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ORIGINAL ARTICLE

Detection of EBV DNA in Non-Hodgkin Lymphoma Patients in Bulgaria

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Abstract Epstein–Barr virus is the first human oncogenic virus associated with a broad range of different malignant diseases but its role in non-Hodgkin lymphomas (NHL) development still needs to be fully understood. High expression levels of EBV major genes are found in NHL tumor cells and free viral DNA circulates in the plasma of such individuals. In the current study we detected EBV DNA levels in plasma samples from NHL patients in order to validate its significance as a laboratory marker for disease monitoring. We investigated a cohort of 52 patients diagnosed with NHL in The University Hospital "St. Marina" Varna, Bulgaria. Viral DNA was extracted from single plasma samples using Kit Ribo Virus (Sacace Biotechnologies S.r.l., Como, Italy) and amplified with EBV Real-TM Quant (Sacace Biotechnologies S.r.l., Como, Italy). Plasma samples of the same patients were tested for presence of EBV VCA IgM/IgG antibodies with indirect ELISA tests (Euroimmun, Luebeck, Germany). We found 15.4% (95% CI 6.9–28.1%, n = 8) of the samples from NHL patients to be positive in quantitative PCR (range 674–221,333 copies/ml). The diffuse large B cell lymphomas and peripheral T cell lymphomas were most often associated (although not statistically significant, $p = 0.167$) with detectable plasma EBV DNA levels. To our knowledge, this is the first study about the role of EBV in NHL development in Bulgaria. The results we have obtained should stimulate new, larger investigations to apply the quantitative PCR technique in the routine laboratory EBV diagnosis.

Keywords Epstein–Barr virus · Non-Hodgkin lymphoma · EBV DNA

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Introduction

Non-Hodgkin lymphomas (NHL) consist of a broad range of diseases with different level of aggressiveness but all affecting B or T lymphocyte population. In addition to their clinical and morphological heterogeneity, they have variable etiology and geographic distribution. The incidence of NHL is around 14 per 100,000 person-years in USA and Canada, 10 per 100,000 in Denmark and Sweden and 3 per 100,000 in South Central Asia [1]. According to the database of the National Statistical Institute of Bulgaria, approximately 400 new cases appear each year (341 in 2016) and the total number of NHL registered cases at the end of 2016 was 3598 [2]. Thus, the NHL prevalence in the country is 50.5 cases per 100,000 and average annual incidence is 4.8 per 100,000 population (data for 2016).

Three groups of infectious agents can participate in the development of NHL: (1) viruses that directly transform lymphocytes, damage their normal cell functions and stimulate cell division (Epstein–Barr virus (EBV), Human herpesvirus 8, Human T lymphotropic virus); (2) Human immunodeficiency virus (HIV), which exhausts the CD4+ T lymphocytes and (3) infectious agents leading to chronic immune stimulation (Hepatitis C virus, Hepatitis B virus, *Helicobacter pylori*, *Borrelia afzelii* etc.) [3]. Among these infectious causes, EBV most often associates with malignant lymphomas. After the primary infection (usually asymptomatic or clinically present as infectious mononucleosis) this gamma herpes virus persists in latent state in the B lymphocytes and under primary or secondary immune deficiency can activate their proliferation and provoke Burkitt's lymphoma, Hodgkin lymphoma or post-transplant lymphoproliferative disorder [4–6].

To our knowledge, data about the role of EBV in NHL development are not available for the Bulgarian population. In addition, studies measuring the level of EBV DNA, especially in plasma samples from NHL patients are rare in Europe. Therefore, we analyzed samples from patients with different subtypes of NHL for presence of EBV DNA with the purpose to collect epidemiological data and to improve the clinical practice for diagnosis and therapy of such patients.

Materials/Methods

The study was approved by the Ethic committee of Medical University Varna and was funded by the University fund "Science" (No. 16003/2016).

Patients and Clinical Samples

We investigated 52 single plasma samples of NHL patients from the Haematology Clinic of the "St. Marina" University Hospital, Varna, Bulgaria, obtained between November 2016 and August 2017. The blood samples were collected in EDTA vacutainers and the resulted plasma was stored at − 20 C° before DNA extraction and further analysis, both performed in September 2017. The blood from the enrolled patients was obtained in the first 3 days after the hospital admission in the cases of ongoing therapy or progression control and in the first 2–5 days in the cases of newly diagnosed patients. At the same time points, the clinical characteristics of the enrolled individuals were also detected and summarized (Table 1).

PCR Methods

DNA was extracted from 150 µl plasma using Kit Ribo Virus (Sacace Biotechnologies S.r.l., Como, Italy). Amplification was performed with EBV Real-TM Quant (Sacace Biotechnologies S.r.l., Como, Italy) following the standard manufacturer's instructions in reaction volumes of 25 µl and using the QuantStudio Dx Real-Time PCR Instrument (Thermo Fisher Scientific, Waltham, MA USA). The target amplification region is the *latent membrane protein (LMP) gene* of EBV and the sensitivity of the kit is reported to be > than 200 copies/ml or 5 copies of EBV DNA per 10^5 cells with a linear range of 500-10^6 EBV DNA copies/ml.

ELISA Methods

The plasma samples of the patients were tested for the presence of EBV VCA IgM/IgG antibodies with indirect ELISA tests (Euroimmun, Luebeck, Germany) according to the standard instructions of the manufacturer. When calculating the IgM results, the semiquantitative method was applied: Ratio = Extinction of the sample/Extinction of calibrator. Positive samples had a ratio > 1.1; negative samples had a ratio of < 0.8; and ratios between 0.8 and 1.1 were considered borderline. For IgG, we used the quantitative method for defining positive and negative samples by constructing a calibration curve (Cal 1 = 200 RU/ml, Cal 2 = 20 RU/ml, Cal 3 = 2 RU/ml, where RU/ml is relative units/ml). Positive results were ≥ 22 RU/ml; negative samples < 16 RU/ml; and the borderline were between 16 and 22 RU/ml.

Statistical Methods

The results obtained were processed with the statistical program SPSS, versus 23 to calculate the average age of

Table 1 Dependence of EBV DNA positivity on the clinical characteristics of NHL patients

Variable	N (%)	Positive patients (n)	Negative patients (n)	*p* value (Fisher's exact test)
Subtype				
DLBCL	25 (48.1)	3	22	
MZL	2 (3.8)	0	2	
T-cell lymphoma (peripheral and precursor)	9 (17.3)	3	6	
FL	7 (13.5)	1	6	0.58
WM	1 (1.9)	0	1	
CLL/SLL	3 (5.8)	1	2	
Unspecified	5 (9.6)	0	5	
Ann Arbor stage				
I–II	6 (11.5)	0	6	
III–IV	34 (65.4)	6	28	0.72
No data	12 (23.1)	2	10	
Therapy				
On therapy	44 (84.6)	8	36	
No therapy	8 (15.4)	0	8	0.33
LDH				
> 378 U/L	33 (63.5)	6	27	
< 378 U/L	19 (36.5)	2	17	0.69
Lymphocytes (%)				
< 20%	20 (38.5)	3	17	
> 44%	5 (9.6)	0	5	0.88
Normal	27 (51.9)	5	22	

DLBCL diffuse large B cell lymphoma, *MZL* marginal zone lymphoma, *FL* follicular lymphoma, *WM* Waldenström macroglobulinemia, *CLL/SLL* chronic lymphocytic leukemia/small lymphocytic lymphoma, *LDH* lactate dehydrogenase

the sample, the relative proportions and the confidence intervals. We used Fisher's exact test to measure the significance of association of viral DNA with the different clinical characteristics of NHL patients and considered $p < 0.05$ as statistically significant.

Results

Most of the patients included in the study were males – 57.7% (n = 30) and individuals aged above 45 years predominated in the sample (75.0%, n = 39). The average age was defined as 54.5 (SD ± 13.42; range of 23-78 years). B cells lymphomas represented the majority of the cases (73.1%), while T cell (peripheral and precursor) lymphomas represented 17.3% (Table 1). Unfortunately, patients with advanced stages predominated, as more than the half of the sample (> 60%) was in III–IV stage according to the Ann Arbor staging system (65.4%) and/or had high LDH (lactate dehydrogenase) levels (Table 1).

According to the serological analysis, 96.2% (95% CI 86.8–99.5, n = 50) of the patients were anti-VCA IgG positive and 7.7% (95% CI 2.1–18.5, n = 4) were anti-VCA IgM positive. Only one of the tested patients was negative for both of the markers and one was positive only for anti-VCA IgM. The majority of the tested individuals (80.8%; 95% CI 67.5–90.4) had anti-VCA IgG > 100 RU/ml and no correlation was shown to exist between the amount of anti-VCA IgG and the age of the tested persons (Fig. 1).

We found positive PCR results (range 674–221,333 copies/ml and average viral load 37115.13 ± 78149.96 copies/ml) in 8 out of 52 tested samples (15.4%; 95% CI 6.9–28.1). Six of the positive samples were from men and six were from patients above 45 years. We detected the following clinical subtypes of NHL in patients positive for EBV DNA: diffuse large B cell lymphoma (DLBCL) (3 patients), peripheral T cell lymphoma (3 patients), follicular lymphoma (FL) (1 patient) and chronic lymphocytic leukemia (CLL) (1 patient). In PCR negative patients, the predominant subtypes of NHL were also the DLBCL (in 50% of the negative patients), the T-cell lymphoma (in 14%) and FL (in 14%).

All of the positive patients showed advanced disease and all were on therapy during the study, while the negative

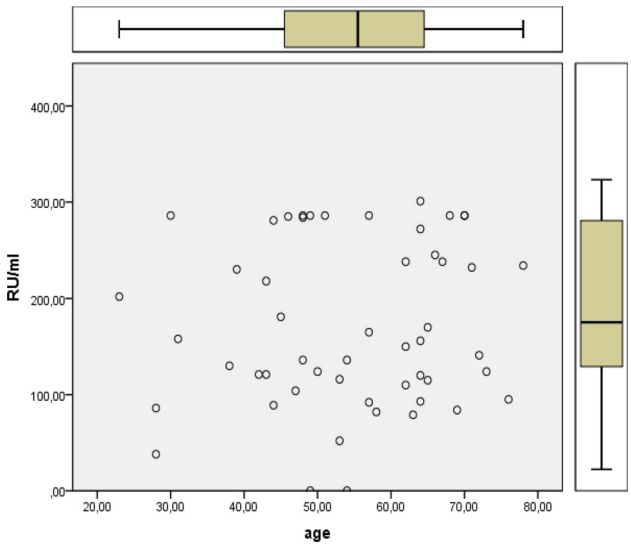

Fig. 1 Correlation of anti-VCA IgG numbers (in RU/ml) with the age of the NHL patients

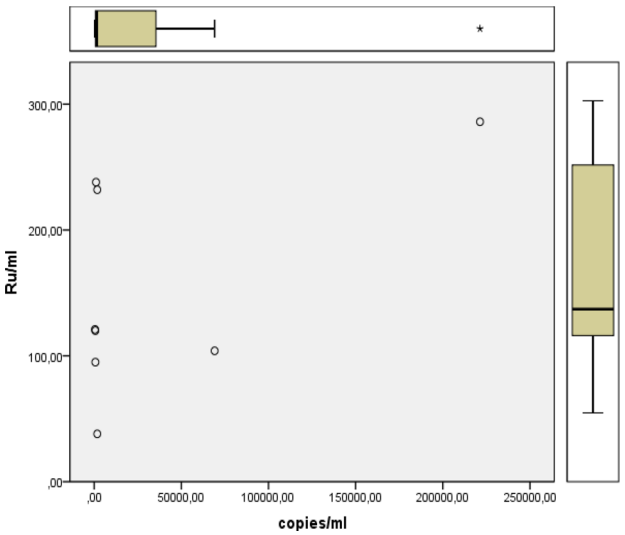

Fig. 2 Correlation of anti-VCA IgG (in RU/ml) with the plasma EBV DNA (copies/ml) in patients with NHL

patients were classified either as III–IV Ann Arbor stage (63.6%) or as I–II stage (13.6%) and 8 of them (18%) did not undergo any treatment during the study (Table 1). The LDH level showed elevated activity (> 378 U/L) in 75% of the EBV DNA positive samples and in only 61% of the negative ones, but the difference was not significant ($p = 0.69$). The proportions of patients with decreased number of lymphocytes (< 20%) did not differ significantly between the two groups (37.5% in the EBV DNA positive samples and 38.6% in the EBV DNA negative samples), while high level of lymphocytes (> 44%) was observed in 5 of the negative patients (11.4%) and was not observed among the positive individuals. However, statistically significant dependence between the EBV DNA positivity and all of the tested clinical variables of the patients was not found—the p values from the Fisher's exact test were > 0.05 (Table 1).

Individuals with detectable EBV DNA were all anti-VCA IgG positive and anti-VCA IgM negative, but the viral load in their sample did not correlate with the amount of anti-VCA IgG (Fig. 2).

Interestingly, two of the patients (at age of 23 and 28 respectively) positive for anti-VCA IgM and therefore susceptible for primary infection were with undetectable plasma EBV DNA levels.

Discussion

In this study we used the PCR method to analyze the correlation between the presence of detectable EBV DNA in plasma and NHL development.

The standard diagnostic EBV protocol in cases of HL and NHL is to measure the levels of EBV-encoded small RNA (EBER-RNA) in tumor cells via in situ hybridization or to find the latent membrane protein (LMP) via immunohistochemical staining [7, 8]. However, the PCR is a fast and easier method, which may also have an important role as a first choice for diagnosis [8–10] or as a tool for prognosis and therapy control [11–13].

The usual procedure in Bulgaria is to define only the EBV serostatus of the lymphoma patients after their clinical diagnosis. Some authors link the higher titers of EBV antibodies (anti-VCA IgG and anti-EA-D IgG) with NHL development (especially with CLL and FL) [14–16]. We found 96.2% anti-VCA IgG positive samples among 52 patients with HNL, while the seroprevalence of anti-VCA IgG in the Bulgarian population is around 83% [17] ($p = 0.012$). Furthermore, most of the NHL patients have anti-VCA IgG higher than 100 RU/ml (Fig. 1), but we should stress that the current study used only one plasma sample and we cannot discuss the antibody response dynamics.

In the current study, we detected EBV DNA in the plasma of 15.4% of the tested NHL patients. Among the published data, various proportions exist, ranged from 10% [5] to 24.6% [10]. Positive results were shown in different subtypes of NHL but Burkitt's lymphoma, peripheral T-cell, NK/T-cell and DLBCL were most often investigated [5, 10]. Usually, EBV associates with 5% of NHL, especially with extranodal NK/T lymphoma, nasal type and endemic Burkitt's lymphoma (> 95%). Around 40% of cases with peripheral T-cell lymphoma and 15% of DLBCL cases depend on EBV persistence [18]. We confirmed the presence of EBV DNA in 33.3% of the plasma

samples from patients with peripheral T-cell lymphoma and in 12% of those with DLBCL, but no statistical difference was found between the positivity of B and T-cell subtypes, as also shown by others [5].

In patients with peripheral T-cell lymphoma, whole blood EBV DNA was found in 42.7% of the samples with statistically significant correlation between the positivity and the disease stage, the LDH and the albumin levels [12]. Others also reported positive correlation with the disease stage and the LDH levels [10, 19] but in contrast our study did not show any dependence of the EBV DNA positive findings with the clinical or demographic variables of the patients, maybe because of the small sample size and the significant number of patients with not defined disease stage.

The PCR EBV target regions and the type of clinical material for analysis are still disputable among the scientific literature. The LMP genes, that we used in the present study are considered as highly sensitive [20]. No consensus exists regarding the type of clinical specimen to be analyzed in PCR. In non-fractionated whole blood, all blood elements that may contain EBV are present [21], which makes the whole blood suitable for analysis; peripheral blood mononuclear cells, as well as paraffin-embedded tissues also have been shown to represent a good source for quantitative PCR analysis [20, 22]. However, plasma EBV DNA levels correlate highly with the in situ hybridization and immunohistochemistry results in HL patients [9, 23] and plasma is even better specimen to detect EBV-associated diseases via PCR [24].

We found an average viral load of $3.7 \times 10^4 \pm 7.8 \times 10^4$ copies/ml (range 6.7×10^2–2.2×10^5 copies/ml), which is higher than the load found in patients with primary EBV infection ($1.0 \times 10^4 \pm 2.1 \times 10^4$ copies/ml, range 1.4×10^2–1.1×10^5 copies/ml) (data not shown). This confirms the need of quantitative determination of EBV DNA in NHL patients but the disease monitoring and prognostics require determination of viral load dynamics.

In conclusion, with the current study we add useful epidemiological data regarding the possible application of the quantitative PCR measurement of EBV DNA in plasma of patients with NHL. Plasma EBV DNA can be used as an additional marker in diagnosis and therapy control in NHL patients but larger studies are needed to evaluate its clinical significance.

Compliance with Ethical Standards

Conflict of interest Authors declare absence of any conflict of interest related to this manuscript.

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Ethical Approval The study was approved by the Ethics Committee of the Medical University Varna.

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