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ORIGINAL RESEARCH REPORT

Plasma Epstein Barr viral load in adult-onset Hodgkin lymphoma in South India

Mahua Sinha [a,*], Clementina Rama Rao [b,1], Mohammed Shafiulla [a], Bharatnur Shankaranand [b], Belathur K. Viveka [c,2], Kuntegowdanahalli C. Lakshmaiah [c], Linu Abraham Jacob [c], Govind K. Babu [c], Rudrapatna S. Jayshree [a]

[a] Department of Microbiology, Kidwai Memorial Institute of Oncology, Bangalore, India
[b] Department of Pathology, Kidwai Memorial Institute of Oncology, Bangalore, India
[c] Department of Medical Oncology, Kidwai Memorial Institute of Oncology, Bangalore, India

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Abstract
Objective/background: Epstein Barr Virus (EBV) DNA load is increasingly being used as a non-invasive biomarker for detecting EBV association in lymphomas. Since there is a need of data from India, we undertook to prospectively evaluate plasma EBV DNA load as a marker of EBV association in newly diagnosed adult-onset Hodgkin lymphoma (HL).
Methods: EBV DNA was quantified using real-time polymerase chain reaction. In a subset of patients, an assay was validated qualitatively with EBV latent membrane protein-1 (LMP1) immunohistochemistry (IHC). Wherever possible, follow-up plasma samples post three cycles of chemotherapy were obtained.
Results: Over a period of 10 months, 33 newly diagnosed adult-onset HL were enrolled in the study. Pretherapy plasma EBV DNA was detectable in ~49% (16/33) patients (viral loads range, 1.0–51.2 $\times$ 10^3 copies/mL) and undetectable in 30 voluntary blood donors. LMP1 IHC was positive in 56% of cases tested (14/25). Sensitivity and specificity of plasma EBV DNA with respect to LMP1 IHC were 86% and 100%, respectively. Of the eight patients in whom follow-up plasma was available, in five EBV baseline-positive patients EBV load reverted to negative postchemotherapy and corroborated with clinical remission.

* Corresponding author at: Department of Microbiology, Room 114, Main Block, Kidwai Memorial Institute of Oncology, Dr. M.H. Marigowda Road, Bangalore 560011, Karnataka, India.
E-mail address: mahuasinha@gmail.com (M. Sinha).
[1] This author has recently retired and is not currently practicing.
[2] This author left the institute after obtaining his super specialty degree (DM) in medical oncology and is currently privately practicing in Bangalore.

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Conclusion: Plasma EBV DNA load estimation may be useful in detecting EBV-association and possibly monitoring the response to therapy in EBV-related HL especially in our country where EBV association of HL is higher than in developed nations.

Introduction

Epstein Barr Virus (EBV) is associated with a variety of human malignancies: Burkitt's lymphoma, lymphomas associated with immunosuppression, nonHodgkin lymphomas, Hodgkin lymphoma (HL), and nasopharyngeal carcinoma [1,2]. Seroepidemiologic studies indicate that EBV infects >95% of the human population by adulthood and is maintained latently in a small fraction of memory B cells [1—3]. While EBV DNA can be detected in the serum or plasma of most patients with EBV-associated malignancies, it remains undetectable in healthy individuals even though they are latently infected [4—6]. Hence, a serum/plasma EBV DNA detection assay can be used as a marker of EBV-associated malignancies. In recent years, plasma EBV-DNA assays are increasingly being used as a noninvasive biomarker for EBV-association in lymphomas [5,7—14].

In order to validate its utility in determining EBV association, there is a need for studies from different geographical regions. In overcrowded living and unsanitary conditions of developing countries like India, primary infections occur very early in life [2]. This is partly responsible for greater EBV-associated HL in developing countries than in developed countries [2]. Since studies on EBV load in malignancies and HL from India are limited, the aim of this study was to investigate the utility of plasma Epstein Barr viral load as a marker of EBV association in adult-onset HL patients.

Materials and methods

Type of study

This was a prospective study carried out at a tertiary care cancer center in south India. The study was approved by the institutional ethics committee and was performed as per the Helsinki Declaration 2000. A written, informed consent was obtained from all patients enrolled in the study.

Patient selection

The target population included patients with a diagnosis of HL following a detailed histopathological examination and immunohistochemistry (IHC). Staging evaluation included a complete history, thorough physical examination, imaging (computed tomography [CT] scan of the thorax, abdomen, and pelvis), and bone marrow aspiration and biopsy. Data regarding demography, clinical staging, International Prognostic Score (IPS), chemotherapy administered, and clinical outcome were recorded. Response assessment was done based on standard criteria [17,18]. Positron emission tomography-CT scan was not done on any patient as it was not available at our center due to the poor affordability of the patients we cater to.

Sample collection

Peripheral blood samples were collected before initiation of anticancer chemotherapy in newly diagnosed HL patients and in healthy voluntary blood donors. In HL patients, follow-up plasma samples at the end of three courses of chemotherapy were collected whenever possible. In order to validate the performance characteristics of the plasma viral DNA assay, EBV latent membrane protein-1 (LMP1) IHC was also performed in a subset of cases.

Laboratory protocol

All samples were handled with sterile precautions. Plasma was separated from all blood samples (pretherapy, posttherapy, or blood donors) within a few hours of collection by centrifugation at 1200*g* for 2 min and stored frozen at −20 °C until DNA extraction. All samples were found to be seronegative for human immunodeficiency virus and hepatitis B virus.

Real-time polymerase chain reaction

Nucleic acid was extracted from 250 μL of plasma with a silica based manual extraction protocol, and eluted with 50 μL elution buffer. The manual method was chosen over commercial extraction columns as it gave better and more consistent results (data not shown) [16]. Plasma EBV DNA in samples was quantified using real-time polymerase chain reaction (PCR) as per previously published protocol [13]. The standard curve was constructed using EBV DNA of known concentration diluted 10-fold (a kind gift from Professor Y.L. Kwong, University Department of Medicine, Queen Mary Hospital, Pok Fu Lam, Hong Kong). As optimized and validated in other major studies, EBV DNA load more than 500 copies/mL was considered as positive [8,14]. In order to validate the performance characteristics of the plasma viral DNA assay, in addition to routine IHC for confirmation of lymphoma diagnosis, EBV LMP1 IHC could be performed in 25 HL cases.

Results

Over a period of 10 months, 130 histologically confirmed cases of adult-onset lymphomas were diagnosed of which 33 were HL, while the remaining were nonHodgkin lymphomas. Thirty age-matched healthy blood donors (all men) were included as controls. Of the 33 patients with HL, 27

patients received treatment at our center, while four patients did not opt for treatment and two patients defaulted after the second chemotherapy cycle and were lost to follow-up.

Of the 33 histologically confirmed, newly diagnosed, adult-onset HLs, 25 were men and eight were women, with a mean age of 39.5 ± 16.8 years (range, 18—77 years; Table 1). The most common lymph nodes involved at presentation were the cervical lymph nodes. The spleen was involved in four patients while bulky mediastinal tumor was found in two patients. None of the patients had bone marrow involvement as evidenced with bone marrow biopsy/aspiration. All patients received therapy with doxorubicin, bleomycin, vinblastine, and dacarbazine in all stages of disease as part of the institutional protocol. In addition, involved field radiation therapy was administered in seven patients with Stage 1 or 2 HL.

Pretherapy plasma EBV DNA was detectable in 16 of 33 HL patients (48.5%). The viral load of the samples ranged from 1.0×10^3 copies/mL to 51.2×10^3 copies/mL (mean, 18×10^3 copies/mL; Table 1). Pretherapy EBV DNA was detected in six of the 16 nodular sclerosis subtype (37.5%) and six of the 10 mixed cellularity subtype (60%). Of the 33 HL cases included in the study, EBV LMP1 IHC could only be performed in 25, as tissue blocks were inadequate/unavailable in eight cases. Of these, 14 were LMP1 positive (56%), 12 of whom had detectable plasma EBV DNA using PCR. The 11 LMP1 negative cases were also negative for plasma EBV DNA.

In the eight HL patients in whom follow-up samples were available, only five patients had detectable pre-therapy plasma EBV DNA (Table 2). In them, viral load reverted to negative postchemotherapy and was associated with clinical remission in four patients and partial remission in one. Unlike in patients with complete response, the patient with partial response had a poor IPS of 5 at diagnosis. In the three follow-up patients without pretherapy plasma EBV DNA, post-therapy EBV DNA levels remained negative. EBV DNA was undetectable in plasma samples from all 30 healthy blood donors.

Discussion

Increased use of Epstein—Barr viral loads in assessing viral association, in risk stratification, and in assessing

Table 1 Adult-onset Hodgkin lymphoma patients' characteristics.

	Variables	All patients (n = 33)
Age (y)	Mean (range)	39.5 (18—77)
Sex	Men:women	25:8
Histological subtype	Nodular sclerosis	16
	Mixed cellular	10
	Nodular lymphocyte predominant	1
	Not classifiable	6
Staging	1	4
	2	13
	3	10
	4	1
	Data not available	5[a]
B symptoms	Yes	11
	No	17
	Data not available	5[a]
IPS	0—2	20
	3—7	8
	Data not available	5[a]
LMP1 IHC	LMP1 positive	14
	LMP1 negative	11
	Not done	8
Pretherapy plasma EBV DNA	EBV DNA present	16
	EBV DNA absent	17
Outcome after therapy	Clinical remission	19
	Partial remission	3
	Default after 2 cycles	2
	No follow-up/therapy taken	4
	Data not available	5[a]

Note. EBV = Epstein Barr Virus; IHC = immunohistochemistry; IPS = International Prognostic Score; LMP1 = latent membrane protein-1; y = year.

[a] Patients in whom staging, IPS, and outcome could not be determined due to discharge before staging/before therapy could be started.

Table 2 Follow-up cases of adult Hodgkin lymphoma.

Serial number	Age (y)/sex	Type of lymphoma	Ann arbor stage	IPS	LMP1 IHC	Prechemotherapy viral load (copies/mL)	Postchemotherapy viral load (copies/mL)	Clinical outcome
1	20/F	HL ns	2B	2	Negative	0	0	CR
2	32/M	HL ns	2B	2	Positive	1024	0	CR
3	22/F	HL mc	1A	2	Negative	0	0	CR
4	53/M	HL unclassified	3A	2	Not done	0	0	CR
5	65/M	HL mc	3A	5	Positive	1238	0	PR
6	38/M	HL ns	2A	1	Positive	7547	0	CR[a]
7	21/M	HL ns	2A	1	Positive	29,916	0	CR
8	26/M	HL ns	2A	1	Positive	30,190	0	CR[a]

Note. CR = complete remission; F = female; HL = Hodgkin lymphoma; IPS = International Prognostic Score; LMP1 IHC = latent membrane protein-1 immunohistochemistry; M = male; mc = mixed cellularity; ns = nodular sclerosis; PR = partial remission; y = year.
[a] Radiation therapy in addition to chemotherapy.

therapeutic response, has redefined the scope of quantitative PCR in detecting and monitoring HL [5,7—9,14,19]. The present study highlights the utility of plasma EBV DNA in determining EBV association in adult onset HL in the Indian context.

In this study, the mean age of onset of HL was 39.5 years with a male preponderance. These demographics are concordant with studies on adult HL from India and abroad [1,3,15]. In this study, 39% of the patients had constitutional ''B" symptoms. In developed countries, ''B" symptoms are found in 25—30% of HL, whereas in developing countries it is found in about 50% of cases [20,21].

Similar to earlier studies from our Institute and abroad, nodular sclerosis was the most common subtype identified in this study [8,9,14,22—24]. However, clinico-epidemiological studies from India show a predisposition towards mixed cellularity subtype [15,21]. Not all subtypes of HL harbor EBV to the same degree. Studies have shown EBV association in ~70% of mixed cellularity, 10—40% of nodular sclerosis, >95% of lymphocyte-depleted, and is almost always EBV negative in lymphocyte-predominant HL [19,25]. In the present study, EBV DNA was detectable in 37.5% of nodular sclerosis and 60% of the mixed cellularity and was negative in the single patient with lymphocyte predominant subtype.

EBV positivity in HL varies with geographical location; 20—50% in the West, 57—64% in the Far East, and up to 90% in developing countries [2,3,19,25,26]. Using plasma EBV DNA estimation in adult-onset HL, studies from Australia, North American, and Europe found 15—20% EBV association, while a study from Brazil revealed a 43% EBV association [7,8,14,24]. In this study, plasma EBV DNA was detectable in ~49% of adult HL patients. Based on LMP1 IHC, our study found EBV association in 56% of HL patients. Similar studies from India (based on either LMP1 IHC or Epstein—Barr encoding region *in situ* hybridization) also revealed a 58—77% EBV association in adult HL [20,22,27].

In our study, the sensitivity of EBV real-time PCR with respect to LMP1 IHC was 86% while its specificity was 100%. Studies estimate sensitivity of EBV PCR to be 65—100% [8—10,14]. The inability to detect EBV DNA in all cases of LMP1-positive HL could be due to the low copy numbers of DNA in these cases [5,19]. Sensitivity of PCR and viral load depends on patients' sample, efficiency of DNA extraction, target gene amplified, and the PCR technique used [5]. In this study, the source of sample was patients' plasma as it has been shown to correlate with EBV-associated lymphomas more closely than whole blood or PBMCs [5—8]. DNA extraction was performed using a manual, silica-based protocol as it was found to be more effective than commercially available extraction columns as has been noted in other studies [12,28]. *EBNA1* was the target gene amplified in our study as it is detectable uniformly in HL and is not present in multicopies. This helped determine viral load unlike multicopy genes like *BamH1-W* which may improve sensitivity but may not reflect the true viral load. Real-time PCR, as used in the present study, has been used for HL since 1999 and its speed, accuracy, and high throughput has outdone other quantitative and conventional PCR methods and is hence widely used for measuring EBV load [10—12].

Epstein Barr viral load is increasingly being used as a biomarker not only to estimate EBV association at diagnosis, but also for monitoring response to therapy in EBV-positive lymphomas [7,8,13,14]. Several studies have conclusively found therapeutic response to be associated with decline in viral load [7,14]. Despite our efforts, follow-up plasma postchemotherapy could be obtained in very few patients with pretherapy EBV positivity. Reversion of viral load to undetectable levels in these patients was associated with response to chemotherapy. Considering the fact that EBV is almost uniformly present latently in the Indian adult population, EBV reactivation did not occur in this study. This is evidenced by the few follow-up patients in whom EBV-negative status at diagnosis remained negative postchemotherapy despite dipping white blood cell count secondary to chemotherapy. Cell-free circulating EBV DNA has been found to correlate to parameters of disease activity, such as stage and IPS [7,9]. However, no such correlation was found, probably due to the modest number of cases in this study.

Considering a high EBV association in adult-onset HL and also postchemotherapy reversal of plasma viral loads in remission, viral load estimation maybe a useful tool for

detecting EBV association and its variations in this region. Future studies on larger patient samples with better follow-up sampling are needed to validate the prognostic use of the assay in EBV-associated lymphomas.

Conclusion

Our study reveals ~49% of newly diagnosed, adult-onset HL to be EBV-associated based on plasma EBV DNA quantitation. However, EBV LMP1 IHC revealed a 56% EBV association in a subset of the patients. Sensitivity and specificity of plasma EBV DNA detection (with respect to LMP1 IHC) was 86% and 100% respectively. Although plasma EBV DNA load did not correlate with parameters of disease activity, clearance of EBV DNA postchemotherapy correlated well with remission in all patients sampled. The ease of sampling and performance of the viral load assay may render it a useful tool for detecting EBV-association and for monitoring response to therapy in virus-related HL. In the Indian context, where a high percentage of patients have EBV-positive HL and may not be able to afford the expensive positron emission tomography and CT scanning for assessing therapeutic response to anticancer chemotherapy, the EBV DNA quantitation assay may, in future, prove to be a valuable tool to assess therapeutic response and help decide adequacy of the number of chemotherapy cycles required. There is, however, a need for well-designed follow-up studies to determine the utility of this assay in prognosticating EBV-positive HL.

Conflicts of interest

The authors declare no conflicts of interest.

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