

# Molecular Prognostication of Nasopharyngeal Carcinoma by Quantitative Analysis of Circulating Epstein-Barr Virus DNA<sup>1</sup>

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## Abstract

We investigated the prognostic implication of pretreatment plasma/serum EBV DNA concentration, as measured by real-time quantitative PCR, in nasopharyngeal carcinoma (NPC). In 91 prospectively recruited NPC patients, those with recurrence or metastasis within the first year after treatment had a higher median plasma EBV DNA concentration than those without events (41,756 copies/ml versus 5,807 copies/ml;  $P < 0.001$ , Mann-Whitney rank-sum test). In multivariate logistic regression analysis, plasma EBV DNA was an independent prognostic indicator for early clinical events [relative risk = 3.8 (95% confidence interval, 1.6–9.2 for each 10-fold increase in plasma EBV DNA concentration;  $P = 0.003$ )]. In a second cohort of 139 NPC patients followed-up for a median period of 2,027 days (interquartile range, 597–2,335 days), serum EBV DNA was found to be a significant variable associated with NPC-related death in multivariate Cox's regression analysis [relative risk = 1.6 (95% confidence interval, 1.1–2.1 for each 10-fold increase in serum EBV DNA concentration;  $P = 0.007$ )]. The quantitation of circulating EBV DNA may thus allow improved prognostication of NPC.

## Introduction

NPC<sup>3</sup> is one of the five most common cancers affecting Hong Kong Chinese and is also prevalent in the southern part of mainland China and Southeast Asia (1). Although the etiological factors leading to the development of NPC are incompletely understood, it has been well established that there is a strong association between infection with EBV and NPC (2). Radiotherapy is the main treatment modality for all locally and regionally confined disease (1). At present, the prognostication of NPC has been accomplished mainly by a variety of clinical and radiological parameters (3, 4). These clinical parameters have contributed to the development of the revised (1997) AJCC/UICC staging system for this cancer (5) that has been shown to be of prognostic significance (4, 6). Thus, 10-year disease-specific survival figures for stages I, II, III, and IV have been reported to be 77%, 65%, 54%, and 29%, respectively (4). Recently, it has been demonstrated that cell-free EBV DNA can be found in the plasma and serum of NPC patients (7, 8). It has further been shown that the serial analysis of circulating EBV DNA offers a powerful method for monitoring the

progress of these patients (9–11). It is currently unknown, however, whether the level of plasma/serum EBV DNA at presentation may be of prognostic value. We explored this issue by examining the relationship between pretreatment circulating EBV DNA concentration and early clinical events (within the first year after treatment) and also with long-term survival. In particular, we tested whether this potential prognostic effect of plasma/serum EBV DNA concentration could be discerned independent of the current staging system.

## Materials and Methods

**Study Populations.** Patients with histologically confirmed NPC attending the Department of Clinical Oncology at the Prince of Wales Hospital were recruited with informed consent. The study was approved by the Ethics Committee of The Chinese University of Hong Kong. All patients were investigated uniformly with endoscopic examination of the nasopharynx and computed tomography of the nasopharynx and neck. Disease staging was performed in accordance with the AJCC/UICC stage classification (5). Three ml of blood were drawn from each recruited subject into either an EDTA (for the early event study module) or a plain tube (for the extended follow-up survival module) before radiotherapy. All recruited patients were treated with a uniform radiotherapy protocol (3). The radiation portals encompassed the nasopharynx and adjacent regions at risk of local spread of tumor and both sides of the neck.

For the early event study module, subjects were prospectively recruited between January 1998 and June 1999. All subjects were followed-up for 1 year from the date of completion of radiotherapy.

For the extended follow-up survival study module, subjects were recruited between January 1993 and July 1994 and followed-up until June 2000. The duration of clinical follow-up was measured from the date of blood sampling for EBV DNA measurement. These patients were followed-up for a median period of 2,027 days (interquartile range, 597–2,335 days).

**DNA Extraction from Plasma/Serum Samples.** Plasma/serum samples were harvested from the patients according to previously described protocols (8). The samples were stored at  $-20^{\circ}\text{C}$  until further processing. DNA from plasma/serum samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the "blood and body fluid protocol" as recommended by the manufacturer (12). A total of 400–800  $\mu\text{l}$  of the plasma/serum samples were used for DNA extraction per column. The exact amount was documented for the calculation of the target DNA concentration. A final elution volume of 50  $\mu\text{l}$  was used to elute the DNA from the extraction column.

**Real-time Quantitative EBV DNA PCR.** Circulating EBV DNA concentrations were measured using a real-time quantitative PCR system that amplified aDNA segment in the *BamHI*-W fragment region of the EBV genome (8). The principles of real-time quantitative PCR and reaction set-up procedures were as described previously (8). Data were collected using an ABI Prism 7700 Sequence Detector and analyzed using the Sequence Detection System software (version 1.6.3) developed by PE Biosystems. Results were expressed as copies of EBV genomes per milliliter of plasma/serum.

All plasma/serum DNA samples were also subjected to real-time PCR analysis for the  $\beta$ -globin gene (8), which gave a positive signal on all tested samples, thus demonstrating the quality of the extracted DNA. Multiple negative water blanks were included in every analysis.

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<sup>3</sup> The abbreviations used are: NPC, nasopharyngeal carcinoma; CI, confidence interval; AJCC/UICC, American Joint Committee on Cancer/International Union Against Cancer.

**Statistical Analysis.** For the early event study module, an “event” was scored when the patient developed either local recurrence or distant metastasis within the first year after treatment. The levels of plasma EBV DNA in patients with and without early events were compared using the Mann-Whitney rank-sum test. In addition, binary logistic regression was used to estimate the prognostic effects of plasma EBV DNA and other variables. For the extended follow-up study module, the end point was death attributed directly to NPC. The Kaplan-Meier method was used for univariate survival analysis, and the log-rank test was used to assess the difference between survival curves. Cox’s proportional hazards analysis was used to estimate the prognostic effects of various variables. A *P* of less than 0.05 was considered statistically significant. These statistical calculations were performed using SPSS version 10.0 and MedCalc version 5.0 software.

## Results

**Early Event Study.** To study the prognostic relationship between the plasma EBV DNA level at presentation and early (within 1 year of treatment) clinical events, 91 NPC patients were recruited. The clinical characteristics of these subjects are listed in Table 1. Within the 1-year follow-up period, 19 subjects developed either local recurrence or distant metastasis, whereas the remaining 72 subjects had no clinical events. The median plasma EBV DNA concentrations in those with and without events were 41,756 copies/ml (interquartile range, 13,931–209,272 copies/ml) and 5,807 copies/ml (interquartile range, 1,103–18,387 copies/ml), respectively (Fig. 1). The difference between these two groups is statistically significant (Mann-Whitney rank-sum test, *P* < 0.001). This analysis was confirmed by univariate binary logistic regression analysis, which once again demonstrated a relationship between plasma EBV DNA and early clinical events (*P* < 0.0005). The relative risk for each 10-fold increase (*i.e.*, one logarithmic unit) in plasma EBV DNA concentration was 4.5 (95% CI, 1.9–10.4).

Univariate binary logistic regression analysis was also carried out for each of the other patient variables, namely, stage, age, and sex. Only stage achieved statistical significance (*P* = 0.008), with a relative risk of 8.0 (95% CI, 1.7–37.3) for advanced-stage disease (stages III and IV) compared with early-stage disease (stages I and II). The other two variables, age and sex, did not achieve statistical significance and had *P*s of 0.23 and 0.11, respectively.

To investigate whether plasma EBV DNA was a predictor of early clinical events independent of stage, multivariate binary logistic regression analysis was performed using EBV DNA and stage as variables. This analysis revealed that plasma EBV DNA was indeed an independent prognostic indicator with a *P* of 0.003. The relative risk for every 10-fold increase in plasma EBV DNA level was 3.8 (95% CI, 1.6–9.2). On the other hand, the prognostic significance of stage was borderline in this multivariate analysis, with a *P* of 0.07 and a relative risk of 4.6 (95% CI, 0.89–23.5) for advanced-stage disease compared with early-stage disease.

Table 1 Clinical characteristics of patients recruited for the early event study

Features	No. of subjects ( <i>n</i> = 91)
Age (yrs)	
<40	19
≥40	72
Sex	
Male	74
Female	17
Stage	
Early (AJCC/UICC stages I and II)	37
Advanced (AJCC/UICC stages III and IV)	54
Event within 1 year after treatment	
With event	19
Without event	72

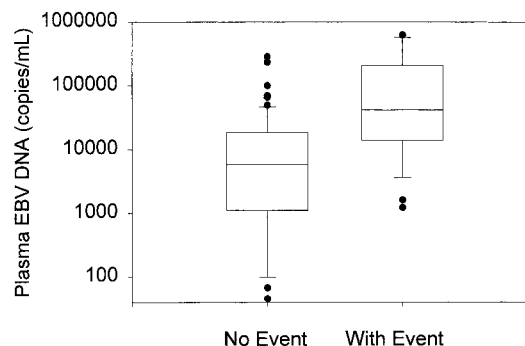


Fig. 1. Plasma EBV DNA concentrations in NPC patients with or without clinical events within the first year after radiotherapy. The subject categories are shown on the X axis. Plasma EBV DNA concentrations (copies/milliliter) as determined by real-time quantitative PCR are plotted on the Y axis (common logarithmic scale). The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. The filled circles mark the data points outside the 10th and 90th percentiles.

Table 2 Clinical characteristics of patients recruited for the survival study

Features	No. of subjects ( <i>n</i> = 139)
Age (yrs)	
<40	36
≥40	103
Sex	
Male	104
Female	35
Stage	
Early (AJCC/UICC stages I and II)	45
Advanced (AJCC/UICC stages III and IV)	94
Status	
Died of disease	55
Censored	84

**Survival Study.** To investigate whether the effect of circulating EBV DNA on early clinical events might translate into an effect on survival, a second independent cohort of 139 NPC patients was studied. The clinical characteristics of these subjects are listed in Table 2.

Univariate Cox’s regression analysis indicated that serum EBV DNA level was a prognostic indicator for death directly related to NPC (*P* = 0.001). The relative risk for every 10-fold increase in serum EBV DNA level was 1.7 (95% CI, 1.3–2.4). Plotting of the levels of serum EBV DNA against clinical outcome indicated that the best discrimination of patients who died of disease and those who were censored at the end of the study was obtained with a serum EBV DNA level of 40,568 copies/ml (data not shown). Kaplan-Meier analysis of patient survival using this cutoff serum EBV DNA level is shown in Fig. 2A. The difference in the survival between patients with serum EBV DNA above or below 40,568 copies/ml was highly statistically significant (log-rank test, *P* < 0.0005).

Univariate analysis also indicated that disease stage was also a prognostic variable for NPC-related death (*P* = 0.001; relative risk, 3.3; 95% CI, 1.6–6.8). The remaining variables, age and sex, did not achieve statistical significance and had *P*s of 0.51 and 0.53, respectively.

Multivariate Cox’s regression analysis was then carried out for the two variables that were significant in the univariate analysis, namely, serum EBV DNA and disease stage. The results indicated that both of these variables were independent prognostic indicators of survival for patients with NPC. For serum EBV DNA, the *P* of the multivariate analysis was 0.007, with a relative risk of 1.6 (95% CI, 1.1–2.1) for every 10-fold increase in serum EBV DNA. For disease stage, the *P* was 0.016, with a relative risk of 2.5 (95% CI, 1.2–5.1) for advanced-

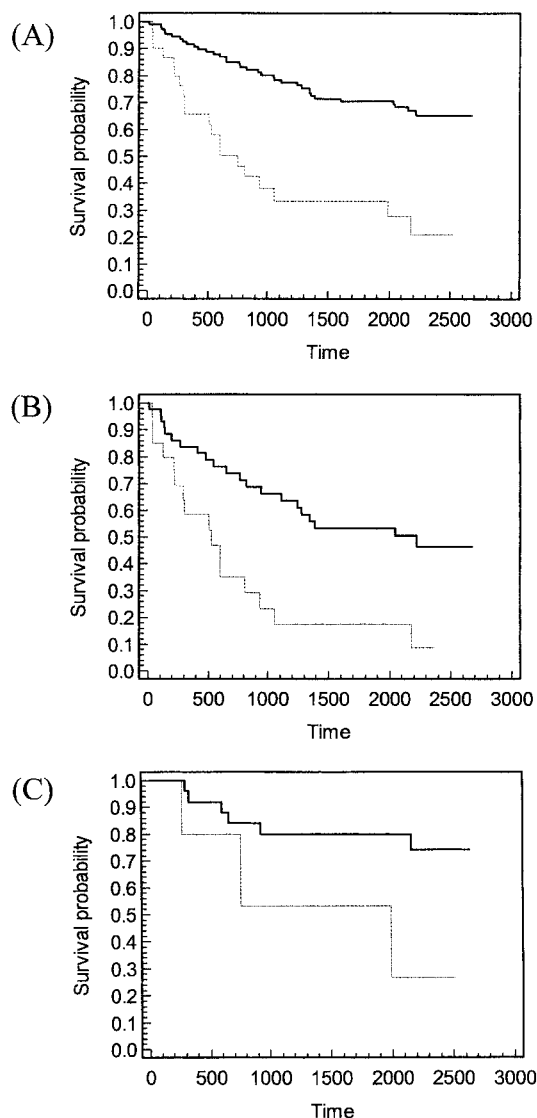


Fig. 2. Kaplan-Meier survival analysis for NPC patients: A, all AJCC/UICC stages; B, stage IV only; and C, stage III only. The solid and dotted lines indicate results from patients with serum EBV DNA < 40,568 and  $\geq$  40,568 copies/ml, respectively.

stage disease (stages III and IV) when compared with early-stage disease (stages I and II).

The independent prognostic value of serum EBV DNA over disease stage was further explored by Kaplan-Meier analysis for patients of the same stage. Because most of the NPC-related deaths occurred in stages III and IV (46 of the 55 deaths), this analysis was only performed for these two advanced stages. The number of patients with stage III and stage IV disease was 31 and 63, respectively. The results in Fig. 2, B and C, indicate that serum EBV DNA is prognostically significant for both stage III (log-rank test,  $P = 0.04$ ) and stage IV disease (log-rank test,  $P = 0.0006$ ).

## Discussion

The recent demonstration of circulating EBV DNA in the plasma and serum of NPC patients has opened up new possibilities for detecting and monitoring this common cancer in south China and Southeast Asia (7–9). In this study, we have explored the prognostic implication of the plasma/serum EBV DNA level at presentation. Excitingly, our data clearly indicate that plasma/serum EBV DNA concentration is an independent prognostic factor for this disease. This

prognostic effect could be seen with regard to both recurrence and metastasis within the first year after treatment and ultimate survival from the disease. Indeed, for prognostication within the first year after treatment, plasma EBV DNA level was a more powerful prognosticator than disease stage. The latter only achieved borderline statistical significance in multivariate analysis in which plasma EBV DNA was also entered as a variable.

The biological explanation for the strong prognostic effect of plasma/serum EBV DNA requires further study. One possibility is that plasma/serum EBV DNA concentration provides a measure of the tumor load that is present in a NPC patient's body. Potentially, this type of measurement could provide a more accurate reflection of tumor burden than conventional staging systems. Our data indicate that additional studies should explore the possibility of incorporating plasma/serum EBV DNA level into future revisions of the staging system for NPC. This would potentially further enhance the precision with which prognostication could be performed for this tumor.

The real-time PCR system used in this study is very well suited to routine clinical application because the assay is rapid, accurate, and easily adapted for large throughput analysis. In addition, because no post-PCR processing is needed, the assay is much more resistant to product contamination than other PCR systems. We have chosen to use the *Bam*HI-W fragment as the target for our EBV DNA PCR because the repetitive nature of this region would enhance the sensitivity of the assay. We have previously validated the accuracy of this target with a single copy EBV target (*EBNA-1*) and found good correlation between the quantitative results obtained by the two assays (8).

In addition to NPC, circulating EBV DNA has also been found in other EBV-associated malignancies, e.g., Hodgkin's disease (13, 14). Our data suggest that it would be interesting to study whether plasma/serum EBV DNA is also an independent prognostic variable for these malignancies.

Finally, plasma/serum EBV DNA in NPC is just one facet of the increasingly recognized phenomenon of circulating tumor-derived DNA in plasma and serum (12, 15–17). Our results indicate that highly clinically relevant information would be obtained from further quantitative analysis of such tumor-associated DNA in the circulation.

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