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SHORT COMMUNICATION

Patients with Plasma Cell Disorders Have High EBV DNA in Peripheral Blood than the General Population

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Abstract

Epstein-Barr virus (EBV) is involved in the development of a wide range of B cell lympho-proliferative disorders. Its association with plasma cell disorders (PCD) however is not clear, especially in immunocompetent patients. To explore any relationship, 39 patients of suspected PCD with positive M-band on electrophoresis and 50 healthy controls were enrolled. EBV DNA in peripheral blood was quantified using quantitative Real Time Polymerase Chain Reaction (qPCR). Of 39 patients, 15 (38.5%) had EBV DNA compared to 8/50 (16%) controls ($p = 0.0008$). The mean viral copy number was found to be significantly high in patients compared to controls (1.8×10^5; range = 2.6×10^3–7.6×10^5 copies/ml and 1.7×10^4; range = 7.0×10^2–6.1×10^4 copies/ml respectively; $p = 0.003$). This is the first study, which characterizes the frequency of EBV in circulation in patients of PCD. The significance of increased prevalence of circulating EBV and a higher viral load in our immunocompetent patients however, needs further evaluation.

Keywords Epstein - Barr virus · Plasma cell disorders · Quantitative real time PCR · Immunocompetent

Introduction

Plasma cell disorders (PCD) is a group of chronic and debilitating diseases associated with monoclonal proliferation of plasma cells and characteristic secretion of homogenous monoclonal proteins in blood and/or urine detectable as M-band in electrophoresis [1]. Despite several proposed aetiologies, i.e. radiation or workplace exposure, diet, socioeconomic status, chronic antigenic stimulation by infections, inflammatory conditions, autoimmune connective tissue and allergy related disorders, the mechanism of development of PCD remains elusive [2].

Epstein-Barr virus (EBV) is one of the most common human viruses with a proven role in the development of a wide range of B cell lympho-proliferative disorders. These include Burkitt's and Hodgkin's lymphoma, and lymphomas arising in immuno-compromised individuals (post-transplant & HIV-associated lympho-proliferative disorders). In view of its

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affinity for B cell, it may be interesting to study the role of EBV, if any, in the causation of PCD originating from terminally differentiated B cells.

Common tools employed for detection of EBV infection are serology and immunohistochemistry (IHC) along with in situ hybridization (ISH). Serology may detect antibodies against EBV despite the individual being EBV negative. Hence, it indicates a recent or active infection but does not confirm development of an EBV associated disease. Though specific, IHC and ISH are invasive as they need tissue biopsy and the studies using these techniques have shown a lower rate of EBV detection in plasma cell neoplasms. Hsieh et al., reported 2/18 (11%) cases of multiple myeloma (MM) while Chang et al., found only 7% (4/54) cases of plasma cell neoplasms positive for EBV in bone marrow biopsy [3, 4]. In another study, IHC and ISH done for EBV on 147 fresh frozen paraffin embedded tissues from different plasma cell neoplasms failed to reveal any association [5]. Recent studies opine that the detection of EBV in peripheral blood samples might be helpful not only for detection of acute infection but also in predicting a risk of development of an EBV associated disease. Qualitative PCR has been employed for this purpose but its high false positive rate limits its use [6]. We therefore, in this preliminary study, aim to assess the importance of real time quantification of EBV load in peripheral blood of patients with PCD.

Patients and Methods

Patient Selection and Sample Preparation

The department of Immunopathology in collaboration with departments of Haematology and Internal Medicine conducted this prospective, case control study. Fifty clinically suspected and M-band positive cases of PCD were recruited after excluding patients with primary or secondary immunodeficiency, autoimmune disorder, on immunosuppressive therapy or with history of any organ transplantation. Fifty M-band negative, healthy volunteers of comparable age from general population were enrolled as controls. An approval by the institute's human ethics committee and informed written consent from each study subject were obtained prior to their recruitment.

About 3 ml peripheral blood was collected in EDTA containing vacutainer from both patients and controls using standard venepuncture procedure. Whole blood DNA was extracted using AxyPrep blood genomic DNA miniprep kit as per the instructions from manufacturer (Axygen Biosciences, USA). After testing genomic DNA quality and quantity by 1% agarose gel electrophoresis and spectrophotometer (Genova Nano, Janway, Cole-Parmer Ltd., UK), samples were stored at −20 °C until analysis by quantitative real time PCR (qPCR).

Quantitative Real Time PCR for EBV Virus to be deleted Viral Load

qPCR was done by using a set of primers specific for nonglycosylated membrane protein BNRF1-p143 and TaqMan probe. The sequences of primers and probe used were F5'-GGAACCTGGTCATCCTTGC-3′, R5'-ACGTGCATGGACC GGTT-AAT-3′ and FAM-CGCA-GGCACTCGTACTGC TCGCT–BBQ respectively. The PCR mix contained 2 μl primer-probe mix (TIB MOLBIOL Synthaselabor, GmbH, Berlin); 3 μl PCR grade water (Invitrogen, ThermoFisher Scientific), 10 μl TaqMan universal master mix (Roche Molecular Systems Inc.), and 5 μl genomic DNA template. The mixture was dispensed in 96 well optical micro titre plates (Roche Molecular Systems Inc.), centrifuged for 1 min at 1500 g and loaded on to real time machine (Light Cycler LC480 Real Time PCR system, Roche applied Science, USA). The cycling parameters used were pre-incubation, 10 min at 95 °C; amplification, 45 cycles at 95 °C and 60 °C for 30 s and 72 °C for 1 min.

Commercially available, cloned standard containing 10^{10} EBV genome equivalents (geEq)/mL was used as an internal control in 8 point serial logarithmic dilutions, each in duplicate, up to 10^{1} EBV DNA geEq/mL (Cat No. 1030–8140-01 EBV; TIB MOLBIOL Synthaselabor, GmbH, Berlin). A standard curve was plotted and the linearity (R^2) was found to be equal to 0.995. The slope of the standard curve and efficiency of the PCR were − 3.347 and 1.9 respectively. The assay was optimized to reach PCR efficiency as close to 100%.

Each sample was run in duplicate along with a standard, a positive and a negative control. The viral load was extrapolated from the standard curve and results were expressed as EBV load copies/ml. An assay result of <50 geEq/mL was considered as negative.

Statistical Analysis

Analysis was done using IBM SPSS (Version 22.0). Non-Parametric test (Mann-Whitney), Chi-square test, unpaired t test and odd's ratio were used for comparative analysis. All the statistical tests were two sided and were performed at a significance level of $p = 0.05$.

Results

Patient Characteristics

Of the 50 M-band positive patients, only 39 were diagnosed as PCD after thorough investigation. The remaining 11 cases did not fit into diagnosis of PCD and therefore were excluded from further analysis. Among confirmed cases of PCD, 31 had MM, 6 plasmacytoma (PL), 1 patient each of monoclonal gammopathy of undetermined significance (MGUS), and

POEM syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy and skin changes). In the excluded cases, 8 remained unclassified and 1 patient each had lymphoplasmacytic lymphoma (LPL), Non-Hodgkins lymphoma (NHL), and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).

Age range was 30–83 years (mean = 54.6 years) for patients & 39–70 years (mean = 50.4 years) for the controls. Male-female ratio was 2:1 (26:13) in patient group and 1.2:1 (27:23) in control group. The presenting symptoms in decreasing order were backache 19(48.7%), joints and bone pain 12(30.7%), fever 8(20.5%), and altered sensorium in 4(10.2%). Laboratory and radiological findings are summarised in Table 1, S1 and S2.

EBV Load

qPCR showed EBV nucleic acid in 38.5% (15/39) of patients compared to 16% (8/50) controls. EBV positive patients had MM (12/15), PL (2/15) and MGUS (1/15) and EBV negative patients were of MM (19/24), PL (4/24), and POEM syndrome (1/24).

The frequency of EBV positivity in patients was statistically significant ($p = 0.0008$; Fig. 1). In addition, the odds ratio for viral load was 3.5 times higher in patient than the control group. The Viral load varied from 2.7×10^3 to 7.6×10^5 copies/ml (mean ± SD = $1.8 \times 10^5 \pm 1.8 \times 10^5$ copies/ml) in patients and 7.0×10^2 to 6.1×10^4 copies/ml (mean ± SD = $1.7 \times 10^4 \pm 2.1 \times 10^4$ copies/ml) in controls ($p = 0.003$; Fig. 2). Also, a very high EBV copy number (>2.6×10^5/ml) was observed in 6 patients of MM and 1 patient with PL.

Laboratory and Clinical Correlation

M-protein quantification done in all EBV positive patients ranged from 0.1 to 7.05 g/dl. Immunofixation study showed IgGκ in 19/30 and IgGλ in 6/39 cases. The plasma cell number in bone marrow aspiration studies varied from 2% to 88%. The CRAB (Hypercalcemia, renal lesions, anaemia and bony lesions) analysis was available in 34/39 cases of PCD. None of these parameters showed any association with EBV infection as p value remained non-significant ($p = \geq 0.05$). Other factors e.g. Type of PCD ($p = 0.8$), difference in plasma cells percentage in the bone marrow in the EBV positive and EBV negative cases ($p = 0.067$), M-protein quantity ($p = 0.36$), M-protein component ($p = 0.63$), haematological, biochemical and radiological findings also remained non-significant (Table S1).

Discussion

Role of EBV in the pathogenesis of several B cell malignancies is well established particularly in patients after organ transplantation or those with an immunocompromised state [1, 2, 7]. Plasma cell is an end to B cell differentiation whose development usually is triggered by infections. Thorley-

Table 1 Patient characteristics and laboratory parameters in PCD

Patient characteristics	EBV positive	EBV negative
Total number	15	24
Age range	38–83 years	30–81
M:F ratio	2:1	2:1
Clinical		
Commonest presenting symptom	Backache	Backache
Radiological		
Lytic lesions	Skull, vertebra, long bones	Vertebra, long bones, skull
Expansile/mass lesions	Frequent	Less frequent
Systemic organ involvement	Kidney, lungs, liver	Lungs, liver, kidney
Haematological (mean ± SD)		
Bone marrow plasma cells (%)	39.8 ± 27.5	35.1 ± 25.9
Haemoglobin (gm/dl)	10.0 ± 1.8	9.3 ± 3.3
Total leukocyte count (10^3/μl)	8.8 ± 3.5	9.2 ± 7.9
Platelet count (10^3/μl)	230 ± 103	190 ± 125
Immunological		
Protein electrophoresis	M band positive	M band positive
Immunofixation	IgG κ (70%)	IgG κ (60%)
M protein quantification (mean ± SD)	2.3 ± 1.2	2.5 ± 2.2
Biochemical (mean ± SD)		
Serum calcium (mg/dl)	10.5 ± 5.1	9.8 ± 2.2
AST (IU/l)	37.7 ± 41.9	37.9 ± 25.3
ALT (IU/l)	37.9 ± 25.3	32.3 ± 14.7
Urea (mg/dl)	55.3 ± 21.6	65.6 ± 34.9
Creatinine (mg/dl)	1.8 ± 1.9	2.4 ± 2.1

AST, Aspartate aminotransferase; *ALT*, Alanine aminotransferase

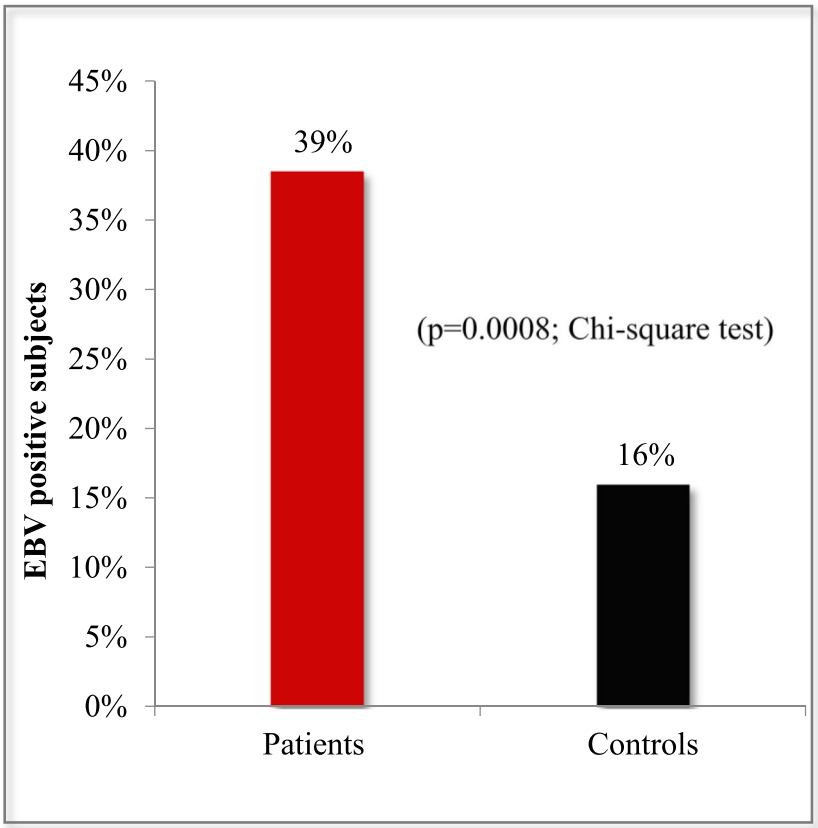

Fig. 1 Comparison of EBV infection in patient and control groups

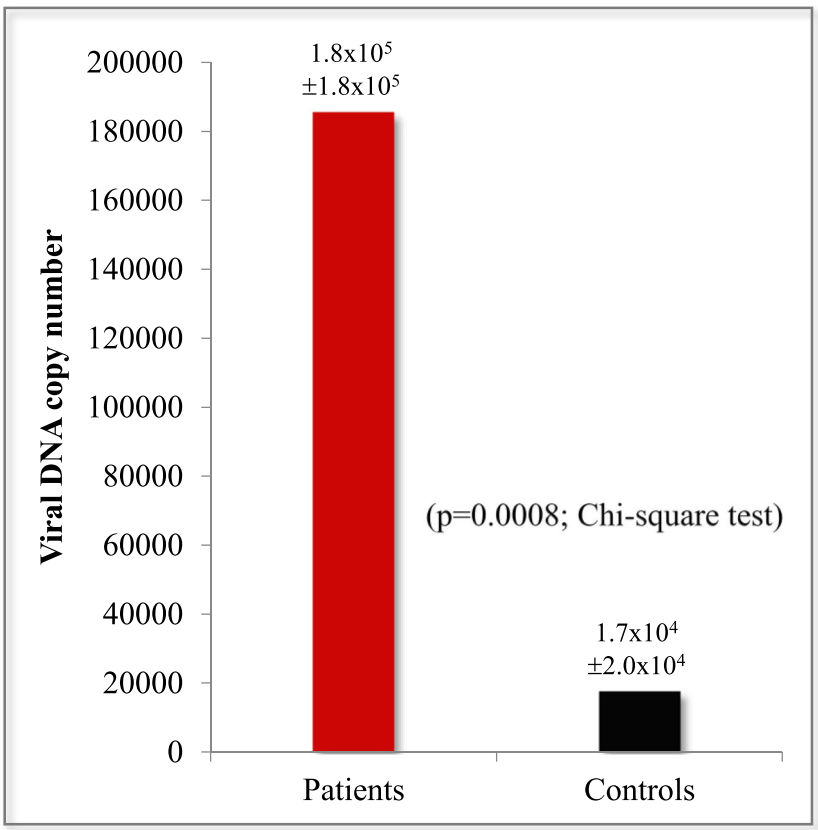

Fig. 2 EBV copy number in patients and controls

Lawson and Babcock in their EBV infection model described how EBV infects and completes its life cycle in B cells [8]. They proposed that during lifetime persistent EBV infection, latently infected memory B cells become active while passing through the mucosal lymphoid tissue. Some of these cells exit the cell cycle and replenish the pool of infected memory B cells while others may terminally differentiate and release infectious virus. Hence, differentiation of infected B cells in to plasma cells is theoretically possible. However, such a link between EBV infection and occurrence of neoplasms of plasma cells in immunocompetent patients is not well studied.

EBV DNA quantification by qPCR in our immunocompetent patients of PCD showed high frequency of EBV positivity. Hence, there is a possibility of high proliferation rate as demonstrated in another study by Chang et al. who found plasmablastic cytomorphological features in immunocompetent patients with plasma cell neoplasms [4]. Other studies have also shown certain features in EBV infected plasmacytic tumours like increase in latency phase, reduction in expression of plasmacytic markers CD38 and CD138 and regression to an immature B cell phenotype, and production of interleukin-6 [9–11]. Although, 16% of our controls also showed EBV DNA in their blood indicating a high EBV prevalence rate general population but very high viral load in patients (3.5 times compared to controls) suggests a possible link between EBV and PCD.

A previous study, in which EBV DNA was quantified by qPCR in paraffin sections of bone marrow biopsies in 30 patients of MM, showed EBV in 33% of patients compared to only 10% in control group with a p value of 0.03 [12]. Our results of quantification from peripheral blood are comparable to this study from tissue biopsy suggesting utility of qPCR as an ancillary technique for detection and monitoring of EBV infection. All related previous studies except one have done ISH directly on tissue biopsy to detect EBV infection [13]. It is apparent from present study that despite a lower specificity, quantification of EBV load in peripheral blood using qPCR is more sensitive and quite useful for defining infection status particularly in immunocompetent patients of PCD. Moreover it is relatively easier and faster than the commonly employed invasive techniques and advantageous for many reasons e.g. provides status of viral genome particularly during disease monitoring or after therapy, has high throughput screening capacity, requires minimal sample quantity and generates results in just few hours [14]. The major limitation of this approach however is that it merely shows the presence of EBV DNA and does not establish the diagnosis of EBV disease. This issue however may be resolved by combining it with routine serological assays.

In contrast to published literature, in our study, EBV infection was more common in MM than PL or other PCD. One reason for this could be the recruitment process as MM outnumbered the cases of PL or other PCD. We found EBV DNA in cases of non-PCD, which may or may not have any pathological association. NHL, a lymphoproliferative disorder, is well associated with EBV. We also found EBV DNA in a case of CIDP. Previously also an increased frequency of EBV infection has been reported in CIDP. Lunemann et al. suggested role of EBV in pathogenesis of CIDP as they found circulating EBV DNA in 92% (11/12) of CIDP cases [15]. Two of our EBV positive patients of MM also had amyloidosis. Though there are few case reports of EBV infection in amyloidosis, its causative association needs to be further explored [16]. Two of unclassified cases that showed circulating EBV DNA, one had chronic kidney disease, chronic obstructive airway disorder and diabetes mellitus. Both these cases were lost to follow up and no hospital records were available to delineate their further course.

To summarise, the present study shows an increased frequency, higher odd's ratio and heavy viral load in the entire spectrum of PCD including MGUS, PL and MM. The association between EBV and PCD therefore is likely to be more than an incidental observation. A large study including entire range of PCD with long follow up however, if carried out, may explain the actual role of EBV in PCD etiopathogenesis. Also for detection and monitoring of EBV infection in such patients, viral load quantification in peripheral blood by qPCR may be considered a good alternative to other ancillary techniques as it is sensitive, non-invasive, faster and may help in decision making as serology sometimes is indecisive particularly in EBV associated tumours.

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Authors Contributions YK conceived and designed study, received funds, analysed data, and wrote the manuscript. KS collected and analysed the data. RWM, NV critically evaluated manuscript. SV provided clinical sample. SA performed the lab work. All authors read and approved the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors have declared that no conflict of interest exists.

Ethical Statement This study was approved by the Institute's Ethics Committee of PGIMER, Chandigarh, India.

Informed Consent Written informed consent was obtained from all the study subjects with respect to the use of their blood for scientific purposes

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