Alteration of β2-microglobulin in EBV infected diffuse large B-cell lymphoma and its impact on clinical outcome of the patients

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Abstract

Cause(s) of diffuse large B-cell lymphoma (DLBCL) in patients without immunodeficiency is still unclear. This study focused on genetic pathogenesis and tumor microenvironment of Epstein-Barr virus (EBV) positive DLBCL in these patients. DNA samples from these cases were sequenced by next generation sequencing (NGS) using a selected gene panel. Results revealed that most gene mutations were not specific for EBV positive DLBCL. However, a mutation of β2-microglobulin (B2M) was significantly increased while HLA-I or HLA-II (human leukocyte antigen I or II) expression was decreased in these cases. The B2M mutation and dysregulation of B2M expression were further confirmed by Sanger sequencing and immunohistochemistry, respectively. In the tumor tissues, the infiltration of CD8+ T lymphocytes HLA-I and/or HLA-II expression were decreased. The overall survival of the EBV positive DLBCL patients with B2M inactivation was shorter than those with EBV negative DLBCL. These results suggest that the mutation of B2M could cause the disruption of the expression and functions of HLA molecules, leading to decreasing the expression of HLA-I or HLA-II and subsequently to reducing T lymphocyte infiltration in these tumor tissues. The consequence of the events could lessen the recognition and elimination of EBV+ tumor cells by host immunity. It might be one of mechanisms for the virus to obtain the ability of the host immune tolerance to EBV+ tumor cells by evading immune recognition and escaping the T lymphocyte killing.

Introduction

Epstein-Barr virus (EBV) positive diffuse large B-cell lymphoma (DLBCL) is a proposed entity as a subtype of DLBCL without age limitation in the recently revised World Health Organization (WHO) classification of lymphoid neoplasm [1-4]. It accounts for 3% to 15% of total DLBCL cases reported from different countries in different age groups [1, 2, 5-7]. Although the importance of the EBV positive DLBCL has been well established, data regarding outcome of this tumor are conflicting as to its special etiology and pathogenesis [7-9].

Human is typically infected by EBV in their early life as a subclinical illness [10]. Most people tolerate latent EBV infection without adverse effects [10]. EBV is highly immunogenic. EBV specific CD8+ T cells are critical for the human immune response to the viral infection. EBV is causally associated with some malignancies in certain individuals [11]. By regulating different pathways, EBV promotes survival of B cells and is implicated in the pathogenesis of DLBCL [5, 6, 12, 13]. Although intrinsic mechanism of EBV carcinogenesis is unknown in the human being, immunoreaction with this virus may play essential roles in EBV positive DLBCL development [10-13]. The etiology might be associated with the immune tolerance induced by EBV, but the mechanism is far from clear [7-9].

Tumor microenvironment (TME) and limited immune surveillance play important roles in the lymphoma pathogenesis and clinical outcome [13, 14]. In EBV positive DLBCLs, this microenvironment could be essential for tumor cells to interact with a variety of non-malignant cells in the EBV positive milieu and to create a special immunosuppressive microenvironment [15, 16]. The immune escape of EBV from important immune cells such as T-lymphocytes and macrophages could lead to the evasion of immune recognition [17]. However, the connection

of the immune microenvironment to the pathogenesis and tumor survival of EBV positive DLBCL is still poorly understood [14, 18, 19]. Understanding the mechanism could be valuable to future DLBCL treatment.

The aim of present study is to investigate the impact of EBV infection on clinical outcome of DLBCL patients and tumor microenvironment and molecular mechanisms of the immune tolerance in EBV positive DLBCLs.

Material and methods

Case selection

Three hundred forty-six cases of diffuse large B-cell lymphoma patients were identified from Pathology database of Department of Pathology, Changhai Hospital (Shanghai, China) over a 4-years period (2013.01-2016.12). Nearly all patients were residing in the east part of China. Patients with impaired immune system, secondary to primary immunodeficiency, HIV infection, transplantation and autoimmune disease, or with previous solid malignant tumor were excluded. Other exclusion criteria included evidences of acute or recent EBV infection (the presence of serum IgM specifically against EBV) and specific lymphoma subtypes known to be associated with EBV, such as Burkitt's lymphoma, classical Hodgkin lymphoma, lymphomatoid granulomatosis, primary effusion lymphoma and plasmablastic lymphoma. The clinical and laboratory data were collected in the hospital patients' data system, including disease location, B symptoms, lactate dehydrogenase (LDH), β2-microglobulin (B2M), peripheral blood counts, EBV serology, clinical stage, primary treatment, relapse, secondary treatment, current status, and date of last follow-up. Three pathologists reviewed all cases and a consensus was reached in terms of morphological subtypes according to the updated World Health

Organization (WHO) classification of tumors of hematopoietic and lymphoid tissue [1, 2]. This study was approved by the Institutional Review Board of Changhai Hospital. All patients were provided with a written consent of sample collection for research use. The study was performed in accordance with the Declaration of Helsinki and was approved by the local ethics review committee.

Immunohistochemistry and *in situ* hybridization studies

The immunohistochemical (IHC) and *in situ* hybridization analyses were performed on formalin-fixed paraffin-embedded (FFPE) tissues of all enrolled 346 DLBCL cases. Among these cases, 180 were prepared on tissue microarrays (TMA). The remaining 166 cases (samples) were on individual 3 μm FFPE tissue slides. Besides the routine diagnostic panel, IHC antibodies used in this study included CD3, CD4, CD8, CD68, CD163, B2M, PD1, PDL1, EBNA2, LMP1, HLA-I (human leukocyte antigen-I, HLA-ABC), HLA-II (HLA-DR) and Ki-67. Particularly, the mismatch repair (MMR) markers containing MLH1, MSH2, MSH6 and PMS2 were investigated. Sources and dilutions of all antibodies used in this study are listed in Supplemental Table 1. Immunohistochemistry was performed using EnVision Plus two-step system on an auto-Immunostainer (Bond, Leica, Germany). *In situ* hybridization for Epstein-Barr virus (EBV)-encoded small RNAs (EBERs) was conducted with an FITC-labeled oligonucleotide probe supplied by Ventana on an automated stainer (Ventana Benchmark, Tuscon, AZ, USA). Positive and negative controls were run along with all cases. The negative control sections were incubated with pre-immune serum. Three representative microscopic fields from each case were photographed with a 20X objective lens that covered a photographic area of 0.57 mm^2. EBERs positivity was scored when positive neoplastic cells

reached the 50% threshold. Immunohistochemistry results were evaluated by Allred score [20].

Genomic DNA extraction and next generation sequencing

Genomic DNA was extracted from EBV positive and negative DLBCL tumor tissues. Next generation sequencing (NGS) was conducted [21] using an 82 genes panel (1914 coding exons, Yuanqi BioPharmaseutics, Shanghai, China). Among the 82 tested, at least 11 genes (*PTEN, PIK3CA, ID3, PTPN6, B2M, CDX2NA, KMT2D, TCF3, MYD88, MYC, TP53*) have been reported to be linked to EBV associated malignancies, including nasopharyngeal carcinoma, gastric cancer, Hodgkin's lymphomas and other lymphomas [22-24]. DNA sequencing was performed on Illumina Hiseq1500TM system. The target genes in the NGS panel are listed in the supplemental Table 2. The coverage of every target region of the samples was internally normalized, compared to normalized control data with other samples in the same run.

DNA sequence verification

In order to verify specific and interesting gene mutations obtained from NGS, the PCR products were purified using Exo SAP-IT (Affymetrix/USB, Santa Clara, Calif) and Qiaquick PCR Purification Kit (QIAGEN, Valencia, CA). The purified samples were directly sequenced from both directions on Applied Biosystems3130 Genetic Analyzer (Thermo Fisher Scientific Inc). Negative controls with wild-type genes were included in Sanger sequencing. As controls, the Burkitt's lymphoma cell line DAUDI and DLBCL cell line LY10 were also used for detecting B2M mutation.

Statistical methods

Gene mutations and verification data were analyzed along with the immunohistochemical

results and patients' follow-up information. Overall survival (OS) was defined from the date of the diagnosis until the date of death or last follow-up. Survival distributions were estimated with the Kaplan-Meier method and significance of difference between pairs was determined by the log-rank test. Analyses were performed using SPSS23 (SPSS Institute, Inc, Chicago, US). Two-tailed student's test was used for statistical analysis for the comparison between two group. $P<0.05$ was considered statically significant.

Results

Clinical and Morphological Characteristics

The current study included 346 patients with DLBCL diagnosed in Changhai Hospital. All specimens were obtained at the time of the initial presentation. Clinical data were summarized in Table1.

All patients presented with lymphadenopathy that was either localized (n=52) in a single location or occurred at multiple sites (n=111). Mediastinal disease was present in 26 patients (7.6%). Extranodal non-lymphoid organ involvement was observed in 175 patients (50.5%), which included bone (n=26), liver (n=15), and lung (n=7). Clinically, 8% of patients (28/346) presented with splenomegaly, 47.4% had B symptoms, 28.9% had increased LDH, and 34.1% had elevated serologic B2M. Morphologically, there were two main histological patterns according to the WHO classification, which included: DLBCL- NOS (not otherwise specified) (n=344) and T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL)-like (n=2). Immunohistochemically, tumor cells had an intact B-cell phenotype with the expression of CD20, CD79a and PAX5. All 346 cases were analyzed for CD10, Bcl-6 and MUM1 expression in order to evaluate their B cell origin. About 51.7% of the cases were GCB (germinal-center

B-cell–like) subtype and the remaining cases were non-GCB subtype. Treatment and follow-up information were obtained for 346 patients (100%). The clinical stages of these cases at the initial diagnosis were: 52 at stage I, 56 stage II, 93 stage III, and 145 stage IV. About 68.8% of patients were presented at advanced stage of the disease when they visited hospital first time. All patients were treated with the combination of rituximab and other drugs. The most common regimen used was rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (346/346, 100%). Local radiation therapy was also applied to 37 patients (10.7%, Table 1)

EBV detection and following-up

We observed EBV infection status and clinico-pathological features of the 346 DLBCLs cases. One of the important criteria for the inclusion of EBV positive DLBCL cases in the study is that enrolled patients must be lack of history of immunodeficiency or immune compromise conditions. The evidence of EBV positive DLBCL is that more than 50% of the viable lymphoma cells are positive for EBV-encoded small RNAs (EBERs) by ISH (Figure 1B). By this criterion, the positivity for EBERs was found 10.7% (36/346) including DLBCL-NOS (diffuse large B-cell lymphoma, not otherwise specified) (n=34) and THRLBCL-like LBCL (n=2) cases. EBV latent membrane protein 1 (LMP1) was present in 18/346 (5.3%) of all analyzed cases. EBNA2 (EBV nuclear antigen 2) was negative in 34/36 (94%) cases. These results were consistent with EBV latency type II. EBV serologic data were available in 24/36 patients. All showed evidences of past infection: EBV viral capsid antigen IgG positive and EBV viral capsid antigen IgM negative. Twenty cases tested were also EBNA1 IgG positive. Among these 24 patients, 15 were positive for EBV early antigen IgG that was an indicator of virus reactivation.

The three common sites of EBV positive DLBCL were cervical (n=11), axillary (n=4), and supraclavicular (n=3). Five out of 36 EBV positive DLBCL cases (13.9%) had bone marrow involvement. For the treatment, only two patients received high-dose of carmustine, etoposide and cytarabine, as well as autologous stem cell transplantation (ASCT). Overall, with a median follow-up of 34 months (range 1-83 months). Among them, 66.7% (24/36) patients were alive with no evidence of the disease, 2.8% (1/36) were alive with lymphoma, and 30.3% (11/36) died of the disease. Surprisingly, there were no significant clinical differences between EBV positive DLBCLs and EBV negative DLBCL cohort in terms of age, gender, subtype, revised International Prognostic Index (R-IPI), Eastern Cooperative Oncology Group (ECOG) score, Ann Arbor stage, B symptoms, bone marrow involvement, serologic LDH and B2M, chemotherapeutic regimen and double/triple gene alteration (C-MYC, BCL2 and/or BCL6) detected by FISH and/IHC. EBV detection and clinical data are summarized in Table 1.

Gene mutations found by NGS in EBV positive and negative DLBCLs

In order to further understand the genetic changes of EBV positive DLBCL, nine cases with more than 80% EBV positive cells in tumor tissue were selected from all 36 EBV positive cases for detecting gene mutation by NGS. For the comparison, nine cases of EBV negative DLBCL were also evaluated by NGS at the same time. NGS results from EBV positive DLBCLs revealed the presence of recurrent alterations in genes *B2M* (6/9; 66.7%) and *PTPN6* (3/9; 33.3%), which are related to the tumor microenvironment and JAK-STAT pathways, respectively. The other mutated genes in EBV positive DLBCL were *MYC* (2/9; 3%), *TP53* (3/9; 33.3%), *KMT2D* (2/9; 22.2%), *MYD88* (2/9; 22.2%), *PIK3CA* (2/9; 22.2%), *NOTCH 1* (2/9; 22.2%) and *TCF3* (1/9; 11.1%). Mutations found in nine cases of EBV negative DLBCLs

sequenced by NGS included *TP53* (3/9; 33.3%), *TCF3* (2/9; 22.2%), *CDKN2A* (3/9; 33.3%), *MYC* (2/9; 22.2%), *ID3* (2/9; 22.2%), *PTEN* (2/9; 22.2%) and *B2M* (1/9; 11.1%). A mutation with the depth of NGS ≥350 leads and variant allele frequency (VAF) ≥10% was considered as a real mutation. The average of VAF of the mutations found in this study was 23.5% with a range of 10.5 to 46.3%. The results showed that mutations on *PTEN, ID3, CDKN2A* and *NOTCH1* genes were not specific to EBV positive DLBCLs. Although *PTPN6* and *TP53* had relatively high mutation rates, there was no statistically significant difference in these mutation rates between EBV positive and negative DLBCLs. B2M was the only gene to stand out regarding the mutation occurrence among the most high-frequently mutated genes between these two groups. Interesting, only one *B2M* mutation (NM_004048:exon1:c.67+2T>G) was detected (Figure 1E). This mutation was found 66.7% (6/9) in EBV positive cases and only 11.1% (1/9) in EBV negative cases were (Figure 1C, 1D left part) in the initial NGS sequenced 9 cases of EBV+ DLBCL and 9 cases of EBV- DLBCL. Later, we further verified and expanded the B2M mutation study by Sanger sequencing and IHC as shown in Figure 2.

B2M mutation verification

Because B2M gene is an invariant subunit of the class I human leukocyte antigen complex (HLA-I) involved in the process of presenting antigenic peptides derived from degraded self- (endogenous) or non-self (endogenous) proteins, including viral- or tumor- associated antigens [14, 25, 26]. It was reported that EBV negative lymphomas had more mutations of *KMT2D, B2M* and *MYD88* genes, and more antigen presentation aberrations as well as lower mutation burden than EBV+ lymphomas. EBV- Hodgkin lymphoma (HL), a typically nodular sclerosing tumor, is rich in *B2M* mutations. Mutations of *MYC*, and *JAK–STAT3* and

RAS–MAPK are common in plasmablastic lymphomas [27] and in HIV+ Plasmablastic lymphomas which occurs typically in the context of EBV latency I [28]. This study compared the mutational profile in EBV+ vs EBV- DLBCL. From our NGS data, we noticed that *B2M* mutation rate was higher in EBV positive DLBCLs than in negative cases. Then, by using Sanger sequencing technique, we verified the *B2M* mutation in 6 out of 9 EBV positive DLBCL cases and 1 out of 9 EBV negative cases previously sequenced by NGS. This special point mutation (exon1: c.67+2T>G) is expected to inactivate protein function of B2M based on the PolyPhen prediction algorithm, resulting in a transcript that encodes a truncated B2M protein. Additional 20 cases of EBV negative DLBCL and 27remaining unsequenced cases among the 36 EBV positive DLBCL enrolled in this study were also sequenced for the *B2M* mutation by Sanger sequencing. Results showed that this missense mutation NM_004048:exon1:c.67+2T>G (n=15) affected the start codon for methionine and converted it to arginine, lysine, or threonine (ATG to AGG/AAG/ACG) [29]. Overall, the *B2M* mutation was found by Sanger sequencing in about 41.7% (15/36) of the EBV positive DLBCL cases and only 20.7% (n=6/29) in the EBV negative DLBCL samples (Fig. 1D). The control Burkitt's lymphoma cell line DAUDI was found having the same missense mutation ATG to AGG as previously documented [29], while the DLBCL cell line LY10 was negative for the *B2M* gene mutation.

Decreased B2M expression and T cell infiltration in EBV+DLBCL with *B2M* gene alterations

It has been reported that the majority of the B2M mutations identified were unambiguously inactivating events with immune abnormality [14, 30]. The association of *B2M* gene alteration with defective HLA-I expression has been reported in several cancers, including colorectal

carcinoma and lymphomas rather than DLBCL. Furthermore, the B2M expression was also studied by immunohistochemistry in both EBV positive and negative malignancy cases. The lack of cell surface HLA-I expression due to different mechanisms such as abnormal DNA mismatch repair (MMR) allows tumor cells to escape from immune recognition by T lymphocytes in colorectal carcinoma [31]. Therefore, we particularly analyzed the expression of B2M, HLA-I, HLA-II, PD1, PDL1, M2 macrophages infiltration, MLH1, MSH2, MSH6 and PMS2 for MMR status in both EBV positive and negative DLBCL cases. We used the double staining of CD20 and CD8 or CD4 to highlight infiltrated T-lymphocyte in tumor tissues.

The expression of *B2M* was down-regulated. The decreased expression of B2M protein was found in 47.2% (17/36) of EBV positive DLBCL cases (Figure 1H, compared to Figure 1I in EBV-DLBCL). The expression of HLA-I or HLA-II was also significantly decreased in the B2M expression-impaired cases (13/17, 76.4%, Figure 1 J and L), compared to those in EBV-DLBCL Figure 1J and M, respectively). Immunohistochemical staining results revealed decreased infiltration of cytotoxic T cells (< 5%) in most of these EBV+ cases (16/17) (Figure 1N), compared with those in EBV- cases (Figure 1O). The B2M mutation and decrease of cytotoxic T cells in EBV positive DLBCL were correlated with patient's poor prognosis compared to EBV negative and B2M normally expressed cases (p=0.043) (Figure 1F). Among EBV negative DLBCLs, only a few cases showed HLA-I or HLA-II negative (n=2), and decreased infiltration of CD8+ T cell (< 5%，n=3). Interestingly, the serum level of B2M did not show any correlation with the cases of B2M negativity in tumors, and it did not reach significant difference between EBV positive and negative group either. On the other hand, the percentage of both CD20-/CD4+ T cells and CD20-/CD8+ T cells in EBV positive DLBCLs were much

lower than those in EBV negative cases. Additionally, the ratio of CD4: CD8 T cells was higher in EBV positive cases than EBV negative ones, which was related to the less infiltration of CD8+T lymphocytes (Figure 1G and 1N) in EBV+ cases. In this study, the M2 macrophages (CD163+ and CD68+) displayed the similar infiltration pattern in EBV positive and negative cases as shown in Figure 1P and 1O, and there was no difference in the percentage of the infiltrated M2 macrophages in tissues between these two groups of the cases (15/36 Vs 12/29, $p>0.05$). Meanwhile, we found the deficiency of MMR (dMMR) in one EBV positive DLBCL and two EBV negative DLCBL cases. These cases also had MSH2 and MSH6 deletions. In addition, two groups did not show significant difference in the expression of PD1 and PDL1 (8 /36 Vs 7/29; 10 /36 Vs 8/29. Images were not shown in Figures).

The overall consort diagram of this study was presented in Figure 2. The multivariable statistics showed that patient's age, B2M mutation and expression, HLA-I or HLA-II decreased expression, and infiltration of CD8+T lymphocytes were significantly associated with patient survival (Table 2).

Discussion

EBV has been closely linked to various malignant tumors. However, the detail pathogenesis is still not very clear [24]. The development of EBV associated tumors might be related to virus induced gene abnormalities and microenvironment changes [11]. EBV positive DLBCLs have been re-recognized recently because of controversial clinical outcomes from different areas [4, 5, 7, 8]. Here, we present a systematic evaluation of EBV status and clinicopathological features of 346 DLBCL patients resided in Eastern China. EBERs were found 10.7% of all tested cases. Ninety four percent of these EBV+ DLBCLs were EBV latency II (EBNA2

negative) without immunodeficiency. Latency II is defined by the expression of *EBNA1, LMP1,* and *LMP2A/B* and is most closely associated with Hodgkin lymphoma, NPC, and T/NK cell lymphomas. In order to better understand this type of special EBV related tumors, we initially studied gene mutations by NGS in 9 cases of EBV positive DLBCL, in which more than 80% tumor cells were EBER positive and in 9 cases of EBV negative DLBCL. Target genes in NGS panel were reported in literatures to be associated with EBV positive tumors including nasopharyngeal carcinoma, gastric cancer, Hodgkin's lymphomas and other lymphomas reported [21, 23, 32]. Unfortunately, the results showed that there was no difference in the profile of specific gene mutations between EBV positive DLBCLs and other EBV associated tumors [21, 23, 32]. Although PTPN6 and TP53 were found to be mutated with relative high percentages, the results did not reach statistical significance between EBV positive and negative DLBCL cases. However, we noticed that *B2M* mutation stood out among other gene mutations. Its mutation rate of *B2M* gene was much higher than other genes studied. The *B2M* mutation was significantly higher in EBV positive cases (66.7%) than in EBV negative cases (11.1%). We further confirmed the B2M mutation from NGS data and investigated the mutation in the rest of EBV positive DLBCL cases and some EBV negative DLBCL cases by Sanger sequencing. Results from Sanger sequencing confirmed B2M mutation by NGS. It also shown that B2M mutations occurred in in 41.7% (15/36) of EBV positive cases and in 20.7% (6/29) of EBV negative ones (Figure 1D).

B2M protein expression detected by Immunohistochemistry revealed that only 2.7% of these *B2M* mutated EBV positive cases had normal B2M expression level, significantly lower than that in EBV+ but B2M un-mutated cases and EBV negative cases. Although the cases number

is limited in this study, *B2M* gene alteration and decreased expression in EBV positive cases were correlated with poor prognosis of the patients, compared to EBV negative cases (p=0.043). These data present a special finding in EBV positive DLBCL patients, comparing with those reported in previous literature [4-7].

Tumor microenvironment is very intriguing. It plays a critical role in the regulation of tumor cell survival and proliferation as well as fostering immune escape [15, 16, 28]. *B2M* gene encodes the protein B2-microglobulin that is essential for MHC class I complex formation and its function [25, 26]. The MHC-I molecule is composed of HLA -I and B2M protein that acts as a stabilizing scaffold. Missense mutations and deletions in the B2M gene could crucially impair MHC-I assembly and cell surface expression [29, 30]. In particular, B2M gene alterations associated with defective HLA-I expression have been reported in a small number of lymphoma cases [31]. Furthermore, we found that the expression of HLA-I or HLA-II was much severely down regulated in EBV positive DLBCL than in negative ones that also had B2M mutation(s). However, there was no significant difference of B2M protein in blood between these two groups of DLBC lymphomas. The reason for this phenomenon is that the seral B2M proteins were expressed by all nucleated cells. Muted *B2M* cells are only accounted for a very small portion of entire nucleated cell population in the body. The decreased *B2M* expression in the small amount of the *B2M* mutated cells had a littler impact on the large pool of the seral B2M level. Miyashita K. and his colleagues previously reported that *B2M* mutations were linked to decreased MHC -1 expression and decreased patient survival in DLBCLs [33]. Because B2M is involved in the presentation of antigenic peptides derived from degraded self- or non-self-proteins, including viral- or tumor-associated antigens [23, 25, 26, 29, 30]. We

believe that in EBV infected DLBCLs, B2M mutations could be one of mechanisms that cause HLA-I and/or HLA-II associated abnormal immune tolerance to viral infection, leading to the development and progress of EBV infected DLBCL.

T lymphocytes play a major role in cell-mediated immunity. As part of HLA molecules, B2M is essential for MHC-I complex formation and peptide presentation [25, 26, 29]. The MHC-I molecules display extracellular peptides on the cell surface for the “inspection” by CD8+ T cells [34-36]. These T cells have the potential to recognize the MHC-I peptide complex and become activated cytotoxic T cells. In general, the immune evasion is a pathogenic mechanism used by several types of cancers in their development in order to escape from CD8 + T cells recognition. One of the main functions of CD4+ T cells is the regulation of antitumor immune responses by suppressing the proliferation of CD8+ T cells, which may lead to an immune escape and to the proliferation of cancer cells. In addition, we used double staining of CD20 and CD8 or CD4 highlighting the infiltrated T-lymphocyte in tumor tissues. Our results indicated that both CD20-/CD4+ T cells and CD20-/CD8+ T cells infiltrated in EBV positive DLBCLs were fewer than those in EBV negative cases, while the ratio of CD4:CD8 T cells in EBV positive DLBCLs was higher than that in EBV negative cases. This could be related to the decreased infiltration of CD8 + T cells within lymphoma tissues in EBV positive DLBCL cases. Meanwhile, in another study, it was found that the loss of HLA class I and II expression on DLBCL cells was associated with low infiltration by CD8+ T cells in tumor tissues [36]. We recognized that reduced membranous staining of HLA-I and HLA-II molecules in EBV positive B2M mutated DLBCLs was correlated with the low level of CD8+ T-cell infiltration. These results suggest that defects in HLA complexes due to B2M inactivation might

impair the recruitment of the tumor-infiltrating CD8+ T-lymphocytes in EBV positive DLBCLs, which induces host immunotolerance to viral infection and tumor cells, and leads to tumor development and progression as presented in previous studies in EBV infection [4, 35, 36].

B2M gene alterations have been reported to associate with defective HLA-I expression in colorectal carcinoma. Through heterogeneous mechanisms such as MMR, tumor EBV + DLBCL cells could enable the tumor cells to escape from immune recognition by T lymphocytes [36]. We particularly analyzed the MMR and T lymphocyte infiltration in both EBV positive and negative cases. We found dMMR in one case of EBV positive DLBCL and in two cases of the EBV negative ones. All of them showed MSH2 and MSH6 deletion without correlation to T lymphocyte infiltration. NK cell and macrophages are very important in tumor microenvironment [15-17, 36]. However, we found that there was no significant difference in the number of infiltrated CD56+ NK cells, CD163 and CD68+ M2 macrophages between EBV positive and negative groups except slight M2 macrophage polarity in EBV positive cases ($p>0.05$). The expression of PD1 and PDL1 between two groups did not show significant difference in our cases [37, 38]. Therefore, B2M gene dysfunction played a crucial role in impairing MHC class I assembly and HLA cell surface expression, which helps tumor cells escape from the recognition by cytotoxic T cells [36, 39]. This cascade event is closely related to HLA -I expression and CD8+ T lymphocytes infiltration, and partially HLA -II expression [40], which induce the immune toleration. However, there was no definite correlation to macrophages infiltration in tumor microenvironments of EBV positive DLBCL. Though *B2M* mutation is strongly associated with dMMR in colorectal carcinoma [34], we did not get significant mismatch repair events in EBV related DLBCLs. We also noticed that there was no

relationship between B2M mutation and the expression with serologic B2M level in EBV positive DLBCLs, which could be due to the secretion of large amount of B2M protein by the vast number of unaffected cells in the body as described above.

In summary, EBV represents a foreign antigen against host cytotoxic T lymphocyte immunity [15, 16, 36]. EBV within the lymphoma cells is strongly implicated in mechanisms of immune evasion by complex mechanisms [9, 13, 14]. The present consort prompts us to believe that B2M mutations in EBV positive DLBCLs might pave the way for the host immunotolerance to tumor cells through the deregulation of HLA expression and impairment of CD8+ T lymphocyte infiltration. The consequence of this could allow the tumor cells to evade human immunity and contribute to the development and progression of tumors, leading to the poor outcome of DLBCL patients. The results presented herein are novel and emphasize the importance of B2M mutation as one of mechanisms regulating host immune toleration to EBV+ tumor cells, which evade immune recognition and escape the T lymphocytes killing in EBV positive DLBCLs.

Supplementary Materials

Supplementary Table 1. Antibodies used for immunohistochemistry; Supplementary Table 2: Gene list in NGS target panel.

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Competing interests

The authors declare that no competing financial interest exists.

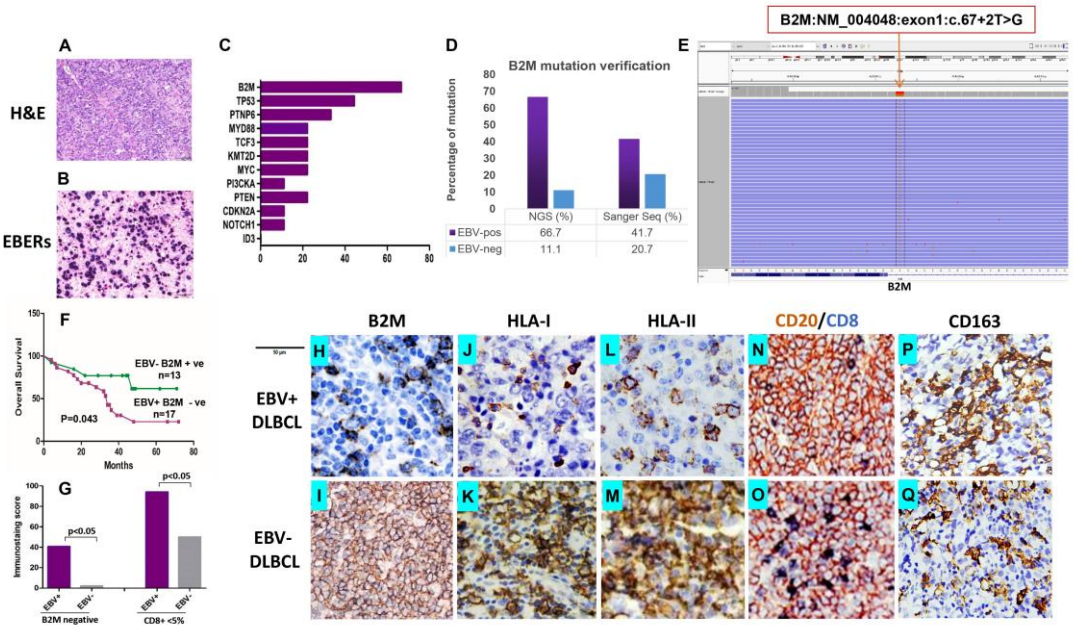

Figure 1. B2M mutation, micro-environment of EBV positive DLBCLs and impact on patients' clinical outcome

A. H & E staining of EBV positive DLBCL showed a typical morphology of DLBCL-NOS (H&E×400). **B.** Tumor cells were positive for EBERs detected by *in situ* hybridization (ISH×400). **C and D.** The percentage of gene mutations identified by NGS and PCR in EBV positive DLBCLs, respectively. **E.** A representative - *B2M* mutation (exon1, c.67+2T>G) in EBV positive DLBCL tissue showed by NGS. **F.** The overall survival of EBV positive DLBCL patients with B2M inactivation is shorter than those with EBV negative DLBCL and no *B2M* mutation (p=0.043). **G.** A comparison of the CD8 + infiltrated T Lymphocyte immunostaining score in tumor tissues between EBV positive DLBCL and negative ones (p<0.05) with and without *B2M* mutation. The data show that the immunostaining score in EBV+ DLBCLs was significantly higher than that in EBV- ones. *B2M* mutation(s) further increased the immunostaining score in both types of DLBCL tissues. **H-M.** Representative images of the expression of B2M, HLA-I and HLA-II in EBV + (**H, J** and **L**) and EBV - DLBCLs (**I, K** and **M**). The expression of these three molecules in EBV positive DLBCLs detected by IHC was much less than that in negative ones (IHC×400). **N** and **O**. Double immunostaining revealed that CD8 + infiltrated T

Lymphocytes (blue stained) in EBV positive DLBCL tumor tissues (**N**) were less than in negative ones (**O**) (IHC×400), while CD20 staining as a control was similar between EBV positive and negative DLBCL tumor tissues (not show here).

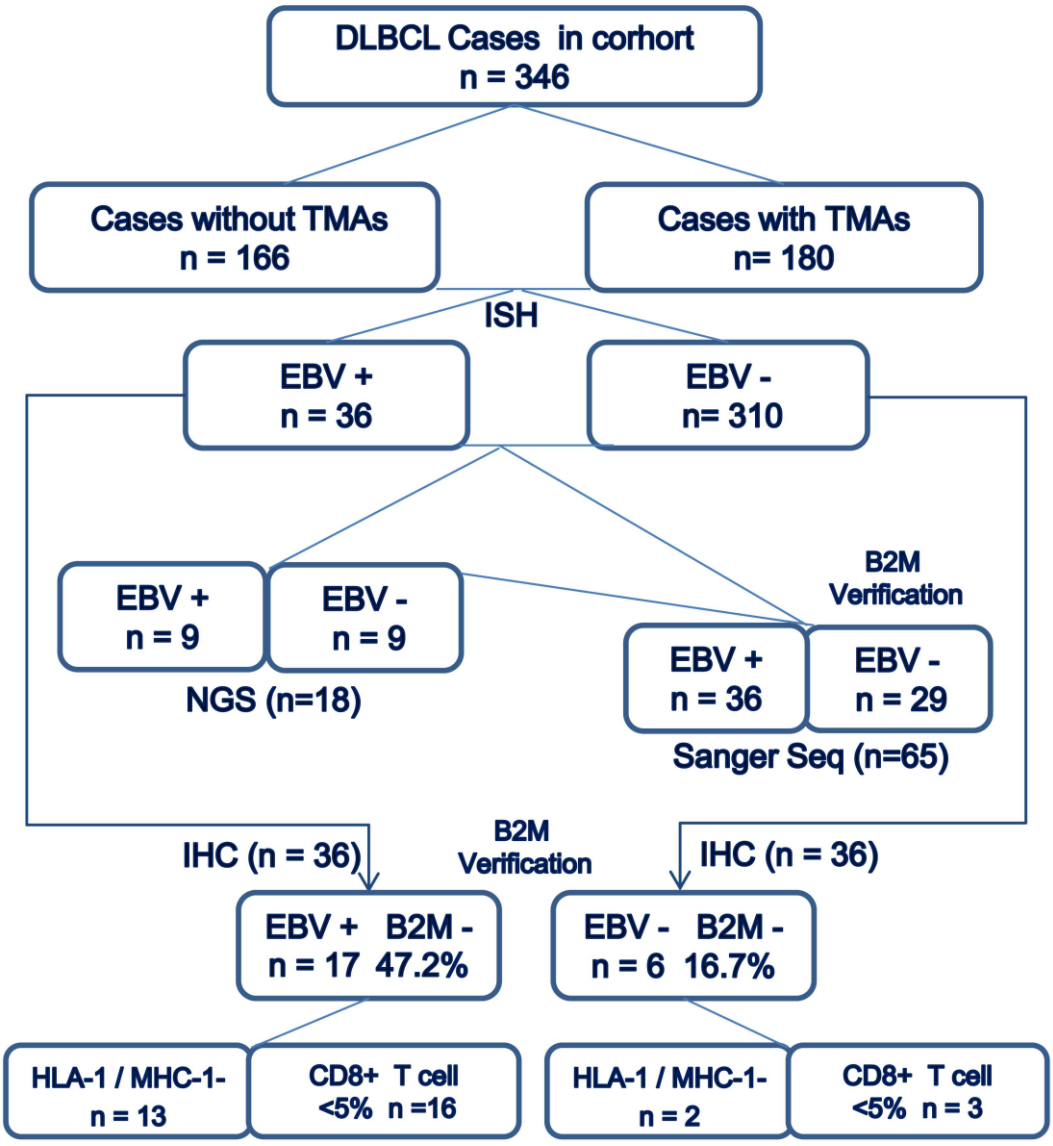

Figure 2. Consort diagram of the present study.

Table 1. Clinical characteristics of 346 cases of DLBCL patients

Parameters		EBERs		
	Total	Positive	Negative	*P* value
Overall	346(100%)	36	310	
Age				0.064
＜50y	104(53.2%)	6(16.7%)	98(31.6%)	
≥50y	242(46.8%)	30(83.3%)	212(68.4%)	

Gender				0.145
Female	155(44.8%)	17(47.2%)	138(44.5%)	
Male	191(55.2%)	19(52.8%)	172(55.5%)	
Subtype *				0.419
GCB	178(51.7%)	15(44.1%)	163(52.9%)	
Non-GCB	166(48.3%)	19(55.9%)	145(47.1%)	
IPI score				0.987
0-1	133(38.5%)	14(38.9%)	119(38.4%)	
2-3	152(43.9%)	16(44.4%)	136(43.9%)	
4-5	61(17.6%)	6(16.7%)	55(17.7%)	
ECOG				0.222
0-1	242(69.9%)	22(61.1%)	220(71.0%)	
≥2	104(30.1%)	14(38.9%)	90(29.0%)	
Ann Arbor stage				0.928
I-II	108(31.2%)	11(30.6%)	97(31.3%)	
III-IV	238(68.8%)	25(69.4%)	213(68.7%)	
B symptoms				0.165
Yes	164(47.4%)	21（58.3%）	143(46.1%)	
No	182(52.6%)	15（41.7%）	167(53.9%)	
Bone marrow involvement				0.711
Yes	37(10.7%)	5(13.9%)	32(10.3%)	
No	309(89.3%)	31(86.1%)	278(89.7%)	
LDH				0.875
＜310	246(71.1%)	26（72.2%）	220（71.0%）	
≥310	100(28.9%)	10（27.8%）	90（29.0%）	
B2M				0.312
＜2.8	228（65.9%）	21（58.3%）	207(66.8%)	
≥2.8	118(34.1%)	15（41.7%）	103（33.2%）	
Chemotherapeutic regimen				0.662
RTX-containing	229(66.2%)	25(69.4%)	204(65.8%)	
RTX-absent	117(33.8%)	11(30.6%)	106(34.2%)	

Abbreviations: DLBCL: diffuse large B-cell lymphoma; GCB: germinal center B-cell; GCB: germinal center B-cell; IPI: International Prognostic Index; ECOG: Eastern Cooperative Oncology Group; LDH: lactate dehydrogenase; B2M: β2-microglobulin and RTX: rituximab.

* This subtype did not include 2 cases of THRBCL.

Table 2. Multivariate Cox regression survival analysis of multiplex data

Variables	Hazard ratio(HR)	95% Confidence Interval(CI)	*P value*
Ages	3.375	1.162-7.886	0.024
EBERs +	1.421	0.765-3.636	0.275
B2M mutation	3.637	1.174-4.657	0.001
B2M expression	4.705	1.485-10.657	0.006
Serologic B2M	1.968	0.893-10.125	0.146
HLA-I or II –	4.105	2.203-8.967	0.017
CD4 +	3.872	1.715-12.149	0.095
CD8 +	5.612	1.956-14.312	0.002
PDL1 +	1.872	0.852-11.234	0.115
PD1 +	3.872	2.412-5.113	0.317
CD163 + M2	4.627	0.994-13.785	0.054
dMMR	0.159	0.632-1.763	0.569

Abbreviations: EBERs: Epstein-Barr virus -encoded small RNAs; B2M: β2-microglobulin; M2: microphage 2; HLA: human leukocyte antigen complex and dMMR: Deficiency of mismatch repair.

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