# Plasma Epstein-Barr Virus DNA and Residual Disease After Radiotherapy for Undifferentiated Nasopharyngeal Carcinoma

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***Background:*** **Epstein-Barr virus (EBV) DNA can be detected and quantified in the plasma of patients with EBV-related tumors, such as nasopharyngeal carcinoma (NPC). Although NPC at early stages can be cured by radical radiotherapy, there is a high recurrence rate in patients with advanced NPC. The pretreatment level of circulating EBV DNA is a prognostic factor for NPC, but the prognostic value of post-treatment EBV DNA has not been studied. We designed a prospective study in Hong Kong, China, to investigate the value of plasma EBV DNA as a prognostic factor for NPC.** ***Methods:*** **One hundred seventy NPC patients, without metastatic disease at presentation, were treated with a uniform radiotherapy protocol. Circulating EBV DNA was measured by real-time quantitative polymerase chain reaction before treatment and 6–8 weeks after radiotherapy was completed. Risk ratios (RRs) were determined with a Cox regression model, and associations of various factors with progression-free and overall survival and recurrence rates were determined with a stepwise Cox proportional hazards model. All statistical tests were two-sided.** ***Results:*** **Ninety-nine percent of patients achieved complete clinical remission. Levels of post-treatment EBV DNA dominated the effect of levels of pretreatment EBV DNA for progression-free survival. The RR for NPC recurrence was 11.9 (95% confidence interval [CI] = 5.53 to 25.43) for patients with higher post-treatment EBV DNA and 2.5 (95% CI = 1.14 to 5.70) for patients with higher pretreatment EBV DNA. Higher levels of post-treatment EBV DNA were statistically significantly associated with overall survival (*P*<.001; RR for NPC recurrence = 8.6, 95% CI = 3.69 to 19.97). The positive and negative predictive values for NPC recurrence for a higher level of post-treatment EBV DNA were 87% (95% CI = 58% to 98%) and 83% (95% CI = 76% to 89%), respectively.** ***Conclusion:*** **Levels of post-treatment plasma EBV DNA in patients with NPC appear to strongly predict progression-free and overall survival and to accurately reflect the post-treatment residual tumor load. [J Natl Cancer Inst 2002;94: 1614–9]**

Nasopharyngeal carcinoma (NPC) is endemic in southern China, and nearly all patients harbor Epstein-Barr virus (EBV) in their tumor tissues *(1,2).* Most patients with early-stage NPC will achieve a complete clinical remission after radiotherapy, with or without concurrent chemotherapy, with no clinical evidence of residual disease *(3–7).* Despite this high rate of initial local control, disease will subsequently recur in 30%–40% of patients with advanced NPC at the local site or with distant metastases *(3).* Such treatment failures, presumably, represent residual microscopic disease that is not detected by current imaging procedures. If such patients with a high risk of recurrence could be identified, they might benefit from more intensive primary treatment or adjuvant therapy, and those at low risk of recurrence could be spared such potentially toxic treatment.

Prompted by reports that tumor-derived DNA can be detected in the plasma and serum of cancer patients *(8,9),* we developed a real-time quantitative polymerase chain reaction (PCR) assay for measuring circulating tumor-derived EBV DNA in patients with NPC *(10–12).* The level of pretreatment EBV DNA is strongly associated with overall survival and is a more powerful prognostic factor than stage *(13).* In a small case–control study *(11),* we have previously observed that patients who relapsed after radiotherapy often had residual high levels of EBV DNA, whereas patients with continuous clinical remission had continuous low or undetectable levels of EBV DNA.

We now report a large prospective study involving 170 patients with a median follow-up of more than 2 years after radiotherapy. We sought to more rigorously test our original hypothesis that the levels of EBV DNA after completion of conventional treatment, perhaps in combination with pretreatment levels, might be associated with the presence or absence of residual disease. We assumed that residual disease would eventually be detected as disease recurrence and that because such disease is usually incurable, it would ultimately be reflected by a statistically significant decrease in survival.

## Patients and Methods

### Patients

One hundred seventy patients with newly diagnosed NPC were recruited to the study between September 25, 1997, and October 5, 1999. The study was approved by the Ethics Committee of the Chinese University of Hong Kong. Patients were investigated uniformly with endoscopic examination of the nasopharynx and computed tomography of the nasopharynx and neck. In patients with advanced disease that had metastasized to the supraclavicular lymph nodes [stage N3b, according to the

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*See* "Notes" following "References."

American Joint Committee on Cancer/International Union Against Cancer stage classification *(14)*] who had abnormal liver function tests or an increased serum alkaline phosphatase level, a thoracic computed tomography scan, liver ultrasound, and bone scintigram were also performed. Written informed consent was obtained from all patients. Those with evidence of metastatic disease at presentation were excluded.

All recruited patients were treated with a uniform radiotherapy protocol *(3)*. Fifteen patients received cisplatin at 40 mg/m$^2$ weekly concurrently with radiotherapy as part of a prospective randomized study of concurrent chemotherapy and radiotherapy compared with radiotherapy alone in locally advanced NPC *(6)*. The patients were assessed by nasopharyngoscopy and clinical examination 6–8 weeks after radiotherapy and were, thereafter, followed up every 8–12 weeks. Complete remission was defined as complete disappearance of locoregional disease by physical examination and endoscopic examination. Patients who developed symptoms or signs suspicious of local recurrence or metastasis were investigated further with nasopharyngeal biopsy and imaging, as appropriate. Three milliliters of blood was drawn before treatment and 6–8 weeks after radiotherapy was completed. All patients were followed up until January 31, 2001. The duration of follow-up was measured from the date of diagnosis of NPC.

### DNA Extraction From Serum Samples

Serum samples were harvested from the patients as described *(10)*. The samples were stored at –20 °C until further processing. DNA from plasma and/or serum samples was extracted with a QIAamp blood kit (Qiagen, Hilden, Germany) by the blood and body fluid protocol, as recommended by the manufacturer *(2)*. A total of 400–800 μL per column of the plasma and/or serum sample was used for DNA extraction. All buffers used were provided by Qiagen. The exact amount was documented for the calculation of the target DNA concentration. Fifty microliters of distilled water was used to elute the DNA from the extraction column.

### Real-Time Quantitative PCR for EBV DNA

Levels of circulating EBV DNA were measured with a real-time quantitative PCR system that amplified a DNA segment in the region of the *Bam*HI-W endonuclease fragment of the EBV genome *(10)*. The principles of real-time quantitative PCR and reaction setup procedures were as described *(10)*. Data were collected with an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) and analyzed with Sequence Detection System software (version 1.6.3) developed by Applied Biosystems. Results were expressed as the number of copies of EBV genomes per milliliter of serum.

All serum DNA samples were also subjected to real-time PCR analysis for the β-globin gene *(10)*, which gave a positive signal on all tested samples, thus demonstrating that the quality of the extracted DNA was good. To further demonstrate the specificity of the EBV and β-globin primer and probe combinations, the EBV primers were deliberately used with the β-globin probe and vice versa. No fluorescence signal was detected by the ABI PRISM 7700 sequencer detector in these deliberately mismatched reactions. As negative controls for the EBV PCR system, buffy coat and paired plasma isolated from cord blood samples from five healthy newborns were analyzed with the EBV primer–probe combination. No amplification signals were seen. In addition, multiple negative water blanks were included in every analysis.

### Statistical Methods

We undertook our statistical analysis in two phases. We first investigated the extent to which the level of post-treatment EBV DNA, presumably through its association with disease relapse, impinged on disease-free and overall survival. We then assessed the extent to which an increased level of EBV DNA after treatment was associated with subsequent disease relapse by determining the positive and negative predictive values of the levels of pretreatment and post-treatment EBV DNA for either local recurrence or distant metastasis.

We postulated that a high level of post-treatment EBV DNA would lead to a 2.5-fold to 3-fold increase in the hazards ratio compared with a low level of post-treatment EBV DNA. We would need to observe about 40 events to have 80% power of detecting this effect with a two-sided test at a level of 5%. The Kaplan–Meier method was used to analyze time-to-event end points. The primary end points of this study were progression-free survival and overall survival. Progression-free survival was defined as the time from diagnosis to the date of progression or the date of death or when censored at the last report date. Survival was defined as the time from diagnosis to the date of death or when censored at the last report date if patients were still alive. The time to first local recurrence and the time to first distant recurrence were also analyzed to determine the importance of the level of post-treatment EBV DNA as a prognostic factor for local control and distant metastasis. The time to first local recurrence was defined as the time from diagnosis to the time of local recurrence if local recurrence occurred before the distant metastasis or death; otherwise, it would be censored at the date of the first event or the last report date. Similarly, the time to first distant recurrence was defined as the time from diagnosis to the time of distant recurrence, if distant recurrence occurred before or on the same day as local recurrence; otherwise, it would be censored at the day of first recurrence or death or the date of last report.

To select the cutoff points for the levels of pre- and post-treatment EBV DNA, we used classification and regression tree analysis that permitted us to maximize the difference in censored survival data between groups of patients represented by nodes in a binary tree. The log-rank test statistic was chosen as a dissimilarity measure in the splitting process. The efficacy of the log-rank splitting statistic, as assessed by LeBlanc and Crowley *(15)* in their simulation study, showed that the log-rank statistic detects the structure well, and the performance for data with 20%–50% censoring remains good compared with that for uncensored data.

A stepwise Cox proportional hazards model was used to examine the association of various prognostic factors, including the levels of pre- and post-treatment EBV DNA, age, sex, tumor (T) stage, and node (N) stage, with both progression-free survival and overall survival. T classification was used as a continuous variable, and N classification was used as a binary variable. The Cox regression analyses for levels of pretreatment and post-treatment EBV DNA were undertaken with both continuous and dichotomized variables. The conclusions of the study by using either the continuous variables or the dichotomized variables for the levels of EBV DNA were identical, and the effect of EBV DNA as a prognostic factor did not depend on the choice

of the cutoff point. Therefore, we present only the results from dichotomized variables analyses. All statistical tests were two-sided. The risk ratios (RRs) from the Cox regression model are also reported. The RR represents the ratio of the hazards for the patients with poor prognosis to those with a better prognosis for the particular factor being considered in the Cox model. The proportional hazards assumption for the Cox model was assessed by both the methods of log-minus-log survival plots and the time-dependent covariate analysis on the final model with pretreatment and post-treatment EBV DNA variables.

## RESULTS

The patient characteristics of the 170 patients are listed in Table 1. One hundred sixty-eight patients (99%) achieved clinical complete remission after radiotherapy with or without chemotherapy. The median follow-up of these patients was 116 weeks (range = 37–239 weeks). Eighteen patients developed local recurrences, and 27 developed distant metastases. Five patients had both local and distant metastases, so that there was a total of 40 progression events. There were 24 deaths, all related to tumor progression.

The median and 75$^{th}$ percentile levels of pretreatment EBV DNA were 2352 copies per milliliter and 9042 copies per milliliter, respectively (range = 0–419 778 copies per milliliter). The median and 75$^{th}$ percentile levels of post-treatment EBV DNA were 0 copies per milliliter and 27.5 copies per milliliter, respectively (range = 0–11 454 730 copies per milliliter). For the statistical analysis, patients were classified into two groups on the basis of their level of EBV DNA. Cutoff values for EBV DNA were chosen on the basis of a measure of heterogeneity with the log-rank test statistic with respect to progression-free survival and overall survival. For pretreatment EBV DNA, a cutoff value of 4000 copies per milliliter was optimal for distinguishing the two groups and demonstrated a highly statistically significant difference in progression-free survival ($P$<.001; log-rank statistic = 26.2). For post-treatment EBV DNA, a cutoff value of 500 copies per milliliter was obtained for progression-free survival ($P$<.001; log-rank statistic = 89.9) (Fig. 1, A). The results on overall survival (Fig. 1, B) and progression-free survival were similar. The levels of pre- and post-treatment EBV DNA are presented in Table 2.

**Table 1.** Characteristics of patients

| Characteristic | Value |
|---|---|
| Age, y | |
| Median (range) | 46 (18–80) |
| Sex, No. (%) | |
| Male | 143 (84.1) |
| Female | 27 (15.9) |
| Overall stage, No. (%) | |
| I | 20 (11.8) |
| II | 50 (29.4) |
| III | 43 (25.3) |
| IV | 57 (33.5) |
| Tumor classification, No. (%)* | |
| 1 | 33 (19.4) |
| 2 | 88 (51.8) |
| 3 | 16 (9.4) |
| 4 | 33 (19.4) |
| Node classification, No. (%)* | |
| 0 | 53 (31.2) |
| 1 | 45 (26.5) |
| 2 | 41 (24.1) |
| 3 | 31 (18.2) |

**See (14).*

### Progression-Free Survival and Overall Survival Analysis

Levels of post-treatment EBV DNA dominated the effect of pretreatment EBV DNA for both progression-free survival ($P$<.001) (Fig. 2, A) and overall survival ($P$<.001) (Fig. 2, B). Patients with high levels of post-treatment EBV DNA (≥500 copies per milliliter) had the poorest prognosis (progression-free rate at 1 year = 48%, 95% CI = 22% to 74%). A subgroup of patients, although the number is relatively small, had a low level of pretreatment EBV DNA (<4000 copies per milliliter), which would have been classified as good prognosis, but the post-treatment levels failed to decrease to less than 500 copies per milliliter after the completion of treatment. These patients also had a poor prognosis. Patients with low post-treatment levels (<500 copies per milliliter) had an excellent prognosis (progression-free rate at 1 year = 93%, 95% CI = 89% to 97%). Among them, a subgroup of patients had a pretreatment EBV DNA level of greater than 4000 copies per milliliter, which would have been classified as poor prognosis without the post-treatment EBV DNA level.

Univariate analysis showed that the level of pretreatment EBV DNA ($P$<.001), the level of post-treatment EBV DNA ($P$<.001), T classification ($P$ = .004), and N classification ($P$<.001) were all statistically significantly associated with progression-free survival. When a stepwise Cox regression model was used, pretreatment EBV DNA with a cutoff value at 4000 copies per milliliter ($P$ = .023; RR = 2.5, 95% CI = 1.14 to 5.70), post-treatment EBV DNA with a cutoff value at 500 copies per milliliter ($P$<.001; RR = 11.9, 95% CI = 5.53 to 25.43), and lymph nodal status ($P$ = .001; RR = 1.8, 95% CI = 1.26 to 2.62) were statistically significantly associated with progression-free survival. Univariate analysis showed that pretreatment EBV DNA ($P$<.001), post-treatment EBV DNA ($P$<.001), age ($P$ = .015), T classification ($P$ = .007), and N classification ($P$ = .001) were all statistically significantly associated with overall survival. In a Cox regression analysis, only pretreatment EBV DNA with a cutoff value at 4000 copies per milliliter ($P$<.001; RR = 6.3, 95% CI = 2.14 to 18.82), post-treatment EBV DNA with a cutoff value at 500 copies per milliliter ($P$<.001; RR = 8.6, 95% CI = 3.69 to 19.97), and age ($P$ = .039; RR for a 10-year increment = 1.36, 95% CI = 1.02 to 1.83) were statistically significantly associated with overall survival.

To assess the performance of the levels of pre- and post-treatment EBV DNA as predictors for important clinical outcomes by use of similar cutoff points, we evaluated the positive and negative predictive values for detecting disease progression within 3 years after treatment. For the level of pretreatment EBV DNA, the positive predictive value was 41% (95% CI = 30% to 54%), and the negative predictive value was 93% (95% CI = 82% to 95%). For the level of post-treatment EBV DNA, the positive predictive value was 87% (95% CI = 58% to 98%), and the negative predictive value was 83% (95% CI = 76% to 89%).

For the time to first local recurrence, there were 17 events in the analysis. Univariate analysis showed that T classification ($P$ = .003), N classification ($P$ = .020), and the level of pretreatment EBV DNA with a cutoff value at 4000 copies per

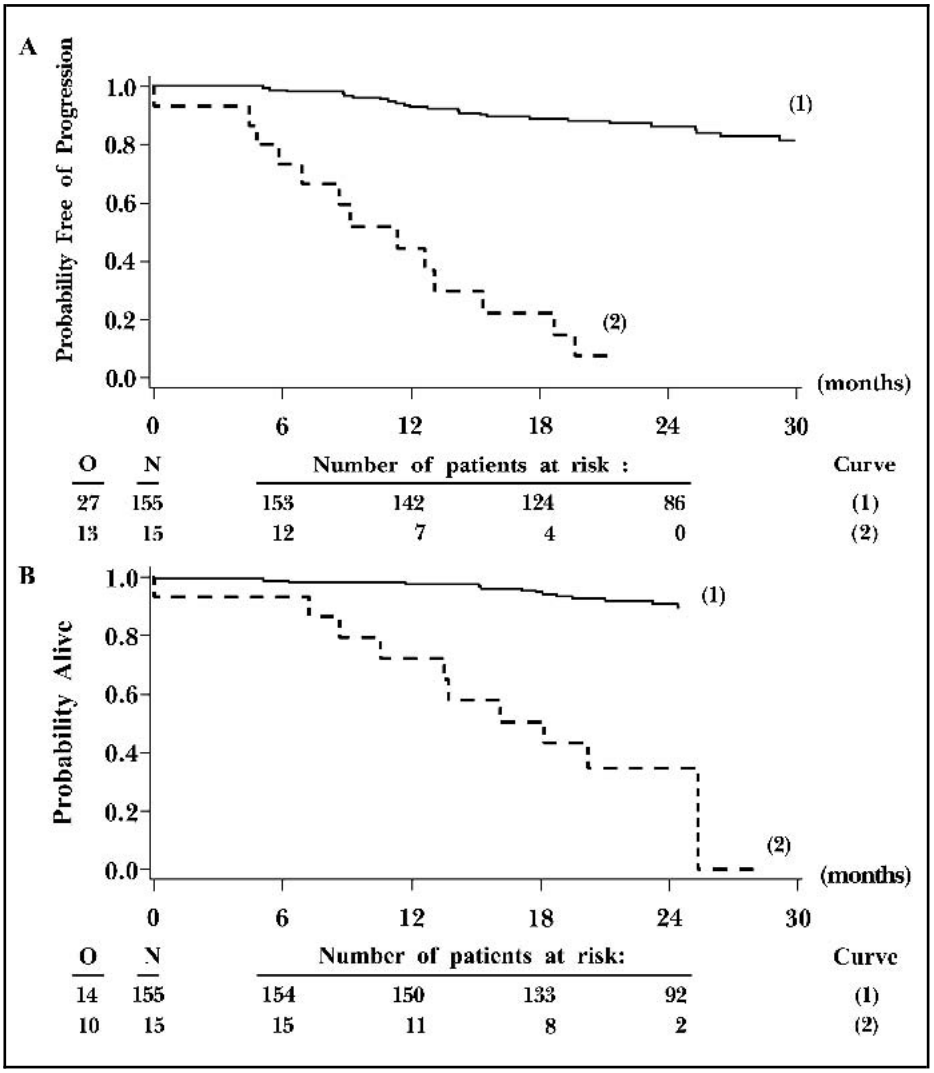


**Fig. 1. A**) Progression-free survival analysis for a post-treatment Epstein-Barr virus (EBV) DNA cutoff value of 500 copies per milliliter. Progression-free rate at 1 year for an EBV DNA value less than 500 copies per milliliter is 93% (95% CI = 89% to 97%) and that for an EBV DNA value greater than 500 copies per milliliter is 48% (95% CI = 22% to 74%). **B**) Overall survival analysis with a post-treatment EBV DNA cutoff value of 500 copies per milliliter. Overall survival rate at 1 year for an EBV DNA value less than 500 copies per milliliter is 97% (95% CI = 94% to 99.5%) and that for an EBV DNA value greater than 500 copies per milliliter is 76% (95% CI = 54% to 98%). Curves: **(1)** = a post-treatment EBV DNA value less than 500 copies per milliliter; **(2)** = post-treatment EBV DNA value greater than 500 copies per milliliter. O = observed events; N = number of patients at risk.

**Table 2.** Pretreatment and post-treatment levels of Epstein-Barr virus (EBV) DNA

| Pretreatment EBV DNA levels* | No. of patients | | | | | |
|---|---|---|---|---|---|---|
| | Post-treatment EBV DNA levels* | | | | | |
| | 0 | 0–499 | 500–3999 | 4000–9999 | ⩾10 000 | Total |
| 0 | 13 | 2 | 0 | 0 | 0 | 15 |
| 0–499 | 35 | 6 | 1 | 0 | 0 | 42 |
| 500–3999 | 32 | 8 | 2 | 0 | 1 | 43 |
| 4000–9999 | 19 | 7 | 2 | 1 | 1 | 30 |
| ⩾10 000 | 22 | 11 | 3 | 0 | 4 | 40 |
| Total | 121 | 34 | 8 | 1 | 6 | 170 |

*Number of copies of EBV DNA per milliliter.

milliliter ($P = .005$) were statistically significantly associated with the time to first local recurrence. The level of post-treatment EBV DNA with cutoff at 500 copies per milliliter ($P = .572$) was not statistically significantly associated with the time to first local recurrence. In the stepwise Cox regression analysis, only the T classification ($P = .046$; RR = 1.7, 95% CI = 1.01 to 2.73) was statistically significantly associated with the time to first local recurrence. Neither the level of pretreatment EBV DNA ($P = .064$; RR = 2.9, 95% CI = 0.94 to 8.67) nor the level of post-treatment EBV DNA ($P = .913$; RR = 1.1, 95% CI = 0.14 to 8.88) was statistically significant.

For the time to first distant recurrence analysis, there were 23 events in the analysis. Univariate analysis showed that the level of pretreatment EBV DNA with a cutoff value at 4000 copies per milliliter ($P<.001$), the level of post-treatment EBV DNA with a cutoff value at 500 copies per milliliter ($P<.001$), and the N classification ($P<.001$) were statistically significantly associated with the time to first distant recurrence analysis. Under the stepwise Cox regression analysis, only the level of post-treatment EBV DNA with a cutoff value at 500 copies per milliliter ($P<.001$; RR = 33.4, 95% CI = 12.03 to 92.62) and the N classification ($P = .001$; RR = 2.5, 95% CI = 1.43 to 4.27) were statistically significantly associated with the time to first distant recurrence analysis. The level of pretreatment EBV DNA with a cutoff at 4000 copies per milliliter ($P = .182$; RR = 2.1, 95% CI = 0.70 to 6.58) was not statistically significantly associated with the time to first distant recurrence.

## Discussion

With standard radical radiotherapy, approximately 80% of patients with early-stage (I and II) NPC appear to be cured of their disease. However, in patients with more advanced disease

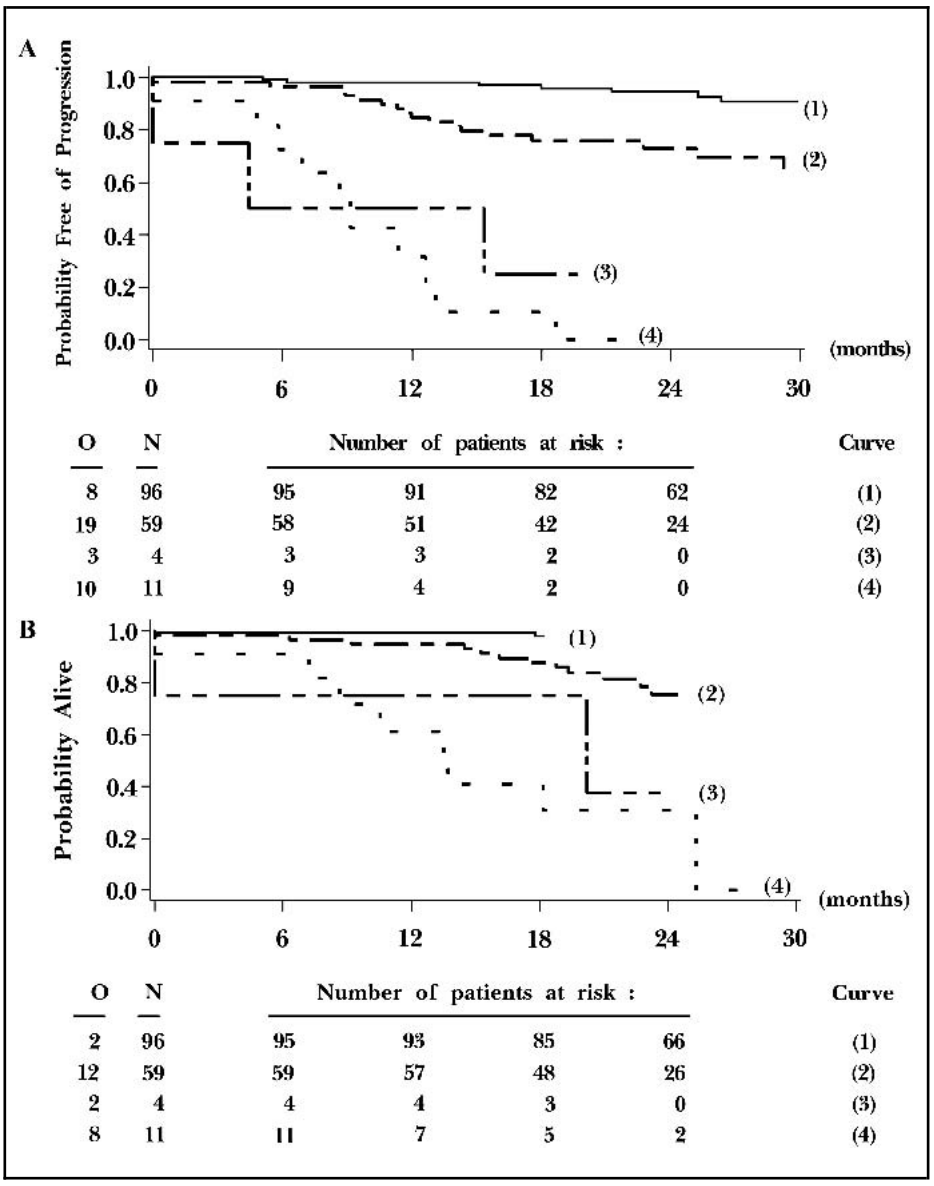


**Fig. 2. A**) Progression-free survival analysis combining levels of pretreatment Epstein-Barr virus (EBV) DNA (cutoff = 4000 copies per milliliter) and levels of post-treatment EBV DNA (cutoff = 500 copies per milliliter). Progression-free rate at 1 year for a pretreatment EBV DNA value less than 4000 copies per milliliter and a post-treatment EBV DNA value less than 500 copies per milliliter is 97% (95% CI = 93% to 100%), that for a pretreatment EBV DNA value greater than 4000 copies per milliliter and a post-treatment EBV DNA value less than 500 copies per milliliter is 85% (95% CI = 76% to 94%), that for a pretreatment EBV DNA value less than 4000 copies per milliliter and a post-treatment EBV DNA value greater than 500 copies per milliliter is 50% (95% CI = 1% to 99%), and that for a pretreatment EBV DNA value greater than 4000 copies per milliliter and a post-treatment EBV DNA value greater than 500 copies per milliliter is 32% (95% CI = 3% to 61%). **B**) Overall survival analysis combining pretreatment levels of EBV DNA (cutoff = 4000 copies per milliliter) and post-treatment levels of EBV DNA (cutoff = 500 copies per milliliter). Overall survival rate at 1 year for a pretreatment EBV DNA value less than 4000 copies per milliliter and a post-treatment EBV DNA value less than 500 copies per milliliter is 98% (95% CI = 95% to 100%), that for a pretreatment EBV DNA value less than 4000 copies per milliliter and a post-treatment EBV DNA value greater than 500 copies per milliliter is 95% (95% CI = 89% to 100%), that for a pretreatment EBV DNA value less than 4000 copies per milliliter and a post-treatment EBV DNA value greater than 500 copies per milliliter is 75% (95% CI = 33% to 100%), and that for a pretreatment EBV DNA value greater than 4000 copies per milliliter and a post-treatment EBV DNA value greater than 500 copies per milliliter is 61% (95% CI = 31% to 91%). Curves: **(1)** = a pretreatment EBV DNA value less than 4000 copies per milliliter and a post-treatment EBV DNA value less than 500 copies per milliliter; **(2)** = a pretreatment EBV DNA value greater than 4000 copies per milliliter and a post-treatment EBV DNA value less than 500 copies per milliliter; **(3)** = a pretreatment EBV DNA value less than 4000 copies per milliliter and a post-treatment EBV DNA value greater than 500 copies per milliliter; **(4)** = a pretreatment EBV DNA value greater than 4000 copies per milliliter and a post-treatment EBV DNA value greater than 500 copies per milliliter. O = observed events; N = number of patients at risk.

stages (III and IV), recurrence is common and, consequently, 5-year survival rates are only 45%–60%. To improve this situation, more intensive regimens are being designed and, of these, combined chemoradiotherapy is the most promising. The Head and Neck Intergroup 0099 study *(7)* demonstrated statistically significant improvement in both overall and progression-free survival with concurrent cisplatin radiotherapy followed by adjuvant cisplatin and 5-fluorouracil compared with radiotherapy alone in a nonendemic NPC population. However, only 50% of patients completed the adjuvant chemotherapy because of toxicities after completion of intensive chemoradiation. A study in an area of the world where NPC is endemic *(6)* has demonstrated the improvement of progression-free survival with concurrent cisplatin and radiotherapy compared with radiotherapy alone, and the improvement was statistically significant in patients with advanced disease. Although improving the outlook in patients with advanced disease, these benefits come at the cost of considerable toxicity. Further improvement in treatment results will probably come from more intensive therapy after concurrent chemoradiation, but such approaches will require better selection of patients to confine treatment to those who will benefit most and to avoid toxic therapy in those who are likely to be already cured.

We have previously demonstrated *(13)* and confirm in this study that it is possible to identify a group of patients who have a poor prognosis by measuring levels of pretreatment circulating EBV DNA—high levels of EBV DNA being an independent prognostic adverse factor for survival. In this study, we show the importance of post-treatment EBV DNA levels in predicting post-treatment recurrence. Such recurrences, in patients who by conventional criteria have achieved complete clinical remission, presumably reflect residual disease, either local or distant, that cannot be detected by currently used imaging modalities. The most reasonable explanation for these observations is that the levels of EBV DNA provide an accurate reflection of the tumor load in NPC patients.

We observed that a level of post-treatment EBV DNA greater than 500 copies per milliliter was highly statistically signifi-

cantly associated with the poorest outcome. Such a subgroup of patients may benefit from further treatment after radiotherapy, which can be monitored by sequential measurements of EBV DNA. Similarly, those who achieve a post-treatment level of less than 500 copies per milliliter have an excellent prognosis with a 2-year survival of greater than 80% and might be spared adjuvant therapy. Future studies will be needed to evaluate the role of adjuvant treatment in the subgroup of patients with high post-treatment levels, but our data strongly suggest that monitoring EBV DNA after treatment allows molecular detection of subclinical residual disease in patients with NPC. Moreover, several other cancers, particularly the lymphoid malignancies, are associated with EBV infection, and patients with such cancers have detectable levels of EBV DNA in plasma *(16,17).* Approaches similar to those described for NPC should also be applicable to these cancers.

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## NOTES


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