Human Pathology (2019) **88**, 60–65

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Human PATHOLOGY

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Original contribution

Epstein-Barr virus incidental expression in bone marrow cells: a study of 230 consecutive bone marrow biopsy samples☆,☆☆

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Received 17 September 2018; revised 19 November 2018; accepted 30 November 2018

Keywords:
EBV;
Bone marrow;
B cells;
T cells;
HIV;
Immunocompromised

Summary Epstein-Barr virus (EBV) is associated with many neoplastic hematologic conditions, but scattered EBV-positive cells can be detected in lymph nodes of healthy individuals and they usually represent latently infected lymphocytes. The incidence of EBV detection in normal bone marrow samples has not been studied and is largely unknown. The lack of knowledge regarding the true incidence of encountering bystander latent EBV-positive cells in the bone marrow may potentially lead to a diagnostic dilemma when assessing a staging bone marrow for a patient with an EBV-positive B or T/NK-cell lymphoma. The aim of our study was to investigate the rate of detection of EBV expression in bone marrow samples and correlate any positive findings with various clinical parameters including patient's age, sex, clinical history, immune status, and any neoplastic transformation if follow-up data are available. We retrospectively studied 230 consecutive bone marrow biopsies performed in 2013 and found 5 cases (2.17%) with scattered EBV-positive cells by in situ hybridization. The observed scattered EBV-positive cells are largely small in size and likely represent bystander, latently infected cells. The rate of detection of EBV-positive cells in the bone marrow appears to be slightly higher in immunodeficient individuals (3%) than in immunocompetent patients (1%).

1. Introduction

Epstein-Barr virus (EBV) is a linear, double-stranded DNA virus belonging to the human herpes virus family [1]. EBV infection rate is estimated at over 90% of the adult population worldwide and accounts for the majority of cases of infectious mononucleosis [1,2]. EBV was the first known oncogenic virus and has been linked since to many human neoplasms including hematopoietic, epithelial, and mesenchymal tumors [1]. EBV infection occurs through oral transmission, with oropharyngeal

☆ Competing interests: The authors declare that there is no conflict of interests regarding the publication of this article and there have been no significant financial contributions for this work that could have influenced its outcome.

☆☆ Funding/Support: The authors also declare that not external funding was used for this study. Only departmental resources were utilized.

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https://doi.org/10.1016/j.humpath.2018.11.034
0046-8177/

infection resulting in a primary lytic (productive) phase that is asymptomatic in most individuals. Following the lytic phase, subsequent infection of B cells leads to the persistence of viral DNA in an unintegrated episome, and the establishment of latent infection [1]. Rare latent infection of T and NK cells has also been shown to occur following the lytic phase of EBV infection [3].

Three distinct latency programs of EBV infection, latency types I, II and III, have been characterized in correlation with the differentiation states of latently infected B cells [4]. The most common progression of latency programs in B cells is from type III to type II, seen in tonsillar naïve and germinal center B cells, respectively, and finally to type I, found in peripheral memory B cells [5]. Depending on an individual's immune status and function, the presence of occasional small, EBV-positive B cells within lymph nodes and mucosa-associated lymphoid tissue has been associated with latent EBV infection [6]. The presence of EBV-positive latent lymphocytes in the bone marrow has not been studied before and the incidence of EBV detection in bone marrow cases is largely unknown. The aim of this study is to investigate the expression of EBV in bone marrow cells and correlate the cases containing EBV-positive cells with certain parameters, including age, sex, clinical features, immune-status, type of cell infected, any neoplastic association, and any follow-up data available.

2. Materials and methods

2.1. Case selection

The University of California Irvine Medical Center (UCIMC) pathology records were searched for bone marrow biopsy cases from 2013 and early 2014 to select sequential bone marrow biopsies performed for any indication with formalin-fixed, paraffin-embedded (FFPE) clot sections. Inclusion criteria included presence of a clot section to avoid possible RNA degradation due to the decalcification process on the core sections. Any marrow biopsies from pregnant women and pediatric patients less than 18 years of age were excluded from the case selection due to an Institutional Review Board (IRB) requirement. Out of 242 sequential bone marrow biopsies with FFPE clot sections, 1 was excluded for a patient age less than 18 years, with the remaining 241 cases being selected for analysis (236 cases from 2013 and 5 cases from 2014).

2.2. In situ hybridization

Epstein-Barr encoding region by in situ hybridization (EBER-ISH) analysis was performed on FFPE clot sections for all selected cases using the Ventana ISH kit (EBER1, Analyte Specific Reagent/RTU, Ventana, Tucson, AZ) in accordance with manufacturer protocol including appropriate positive and negative controls. PolyT probe (U6 DNP, Analyte Specific Reagent/RTU, Ventana, Tucson, AZ) was used as a control for RNA preservation for all cases. Cases with no viable RNA as determined by the U6 probe or that lost cellular particles on deeper sections were excluded (11 cases). Dual staining with EBER/CD3 and EBER/CD20 was performed in select case in accordance with previously published staining protocol [7].

2.3. Immunohistochemistry

A panel of immunohistochemical stains was performed on all bone marrow clot sections that contained EBV-positive

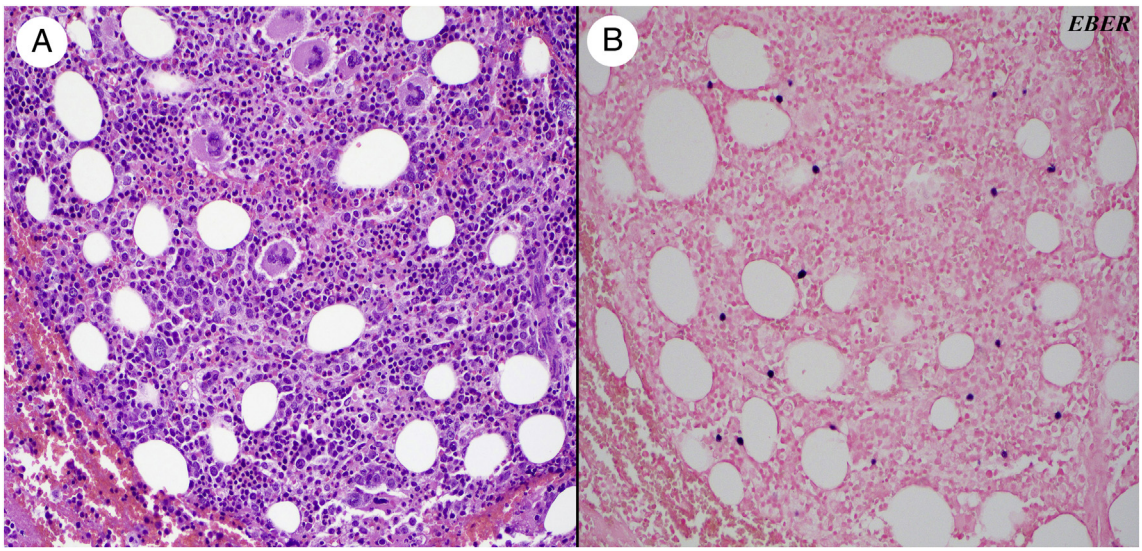

Fig. 1 The composite picture from case 4 in the Table shows the same cellular particle from the bone marrow clot section. A, Evidence of trilineage hematopoiesis by H&E stain. B, Scattered cells positive for EBER by in situ hybridization. All pictures were taken at 40× magnification.

Table Features of bone marrow cases containing EBV-positive cells.

Case	Age, sex, ethnicity	BMB diagnosis	History	Immune status	Follow up	EBER	PAX-5	CD3
1	68 M Korean	Slightly hypercellular marrow with active trilineage hematopoiesis. No evidence of lymphoma	Recently diagnosed EBV-negative diffuse large B-cell lymphoma in maxillary sinus	Preserved	5 year follow-up available. Currently alive and in remission.	Rare cells positive	Scattered cells positive	Scattered cells positive
2	48 M Caucasian	Normocellular marrow with active trilineage hematopoiesis and marked erythroid hyperplasia	HIV infection, on HAART therapy, history of ITP	HIV positive	5 year follow-up available. Currently alive with no development of any hematopoietic/EBV-related disorders.	Rare cells positive	Scattered cells positive	Scattered cells positive
3	54 M Caucasian	Moderately hypocellular marrow with active trilineage hematopoiesis, left-shifted myeloid hyperplasia, and megakaryocytic hyperplasia	Recently diagnosed acute myeloid leukemia (AML) status post–induction chemotherapy	Day 14 status post–induction chemotherapy	Only 3 month follow-up data available. Negative for residual AML or any other hematopoietic/EBV-related disorder in that time period.	1 cell positive	Negative	Scattered cells positive
4	47 M Hispanic	High normocellular marrow with active trilineage hematopoiesis, negative for lymphoma involvement, with scattered EBV cells of undetermined significance. Flow cytometry and IHC showed no evidence of lymphoma	HIV infection, on HAART therapy, Kaposi sarcoma, recently diagnosed plasmablastic lymphoma	HIV positive	5 year follow-up available. Currently alive and in remission for both plasmablastic lymphoma and Kaposi sarcoma. Negative for any hematopoietic/EBV-related disorder in that time period.	Scattered cells positive (average 4-5/HPF)	Scattered cells positive	Scattered cells positive
5	24 M Hispanic	Mildly hypocellular marrow with mild myeloid and megakaryoid hyperplasia and erythroid hypoplasia	HIV infection, on HAART therapy, Kaposi sarcoma	HIV positive	Only 5 month follow-up data available. Negative for any hematopoietic/EBV-related disorder in that time period.	Rare cells positive	Negative	Scattered cells positive

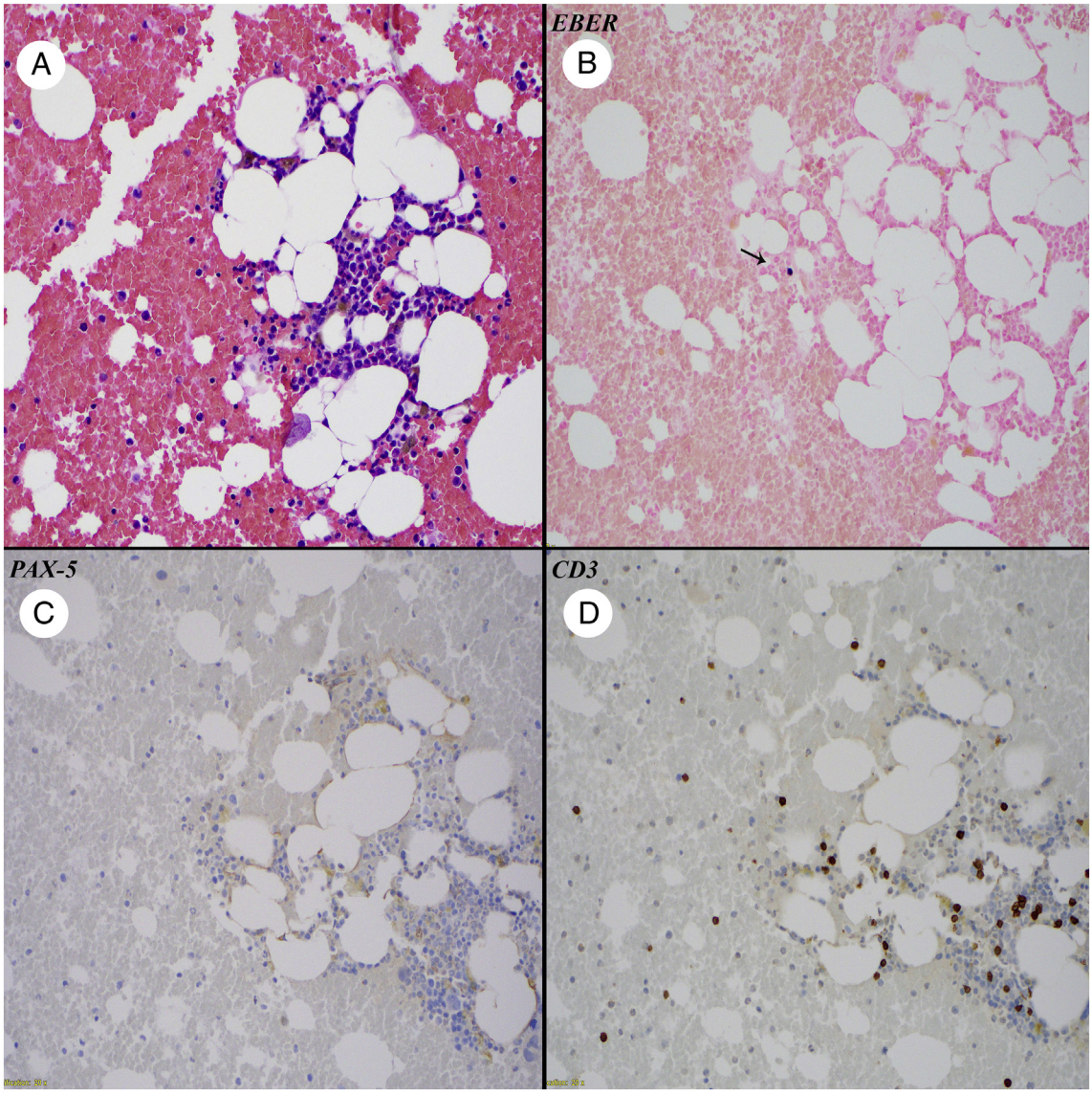

Fig. 2 The composite picture from case 3 in the Table shows the same cellular particle from the bone marrow clot section. A, Evidence of hematopoiesis by H&E stain. B, One individual cell positive (arrow) for EBER by in situ hybridization. C, Negative PAX-5 staining by immunohistochemistry. D, Scattered positive T lymphocytes for CD3 by immunohistochemistry. All pictures were taken at 40× magnification.

cells as determined by EBER-ISH analysis. The panel of stains included: PAX-5 as a pan B cell marker, CD3 as a pan T cell marker, CD4, CD8, human herpes virus-8 (HHV8), and EBV LMP-1. All immunohistochemical stains were performed using an automated Ventana BenchMark ULTRA immunostainer according to the manufacturer's protocol, with antigen-retrieval applied as needed for each antibody. All antibodies were pre-diluted and ready to use (RTU) by the manufacturer. The manufacturer information for each used antibody (Clone, Vendor and City) is as follows: PAX-5 (SP34, Ventana, Tucson, AZ), CD3 (2GV6, Ventana, Tucson, AZ), CD4 (SP35, Cell Marque, Rocklin, CA), CD8 (C8/144B, Cell Marque, Rocklin, CA), HHV8 (13B10, Cell Marque, Rocklin, CA), EBV Latent Membrane Protein 1 (LMP-1) (CS1-4, Cell Marque, Rocklin, CA). Appropriate positive and negative control samples were used for all immunohistochemical stains.

3. Results

Out of 241 selected cases, 11 were excluded due to absence of cellular particles on deeper sections or lack of RNA preservation on the U6 control probe. The 230 remaining cases represent 160 different patients since many patients had repeat bone marrow biopsies during that time interval. The 160 patients represented different ethnicities with 67

Caucasian patients (41.9%), 59 Hispanic patients (36.9%), 21 Asian patients (13.1%), 11 Middle-Eastern patients (6.9%) and 2 African-American patients (1.3%). They were mostly males with 108 male patients (67.5%) and 52 female patients (32.5%). A total of 86 patients (53.8%) with 98 bone marrow biopsies had preserved immune status, while 69 patients (43.1%) with 132 bone marrow biopsies were immunocompromised for different reasons. A total of 5 human immunodeficiency virus (HIV)-positive patients (3.1%) were present in our study.

Of the total 230 cases, only 5 cases (2.17%), for 5 different patients, demonstrated positive EBV expression by in situ hybridization. The patients from these 5 positive cases showed ethnic diversity with 2 Caucasian patients, 2 Hispanic patients, and 1 Asian (Korean) patient. EBV expression varied among positive cases, with the quantity of EBV-positive cells per section ranging from an average of 1 cell per high power field to an average of 4 to 5 cells per high power field (Fig. 1). The EBV-positive cells displayed variable size but overall represented small to medium lymphocytes. All patients were males with ages ranging from 24 to 68 years. Three of the five cases were from HIV-positive patients. One patient had a history of acute myeloid leukemia (AML) and was day 14 status post–induction chemotherapy at the time of the bone marrow biopsy. The fifth patient had a recent diagnosis of EBV-negative diffuse large B cell lymphoma involving the right maxillary sinus and had not received treatment prior to the bone marrow biopsy. Notably, all bone marrow biopsy diagnoses were negative for leukemia, involvement by lymphoma, or metastatic disease (Table). Upon review of UCIMC electronic medical records, follow-up data of more than 1 year were available for 3 of the 5 patients and demonstrate no subsequent development of hematologic or any EBV-associated disorder.

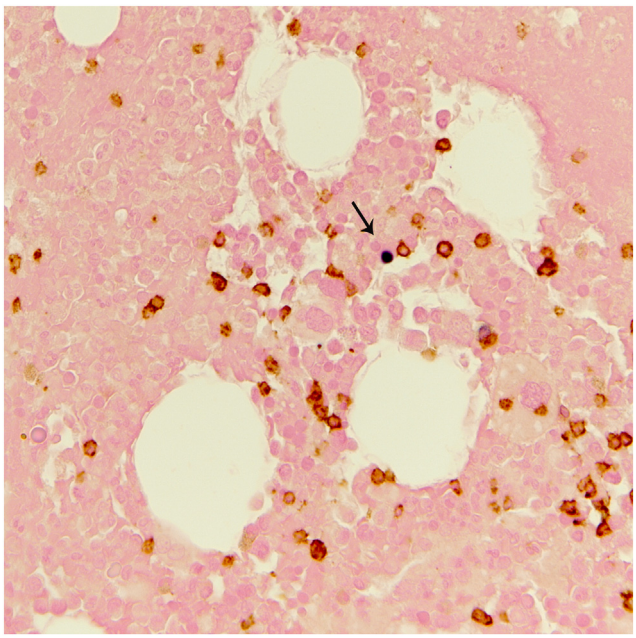

Fig. 3 The picture from case 3 in the Table shows dual staining for EBER by in situ hybridization and CD3 by immunohistochemistry. One EBER-positive cell is seen in the middle (arrow) and scattered CD3-positive T cells are noted. No evidence of positive dual staining is seen. The picture was taken at 40× magnification.

In all 5 cases containing EBV-positive cells, EBV LMP-1 and HHV8 were negative by immunohistochemistry. CD3 immunostaining showed scattered positive T cells in the region with EBER-positive cells in each case. CD4 and CD8 immunostains similarly show scattered T cells in all cellular particles. PAX-5 immunostaining showed few scattered B cells in the same cellular region in 3 of the 5 cases, while the other 2 cases were completely negative for PAX-5 in the areas with EBV-positive cells (Fig. 2). Dual staining for EBER/CD3 and EBER/CD20 showed no evidence of positive dual staining for either EBER/CD3 (Fig. 3) or EBER/CD20.

4. Discussion

EBV has been linked to various hematopoietic and non-hematopoietic disorders. While EBV is known to be able to infect epithelial cells in the lytic (productive) cycle, it has a predilection for B lymphocytes, which serve as the virus's primary reservoir [1,8]. The virus can also infect T and NK cells. Following the initial lytic phase, the latent phase of EBV infection allows the virus to persist and evade the host immune response. In latent infection, small EBV-positive cells, mainly B cells have been detected in lymph nodes and mucosa-associated lymphoid tissue and they have been reported to represent bystander reactive cells and not associated with a neoplastic or an acute infectious process [5,6]. However, detection of these EBV-positive cells has not been studied in bone marrow samples. The absence of reportable data regarding the true incidence of encountering bystander latent EBV-positive cells in the bone marrow may potentially lead to a diagnostic dilemma when assessing a staging bone marrow for a patient with an EBV-positive B or T/NK-cell lymphoma. We sought to investigate the frequency of detecting EBV-positive cells in bone marrow biopsies and correlate the positive findings with relevant patient clinical data and follow-up data if available.

Our study evaluated 230 sequential bone marrow biopsies for EBV expression by in situ hybridization. These cases represented an ethnically-diverse population with 42% Caucasians, 37% Hispanics, 13% Asians, 7% Middle Easterners, and 1% African-Americans. About 54% of the patient population was immunocompetent, while 46% of the patients were immunocompromised, including 5 patients with HIV infection (3% of total patients). Of the 230 cases, only 5 cases (2.17%) contained scattered EBV-positive cells (Table). The number of positive cells in each case ranged from an average of 1 cell per high power field in 4 cases to an average of 4 to 5 cells per high power field in one case. All positive cases were from male patients, ranging in age from 24 to 68 years. The patients with EBV-positive cells showed ethnic diversity

with 2 Caucasian patients, 2 Hispanic patients, and 1 Asian (Korean) patient.

Four of the 5 patients with EBV-positive cells were immunocompromised at the time of biopsy (3 HIV-positive patients and 1 patient status post–induction chemotherapy for AML). All bone marrow biopsy diagnoses were negative for any leukemia, lymphoma, or an acute infectious process. The bone marrows of the 3 HIV-positive patients were also negative for involvement by Kaposi sarcoma or any other HIV-related disorder. One of the 3 HIV-positive patients had an IgG kappa monoclonal gammopathy demonstrated by serum electrophoresis. A potential role that HIV and EBV replication may play in long term persistence of monoclonal gammopathy in patients on antiretroviral therapy has been postulated by some scholars [9]. All 5 bone marrows demonstrated evidence of trilineage hematopoiesis, with marrow cellularity ranging from moderately hypocellular to slightly hypercellular. Follow-up data for 4 years in UCIMC electronic medical records show no neoplastic transformation or development of an EBV-associated disorder for 3 patients; however only limited follow-up data of 3 to 5 months was available for the remaining 2 patients that also showed no evidence of neoplasia or an EBV-related disorder during that brief time interval (Table).

EBV LMP-1 immunostaining was negative in all 5 cases, in discordance with the positive EBER results. These findings suggest that the observed EBV-positive cells seen in our study are in a more restricted latency pattern, likely latency type I, which is seen in peripheral B cells and associated with the expression of EBER and EBNA1 antigens but not LMP-1 [6,10]. Immunohistochemical staining for CD3 showed scattered T lymphocytes in all cellular particles demonstrating EBV-positive cells. PAX-5 immunostaining, however, showed rare B cells in the corresponding particles of only 3 out of the 5 positive cases. For the 2 CD3-positive/PAX-5 negative cases, the overall immunophenotype may suggest that the observed EBV-positive cells represent latently infected T cells; however, dual staining for EBER/CD3 and EBER/CD20 showed no evidence of positive dual staining between EBER and CD3 or EBER and CD20. EBV has been reported to more likely infect T cells and NK cells in patients with defective immunity [11]. Interestingly, recent studies have demonstrated that two genetically-distinct major strains of EBV, EBV-1, and EBV-2, exhibit significant functional differences, with EBV-1 preferentially infecting B cells and EBV-2 latently infecting T cells [12]. Further studies elucidating the roles of these different EBV strains in latent infection may aid in clarifying the role of immune status and incidental findings in non-neoplastic bone marrows.

In conclusion, our study is the first to investigate and characterize the incidence of detection of EBV-positive cells in bone marrow cells. Out of 230 sequential bone marrow samples, 5 cases (2.17%) had clot sections with identifiable EBV-positive cells. The observed scattered EBV-positive cells are largely small in size and likely represent bystander, latently infected cells, as previously described in lymph nodes and mucosa-associated lymphoid tissues. The rate of detection of EBV-positive cells in the bone marrow appears to be slightly higher in immunodeficient individuals (3%) than in immunocompetent patients (1%). Although it is a small sample size, but the detection of EBV positivity in 3 out of 5 total HIV-positive patients in our study may represent truly increased incidence of EBV detection in patients with HIV infection, similar to that observed in other sites.

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