence of the CD40 receptor in its active state, which leads to the stimulation of cell proliferation [22]. LMP-2 shows a homologous function to the B-cell receptor and LMP-2 signaling plays a key role in the immortalization HRS cells [28]. EBV uses the cellular signaling pathways (NF-kB, JAK/STAT and phosphatidylinositol 3-kinase/AKT) through LMP-1, which adds a survival and proliferation advantage to HRS cells [22, 29]. Evidence suggests that EBV impede cytotoxic T-cell response [30].

The mean age of the patients enrolled in the study was of 12 years. Literature suggests an early onset of the EBV infection in developing countries, where population density is higher [31].

Patient age is a determining factor in EBV-positive classic Hodgkin lymphoma (cHL) prognosis. Studies have shown that EBV+cHL have a worse prognosis especially in the elderly [32–36]. For young adults (16 to 34 years of age), some studies have shown a survival advantage in EBV+ cases [37]. For EBV-associated Burkitt's lymphoma, it seems that age does not influence the presentation type abdominal *versus* extra abdominal [38]. Overall survival is statistical influenced by age only in univariate analysis [39]. In contrast, in other studies, the presence of EBV itself represents a negative prognostic factor for overall and progression free survival of diffuse large cell lymphoma [40]. Moreover, the presence of EBV in strong enough as prognostic factor for both groups of patients: young and aged. Non-Hodgkin's lymphoma represents only 6–10% of pediatric neoplasia [40]. In pediatric population the EBV-associated lymphoma has a significantly higher incidence among patients <10 years and immunosuppressed [41]. EBV could be implicated in

approximately 30% of sporadic Burkitt's lymphoma compared to 25–40% in immunocompromised hosts [42]. In Indian population like other developing countries, Epstein–Barr virus (EBV) association of malignant hemopathies confirmed by Epstein–Barr virus encoded RNA (EBER) RISH or EBV-LMP-1 IHC revealed an EBV association of 93% [43]. In Western countries, the EBV etiology of Hodgkin's disease is met more lately and with a lower frequency – 20–40% [16].

There was no statistically significant correlation between the EBV infection intensity and the EBER status for the group under study, although there are studies that establish a connection between EBV positivity and high antibody levels [44].

Nowadays is little known regarding the correlation, if any, between EBV-negative DNA load and EBER status. In a retrospective study of 140 patients [45], EBV DNA load was significantly higher in EBER positive than in negative lymphomas. Despite this fact, a significant DNA load (more than median number of copies) was present also in EBER-negative patients (>70% of patients). DNA intense load in EBER-negative patients altered the overall and progression free survival, underlying that DNA load is not a surrogate for EBER status, on the contrary, it could represent an independent prognostic factor [45]. In Hodgkin's lymphoma patients, the serum level of EBV DNA seems to be correlated with response to chemotherapy [15]. Even in immunocompromised hosts, Epstein–Barr DNA is not detected in malignant cells of all lymphoma subtypes. In AIDS-related lymphoma, EBV viral load had a significant decrease after chemotherapy treatment [46], but this viral load remains significantly higher in EBER-positive patients than in EBER-negative. EBV viral load could be predictive for oncological response; meanwhile, EBER expression was associated to advanced stages of disease and worse immune status. EBV DNA in plasma has a high specificity (90%) but low sensitivity (65%) in order to certify the EBV association in HL [16]. EBV viral load correlate with high infiltration of the tumor with macrophage and low serum level of EBNA1 and lymphocytes, which may suggest a reduction of immunosurveillance needed for expansion of Reed–Sternberg cells.

The standard method applied to identify EBER is *in situ* hybridization (ISH). Automated analysis is more effective, with a sensitivity of 94%, a specificity of 94% and a global accuracy of 83% [47]. The positivity rate for Hodgkin lymphomas varies significantly, ranging from 20 to 70% [48]. The comparison between immunohistochemistry (IHC) for LMP-1, PCR for EBER-1 and PCR for BamHI W fragment revealed the superiority of the PCR-based method [48].

In cytoplasm, EBER is sometimes represented by over one million copies/cell, while LMP-1 is found in cytosol and on the surface of the cytoplasmic membrane [24]. Their expression on the Reed–Sternberg (RS) cells abides by the "all or nothing" rule. If equivocal results are obtained, the material is likely to have been degraded during the preparation stage [49]. The major advantage of EBER technique is represented by a particularity of these particles, which consist in being amplified and present at high levels in all latency forms of EBV infection. By this, they are ideal targets for ISH, which is widely considered the gold standard for the detection of EBV latent infection, more sensitive than the immunohistochemical evaluation of LMP-1 expression [42].

Plasma EBV-DNA could serve as a surrogate for EBER-ISH and need further studies to explore its prognostic utility in HL. In a published study, *Cancer Cooperative Intergroup Trial E2496*, a cut-off value of 60 copies/100 μL plasma yielded 96% concordance with positivity of EBER on ISH [50].

No correlation was established between EBER status and response to chemotherapy in our study. The prognostic role of the EBV infection remains controversial despite the description of distinct entities of EBV-positive lymphoproliferations. A possible explanation lies in the role played by EBV in maintaining the RS cells in the cycle, which renders them highly sensitive to chemotherapy and radiotherapy, thus increasing the chances of achieving complete remission [51]. Other studies reports that EBER-positive status of lymphoma is correlated with poor survival [45]. The positivity of EBER is associated with male gender, poor performance status and lower response to the first line of chemotherapy [40]. EBER positive status is linked to more advanced stage, more than one extranodal involvement, higher International Prognostic Index (IPI) risk group, presence of B-symptom [52].

Classical Hodgkin's lymphoma (cHL) is characterized by a small number of neoplastic cells in a background of reactive cells. In a population of 100 children with HL, a morphological analysis revealed that children <10 years and EBV-positive cases have an intense T-cell infiltrate, exhibiting a cytotoxic/Th1 profile, characterized by higher numbers of CD3+, CD8+, TIA1+ and TBET+ lymphocytes [53]. That could be explained by physiological changes of the immunity depending on time of developing of the disease and by interactions with EBV.

The survival rate of the patients included in the study was not influenced by the EBER status. Other studies on pediatric patients confirm these results [54]. However, EBER seems to be correlated with the tumor proliferative index [54]. EBV appears to be absent in lymphomas of low malignancy [55]. In nasopharyngeal carcinomas, where EBV has a very strong causative role, the survival rate of EBV-positive patients being statically higher than the survival rate of EBV-negative patients. By contrast, in aggressive non-Hodgkin's lymphomas, the presence of EBV may be a negative prognostic factor, especially in elderly patients [56].

The presence of EBV in hematological hemopathies could represent a factor, which aggravates the clinical behavior of a tumor. This influence can affect recipients with normal or altered immunity. EBV favors a window in immunological surveillance, which permits proliferation of EBV-transformed B-cells. Existing published report present infaust prognostic for patients with coinfection of EBV after hematopoietic stem cell transplantation by the developing of post-transplant lymphoproliferative disorder [57]. Chemotherapy used in such cases include a target therapy consisted in Rituximab an anti-CD20 antibody. Despite aggressive treatment, the clinical evo-

lution could be aggressive with little or no response to chemotherapy regimen. The tumor suffers genetic and clonal changes induced by chemotherapy action, for example, CD20-positive could disappear and be replaced by CD19-negative CD20-negative EBER-positive, more aggressive and less responsive to treatment. In a larger analysis of bone marrow transplants recipients, negative prognostic factors linked with high rate of mortality were (amongst others): involvement of extralymphoid tissue, acute graft-*versus*-host disease (GVHD), and a lack of reduction of immunosuppression upon post-transplant lymphoproliferative disease (PTLD) diagnosis [58]. In immunocompetent patients, the appearance of an EBV-positive lymphoma (especially NK or T) is characterized by an aggressive evolution. In the vast majority of them, they will present B-symptoms (80%), an advanced Ann Arbor stage (III, IV) (87%) and an *International Prognostic Index* high or high/intermediate (87%) [59]. The association of EBV (quantified by positivity of EBER) with NK and peripheral T-cell lymphomas not otherwise specified (PTCL NOS) was underlined by a morphopathological analysis [60]. The mismatch EBV between donor and recipient it is thought to be a risk factor [61].

In order to minimize the risk of PTLD it is necessary to closely monitor the cytomegalovirus (CMV) reactivation. In multivariate analysis, the reactivation of CMV is strongly related with EBV reactivation [62]. A single administration of Rituximab in patients with EBV DNA more than 10 000 copies/100 μL seems to do the viral clearance and to prevent the risk of progression into EBV-related PTLD [62].

Closely monitoring the viral load of EBV could identify the patients at risk for lymphoproliferative disorders [63]. Nearly 75% of those with less than 1000 copies/100 μL will not have any EBV-associated lymphoproliferative disease. Monitoring the viral load of EBV could help physicians for an early intervention in EBV-associated lymphoproliferative disease and also for follow-up of the efficacy of the antiviral therapy.

Conclusions

The results of this study did not highlight the prognostic factor of EBER in the evolution of children neoplasms. There were no statistically significant correlations between infection intensity, measured using the anti-VCA IgG titer and EBER, between EBER and patient response to oncological treatment or between EBER and the disease-free survival rate. The results of this study must be interpreted cautiously, given the low number of patients included in the study, the histological heterogeneity of the neoplasms studied as well as the potential presence of false-negative reactions caused by the degradation of viral miRNA during the processing stage.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

This paper was published under the frame of European Social Fund, Human Resources Development Operational Program 2007–2013, Project No. POSDRU/159/1.5/138776.

References

[1] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer, 2015, 136(5):E359–E386.
[2] AIRTUM Working Group; CCM; AIEOP Working Group. Italian cancer figures, report 2012: cancer in children and adolescents. Epidemiol Prev, 2013, 37(1 Suppl 1):1–225.
[3] Mellinger JL, Rossaro L, Naugler WE, Nadig SN, Appelman H, Lee WM, Fontana RJ. Epstein–Barr virus (EBV) related acute liver failure: a case series from the US Acute Liver Failure Study Group. Dig Dis Sci, 2014, 59(7):1630–1637.
[4] Chesnokova LS, Hutt-Fletcher LM. Epstein–Barr virus infection mechanisms. Chin J Cancer, 2014, 33(11):545–548.
[5] Grywalska E, Rolinski J. Epstein–Barr virus-associated lymphomas. Semin Oncol, 2015, 42(2):291–303.
[6] Shinozaki-Ushiku A, Kunita A, Fukayama M. Update on Epstein–Barr virus and gastric cancer (review). Int J Oncol, 2015, 46(4):1421–1434.
[7] Liebowitz D, Kieff E. Epstein–Barr virus. In: Roizman B, Whitley RJ, Lopez C (eds). The human herpesviruses. 1st edition, Raven Press, New York, 1993, 107–172.
[8] Thorley-Lawson DA, Gross A. Persistence of the Epstein–Barr virus and the origins of associated lymphomas. N Engl J Med, 2004, 350(13):1328–1337.
[9] Shen ZC, Luo B, Chen JN, Chao Y, Shao CK, Liu QQ, Wang Y. High prevalence of the EBER variant EB-8m in endemic nasopharyngeal carcinomas. PLoS One, 2015, 10(3):e0121420.
[10] Yau TO, Tang CM, Yu J. Epigenetic dysregulation in Epstein–Barr virus-associated gastric carcinoma: disease and treatments. World J Gastroenterol, 2014, 20(21):6448–6456.
[11] Takada K. Role of EBER and BARF1 in nasopharyngeal carcinoma (NPC) tumorigenesis. Semin Cancer Biol, 2012, 22(2):162–165.
[12] Nanbo A, Yoshiyama H, Takada K. Epstein–Barr virus-encoded poly(A)-RNA confers resistance to apoptosis mediated through Fas by blocking the PKR pathway in human epithelial intestine 407 cells. J Virol, 2005, 79(19):12280–12285.
[13] Ling W, Cao SM, Huang QH, Li YH, Deng MQ. Prognostic implication of pretreatment titer of serum immunoglobulin A against Epstein–Barr virus capsid antigen in nasopharyngeal carcinoma patients in Sihui, Guangdong. Ai Zheng, 2009, 28(1):57–59.
[14] Song C, Yang S. A meta-analysis on the EBV DNA and VCA-IgA in diagnosis of nasopharyngeal carcinoma. Pak J Med Sci, 2013, 29(3):885–890.
[15] Spacek M, Hubacek P, Markova J, Zajac M, Vernerova Z, Kamaradova K, Stuchly J, Kozak T. Plasma EBV-DNA monitoring in Epstein–Barr virus-positive Hodgkin lymphoma patients. APMIS, 2011, 119(1):10–16.
[16] Hohaus S, Santangelo R, Giachelia M, Vannata B, Massini G, Cuccaro A, Martini M, Cesarini V, Cenci T, D'Alo F, Voso MT, Fadda G, Leone G, Larocca LM. The viral load of Epstein–Barr virus (EBV) DNA in peripheral blood predicts for biological and clinical characteristics in Hodgkin lymphoma. Clin Cancer Res, 2011, 17(9):2885–2892.
[17] Uccini S, Al-Jadiry MF, Scarpino S, Ferraro D, Alsaadawi AR, Al-Darraji AF, Moleti ML, Testi AM, Al-Hadad SA, Ruco L. Epstein–Barr virus-positive diffuse large B-cell lymphoma in children: a disease reminiscent of Epstein–Barr virus-positive diffuse large B-cell lymphoma of the elderly. Hum Pathol, 2015, 46(5):716–724.
[18] Duan YL, Zhang YH, Jin L, Yang J, Zhang R, Zhou CJ. Clinical characteristics of 34 children with Hodgkin lymphoma and efficacy of treatment with chemotherapy plus low dose radiotherapy on involved sites. Zhonghua Er Ke Za Zhi, 2010, 48(9):698-702.
[19] Fatima S, Ahmed R, Ahmed A. Hodgkin lymphoma in Pakistan: an analysis of subtypes and their correlation with Epstein Barr virus. Asian Pac J Cancer Prev, 2011, 12(6):1385–1388.
[20] de Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, Plummer M. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. Lancet Oncol, 2012, 13(6):607–615.
[21] Khan G, Hashim MJ. Global burden of deaths from Epstein–Barr virus attributable malignancies 1990–2010. Infect Agent Cancer, 2014, 9(1):38.

[22] Kumar V, Abbas AK, Aster JC (eds). Robbins & Cotran pathologic basis of disease. Chapter 7: Neoplasia. 9th edition, Elsevier Inc., Philadelphia, 2014, 265–340.
[23] Shah KM, Young LS. Epstein–Barr virus and carcinogenesis: beyond Burkitt's lymphoma. Clin Microbiol Infect, 2009, 15(11): 982–988.
[24] Raab-Traub N, Flynn K. The structure of the termini of the Epstein–Barr virus as a marker of clonal cellular proliferation. Cell, 1986, 47(6):883–889.
[25] Korcum AF, Özyar E, Ayhan A. Epstein–Barr virus genes and nasopharyngeal cancer. Turk J Cancer, 2006, 36(3):97–107.
[26] Shao Y, Jiang H, Wu X, Luo Y, Tang W. p16 promoter hypermethylation is associated with increased risk of nasopharyngeal carcinoma. Mol Clin Oncol, 2014, 2(6):1121–1124.
[27] Hutajulu SH, Indrasari SR, Indrawati LP, Harijadi A, Duin S, Haryana SM, Steenbergen RD, Greijer AE, Middeldorp JM. Epigenetic markers for early detection of nasopharyngeal carcinoma in a high risk population. Mol Cancer, 2011, 10:48.
[28] Jaffe ES, Harris NL, Vardiman JW, Campo E, Arber DA. Hematopathology. 1st edition, Saunders/Elsevier, Philadelphia, 2011, 124–144.
[29] Vockerodt M, Cader FZ, Shannon-Lowe C, Murray P. Epstein–Barr virus and the origin of Hodgkin lymphoma. Chin J Cancer, 2014, 33(12):591–597.
[30] Asano N, Yamamoto K, Tamaru J, Oyama T, Ishida F, Ohshima K, Yoshino T, Nakamura N, Mori S, Yoshie O, Shimoyama Y, Morishima Y, Kinoshita T, Nakamura S. Age-related Epstein–Barr virus (EBV)-associated B-cell lymphoproliferative disorders: comparison with EBV-positive classic Hodgkin lymphoma in elderly patients. Blood, 2009, 113(12): 2629–2636.
[31] Chabay PA, Barros MH, Hassan R, De Matteo E, Rey G, Carrico MK, Renault IZ, Preciado MV. Pediatric Hodgkin lymphoma in 2 South American series: a distinctive epidemiologic pattern and lack of association of Epstein–Barr virus with clinical outcome. J Pediatr Hematol Oncol, 2008, 30(4): 285–291.
[32] Elsayed AA, Asano N, Ohshima K, Izutsu K, Kinoshita T, Nakamura S. Prognostic significance of CD20 expression and Epstein–Barr virus (EBV) association in classical Hodgkin lymphoma in Japan: a clinicopathologic study. Pathol Int, 2014, 64(7):336–345.
[33] Koh YW, Yoon DH, Suh C, Huh J. Impact of the Epstein–Barr virus positivity on Hodgkin's lymphoma in a large cohort from a single institute in Korea. Ann Hematol, 2012, 91(9): 1403–1412.
[34] Flavell KJ, Biddulph JP, Constandinou CM, Lowe D, Scott K, Crocker J, Young LS, Murray PG. Variation in the frequency of Epstein–Barr virus-associated Hodgkin's disease with age. Leukemia, 2000, 14(4):748–753.
[35] Flavell KJ, Billingham LJ, Biddulph JP, Gray L, Flavell JR, Constandinou CM, Young LS, Murray PG. The effect of Epstein–Barr virus status on outcome in age- and sex-defined subgroups of patients with advanced Hodgkin's disease. Ann Oncol, 2003, 14(2):282–290.
[36] Kwon JM, Park YH, Kang JH, Kim K, Ko YH, Ryoo BY, Lee SS, Lee SI, Koo HH, Kim WS. The effect of Epstein–Barr virus status on clinical outcome in Hodgkin's lymphoma. Ann Hematol, 2006, 85(7):463–468.
[37] Jarrett RF, Stark GL, White J, Angus B, Alexander FE, Krajewski AS, Freeland J, Taylor GM, Taylor PR; Scotland and Newcastle Epidemiology of Hodgkin Disease Study Group. Impact of tumor Epstein–Barr virus status on presenting features and outcome in age-defined subgroups of patients with classic Hodgkin lymphoma: a population-based study. Blood, 2005, 106(7):2444–2451.
[38] Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB. Differences in B cell growth phenotype reflect novel patterns of Epstein–Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J, 1987, 6(9):2743–2751.
[39] Chen BJ, Chang ST, Weng SF, Huang WT, Chu PY, Hsieh PP, Jung YC, Kuo CC, Chuang YT, Chuang SS. EBV-associated Burkitt lymphoma in Taiwan is not age-related. Leuk Lymphoma, 2015, Sep 30:1–10.
[40] Lu TX, Liang JH, Miao Y, Fan L, Wang L, Qu XY, Cao L, Gong QX, Wang Z, Zhang ZH, Xu W, Li JY. Epstein–Barr virus positive diffuse large B-cell lymphoma predict poor outcome, regardless of the age. Sci Rep, 2015, 5:12168.
[41] Cohen M, De Matteo E, Narbaitz M, Carreño FA, Preciado MV. Epstein–Barr virus presence in pediatric diffuse large B-cell lymphoma reveals a particular association and latency patterns: analysis of viral role in tumor microenvironment. Int J Cancer, 2013, 132(7):1572–1580.
[42] Pannone G, Zamparese R, Pace M, Pedicillo MC, Cagiano S, Somma P, Errico ME, Donofrio V, Franco R, De Chiara A, Aquino G, Bucci P, Bucci E, Santoro A, Bufo P. The role of EBV in the pathogenesis of Burkitt's lymphoma: an Italian hospital based survey. Infect Agent Cancer, 2014, 9(1):34.
[43] Zameer MA, Premalata CS, Arunakumari B, Appaji L, Rao CR. Pediatric Hodgkin lymphoma in a South Indian regional cancer center: its immunomorphology, tumor-associated macrophages, and association with Epstein–Barr virus. Pediatr Hematol Oncol, 2015, 32(4):229–238.
[44] Axdorph U, Porwit-MacDonald A, Sjöberg J, Grimfors G, Ekman M, Wang W, Biberfeld P, Björkholm M. Epstein–Barr virus expression in Hodgkin's disease in relation to patient characteristics, serum factors and blood lymphocyte function. Br J Cancer, 1999, 81(7):1182–1187.
[45] Okamoto A, Yanada M, Inaguma Y, Tokuda M, Morishima S, Kanie T, Yamamoto Y, Mizuta S, Akatsuka Y, Yoshikawa T, Mizoguchi Y, Nakamura S, Okamoto M, Emi N. The prognostic significance of EBV DNA load and EBER status in diagnostic specimens from diffuse large B-cell lymphoma patients. Hematol Oncol, 2015, Jul 14.
[46] Tanaka PY, Ohshima K, Matsuoka M, Sabino EC, Ferreira SC, Nishya AS, de Oliveira Costa R, Calore EE, Perez NM, Pereira J. Epstein–Barr viral load is associated to response in AIDS-related lymphomas. Indian J Hematol Blood Transfus, 2014, 30(3):191–194.
[47] Fanaian NK, Cohen C, Waldrop S, Wang J, Shehata BM. Epstein–Barr virus (EBV)-encoded RNA: automated *in-situ* hybridization (ISH) compared with manual ISH and immunohistochemistry for detection of EBV in pediatric lymphoproliferative disorders. Pediatr Dev Pathol, 2009, 12(3):195–199.
[48] Qi ZL, Han XQ, Hu J, Wang GH, Gao JW, Wang X, Liang DY. Comparison of three methods for the detection of Epstein–Barr virus in Hodgkin's lymphoma in paraffin-embedded tissues. Mol Med Rep, 2013, 7(1):89–92.
[49] Gulley ML, Glaser SL, Craig FE, Borowitz M, Mann RB, Shema SJ, Ambinder RF. Guidelines for interpreting EBER *in situ* hybridization and LMP1 immunohistochemical tests for detecting Epstein–Barr virus in Hodgkin lymphoma. Am J Clin Pathol, 2002, 117(2):259–267.
[50] Kanakry JA, Li H, Gellert LL, Lemas MV, Hsieh WS, Hong F, Tan KL, Gascoyne RD, Gordon LI, Fisher RI, Bartlett NL, Stiff P, Cheson BD, Advani R, Miller TP, Kahl BS, Horning SJ, Ambinder RF. Plasma Epstein–Barr virus DNA predicts outcome in advanced Hodgkin lymphoma: correlative analysis from a large North American cooperative group trial. Blood, 2013, 121(18):3547–3553.
[51] Naresh KN, Johnson J, Srinivas V, Soman CS, Saikia T, Advani SH, Badwe RA, Dinshaw KA, Muckaden M, Magrath I, Bhatia K. Epstein–Barr virus association in classical Hodgkin's disease provides survival advantage to patients and correlates with higher expression of proliferation arkers in Reed–Sternberg cells. Ann Oncol, 2000, 11(1):91–96.
[52] Park S, Lee J, Ko YH, Han A, Jun HJ, Lee SC, Hwang IG, Park YH, Ahn JS, Jung CW, Kim K, Ahn YC, Kang WK, Park K, Kim WS. The impact of Epstein–Barr virus status on clinical outcome in diffuse large B-cell lymphoma. Blood, 2007, 110(3):972–978.
[53] Barros MH, Vera-Lozada G, Soares FA, Niedobitek G, Hassan R. Tumor microenvironment composition in pediatric classical Hodgkin lymphoma is modulated by age and Epstein–Barr virus infection. Int J Cancer, 2012, 131(5):1142–1152.
[54] Aktaş S, Kargi A, Olgun N, Diniz G, Erbay A, Vergin C. Prognostic significance of cell proliferation and apoptosis-regulating proteins in Epstein–Barr virus positive and negative pediatric non-Hodgkin's lymphoma. Pathol Oncol Res, 2009, 15(3):345–350.
[55] Yip KW, Shi W, Pintilie M, Martin JD, Mocanu JD, Wong D, MacMillan C, Gullane P, O'Sullivan B, Bastianutto C, Liu FF. Prognostic significance of the Epstein–Barr virus, p53, Bcl-2, and survivin in nasopharyngeal cancer. Clin Cancer Res, 2006, 12(19):5726–5732.

[56] Uner A, Akyurek N, Saglam A, Abdullazade S, Uzum N, Onder S, Barista I, Benekli M. The presence of Epstein–Barr virus (EBV) in diffuse large B-cell lymphomas (DLBCLs) in Turkey: special emphasis on 'EBV-positive DLBCL of the elderly'. APMIS, 2011, 119(4–5):309–316.
[57] Yamamoto N, Nishimura N, Takeuchi M, Ito T, Yokozaki H, Hirase S, Kubokawa I, Mori T, Yanai T, Hayakawa A, Takeshima Y, Nishio H, Matsuo M, Imadome K, Iijima K. The emergence of CD20-/CD19- tumor cells after rituximab therapy for Epstein–Barr virus-associated post-transplant lymphoproliferative disorder complicated with hemophagocytic lymphohistiocytosis. Eur J Pediatr, 2014, 173(12):1615–1618.
[58] Styczynski J, Gil L, Tridello G, Ljungman P, Donnelly JP, van der Velden W, Omar H, Martino R, Halkes C, Faraci M, Theunissen K, Kalwak K, Hubacek P, Sica S, Nozzoli C, Fagioli F, Matthes S, Diaz MA, Migliavacca M, Balduzzi A, Tomaszewska A, Camara Rde L, van Biezen A, Hoek J, Iacobelli S, Einsele H, Cesaro S; Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Response to rituximab-based therapy and risk factor analysis in Epstein Barr virus-related lymphoproliferative disorder after hematopoietic stem cell transplant in children and adults: a study from the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Clin Infect Dis, 2013, 57(6):794–802.
[59] Jeon YK, Kim JH, Sung JY, Han JH, Ko YH, Hematopathology Study Group of the Korean Society of Pathologists. Epstein–Barr virus-positive nodal T/NK-cell lymphoma: an analysis of 15 cases with distinct clinicopathological features. Hum Pathol, 2015, 46(7):981–990.
[60] Karaarslan S, Hekimgil M, Soydan S, Ertan Y, Doğanavşargil B. Evaluation of the role of Epstein–Barr virus in cases of nodal or extranodal T- and NK-cell lymphoma using EBER *in situ* hybridization. Pol J Pathol, 2015, 66(2):161–169.
[61] Uhlin M, Wikell H, Sundin M, Blennow O, Maeurer M, Ringden O, Winiarski J, Ljungman P, Remberger M, Mattsson J. Risk factors for Epstein–Barr virus-related post-transplant lymphoproliferative disease after allogeneic hematopoietic stem cell transplantation. Haematologica, 2014, 99(2):346–352.
[62] Zallio F, Primon V, Tamiazzo S, Pini M, Baraldi A, Corsetti MT, Gotta F, Bertassello C, Salvi F, Rocchetti A, Levis A. Epstein–Barr virus reactivation in allogeneic stem cell transplantation is highly related to cytomegalovirus reactivation. Clin Transplant, 2013, 27(4):E491–E497.
[63] Meerbach A, Wutzler P, Häfer R, Zintl F, Gruhn B. Monitoring of Epstein–Barr virus load after hematopoietic stem cell transplantation for early intervention in post-transplant lymphoproliferative disease. J Med Virol, 2008, 80(3):441–454.

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Received: April 6, 2015

Accepted: December 9, 2015