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RESEARCH ARTICLE

Epstein–Barr virus DNA quantification and follow-up in Tunisian nasopharyngeal carcinoma patients

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Abstract

The prognostic value of the Epstein–Barr virus (EBV) DNA load in sera of nasopharyngeal carcinoma (NPC) patients measured before any treatment, after treatment and before relapse was assessed. The real-time polymerase chain reaction was used to detect the viral load levels among 74 NPC subjects. Patients were followed up for a period going from 1 to 6 years (median 4 years). Before treatment, the EBV DNA load was correlated with lymph node involvement and advanced stages. After treatment, the viral load level declined significantly and patients presenting a viral load level lower than 1000 copies/ml showed a better overall survival (OS). Moreover, a significant result was found when the 6-year OS rates of patients having fewer or more than 15,000 copies/ml of viral load before relapse were compared. These results suggest that the EBV DNA load quantification after treatment may be a useful predictor of disease progression and survival.

Keywords: EBV DNA load, NPC, prognosis, survivals

Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck epithelial malignancy with distinct clinical, demographic and geographical features (Yu and Yuan, 2002). In most parts of the world, this cancer has an annual incidence below 1 per 100,000 inhabitants. High incidence foci are located in Asia, especially in native Sarawak (32 per 100,000) and Cantonese people (25 per 100,000) (Parkin et al., 2002; Devi et al., 2004). There are also large areas of intermediate incidence (3–8 per 100,000) including North African countries (Tunisia, Algeria and Morocco) and countries such as Vietnam and Indonesia in Southeastern Asia, Somalia and Kenya in Eastern Africa (Ferlay et al., 2001). The particular geographical distribution of NPC incidence is mainly due to its etiological factors including both environmental and genetic ones. However, regardless of the NPC incidence area, this cancer has always been associated to Epstein–Barr virus (EBV) infection. In NPC, EBV DNA occurrence was firstly detected in tumor biopsies (Chang et al., 1990). Subsequently, circulating EBV DNA was detected in NPC patients' sera and plasma using quantitative real-time polymerase chain reaction (PCR) assays (Lo et al., 1999a). The viral load was correlated with disease stage, and presented a promising marker for NPC patient's prognosis and monitoring (Lo et al., 1999b; Lo et al., 2000a; Lin et al., 2004). Most of these previous studies aimed at evaluating the usefulness of EBV DNA quantification at diagnosis and/or after treatment on populations from high NPC incidence areas. Because EBV DNA load may fluctuate from an area to another, herein the circulating EBV DNA load value quantification was assessed on sera from North African NPC patients. Circulating EBV DNA load measurement was performed using real-time PCR technology on NPC

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patients' sera at three different times: on diagnosis, after treatment and before relapse. Then, a correlation analysis was carried out according to patient's clinical features and survival for each time.

Materials and methods

Subjects

One hundred and five unrelated subjects belonging to the same population living in the Middle coast of Tunisia were enrolled in this study. Informed consent was obtained from each participant and approval for the study was given by the National Ethics Committee (Monastir University, Monastir, Tunisia).

Seventy-five patients with confirmed NPC were recruited from the Department of Cancerology and Radiotherapy of CHU Farhat Hached, Sousse, Tunisia (Table 1). The cancer diagnosis was confirmed by a histopathological analysis. All of them had the undifferentiated NPC histological type (type III, World Health Organization classification), which is the most frequently observed in Tunisia. Patients were aged between 10 and 70 years with 20% younger than 30. According to the Alternate Joint Communications Center/Union Internationale Contre le Cancer (1997) and through computed tomography and/or magnetic resonance imaging scans, 95% of the patients were diagnosed at advanced stages (stage III or IV) and 70% had lymph node metastasis. The majority of patients were treated with concomitant chemotherapy and radiotherapy (86%), eight patients by chemotherapy only and one patient by radiotherapy only. Patients were followed up for a period going from 1 to 6 years (median 4 years). During this period, 24 patients relapsed and 4 of them died. Two other patients diagnosed with a clinical stage IV before treatment died due to tumor progression. Sera were harvested from the patients before any treatment and 5–8 weeks after treatment was completed. Available sera from relapsed patients ($n = 22$) who presented at the Department of Cancerology and Radiotherapy for clinical control 4–6 weeks before relapse were also included in the study.

Table 1. Characteristics of 75 NPC patients.

Characteristic	Number of patients
Gender (male/female)	60/15
Tumor size	
T1	6
T2	21
T3	26
T4	22
Lymph node status	
N0	22
N+	53
Metastasis	
M0	62
M+	3
Mx	10
Overall stage	
I	1
II	3
III	18
IV	53

The 30 healthy controls were blood donors with no evidence of any personal or family history of cancer or any apparent other serious illnesses. Sera samples from patients and controls had been collected consecutively since 1998 and conserved at –80°C until use.

DNA extraction and real-time quantitative EBV DNA PCR

DNA was extracted from sera samples and Raji cells using the QIAamp DNA Mini Kit (Qiagen; Courtaboeuf, France) and the protocol recommended by the manufacturer. A total of 200 µl of the serum samples (V_{ext}) per column was used, and DNA was eluted with 50 µl of elution buffer provided in the kit (V_{DNA}). The extracted DNA quality was controlled in all tested samples by conventional PCR amplification of the β-globin gene using the PCO3/PCO4 primers (Saiki et al., 1985).

The iCycler iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, CA) was used to quantify the viral load. The PCR primers were designed for optimal hybridization using the beacon designer software, version 7.0 (Premier Biosoft International CA). Selected primer sequences amplified a 108 pb target sequence specific for the C1 exon, an Epstein–Barr nuclear antigen genes promoter [C promoter (Cp)] of the EBV genome (forward 5′-ATA GAG ACA AGG ACA CCG AAG AC-3′ and reverse 5′-ATG ATG AAT GGA GAG GCG TAG G-3′). A standard curve was constructed with DNA dilutions extracted from Raji cell line containing 50 copies of EBV genome per cell. Each 25-µl PCR reaction included a 5-µl DNA sample (V_{PCR}), 0.1 µM of each primer and 12.5 µl of 2 × SYBR Green PCR Supermix (Bio-Rad), containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 50 units/ml iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM SYBR Green I fluorescent dye and stabilizers. The thermal cycling conditions were 95°C for 5 min and 60 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec. For each run, three negative controls without any DNA template were included. The EBV DNA load was calculated using the following equation (Lo et al., 1999a): $C = Q \times (V_{DNA}/V_{PCR}) \times (1/V_{ext})$; where C corresponds to EBV DNA load (copies/ml of serum) and Q to EBV DNA copies determined from the standard curve. All the analyses were tested in duplicate and the results were averaged. The samples were defined as negative if their threshold cycle values exceeded 60 cycles. After amplification, a melting curve analysis was performed by increasing the temperature from 75 to 90°C. The melting temperature (T_m) of each sample was used for EBV DNA identification. The samples that showed the same T_m point with DNA Raji cell amplicons were considered as containing the EBV genome. The quantification lower limit was determined as the lowest positive point of the standard curve. The lower detection limit was 20 copies per reaction. To evaluate the intra-assay repeatability and the interassay reproducibility coefficients of variation

(CV) the Ct values of six different standard dilutions were compared. Each dilution was repeated three times for intra-assay variability and 10 times for interassay variability. Intra- and interassay CV ranged from 0.1 to 0.5% and 0.9 to 5%, respectively.

Statistical analysis

The Mann–Whitney U test, a nonparametric test, was used to evaluate the differences in EBV DNA load levels between the several groups of the study. The results were considered statistically significant when P value <0.05. The multivariate analysis was made using the SPSS 13.0 software (SPSS Inc.; Chicago, IL).

Disease-free survival (DFS) was defined as the time from the date of diagnosis to the first local or distant recurrence or to the last contact. Overall survival (OS) was defined as the time from the date of diagnosis to death if the patient died from NPC or to the last contact. Six-year survival rates were estimated, and survival curves were plotted according to Kaplan and Meier. The differences between the groups were calculated by the log-rank test and the statistics were performed using the SEM-STATISTIQUES software (Centre Jean Perrin; Clermont-Ferrand, France).

Results

EBV DNA load in controls and pretreated NPC patients

Out of the 75 samples collected before any treatment, one sample was ineligible for real-time PCR analysis. Therefore, 74 NPC sera samples were included in the subsequent analysis. Seventy-three (98%) of them showed detectable EBV DNA with a median EBV DNA load of 10,950 copies/ml (Table 2). Among the 30 healthy control sera samples, 9 were positive (30%) for EBV DNA and the median viral load was 0 copy/ml (range 0–6850). The viral load was approximately 100 times higher in untreated NPC patients than in controls ($P < 0.001$).

According to the patient's clinicopathological characteristics, the EBV DNA load was significantly elevated in patients with lymph node positive status ($P = 0.023$) (Table 2), but did not significantly vary with age, gender, tumor size or metastasis status. The median EBV DNA load was for the I–III and for the IV stage groups 7175 copies/ml and 19,575 copies/ml, respectively (Table 2), without any significant differences ($P = 0.061$). However, the difference was significant when stages I–II and III–IV were compared ($P = 0.024$).

The median EBV DNA load of relapsed and unrelapsed patients at diagnosis were 21,000 copies/ml and 8075 copies/ml, respectively. Evaluated by the Mann–Whitney U test, the difference in EBV DNA load levels between the two groups was not significant ($P = 0.611$).

Post-treatment EBV DNA load

Five to eight weeks after treatment, EBV DNA was undetectable (0 copy/ml) in 43% of patients' sera (32/74). After treatment the median EBV DNA load decreased to 236 copies/ml (range 0–2,470,000 copies/ml). The comparison of EBV DNA load levels of pretreated and post-

Table 2. Pretreatment and post-treatment plasma EBV DNA loads in NPC patients.

		Pretreated NPC patients		Post-treated NPC patients	
	N	Median (copies/ml)	Range (copies/ml)	Median (copies/ml)	Range (copies/ml)
Overall patients[a]	74	10,950	0–680,000	236	0–2,470,000
Age (years)					
≤30[a]	16	5475	191–345,500	0	0–6200
>30[a]	58	14,450	0–680,000	337	0–2,470,000
Gender					
Male[a]	59	9200	0–680,000	225	0–2,470,000
Female[a]	15	15,700	730–345,500	375	0–33,750
Tumor stage					
I–III[a]	22	7175	0–83,000	86	0–33,750
IV[a]	52	19,575	191–680,000	337	0–2,470,000
Tumor size					
T1–T2[a]	27	9200	0–397,000	248	0–2,470,000
T3–T4[a]	47	15,300	191–680,000	225	0–79,000
Lymph node status					
N0[a]	21	6800	399–61,500	454	0–33,750
N+[a,b]	53	23,450	0–680,000	121	0–2,470,000
Metastasis					
M0[a]	61	11,800	191–397,000	225	0–2,470,000
M+	3	35,650	600–6600	0	0–970
Relapsed[a]	24	21,000	600–110,000	196	0–79,000
Unrelapsed[a]	48	8075	0–680,000	248	0–33,750

EBV, Epstein–Barr virus; NPC, nasopharyngeal carcinoma.
[a]$P < 0.001$ value of EBV DNA load before treatment vs. after treatment comparison.
[b]$P = 0.024$ value of before treatment EBV load N0 vs. N+ comparison.

treated patients sera showed a highly significant decline ($P < 0.001$) (Table 2). Compared to healthy controls, the EBV DNA load levels among post-treated patients remained statistically higher ($P = 0.021$). The EBV DNA load decreased in most treated patients except for five where the level markedly increased. Among them three patients died during follow-up (3/5), two died after relapse and the third patient showing the greatest EBV DNA load increase (2,421,000 copies/ml) died 1 month after treatment was completed. When post-treatment EBV DNA loads between patient subgroups were compared (stages I–III vs. IV, T1–T2 vs. T3–T4, N0 vs. N+ and M0 vs. M+), no significant differences were found. Moreover, no significant differences were found when EBV DNA load of post-treated patients with or without relapse were compared (median 196 and 248 copies/ml, respectively) (Table 2). The multivariate analysis did not show any significant independent prognostic influence, and no significant results were observed when patients were compared according to nodal status or disease stage (data not shown).

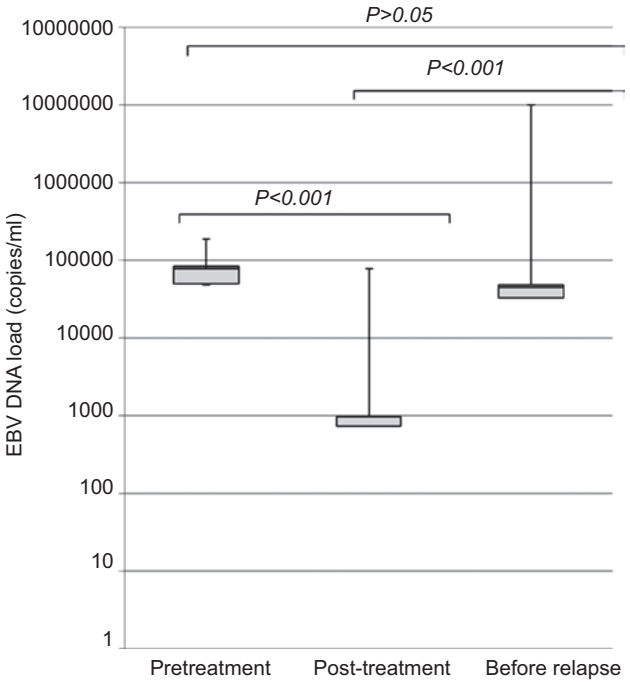

Figure 1. Epstein–Barr virus (EBV) DNA loads (copies/ml) of 22 relapsed nasopharyngeal carcinoma patients (pretreatment, post-treatment and before relapse).

EBV DNA load among relapsed patients

Among the relapsed patients, 22 sera were available for analysis. The median value of the pretreatment EBV DNA load in the 22 relapsed patients was 30,375 copies/ml (range 600–110,000 copies/ml). After treatment, the median decreased to 236 copies/ml (range 0–79,000 copies/ml) then increased to reach 11,775 copies/ml before relapse (range 0–10,150,000 copies/ml) (Figure 1). When compared to post-treatment EBV DNA load, the pretreatment and the pre-relapse EBV DNA load levels were both found to be significantly higher ($P < 0.001$ for both). When pretreatment and pre-relapse viral loads were compared, no statistical differences were found ($P > 0.05$). Eight patients showed higher EBV DNA load before relapse than before treatment, and 50% (4/8) died during follow-up with a median EBV DNA load of 225,750 copies/ml (range 29,300–10,150,000 copies/ml).

EBV DNA load and survivals

To investigate correlations between EBV DNA load in NPC patients and OS or DFS, survival rates were estimated for a 6-year period (4 years median period follow-up) and survival curves were plotted according to Kaplan and Meier. When OS rates were evaluated according to pretreatment EBV DNA load, nonsignificant results were found.

When compared, the 6-year OS rates of post-treatment EBV DNA load of negative and positive patients showed a significant difference (100 and 85.5%, respectively, $P < 0.05$) (Figure 2). Moreover, treated patients presenting an EBV DNA load with fewer than1000 copies/ml had a better survival rate than those having more than 1000 EBV DNA copies/ml (96 vs. 76.5%, $P < 0.01$) (Figure 2). The survival rates decreased to 37.5% among patients having fewer or more than 20,000 copies/ml of post-treatment EBV DNA load vs. 100% among those having a lower level ($P < 0.001$). The 6-year OS rates

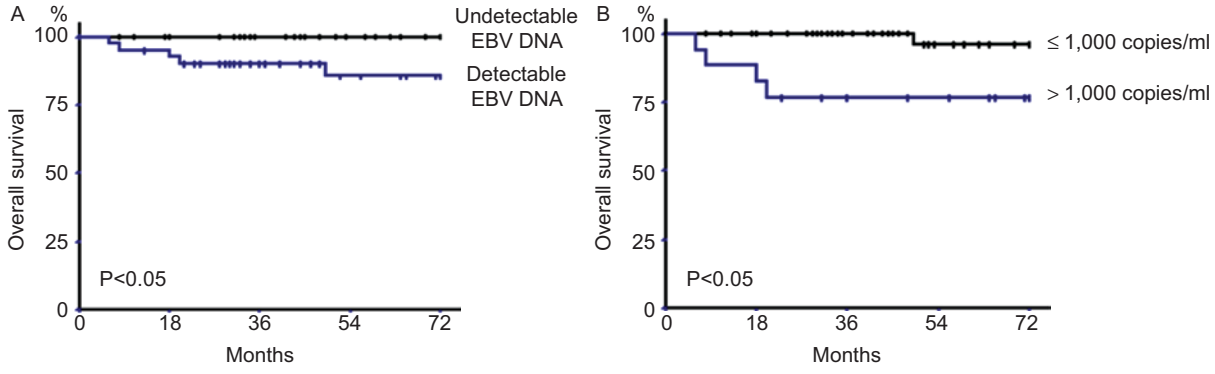

Figure 2. Overall survival of nasopharyngeal carcinoma patients according to their post-treatment Epstein–Barr virus (EBV) DNA load levels ($n = 74$); (A) undetectable vs. detectable EBV DNA and (B) EBV DNA load inferior or equal to 1000 copies/ml vs. superior to 1000 copies/ml. *P* denotes the log-rank test value.

were significantly lower among NPC patients having stage IV or lymph node positive status with detectable EBV DNA after treatment, 79 and 85%, respectively vs. 100% among those with undetectable EBV DNA load (Figure 3). Among the same patient subgroups, OS rates were also lower when the post-treatment EBV DNA load was superior to 1000 copies/ml (69 and 66%, respectively) (Figure 3).

The 6-year OS rates were not statistically different between pre-relapse EBV DNA positive and negative patients ($P > 0.37$) (Figure 4). However, a significant result was found when OS rates of patients having fewer or more than 15,000 copies/ml of EBV DNA load before relapse were compared ($P < 0.03$) (Figure 4). Moreover, when pre-relapse EBV DNA load exceeded 30,000 copies/ml the OS rates decreased to 0% vs. 100% among patients having fewer than 30,000 copies/ml ($P < 0.01$).

The OS rate was 100% among pretreated EBV DNA positive patients whose viral load was annulled after treatment and 85.5% among those who remained positive after treatment, but the difference was not significant ($P < 0.06$).

Nonsignificant results were found when DFS rates were correlated to pretreatment, post-treatment and pre-relapse EBV DNA loads.

Discussion

EBV infection is one of the main etiological factors of NPC. Evidence supporting an EBV-NPC etiological relationship included serological data and the detection in biopsies of EBV antigens and DNA. In this study, the EBV DNA load in the sera of Tunisian NPC patients was quantified using real-time PCR. The plasma EBV DNA load level prognostic value was estimated by comparing patients according to their clinical features and by comparing their pretreatment, post-treatment and pre-relapse viral load levels.

Our findings corroborate the relationship between EBV infection and NPC in the intermediate incidence risk area. In accordance with previous reports, the plasma EBV DNA load levels and DNA positivity were both significantly higher in NPC patients than in healthy blood donors (Lo et al., 1999a; Stevens et al., 2005; Tan et al., 2006). On diagnosis, the median viral load was significantly higher in NPC patients than in blood donors (10,950 copies/ml vs. 0 copy/ml). Among patients, the EBV DNA 98% positivity rate is very close to those reported in Chinese, Taiwanese and Italian NPC patients where EBV DNA was detected in 96, 94 and 74%, respectively (Lo et al., 1999a; Lin et al., 2004; Bortolin et al., 2006). Among our healthy blood donors, a 30% (9/30) positivity rate was observed, and 6% (2/30) had an EBV DNA load with more than 1000 copies/ml. In endemic areas, levels higher than 1000 copies/ml were also detected among healthy controls (Lo et al., 1999a; Lo et al., 2001). The clinical significance of the circulating EBV DNA in subjects without clinical evidence of NPC is still unclear and needs to be further investigated. It would be interesting to follow up these EBV DNA positive blood donors to detect if NPC or any EBV-related diseases could develop later.

Circulating EBV DNA detection and viral load quantification among NPC patients from both high and low incidence NPC areas were previously assessed (Lo et al., 1999b; Lo et al., 2000a; Lin et al., 2004; Stevens et al., 2005; Kalpoe et al., 2006). The results strongly support the useful application of EBV DNA load quantification assays as a new prognostic tool. Herein high EBV DNA load levels at diagnosis were found to correlate with lymph node involvement. In patients with lymph node positive status, the disease has spread beyond the primary site, suggesting that the plasma EBV DNA load level may reflect a worst disease progression. The same observation was found by Bortolin et al. (2006) who suggested that the release of the EBV DNA in serum might be related to the nodal invasion. However, the EBV DNA load level correlation with the lymph node involvement was not usually established among high-risk incidence areas probably because of the high incidence of lymph node positive status in these regions. When correlated to clinical stages, pretreatment EBV DNA load levels were found to be significantly higher among advanced stages (III–IV) NPC patients than those with early stages (I–II). Although the correlation was not strong because of the small number sample, it was consistent with previous reports both in high and low risk NPC areas (Lo et al., 1999a; Lo et al., 2000a; Bortolin et al., 2006). For instance, among Chinese advanced and early stage NPC patients, the median EBV DNA loads were 47,047 and 5918 copies/ml, respectively (Lo et al., 1999a). Among Italian NPC patients the median EBV DNA loads of stages I–II, III and IV were 772, 8417 and 11,495 copies/ml, respectively (Bortolin et al., 2006). However, while pretreatment EBV DNA load levels were usually higher among advanced stage patients, statistical significance was not reached for all studies conducted on NPC patients (Stevens et al., 2005; Tan et al., 2006). Moreover, in a multivariate analysis Lo et al. (2000a) showed that circulating EBV DNA load level is a stronger significant independent prognostic factor than disease staging, suggesting that pretreatment EBV DNA quantification should be incorporated into routine clinical assays. Altogether, these data provide more evidence that plasma EBV DNA load on diagnosis could be an indicator of NPC disease severity that needs to be validated throughout longer follow-ups and larger scale studies.

This study further highlighted the value of post-treatment plasma EBV DNA detection in predicting OS especially among lymph node positive metastasis patients. In our patient cohort, the 5–8-week post-treatment plasma EBV DNA load levels clearly decreased and became undetectable in 46% (34/74), and no mortality has so far been reported among these patients. However, this decrease was not observed in all patients: 7% (5/74) have showed higher post-treatment viral load and 60% (3/5) of them died. This result let us suppose that a higher

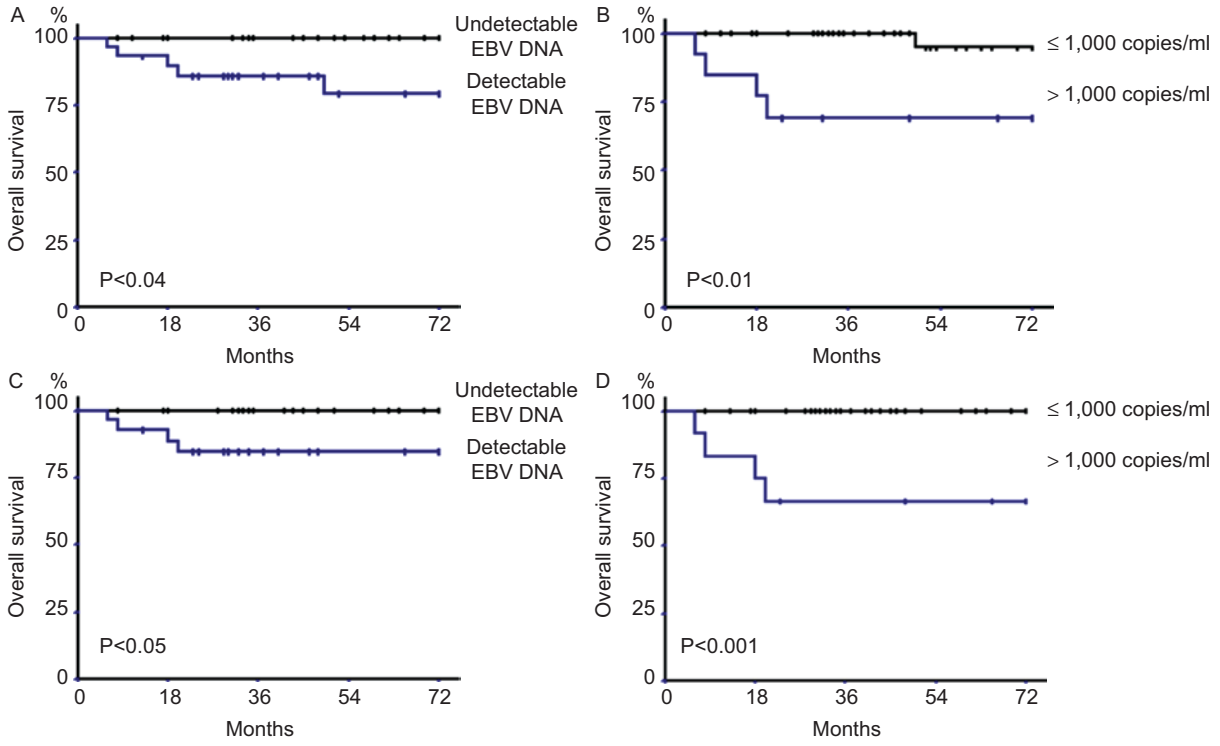

Figure 3. Overall survival of stage IV ($n=52$) (A and B) and N+ nasopharyngeal carcinoma patients ($n=53$) (C and D) according to their post-treatment Epstein–Barr virus (EBV) DNA load levels. A and C undetectable vs. detectable EBV DNA, and B and D EBV DNA load inferior or equal to 1000 copies/ml vs. superior to 1000 copies/ml. *P* denotes the log-rank test value.

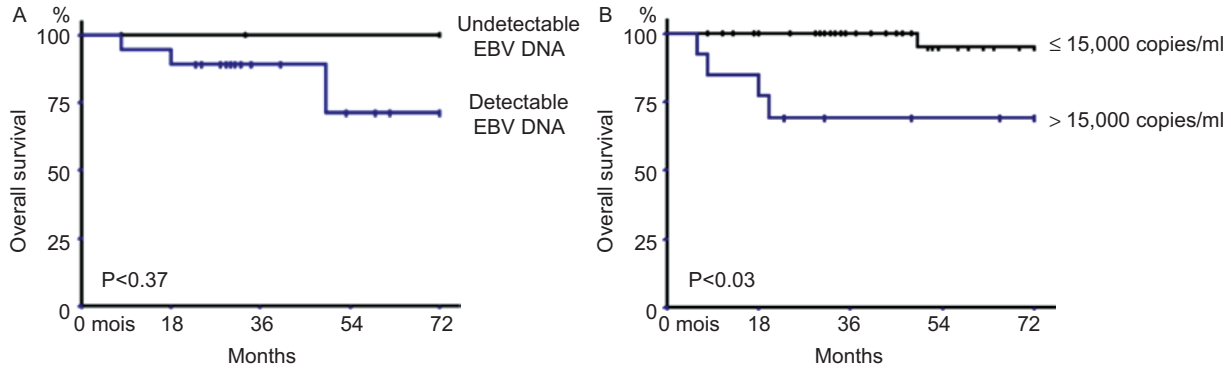

Figure 4. Overall survival of nasopharyngeal carcinoma patients according to their pre-relapse Epstein–Barr virus (EBV) DNA load levels ($n=22$); (A) undetectable vs. detectable EBV DNA and (B) EBV DNA load inferior or equal to 15,000 copies/ml vs. superior to 15,000 copies/ ml. *P* denotes the log-rank test value.

post-treatment viral load level reflects tumor resistance to therapy suggesting that post-treatment EBV DNA quantification can give an idea about the responsiveness of the tumor to the therapy. Moreover, a post-treatment EBV DNA load level >1000 copies/ml significantly decreased OS. The decrease is more pronounced in patients with lymph node involvement as well as stage IV patients. Although the prognostic value of the EBV DNA load is ambiguous at diagnosis, in numerous studies the plasma EBV DNA load was found to strongly correlate with response to treatment (Lo et al., 1999a; Lo et al., 1999b; Lo et al., 2000b). Indeed, among Chinese NPC patients Lo et al. (1999a) found that the persistence of circulating EBV DNA 1 month after the end of treatment was an indicator of poor prognosis, and suggested that the post-treatment EBV DNA load level measurement should be important for monitoring treated patients (Lo et al., 1999b). Furthermore, Chan et al. (2002) have previously demonstrated that, by quantifying pretreatment as well as post-treatment plasma EBV DNA levels, it is possible to identify a group of patients who have a lower survival rate, suggesting that these patients might be closely followed up through EBV DNA level quantification and further treatment. More recently, Wang et al. (2010) found that patients with shorter half-life values of plasma EBV DNA clearance among metastatic-recurrent NPC patients have significantly higher complete response and OS rates compared to those with longer half-life values.

Though not statistically significant, our findings are in accordance with those reported by Lin et al. (2004) who

found that relapsed patients had a greater pretreatment EBV DNA load level than those who did not relapse. They also found that OS was significantly lower among patients with an EBV DNA load level superior to 1500 copies/ml. In this study, the small number of relapsed patients did not allow any correlation with OS according to pretreatment as well as post-treatment plasma EBV DNA load levels. However, among them the 6-year-survival rate was 100% among relapsed patients whose EBV DNA load was annulled after treatment vs. 63.5% among EBV DNA positive relapsed patients. On the other hand, if we consider the pre-relapse EBV DNA load level among relapsed patients, a level superior to 15,000 copies/ml presents a significantly poor survival rate. Thus, the post-treatment follow-up of patients whose EBV DNA load was not annulled is of great importance to predict relapse.

To conclude, our data suggest that circulating EBV DNA quantification can be a helpful indicator of disease progression especially for patients whose viral load remained high after treatment. Although limited by the small number of patients, this study clearly shows the valuable use of post-treatment EBV DNA load quantification for selecting patients who need more surveillance. Indeed, a systematic post-treatment EBV DNA quantification for NPC patients would be of great importance in monitoring relapse and choosing an adequate treatment. In conjunction with clinical follow-up, supplementary studies on large cohort of NPC patients would provide a better evaluation of the usefulness of the EBV quantification.

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Declaration of interest

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