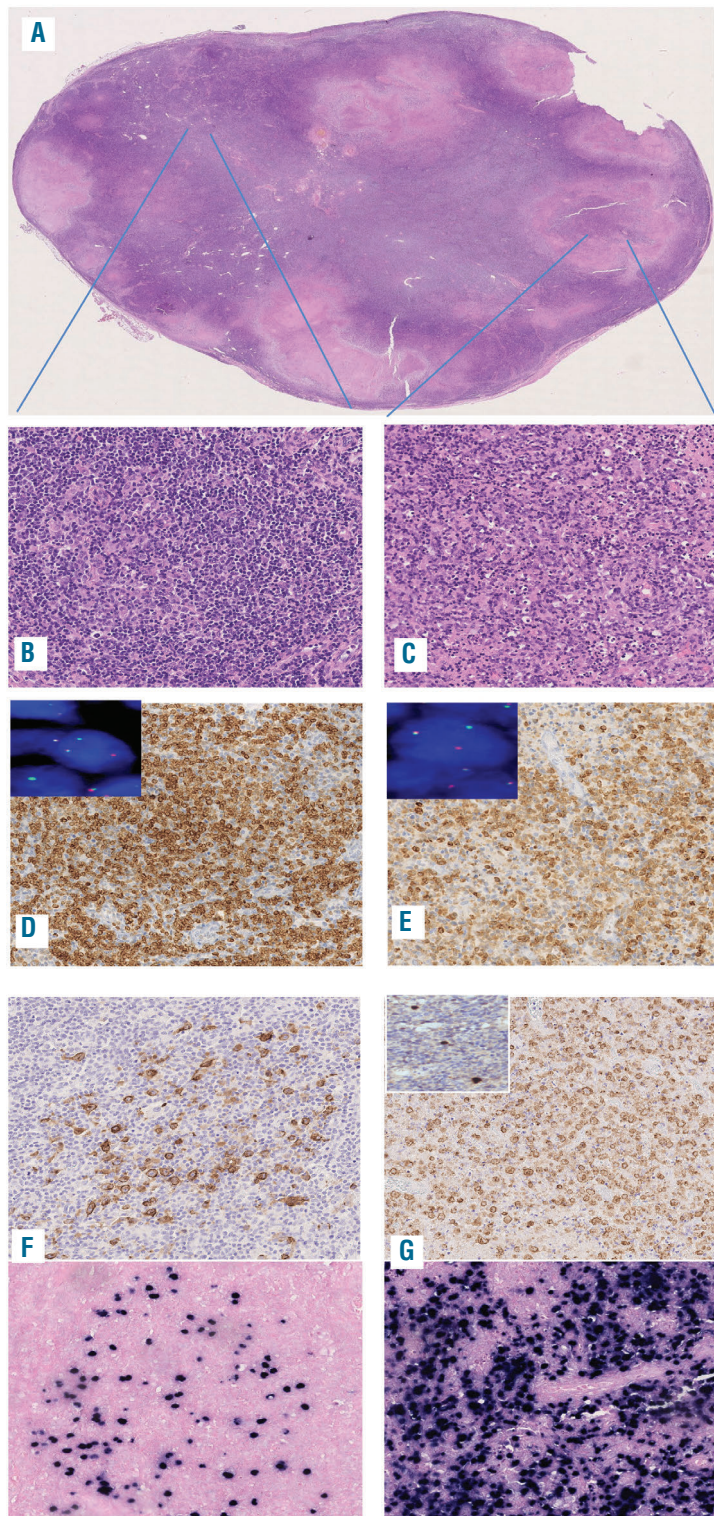


### Role of Epstein-Barr virus in transformation of follicular lymphoma to diffuse large B-cell lymphoma: a case report and review of the literature

The Epstein-Barr virus (EBV) is the most common human virus, implicated in the pathogenesis of several human tumors, particularly B-cell lymphomas.<sup>1</sup> EBV-induced lymphoproliferative disorders are the result of the outgrowth of EBV-infected B cells that would normal-

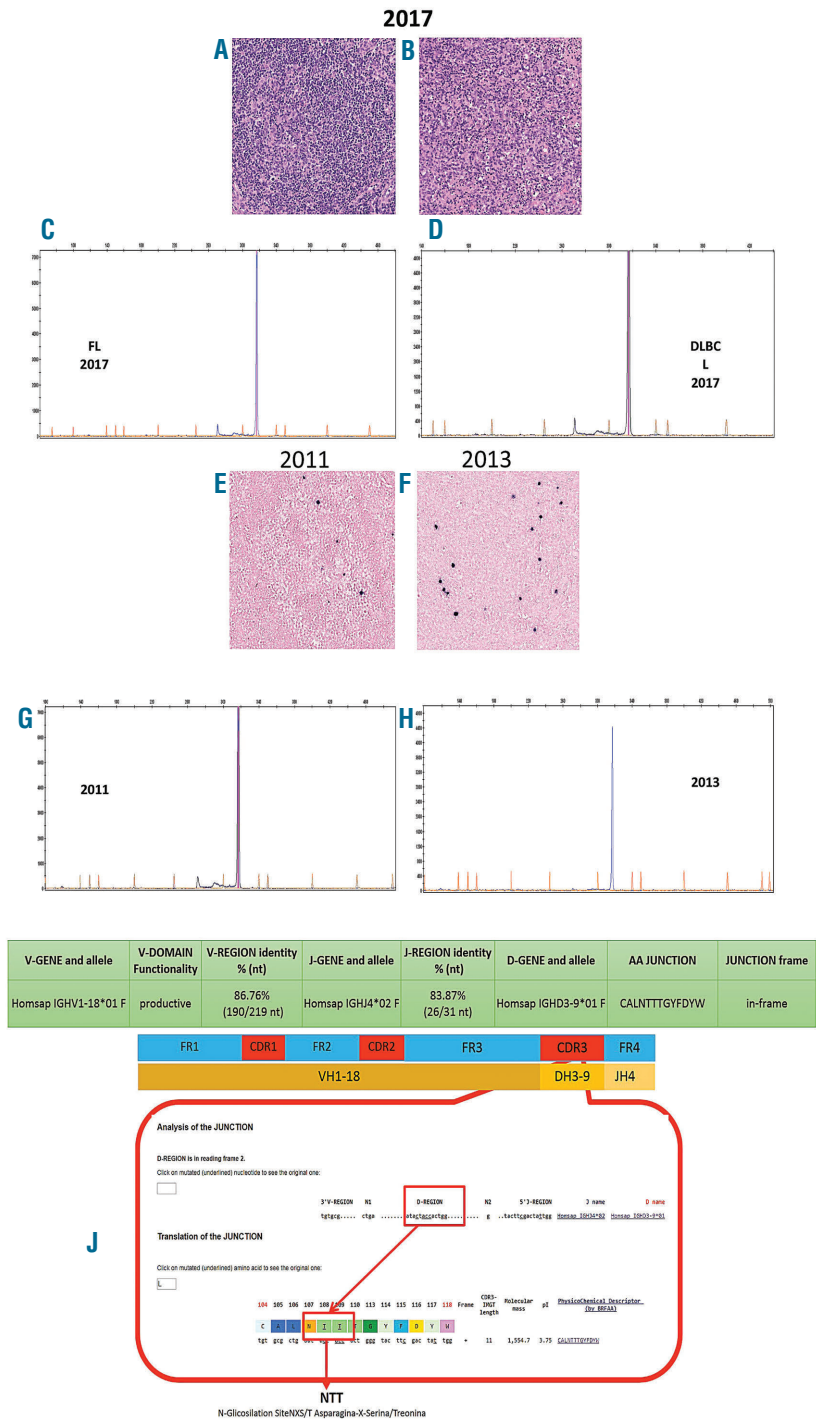
ly be controlled by an effective EBV-specific cytotoxic T-cell response.<sup>2,3</sup> The association between EBV and follicular lymphoma (FL) is not well recognized and only ten cases have been previously described.<sup>4-7</sup> Nine of them showed EBV-encoded small RNA (EBER)-positivity in >75% of the tumor cells and one case in ~5–10%. EBV-positivity was associated with grade 3 FL (9/10 cases), CD30 expression and rapid progression of disease.<sup>5</sup> However, it is still unclear whether EBV infection is an early event in the development of FL or a late event that



**Figure 1. Histological examination of the 2017 specimen.** (A-C) Effacement of the nodal architecture and cellular necrosis (A) with areas of grade 2 follicular lymphoma (B) intermingled with a diffuse proliferation of blastic elements (C) were seen. (D,E) The neoplastic cells of the follicular component expressed BCL-2 (D) as did the diffuse component (E). Fluorescence *in situ* hybridization using break-apart probes was positive for the *BCL2* translocation in both the follicular (inset 1D) and diffuse (inset 1E) areas. (F,G) Positivity for LMP-2A and EBV-encoded RNA *in situ* hybridization was present in scattered neoplastic cells within the follicles of the follicular areas (F) and in 90% of the neoplastic cells in the diffuse areas (G). Immunohistochemistry for Zebra was also positive in scattered larger cells in the diffuse areas (inset 1G). Original magnification: (A) 2.5x; (B-E) 10x; (F,G) 20x.

may contribute to the progression of a pre-existing lymphoma. We describe a case of EBV-positive diffuse large B-cell lymphoma (DLBCL) that evolved from a previous FL. In April 2011, a 73-year old woman presented with systemic lymphadenopathy and a history of weakness. A right inguinal lymph node was removed for histological examination which showed complete effacement of the nodal architecture by closely packed follicles, with attenuated mantle zones, loss of polarity and absence of tingible body macrophages. The tumor cells expressed CD20, CD10, BCL-2, BCL-6 and, focally, CD30. The proliferation index (Ki-67) was about 20%. Fluorescence *in situ*

hybridization using break-apart probes identified the typical *BCL2* translocation. Due to the involvement of multiple extranodal sites, a diagnosis of FL, grade 2, stage IVA (Ann Arbor system) was made. The patient was treated with four cycles of rituximab plus fludarabine, mitoxantrone, and dexamethasone, followed by maintenance therapy (rituximab). The treatment was discontinued in 2013 because the disease was progressing. Biopsy of a right axillary lymph node was performed, confirming the previous diagnosis with an increased proliferative index (40%). The patient was placed in a "watch and wait" program. In March 2016, a follow-up computed tomography

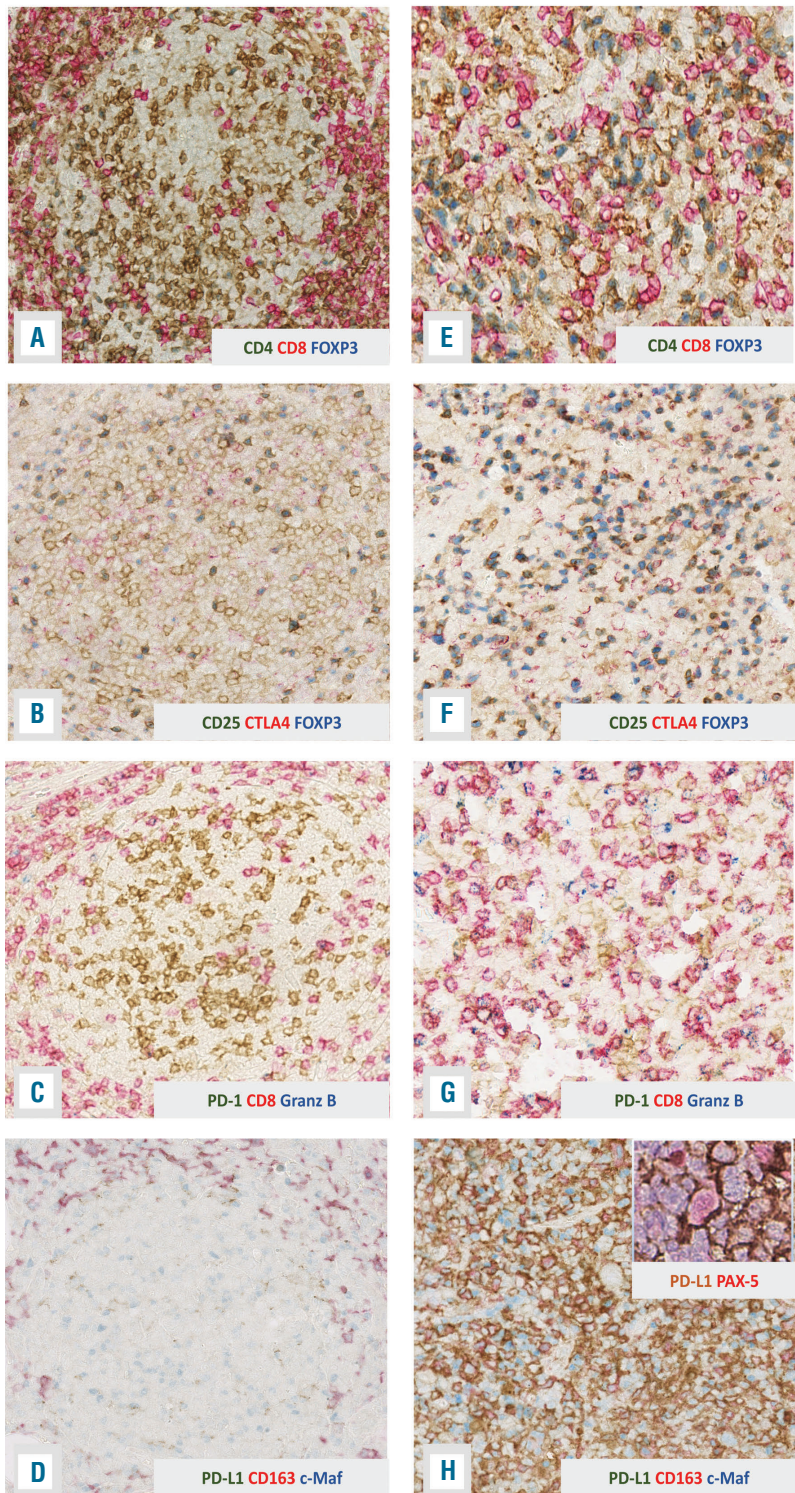


**Figure 2.** B-cell clonality, an identical N-glycosylation motif and Epstein-Barr virus positivity were demonstrated in all the specimens. (A,B) Laser capture microdissection of the follicular and diffuse areas of the 2017 lymph node biopsy. (C,D) A peak of 316 base pairs (bp) in length was identified in both the follicular and diffuse components. (E,F) EBV-encoded RNA *in situ* hybridization was positive in the 2011 and 2013 follicular lymphoma samples of the patient, increasing from the first (5%) to the second (10%) biopsy. (G,H) The clonal analysis of the 2011 and 2013 follicular lymphoma samples identified a peak with the same length as that of the 2017 sample (316 bp). (I) The corresponding FR1-JH polymerase chain reaction products of all the samples were directly sequenced and a productive *IGH-VDJ* rearrangement (V1-18\*01, D3-9\*01 and J4\*02) with 86.76% homology with the germ-line sequence was detected, confirming the clonal relationship. (J) Furthermore, in all the biopsies, we detected an identical somatic hypermutation pattern and the same N-glycosylation motif (NTT) in the HCDR3 region, characteristic of follicular lymphoma. Original magnification: (A,B,E,F) 10x. FL: follicular lymphoma; DLBCL: diffuse large B-cell lymphoma.



scan revealed enlargement of mediastinal and abdominal lymph nodes and laboratory tests showed increased serum lactate dehydrogenase levels. The patient started the rituximab plus cyclophosphamide, vincristine and prednisolone protocol which was interrupted in March 2017 because of pulmonary intolerance. A new regimen with rituximab and fludarabine *per os* was started. In November 2017, the patient was hospitalized for recurrent non-infectious fever and a left inguinal-crural lymph

node biopsy was performed. Histological examination showed the presence of a diffuse proliferation of large lymphoid cells intermingled with areas of FL grade 2 and areas of necrosis (Figure 1A-C). The neoplastic cells of both components expressed PAX-5, CD10, BCL-2, BCL-6, MUM-1 and CD30. The proliferative index was 70% in the areas of diffuse proliferation. Fluorescence *in situ* hybridization using break-apart probes identified the *BCL2* translocation in both areas (Figure 1D,E). Ninety



**Figure 3. Time trend of evolution of the tumor microenvironment evolution.** (A-D) In the follicular lymphoma sample, the neoplastic follicles showed a low number of regulatory T cells (A,B) and exhausted T cells (C). Numerous M2 macrophages were also found in the interfollicular areas accompanied by occasional M2 macrophages expressing PD-L1 (D). (E-H) The diffuse large B-cell lymphoma biopsy showed increased regulatory T cells (E,F) and exhausted T cells (G). The number of M2 macrophages was increased and the great majority of them expressed PDL-1 (H). PD-L1 expression was also demonstrated in scattered neoplastic cells by double staining with PAX-5 and PD-L1 (H inset). Original magnification: (A-H) 20x.

percent of the neoplastic cells in the diffuse area and scattered cells in the follicular area were positive for Epstein-Barr latent membrane protein (LMP)-2A and EBV *in situ* hybridization (Figure 1F,G). A diagnosis of DLBCL transformed from FL was proposed. Due to the patient's critical clinical condition and high viral loads of cytomegalovirus (32,461 copies) and EBV (24,212 copies) in the serum, the patient was treated with rituximab and lenalidomide plus valgancyclovir for the cytomegalovirus infection. To evaluate whether we were dealing with the transformation of the pre-existing FL, a comparative evaluation of the original FL and DLBCL specimens was made in terms of clonality, EBV status and tumor microenvironment. To assess B-cell clonality, *IGH-VDJ* rearrangements were studied in all the specimens according to the BIOMED-2 protocol. Specifically, to evaluate whether the EBV-positive population observed in the histological sections of DLBCL corresponded to the follicular population, we performed laser capture microdissection of the diffuse and follicular areas of the 2017 biopsy (Figure 2A,B). A peak of 316 base pairs (bp) in length was identified in both (Figure 2C,D). We then checked the EBV status also in the 2011 and 2013 FL samples and found the virus in 5% (2011) and 10% (2013) of neoplastic cells within the follicles (Figure 2E,F). After laser capture microdissection, the clonal analysis of the 2011 and 2013 FL samples identified a peak with the same length as the 2017 one (316 bp) (Figure 2G,H). The corresponding FR1-JH polymerase chain reaction (PCR) products of all the samples were directly sequenced and a productive *IGH-VDJ* rearrangement (V1-18\*01, D3-9\*01 and J4\*02) with 86.76% homology of the germline sequence was detected, confirming the clonal relationship (Figure 2I). Furthermore, in all the biopsies, we detected an identical somatic hypermutation pattern and the same N-glycosylation motif (NTT) in the HCDR3 region (Figure 2J). Since the oncogenic role of EBV in leading the transformation of FL to a high-grade form is still controversial, a complete genetic assessment of the virus latency by quantitative reverse transcription PCR and immunohistochemistry was performed. The expression of EBV nuclear antigen (EBNA)-1 and the negativity for LMP-1 and EBNA-2 in the FL specimen suggested a type I latency. In the DLBCL sample, we detected a non-canonical latency pattern of neoplastic cells characterized by the expression of LMP-2A along with some genes/proteins involved in the lytic cycle [BMRF1/Ea-D, BHRF1/Ea-R/p17 and BZLF1/Zebra (Figure 1G inset)]. Finally, we studied the tumor microenvironment of the FL and DLBCL specimens to identify a possible alteration of the EBV-host balance responsible for the evolution. In the FL sample, the neoplastic follicles showed a low number of regulatory T cells (CD4<sup>+</sup>/FOXP3<sup>+</sup> and CD25<sup>+</sup>/FOXP3<sup>+</sup>/CTLA-4<sup>+</sup>) (Figure 3A,B) and CD8<sup>+</sup>/PD1<sup>+</sup>/Granzyme B<sup>+</sup> T cells (Figure 3C). The scenario was different in the transformed DLBCL biopsy, which showed increased regulatory and CD8<sup>+</sup>/PD1<sup>+</sup>/Granzyme B<sup>+</sup> T cells (Figure 3E-G). The latter were concentrated at the periphery of the necrotic areas; the center of the necrotic area showed a significant increase of CD8<sup>+</sup> T cells. In the FL sample numerous CD163<sup>+</sup>/c-MAF<sup>+</sup> macrophages (M2 macrophages) were found in the interfollicular areas accompanied by occasional CD163<sup>+</sup>/PD-L1<sup>+</sup> cells (Figure 3D). In DLBCL, the M2 macrophages increased and the great majority expressed PD-L1 (Figure 3H). PD-L1 expression was also demonstrated in scattered neoplastic cells (Figure 3H inset).

The patient's clinical condition worsened and she died in June 2018.

The process of transformation has some confounding factors: the impact of chemotherapy on the immune microenvironment; the role of the immune system; the proliferative advantage provided by EBV; the impact of EBV latency program modification and expression of additional EBV products; and the natural evolution of FL independently of EBV. Our case favors the hypothesis that EBV was the trigger responsible for the transformation of FL in DLBCL, under permissive immunological conditions. Indeed, the evidence of EBV lytic cycle reactivation in the DLBCL sample and the expression of a non-canonical latency program, as demonstrated by quantitative reverse transcription PCR and immunohistochemistry, may shed light on the effective role of the virus in the transformation of the disease and in the maintenance of an immunosuppressive niche. Conventionally, EBV-driven lymphomagenesis is thought to involve primarily the latent cycle, but there is increasing evidence that lytic gene products contribute to the development and maintenance of malignancies through the induction of growth factors, oncogenic cytokine production such as interleukin-10, transforming growth factor- $\beta^{9,11}$  and the evasion of the inflammatory response by attenuation of interferon- $\gamma$ .<sup>12</sup> Furthermore, we were able to demonstrate the clonal relationship between the diffuse areas and follicular components in all three biopsies and direct sequencing of the FR1-JH PCR products showed an identical *IGH-VDJ* rearrangement. These findings were also consolidated by the identification of an identical somatic hypermutation pattern and the same NTT which are characteristic of FL and uncommon in other B-cell malignancies.<sup>8</sup>

The role of the virus in the transformation is further supported by the changes induced in the tumor microenvironment. In fact, a valuable CD4/PD-1 T-cell population controls the response against the virus in the follicular components.<sup>2</sup> Following chemotherapy, the balance between EBV and the host immune system might have been disrupted leading to unchecked EBV activation resulting in non-canonical latency of the virus in the DLBCL sample and changes in the inflammatory microenvironment.<sup>9</sup> In fact, in the diffuse areas, we found an increased number of T-regulatory cells and CD8<sup>+</sup>/PD1<sup>+</sup>/Granzyme B<sup>+</sup> T cells as well as the expression of PD-L1 on M2 macrophages and lymphoid cells. The PD-1/PD-L1 pathway is a key check-point in controlling the re-education of the tumor microenvironment,<sup>15</sup> diminishing activation of tumor infiltrating lymphocytes and inhibiting phagocytosis and tumor immunity, promoting lymphoma growth.<sup>14</sup> This pattern seems to be different from EBV-negative DLBCL in which the number of M2 macrophages expressing PD-L1 is lower than in our case (preliminary, unpublished results from Marafioti *et al.*).

Notwithstanding this evidence, the precise role of EBV lytic activity and its ability to reshape the tumor microenvironment remains elusive and further investigation is warranted. However, our findings expand the spectrum of recognized EBV-associated B-cell lymphomas and raise the question as to whether EBV association should be investigated in cases of FL to establish its relationship with lymphomagenesis or transformation.

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doi:10.3324/haematol.2018.215053

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

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