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ORIGINAL ARTICLE

Epstein-Barr virus DNA loads in the peripheral blood cells predict the survival of locoregionally-advanced nasopharyngeal carcinoma patients

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ABSTRACT

Objective: Circulating cell-free Epstein-Barr virus (EBV) DNA has been shown to be a valuable biomarker for population screening and prognostic surveillance for nasopharyngeal carcinoma (NPC). Despite important insights into the biology of persistence, few studies have addressed the clinical significance of cell-based EBV-DNA loads in peripheral blood cells (PBCs).

Methods: A prospective observational cohort study was conducted involving 1,063 newly diagnosed, locoregionally-advanced NPC patients at Sun Yat-sen University Cancer Center from 2005 to 2007. Cox regression analysis was conducted to identify the association of PBC EBV DNA loads to overall survival (OS) and other prognostic outcomes. Prognostic nomograms were developed based on PBC EBV DNA loads to predict survival outcomes for NPC patients.

Results: After a median follow-up of 108 months, patients with higher PBC EBV-DNA loads had significantly worse OS [hazard ratio (HR) of medium, medium-high, and high *vs*. low were 1.50, 1.52, and 1.85 respectively; $P_{trend} < 0.001$]. Similar results were found for progression-free survival and distant metastasis-free survival. The concordance index of the prognostic nomogram for predicting OS in the training set and validation set were 0.70 and 0.66, respectively. Our data showed that the PBC EBV DNA load was an independent and robust survival biomarker, which remained significant even after adjusting for plasma EBV DNA loads in a subset of 205 patients of the cohort (HR: 1.88; $P = 0.025$). Importantly, a combination of PBC EBV DNA load and plasma EBV DNA load improved the predicted OS.

Conclusions: The EBV-DNA load in PBCs may be an independent prognosis marker for NPC patients.

KEYWORDS Nasopharyngeal carcinoma; Epstein-Barr virus DNA; prognosis; peripheral blood cells; nomogram

Introduction

Epstein-Barr virus (EBV) infects more than 95% of adults worldwide and is associated with a diverse range of tumors of both lymphoid and epithelial origins, such as Burkitt lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma (NPC)[1]. Among these diseases, NPC is characterized by its distinct geographical distribution and is particularly prevalent in southern China, the Arctic, and the middle/northern regions of Africa[2]. The incidence of NPC in southern China is approximately 25–50/100,000 person-years, which is a 20–50-fold increase when compared with Western countries[3-6]. Studies have reported that NPC patients with the same clinical stage receiving similar treatment strategies exhibited distinct clinical outcomes[7], and that treatment failures were due to high percentages of recurrences and distant metastases[8]. An accurate prognosis in the early treatment stage is therefore necessary to guide precise treatment and follow-up monitoring to reduce the disease burden.

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Extensive efforts have been made over the past few decades to discover novel NPC biomarkers for use in clinical practice. Recently, the quantitative assessment of EBV infection has emerged as a promising tool for epidemiological screening, clinical diagnosis, and surveillance of recurrence. The virus is able to infect both B cells and epithelial cells, and shuttle between these 2 cells, facilitating its persistence and transmission in humans[9-11]. Samples from different blood compartments [e.g., serum/plasma, peripheral blood mononuclear cells (PBMCs), and whole blood] are therefore often used to monitor EBV infection in clinical or epidemiological studies of NPC[12,13]. The measurement of viral DNA from different blood compartments, however, yields very different information in certain situations due to the complexity of EBV kinetics, which affects the distribution, persistence, and interchange of EBV among plasma and PBMCs[14]. For example, the plasma cell-free EBV DNA (cfEBV DNA), which is mainly derived from tumor cells, appears to correlate closely with the presence of residual tumors. Thus, measuring plasma cfEBV DNA provides an almost real-time readout of the tumor burden, and is useful for monitoring the recurrence, prognostication, treatment response prediction, and disease surveillance of NPC[8,15-22]. However, the EBV DNA in PBMCs, typically harbored within latently infected B lymphocytes[9,23,24], does not appear promising for either tumor diagnoses or therapeutic effect evaluations in NPC clinical practice due to its lower detection[25]. Nevertheless, measuring EBV copy number in PBMCs has provided important insights into the biology of persistence and the role of the resting memory B cells. It might serve as a general marker of immune function, which may have prognostic significance among patients regardless of tumor EBV status[26]. Indeed, a previous study reported that 4 of 9 patients with detectable EBV DNA in peripheral blood cells (PBCs) had tumor relapses, whereas none of the 4 patients without detectable EBV DNA in PBCs developed a tumor relapse, suggesting that EBV DNA in PBCs could be a prognostic biomarker for NPC[27].

However, few studies have reported the clinical application of EBV DNA loads in PBCs, particularly its prognostic value in NPC, so we conducted the first prospective observational cohort study with a relatively large sample size and longer follow-up. In this study, we aimed to determine whether EBV DNA loads in PBCs could be used as a prognostic marker. If so, we aimed to determine if it was correlated with current prognostic markers such as plasma EBV DNA loads, and if the combination of EBV DNA loads in both plasma and PBCs could improve the predictive value for prognoses.

Materials and methods

Study population

The present hospital-based cohort study was conducted on the basis of the EPI-NPC-2005 project during October 2005 and October 2007, which was designed to assess the interaction among EBV, environmental factors, and genetic determinants in the pathogenesis and progression of NPC. Briefly, NPC patients were recruited from the Sun Yat-sen University Cancer Center (SYSUCC; Guangzhou, China), according to the following criteria: 1) newly-diagnosed NPC patients without other malignancy histories; 2) pathologically confirmed as World Health Organization type II and III NPC patients; 3) under 80 years of age; and 4) residing in the Guangdong Province for at least 5 years. This study further included patients from the EPI-NPC-2005 according to the following criteria: 1) diagnosed as locoregionally-advanced NPC patients with $T_xN_{2-3}M_0$ or $T_{2-4}N_{0-3}M_0$ (6th AJCC/UICC TNM staging systems); 2) had been treated with radiotherapy; and 3) having blood samples collected prior to radiotherapy or within 2 weeks after the start of radiotherapy. Finally, a total of 1,063 eligible patients were included. The flow chart of patient inclusion is shown in **Supplementary Figure S1**. Written informed consent was obtained from each participant at enrollment. The study was approved by the Ethics Committee of SYSUCC (YB2005001).

Data collection

Personal information was collected using a structured questionnaire through face-to-face interviews by trained staff. The collected information mainly included demographics (age, gender, and levels of education), lifestyle habits (smoking behavior, alcohol drinking, etc.) and disease history (hypertension, diabetes, and heart disease). Medical information included clinical stage, T and N stage, radiotherapy technology [2-dimensional radiotherapy (2D-RT), 3-dimensional conformal radiotherapy (3D-CRT), and intensity modulated radiation therapy (IMRT)], chemotherapy (induced chemotherapy, concurrent chemotherapy, and adjuvant chemotherapy), and other information, which was retrieved from the hospital information system of SYSUCC. The raw data for this

study have been uploaded to the Research Data Deposit with a number of RDDA2020001667.

Blood processing and DNA extraction

Peripheral blood (4 mL) was collected from each patient in an EDTA tube. The whole blood was centrifuged at 3,500 × *g* for 10 min, and the supernatant was removed. Cell pellets were transferred into 1.5 mL centrifuge tubes and centrifuged at 850 × *g* for 5 min, and then washed 3 times with phosphate-buffered saline for PBC DNA extractions. The DNA of PBCs was extracted using a QIAamp DNA Blood MiniKit (Qiagen, Hilden, Germany). The final volume was 50 μL.

The real-time quantitative polymerase chain reaction

The EBV DNA loads were measured using a real-time quantitative PCR assay targeting the *BamHI-W* region of the EBV genome. The detailed method has been reported in our previous study[28]. The *BamHI-W* system consisted of the amplification primers W-44F: (5′-CCC AAC ACT CCA CCA CACC-3′) and W-119R: (5′-TCT TAG GAG CTG TCC GAG GG-3′) and the dual-labeled fluorescent probe W-67T: [5′-(FAM) CAC ACA CTA CAC ACA CCC ACC CGT CTC (TAMRA)-3′]. Real-time quantitative PCR for the β-globin gene consisted of the amplification primers β-globin-354F: (5′-GTG CAC CTG ACT CCT GAG GAG-3′) and β-globin-455R: (5′-CCT TGA TAC CAA CCT GCC CAG-3′) and the dual-labeled fluorescent probe β-globin-402T: [5′-(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)-3′][29]. The fluorescent probes and PCR primers were custom-synthesized by Life Technologies (Carlsbad, CA, USA). Standard samples that contained the *BamHI-W* gene or β-globin gene were constructed from the pMD19-T simple vector (TaKaRa, Shiga, Japan), termed pMD19-T-BamHI-W and pMD19-T-β-globin, respectively. The standard curves were generated with serially diluted standard samples at 10^3,10^4, 10^5, 10^6, and 10^7 copies per 2 μL.

Each PCR reaction was conducted in a total volume of 8 μL, which contained 2× MasterMix (Roche, Basel, Switzerland), 4 μL of 100 nM of each of the amplification primers, 100 nM of the corresponding fluorescent probe, and 2 μL of DNA templates. Duplicate amplification reactions were performed in 384-well microplates using a Roche LightCycler 480 amplifier. Standard curves were run in parallel and in duplicate with each assay. Thermal cycling was initiated at 10 min at 95 °C for the first denaturation, then followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 45 s. The β-globin gene quantitative PCR results were used to normalize the quantity of EBV DNA. The EBV DNA load in PBCs was expressed as 10^6-fold of the ratio of EBV DNA copies to the β-globin copies in the same tested samples[29,30]. Multiple blanks were used as negative controls. The mean quantity of each duplicate was used for further calculations for the concentrations of EBV DNA. The equation to calculate EBV copies per β-globin was:

$$\text{PBC EBV copies}/10^6 \text{ globin} = (\text{EBV mean quantity}/\beta\text{-globin mean quantity}) \times 10^6.$$

Follow-up and outcomes

Patients were followed-up mainly by telephone interviews, retrieving the HIS medical records, and accessing death registration data from the public security bureau. The follow-up period ended in September 2019. The main endpoints of this study were death, disease progression, distant metastasis, and recurrence. Distant metastasis and recurrence were defined as the appearance of a newly detected local/regional recurrence or distant metastasis, and were confirmed by nasopharyngeal biopsy or 2 kinds of imaging diagnoses. Overall survival (OS) was determined as the date of diagnosis to the date of death or to the date of the last follow-up visit. Progression-free survival (PFS) was defined as the time from the date of diagnosis to the first day of local recurrence or distant metastasis or to the date of the last follow-up visit. Distant metastasis-free survival (DMFS) was calculated from the date of diagnosis to the date of distant metastasis or the date of the last follow-up visit. Recurrence-free survival (RFS) was calculated from the date of diagnosis to the date of local and/or regional recurrence or the date of the last follow-up visit.

Statistical analysis

Life table estimation was performed using the method of Kaplan-Meier. Univariate comparison of survival curves was performed using the log-rank test. Univariate Cox regression was performed to screen for significant variables. The multivariate Cox proportional hazards model was used to estimate hazard ratios and 95% confidence intervals. Adjusted variables in the model included age, gender, smoking status, clinical

stage, radiotherapy technology, chemotherapy, and other factors that were significant in univariate Cox regression. To properly display the dose-response relationship between EBV DNA loads in PBCs and the prognoses of NPC patients, we defined cut-off values based on the quartiles of patients with detectable PBC EBV DNA. We classified these patients with 0 copies/10^6 globin and the first quartile of PBCs EBV DNA as the low group (≤ 392 copies/10^6 globin), second quartile as the medium group (> 392 copies/10^6 globin and ≤ 581 copies/10^6 globin), third quartile as the medium-high group (> 581 copies/10^6 globin and ≤ 918 copies/10^6 globin), and the top quartile as the high group (> 918 copies/10^6 globin). Using subgroup analysis by clinical stage, the patients were categorized into low or high groups (≤ 392 copies/10^6 globin and > 392 copies/10^6 globin, respectively) because of the limited sample size in each subgroup. All statistical tests were 2-sided, and $P < 0.05$ was considered statistically significant. Analyses were performed using R 3.6.1 software (The R Project for Statistical Computing, Vienna, Austria).

The nomograms were developed to predict the status of OS and PFS based on the results of univariate Cox regression analyses. Two-thirds of 1,063 patients were randomly assigned to the training set and one-third to the validation set. In addition, the 2 markers of lactate dehydrogenase (LDH) and neutrophil to lymphocyte ratio (NLR) that were associated with prognosis of NPC patients in previous publications were also included in the nomogram[31,32]. The performance of the nomogram was evaluated by the concordance index (C-index) in the training and validation sets. A calibration curve of the nomogram was used to estimate agreement between the predictions and observations regarding the probabilities of 3-, 5-, and 10-year survivals.

To study the relationship between plasma EBV DNA and PBC EBV DNA loads, 205 of 1,063 samples with available pretreatment plasma EBV DNA loads were retrieved from the HIS of SYSUCC. The correlations were tested using Spearman's rank correlation. We classified plasma EBV DNA and PBC EBV DNA loads into the high and low categories, respectively. The optimal threshold of plasma EBV DNA loads was 128,000 copies/mL based on receiver operating characteristic curve analyses and the cut-off value of PBC EBV DNA loads was consistent with that of the subgroup analyses (392 copies/10^6 globin). We further combined the 2 markers and classified the population into a low (both markers were low), medium (1 of the 2 markers was low), and high (both markers were high) groups. The C-index was calculated to evaluate the prognostic effect of the 2 markers, alone and in combination.

Results

Patient characteristics and follow-up

The baseline characteristics of the 1,063 locoregionally-advanced NPC patients are summarized in **Supplementary Table S1**. The median age of the population was 45 years [interquartile range (IQR): 38–54 years], with a male-to-female ratio of 2.7 (775 males and 288 females). Based on the 6th edition of AJCC/UICC staging system, 178 patients (16.7%) were in stage II, 601 patients (56.5%) in stage III, and 284 patients (26.7%) in stage IV. The detection percentage of the PBC EBV DNA loads was 38.9% (413/1,063) in all patients. The median PBC EBV DNA loads were 0 copies/10^6 globin (IQR = 0–0), 469 copies/10^6 globin (IQR = 425–521), 714 copies/10^6 globin (IQR = 641–793), and 1,428 copies/10^6 globin (IQR = 1,103–2,342) in the low (752 patients), medium (104 patients), medium-high (103 patients), and high (104 patients) groups, respectively (**Supplementary Figure S2**).

The median follow-up time was 107.93 months (range: 2.47–163.53 months), and 431 (40.5%) patients died, 466 (43.8%) experienced disease progression, 196 (18.4%) developed distant metastasis, and 137 (12.9%) developed local and/or regional recurrences during the follow-up period.

Survival status and factors associated with the OS, PFS, DMFS, and RFS of NPC patients

The overall survival rates of NPC patients at 3-, 5-, and 10-years were 89%, 79%, and 61%, respectively. The PFS rates at 3-, 5-, and 10-years were 80%, 72%, and 58%, respectively. The DMFS rates at 3-, 5-, and 10-years were 87%, 83%, and 80%, respectively, and the RFS rates at 3-, 5-, and 10-years were 93%, 90%, and 85%, respectively.

Univariate analyses indicated that age, gender, education levels, smoking status, clinical stage, T stage, N stage, radiotherapy technology (2D-RT, 3D-CRT, and IMRT), induced chemotherapy, and concurrent chemotherapy were significantly associated with the OS and PFS. Of these parameters, age, smoking status, clinical stage, T stage, N stage, and induced chemotherapy were risk factors, while higher education levels, female, using radiotherapy technology of intensity-modulated radiation therapy, and concurrent chemotherapy

were protective factors. In addition, gender, smoking status, clinical stage, and concurrent chemotherapy were correlated with both the DMFS and RFS. The T stage and N stage were also correlated with the DMFS (**Supplementary Table S2**).

Multivariate analyses showed that age, gender, clinical stage, and concurrent chemotherapy were associated with the OS and PFS. High levels of education were a protective factor for OS. Gender, clinical stage, and concurrent chemotherapy were independent factors for the DMFS. Only clinical stage and concurrent chemotherapy were significant for RFS when using multivariate analysis (**Supplementary Table S3**).

The prognostic value of EBV DNA loads in PBCs

We found positive associations between PBC EBV-DNA loads and clinical stage characteristics in NPC patients. A significant association was found between high levels of PBC EBV-DNA and overall clinical stage progressing [III *vs.* II: odds ratio (OR): 1.71, 95% confidence interval (CI): 1.05–2.79; P = 0.032; IV *vs.* II: OR: 2.45; 95% CI: 1.41–4.24; $P < 0.001$; $P_{trend} < 0.001$). Patients with a body mass index (BMI) more than 24 had significantly lower PBC EBV DNA loads than those with a normal BMI (OR: 0.70; 95% CI: 0.50–0.97; $P = 0.033$). The results are summarized in **Supplementary Table S4**.

The overall survival at 5 years of patients in the low, medium, medium-high, and high groups of EBV DNA loads were 83.9%, 73.4%, 67.9%, and 61.1%, respectively, which showed a gradually decreased EBV DNA load with a stepwise rise. Similar results at 5 years were also observed for the PFS (76.7%, 67.6%, 60.2%, and 53.3% in the low, medium, medium-high, and high groups, respectively), DMFS (85.7%, 81.3%, 74.5%, and 70.6% for the low, medium, medium-high, and high groups, respectively) and RFS (90.7%, 90.8%, 84.1%, and 86.3% for the low, medium, medium-high, and high groups, respectively). The survival curves are showed in **Figure 1**.

Multivariate Cox regression analyses identified EBV DNA loads in PBCs as an independent prognostic factor for the OS. Compared with the low group of EBV DNA loads in PBCs, the HRs in the medium, medium-high, and high EBV DNA loads were 1.50 (95% CI: 1.10–2.05; $P = 0.010$), 1.52 (95% CI: 1.12–2.07; $P = 0.007$), and 1.85 (95% CI: 1.40–2.46; $P < 0.001$), respectively, $P_{trend} < 0.001$. Similar positive associations were also found for the PFS [medium, medium-high, high *vs.* low: HR: 1.50 ($P = 0.007$); 1.46 ($P = 0.013$); 1.85 ($P < 0.001$), respectively, $P_{trend} < 0.001$] and the DMFS [medium, medium-high, high *vs.* low: HR: 1.31 ($P = 0.272$); 1.76 ($P = 0.009$); 2.37 ($P < 0.001$), respectively; $P_{trend} < 0.001$]. Only the high PBC EBV DNA group had a significantly worse RFS relative to the low PBC EBV DNA groups (HR: 1.70; $P = 0.047$), but there was a significantly linear trend with a greater probability of recurrence with increasing EBV DNA loads in PBCs ($P_{trend} = 0.023$) (**Table 1 and Supplementary Table S3**).

To further investigate the prognostic value of EBV DNA loads in PBCs, we performed subgroup analyses based on clinical stages. Multivariate Cox regression analyses in the subgroup of clinical stage II indicated that PBC EBV DNA was significantly correlated with worse outcomes of the OS (HR: 2.72; $P = 0.008$), PFS (HR: 3.38; $P < 0.001$), DMFS (HR: 4.96; $P = 0.003$), and RFS (HR: 3.31; $P = 0.039$). The PBC EBV DNA was significantly associated with the OS in clinical stage subgroup III ($P = 0.025$). In the clinical stage IV group, which had the worse prognosis, patients with a higher EBV DNA load in the PBCs had a worse OS (HR: 1.92; $P < 0.001$), PFS (HR: 1.93; $P < 0.001$), DMFS (HR: 2.07; $P = 0.002$), and RFS (HR: 2.15; $P = 0.015$) (**Table 2, Supplementary Figure S3, and Supplementary Table S5–S7**).

We conducted subgroup analyses using different treatment stages to further determine whether the PBC EBV DNA loads were correlated with NPC survival in different treatment stages. In our study, 630 patients (59.3%) were recruited before any treatment, 247 (23.2%) were recruited during induced chemotherapy, and 186 (17.5%) were recruited within 2 weeks after the start of radiotherapy. We conducted multivariate Cox regression analyses according to different treatment stage subgroups and found that PBC EBV DNA loads were significantly associated with the OS and PFS within each subgroup of the treatment stages (**Supplementary Figure S4**).

Nomogram development using the EBV DNA loads in PBCs

To investigate the clinical prognostic value of EBV DNA loads in PBCs, we constructed the prognostic nomograms using the training set to predict the 3-, 5-, and 10-year OS using the variables of EBV DNA loads in PBCs, age, gender, education level, T stage, N stage, concurrent chemotherapy, BMI, smoking status, LDH, and NLR (**Figure 2A**). The calibration plot for the probability of 3-, 5-, and 10-year OS revealed favorable agreement between predictions by the nomogram and actual observations in both the training and validation

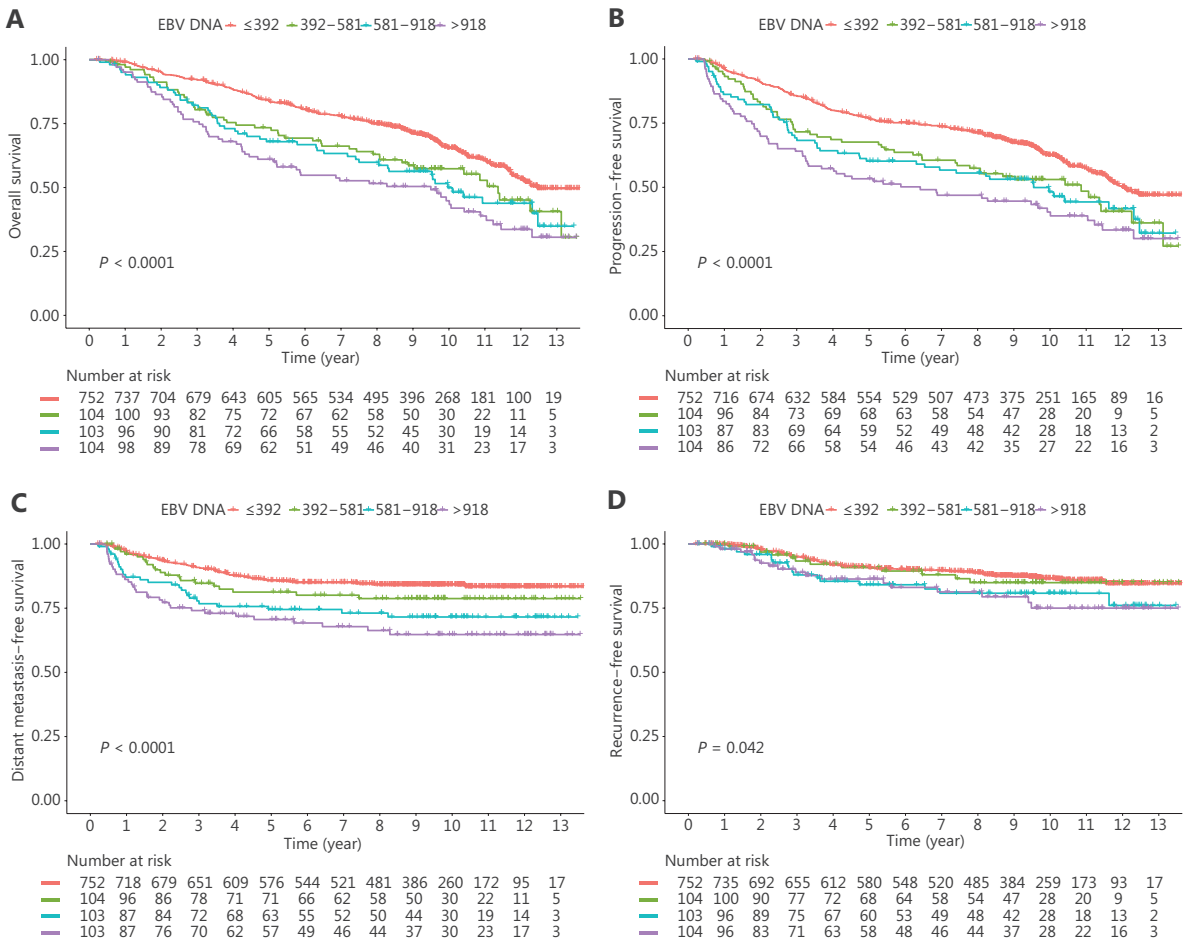

Figure 1 Kaplan-Meier survival curves of Epstein-Barr virus DNA in peripheral blood cells for (A) overall survival, (B) progression-free survival, (C) distant metastasis-free survival, and (D) recurrence-free survival in all patients.

sets (**Figure 2B**). The C-indices for the OS in the training and validation sets were 0.70 (95% CI: 0.66–0.73) and 0.66 (95% CI: 0.61–0.71), respectively, indicating that the nomogram with PBC EBV DNA loads had good prognostic stratification. Moreover, patients in the training set were categorized into 3 risk groups at the 25th and 75th percentiles of the total score distribution of the training set: low risk group (total score: 0–175), medium risk group (total score: 176–290), and high risk group (total score ≥ 291), and each group showed a distinct prognosis (**Supplementary Table S8**). In the validation set, the 3-, 5-, and 10-year OS rates were 94%, 90%, and 77%, respectively, in the low risk group, 89%, 80%, and 68% in the medium risk group, respectively, and 76%, 55%, and 31% in the high risk group, respectively. **Figure 2C** shows the Kaplan-Meier survival curves of different risk groups for the OS in the training and validation sets. In addition, similar results for the PFS were also found (**Supplementary Table S8 and Supplementary Figure S5**).

The relationship between plasma EBV DNA and PBC EBV DNA loads

To determine whether PBC EBV DNA loads were independent of plasma EBV DNA loads, a correlation analysis was conducted of 205 patients who had available data on both of the 2 markers in our cohort. Notably, we observed a weak correlation between plasma EBV DNA and PBC EBV DNA loads (**Figure 3A**; R^2: 0.32; $P < 0.001$). Multivariate Cox regression including the 2 EBV DNA loads and other adjusting factors showed PBC EBV DNA loads were independent of plasma EBV DNA loads for the OS (HR: 1.88; $P = 0.025$; **Supplementary Table S9**). We further compared the predictive effects of the 2

Table 1 Multivariate Cox regression analysis of peripheral blood cell Epstein-Barr virus DNA loads in all patients

Characteristics	Overall survival[a]			Progression-free survival[a]			Distant metastasis-free survival[b]			Recurrence-free survival[b]		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
≤ 392 copies/10^6 globin	269/752	Reference	–	294/752	Reference	–	115/752	Reference	–	90/752	Reference	–
392–581 copies/10^6 globin	49/104	1.50 (1.10–2.05)	0.010	54/104	1.50 (1.12–2.01)	0.007	20/104	1.31 (0.81–2.14)	0.272	12/104	1.05 (0.56–1.96)	0.889
581–918 copies/10^6 globin	51/103	1.52 (1.12–2.07)	0.007	54/103	1.46 (1.08–1.96)	0.013	27/103	1.76 (1.15–2.69)	0.009	17/103	1.61 (0.95–2.72)	0.077
> 918 copies/10^6 globin	62/104	1.85 (1.40–2.46)	< 0.001	64/104	1.85 (1.41–2.44)	< 0.001	34/104	2.37 (1.61–3.51)	< 0.001	18/104	1.70 (1.01–2.87)	0.047
P_{trend}			< 0.001			< 0.001			< 0.001			0.023

n: the number of events; *N*: the total number of patients in each group. a: Adjusted for age, gender, smoking status, T stage, N stage, radiotherapy technology, chemotherapy (induced chemotherapy, concurrent chemotherapy, and adjuvant chemotherapy), and education levels. b: Adjusted for age, gender, smoking status, T stage, N stage, radiotherapy technology, and chemotherapy (induced chemotherapy, concurrent chemotherapy, and adjuvant chemotherapy).

markers alone and in combination. The C-index of PBC EBV DNA loads, plasma EBV DNA loads, and combination of the 2 markers were 0.56, 0.59, and 0.61, respectively, indicating that the addition of PBC EBV DNA loads improved the predictive accuracy of plasma EBV DNA loads. Kaplan-Meier curve analyses indicated that the combination of 2 markers further identified patients with worse prognoses than either of the 2 markers alone (**Figure 3B–3D**).

Discussion

In the present study, we observed a quantitative correlation between PBC EBV DNA loads and the prognoses of locoregionally-advanced NPC patients. Importantly, nomograms with PBC EBV DNA loads and other relative factors showed an improved accuracy in the prediction for OS and PFS, which could be utilized as clinical tools to further distinguish subgroups of NPC patients with different prognoses.

Previous studies have reported that high levels of EBV DNA in PBMCs were associated with increased risks of developing EBV-associated diseases, such as AIDS-related systemic B lymphoma and post-transplant lymphoproliferative disease[33-35]. Lin et al.[36] detected the presence of EBV DNA in PBCs by nested PCR among 124 NPC patients, and qualitatively showed that EBV DNA positive patients had an inferior OS and DMFS, when compared with patients with a negative EBV DNA in their PBCs. In the present study, we used a more sensitive method to detect EBV DNA loads in PBCs, to establish a quantitative relationship between EBV DNA loads and the survival outcomes of NPC patients, using our NPC cohort with larger sample sizes and a longer follow-up. The survival data of patients (OS, PFS, DMFS, and RFS) were significantly decreased with increased EBV DNA loads in their PBCs, and these associations remained consistent in each subgroup of patients with clinical stages II, III, and IV. Moreover, our nomogram with PBC EBV DNA loads and other risk factors showed good predictive performance in identifying subgroups of patients with poor survivals. Using our scoring system, physicians could predict individual survival, which would improve treatment and health care. The impact of EBV-infected PBCs on the prognoses of NPC patients may be related to the ability of the virus to counteract and evade host immunity by modulating cellular signaling pathways, blocking antiviral cytokines, impairing the antigen-presenting HLA I or the HLA II pathway, and switching-off immunodominant viral antigen expression as well as regulation of immune-inhibitory biomolecules[37]. The detailed molecular mechanisms underlying

Table 2 Multivariate Cox regression analysis of peripheral blood cell Epstein-Barr virus DNA loads in subgroups of clinical stages

Characteristics	Overall survival[a]			Progression-free survival[a]			Distant metastasis-free survival[b]			Recurrence-free survival[b]		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Subgroup of clinical stage II												
≤ 392 copies/10^6 globin	27/148	Reference	–	29/148	Reference	–	9/148	Reference	–	11/148	Reference	–
> 392 copies/10^6 globin	11/30	2.72 (1.29–5.73)	0.008	14/30	3.38 (1.72–6.65)	< 0.001	7/30	4.96 (1.71–14.34)	0.003	5/30	3.31 (1.06–10.31)	0.039
Subgroup of Clinical stage III												
≤ 392 copies/10^6 globin	153/428	Reference	–	169/428	Reference	–	68/428	Reference	–	56/428	Reference	–
> 392 copies/10^6 globin	76/173	1.38 (1.04–1.82)	0.025	80/173	1.27 (0.97–1.67)	0.081	36/173	1.42 (0.94–2.14)	0.093	20/173	0.97 (0.58–1.63)	0.919
Subgroup of Clinical stage IV												
≤ 392 copies/10^6 globin	89/176	Reference	–	96/176	Reference	–	38/176	Reference	–	23/176	Reference	–
> 392 copies/10^6 globin	75/108	1.92 (1.40–2.63)	< 0.001	78/108	1.93 (1.41–2.63)	< 0.001	38/108	2.07 (1.30–3.29)	0.002	22/108	2.15 (1.16–3.98)	0.015

n: the number of events; *N*: the total number of patients in each group. a: Adjusted for age, gender, smoking status, T stage, N stage, radiotherapy technology, chemotherapy (induced chemotherapy, concurrent chemotherapy, and adjuvant chemotherapy), and education levels. b: Adjusted for age, gender, smoking status, T stage, N stage, radiotherapy technology, and chemotherapy (induced chemotherapy, concurrent chemotherapy, and adjuvant chemotherapy).

the role of EBV infected PBCs in NPC prognosis therefore need further investigation.

An early study using a prognostic model for NPC indicated that including plasma EBV DNA loads in the prognostic prediction significantly increased the predictive accuracy and discriminative ability[16,38]. Because plasma EBV DNA is mainly released by tumor cells, it has been useful for the diagnosis, occurrence, development, and prognoses of EBV-associated diseases, including NPC[39]. Significant progress was recently made by Chan et al.[15], who used plasma EBV DNA in screening for early NPC, with the sensitivity and specificity as high as 97.1% and 98.6%, respectively. Moreover, Lv et al.[18] successfully regrouped NPC patients into different prognostic phenotypes by monitoring cfEBV DNA longitudinally, and Kanakry et al.[40] also showed that plasma EBV DNA had prognostic utility for Hodgkin lymphoma patients.

To further explore the impact of plasma EBV DNA and PBC EBV DNA loads on the accuracy of NPC prognostic predictions, we selected 205 patients with pretreatment plasma EBV DNA loads among all patients. Our analyses showed a weak correlation between the 2 markers, showing that PBC EBV DNA loads were independent of plasma EBV DNA loads. The significant association between PBC EBV DNA loads and the prognoses of NPC patients was confirmed after adjusting the plasma EBV DNA loads and other parameters. Shao et al.[25] reported that plasma EBV DNA loads were not correlated with PBC EBV DNA loads in NPC patients before treatment, and Gandhi et al.[41] also reported that EBV DNA loads of EBV-positive Hodgkin's lymphoma patients in matched plasma/PBMC samples were not correlated. Consistent with these previous studies, we hypothesized that cell-based EBV DNA in PBCs may be slightly associated with plasma EBV DNA loads, and that PBC EBV DNA loads could be an independent prognostic factor relative to plasma EBV DNA loads.

Previous studies focused on which blood compartments for detection and quantification of EBV DNA could better reflect the diagnosis and prognosis of EBV-associated disease[26,42,43]. However, EBV DNA loads from different blood compartments may have different biological significances. For example EBV DNA loads in the plasma reflect tumor burden in NPC patients[20], and EBV DNA loads in PBMCs may be more related to body immunity in post-transplant lymphoproliferative disorders[44]. The combined use of EBV DNA from different blood compartments may be able to more comprehensively reflect the status of the disease. In our study, the combination of PBC EBV DNA and pretreatment plasma EBV DNA loads further differentiated

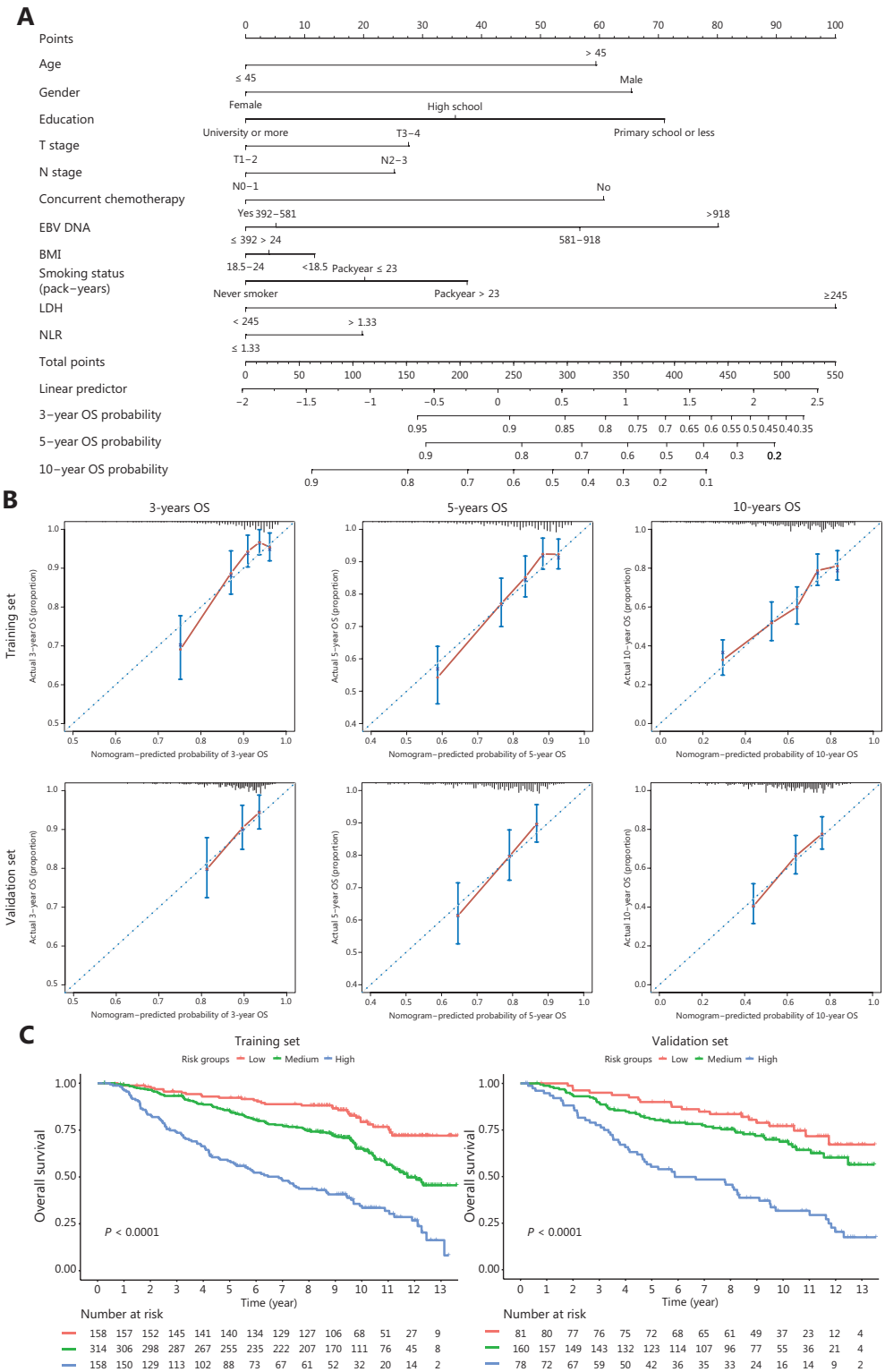

Figure 2 Nomogram (A), including age, gender, education level, T stage, N stage, concurrent chemotherapy, body mass index, smoking status, serum lactate dehydrogenase levels, and neutrophil to lymphocyte ratio for 3-, 5-, and 10-year overall survival (OS) in patients with nasopharyngeal carcinoma. The calibration curve (B) of the nomogram for predicting OS. The Kaplan-Meier curves (C) of different risk groups in the training and validation sets according to the score system for the OS.

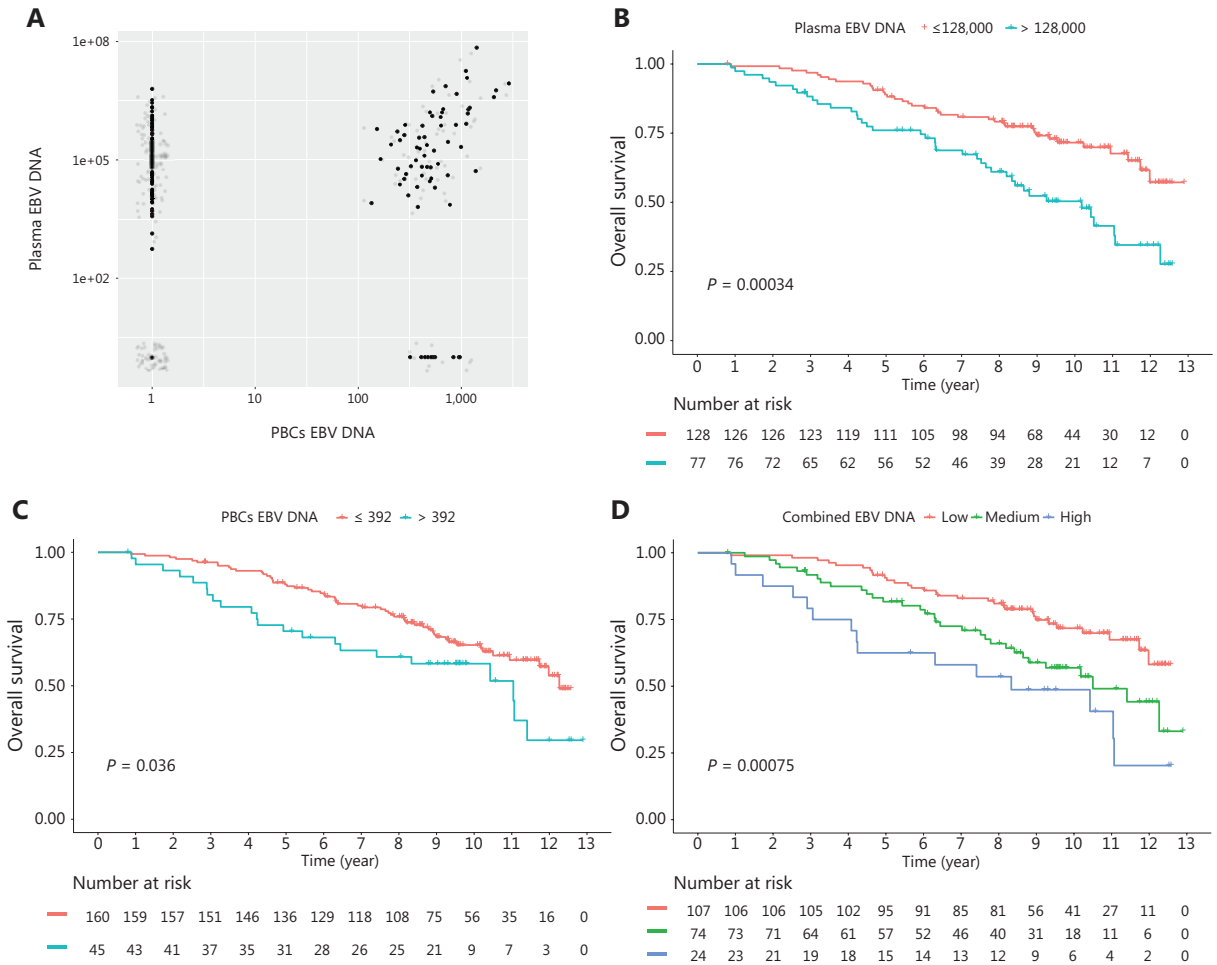

Figure 3 A scatter plot (A) indicating the correlation between plasma Epstein-Barr virus (EBV) DNA and peripheral blood cell (PBC) EBV DNA. Grep points are the jittered points. Kaplan-Meier survival curves of overall survival. (B) plasma EBV DNA alone, (C) PBC EBV DNA alone, (D) The combination of plasma EBV DNA and PBC EBV DNA.

between higher risk groups and showed better performance in NPC prediction than plasma EBV DNA loads alone.

Our study had some limitations. First, because 2-dimensional radiation therapy was the standard radiation technique during enrollment, only a small percentage of patients underwent radiotherapy using intensity-modulated radiation therapy. Second, although the relationship between PBC EBV DNA loads and prognosis was significant, based on our cohort with a large sample size and longer follow-up times, we suggest the future use of multi-center studies with replication results to better verify these associations. In addition, our available data and samples for simultaneous EBV DNA load detection for both plasma and PBCs were relatively limited, and the conclusions from these samples needs to be confirmed, using larger sample sizes and other well-designed studies.

Conclusions

In summary, our study showed that EBV DNA loads in PBCs may be an important independent prognostic marker for locoregionally-advanced NPC patients relative to plasma EBV DNA loads. The proposed nomogram with PBC EBV DNA loads in this study provided good discrimination in predicting OS and disease progression.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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Supplementary materials

Table S1 Patient demographics and clinical characteristics

Characteristics	No. of patients	%
Age (years)		
≤ 45	565	53.2
> 45	498	46.8
Gender		
Male	775	72.9
Female	288	27.1
BMI		
18.5–24.0	576	59.0
> 24.0	301	30.8
< 18.5	100	10.2
Education		
Primary school or less	244	23.0
High school	663	62.4
University or more	156	14.7
Smoking status (pack-years)		
Never smoker	493	46.6
≤ 23	288	27.2
> 23	277	26.2
Alcohol drinking		
Nondrinker	628	59.1
≤ 1 drink per day	251	23.6
> 1 drink per day	184	17.3
Clinical stage		
II	178	16.7
III	601	56.5
IV	284	26.7
T stage		
T_{1-2}	297	27.9
T_{3-4}	766	72.1
N stage		
N_{0-1}	670	63.0
N_{2-3}	393	37.0
Radiotherapy technology		
2D-RT	869	82.3
3D-CRT	14	1.3
IMRT	173	16.4
Induced chemotherapy		
No	548	51.6
Yes	515	48.4
Concurrent chemotherapy		
No	621	58.4
Yes	442	41.6
Adjuvant chemotherapy		
No	1,017	95.7
Yes	46	4.3
Groups by PBC EBV DNA loads (copies/10^6 globin)		
0	650	61.1
(0–392]	102	9.6
(392–581]	104	9.8
(581–918]	103	9.7
> 918	104	9.8
History of hypertension		
No	994	93.5
Yes	69	6.5
History of diabetes		
No	1,031	97.0
Yes	32	3.0
History of heart disease		
No	1,055	99.2
Yes	8	0.8
Overall survival status		
No	632	59.5
Yes	431	40.5
Progress-free survival status		
No	597	56.2
Yes	466	43.8

Table S1 Continued

Characteristics	No. of patients	%
Distant metastasis-free survival status		
No	867	81.6
Yes	196	18.4
Recurrence-free survival status		
No	926	87.1
Yes	137	12.9

Table S2 Univariate Cox regression analysis of all patients

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Age (years)												
≤ 45	176/565	Reference	–	201/565	Reference	–	95/565	Reference	–	72/565	Reference	–
> 45	255/498	1.86 (1.53–2.25)	< 0.001	265/498	1.64 (1.36–1.97)	< 0.001	101/498	1.26 (0.95–1.67)	0.108	65/498	1.09 (0.78–1.53)	0.612
Gender												
Male	347/775	Reference	–	371/775	Reference	–	164/775	Reference	–	109/775	Reference	–
Female	84/288	0.56 (0.44–0.71)	< 0.001	95/288	0.59 (0.47–0.74)	< 0.001	32/288	0.49 (0.33–0.71)	< 0.001	28/288	0.61 (0.40–0.93)	0.021
BMI												
18.5–24.0	235/576	Reference	–	258/576	Reference	–	111/576	Reference	–	73/576	Reference	–
> 24.0	116/301	0.88 (0.70–1.10)	0.259	122/301	0.84 (0.68–1.05)	0.120	48/301	0.79 (0.56–1.11)	0.174	38/301	0.94 (0.64–1.39)	0.759
< 18.5	39/100	0.90 (0.64–1.26)	0.545	43/100	0.90 (0.65–1.24)	0.503	18/100	0.92 (0.56–1.51)	0.742	12/100	0.91 (0.49–1.68)	0.762
Education												
Primary school or less	119/244	Reference	–	125/244	Reference	–	48/244	Reference	–	31/244	Reference	–
High school	268/663	0.77 (0.62–0.95)	0.017	288/663	0.80 (0.65–0.99)	0.041	127/663	0.96 (0.69–1.34)	0.819	85/663	0.95 (0.63–1.44)	0.812
University or more	44/156	0.47 (0.34–0.67)	< 0.001	53/156	0.56 (0.40–0.77)	< 0.001	21/156	0.64 (0.38–1.07)	0.086	21/156	0.92 (0.53–1.60)	0.765
Smoking status (pack-years)												
Never smoker	157/493	Reference	–	176/493	Reference	–	67/493	Reference	–	51/493	Reference	–
≤ 23	122/288	1.48 (1.17–1.88)	0.001	132/288	1.44 (1.15–1.81)	0.001	62/288	1.66 (1.18–2.35)	0.004	41/288	1.51 (1.00–2.28)	0.049
> 23	149/277	2.16 (1.73–2.71)	< 0.001	155/277	2.00 (1.61–2.48)	< 0.001	67/277	1.99 (1.42–2.80)	< 0.001	44/277	1.85 (1.23–2.77)	0.003
Alcohol drinking												
Nondrinker	236/628	Reference	–	257/628	Reference	–	109/628	Reference	–	74/628	Reference	–
≤ 1 drink per day	110/251	1.21 (0.97–1.52)	0.099	119/251	1.21 (0.97–1.50)	0.092	53/251	1.28 (0.92–1.78)	0.139	37/251	1.30 (0.88–1.93)	0.192
> 1 drink per day	85/184	1.26 (0.98–1.61)	0.070	90/184	1.22 (0.96–1.55)	0.109	34/184	1.09 (0.74–1.60)	0.666	26/184	1.22 (0.78–1.91)	0.383
Clinical stage												
II	38/178	Reference	–	43/178	Reference	–	16/178	Reference	–	16/178	Reference	–
III	229/601	1.96 (1.39–2.76)	< 0.001	249/601	1.92 (1.39–2.66)	< 0.001	104/601	2.07 (1.22–3.50)	0.007	76/601	1.53 (0.89–2.63)	0.121
IV	164/284	3.63 (2.55–5.17)	< 0.001	174/284	3.46 (2.48–4.84)	< 0.001	76/284	3.47 (2.02–5.95)	< 0.001	45/284	2.21 (1.25–3.90)	0.007

Table S2 Continued

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
T stage												
T_{1-2}	91/297	Reference	–	98/297	Reference	–	38/297	Reference	–	30/297	Reference	–
T_{3-4}	340/766	1.55 (1.23–1.95)	< 0.001	368/766	1.59 (1.27–1.98)	< 0.001	158/766	1.73 (1.21–2.47)	0.003	107/766	1.47 (0.98–2.20)	0.064
N stage												
N_{0-1}	242/670	Reference	–	269/670	Reference	–	105/670	Reference	–	87/670	Reference	–
N_{2-3}	189/393	1.47 (1.21–1.78)	< 0.001	197/393	1.40 (1.16–1.68)	< 0.001	91/393	1.63 (1.23–2.17)	0.001	50/393	1.11 (0.78–1.57)	0.562
Radiotherapy technology												
2D-RT	372/869	Reference	–	396/869	Reference	–	160/869	Reference	–	114/869	Reference	–
3D-CRT	3/14	0.41 (0.13–1.28)	0.125	3/14	0.38 (0.12–1.17)	0.092	2/14	0.72 (0.18–2.91)	0.648	0/14	0 (0-Inf)	0.994
IMRT	51/173	0.58 (0.43–0.77)	< 0.001	61/173	0.67 (0.52–0.88)	0.004	31/173	0.94 (0.64–1.38)	0.757	19/173	0.76 (0.47–1.23)	0.262
Induced chemotherapy												
No	198/548	Reference	–	216/548	Reference	–	80/548	Reference	–	71/548	Reference	–
Yes	233/515	1.41 (1.17–1.71)	< 0.001	250/515	1.40 (1.16–1.67)	< 0.001	116/515	1.67 (1.26–2.23)	< 0.001	66/515	1.10 (0.78–1.53)	0.595
Concurrent chemotherapy												
No	274/621	Reference	–	293/621	Reference	–	127/621	Reference	–	89/621	Reference	–
Yes	157/442	0.70 (0.58–0.86)	< 0.001	173/442	0.74 (0.61–0.89)	0.002	69/442	0.73 (0.54–0.97)	0.032	48/442	0.69 (0.48–0.97)	0.036
Adjuvant chemotherapy												
No	409/1,017	Reference	–	443/1,017	Reference	–	185/1,017	Reference	–	133/1,017	Reference	–
Yes	22/46	1.32 (0.86–2.02)	0.209	23/46	1.26 (0.83–1.92)	0.280	11/46	1.44 (0.78–2.64)	0.244	4/46	0.70 (0.26–1.89)	0.479
EBV DNA (copies/10^6 globin)												
≤ 392	269/752	Reference	–	294/752	Reference	–	115/752	Reference	–	90/752	Reference	–
392–581	49/104	1.49 (1.10–2.02)	0.010	54/104	1.49 (1.12–1.99)	0.007	20/104	1.37 (0.85–2.20)	0.193	12/104	1.10 (0.60–2.01)	0.759
581–918	51/103	1.64 (1.22–2.21)	0.001	54/103	1.60 (1.20–2.14)	0.001	27/103	1.98 (1.30–3.01)	0.001	17/103	1.65 (0.98–2.78)	0.057
> 918	62/104	2.04 (1.55–2.69)	< 0.001	64/104	2.01 (1.53–2.63)	< 0.001	34/104	2.57 (1.75–3.79)	< 0.001	18/104	1.84 (1.11–3.06)	0.018

Table S2 Continued

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
History of hypertension												
No	397/994	Reference	–	431/994	Reference	–	188/994	Reference	–	132/994	Reference	–
Yes	34/69	1.34 (0.95–1.91)	0.100	35/69	1.20 (0.85–1.70)	0.290	8/69	0.60 (0.30–1.22)	0.158	5/69	0.55 (0.22–1.34)	0.187
History of diabetes												
No	413/1,031	Reference	–	448/1,031	Reference	–	189/1,031	Reference	–	134/1,031	Reference	–
Yes	18/32	1.56 (0.97–2.50)	0.066	18/32	1.36 (0.85–2.17)	0.205	7/32	1.22 (0.58–2.60)	0.599	3/32	0.75 (0.24–2.34)	0.616
History of heart disease												
No	428/1,055	Reference	–	463/1,055	Reference	–	195/1,055	Reference	–	136/1,055	Reference	–
Yes	3/8	0.95 (0.31–2.97)	0.934	3/8	0.84 (0.27–2.62)	0.765	1/8	0.66 (0.09–4.69)	0.675	1/8	0.99 (0.14–7.06)	0.990

n: the number of events; *N*: the total number of patients in each group.

Table S3 Multivariate Cox regression analysis of all patients

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Age												
≤ 45	176/565	Reference	–	201/565	Reference	–	95/565	Reference	–	72/565	Reference	–
> 45	255/498	1.50 (1.22–1.86)	< 0.001	265/498	1.36 (1.11–1.66)	0.003	101/498	1.11 (0.82–1.5)	0.505	65/498	0.89 (0.62–1.28)	0.532
Gender												
Male	347/775	Reference	–	371/775	Reference	–	164/775	Reference	–	109/775	Reference	–
Female	84/288	0.63 (0.45–0.87)	0.005	95/288	0.66 (0.49–0.90)	0.009	32/288	0.60 (0.37–0.97)	0.038	28/288	0.83 (0.48–1.44)	0.509
Education												
Primary school or less	119/244	Reference	–	125/244	Reference	–	48/244	–	–	31/244	–	–
High school	268/663	0.79 (0.62–1.00)	0.050	288/663	0.80 (0.64–1.01)	0.062	127/663	–	–	85/663	–	–
University or more	44/156	0.64 (0.44–0.93)	0.018	53/156	0.72 (0.51–1.02)	0.067	21/156	–	–	21/156	–	–

Table S3 Continued

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Smoking status (pack-years)												
Never smoker	157/493	Reference	–	176/493	Reference	–	67/493	Reference	–	51/493	Reference	–
≤ 23	122/288	1.06 (0.79–1.41)	0.698	132/288	1.05 (0.80–1.39)	0.706	62/288	1.14 (0.75–1.72)	0.542	41/288	1.30 (0.78–2.17)	0.312
> 23	149/277	1.25 (0.93–1.67)	0.137	155/277	1.25 (0.94–1.66)	0.119	67/277	1.26 (0.82–1.93)	0.297	44/277	1.57 (0.92–2.67)	0.097
Clinical stage												
II	38/178	Reference	–	43/178	Reference	–	16/178	Reference	–	16/178	Reference	–
III	229/601	2.10 (1.45–3.05)	< 0.001	249/601	2.03 (1.43–2.89)	< 0.001	104/601	2.18 (1.23–3.86)	0.008	76/601	1.86 (1.03–3.35)	0.040
IV	164/284	3.30 (2.20–4.96)	< 0.001	174/284	3.22 (2.19–4.72)	< 0.001	76/284	2.86 (1.54–5.30)	0.001	45/284	2.40 (1.23–4.67)	0.010
Radiotherapy technology												
2D-RT	372/869	Reference	–	396/869	Reference	–	160/869	Reference	–	114/869	Reference	–
3D-CRT	3/14	0.74 (0.24–2.34)	0.610	3/14	0.62 (0.20–1.96)	0.421	2/14	1.18 (0.29–4.88)	0.817	0/14	0 (0-Inf)	0.994
IMRT	51/173	0.76 (0.55–1.05)	0.096	61/173	0.88 (0.65–1.19)	0.399	31/173	1.22 (0.79–1.87)	0.373	19/173	0.96 (0.56–1.64)	0.881
Induced chemotherapy												
No	198/548	Reference	–	216/548	Reference	–	80/548	Reference	–	71/548	Reference	–
Yes	233/515	0.94 (0.76–1.17)	0.589	250/515	0.94 (0.77–1.16)	0.587	116/515	1.14 (0.83–1.58)	0.415	66/515	0.78 (0.53–1.15)	0.216
Concurrent chemotherapy												
No	274/621	Reference	–	293/621	Reference	–	127/621	Reference	–	89/621	Reference	–
Yes	157/442	0.69 (0.55–0.86)	0.001	173/442	0.69 (0.56–0.86)	0.001	69/442	0.57 (0.40–0.81)	0.002	48/442	0.65 (0.44–0.98)	0.038
Adjuvant chemotherapy												
No	409/1,017	Reference	–	443/1,017	Reference	–	185/1,017	Reference	–	133/1,017	Reference	–
Yes	22/46	1.53 (0.98–2.38)	0.063	23/46	1.41 (0.91–2.18)	0.122	11/46	1.62 (0.86–3.07)	0.138	4/46	0.69 (0.25–1.90)	0.472
EBV DNA (copies/10^6 globin)												
≤ 392	269/752	Reference	–	294/752	Reference	–	115/752	Reference	–	90/752	Reference	–
392–581	49/104	1.50 (1.10–2.05)	0.010	54/104	1.50 (1.12–2.01)	0.007	20/104	1.31 (0.81–2.14)	0.272	12/104	1.05 (0.56–1.96)	0.889
581–918	51/103	1.52 (1.12–2.07)	0.007	54/103	1.46 (1.08–1.96)	0.013	27/103	1.76 (1.15–2.69)	0.009	17/103	1.61 (0.95–2.72)	0.077
> 918	62/104	1.85 (1.40–2.46)	< 0.001	64/104	1.85 (1.41–2.44)	< 0.001	34/104	2.37 (1.61–3.51)	< 0.001	18/104	1.70 (1.01–2.87)	0.047

n: the number of events; *N*: the total number of patients in each group.

Table S4 Relationship between peripheral blood cell Epstein-Barr virus DNA loads and other factors

Characteristics	Low group (≤ 392 copies)	High group (> 392 copies)	OR (95%CI)[a]	P^a	OR (95%CI)[b]	P^b
Age						
≤ 45	400 (70.8%)	165 (29.2%)	Reference	–	Reference	–
> 45	352 (70.7%)	146 (29.3%)	1.00 (0.77–1.31)	0.968	0.98 (0.73–1.32)	0.888
Gender						
Male	545 (70.3%)	230 (29.7%)	Reference	–	Reference	–
Female	207 (71.9%)	81 (28.1%)	0.93 (0.69–1.25)	0.621	1.19 (0.77–1.83)	0.426
BMI						
18.5–24.0	392 (68.1%)	184 (31.9%)	Reference	–	Reference	–
> 24.0	230 (76.4%)	71 (23.6%)	0.66 (0.48–0.90)	0.010	0.70 (0.50–0.97)	0.033
< 18.5	69 (69.0%)	31 (31.0%)	0.96 (0.61–1.51)	0.852	1.00 (0.62–1.60)	0.994
Education						
Primary school or less	166 (68.0%)	78 (32.0%)	Reference	–		
High school	476 (71.8%)	187 (28.2%)	0.84 (0.61–1.15)	0.270		
University or more	110 (70.5%)	46 (29.5%)	0.89 (0.57–1.38)	0.601		
Smoking status (pack-years)				0.407		
Never smoker	357 (72.4%)	136 (27.6%)	Reference	–	Reference	–
≤ 23	202 (70.1%)	86 (29.9%)	1.12 (0.81–1.54)	0.497	1.13 (0.74–1.73)	0.571
> 23	188 (67.9%)	89 (32.1%)	1.24 (0.90–1.71)	0.184	1.21 (0.78–1.87)	0.392
Alcohol drinking				0.293		
Nondrinker	455 (72.5%)	173 (27.5%)	Reference	–		
≤ 1 drink per day	174 (69.3%)	77 (30.7%)	1.16 (0.84–1.60)	0.353		
> 1 drink per day	123 (66.8%)	61 (33.2%)	1.30 (0.92–1.86)	0.141		
Clinical stage						
II	148 (83.1%)	30 (16.9%)	Reference	–	Reference	–
III	428 (71.2%)	173 (28.8%)	1.99 (1.30–3.07)	0.002	1.71 (1.05–2.79)	0.032
IV	176 (62.0%)	108 (38.0%)	3.03 (1.91–4.79)	< 0.001	2.45 (1.41–4.24)	0.001
T stage						
T_{1-2}	225 (75.8%)	72 (24.2%)	Reference	–		
T_{3-4}	527 (68.8%)	239 (31.2%)	1.42 (1.04–1.93)	0.026		
N stage						
N_{0-1}	518 (77.3%)	152 (22.7%)	Reference	–		
N_{2-3}	234 (59.5%)	159 (40.5%)	2.32 (1.77–3.04)	< 0.001		

Table S4 Continued

Characteristics	Low group (≤ 392 copies)	High group (> 392 copies)	OR (95%CI)[a]	*P*[a]	OR (95%CI)[b]	*P*[b]
Radiotherapy technology				0.799		
2D-RT	614 (70.7%)	255 (29.3%)	Reference	–	Reference	–
3D-CRT	11 (78.6%)	3 (21.4%)	0.66 (0.18–2.37)	0.521	0.70 (0.19–2.63)	0.603
IMRT	122 (70.5%)	51 (29.5%)	1.00 (0.70–1.44)	0.971	0.96 (0.64–1.45)	0.864
Induced chemotherapy						
No	415 (75.7%)	133 (24.3%)	Reference	–	Reference	–
Yes	337 (65.4%)	178 (34.6%)	1.63 (1.25–2.13)	< 0.001	1.25 (0.92–1.72)	0.159
Concurrent chemotherapy						
No	445 (71.7%)	176 (28.3%)	Reference	–	Reference	–
Yes	307 (69.5%)	135 (30.5%)	1.11 (0.85–1.45)	0.437	1.07 (0.77–1.49)	0.665
Adjuvant chemotherapy						
No	722 (71.0%)	295 (29.0%)	Reference	–	Reference	–
Yes	30 (65.2%)	16 (34.8%)	1.31 (0.70–2.43)	0.401	1.20 (0.61–2.36)	0.595
History of hypertension						
No	705 (70.9%)	289 (29.1%)	Reference	–		
Yes	47 (68.1%)	22 (31.9%)	1.14 (0.68–1.93)	0.620		
History of diabetes						
No	730 (70.8%)	301 (29.2%)	Reference	–		
Yes	22 (68.8%)	10 (31.3%)	1.10 (0.52–2.36)	0.801		
History of heart disease						
No	745 (70.6%)	310 (29.4%)	Reference	–		
Yes	7 (87.5%)	1 (12.5%)	0.34 (0.04–2.80)	0.318		

a: the results of univariate analysis. b: the results of multivariate analysis.

Table S5 Multivariate Cox regression analysis in the clinical stage II subgroup

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Age												
≤ 45	15/100	Reference	–	18/100	Reference	–	8/100	Reference	–	7/100	Reference	–
> 45	23/78	2.58 (1.15–5.77)	0.021	25/78	2.11 (1.02–4.36)	0.043	8/78	1.90 (0.58–6.16)	0.287	9/78	1.82 (0.58–5.69)	0.301
Gender												
Male	31/127	Reference	–	34/127	Reference	–	12/127	Reference	–	13/127	Reference	–
Female	7/51	0.81 (0.29–2.25)	0.693	9/51	0.94 (0.37–2.37)	0.898	4/51	2.86 (0.45–18.11)	0.263	3/51	0.77 (0.17–3.44)	0.729
Education												
Primary school or less	10/31	Reference	–	10/31	Reference	–	4/31	–	–	3/31	–	–
High school	23/110	0.72 (0.32–1.64)	0.438	26/110	0.82 (0.37–1.83)	0.633	12/110	–	–	9/110	–	–
University or more	5/37	0.65 (0.21–2.01)	0.452	7/37	0.93 (0.34–2.59)	0.894	0/37	–	–	4/37	–	–
Smoking status (pack-years)												
Never smoker	19/87	Reference	–	22/87			8/87			10/87		
≤ 23	10/60	1.67 (0.68–4.06)	0.261	11/60	1.65 (0.71–3.83)	0.242	2/60	5.22 (0.88–30.94)	0.069	3/60	1.36 (0.36–5.07)	0.650
> 23	7/16	1.74 (0.60–5.06)	0.312	8/16	1.78 (0.66–4.83)	0.257	5/16	4.15 (0.54–31.74)	0.170	3/16	1.33 (0.28–6.46)	0.721
Radiotherapy technology												
2D-RT	33/151	Reference	–	38/151	Reference	–	14/151	Reference	–	13/151	Reference	–
3D-CRT	0/5	0 (0-Inf)	0.996	0/5	0 (0-Inf)	0.996	0/5	0 (0-Inf)	0.998	0/5	0 (0-Inf)	0.998
IMRT	4/21	1.23 (0.40–3.76)	0.717	6/25	1.03 (0.34–3.07)	0.965	1/21	0.89 (0.11–7.47)	0.912	2/21	1.33 (0.27–6.55)	0.723
Induced chemotherapy												
No	34/165	Reference	–	39/165	Reference	–	14/165	Reference	–	15/165	Reference	–
Yes	4/13	2.28 (0.67–7.80)	0.188	4/13	1.60 (0.49–5.22)	0.437	2/13	1.59 (0.31–8.28)	0.579	1/13	1.25 (0.14–11.32)	0.842
Concurrent chemotherapy												
No	33/147	Reference	–	38/147	Reference	–	15/147	Reference	–	12/147	Reference	–
Yes	5/31	0.62 (0.23–1.68)	0.350	5/31	0.54 (0.20–1.42)	0.209	1/31	0.31 (0.04–2.47)	0.267	4/31	1.42 (0.42–4.75)	0.569

Table S5 Continued

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Adjuvant chemotherapy												
No	38/178	–	–	43/178	–	–	16/178	–	–	16/178	–	–
Yes	0/0	–	–	0/0	–	–	0/0	–	–	0/0	–	–
EBV DNA (copies/10^6 globin)												
≤ 392	27/148	Reference	–	29/148	Reference	–	9/148	Reference	–	11/148	Reference	–
> 392	11/30	2.72 (1.29–5.73)	0.008	14/30	3.38 (1.72–6.65)	< 0.001	7/30	4.96 (1.71–14.34)	0.003	5/30	3.31 (1.06–10.31)	0.039

n.: the number of events; *N.*: the total number of patients in each group.

Table S6 Multivariate Cox regression analysis in the clinical stage III subgroup

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Age												
≤ 45	101/326	Reference	–	116/326	Reference	–	54/326	Reference	–	46/326	Reference	–
> 45	128/275	1.40 (1.05–01.86)	0.023	133/275	1.22 (0.93–1.60)	0.161	50/275	1.13 (0.75–1.70)	0.562	30/275	0.66 (0.41–1.08)	0.099
Gender												
Male	171/406	Reference	–	182/406	Reference	–	86/406	Reference	–	54/406	Reference	–
Female	58/195	0.58 (0.38–0.89)	0.013	67/195	0.68 (0.45–1.01)	0.058	18/195	0.48 (0.25–0.91)	0.026	22/195	0.99 (0.49–2.00)	0.980
Education												
Primary school or less	73/149	Reference	–	78/149	Reference	–	26/149	–	–	18/149	–	–
High school	130/362	0.73 (0.53–1.00)	0.050	142/362	0.74 (0.55–1.01)	0.057	68/362	–	–	47/362	–	–
University or more	26/90	0.63 (0.39–1.04)	0.070	29/90	0.63 (0.39–1.01)	0.055	10/90	–	–	11/90	–	–

Table S6 Continued

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Smoking status (pack-years)												
Never smoker	94/304	Reference	–	106/304	Reference	–	37/304	Reference	–	34/304	Reference	–
≤ 23	58/142	1.14 (0.76–1.72)	0.512	63/142	1.24 (0.84–1.83)	0.281	36/142	1.48 (0.85–2.58)	0.165	20/142	1.53 (0.75–3.12)	0.238
> 23	76/153	1.28 (0.85–1.91)	0.237	79/153	1.33 (0.90–1.98)	0.150	31/153	1.23 (0.68–2.21)	0.495	22/153	1.85 (0.89–3.82)	0.099
Radiotherapy technology												
2D-RT	201/488	Reference	–	215/488	Reference	–	84/488	Reference	–	65/488	Reference	–
3D-CRT	1/5	0.47 (0.07–3.46)	0.463	1/5	0.42 (0.06–3.04)	0.388	1/5	1.58 (0.21–11.91)	0.656	0/5	0 (0-Inf)	0.994
IMRT	26/105	0.59 (0.38–0.93)	0.024	31/105	0.69 (0.45–1.06)	0.088	19/105	1.32 (0.74–2.36)	0.347	10/105	0.86 (0.41–1.81)	0.685
Induced chemotherapy												
No	117/310	Reference	–	129/310	Reference	–	47/310	Reference	–	44/310	Reference	–
Yes	112/291	1.13 (0.87–1.48)	0.363	120/291	1.09 (0.84–1.41)	0.500	57/291	1.26 (0.85–1.87)	0.245	32/291	0.76 (0.47–1.2)	0.239
Concurrent chemotherapy												
No	138/324	Reference	–	149/324	Reference	–	65/324	Reference	–	50/324	Reference	–
Yes	91/277	0.72 (0.53–0.97)	0.030	100/277	0.73 (0.55–0.98)	0.038	39/277	0.52 (0.32–0.84)	0.008	26/277	0.55 (0.32–0.95)	0.031
Adjuvant chemotherapy												
No	217/571	Reference	–	236/571	Reference	–	97/571	Reference	–	75/571	Reference	–
Yes	12/30	1.52 (0.83–2.79)	0.176	13/30	1.38 (0.77–2.48)	0.274	7/30	1.97 (0.87–4.45)	0.104	1/30	0.30 (0.04–2.20)	0.236
EBV DNA (copies/10^6 globin)												
≤ 392	153/428	Reference	–	169/428	Reference	–	68/428	Reference	–	56/428	Reference	–
> 392	76/173	1.38 (1.04–1.82)	0.025	80/173	1.27 (0.97–1.67)	0.081	36/173	1.42 (0.94–2.14)	0.093	20/173	0.97 (0.58–1.63)	0.919

n.: the number of events; *N.*: the total number of patients in each group.

Table S7 Multivariate Cox regression analysis in the clinical stage IV subgroup

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Age												
≤ 45	60/139	Reference	–	67/139	Reference	–	33/139	Reference	–	19/139	Reference	–
> 45	104/145	1.55 (1.08–2.23)	0.017	107/145	1.43 (1.01–2.02)	0.043	43/145	1.02 (0.60–1.72)	0.940	26/145	1.06 (0.54–2.09)	0.863
Gender												
Male	145/242	Reference	–	155/242	Reference	–	66/242	Reference	–	42/242	Reference	–
Female	19/42	0.80 (0.43–1.49)	0.480	19/42	0.61 (0.34–1.12)	0.113	10/42	0.75 (0.33–1.68)	0.484	3/42	0.51 (0.13–2.00)	0.336
Education												
Primary school or less	36/64	Reference	–	37/64	Reference	–	18/64	–	–	10/64	–	–
High school	115/191	0.92 (0.61–1.41)	0.712	120/191	0.91 (0.61–1.37)	0.652	47/191	–	–	29/191	–	–
University or more	13/29	0.69 (0.35–1.36)	0.278	17/29	0.90 (0.48–1.67)	0.734	11/29	–	–	6/29	–	–
Smoking status (pack-years)												
Never smoker	48/91	Reference	–	52/91	Reference	–	25/91	Reference	–	10/91	Reference	–
≤ 23	49/95	0.91 (0.56–1.45)	0.680	53/95	0.77 (0.49–1.20)	0.242	18/95	0.56 (0.28–1.12)	0.099	15/95	1.08 (0.44–2.69)	0.862
> 23	66/97	1.15 (0.72–1.84)	0.567	68/97	1.02 (0.65–1.59)	0.935	33/97	1.06 (0.55–2.06)	0.857	19/97	1.32 (0.52–3.35)	0.554
T stage												
T_{1-2}	10/20	Reference	–	11/20	Reference	–	8/20	Reference	–	4/20	Reference	–
T_{3-4}	154/264	1.51 (0.72–3.14)	0.272	163/264	1.29 (0.64–2.60)	0.480	68/264	1.00 (0.41–2.47)	0.999	41/264	0.97 (0.27–3.45)	0.960
N stage												
N_{0-1}	90/162	Reference	–	97/162	Reference	–	37/162	Reference	–	25/162	Reference	–
N_{2-3}	74/122	1.33 (0.94–1.87)	0.105	77/122	1.20 (0.86–1.68)	0.275	39/122	1.46 (0.88–2.41)	0.144	20/122	1.16 (0.59–2.26)	0.666
Radiotherapy technology												
2D-RT	138/230	Reference	–	143/230	Reference	–	62/230	Reference	–	36/230	Reference	–
3D-CRT	2/4	1.12 (0.26–4.78)	0.879	2/4	1.00 (0.24–4.23)	0.999	1/4	0.98 (0.13–7.56)	0.983	0/4	0 (0-Inf)	0.997
IMRT	21/47	1.00 (0.60–1.67)	0.993	26/47	1.25 (0.78–2.01)	0.348	11/47	1.12 (0.55–2.29)	0.755	7/47	1.34 (0.54–3.35)	0.532

Table S7 Continued

Characteristics	Overall survival			Progression-free survival			Distant metastasis-free survival			Recurrence-free survival		
	n./N.	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*	*n./N.*	HR (95%CI)	*P*
Induced chemotherapy												
No	47/73	Reference	–	48/73	Reference	–	19/73	Reference	–	12/73	Reference	–
Yes	117/211	0.65 (0.45–0.93)	0.020	126/211	0.70 (0.49–1.00)	0.052	57/211	0.87 (0.50–1.51)	0.613	33/211	0.88 (0.42–1.85)	0.728
Concurrent chemotherapy												
No	103/150	Reference	–	106/150	Reference	–	47/150	Reference	–	27/150	Reference	–
Yes	61/134	0.66 (0.45–0.96)	0.031	68/134	0.67 (0.46–0.96)	0.030	29/134	0.66 (0.38–1.15)	0.145	18/134	0.74 (0.36–1.52)	0.412
Adjuvant chemotherapy												
No	154/268	Reference	–	164/268	Reference	–	72/268	Reference	–	42/268	Reference	–
Yes	10/16	1.37 (0.70–2.69)	0.364	10/16	1.23 (0.63–2.41)	0.543	4/16	1.20 (0.42–3.45)	0.733	3/16	1.26 (0.36–4.35)	0.720
EBV DNA (copies/10^6 globin)												
≤ 392	89/176	Reference	–	96/176	Reference	–	38/176	Reference	–	23/176	Reference	–
> 392	75/108	1.92 (1.40–2.63)	< 0.001	78/108	1.93 (1.41–2.63)	< 0.001	38/108	2.07 (1.30–3.29)	0.002	22/108	2.15 (1.16–3.98)	0.015

n.: the number of events; *N.*: the total number of patients in each group.

Table S8 The scoring system of the nomogram for overall survival and progression-free survival

Characteristics	Overall survival score	Progression-free survival score
Age		
≤ 45	0.0	0.0
> 45	59.4	59.8
Gender		
Male	65.4	66.1
Female	0.0	0.0
BMI		
18.5–24.0	0.0	10.6
> 24.0	4.0	0.0
< 18.5	11.7	11.3
Education		
Primary school or less	71.0	67.6
High school	35.6	28.0
University or more	0.0	0.0
Smoking status (pack-years)		
Never smoker	0.0	0.0
≤ 23	20.2	21.2
> 23	37.6	48.9
T stage		
T_{1-2}	0.0	0.0
T_{3-4}	27.6	26.5
N stage		
N_{0-1}	0.0	0.0
N_{2-3}	25.2	16.9
Concurrent chemotherapy		
No	60.7	61.2
Yes	0.0	0.0
EBV DNA (copies/10^6 globin)		
≤ 392	0.0	0.0
392–581	5.1	19.2
581–918	56.7	53.0
> 918	80.1	86.9
LDH		
< 245	0.0	0.0
≥ 245	100.0	100.0
NLR		
≤ 1.33	0.0	0.0
> 1.33	19.8	26.4

Table S9 Multivariate Cox analysis after adjusted plasma Epstein-Barr virus DNA loads in 205 patients

Characteristics	*n./N.*	HR (95%CI)	*P*
Age			
≤ 45	30/111	Reference	–
> 45	48/94	1.15 (0.67–1.96)	0.607
Gender			
Male	65/153	Reference	–
Female	13/52	0.82 (0.37–1.79)	0.612
Smoking status (pack-years)			
Never smoker	27/96	Reference	–
≤ 23	21/55	1.09 (0.55–2.18)	0.802
> 23	30/54	1.34 (0.68–2.65)	0.394
Clinical stage			
II	4/32	Reference	–
III	37/115	2.74 (0.90–8.36)	0.076
IV	37/58	5.32 (1.67–17.00)	0.005
Radiotherapy technology			
2D-RT	64/146	Reference	–
3D-CRT	0/0	–	–
IMRT	13/56	0.47 (0.23–0.96)	0.038
Induced chemotherapy			
No	27/96	Reference	–
Yes	51/109	0.88 (0.49–1.58)	0.672
Concurrent chemotherapy			
No	49/115	Reference	–
Yes	29/90	0.67 (0.37–1.20)	0.179
Adjuvant chemotherapy			
No	75/200	Reference	–
Yes	3/5	2.84 (0.80–10.14)	0.108
PBC EBV DNA loads			
≤ 392 copies/10^6 globin	56/160	Reference	–
> 392 copies/10^6 globin	22/45	1.88 (1.08–3.26)	0.025
Plasma EBV DNA loads			
≤ 128,000 copies/mL	38/128	Reference	–
> 128,000 copies/mL	40/77	1.50 (0.90–2.52)	0.121

n.: the number of events; *N.*: the total number of patients in each group.

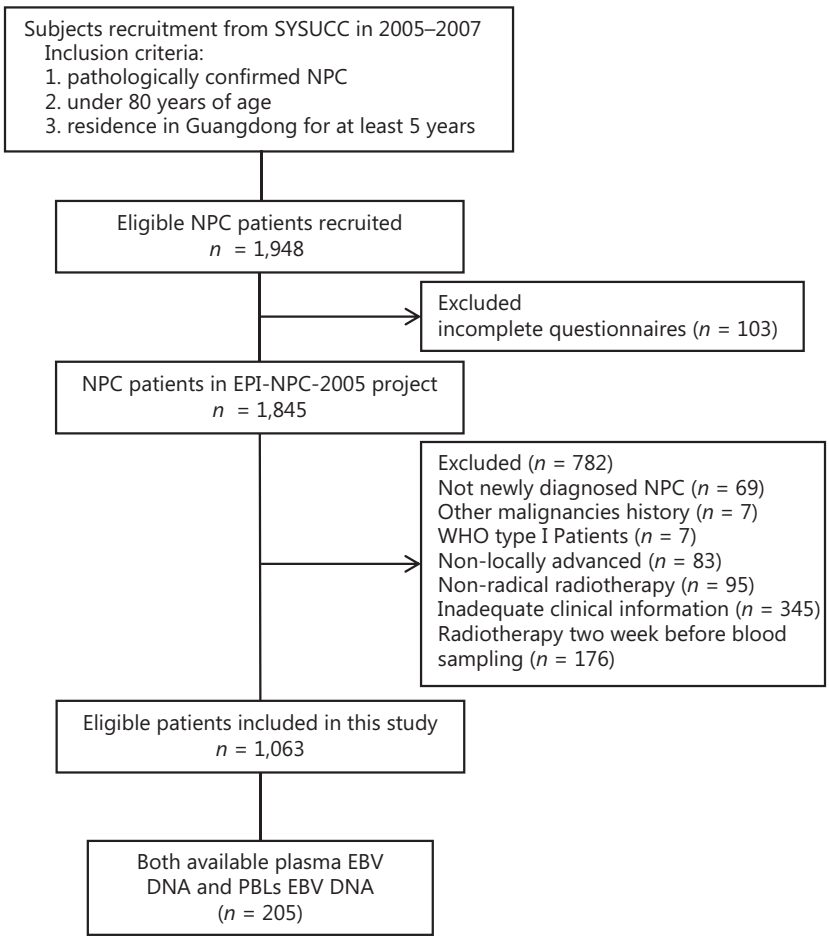

Figure S1 A flow chart of patient inclusion. NPC = nasopharyngeal carcinoma; SYSUCC = Sun Yat-sen University Cancer Center; PBC = peripheral blood cells.

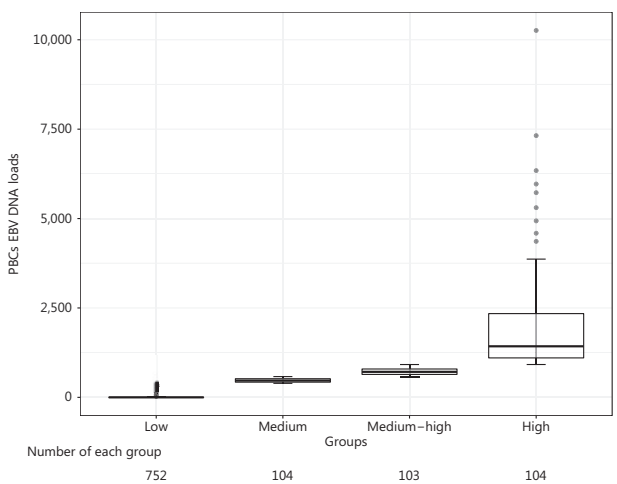

Figure S2 A box plot for peripheral blood cell Epstein-Barr virus DNA loads in the low, medium, medium-high, and high groups.

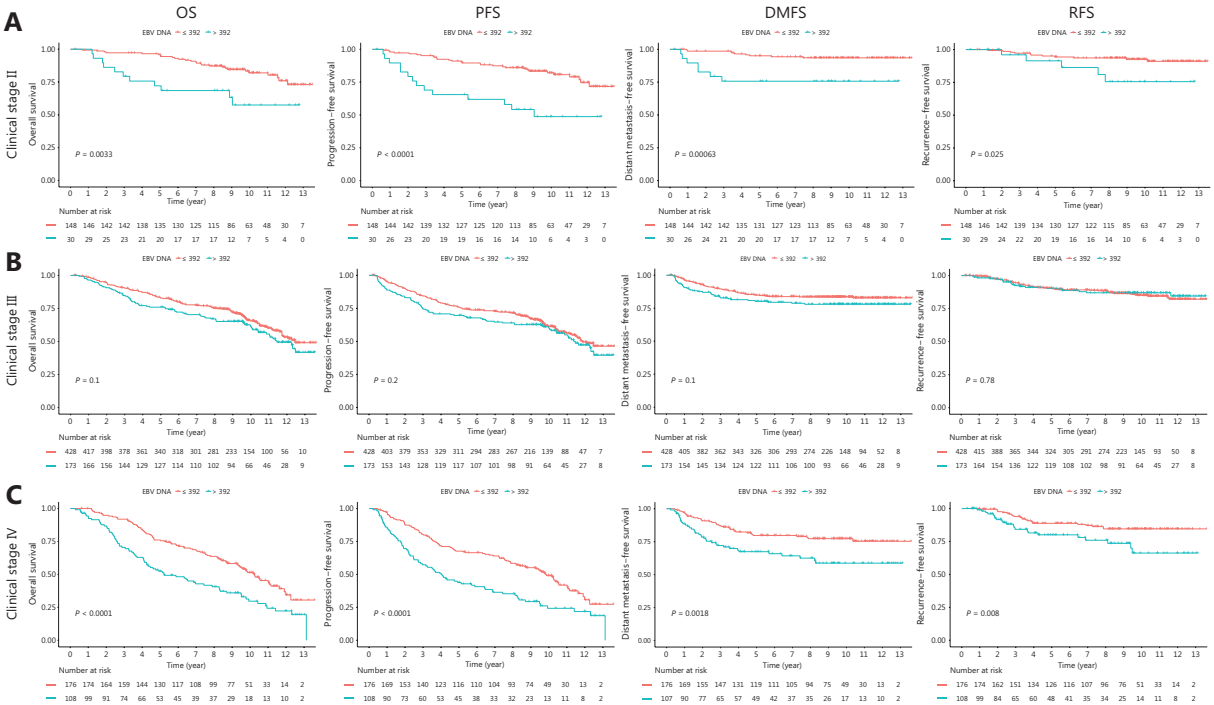

Figure S3 Kaplan-Meier survival curves of Epstein-Barr virus DNA in peripheral blood cells for overall survival, progression-free survival, and distant metastasis-free survival, recurrence-free survival in subgroups of (A) clinical stage II, (B) clinical stage III, and (C) clinical stage IV.

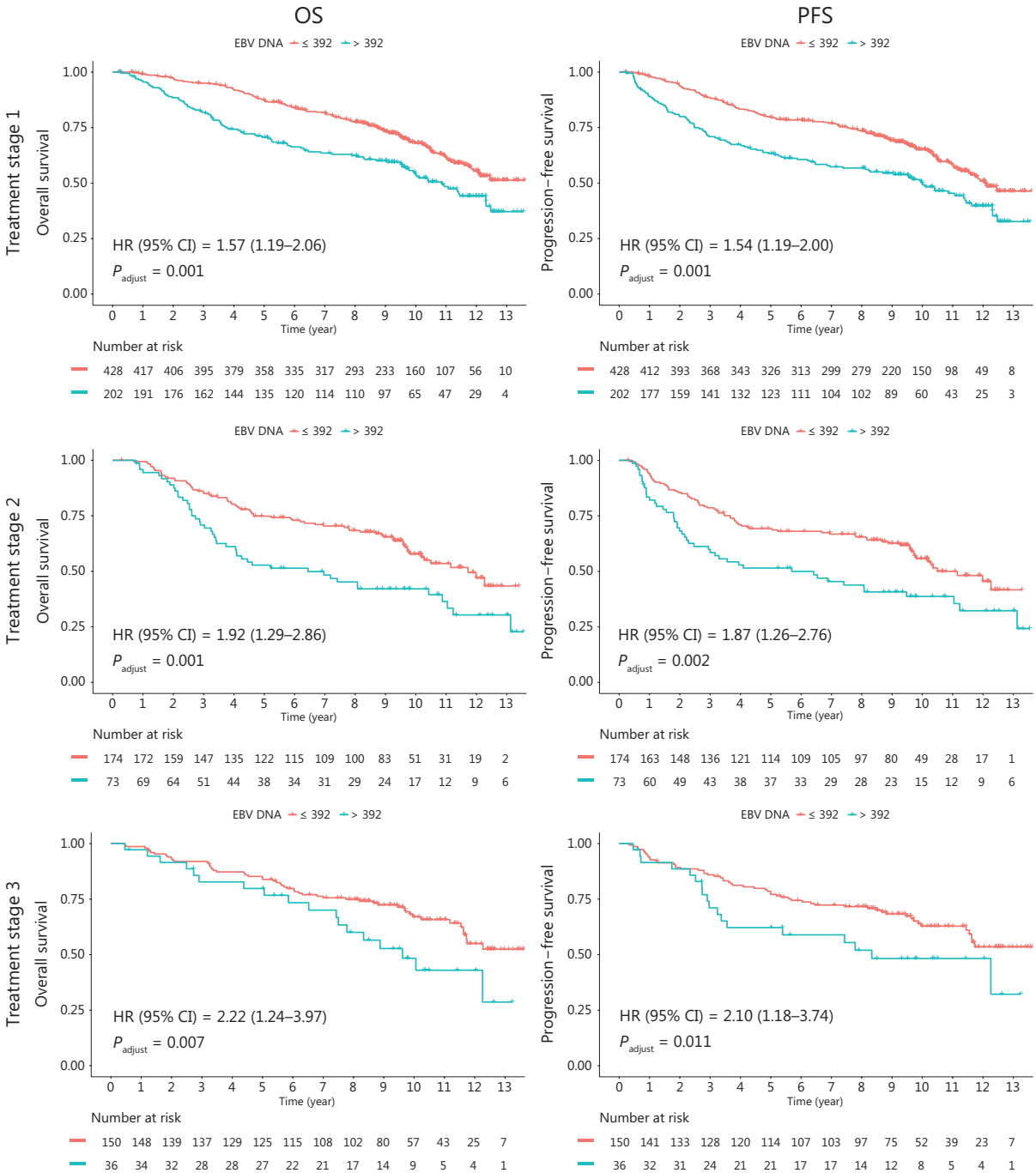

Figure S4 Kaplan-Meier survival curves of Epstein-Barr virus DNA in peripheral blood cells for overall survival and progression-free survival in different treatment stages. The blood samples of patients in treatment stage 1 were collected before any treatment, treatment stage 2 was during induced chemotherapy, and treatment stage 3 was within 2 weeks after the start of radiotherapy. P_{adjust} and hazard ratio (95% confidence interval) were based on the results of multivariable Cox regression that adjusted for age, gender, smoking status, T stage, N stage, radiotherapy technology, chemotherapy (induced chemotherapy, concurrent chemotherapy, adjuvant chemotherapy), and education level.

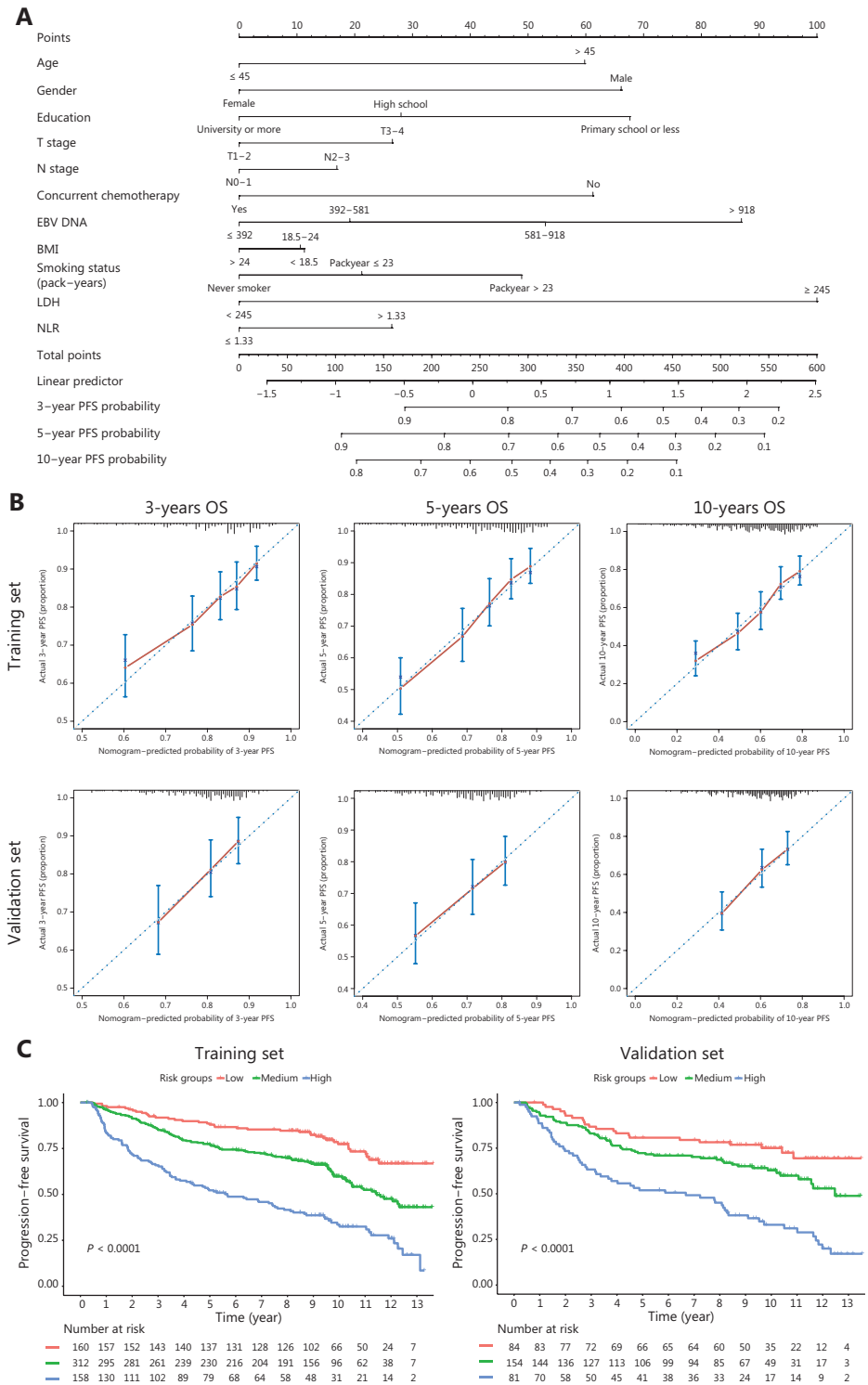

Figure S5 Nomogram (A), including age, gender, education level, T stage, N stage, concurrent chemotherapy, body mass index, smoking status, serum lactate dehydrogenase level, and neutrophil to lymphocyte ratio for 3-, 5-, and 10-year progression-free survival (PFS) for patients with nasopharyngeal carcinoma. The calibration curve (B) of the nomogram for predicting the PFS. The Kaplan-Meier curves (C) for PFS in the training and validation sets.