Original Article

A Feasible Flowchart to Screen EBV-Positive Diffuse Large B Cell Lymphoma of the elderly: A Molecular Approach

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ABSTRACT

Background: Epstein Barr Virus-positive diffuse large B cell lymphoma of the elderly (DLBCL-EBV$^+$-e), initially described in 2003, is a provisional entity in the 4th edition of the World Health Organization (WHO) Classification of tumors of hematopoietic and lymphoid tissues and is defined as an EBV$^+$ monoclonal large B cell proliferation that occurs in patients >50 years of age and in whom there is no known immunodeficiency or history of lymphoma. It is associated with a poor prognosis but could be treated with the association of novel therapeutic approaches. EBER in situ hybridization (EBER-ISH) is the gold standard assay for defining a tumor as EBV-related, however there are no uniform criteria for the percentage of EBV-positive cells in DLBCL-EBV$^+$-e.

Methods: We applied to Formalin Fixed Paraffin Embedded (FFPE) tissue of 43 novel DLBCL over 50 years Q-PCR for the detection of viral DNA in concert with EBER-ISH.

Result: Only one case was defined as true DLBCL-EBV+-e being Q-PCR positive with a value of log EBV DNA/10^5 cells $\geq$ 6 and EBER-ISH positive.

Conclusion: In the current study we applied to FFPE tissue of DLBCL-EBV$^+$-e a reliable and feasible Q-PCR/EBER-ISH combined protocol for the screening of EBV status. Our protocol could help to better identify the DLBCL-EBV$^+$-e patients eligible for novel therapeutic approaches.

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Introduction

Epstein-Barr virus (EBV) is a ubiquitous human DNA γ-herpesvirus. It infects asymptomatically >90% of population worldwide and it is the causative agent of infectious mononucleosis (IM). It is associated with a wide spectrum of epithelial and lymphoproliferative diseases.[1,2] The 4th edition of the World Health Organization (WHO) Classification of tumors of hematopoietic and lymphoid tissues defines the Epstein Barr Virus-positive diffuse large B cell lymphoma of the elderly (DLBCL-EBV$^+$-*e*) as an EBV-positive diffuse large B cell lymphoma (DLBCL) of patients older than 50 years, without any known underlying immunosuppression or prior lymphoproliferative disease.[3] There is a higher prevalence of EBV-positive DLBCL among East Asians (8,7%-11,4%) compared with <5% in Western Countries.[1] Beltran et al. has reported the highest frequency of EBV-positive DLBCL in a Peruvian population, 14,9%.[4]

It has been postulated that DLBCL-EBV$^+$-*e* might be caused by the senescence of the immune system as a part of the normal aging process, based largely on shared features with immunodeficiency-associated lymphoproliferative disorders (LPDs).[5,6] Such lymphoma shows an EBV latency pattern Type II or III.[5] The oncogenic mechanisms of EBV in DLBCL-EBV$^+$-*e* are thought to be attributable predominantly to LMP1, a 63kDa membrane protein that elicits NF-kB, P13K/ATK, MAPK and JAK/STAT pathways by mimicking CD40, which is expressed constitutively on the B-cell membrane involving in B-cell activation and proliferation.[2,5]

Several published studies have shown that DLBCL-EBV$^+$-*e* is associated with a poorer prognosis compared to age-matched DLBCL without EBV infection, independent of the International Prognostic Index (IPI).[1,7] Currently, there is no uniformly accepted treatment for DLBCL-EBV$^+$-*e* beyond the current standard therapy for DLBCL.[6] However, novel therapeutic approaches need to be considered, including EBV-specific adoptive immunotherapy, miRNA-targeted therapy, combination therapy based on EBV lytic phase induction followed by exposure of the tumor cells to anti-herpesvirus drug, and targeting specific signaling pathways such as NF-kB pathway.[5] Therefore, the identification even of few DLBCL-EBV$^+$-*e* cases ensures these patients may benefit from these novel therapies.

The gold standard assay for determining whether a biopsied tumor is EBV-related is the EBER in situ hybridization (EBER-ISH).[6,8] However, the criteria for defining EBV-positive DLBCL vary among the studies and there is no uniform consensus about the cut-off value of EBER-ISH positive cells ranging from 10% of to almost all tumor cells.[1,9,10] Moreover, due to the low prevalence of the EBV-positive DLBCL cases, this approach is not uniformly recommended in all DLBCL patients older than 50 years.[6]

For these reasons and considering the potential prognostic and predictive value of EBV tumoral status, we developed a reliable and feasible Q-PCR/EBER-ISH combined protocol to assess the EBV presence in DLBCL-*e* cells.

Materials and Methods

Forty-three (43) newly diagnosed primary DLBCL > 50 years, without known underlying immunosuppression condition, of whom paraffin blocks were available and well preserved, were tested for EBV presence. Of these patients 28 had nodal and 15 extra-nodal DLBCL. All patients were diagnosed and treated at the Unit of Hematology of the University Hospital Campus Bio-Medico of Rome (UCBM). Collection of cases was in accordance with data safety laws and institutional review board requirements.

DNA was extracted from Formalin Fixed Paraffin Embedded (FFPE) sections using the High Pure FFPET DNA Isolation Kit (Roche) prior microdissection to enrich the tissue of neoplastic cells, and DNA quantity and quality was evaluated spectroscopically.

Specific amplification of the viral DNA was carried out by quantitative real time PCR (Q-PCR), using the EBV Real-TM Quant kit (Dia-Chem) according to manufacture. This real time test provides a qualitative and quantitative detection of LMP-gene of EBV DNA in biological materials (including FFPE samples).

For each control and patient specimen, the concentration of EBV DNA (or number of viral DNA copies per cell) was expressed as logarithm of EBV DNA in 10^5 cells and calculated using the following formula:

log [EBV DNA copies/β-globin DNA copies x $2x10^5$] = log (copies EBV DNA/10^5 cells).

The samples were considered as negative for EBV status if in the channel JOE (Yellow) the value of Ct was ≥ 30 cycles.

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific EBV primers and probes. The specificity of the kit was 100%. The potential cross-reactivity of the kit was tested against the group control (CMV, HSV 1 & 2, HHV6, HHV8, VZV, Parvovirus and other ones). It was not observed any cross-reactivity with other pathogens. The sensitivity of the kit was not less than 200 copies/ml or 50 copies of EBV DNA per 10^5 cells.

EBER-ISH was then performed on paraffin section using a fluorescein-conjugated PNA probe targeting EBER RNA (Dako) according to manufacture. A tumor was considered EBV-related if the EBER signal was localized in more than 10% of neoplastic cells.[1] A case was considered EBER-negative if EBER staining was undetected or was apparently only in benign-appearing lymphoid cells.[11,12]

Two well-known EBER-ISH positive neoplasms, a classical Hodgkin's Lymphoma (cHL) and a B-cell Lymphoma associated with HIV infection, were considered as positive controls.

Result

Using the Q-PCR analysis, twenty-nine (29) out of 43 (67,44%) of the cases examined were negative for the presence of viral DNA, 13/43 (30,23%) of cases were positive having a number of viral DNA copies per cell (log EBV DNA/10^5 cells) less than 6 and 1/43 (2,32%) was positive having a number of viral DNA copies per cell greater than 6 (Table 1).

All cases have been also tested with EBER-ISH. None of Q-PCR negative (Ct ≥ 30 cycles) DLBCL-*e* patients showed EBER-ISH positivity (Table 1 and Figure 1).

Only one case was defined as DLBCL-EBV$^+$-*e* being Q-PCR positive, with a value of log EBV DNA/10^5 cells ≥ 6 and EBER-ISH positive, with a percentage of neoplastic cells > 80% (Figure 1, panel a). In our cohort the prevalence of DLBCL-EBV$^+$-*e* was 2,32%, in agreement with the data reported in the literature for the Western population.[1]

The control cases showed at Q-PCR a copy number higher than 6. EBER-ISH was positive only in the Hodgkin/Reed Sternberg cells of the classical Hodgkin's Lymphoma (Figure 1, panel b) and in > 60% of cells of the B-cell lymphoma associated to HIV infection (Figure 1, panel c).

Table 1: UCBM cohort of primary DLBCLs with > 50 years and without known underlying immunosuppression condition. For each sample is reported the Q-PCR score (expressed both as "DNA EBV copies in 10^5 cells" and "Log EBV copies in 10^5 cells") and EBER-ISH positive (POS) or negative (NEG) result.

Sample number	EBV copies/105 cells	Log EBV/105 cells	EBER-ISH
2002-1	0	/	NEG
2003-1	0	/	NEG
2003-2	0	/	NEG
2003-3	49810,19881	4,7	NEG
2005-1	0	/	NEG
2005-2 (CTRL+)	1790411,71	6,25	POS in RS cells
2006-1	0	/	NEG
2006-2	21180,10767	4,32	NEG
2006-3	0	/	NEG
2007-1	0	/	NEG
2009-1	13283,20495	4,12	NEG
2009-2	0	/	NEG
2009-3	26630,5743	4,42	NEG
2009-4	155469,4994	5,19	NEG
2010-1	0	/	NEG
2010-2	139636,9303	5,14	NEG
2010-3	0	/	NEG
2010-4	0	/	NEG
2010-5	0	/	NEG
2011-1	47465,15021	4,67	NEG
2011-2	0	/	NEG
2011-3	0	/	NEG
2011-4	19076,22415	4,28	NEG
2011-5	0	/	NEG
2011-6	0	/	NEG
2011-7	0	/	NEG
2011-8	0	/	NEG
2011-9	0	/	NEG
2011-10	0	/	NEG
2011-11	0	/	NEG
2011-12	169733,0092	5,23	NEG
2012-1	0	/	NEG

Sample number	EBV copies/105 cells	Log EBV/105 cells	EBER-ISH
2012-2	0	/	NEG
2012-3	0	/	NEG
2012-4	42649,30616	4,63	NEG
2012-5	0	/	NEG
2012-6	20624,66117	4,31	NEG
2012-7	34747,20526	4,54	NEG
2012-8	49263578,61	7,7	POS in > 80% neopl. cells
2013-1	0	/	NEG
2013-2	0	/	NEG
2013-3	25151,3774	4,4	NEG
2013-4	0	/	NEG
2013-5	0	/	NEG
2013-6 (CTRL+)	3642383,665	6,56	POS in > 60% neopl. cells

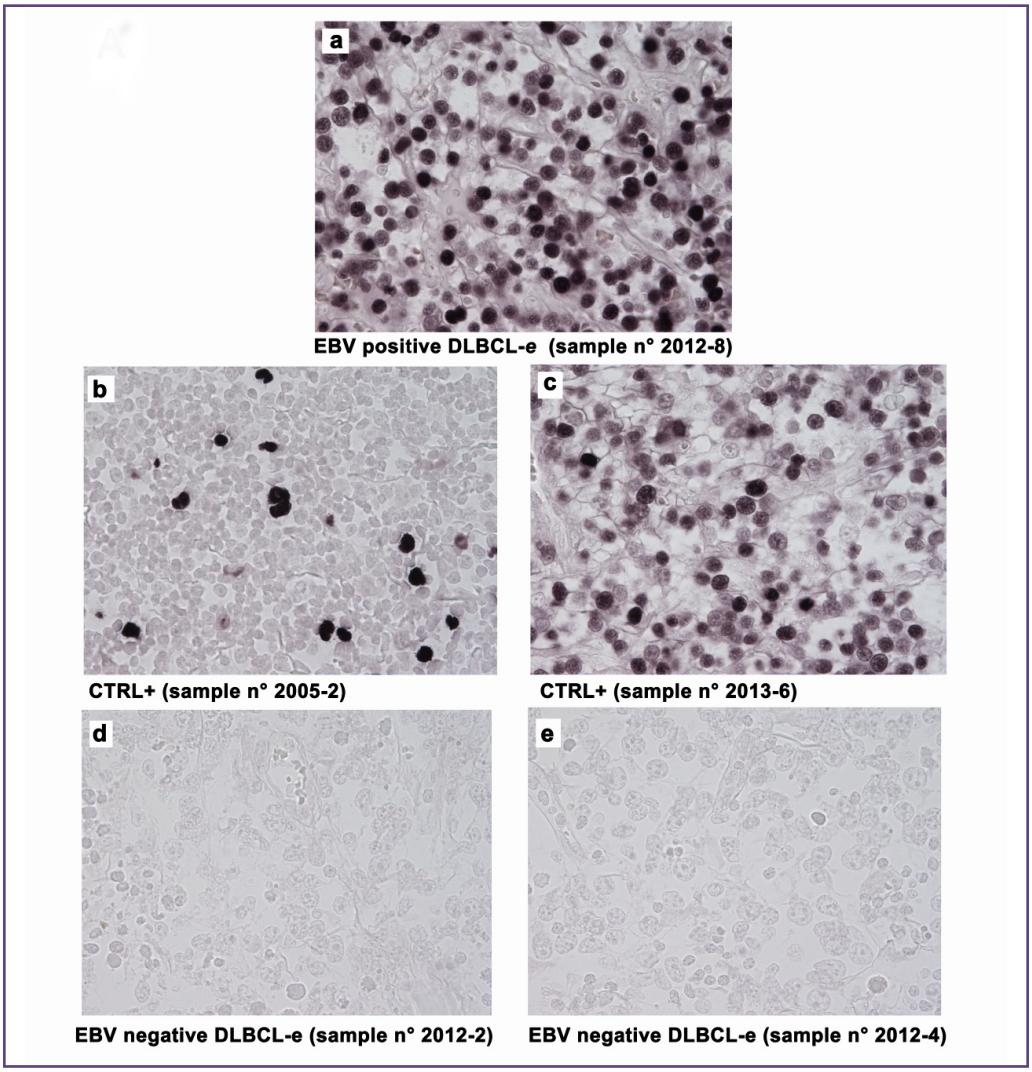

Fig. 1: Representative EBER-ISH images. Representative EBER-ISH images for: (panel a) the unique EBV+ DLBCL-e case (sample 2012-8); (panel b) a classical Hodgkin's Lymphoma as a positive control (CTRL+) that shows the positivity only in the Hodgkin/Reed Sternberg cells (sample 2005-2); (panel c) a B-cell lymphoma associated to HIV infection (CTRL+; sample 2013-6); (panel d) a negative sample both in Q-PCR and in EBER-ISH (sample 2012-2); (panel e) a EBER-ISH negative sample with a Q-PCR score positive <6 (sample 2012-4). The magnification is 40X for each panel.

Discussion

DLBCL-EBV+-*e* is defined as a histologically malignant polymorphic or monomorphic B cell lymphoproliferation in patients older than 50 years without any known immunodeficiency or prior lymphoma. The clinical behavior is aggressive, with frequent extranodal presentation (e.g stomach, lung, tonsils and skin) and overall poor prognosis. There is some morphologic overlap with cHL, also encountered in the elderly but reported to have a better prognosis. The lesion is thought to be related to defective immune surveillance of EBV secondary to immunosenescence, the natural decay of the immune system as a consequence of aging.[13,14]

The cutoff value of 50 years used to define this type of lymphoma is arbitrary, because patients <50 years can also be affected.[5] For this reason, the update of the 4th edition of the WHO Classification of tumors of hematopoietic and lymphoid tissues, expected for mid-2016, likely will define the disease EBV+ DLBCL, NOS, avoiding the term "elderly".

According to the exact definition of this entity at the moment, we restricted our study to patients older than 50 years, considering that in the future our approach could be extended to a larger cohort of DLBCL or other type EBV related neoplasms.

Our results obtained on this selected cohort, show that, using Q-PCR as a screening test, all cases negative for Q-PCR (Ct ≥ 30 cycles) would not need to be subjected to the EBER-ISH (about 70%), with a considerable time, costs and resources saving. Furthermore, we show that Q-PCR positive cases with a value of log EBV DNA/10^5 ≥ 6 would correspond to the real expression of the virus inside of neoplastic cells, while Q-PCR positive cases with a value of log EBV/10^5 cell < 6 would correspond to the presence of EBV-DNA in the latently infected memory B cells (type 0 latency). These normal B cells, virtually present in the tissue and not eliminable with microdissection, are not detected by EBER-ISH.[2,8,11,15] Accordingly, we verified the presence of EBV specific IgG in the sera of the patients with a positive value of log EBV/10^5 cell < 6.

Moreover, the evidence of an association between EBV and cancer is well defined. EBV has a causative role in IM and X linked lymphoproliferative disease and has a strong association with Burkitt's lymphoma, cHL, lymphomatoid granulomatosis, primary effusion lymphoma, plasmablastic lymphoma, posttransplant lymphoproliferative disorders, extranodal NK/T cell lymphoma, nasal type, and nasopharyngeal carcinoma.[16,17]

Dojcinov et al. also described EBV-driven proliferations presenting in cutaneous or mucosal sites, termed mucocutaneous ulcer, that are histologically alarming, but have a much more indolent and often self-limited clinical course.[18]

Then, although it is to be taken with caution, log EBV/10^5 cell = 6 could be considered a cut-off value useful to reveal the presence of the virus inside of neoplastic cells (Figure 2), not only in DLBCL but also in these other types of EBV related diseases.

Of note, using EBER-ISH analysis there are no uniform criteria for percentage of EBV-positive cells in EBV-positive DLBCL. Wada and other authors[19,20] emphasized the need for establishing uniform criteria for EBV positivity, either >20%, >50% or almost all tumor cells. Hofscheier et al[10] interpreted EBER-positive DLBCL with <20% EBER as clonally unrelated EBV transformed B cells or secondary EBV infection in an established B-cell clone.

Therefore, if this approach will be confirmed with a larger cohort of DLBCL or other type EBV related neoplasms, it could not only potentially avoid EBER-ISH analysis, but also solve the difficulties to define uniform criteria for EBER-ISH.

In addition to already mentioned novel therapeutical strategies, several clinical trials are also currently ongoing for the treatment of EBV positive lymphomas. In particular, the combination of arginine butyrate and ganciclovir induced clinical responses in 10 of 15 patients (67%) diagnosed with EBV positive lymphoma.[21] Preclinically, bortezomib and simvastatin have shown to be effective at inducing apoptosis in EBV-positive lymphoblastoid cell lines (LCLs) and at delaying onset of lymphoma and increasing survival in SCID mice injected with EBV-positive LCLs.[22] Both medications seem to exert their effect by blocking NF-kB pathway signaling.

Conclusion

DLBCL-EBV+-*e* reflects the heterogeneity of B-cell neoplasms and the complexity of the WHO classification.

The promising results of our study encourage to confirm the reliability of our new Q-PCR/EBER-ISH combined protocol (Figure 2) on a larger series of EBV+ related processes, to better identify patients eligible for novel therapeutic approaches.

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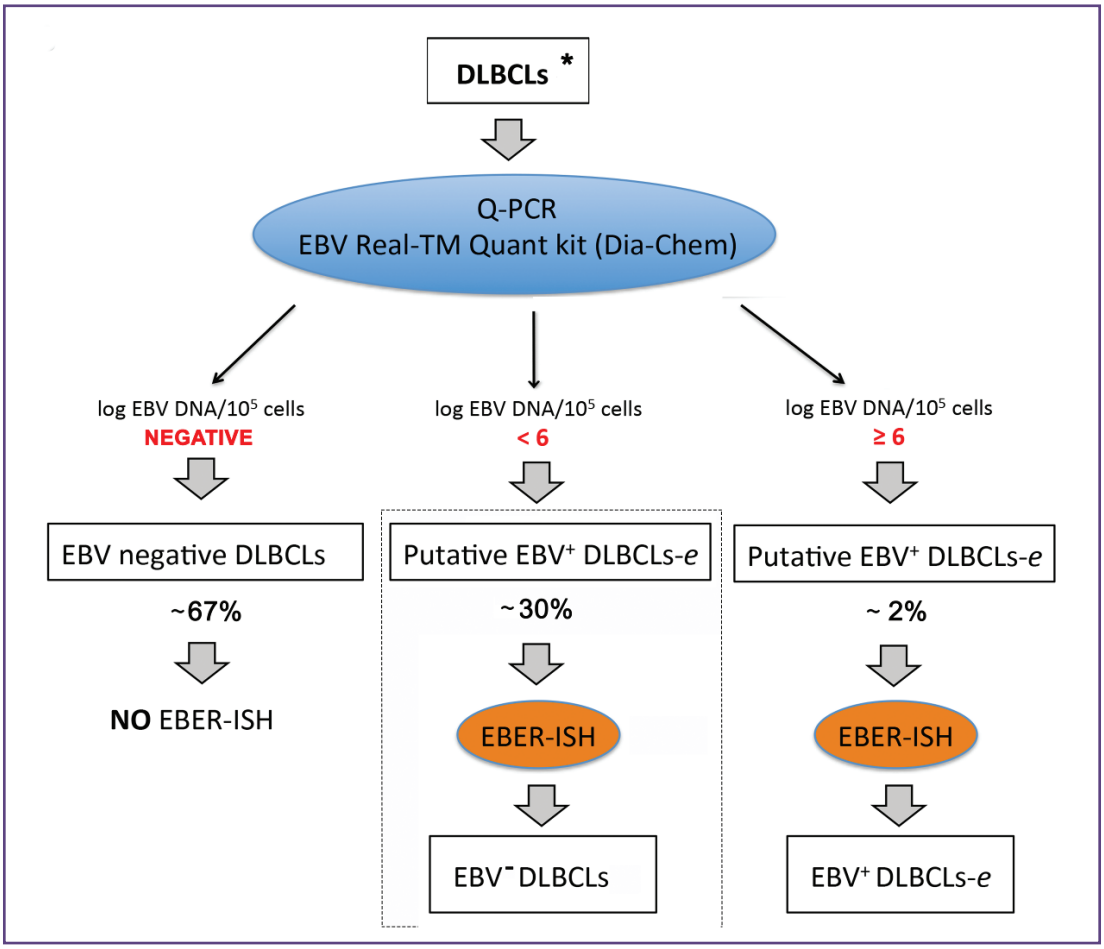

Fig. 2: Flowchart of our diagnostic protocol. Flowchart of our diagnostic protocol. The asterisk indicates DLBCLs with > 50 years and without known underlying immunosuppression condition. The dashed box highlights the optional step according to the cut-off value log EBV/105 cell = 6.

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Competing Interests
None declared

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