RESEARCH PAPER

Plasma Epstein-Barr Virus (EBV) DNA as a Biomarker for EBV-associated Hodgkin lymphoma

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Received: August 01, 2014; Initial review: December 29, 2014; Accepted: June 01, 2015.

Objective: To assess plasma Epstein-Barr virus (EBV) DNA as a biomarker of tumour burden at diagnosis and during therapy in children with Hodgkin lymphoma.

Design: Case-control study, with prospective follow-up of the Hodgkin lymphoma cohort (2007-2012).

Setting: Pediatric Hematology Oncology unit of a tertiary care hospital in Delhi.

Patients: Thirty children with Hodgkin lymphoma and 70 sex and age-matched controls (benign lymphadenopathy 19, non-lymphoid malignancy 29, Burkitt lymphoma 5, healthy children 17).

Intervention: Positive EBV-staining on immunohistochemistry was defined as EBV-associated Hodgkin lymphoma. Plasma EBV real-time quantitative polymerase chain reaction (PCR) was tested at presentation, after first and last chemotherapy cycles, and on follow-up.

Main outcome measures: Plasma EBV quantitative PCR was compared between cases and controls. Its kinetics was assessed during and after chemotherapy.

Results: EBV quantitative PCR was positive in 19 (63%) Hodgkin lymphoma cases (range 500–430,000 copies/mL), with 87.5% accuracy (kappa=0.69) as compared with EBV-immunohistochemistry. Sensitivity and specificity of the quantitative PCR were 87.5% and 81.8%, respectively. Only boys showed positive EBV immunohistochemistry and/or quantitative-PCR positivity. All controls were quantitative-PCR negative. All quantitative-PCR positive cases with follow-up blood sample showed EBV clearance after the first cycle. A quantitative-PCR negative case in long-term remission became positive at relapse. EBV status did not influence survival.

Conclusion: Plasma EBV-DNA, detectable in EBV-associated Hodgkin lymphoma, becomes undetectable early after initiating therapy. It can be used as a biomarker of treatment response in EBV-associated Hodgkin lymphoma.

Keywords: *Follow-up; Immuno-histochemistry; Outcome.*

Epstein-Barr virus (EBV)-associated Hodgkin lymphoma (HL), defined by the presence of EBV proteins or EBV-encoded RNA in tumor cells, is seen in a higher proportion of children from underprivileged regions as compared to developed countries [1]. A large proportion of Indian childhood HL cases are EBV-associated [2]. Free circulating EBV genome fragments have been detected in the plasma/serum of HL patients prior to therapy. They become undetectable in those responding to therapy and persistence of circulating EBV DNA may correlate with either non-response or impending relapse [3,4]. Circulating EBV DNA is usually not detected in non-EBV associated HL cases and in healthy controls. The potential prognostic value of EBV viral load levels remains unknown. There is limited data available on the prognostic significance of circulating EBV DNA during therapy for HL, particularly in children [5].

We conducted this study to understand the significance of plasma detection of cell-free circulating EBV DNA in children with HL and to see if it could be used as a biomarker of EBV-associated HL and of tumour burden. A secondary objective was to assess whether serial monitoring of EBV DNA in children with EBV-positive HL could predict response to therapy and relapse.

METHODS

A case-control study was designed to measure circulating EBV DNA in children with HL and in controls. Secondly, a cohort study was designed over the same time period to follow the HL cohort's response to therapy, EBV load and long-term remission status.

All new cases presenting to the pediatric department of our hospital between 2007 and 2012 with lymphadenopathy that was biopsied and reported as HL

were included in the study. Children below 18 years of age with reactive lymphoid hyperplasia, newly diagnosed malignancies other than HL, and healthy controls, were included in the control group. Sex- and age-matching was done for selection of control subjects. Children with other malignancies were included among controls, in order to assess if EBV plays a pathogenic role in the development of HL or is seen as a consequence of malignancy-associated immune suppression. A minimum sample size of 23 per group was estimated for 2-sided test (2 independent groups, type-I error–5%, power–90%), assuming plasma EBV to be detectable in 50% of cases and 5% of controls.

The study was approved by the institutional ethics committee of our institute and informed consent was taken from patients and controls. Ann Arbor staging of HL was done using either positron-emission tomography (PET)-computed tomography (CT) scan or contrast-enhanced CT (CECT) scan of neck, chest, abdomen and pelvis along with bilateral iliac crest bone marrow biopsies [6]. Tumour burden was assessed in terms of disease stage, disease bulk, number of areas involved, B symptoms and lactate dehydrogenase (LDH) levels. Bulky disease was defined as any extra-thoracic nodal mass with a diameter ≥6 cm or a mediastinal mass with a diameter exceeding one-third of the maximum mediastinal width on chest *X*-ray. WHO classification was used for subtyping of HL based on morphology and CD15, CD30 and CD20 immunohistochemistry (IHC) [7].

Risk-adapted treatment was given using Adriamycin, Bleomycin, Vinblastine, Dacarbazine (ABVD) regimen. Early response was assessed after two (stage I-II) or four ABVD cycles (stage III-IV) by re-evaluating all involved sites. Involved-field radiotherapy (IFRT) was given only to initial bulky sites, to patients with residual disease after six ABVD cycles, and as a part of relapse protocols. End-of-treatment response was assessed by re-staging 4 to 8 weeks after treatment completion. Further follow-up was done 3-monthly for the first year, 6-monthly for the second year, and yearly thereafter.

Two blood samples of 2 mL each were collected from newly diagnosed cases and controls in an EDTA and plain vials. We separated plasma by centrifugation at 2,500 rpm for 20 min at 25ºC, and stored it at –70ºC until processing for DNA extraction. Serum separation was done at 4,500 rpm for 10 min and stored at –70ºC until processing for EBV serology. DNA was extracted using Roche High Pure Viral Nucleic Acid Kit. Plasma EBV real-time quantitative-PCR (qPCR) was assessed in controls and in pre-treatment HL cases using LightCycler EBV Quantitative Kit (Roche Molecular Diagnostics, USA) on a LightCycler 2.0 instrument (Roche). Non-template kit control and EBV calibrators provided by the supplier were used in each procedure. Post-HL chemotherapy, qPCR was reassessed after the first and last ABVD cycles, on further follow-up and whenever relapse was suspected.

EBV IHC was done on pre-treatment paraffin-embedded lymph-node biopsies of HL cases using EBV latent membrane protein-1 (EBV-LMP1) primary antibody (BioGenex, USA) and Novolink polymer detection system (Novocastra, Germany) according to manufacturer's instructions. EBV-associated HL was defined by the presence of unequivocal membrane and/or cytoplasmic staining in a proportion of Reed-Sternberg (RS) cells and mononuclear variants. EBV-LMP1 IHC was not performed in control subjects.

Statistical analysis: Chi-square test/Fisher exact test and binary logistic regression (univariate/ multivariate) were applied to study the association between categorical variables as appropriate. Mann-Whitney-Wilcoxon test and Kruskal-Wallis test were applied to study the association between categorical variables and EBV viral load as appropriate. Spearman rank correlation was used to compare viral loads between cases and controls. Kaplan-Meier survival analysis was done to assess potential prognostic factors.

RESULTS

Thirty-one consecutive children were diagnosed with HL during the study period. Of these, one was excluded as blood was not collected for EBV qPCR prior to initiation of therapy. Thus, 30 children with HL were prospectively included (28 newly diagnosed biopsy-proven HL and 2 presenting with relapsed HL). Stage and subtype distribution of HL is shown in ***Table* I**. HIV serology was negative for all patients. Three cases could not be classified as two were diagnosed on fine needle aspiration cytology and one by bone marrow biopsy as he was clinically unstable to undergo a lymph node biopsy.

Seventy controls were selected: healthy controls 17, children with non-malignant lymphadenopathiy 19, children with non-lymphoid malignancies 29 (brain tumor 9, neuroblastoma 9, Wilms tumor 5, myeloid malignancies 3, rhabdomyosarcoma 2, retinoblastoma 1) and children with Burkitt lymphoma 5.

EBV-LMP1 IHC was positive in 15 HL lymph-node biopsies and 1 bone marrow biopsy out of 27 patients analyzed. EBV association was higher in mixed cellularity (MC) as compared with nodular sclerosis subtype (77.8% *vs.* 16.7%, P=0.015). IHC-defined EBV-

TABLE I CHARACTERISTICS OF CHILDREN WITH HODGKIN LYMPHOMA ACCORDING TO THEIR PLASMA EPSTEIN BARR VIRUS QPCR STATUS (*N*=30)

		Total (n=30)	*EBV positive (n=19)*	*EBV negative (n=11)*	*P value*
Sex		24:6	19: 0	5: 6	0.001[a]
Median (IQR) age, y		8.7 (3.5-18)	8 (5.5-12)	12 (8-15)	0.10[b]
Stage I		4	4 (21.1%)	0	0.37[c]
II		9	6 (31.6)	3 (27.3	
III		4	2 (10.5)	2 (18.2)	
IV		13	7 (36.8)	6 (54.5)	
B symptoms		17	10 (52.6)	7 (63.6)	0.70[c]
Bulky disease		7	5 (26.3)	2 (18.2)	1.0[c]
Involved LN areas	1-2	12	8 (42.1)	4 (36.4)	0.90[c]
	3-4	12	7 (36.8)	5 (45.5)	
	≥5	6	4 (21.1)	2 (18.2)	
Anemia (Hb <10.5 g/dL)		12	8 (42.1)	4 (36.4)	1.00[c]
Immunophenotype	Mixed cellularity	19	15 (78.9)	4 (36.4)	0.05[c]
	Nodular sclerosis	6	2 (10.5)	4 (36.4)	
	Lymphocyte predominant	2	0	2 (18.2)	
	Unclassified	3	2 (10.5)	1 (9.1)	
EBV IHC (*n*=27)	Positive	16	14/16 (87.5)	2 (12.5)	

qPCR: real-time quantitative polymerase chain reaction; [a]Fisher's exact test. [b]Mann Whitney U-test. [c]Pearson's Chi square test. LN: lymph node. IHC: immunohistochemistry.

associated HL was seen in 76.2% of boys (16/21) and in none of the girls (*P*=0.002).

Circulating EBV-DNA: Pre-treatment plasma EBV qPCR was positive in 19 (63.3%) out of 30 children with HL (median 1,800 copies/mL, range 500-430,000), while all 70 controls tested negative (*P*<0.001). Median EBV load was higher in EBV-associated HL (1,065 copies/mL, range 0-50,000) as compared to EBV-negative HL (0 copy/ml, range 0-7,000; *P*=0.002). The patient with highest EBV load (430,000 copies/mL) had no tissue biopsy available for IHC.

Using IHC as a gold standard for diagnosis of EBV-associated HL, the sensitivity [95% CI] and specificity [95% CI] of EBV qPCR were 87.5% 62.7%, 97.8% and 81.8% [51.1%, 96.0%], respectively. The accuracy of q-PCR was 87.5% (kappa coefficient=0.63). Non-concordant results were seen in 4 cases. Two EBV-associated HL cases were negative for EBV qPCR, both having stage IV disease with bone marrow involvement. Two EBV-negative cases on IHC had detectable plasma EBV DNA (620 and 7000 copies/mL, respectively).

One boy was initially diagnosed as EBV-induced lymphoproliferation in view of fever and generalized lymphadenopathy with EBV-LMP1 positive reactive nodal hyperplasia and positive EBV q-PCR (1130 copies/mL). Seven months later, he presented with recurrence of fever and increasing lymphadenopathy after which repeat lymph node biopsy was done that was reported as HL MC. EBV-LMP1 continued to be positive whereas plasma EBV-DNA was no longer detectable. All HL cases with detectable circulating EBV genome were boys, 79.2% of the boys being qPCR positive. None of the 6 girls had detectable EBV DNA (*P*=0.001).

Viral load at diagnosis was higher in advanced stage disease, in cases with bulky disease, and in those with B symptoms, although without reaching statistical significance (***Table* II**).

EBV qPCR and outcome: Out of 19 qPCR positive cases, one died of advanced disease before starting chemotherapy. All 16 patients tested for plasma qPCR after the first ABVD cycle showed EBV clearance, regardless of response and time required to achieve complete remission.

IFRT was given to 8 patients. Twenty-two children were in complete remission and were qPCR negative at the end of therapy; 4 died (2 before initiating therapy, 1 on therapy, 1 refractory disease) and one defaulted treatment. Three children are yet to complete treatment.

WHAT IS ALREADY KNOWN?

- Most cases of Indian childhood Hodgkin lymphoma are Epstein-Barr Virus (ERV)-associated.

WHAT THIS STUDY ADDS?

- Plasma EBV quantitative-PCR is a sensitive and specific marker of EBV-associated Hodgkin lymphoma and becomes negative after the first chemotherapy cycle.

No relapse was observed in the cohort of newly diagnosed cases. A 14-year-old boy with unknown EBV status at diagnosis, in whom qPCR tested negative 10 years after first complete remission, became qPCR positive when he experienced local relapse 7 months later and was then enrolled in the study as a relapsed case.

At a median follow-up of 2.5 years (range 0.6 to 6.3 years), 21 patients are in long term remission and one is lost to follow-up. Five-year overall survival and 5-year event-free surivial was not significantly different in qPCR EBV-positive and negative cases at diagnosis (87.7% *vs.* 72.7%, *P*=0.63; and 82.1% *vs.* 80.8%, *P*=0.98, respectively). Similarly, EBV status in tumor cells did not show any association with outcome.

DISCUSSION

Various Indian studies have reported EBV-association in >95% of pediatric cases [2,8-10]. We observed a lower EBV association, probably due to higher socioeconomic status of patients, and a greater proportion of older patients, both factors being associated with EBV-negative tumors [1]. Viral load was higher in cases with advanced disease and/or B symptoms, as shown in earlier studies [4], and became undetectable after initiating chemotherapy. Though we have only one case suggesting the role of monitoring EBV load during follow-up for early detection of relapse, other reports support this approach, as patients testing positive for EBV PCR at the end of treatment are more likely to relapse [3-5].

TABLE II EBV LOAD IN CHILDREN WITH POSITIVE PLASMA QPCR AT DIAGNOSIS OF HODGKIN LYMPHOMA (*N*=19)

*EBV load*Median (IQR)*	
Stage	
Advanced (III-IV) (*n*=9)	7000 (1,065-33,600)
Early (I-II) (*n*=10)	1030 (500-4,600)
B symptoms	
Present (*n*=10)	5600 (1,097-25,300)
Absent (*n*=9)	1000 (500-4155)
Bulky disease	
Present (*n*=5)	5800 (2,282-29,000)
Absent (*n*=14)	1095 (904-9,525)

**copies/mL; IQR: Interquartile range; EBV: Epstein-Barr virus; qPCR: Quantitative PCR.*
No statistial differences in EBV load between the groups.

In cases of EBV-negative tumors with detectable circulating EBV DNA, scattered lymphocytes harboring EBV are the likely origin of circulating EBV DNA [3]. Viral load can be influenced by any cell undergoing viral lytic replication, leading to release of viral particles in the circulation [11]. Hence measuring EBV-infected circulating B lymphocytes may give more relevant information. The case with detectable circulating EBV DNA months before diagnosis, and negative qPCR when HL was finally diagnosed, may be explained by a "hit and run" mechanism. This was earlier postulated in EBV-negative sporadic Burkitt lymphoma [12] and EBV-negative HL [13]. The influence of EBV status on prognosis has not been clearly established, and may be age-related. Some reports have shown that EBV-positive tumors were associated with better outcome in children [14] and poorer outcome in older adults [15,16]. Large studies are needed to confirm the influence of EBV status on prognosis.

Limitations of our study include the small sample size and missing histopathological data in two cases. Median follow-up is still too short for adequate survival analysis.

Our data suggest that plasma qPCR may prove useful in screening children with persistent lymphadenopathy. A positive result may prompt early lymph node biopsy to diagnose EBV-associated HL, as benign lymphadenopathy would be unlikely. qPCR monitoring as indicator of later relapses in EBV-associated HL may be especially relevant in the developing world where PET-CT may be out of reach for the majority.

Acknowledgement: Alok Bajwa for technical assistance.
Contributors: VD: conceived and designed the study; compiled, analyzed and interpreted the data; and drafted the manuscript. All other authors contributed to data collection and critically reviewed the manuscript. AS: will act as guarantor of the study.
Funding: None; *Competing interests*: None stated.

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