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PLASMA EPSTEIN-BARR VIRUS (EBV) DNA AS A BIOMARKER FOR DIAGNOSIS OF SYRIAN EBV-POSITIVE BURKITT'S LYMPHOMA

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Epstein-Barr virus - positive Burkitt's Lymphoma is defined as the presence of Epstein-Barr virus (EBV) in tumor cells, the standard way to detect (EBV) in Burkitt's Lymphoma is in-situ hybridization (ISH) of EBV-encoded small RNA (EBERs) in tumor cells. The present study aimed to evaluate plasma Epstein-Barr virus (EBV) DNA as a noninvasive biomarker for diagnosis and prognosis of EBV-positive Burkitt's lymphoma. The study included 40 newly diagnosed patients with Burkitt's lymphoma, ranging in age from 4 to 60 years, and 55 sex and age-matched controls. Forty formalin-fixed paraffin-embedded blocks of Burkitt's lymphoma tissue samples were used to investigate the EBV by in-situ hybridization detection of the EBERs. Plasma EBV DNA was quantified by real-time quantitative polymerase chain reaction (PCR) for all Burkitt lymphoma patients prior to therapy and for control. The results showed that (22/40, 55%) of Burkitt lymphoma were positive for histological EBER, whereas plasma EBV DNA was detectable (range from 1.2×10^4 to 4.7×10^6 copies/mL) in all EBV-positive Burkitt lymphoma samples (22/22). EBV DNA was undetectable in all cases of EBV-negative Burkitt lymphomas (18/18) and all healthy control (55/55). It is worth mentioning that our results demonstrated that the EBV DNA load was significantly high in the EBV-positive BL patients suffering poor prognostic state. In conclusion: Plasma EBV-DNA can be used as a noninvasive biomarker for diagnosis and prognosis of EBV-positive Burkitt's lymphoma.

INTRODUCTION

Epstein Barr virus (EBV) is associated with a variety of lymphomas/leukemias, and epithelial malignancies, including Burkitt's lymphoma (BL), Hodgkin lymphoma (HL), and nasopharyngeal carcinoma[1]. EBV-associated malignancies are associated with a latent form of infection[1], 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[1]. The presence of EBV in the tumor cells of EBV-associated malignancies might afford a basis for specific therapy[2]. New research concentrate on application of different new treatment strategies targeting the EBV within tumor cells[3]. The diagnosis of EBV-associated malignancies is principally based on biopsy of the primary tumor, where EBER *in-situ* hybridization is used to determine if there is an association with EBV[1]. However, it can be difficult to perform a biopsy because of difficulties obtaining a biopsy of the tumor or poor patient status. 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[4], which is derived from apoptotic or necrotic tumor cells as naked

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DNA fragments[5&6], while it remains undetectable in non EBV associated tumor or in healthy individuals[7]. Plasma EBV DNA has recently played a more important role in the diagnosis and management of EBV-associated cancers[8], especially in Hodgkin lymphoma (HL)[9&10], and nasopharyngeal carcinoma[11&12], but there is limited data available on the diagnostic and prognostic significance of plasma EBV DNA for Burkitt's lymphoma. 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[4].

Burkitt's lymphoma (BL) is an aggressive, non-Hodgkin lymphoma (NHL)[13]. There are three types of Burkitt's lymphoma which differ in geographic distribution and Epstein-Barr virus (EBV) association[14]: Endemic (eBL), sporadic (sBL), and HIV-associated BL. Endemic BL which is associated with (EBV) in over 95% of cases, and it is predominant in the malaria endemic regions such as Equatorial Africa and Papua New Guinea[13], Common sites of tumor occurrence of eBL are the jaws and other facial bones[13]. Sporadic Burkitt's lymphoma (sBL) has a wide global distribution outside Africa, the rate of its association with EBV ranges from 15 to 80%, common sites of tumor occurrence of sBL are intra-abdominal regions[14&15]. EBV-positive BL is defined as the presence of EBV in tumor cells[13], the expression of EBV genes in BL restricted to the type I latency default programs, where Only EBNA-1 and EBER are expressed in EBV positive cells in BL tumors[13]. The standard way to detect Epstein-Barr virus (EBV) in Burkitt lymphoma is by *in-situ* hybridization (ISH) of EBV-encoded small RNA (EBER) in tumor cells[16]. In this study we aimed to evaluate plasma Epstein-Barr virus (EBV) DNA as a noninvasive biomarker for diagnosis and prognosis of EBV-positive Burkitt's lymphoma.

MATERIALS AND METHODS

Study groups

This prospective cross-sectional study enrolled forty newly diagnosed Burkitt's 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 (40) of BL were confirmed by morphologic, immunohistochemical, and molecular analyses carried out according to the World Health Organization (WHO) classification criteria for Non-Hodgkin's lymphoma[17] and were adopted from their medical records. All showed classic type distinguished by a proliferation of medium-sized cells, basophilic cytoplasm, and numerous mitotic figures with a starry-sky pattern Image, they also were characterized by c-MYC translocation and CD20+/CD10+/bcl-2–/bcl-6+/CD3− with a very high Ki-67 proliferation index.

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 Murphy/St. Jude's staging system for non-Hodgkin[18]. None of the patients had primary or acquired immunodeficiencies. BL patients were treated according to BFM-NHL protocol[19]. Medical records were reviewed for treatment response and clinical state within 20 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 in Damascus University.

Sampling

Formalin-fixed paraffin embedded blocks of Burkitt's lymphoma tissue samples were used to investigate the presence of EBV by detecting EBERs using *in-situ* hybridization method.

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 freezed at -80°C until assay. DNA was extracted from 300 µL of plasma using a QIAamp DNA Blood Minikit (Qiagen, Germany) and eluted in 100 µL AE buffer (Qiagen, Germany), according to manufacturer instructions.

EBV investigation

***In-situ* hybridization for Epstein-Barr virus-encoded RNA**

The presence of EBV was detected by in situ hybridization for EBV-encoded RNA (EBER) by histopathologic examination of the neoplastic cells by using a Biotin-labeled ZytoFast CISH probe, Zyto Fast CISH Implementation Kit AP-NBT/BCIP (Zytovision, Germany), according to manufacturer instructions.

Plasma EBV DNA quantification

Plasma EBV DNA were evaluated in controls and in naive BL patients by a real-time quantitative polymerase chain reaction (qPCR) assay, by using artus EBV LC PCR Kit (QIAGEN, Germany) on LightCycler 2.0 instrument (Roch, 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 EBER-ISH. SPSS Statistics version 25 was used for statistical analysis. P-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Results

Of the 40 BL cases, 29 (72.5%) were males and 11 (27.5%) were females. there was a male predominance in sex distribution (M : F = 2.6:1). Patients ranged in age from 4 to 60 years, distributed in two age groups: pediatric group (4-12 Y, average 8 Y) (37/40, 92.5%), and adult group (48-60 Y, average 54 Y) (3/40, 7.5%). In all Burkitt lymphoma patients, the tumor was located in the abdomen.

EBV was detected in tumor cells by ISH, the results showed that (22/40, 55%) of paraffin-embedded formalin-fixed tumor specimens were positive for EBER (Fig. 1a), while (18/40, 45%) were negative for EBER (Fig. 1b).

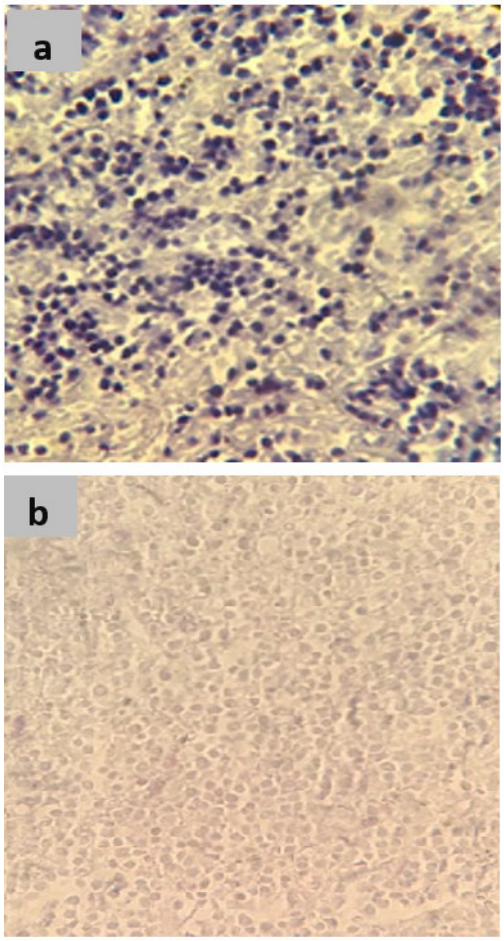

Fig. 1: EBER in situ hybridization: (a) A Case of EBV positive Burkitt lymphoma showing a dark blue-violet positive staining in the nucleus of neoplastic cells (×400); (b) A Case of EBV negative Burkitt lymphoma where no dark blue-violet color appears in the nucleus of tumor cells indicating negative EBER (×400).

Plasma EBV DNA levels ranging from 1.2×10^4 to 4.7×10^6 copies/mL (median: 8.3×10^5 copies/mL) were detected in all cases of EBV-positive BL patients (22/22), meanwhile the EBV DNA was not detected in the plasma of patients who had EBV-negative tumors (18/18).

Our results demonstrated that data obtained from both histopathological and serological detection methods of EBV completely coincided (100%). A cutoff for plasma EBV-DNA >12000 viral copies/mL yielded 100% concordance with EBER-ISH. On the other hand, the EBV in samples (55/55) of healthy individuals was undetectable.

Our samples (40) were distributed according to medical records into (24/40, 16/40) patients in advanced stage and in limited stage respectively. All plasma EBV DNA and EBER positive cases (22/22) were in advanced stage, distributed between stage III and stage IV (18/22, 4/22), respectively. Albeit (16/18) of plasma EBV DNA and EBER negative cases were in limited stage (stage II), (2/18) were in advanced stage (stage III). This give an evidence of significant correlation between EBV presence in BL and advanced stage (p= 0.001).

Treatment responses were evaluated according to international response criteria for lymphoma[20], we assessed all the cases over 20 months after the beginning of treatment. We found that all diagnosed EBV-negative BL who were plasma EBV DNA negative had responded to treatment with complete remission (18/18, 100%). Conversely, the outcomes were worse for EBV-positive BL, whereas (14/22, 63.6%) patients had died, (3/22, 13.7%) patients had Refractory lymphoma (resistant to treatment), (2/22, 9%) patients had relapsed lymphoma, and (3/22, 13.7%) patients had responded to treatment with complete remission.

The median concentration of pretreatment plasma DNA EBV was significantly higher in later deceased patients (3.9×10^6 copies/mL, p= 0.047) as compared with patients with relapse lymphoma (4.9×10^5 copies/mL), patients with Refractory lymphoma (4.3×10^5 copies/mL), and patients who had responded to treatment with complete remission, whose level values were significantly low (7.1×10^4 copies/mL). No significant difference was observed between the plasma EBV DNA levels in patients with relapsed lymphoma (4.9×10^5 copies/mL) and with patients with Refractory lymphoma (4.3×10^5 copies/mL, p= 0.09).

Table 1 demonstrates the distribution of EBV positive BL and EBV negative BL according to clinical stages and treatment responses evaluation over 20 months as well as the Median plasma EBV DNA for each group.

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

	EBV positive Burkitt lymphoma (n= 22)		EBV negative Burkitt lymphoma (n= 18)	
	EBER -ISH* positive (n)	Median plasma EBV DNA† Copies/ml	EBER-ISH* negative (n)	Median plasma EBV DNA† Copies/ml
Clinical stage				
Advanced stage	22/22 (100%)	8.3×10^5	2/18 (11%)	undetectable
Limited stage	0/22 (0%)		16/18 (89%)	undetectable
Treatment responses evaluation (over 20 months)				
Patients had died	14/22 (63.6%)	3.9×10^6	0/18 (0%)	
Patients had Refractory lymphoma	3/22 (13.7%)	4.3×10^5	0/18 (0%)	
Patients had relapsed lymphoma	2/22 (9%)	4.9×10^5	0/18 (0%)	
Patients had responded to treatment	3/22 (13.7%)	7.1×10^4	22/22 (100%)	undetectable

EBER-ISH*: EBV-encoded small RNA (EBER) *in-situ* hybridization
Plasma EBV DNA†: plasma Epstein-Barr Virus DNA

Discussion

In our study, the incidence of Burkitt's lymphoma was higher in children (92.5%) as compared with adults, this corresponds to many studies which report that Burkitt's lymphoma is more common in children[21&22]. The exact cause of the high incidence of Burkitt's lymphoma in children is not known yet. We believe that exposure to EBV infections in childhood may increase a child's risk of having Burkitt lymphoma.

Our data showed that BL incidence among males is significantly higher than in females, and this compatible with many studies which report that Burkitt's lymphoma is more prevalent in males compared to females[22-25].

In concordance with other studies[26], we observed a lower incidence of Burkitt lymphoma between females. We agree with Yakimchuk *et al.* point of view that estrogen has anti-proliferative effect on Burkitt lymphoma cells through estrogen receptor β (ERβ) signaling[26].

Our study is the first study about association of EBV with Burkitt lymphoma in Syria, with respect to the incidence rate of EBV-positive Burkitt lymphoma in our cases (22/40, 55%), it was higher than is reported in developed countries (15-30%)[24], and intermediate between those of Asian countries (50%, 80%, 30.5%) in Iran, Iraq and China respectively[23,24&27], and those of Latin American countries (52%, 37%) in Brazil and Argentina respectively[28&29]. The rate obtained in our study might be due to childhood primary infection of EBV usually occurs in these countries in childhood, and this inappropriate socioeconomic level which may lead to immune impairment and lack of monitoring and control of latent infection with EBV[30&31].

Our 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 (ISH) and serological detection methods (PCR). Similarly, we found compatibility of undetectable plasma EBV DNA levels and EBER-ISH negative cases of BL and healthy control. Our findings agree with those of Westmoreland *et al.*, Machado *et al.* and Kabyemera *et al.*[16,7&32]. This perfect compatibility gave evidence to consider EBV DNA in plasma as a noninvasive biomarker for diagnosing EBV-positive Burkitt lymphoma.

Our results indicated that all plasma EBV DNA and EBER positive Burkitt samples were obtained from patients in an advanced stage (22/22), with a high death rate (63%) and a low response rate to treatment (13%). In contrast, most of the plasma EBV DNA and EBER negative Burkitt samples of those who were in limited-stage (16/18) with a high response rate to treatment (100%), these results indicated a poor prognostic value for EBV presence in Burkitt lymphoma. We agree to the opinion which explains that is due to the role of EBNA and EBERs in apoptosis inhibition and increasing the rate of neoplastic cell proliferation[30]. Previous studies have demonstrated that EBERs induce interleukin-10, an autocrine growth factor for Burkitt's lymphoma cells, by activating RIG-I/interferon regulatory factor 3 pathway[33]. EBERs were also found to bind retinoic acid-inducible gene I (RIG-I) and thus activate its downstream signaling[33]. EBNA is essential for viral DNA replication and episome maintenance during cellular replication at latent stages[34], EBNA1 also contribute to the oncogenic process by up-regulating the apoptosis suppressor protein, survivin in EBV-associated B-lymphoma cells[35], so EBNA1 is associated with the survival of Burkitt's lymphoma cells[34&35].

Although Westmoreland D *et al.*[16], and Wang *et al.*[36] finding is similar to ours, but there are few studies on the prognostic value of EBV in Burkitt lymphoma due to high mortality rate, delay in diagnosis, and rapid tumor spread and proliferation.

Our results demonstrated that outcomes for EBV positive Burkitt's patients were worse in patients who had elevated plasma EBV DNA, where the highest values of plasma DNA EBV were for later deceased patients, then in patients who had relapsed lymphoma or patients with Refractory lymphoma. And on the contrary, the lowest values were in patients who had responded to treatment with complete remission. Hence plasma EBV DNA may be used as prognostic marker. Our result was compatible with the result obtained by Westmoreland *et al.*[16], and Kabyemera[32]. As

well 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[37&38], post-transplant lymphoproliferative disorder[39], nasopharyngeal carcinoma (NPC)[40], and EBV-positive gastric carcinoma[41]. Kanakry JA study 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[42].

We believe that the diagnostic and prognostic value of plasma EBV DNA in EBV positive BL is due to the fact that most EBV-tumor cells in EBV positive Burkitt lymphoma persist in tumor tissues[13], and the plasma EBV-DNA is resulting from apoptotic or necrotic tumor cells which pass into the peripheral blood[43]. Although it was estimated that 95% of the population in the world have an asymptomatic lifelong EBV infection[44], less than 5% have detectable levels of EBV DNA in plasma[44]. The virus remains latent in the B lymphocyte pool with little cell turnover[44], 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[43]. In contrast, there is a much higher cell turnover rate in cancers, e.g., up to 200 000 cancer cells/day in NPC[40], 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[45], suggesting that cell-free EBV-DNA is derived from apoptotic or necrotic EBV-infected cells in tumors[45]. Accordingly, the release of EBV DNA into the circulation is in turn determined by the cancer cell population[43]. As that, plasma EBV-DNA can reflect a patient's tumor burden and the cell damage caused by inflammation or immunity[43]. Hence, plasma EBV DNA constitutes a suitable candidate as a biomarker for evaluating tumor severity and prognosis of EBV positive BL patients.

Conclusions

We recommend plasma EBV-DNA testing in naïve BL patients for diagnosis EBV-positive BL and predicting prognosis. The main limitation is the small number of cases. Further studies are needed to evaluate the use of plasma EBV-DNA as a biomarker for monitoring response to treatment in patients with EBV-positive BL.

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الحمض النووي لفيروس إبشتاين بار (EBV) كمؤشر بيولوجي لتشخيص لمفومة بيركيت إيجابية EBV فى سوريا

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تتميز لمفومة بيركيت إيجابية فيروس إبشتاين بار بوجود فيروس إبشتاين بار (EBV) في جميع الخلايا اللمفاوية الورمية. الطريقة المرجعية لكشف وجود فيروس EBV في النسيج الورمي للمفومة بيركيت هي التهجين في الموضع (ISH) للحمض النووي الريبي الفيروسي EBERs. هدفت دراستنا الحالية إلى تقييم الحمض النووي لفيروس إبشتاين بار EBV DNA الحر في البلازما كواصم حيوي لتشخيص لمفومة بيركيت إيجابية EBV وذلك باستخدام طريقة التفاعل التسلسلً للبولّيميُراز بالوقت الحقيقي (PCR) وهي طريقة غير باضعة.

اشتملت الدراسة على ٤٠ مريضًا تم تشخيصهم حديثًا ، تتراوح أعمارهم بين ٤ إلى ٦٠ عاماً ، بالإضافة إلى ٥٥ شاهد سليم. تم استخدام عينات من النسيج الورمي محفوظة بالبارافين مثبتة بالفورمالين لكشف وجود EBV عن طريق التهجين في الموضع لـ EBERs ، كما تمت معايرة الحمض النووي الفيروسي DNA EBV في البلازما عن طريق التفاعل التسلسلً للبولّيميُراز بالوقت الحقيقي (PCR) لجميع مرضى لمفومة بيركيت قبل العلاج.

أظهرت النتائج أن (٤٠/٢٢ ، ٥٥٪) من حالات لمفومة بيركيت كانت إيجابية لـ EBER في النسيج الورمي. في حين أن plasma EBV DNA كان قابلاً للكشف فقط عند جميع مرضى لمفومة بيركيت إيجابية EBV وتراوحت القيم بين ١٠×١,٢[٤] إلى ١٠×٤,٧[٦] نسخة/مل. بينما كان plasma EBV DNA غير قابل للكشف في جميع حالات لمفومة بيركيت سلبية EBV وعند المجموعة الشاهدة ، ونتيجة لما سبق نجد أن وجود EBV DNA في البلازما يعكس وجود EBV في الخلايا السرطانية ، مع توافق تام (١٠٠٪) بين الطريقة النسيجية والطريقة المصلية. من الجدير بالذكر أن نتائجنا أظهرت أن حمل الحمض النووي EBV DNA كان مرتفعًا بشكل ملحوظ عند مرضى لمفومة بيركيت إيجابيين EBVالذين يعانون من حالة إنذارية سيئة.

الخلاصة: يمكن استخدام EBV-DNA كواصم حيوي غير باضع لتشخيص لمفومة بيركيت إيجابية EBV.