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Original Article

Prognostic significance of circulating plasma Epstein-Barr virus DNA in monitoring aggressive non-Hodgkin's lymphoma

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Abstract: Epstein-Barr virus (EBV) DNA copynumber quantification for blood specimens by real-time polymerase chain reaction (RT-PCR) is often used as a tool of screening for EBV-associated diseases or assessing treatment response. But the clinical value of plasma EBV-DNA has been rarely evaluated in patients with non-Hodgkin lymphoma (NHL). One hundred and eighty-two patients with NHL were recruited. Plasma EBV-DNA positive (≥1000 copies/ml) was detected in 45 patients (PTCL, n = 16; NK/T, n = 13; DLBCL, n = 9; ATCL, n = 3; ALCL, n = 2; ANKL, n = 2). In all cases, plasma EBV-DNA was detectable (10^3-10^7 copies/ml) at diagnosis. Plasma EBV-DNA positive was more common in mature T/NK neoplasms (80%) and related to more advanced disease stage (P = 0.004), presence of B symptoms (P<0.001) and higher lactate dehydrogenase (LDH) level (P<0.001). Plasma EBV-DNA positive showed distinctly worse overall survival (OS) than other patients with NHL and diffuse large B cell lymphoma (DLBCL) (P<0.001, each). In addition, the EBV-DNA positive patients with mature T/NK neoplasms also demonstrated substantially poorer OS and PFS (P = 0.009 and P = 0.008, respectively) compared with EBV-DNA negative (<1000 copies/ml). Multivariate analysis involving EBV DNA parameters demonstrated that it was an independent poor prognostic factor for OS in patients with NHL. These findings indicated that plasma EBV DNA load may be a useful potential biomarker and prognostic indicator in NHL.

Keywords: Lymphoma, Epstein-Barr virus, EBV-DNA, diffuse large B-cell lymphoma, T/NK neoplasms, prognosis

Introduction

Epstein-Barr virus (EBV) which belongs to the herpes virus family (human herpesvirus 8) infects more than 90% human during life and establishes a latent infection of B lymphocytes [1]. If host-virus balance is shifted, virus reactivation might occur and promote the development of lymphomas. EBV was first isolated from Burkitt lymphoma 50 years ago [2]. Subsequently, it was described to drive oncogenic capacities. EBV is associated with hematological malignancies such as Burkitt lymphoma, Hodgkin lymphoma (HL), Natural killer (NK)/T-cell lymphoma and diffuse large B-cell lymphoma (DLBCL), Aggressive NK leukemia, Angioimmunoblastic T-cell lymphoma, Peripheral T cell lymphoma [3-6]. Detection of circulating tumour DNA in the blood specimen of cancer patients has been elevated [7, 8]. The release of free DNA suggested the possibility of blood market that reflected tumour burthen [9]. Recently, EBV load in peripheral blood, as determined by RT-PCR, was considered as useful prognostic factors in HL and non-Hodgkin lymphoma (NHL) [10, 11]. However, no appropriate specimen type has been used for EBV viral load monitoring in lymphoma. Some researchers used whole blood, EBV-infected cells, such as, peripheral blood mononuclear cells (PBMCs), tumor tissues [3, 12, 13]. In patients with EBV-related lymphoma, plasma EBV was more sensitive than that of PBMCs, and the rate of detection was higher [14]. Furthermore, plasma EBV-DNA can be detected by RT-PCR more reliable using plasma than PBMCs in EBV positive HL [12, 15]. At present, a few studies have been applied to testing EBV DNA load by using plasma specimen as prognostic factors in NHL. In this study, we investigated EBV DNA levels in plasma measured from NHL patients by RT-PCR and evaluated whether EBV DNA load in diag-

Table 1. Correlation of clinical characteristics and plasma EBV-DNA levels

Characteristics	Plasma EBV-DNA (copies/ml)		*P*
	<1000, n (%)	≥1000, n (%)	
Age, y			0.391
<60	62 (44.9)	24 (53.3)	
≥60	76 (55.1)	21 (46.7)	
Sex			0.476
Male	85 (61.6)	31 (68.9)	
Female	53 (38.4)	14 (31.1)	
B-symptoms			<0.001
Absence	67 (48.6)	2 (4.4)	
Presence	71 (51.4)	43 (95.6)	
Ann Arbor stage			0.004
I-II	45 (32.6)	5 (11.1)	
III-IV	93 (67.4)	40 (88.9)	
IPI score			0.111
0-2	87 (63.0)	21 (48.8)	
3-5	51 (37.0)	22 (51.2)	
LDH level			<0.001
Normal	82 (59.4)	7 (15.6)	
Elevated	56 (40.6)	38 (84.4)	
β2-Microglobulin, μg/l			<0.001
<2.0	50 (36.2)	1 (2.2)	
≥2.0	88 (63.8)	44 (97.8)	

nostic specimens had a prognostic significance for monitoring the treatment response in patients with NHL.

Patients and methods

A total of 183 newly diagnosed Non Hodgkin's lymphoma patients based on the WHO classification at the Hangzhou First People's Hospital between January 2008 and December 2015 were enrolled. Enrolled patients were divided into mature B-cell neoplasms and mature T/NK neoplasms. The chemotherapy regimen consisted of either CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or a CHOP-like regimen. CEOP [cyclophosphamide, etoposide, vincristine, prednisolone]) chemotherapy, RCHOP (rituximab plus CHOP), EPOCH (etoposide, prednisolone, vincristine, cyclophosphamide, doxorubicin), REPOCH (rituximab plus EPOCH) or others such as SMILE (steroid [dexamethasone], methotrexate, ifosfamide, pegaspargase, and etoposide), GELOX (gemcitabine, pegaspargase and oxaliplatin). All patients detected EBV-DNA in plasma. In this study, we assessed prognostic factors according to EBV-DNA copies in patients with NHL. The following clinical data were collected: age, sex, Ann Arbor stage, B-symptoms, international prognostic index (IPI), LDH, β2-microglobulin, Ki67 and EBV-DNA copies.

Quantification of plasma EBV-DNA load

DNA was extracted from cryopreserved plasma using EBV virus nucleic acid fluorescent quantitative detection reagent kit (Daan Gene company, Guangzhou) according to manufacturer's instructions. Adopting boiling lysis method to extract virus DNA template, DNA samples was amplified by Taq enzyme and TaqMan probe. Nucleic acid amplification program analysed data according to the change of the fluorescence signal. The quantification of EBV DNA loadwas calculated usingan 7500/7300 System (ABI company). Plasma EBV-DAN load ranged from 1×10^3 to 1×10^8 (copies/ml).

Statistical analysis

Overall survival (OS) was defined from diagnosis to death or to the final follow-up date, and progression-free survival (PFS) from the date of diagnosis to progression or relapse, death or last follow-up. Comparisons of clinical characteristics and EBV-DNA levels in plasma were analyzed using the χ^2-test or Fisher's exact test and Mann-Whitney U-test. Survivals were estimated using the Kaplan-Meier estimator, and the different groups were analyzed using the log-rank. A Cox proportional hazard mode was used for univariate and multivariate analysis to identify which factors influenced survival and independent prognostic factor. All statistical analyses were performed SPSS version 19.0 software.

Results

Clinical characteristics and plasma EBV-DNA load

The clinical characteristics of patients were summarized. The median patient age was 62 years (range, 23-86 years), with 53.0% patients older than 60 years. The male to female ratio of

Table 2. The characteristic of plasma EBV-DNA positive lymphma

Diagnoses	Sex		Median age y (range)	Median plasma EBV DNA copies/ml (range)	Medial survival m (range)	Outcome	
	F	M				CR	NR
PTCL	11	5	57 (24-79)	1.74×10^4 (1.00×10^3-2.71×10^7)	7 (1-44)	3	13
NK/T	9	4	63 (24-68)	1.80×10^5 (1.41×10^4-2.21×10^7)	6 (1-58)	5	8
DLBCL	8	1	56 (23-77)	1.57×10^4 (3.10×10^3-3.82×10^6)	10 (1-24)	2	7
ATCL	2	1	71 (66-71)	3.01×10^3 (1.23×10^3-3.82×10^3)	17 (4-21)	1	2
ALCL	1	1	44, 63	1.38×10^3, 3.07×10^7	5, 75	1	1
ANKL	0	2	37, 49	1.41×10^4, 4.17×10^5	1, 2	0	2

cases was 1.73:1. Among the 183 patients enrolled, diffuse large B-cell lymphoma was 122 cases, peripheral T-cell lymphoma was 28 cases, NK/T cell lymphoma was 22 cases, angioimmunoblastic T-cell lymphoma was 5 cases, anaplastic large cell lymphoma was 4 cases, aggressive NK cell leukemia was 2 cases. The majority patients had B symptoms (n = 114) and stage III-IV disease (n = 133). 73 cases (40.0%) were IPI score of 3 to 5, 94 cases (51.4%) had high lactate dehydrogenase level. 132 patients had high level of β2-Microglobulin (≥2.0 ug/ml). Plasma EBV-DNA was detected before treatment in 45 of 183 (24.6%) of patients with NHL

Correlation between clinical features and plasma EBV-DNA levels

To study the correlation between prognostic factors and pretreatment EBV-DNA levels, the comparison of plasma EBV-DNA load and clinical features were listed in **Table 1**. We grouped EBV-DNA load into EBV-DNA level ≥1000 copies/ml and <1000 copies/ml. The two groups were compared with age, sex, stage, B-symptoms, LDH, IPI score, β2-Microglobulin. Plasma EBV-DNA were identified in T/NK cell neoplasms (59.0%). In patients with plasma EBV-DNA positive tended to have B-symptoms (P<0.001), elevated lactate dehydrogenase (LDH) levels (P<0.001) and advanced stage (P = 0.004) compared with those with plasma EBV-DNA negative.

The characteristic of plasma EBV-DNA positive in lymphoma

All of 45 patients with plasma EBV-DNA lymphomas were investigated (**Table 2**). 16 peripheral Tcell lymphoma, 13 NK/T cell lymphoma, 9 diffuse large B-cell lymphoma, 3 angioimmunoblastic T-cell lymphoma, 2 anaplastic large cell lymphoma and 2 aggressive NK cell leukemia were plasma EBV-DNA positive. A total of 45 cases, 12 cases reached CR.

Analysis of the prognostic factors in NHL

The median follow-up time for all patients was 19 months (range, 1-96 months). Of the 183 patients, 70 patients (38.3%) died before the end of the follow-up period. Patients with plasma EBV-DNA positive showed evidently worse progression-free survival (PFS) and overall survival (OS) than others (P<0.001 each) in NHL (**Figure 1**). Likewise, patients with plasma EBV-DNA positive presented inferior OS and PFS compared with plasma EBV-DNA negative in DLBCL (P<0.001 each, **Figure 2**) and in mature T/NK neoplasms (OS, P = 0.009; PFS, P = 0.008, **Figure 3**).

Univariate analysis and multivariate of prognostic factors and plasma EBV-DNA

In univariate analysis, the differences for overall survival in patients with NHL were showed in **Table 3**. Patients with plasma EBV-DNA positive significantly influenced OS (HR, 0.271; 95% CI = 0.168-0.437; P<0.001), B-symptoms (HR, 0.107; 95% CI = 0.046-0.249; P<0.001), high levels of serum LDH (HR, 0.253; 95% CI = 0.153-0.420; P<0.001), stage III and IV (HR, 0.200; 95% CI = 0.086-0.461; P<0.001), high-intermediate/high risk IPI (HR, 0.253; 95% CI = 0.153-0.420; P<0.001). In multivariate analysis, B-symptoms (HR, 0.235; 95% CI = 0.092-0.603, P = 0.003) and plasma EBV-DNA levels as an independent prognostic factor for OS (HR, 0.490; 95% CI = 0.289-0.829, P = 0.008). These results were showed in **Table 3**.

Discussion

At present, more studies suggested that serial copy number changes in plasma were connected with survival in EBV-positive NHL. Besides, researchers suggested that immunosuppressionis associated with an increased EBV repli-

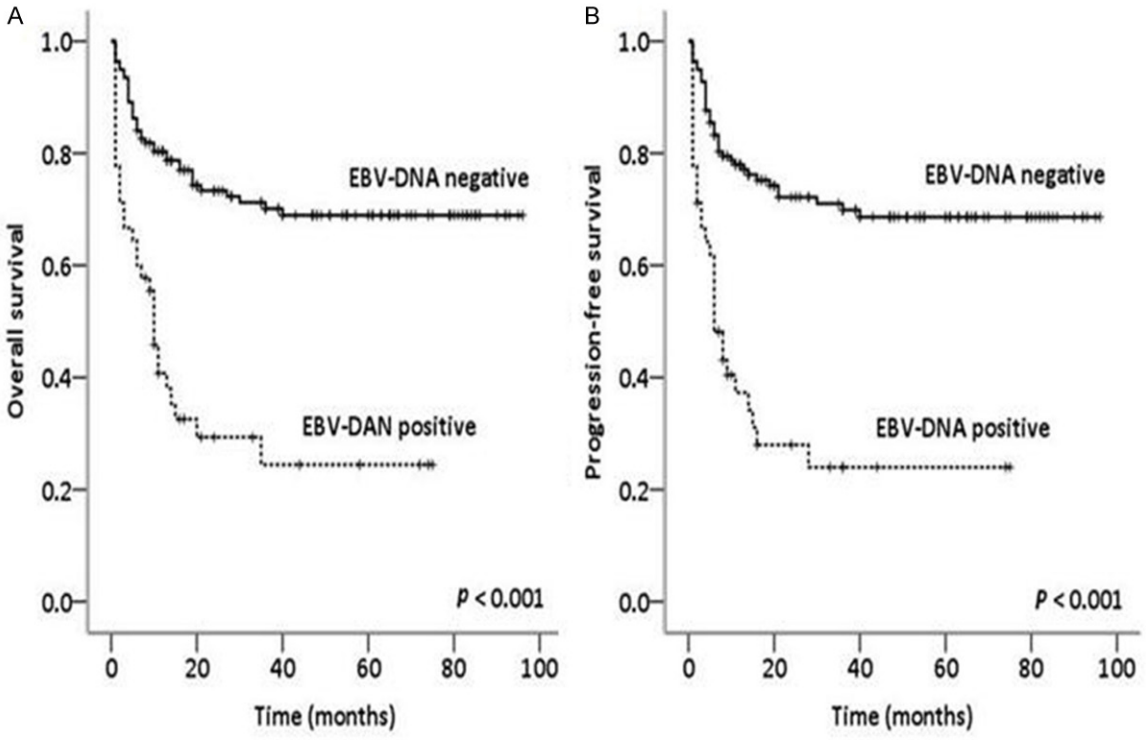

Figure 1. Kaplan-Meier estimates of overall survival (A) and progression-free survival (B) for NHL with (n = 45) or without (n = 138) Epstein-Barr virus (EBV) DNA detected in plasma.

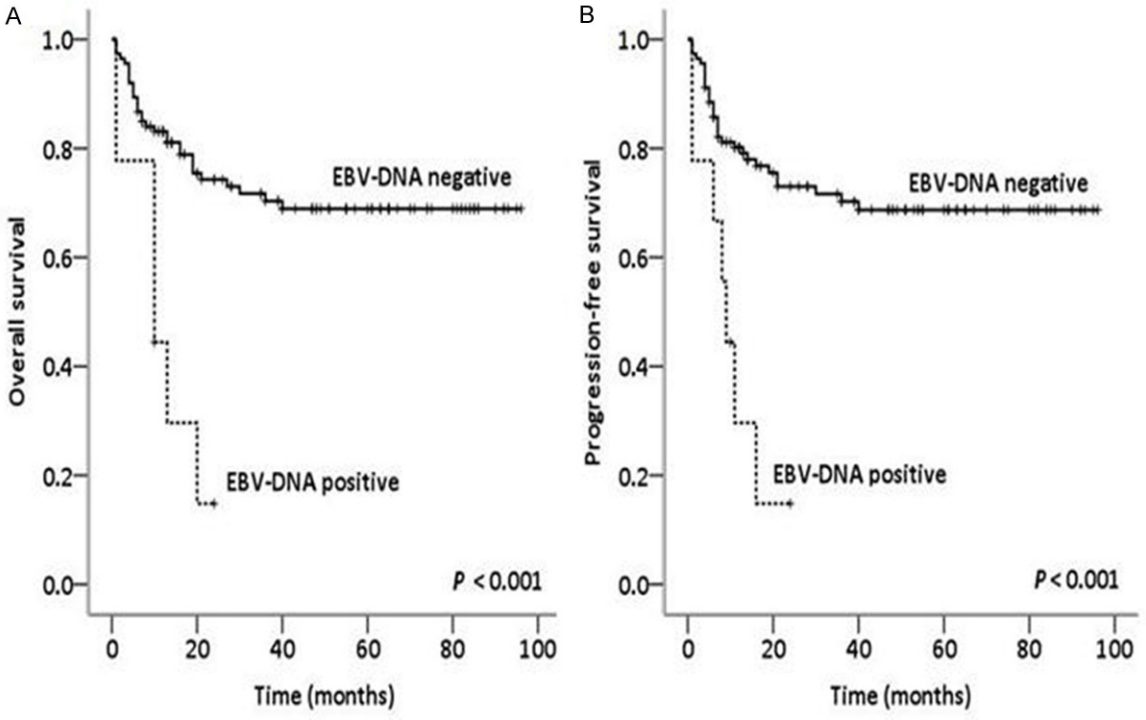

Figure 2. Kaplan-Meier estimates of overall survival (A) and progression-free survival (B) for diffuse large B-cell lymphoma patients with (n = 9) or without (n = 113) Epstein-Barr virus (EBV) DNA detected in plasma.

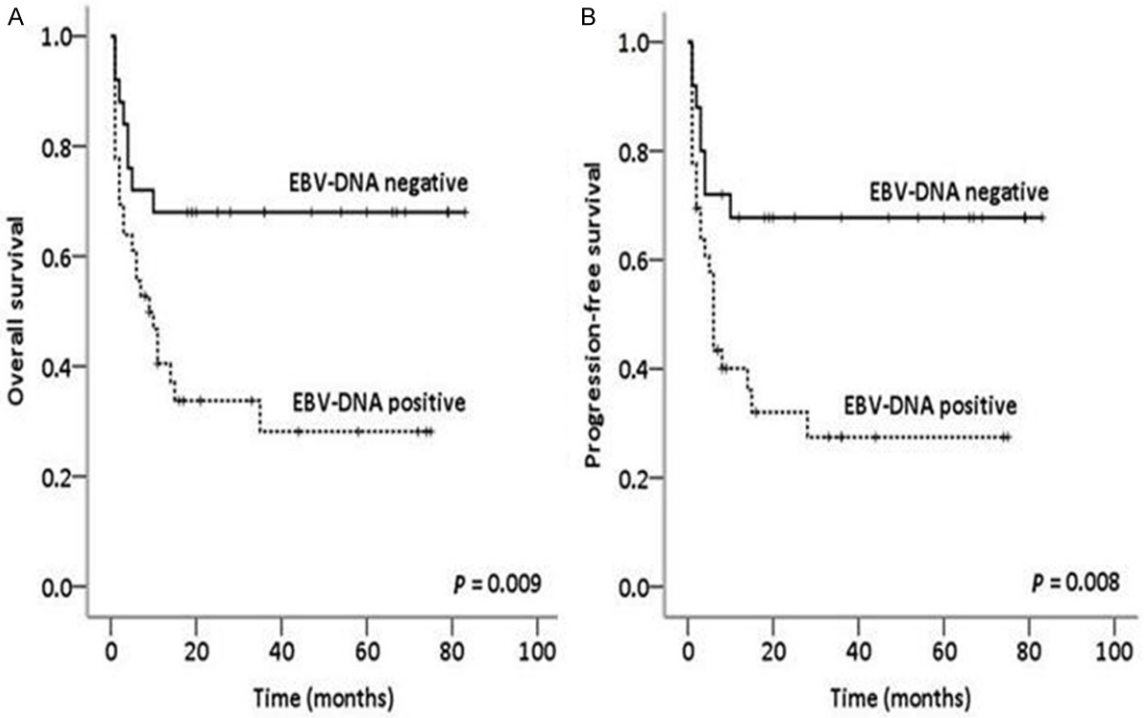

Figure 3. Kaplan-Meier estimates of overall survival (A) and progression-free survival (B) for mature T/NK neoplasms patients with (n = 36) or without (n = 25).

Table 3. Univariate analysis and multivariate of prognostic factors and plasma EBV-DNA in terms of OS rates

Diagnosis	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Plasma EBV-DNA				
≥1000 copies/ml	0.271 (0.168-0.437)	<0.001	0.490 (0.289-0.829)	0.008
Age, y				
>60	1.265 (0.792-2.002)	0.325	1.496 (0.891-2.512)	0.128
B-symptoms				
Presence	0.107 (0.046-0.249)	<0.001	0.235 (0.092-0.603)	0.003
LDH leve				
Elevated	0.253 (0.153-0.420)	<0.001	0.774 (0.341-1.731)	0.532
Ann Arbor stage				
III-IV	0.200 (0.086-0.461)	<0.001	0.463 (0.190-1.130)	0.091
IPI score				
3-5	0.253 (0.153-0.420)	<0.001	0.528 (0.267-1.004)	0.066

cation in NHL patients, in whom immune surveillance against EBV is probably impaired [16]. The current study demonstrated that cell-free (plasma) EBV DNA performs better than cellular (PBMC) EBV DNA as a marker of EBV+ diseases and tracking EBV+ disease response to therapy [14]. The detection of EBV-DNA in plasma may be more convenient and useful than other specimens of Epstein-Barr virus. Moreover, there are some researches that the copies of plasma EBV-DNA could be used as a biomarker for lymphoma [17-20]. In our study, Epstein-Barr virus DNA was detectable in serum from 45 (24.6%) of the 182 NHL patients without underlying immunodeficiency. In addition, plasma EBV-DNA positive was showed a statistical association with B symptom advanced stage, poor clinical response and elevated LDH levels in NHL patients. However, some studies found that EBV viral load showed no clinical correlation with LDH or stage [8, 21], but that was just a small sample study. Therefore, more large sample, randomized, controlled clinical researches were needed to explore the relationship between EBV viral load and LDH, stage of NHL

at the time of diagnosis. In the patients group, there was no significant difference between the two groups of EBV-DNA positive and negative for sex distribution and age in NHL.

The concentration of EBV DNA in the plasma might be a prognostic marker in NHL patients. However, the clinical value of plasma EBV-DNA has been rarely evaluated in patients with NHL. EBV has been known to be strongly associated with extranodal NK/T cell lymphoma (>95%), 10-35% of DLBCLs, 30-60% of aggressive NK leukemia lymphomas, and over 80% of angioimmunoblastic lymphomas, 40% of Peripheral T-cell lymphomas, CD30+ Ki-1 positive anaplastic large cell lymphomas, and others [5, 22].

Several studies have suggested that EBV DNA was associated with the prognosis of lymphoma for several different types of NHL [17-20, 23-25]. Furthermore, our data showed that EBV DNA was closely related to poor prognosis; that is, patients with plasma EBV-DNA positive exhibited evidently inferior PFS and OS compared with those with plasma EBV-DNA negative. The 3-year OS rates were 70.1% and 24.4% in the groups with plasma EBV-DNA negative and plasma EBV-DNA positive respectively in patients with NHL. The 3-year PFS rates were 69.9% and 24.0% in the groups with plasma EBV-DNA negative and plasma EBV-DNA positive, respectively in patients with NHL. In multivariate analysis, plasma EBV-DNA level and B symptom were independent poor prognostic factors for OS in patients with NHL. In this current study plasma EBV-DNA is an independent prognostic factor for NHL. Small sample study suggested that high levels of plasma EBV DNA were detected in cases with EBV-related NHL relapse or progression, but that plasma EBV-DNA was present at undetectable or low levels in the complete remission state [8, 21]. Our findings demonstrated that the concentration of plasma EBV DNA in the plasma was a prognostic marker of NHL and showed poor survival. Therefore, the potential for the advantage with experimental treatment strategies may be greater in these patients.

Several studies reported plasma EBV-DNA load as adverse prognosis on DLBCL [3, 17, 18, 24, 26]. In our context, plasma EBV-DNA was detected in about 7% of patients with DLBCL at diagnosis and had a prognostic relevance. A majority of plasma EBV-DNA positive patients progressed rapidly, and died within 1.8 years. EBV-associated T-cell and NK-cell lymphomas were especially prevalent in Southeast Asia [27]. In addition, EBV infection was significantly associated with overall survival for T-cell and NK-cell lymphomas [28]. Our findings also demonstrated that high EBV DNA load had a poor survival. The 3-year OS was above 60% for EBV-DNA negative in T-cell and NK-cell lymphomas, however, they were less than 30% for EBV-DNA positive patients. Plasma EBV-DNA provided an important means for prognostication of lymphomas. Furthermore, patients with plasma EBV-DNA negative had excellent outcomes could still be in support of the prognostic relevance of EBV DNA load.

When tracked our data, on the one hand, a single center study and the sample size were necessarily limited. On the other hand, it was the sensitivity of PCR to the detection of plasma EBV-DNA loading. Therefore, a larger number of patients and multicenter studies need to be enrolled to be validated the results presented here. However, our study had certain advantages in that we not only compared the prognosis of EBV-DNA positive patients in non Hodgkin's lymphoma. Besides, we discovered useful information regarding the significance of EBV DNA load in NHL patients, it may lay the foundation for prognosis factors of non-Hodgkin's lymphoma. It was also a prognostic indicator in mature B-cell neoplasms and mature T and NK neoplasms. Especially, plasma EBV-DNA load was rarely taken as a parameter to research in mature T and NK neoplasms.

In conclusion, our studies suggest that plasma EBV DNA level may be used as a surrogate marker for therapeutic response and considered to be an independent prognostic indicator of EBV-positive NHLs. Measurement of the plasma EBV-DNA is useful for clinical guidance, maybe the circulating plasma EBV-DNA level serves as a valuable biomarker of tumor load to supplement IPI for acting as a prognostic factor in NHL. However, the level of plasma EBV-DNA as an adverse prognostic marker on non-Hodgkin's lymphoma patients needs to be validated in larger studies.

Disclosure of conflict of interest

None.

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