Original Article

Plasma Epstein-Barr Virus (EBV) DNA as a Biomarker for Diagnosis of EBV-positive Hodgkin Lymphoma in Syria

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Abstract

Introduction: Epstein Barr Virus - positive Hodgkin lymphoma is defined by the presence of Epstein-Barr virus (EBV) in tumor cells. EBV plays an important role in the development and prognosis of Hodgkin's lymphoma. The standard way to detect EBV in Hodgkin lymphoma is immunohistochemistry stains for latent membrane protein-1 (LMP1) in tumor cells. The present study aimed to evaluate plasma Epstein-Barr virus (EBV) DNA as a noninvasive biomarker for diagnosis of EBV-positive Hodgkin lymphoma.

Methodology: The study included 60 newly diagnosed patients with Hodgkin lymphoma, ranging in age from 4 to 60 years, and 55 sex and age-matched controls. (60) Formalin-fixed paraffin embedded blocks of Hodgkin lymphoma tissue samples were used to investigate the EBV by in immunohistochemistry stains for (LMP1) in tumor cells. Plasma EBV DNA was quantified by real-time quantitative polymerase chain reaction (PCR) for all Hodgkin lymphoma patients prior to therapy and for control.

Results: The results showed that (25/60, 41.7%) of Hodgkin lymphoma were positive for histological LMP1, whereas plasma EBV DNA was detectable (range from 1.1×10^3 to 1.5×10^4 copies/mL, median: 1.1×10^4 copies/mL) in all EBV-positive Hodgkin lymphoma samples (25/25). EBV DNA was undetectable in all cases of EBV-negative Hodgkin lymphoma (35/35) and all healthy control (55/55). It is worth mentioning that our results demonstrated that the EBV DNA load was high in the EBV associated Hodgkin lymphoma patients suffering poor prognostic state.

Conclusions: Plasma EBV-DNA can be used as a noninvasive biomarker for diagnosis of EBV- positive Hodgkin lymphoma.

Key words: Hodgkin lymphoma; EBV; plasma EBV DNA; LMP1; immunohistochemistry; Syria.

Introduction

Epstein-Barr virus (EBV) is a double stranded DNA virus that belongs to the Herpesviridae family and Gamma herpesvirinae subfamily [1]. Epstein Barr virus (EBV) is associated with a variety of lymphomas/leukemias, and epithelial malignancies, including Hodgkin lymphoma (HL), non-Hodgkin lymphoma like Burkitt's lymphoma (BL), and nasopharyngeal carcinoma [2]. EBV-associated malignancies are associated with a latent form of infection [2], where EBV expresses restricted sets of proteins called EBV transcription programs (ETPs) in every tumor cell, including six nuclear antigens (EBNAs), three latent membrane proteins (LMP), and untranslated RNA called EBV- encoded small RNA (EBERs), these latent proteins and EBER can mediate cellular transformation [2]. The presence of EBV in the tumor cells of EBV-associated malignancies might afford a basis for specific therapy [3]. New research concentrates on application of different new treatment strategies targeting the EBV within tumor cells [4]. The diagnosis of EBV-associated malignancies is principally based on biopsy of the primary tumor, where EBER in situ hybridization (ISH) or LMP1 by immunohistochemistry (IHC) are used to determine if there is an association with EBV [2]. However, it can be difficult to perform a biopsy because of difficulties obtaining a biopsy of the tumor or poor patient status like patient has refractory or relapsed lymphoma. Many Studies in EBV association tumors including lymphoma have suggested that the EBV-DNA can be detected in the plasma of most patients with EBV-associated malignancies [5], which is derived from apoptotic or necrotic tumor cells as naked DNA fragments [6], while it remains undetectable in non EBV associated tumor or in healthy individuals [7,8]. Plasma EBV DNA has recently played a more important role in the diagnosis and management of EBV-associated cancers [9,10], especially in nasopharyngeal carcinoma [11,12], but there is no data

available on the diagnostic and prognostic significance of plasma EBV DNA for Hodgkin lymphoma in Syria. The World Health Organization has promoted cooperative studies about plasma EBV DNA quantification across organizations and countries to establish appropriate guidelines for EBV associated tumor diagnosis and treatment [5]. Since EBV plays an important role in the development and prognosis of EBV associated tumors [13].

Hodgkin lymphoma (HL) is a lymphoid malignancy of B-cell origin which is classified into either nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) or classical Hodgkin lymphoma (cHL) in accordance with 2008 WHO classification [10]. Since NLPHL is rarely EBV-positive [14], here we will focus on cHL where the link with EBV is well established.

Classic Hodgkin lymphoma (cHL) is a heterogeneous group of tumors characterized by a minority of neoplastic cells (RS cells and variants) in an inflammatory microenvironment rich in non-malignant reactive immune cells. EBV-positive HL is defined as the presence of EBV in tumor cells [13], the expression of EBV genes in HL is restricted to the type II latency pattern, including a relatively restricted set of viral genes (EBNA-1, LMP-1, and LMP-2 latent proteins, together with EBERs and BARTs RNAs) [14]. EBV LMP-1 is a viral protein that acts as an oncogene protein [15]. which causes a signal in a manner similar to an active form of cell surface molecule CD40 on B cells [15]. The standard way to detect Epstein-Barr virus (EBV) in Hodgkin lymphoma is by immunohistochemistry stains (IHC) for latent membrane protein-1 (LMP1) in tumor cells [16]. In this study we aimed to evaluate plasma Epstein-Barr virus (EBV) DNA as a noninvasive biomarker for diagnosis of EBV-associated Hodgkin lymphoma.

Methodology
Patients
This prospective cross- sectional study enrolled sixty newly diagnosed Hodgkin lymphoma patients, During the period from 1/9/2017 to 1/1/2019, from Al-Assad University Hospital, Mouwasat University Hospital, and University Children's Hospital in Damascus.

An age- and sex matched control group of 55 healthy volunteers were also included.

ALL cases (60) of HL were confirmed by morphologic and immunohistochemical analysis carried out according to the World Health Organization (WHO) classification criteria for Hodgkin's lymphoma [17], and were adopted from their medical records.

We obtained patients' clinical data from their medical records, which included gender, age, and tumor localization. All patients underwent a staging assessment according to the Ann Arbor staging system [18]. None of the patients had primary or acquired immunodeficiencies. HL patients were treated according to ABVD protocol (doxorubicin, bleomycin, vinblastine, and dacarbazine) [19]. Medical records were reviewed for treatment response and clinical state within 24 months through a physical exam, chest x-ray, and computed tomography of the chest and abdomen.

All patients and control were informed consent after the approval of the ethical committee (protocol number 1) in Damascus University.

Clinical specimens
Formalin-fixed paraffin embedded blocks of Hodgkin lymphoma tissue samples were used to investigate the presence of EBV by detecting LMP1 using immunohistochemistry stains.

Peripheral venous blood (5 mL) samples were taken from patients and controls into EDTA -treated tubes, and centrifuged at 1200 g for 10 min. Plasma isolation was performed immediately and frozen at -80°C until assay. DNA was extracted from 300 µL of plasma using a QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany) and eluted in 100 µL AE buffer (Qiagen, Hilden, Germany), according to manufacturer instructions.

EBV investigation
EBV immunohistochemistry stains was done on pre-treatment paraffin embedded lymph-node biopsies of HL cases using EBV latent membrane protein-1 (EBV-LMP1) primary antibody (Bio SB, USA), and Mouse/Rabbit PolyDetector DAB HRP Brown detection system (Bio SB, Germany) according to manufacturer's instructions. EBV-associated HL was defined by the presence of clear 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.

Plasma EBV DNA quantification
Plasma EBV DNA were evaluated in controls and in pre-treatment HL patients by a real-time quantitative polymerase chain reaction (qPCR) assay, by using artus EBV LC PCR Kit (QIAGEN, Hilden, Germany) on LightCycler 2.0 instrument (Roch, Mannheim, Germany).

The EBV LC PCR Master reagent kit contained Primers and enzymes for the specific amplification of a

97 bp region of the EBNA1 gene of the EBV genome. Quantification standards were provided with the kit, and were included in each run to generate the standard curve for EBV load determination.

Statistical analysis

Chi-square test/Fisher exact test was applied to compare variables between patient groups. Mann-Whitney- test and Kruskal-Wallis test were also used. A receiver operating characteristic curve was used to determine the cutoff value for plasma EBV-DNA with optimal sensitivity, specificity, and concordance with tumor EBV status by LMP1-IHC. SPSS Statistics version 25 was used for statistical analysis. *p*-value < 0.05 was considered statistically significant.

Results

Of the 60 HL cases, 37 (61.7%) were males and 23 (38.3%) were females. there was a male predominance in sex distribution (M: F = 1.6:1). Patients ranged in age from 4 to 60 years, distributed in two age groups: pediatric group (4-12 Y, average 8 Y) (20/60, 33.3%), and adult group (18-60Y, average 40 Y) (40/60, 66.7%). All of the patients had lymph node enlargement at presentation, with cervical lymphadenopathy being most frequent accounting for 70%.

Nodular sclerosis (NS) subtype was the most common subtype in all age groups and representing 41.7% of the entire group. Mixed cellularity (MC) was the second most common subtype and represented 36.7% of the entire group. While lymphocyte rich (LR) subtype represented 13.3%, and lymphocyte depletion (LD) subtype was the less common subtype and represented 8.3%

EBV was detected in tumor cells by IHC for LMP1, the results showed that 25/60 of paraffin-embedded formalin-fixed tumor specimens were positive for LMP1 (41.7%), while 35/60 were negative for LMP1 (58.3%).

The EBV positive cases were more frequently encountered in the pediatric group (4-12Y) 65% positivity, in contrast to 35% in the young adult group (18-60Y); and this was statistically significant (*p* = 0.03). There were also more positive cases amongst males (45.9%) than females (34.8%), without statistically significant (*p* = 0.07). The histologic subtype was also a factor strongly linked to positivity EBV, with 65% in MC vs. 24% in NS (p = 0.04), while 12% of LR cases were positive EBV, and 8% of LD cases were positive EBV

Plasma EBV DNA levels ranging from 1.1×10^3 to 1.5×10^4 copies/mL (median: 1.1×10^4 copies/mL) were detected in all cases of EBV-positive HL patients (25/25), meanwhile the EBV DNA was not detected in the plasma of patients who had EBV-negative tumors (35/35). The results demonstrated that data obtained from both histopathological and serological detection methods of EBV completely coincided (100%). A cutoff for plasma EBV-DNA >1.1×10^3 viral copies/ mL yielded 100% concordance with LMP1-IHC. On the other hand, the EBV in samples of healthy individuals was undetectable (55/55).

The patients (60) were distributed according to medical records into in advanced stage and in limited stage (42/60, 18/60), respectively. All plasma EBV DNA and LMP1 positive cases 25/25 were in advanced stage (100%), distributed between stage IIB and stage III (7/25, 18/25), respectively. Albeit 17/35 plasma EBV DNA and LMP1 negative cases were in advanced stage (stage IIB) (48.6%), and 18/35 were in limited stage (stage II) (51.4%). This gives an evidence of significant correlation between EBV presence in HL and advanced stage (*p* = 0.001).

Treatment responses were evaluated according to international response criteria for lymphoma [20], we assessed all the cases over 24 months after the beginning of treatment. We found that all diagnosed EBV-negative HL who were plasma EBV DNA negative 35/35 had responded to treatment with

Table 1. Distribution of EBV positive HL and EBV negative HL according to clinical stages, and Treatment responses evaluation over 20 months, besides the median plasma EBV DNA for each group.

	EBV positive Hodgkin lymphoma (n = 25)		EBV negative hodgkin lymphoma(n = 35)	
	LMP1-IHC* positive	Median plasma EBV DNA†	LMP1-IHC * negative	Median plasma EBV DNA†
	(n)	Copies/ml	(n)	Copies/ml
Clinical stage				
Advanced stage	25/25 (100%)	1.1×10^4	17/35(48.6%)	undetectable
Limited stage	0/25 (0%)		18/35(51.4%)	undetectable
Treatment responses evaluation (over 20 months)				
patients had relapsed lymphoma	8/25(32%)	1.3×10^4	0/35(0%)	
patients had Refractory lymphoma	5/25(20%)	1.1×10^4	0/35(0%)	
patients had responded to treatment	12/25 (48%)	1.9×10^3	35/35 (100%)	undetectable

LMP1-IHC *: latent membrane protein-1 (LMP1) immunohistochemistry stains; plasma EBV DNA†: plasma Epstein-Barr Virus DNA.

complete remission (100%). Conversely, the outcomes were worse for EBV- associated HL, whereas 5/25patients had Refractory lymphoma (20%) (resistant to treatment), 8/25patients had relapsed lymphoma (32%), and 12/25patients had responded to treatment with complete remission (48%).

The median concentration of pretreatment plasma DNA EBV was higher in patients with relapse lymphoma ($1.3×10^4$copies/mL), and patients with refractory lymphoma ($1.1×10^4$ copies/mL), as compared with patients who had responded to treatment with complete remission ($1.9×10^3$copies/mL), without statistically significant (*p* = 0.1). Table 1 showed the distribution of EBV positive HL and EBV negative HL according to clinical stages, and Treatment responses evaluation over 20 months, besides the median plasma EBV DNA for each group.

Discussion

The data showed that HL incidence among males is higher than in females, and this compatible with many studies, which report that Hodgkin lymphoma is more prevalent in males compared to females [21-23].

EBV infection rates in HL differ worldwide, with high rates (50-86%) reported from developing countries [23], while rates from developed countries were lower ranging from 30-48% in UK, France and the USA [24-26]. Therefore, the results of 41.7% are closer to those reported in the developed countries, and to near countries like Jordan, Iran and Iraq [27-29]. This may be related to improvement of the living levels over the past two decades in our region, as it is well known that one of the significant determinants of the EBV viral infection is socioeconomic status [28].

The results demonstrated that the presence of EBV DNA in plasma reflects the presence of EBV in tumor cells, with perfect compatibility (100%) when performed by histological detection method (IHC) and serological detection methods (PCR). Similarly, we found compatibility of undetectable plasma EBV DNA levels and LMP1-IHC negative cases of HL and healthy control. This perfect compatibility gave evidence to consider EBV DNA in plasma as a noninvasive biomarker for diagnosing EBV-positive Hodgkin lymphoma. Our findings agree with Spacek *et al.*, Kanapuru *et al.*, and Sinha *et al.* [30-32].

The results indicated that all plasma EBV DNA and LMP1 positive Hodgkin samples were obtained from patients in an advanced stage, with a low response rate to treatment, the results indicated a poor prognostic value for EBV presence in hodgkin lymphoma. The result was compatible with the result obtained by Yin *et*

al., Kwon, Koh [33-35], and with Kanapuru study, which showed that EBV-DNA in plasma is highly correlated with EBV tumor status in HL and is significant for determining the prognosis before therapy and at follow-up after 6 months [31].

The EBV is responsible for the advanced cases, because it contributes to the chronic inflammatory microenvironment that surrounds and supports tumor cells through major viral oncogenes protein LMP1 which stimulates the production of many cytokines and chemokines, including CCL5 (RANTES), CCL17 (TARC), and interleukin-8, by HRS cells [36], in addition LMP1 activates the signaling pathways of nuclear factor-B (NF-B), activating protein 1 (AP-1), and signal transducer and activator of transcription (STAT).In fact, LMP1 can substitute for the signaling of CD40 in B cells [37,38], and induce expression of BCL2, All of that increase the rate of neoplastic cell proliferation and inhibition of apoptosis [36].

The diagnostic and prognostic value of plasma EBV DNA in EBV positive HL is due to the fact that most EBV-tumor cells in EBV positive Hodgkin lymphoma persist in tumor tissues, and the plasma EBV-DNA is resulting from apoptotic or necrotic tumor cells which pass into the peripheral blood [39]. Although it was estimated that 95% of the population in the world have an asymptomatic lifelong EBV infection [40], less than 5% have detectable levels of EBV DNA in plasma [40]. The virus remains latent in the B lymphocyte pool with little cell turnover [40]. that the tenny amounts of EBV DNA in healthy people, if any, released from cell death or viral reactivation would not be sufficient to be detected in the circulation [39]. In contrast, there is a much higher cell turnover rate in cancers, e.g., up to 200,000 cancer cells/day in NPC [41], which would release sufficient cell-free EBV DNA into the circulation to be detected. Moreover, Ryan JL study showed that in patients with EBV-related malignancies, very few of the EBV-DNA in plasma is encapsidated [42], suggesting that cell-free EBV-DNA is derived from apoptotic or necrotic EBV-infected cells in tumors [42]. The quantification of plasma DNA EBV loads has played an important role in the diagnosis and management of other EBV-associated malignancies. Viral load measurement is particularly useful for assessing the prognosis or response to therapy of EBV-associated intractable lymphomas like extranodal NK/T-cell lymphoma [43,44], post-transplant lymphoproliferative disorder [45], EBV-positive nasopharyngeal carcinoma (NPC) [41], and EBV-positive gastric carcinoma [46].

Conclusions

The results indicated a poor prognostic value for EBV presence in Hodgkin lymphoma, so the presence of EBV should be investigated for all patients diagnosed with Hodgkin lymphoma. we recommend the use of plasma Epstein-Barr virus (EBV) DNA for diagnosis of EBV-associated Hodgkin lymphoma, as it offers a convenient, noninvasive, and repeatable approach to the assessment of EBV in tumor cell.

Study limitations

The limitation of our study was small sample size, we recommended more research with larger sample size to ensure that the sample is considered representative of a population and that the statistical result can be generalized to a larger population.

Authors' contributions

Rana Habeeb and Fawza Monem designed the study. Rana Habeeb and Lina al hafar carried out the histopathologic examination. Rana Habeeb and Fawza Monem carried out the serological analysis. Rana Habeeb and Lina al hafar and Fawza Monem analyzed the data and wrote the manuscript. Lina al hafar and Fawza Monem revised the paper. All authors read and approved the final manuscript.

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