Plasma Epstein–Barr Viral Deoxyribonucleic Acid Predicts Worse Outcomes in Pediatric Nonmetastatic Nasopharyngeal Carcinoma Patients

An Observational Study of 89 Cases in an Endemic Area

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Abstract: To evaluate the clinical significance of pretreatment levels of plasma Epstein–Barr virus DNA (pEBV DNA) on prognoses in pediatric nasopharyngeal carcinoma (NPC) patients.

Eighty-nine patients aged 21 years old or younger with nonmetastatic NPC were evaluated to determine the effect of pEBV DNA levels on progression-free survival (PFS), distant metastasis-free survival (DMFS), and overall survival (OS). Survival probabilities in patient groups that were segregated by clinical stage or pEBV DNA load (low or high) were compared.

The median pretreatment concentrations of pEBV DNA were 3440 copies/mL in 35 patients with stage III disease and 14,900 copies/mL in 50 patients with stage IV disease (P = 0.059). The median concentration of pEBV DNA was 34,500 copies/mL in 17 patients with relapse, which was higher than the concentration in 72 patients without relapse, who had a median level of 4985 copies/mL (P = 0.057). Further study showed that pretreatment pEBV DNA load was an independent prognostic indicator in pediatric NPC patients. High pEBV DNA was associated with adverse clinical outcomes, including PFS (3-year PFS rate = 80.5% versus 95.8%, hazard ratio (HR) = 5.00, 95% confidence interval (CI) = 1.08–27.22; P = 0.040), DMFS (3-year DMFS rate = 82.9% versus 95.8%, HR = 5.41, 95% CI = 1.08–27.22; P = 0.040), and OS (3-year OS rate = 82.9% versus 95.8%, HR = 5.41, 95% CI = 1.08–27.22; P = 0.040).

Pretreatment pEBV DNA load was an independent prognostic indicator for PFS, DMFS, and OS in pediatric patients with NPC. Prospective studies, however, are needed to validate these results.

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Abbreviations: Three-dimensional CRT = three-dimensional conformal radiotherapy, CCRT = concurrent chemoradiotherapy, CI = confidence interval, CTV = clinical target volume, DMFS = distant metastasis-free survival, EBV = Epstein–Barr virus, GTV = gross target volume, HR = hazard ratio, IMRT = intensity-modulated radiotherapy, MRI = magnetic resonance imaging, NPC = nasopharyngeal carcinoma, OS = overall survival, pEBV DNA = plasma EBV DNA, PF = cisplatin/5-fluorouracil, PFS = progression-free survival, ROC = the receiver operating characteristic, UIICC/AJCC = International Union against Cancer/ American Joint Committee on Cancer, WHO = World Health Organization.
Plasma Epstein–Barr Virus Deoxyribonucleic Acid Measurement

As described in previous studies,10,16,17 patient pEBV DNA concentrations were routinely measured using quantitative polymerase chain reaction before treatment.

Treatment

All patients received definitive radiation therapy. A total of 24 patients were treated using conventional radiotherapy, 6 patients received three-dimensional conformal radiotherapy (three-dimensional-CRT), and 59 patients were treated with intensity-modulated radiotherapy (IMRT). The median radiation dose was 70 Gy (range from 65 to 76 Gy) to the nasopharynx primary tumor and 64 Gy to the positive cervical lymph node (74 patients were positive for cervical lymph node). For patients who received IMRT, the prescription dose was 65 to 70 Gy/30 to 35 fractions to the nasopharynx gross target volume, 60 to 72 Gy/30 to 36 fractions to the GTVnd (for involved lymph nodes), 60 Gy/30 fractions to the clinical target volume 1, and 54 Gy/30 fractions to the clinical target volume 2. The irradiation doses to the organs at risk were restricted to avoid exceeding their tolerance doses and to prevent sacrificing coverage of the tumor target volumes. For patients who received conventional radiotherapy or three-dimensional-CRT, the prescribed dose to the neck was 50 to 56 Gy/25 to 28 fractions for prophylaxis and 60 to 70 Gy/30 to 33 fractions for therapy.

The treatment modality was determined according to the TNM stage. In all, 37 patients (41.6%) received concurrent chemoradiotherapy, 31 patients (34.8%) were treated with induction and concurrent chemoradiotherapy, 18 patients (20.2%) received induction chemotherapy followed by radiotherapy, and 3 patients (3.4%) received radiotherapy alone, as shown in Table 1. All chemotherapy regimens were either cisplatin-based single drug or 2-drug or 3-drug combinations of fluorouracil, paclitaxel, and gemcitabine+cisplatin. The most commonly used induction chemotherapy regimen was 2 to 3 cycles of cisplatin/5-fluorouracil administered at 3-week intervals, whereas paclitaxel+cisplatin, gemcitabine+cisplatin or paclitaxel, and cisplatin + 5-fluorouracil were administered to a small minority of the included patients. Five to 8 cycles of single cisplatin were administered weekly as the most common regimen that was administered concurrently with radiotherapy.

Clinical Outcome Assessment and Follow-Up

The first assessment of tumor responses was performed 3 months after completion of therapy. The assessment included a complete physical examination, a fiber-optic nasopharyngoscopy, magnetic resonance imaging of the head and neck, hematologic and biochemical profiles, chest radiography, abdominal ultrasonography, whole-body bone scans, and EBV serology. Then, the patients were evaluated once every 3 months during the first 3 years after therapy, once half a year for the following 2 years, and once every year thereafter. When an abnormality was identified, further investigation was arranged. The primary end point was progression-free survival (PFS), which was calculated from the date of diagnosis to the first relapse at any site, death from any cause or the date of the last follow-up visit. Overall survival (OS), as measured from the date of diagnosis to the date of death or the last follow-up, distant metastases-free survival (DMFS), as measured from the date of diagnosis to the date of distant relapse or patient censoring at the date of the last follow-up, were also assessed.

Statistical Analysis

The Statistical Package for the Social Sciences software package (SPSS V. 17.0, SPSS, Inc., Chicago, IL) was used for data analysis. A comparison of pEBV DNA levels, the clinical UIICC staging and the relationship between the pEBV DNA concentration and the relapse rate were evaluated using the Mann–Whitney U test. A receiver operating characteristic curve was used to determine the optimal cutoff value for pEBV DNA load that could be used to predict outcomes with the best trade-off between sensitivity and specificity. Finally, a cutoff level of 7500 copies/mL was chosen to define low and high EBV DNA load. Multivariate analysis using a Cox regression model.
RESULTS

Patient Characteristics

The characteristics of the enrolled patients are listed in Table 1. The median age at diagnosis was 19 years old (range, 6–21 years old). The sex ratio of male/female was 3.05. Histologic types were determined according to the WHO classification system. A total of 85 patients (95.5%) had undifferentiated carcinoma (formerly WHO type III), and 4 patients had nonkeratinizing carcinoma (formerly WHO type II). Consistent with previous studies, 85 patients (95.5%) presented with advanced stage III or stage IV locoregional disease. The median follow-up time of our patients was 44.9 months.

TABLE 1. Patient Characteristics

| Characteristics                          | No. (% or Range) |
|------------------------------------------|------------------|
| Median age (years)                       | 19 (6–21)        |
| Sex                                       |                  |
| Male                                      | 67 (75.3)        |
| Female                                   | 22 (24.7)        |
| World Health Organization Pathologic Classification |       |
| Type II                                   | 4 (4.5)          |
| Type III                                  | 85 (95.5)        |
| Tumor Classification                      |                  |
| T1–T2                                     | 12 (13.5)        |
| T3–T4                                     | 77 (86.5)        |
| Lymph Node Status                         |                  |
| N0–N1                                     | 32 (36.0)        |
| N2–N3                                     | 57 (64.0)        |
| International Union against Cancer Stage  |                  |
| II                                        | 4 (4.5)          |
| III                                       | 35 (39.3)        |
| IVa                                       | 38 (42.7)        |
| IVb                                       | 12 (13.5)        |
| pEBV DNA (copies/mL)                     | 5720 (median)    |
| 0                                         | 16 (18.0)        |
| <10³                                      | 9 (10.1)         |
| <10⁴                                      | 25 (28.1)        |
| <10⁵                                      | 30 (33.7)        |
| <10⁶                                      | 9 (10.1)         |
| Radiotherapy Technology                  |                  |
| IMRT                                      | 59 (66.3)        |
| Three-dimensional-CRT                     | 6 (6.7)          |
| Conventional radiotherapy                 | 24 (27.0)        |
| Mean Total Dose (Gy)                      |                  |
| Nasopharynx gross target volume           | 69.28            |
| Positive neck lymph nodes                 | 62.20            |
| Treatment Modality                        |                  |
| Radiotherapy alone                        | 3 (3.4)          |
| Concurrent chemoradiotherapy              | 37 (41.6)        |
| Induction + radiotherapy                  | 18 (20.2)        |
| Induction + concurrent chemoradiotherapy  | 31 (34.8)        |

Three-dimensional-CRT = three-dimensional conventional radiotherapy, IMRT = intensity-modulated radiotherapy, pEBV = plasma Epstein–Barr virus.

was performed with the following variables in the model: age, sex, pathologic type, clinical stage, radiation technology (IMRT versus concurrent radiotherapy or three-dimensional-CRT), pEBV DNA level (>7500 or ≤7500 copies/mL). Kaplan–Meier plots of OS, PFS, and DMFS were established for patients with high and low EBV DNA levels (indicated as >7500 and ≤7500 copies/mL, respectively), for patient groups with different UICC stages (stage III–stage IV), and for patients with pEBV DNA levels and UICC stages together. Log-rank tests were performed to assess the differences in survival probabilities between patient subgroups. Analyses were repeated for the following end points: PFS, OS, and DMFS. All statistical tests were 2 sided, and a P < 0.05 was considered to indicate statistical significance.

Relationship Between Epstein–Barr Virus Deoxyribonucleic Acid Concentration and Clinical Stage or Relapse

We detected pEBV DNA levels in plasma samples from 73 of 89 patients. Of these, pEBV DNA was undetectable in 16 patients; 10 of these had stage III disease, and 6 had stage IV disease. A total of 55 patients (61.8%) presented with a level of 1000 to 100,000 copies/mL. The median pretreatment concentration of pEBV DNA was 5720 copies/mL (interquartile range, 746–38,850 copies/mL). Of the 89 included patients, 35 presented with stage III disease, 50 presented with stage IV disease, and only 4 patients had stage II disease. The median pretreatment concentration of pEBV DNA was 3440 copies/mL (interquartile range, 0–20,000 copies/mL) and 14,900 copies/mL (interquartile range, 799–47,100 copies/mL) for patients with stage III and stage IV disease, respectively (P = 0.059, Figure 1A). A Spearman correlation analysis further demonstrated that pEBV DNA levels were closely correlated with the node stage and the UICC TNM tumor stage (P < 0.001 and P = 0.057, respectively; Table 2).

In our study, 17 patients ended in disease relapse, including 3 patients with locoregional relapse, 13 patients with distant failure, and 1 patient with locoregional relapse and distant failure. Distant failure was the main mode of failure in pediatric patients, occurring in 82.4% of treatment failures. Patients with disease relapse had a higher level of pEBV DNA then patients without disease relapse, with a marginal level of significance (P = 0.057, Figure 1B). The median concentrations in these patients were 34,500 copies/mL (interquartile range, 34,700–68,000 copies/mL) and 4985 copies/mL (interquartile range, 438–23,800 copies/mL), respectively.

Progression-Free Survival

Kaplan–Meier analysis of PFS was performed. The high EBV DNA group had significantly lower survival than the low EBV DNA group (3-year PFS rate = 80.5% versus 95.8%; P = 0.025, Figure 2A). In multivariate analyses, EBV DNA was the only independent prognostic factor for PFS with a borderline difference (HR = 5.00, 95% CI = 1.00–25.00, P = 0.050, Table 3).

The 3-year survival probabilities for different clinical characteristics (stages from stage III to stage IV) are listed in Table 4. A difference in survival rates for stage III and stage IV patients was observed, but the difference was not significant (P = 0.230). The 3-year survival rates for these patients were 93.9% (95% CI, 85.7%–100.0%) and 85.6% (95% CI, 75.8%–95.4%), respectively. In view of the very small number of patients with stage II disease, stage II disease was not taken into consideration in statistical analyses.

The survival probabilities in patient subgroups defined by low and high EBV DNA levels that had advanced-stage disease (stage III and IV) are also listed in Table 4. Within patients with stage III and IV diseases, high EBV DNA levels strongly predicted a lower survival rate than the rate for the low EBV
DNA group ($P = 0.047$). The 3-year survival rates for the high and low EBV DNA groups containing patients with advanced-stage disease (stage III and stage IV) were 81.6% (95% CI, 69.3%–93.9%) and 95.4% (95% CI, 89.1%–100.0%), respectively.

**Overall Survival**

All analyses were repeated using OS as the end point, and the same conclusions were obtained. The high EBV DNA group had a significantly lower survival rate than the low EBV DNA group (3-year OS rate = 82.9% versus 95.8%; $P = 0.020$, Figure 2B). In the multivariate analyses, pEBV DNA was the only independent prognostic factor for OS that showed a significant difference ($HR = 5.41$, 95% CI = 1.08–27.22, $P = 0.040$, Table 3).

**Distant Metastasis-Free Survival**

Distant failure was the main mode of failure observed in our study of pediatric patients. In all, 15.7% of the cases in our study ended in distant failure, corresponding to 82.4% of all treatment failures. All analyses were repeated using DMFS as the end point, and similar conclusions were obtained. The high EBV DNA group had significantly lower survival than the low EBV DNA group (3-year DMFS rate = 80.5% versus 95.8%; $P = 0.023$, Figure 2C). In multivariate analyses, pEBV DNA was the only independent prognostic factor for DMFS ($HR = 5.20$, 95% CI = 1.04–26.00, $P = 0.045$, Table 3).

**DISCUSSION**

In the current study, the nonkeratinizing, undifferentiated subtype of NPC was the most common histologic variant. Advanced locoregional disease, a higher rate of distant failure, and a close association with high EBV DNA levels were also observed. These findings are in accordance with findings in previous reports.3-9

To the best of our knowledge, this is the first study to evaluate prognostication according to EBV DNA load in pediatric NPC patients. The results of the current study demonstrate that pretreatment levels of pEBV DNA are correlated with the N stage and disease relapse in pediatric patients, which was in agreement with previous studies by Lo et al and Lin et al in adult NPC patients. They demonstrated that circulating pEBV DNA concentrations were correlated with tumor stage,16,20 and the likelihood of recurrence.20,21

We also found that pretreatment levels of pEBV DNA are a powerful prognostic factor that is associated with progression-free, distant metastasis-free, and overall survival. High pEBV DNA levels predicted inferior outcomes for PFS, OS, and DMFS in nonmetastatic pediatric NPC patients. Patients with high pEBV DNA levels displayed a greater than 5-folds increased risk of disease progression, distant failure, and shorter overall survival than patients with low pEBV DNA levels. This effect was independent of the TNM tumor stage, radiotherapy techniques used, age, sex, and pathologic type. In patients with advanced disease (stage III and IV), patient subgroups defined by low and high pEBV DNA levels showed similar results.

Currently, treatment for pediatric patients is generally extrapolated from guidelines tailored for adult patients, but children are usually excluded from adult clinical trials because of strict age cutoffs.22 The optimal treatment for pediatric NPC patients, who represent a small group, is less well defined, partly because of the rarity of the disease. Previous findings related to pEBV DNA in adults might not be reliable or directly applicable in children. Our study demonstrates the prognostic value of pretreatment levels of pEBV DNA in pediatric patients, which may have a bearing on management decisions. In our study, the

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**TABLE 2. Plasma Epstein–Barr Virus Deoxyribonucleic Acid Relationships**

| Variable                        | Spearman Correlation Coefficient | $P$  |
|--------------------------------|----------------------------------|------|
| Age                            | –0.097                           | 0.376|
| Sex (male/female)              | 0.153                            | 0.161|
| WHO pathologic classification (II/III) | 0.004                           | 0.972|
| Tumor stage (3/4)              | 0.062                            | 0.573|
| Node stage (0/1/2/3)           | 0.388                            | <0.001|
| UICC stage (II/III)            | 0.206                            | 0.058|
| Radiotherapy technology (two-dimensional/three-dimensional/IMRT) | 0.169                           | 0.123|

IMRT = intensity-modulated radiotherapy, UICC = International Union against Cancer, WHO = World Health Organization.
level of pEBV DNA was a more powerful indicator than the TNM stage. Moreover, pEBV DNA combined with the TNM stage had a refining effect on the risk stratification for overall survival. Survival probabilities showed that subgroups defined by high pEBV DNA levels and stage III disease had 3-year survival rates similar to those defined in patients with high pEBV DNA levels and stage IV disease. A subset of patients with stage IV disease also had relatively superior survival. Such observations have implications that may allow clinicians to potentially identify candidates who are eligible for aggressive therapy, thereby improving treatment outcomes. Similar results were achieved in previous studies. A study by Leung et al\textsuperscript{11,13} suggested a method for selecting patients for therapy intensification. According to their perspective, the therapeutic ratio could be increased by lowering the intensity of therapy in the low-risk group, whereas intensified therapy should be maintained or further augmented through the adjuvant phase in the high-risk group.\textsuperscript{13} A recent study showed that adjuvant chemotherapy can reduce distant failure and improve overall survival in NPC patients with persistently detectable pEBV DNA after curative radiation therapy + induction/concurrent chemotherapy.\textsuperscript{23} This also provides evidence that supports

**FIGURE 2.** Kaplan-Meier analysis of PFS (panel A), OS (panel B) and DMFS (panel C) of patient groups according to Epstein–Barr virus DNA levels. DMFS = distant metastasis free survival, OS = overall survival, PFS = progression free survival.

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**TABLE 3.** Progression Free, Overall, and Distant Metastasis-Free Survival Analyses Using a Multivariate Cox Proportional Hazards Model

| Parameter | PFS | OS | DMFS |
|-----------|-----|----|------|
| Age: >19 versus ≤19 years | 0.87 (0.18–4.35) 0.968 | 1.00 (0.23–4.43) 0.997 | 0.93 (0.21–4.16) 0.929 |
| Sex: male versus female | 0.53 (0.11–2.48) 0.419 | 0.53 (0.11–2.53) 0.428 | 0.53 (0.11–2.52) 0.425 |
| Pathologic type: III versus II | 0.990 | 0.990 | 0.990 |
| Overall stage: IX versus III + III | 1.41 (0.34–5.94) 0.636 | 1.32 (0.31–5.58) 0.708 | 1.41 (0.34–5.89) 0.636 |
| pEBV DNA: >7500 versus ≤7500 copies/mL | 5.00 (1.00–25.00) 0.050 | 5.41 (1.08–27.22) 0.040 | 5.20 (1.04–26.00) 0.045 |
| IMRT versus two-dimensional-CRT or three-dimensional-CRT | 1.53 (0.31–7.63) 0.604 | 1.75 (0.36–8.61) 0.490 | 1.56 (0.32–7.70) 0.586 |

CI = confidence index, CRT = conventional radiotherapy, DMFS = distant metastasis-free survival, HR = hazard ratio, IMRT = intensity-modulated radiotherapy, OS = overall survival, pEBV = plasma Epstein–Barr virus, PFS = progression free survival.

**TABLE 4.** Survival Probabilities of Patients Groups With Different International Union Against Cancer Stages and With Different Epstein–Barr Virus Deoxyribonucleic Acid Levels Within International Union Against Cancer Stages

| Stage | No. of Patients | 3-Year of PFS (%) | 95% CI (%) | 3-Year of OS (%) | 95% CI (%) | 3-Year of DMFS (%) | 95% CI (%) |
|-------|----------------|------------------|------------|-----------------|------------|-------------------|------------|
| III   | 35             | 93.9             | 85.7–100.0 | 93.8            | 85.4–100.0 | 94.1              | 86.3–100.0 |
| IV    | 50             | 85.6             | 75.8–95.4  | 87.2            | 77.6–96.8  | 85.8              | 76.0–95.6  |
| III, low DNA | 24             | 86.6             | 77.3–100.0 | 90.2            | 75.6–100.0 | 90.4              | 77.7–100.0 |
| III, high DNA | 11             | 79.5             | 68.0–96.2  | 85.2            | 71.9–98.5  | 82.1              | 68.0–96.2  |
| IV, low DNA | 22             | 82.1             | 89.1–100.0 | 95.1            | 84.8–100.0 | 95.5              | 89.4–100.0 |
| IV, high DNA | 28             | 81.6             | 69.3–93.9  | 83.9            | 71.9–95.9  | 81.8              | 69.6–94.0  |
| III + IV, low DNA | 39             | 95.4             | 79.5–100.0 | 95.1            | 84.8–100.0 | 95.5              | 89.4–100.0 |
| III + IV, high DNA | 46             | 95.4             | 89.1–100.0 | 95.1            | 84.8–100.0 | 95.5              | 89.4–100.0 |

Low DNA denotes EBV DNA levels of ≤7500 copies/mL, high DNA denotes EBV DNA levels of >7500 copies/mL.

CI = confidence index, DMFS = distant metastasis-free survival, DNA = deoxyribonucleic acid OS = overall survival, pEBV = plasma Epstein–Barr virus, PFS = progression free survival.
Intensifying treatment in pediatric patients with high pEBV DNA levels.

Similar studies have been performed in untreated NPC patients who show no evidence of distant metastasis. Pretreatment pEBV DNA cutoff values have been defined differently in previous studies. Chan et al. used a pretreatment cutoff value of 4000 copies/mL; however, Lin et al. used a value of 1500 copies/mL. Patients in these studies, however, were not specifically young patients. Given that the patients in our study were children and adolescents with locoregionally advanced disease and a higher level of pEBV DNA concentration, previous cutoff values might not be applicable to our studies. Therefore, we used receiver operating characteristic to determine the optimal cutoff value and to determine the best trade-off between sensitivity and specificity. Taken together, our results and those of previous studies show that pretreatment levels of pEBV DNA can be a useful biomarker for predicting outcomes in NPC patients, not only in adults but also in children and adolescents.

There are some limitations to the current study, such as the relatively small sample size and short follow-up time (median follow-up time was 44.9 months). Second, measurements were recorded in a single center. Hence, a larger-scale and multicenter cohort study is warranted.

CONCLUSIONS

In summary, we have demonstrated that the pretreatment level of pEBV DNA is a promising prognostic biomarker that is associated with progression-free, distant metastasis-free, and overall survival in pediatric patients. Pediatric patients who have a poor prognosis can be identified by measuring levels of pretreatment circulating pEBV DNA, which may indicate that they should receive intensified treatment. A prospective study to evaluate the prognostic value of pEBV DNA is needed.

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