

ORIGINAL ARTICLE

Quantification of Plasma Epstein–Barr Virus DNA in Patients with Advanced Nasopharyngeal Carcinoma

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ABSTRACT

BACKGROUND

We investigated the clinical significance of plasma concentrations of Epstein–Barr virus (EBV) DNA in patients with advanced nasopharyngeal carcinoma.

METHODS

Ninety-nine patients with biopsy-proven stage III or IV nasopharyngeal carcinoma and no evidence of metastasis (M0) received 10 weekly chemotherapy treatments followed by radiotherapy. Plasma samples from the patients were subjected to a real-time quantitative polymerase-chain-reaction assay. EBV genotypes of paired samples from plasma and primary tumor were compared.

RESULTS

Plasma EBV DNA was detectable before treatment in 94 of the 99 patients, but not in 40 healthy controls or 20 cured patients. The median concentrations of plasma EBV DNA were 681 copies per milliliter among 25 patients with stage III disease, 1703 copies per milliliter among 74 patients with stage IV disease, and 291,940 copies per milliliter among 19 control patients with distant metastasis ($P < 0.001$). Patients with relapse had a significantly higher plasma EBV DNA concentration before treatment than those who did not have a relapse (median, 3035 vs. 1202 copies per milliliter; $P = 0.02$). The consistent genotyping of EBV DNA between paired samples of plasma and primary tumor suggested that the circulating cell-free EBV DNA may originate from the primary tumor. Unlike the rebound of plasma EBV DNA concentrations in the patients who had a relapse, the plasma EBV DNA concentration was persistently low or undetectable in patients with a complete clinical remission. Overall survival ($P < 0.001$) and relapse-free survival ($P = 0.02$) were significantly lower among patients with pretreatment plasma EBV DNA concentrations of at least 1500 copies per milliliter than among those with concentrations of less than 1500 copies per milliliter. Patients with persistently detectable plasma EBV DNA had significantly worse overall survival ($P < 0.001$) and relapse-free survival ($P < 0.001$) than patients with undetectable EBV DNA one week after the completion of radiotherapy.

CONCLUSIONS

Quantification of plasma EBV DNA is useful for monitoring patients with nasopharyngeal carcinoma and predicting the outcome of treatment.

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NASOPHARYNGEAL CARCINOMA IS AN endemic carcinoma associated with Epstein-Barr virus (EBV) infection. Radiotherapy is the primary treatment, but studies have supported the use of combined radiotherapy and chemotherapy for advanced cases.¹⁻⁴ Advances in radiation oncology have improved local control, and treatment failure is now due mainly to distant metastasis.¹⁻⁴ The outcome of salvage treatment for relapse is poor. The extent of involvement by the tumor at the time of recurrence is an important determinant of survival,⁵⁻⁷ and the efficacy of salvage treatment is closely related to the tumor burden at the time of relapse. For these reasons, the development of reliable methods to detect relapse at an early stage may improve the outcome of treatment. If used at the time of diagnosis, such methods might also identify high-risk patients who could benefit from early, aggressive treatment.

EBV is present in cells from almost every primary and metastatic nasopharyngeal carcinoma, regardless of the degree of tumor differentiation or the geographic origin of the patient.⁸⁻¹⁵ In this prospective study, we investigated whether the plasma EBV DNA load, measured by real-time quantitative polymerase chain reaction (PCR), correlates with the response to treatment and the likelihood of relapse and survival among patients with nasopharyngeal carcinoma.

METHODS

STUDY SUBJECTS

A total of 101 previously untreated patients with biopsy-proven nasopharyngeal carcinoma and no evidence of distant metastasis (M0) were enrolled between June 1999 and April 2002. The routine staging workup included a detailed clinical examination of the head and neck, fiberoptic nasopharyngoscopy, computed tomography or magnetic resonance imaging of the entire neck from the base of the skull, chest radiography, whole-body bone scanning, abdominal sonography, a complete blood count, and a biochemical profile. Computed tomography of the chest was performed when chest radiography suggested the presence of lung metastasis, and bone marrow biopsy was performed when an abnormal blood count was reported. The cancer stage was defined according to the 1997 American Joint Committee on Cancer tumor-node-metastasis staging system.¹⁶

The following persons were recruited to serve as controls: 40 healthy volunteers, 20 patients who had received radiotherapy and had survived more than five years without evidence of recurrent nasopharyngeal carcinoma, and 19 patients who had distant metastasis. Written informed consent was obtained from each study subject.

TREATMENT

All the patients met at least one of the following criteria: a neck node exceeding 6 cm in diameter; supraclavicular-node metastasis; destruction of the skull base, intracranial invasion, or cranial-nerve palsy; or multiple metastases to the neck, with at least one node exceeding 4 cm in diameter. Treatment consisted of weekly neoadjuvant chemotherapy.² The chemotherapy consisted of intravenous cisplatin, 60 mg per square meter of body-surface area on days 1, 15, 29, 43, and 57, alternating with 2500 mg of fluorouracil per square meter plus 250 mg of leucovorin per square meter, given by continuous intravenous infusion for 24 hours with the use of an ambulatory pump in an outpatient setting, on days 8, 22, 36, 50, and 64. Radiotherapy was started one week after the completion of 10 weekly doses of chemotherapy and was administered in conventional fractionated doses as described previously.² The total dose to the primary tumor was 70 Gy for tumor (T) stage T1, T2, or T3 disease and 74 Gy for T4 disease.

EXTRACTION OF DNA FROM PLASMA

Peripheral blood (10 ml) was obtained from each patient and control, placed in an EDTA-treated tube, and centrifuged at 1000×g for 15 minutes, and the plasma was transferred into 1.5-ml microtubes. The samples were stored at -30°C until further processing. Plasma DNA was extracted with a QIAamp DNA Blood MiniKit (Qiagen). Before DNA extraction, the plasma samples were thawed and centrifuged at 20,000×g for five minutes. About 200 to 400 μl of each sample per column (supplied in the QIAamp kit) was used for DNA extraction. The exact amount of extracted plasma 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.

Blood samples were obtained one day before treatment began, on days 35 and 64 during chemotherapy, and one week after the completion of radiotherapy. During the follow-up period, blood sam-

ples were collected every six months or when tumor recurrence was suspected or clinically evident.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Concentrations of EBV DNA in plasma were measured with the use of a real-time quantitative PCR assay of the *Bam*HI-W region of the EBV genome. The sequences of the forward and reverse primers were 5'CCCAACTCCACCACACC3' and 5'TCT-TAGGAGCTGTCCGAGGG3', respectively. A dual fluorescence-labeled oligomer, 5'(FAM)CA CACA-CTACACACACCCACCCGTCTC(TAMRA)3', served as a probe. The real-time quantitative PCR assay (40 cycles) and the reaction-setup procedures have been described in detail previously.¹⁷ Real-time quantitative PCR was performed with the ABI Prism 7700 Sequence Detection Analyzer (Applied Biosystems). All DNA samples were also subjected to real-time quantitative PCR for the β -globin gene, which served as a control for the extent to which the plasma DNA could be amplified. Multiple water blanks were included in every analysis as a negative control. The EBV and β -globin PCRs were carried out in triplicate. A calibration curve was run in parallel and in duplicate for each analysis with the use of DNA extracted from an EBV-positive cell line, Namalwa (American Type Culture Collection number CRL-1432), as the standard. Concentrations of plasma EBV DNA were expressed as the number of copies of the EBV genome per milliliter of plasma.¹⁷ Samples with an undetectable EBV signal after processing under our real-time quantitative PCR conditions (40 cycles) were considered to have zero copies.

TYPING OF EBV DNA IN PAIRED PLASMA AND TUMOR SAMPLES

In some cases, paired samples of DNA from the primary tumor and plasma from the same patient were randomly selected and subjected to qualitative PCR with the use of primers specific to latent membrane protein 1 (LMP-1) and EBV nuclear antigen 3C (EBNA-3C). Direct sequencing of the PCR products from the primary tumor and plasma was performed for verification.

STATISTICAL ANALYSIS

Relapse-free survival was calculated from the first day of chemotherapy until the date of relapse or the last follow-up visit. Overall survival was calculated from the first day of chemotherapy until death or the

last follow-up visit. Life-table estimation was performed according to the method of Kaplan and Meier. Univariate comparison of survival curves was performed with the use of the log-rank test. The multivariate Cox proportional-hazards model was used to estimate the hazard ratios and 95 percent confidence intervals. Variables in the model included age, sex, Karnofsky performance status, pathological type according to the World Health Organization, response to chemotherapy, T stage, nodal (N) stage, overall stage, pretreatment plasma EBV DNA concentration, and plasma EBV DNA status one week after the completion of treatment. The relation between the plasma EBV DNA concentration and the relapse rate was evaluated with the use of a chi-square test. The concentrations of plasma EBV DNA were compared with the Mann-Whitney rank-sum test for binary categories or the Kruskal-Wallis test for more than two categories. All statistical tests were two-sided, and a P value of less than 0.05 was considered to indicate statistical significance. Analyses were performed with the use of SAS software (version 8.0, SAS Institute).

RESULTS

PRETREATMENT CHARACTERISTICS AND CLINICAL OUTCOME

Of the 101 patients, 2 patients who interrupted radiotherapy prematurely after receiving 24 and 34 Gy were excluded from subsequent analyses. Table 1 lists the pretreatment characteristics of the remaining 99 patients and their tumors.

On completion of chemotherapy, 59 patients (60 percent) had a complete clinical response and 40 (40 percent) had a partial response. A second biopsy of the primary site of the tumor was performed before radiotherapy in 97 patients, 65 of whom were found to have had a pathologically complete response. After a median follow-up of 30 months (range, 14 to 48), 18 patients had had a relapse: 3 relapses were at the site of the primary tumor, 1 was regional, 1 was in the neck and a distant site, and 13 were at distant sites only. The two-year overall survival rate was 92.6 percent. Detailed clinical results for some patients have been reported elsewhere.²

PLASMA EBV DNA IN PATIENTS AND CONTROLS

Using real-time quantitative PCR (Fig. 1A), we detected EBV DNA in plasma samples from 94 of 99 patients, but in none of the plasma samples from 40 healthy volunteers (Fig. 1B) or 20 patients with cured

Table 1. Characteristics of the 99 Patients.

Characteristic	Value
Age — yr	
Median	47
Mean	47
Range	24–74
Sex — no. (%)	
Male	73 (74)
Female	26 (26)
Karnofsky performance status score — no. (%)	
≥80	89 (90)
<80	10 (10)
WHO pathological classification — no. (%) [*]	
Type I	4 (4)
Type II	80 (81)
Type III	15 (15)
Tumor stage — no. (%) [†]	
T1 or T2	30 (30)
T3 or T4	69 (70)
Nodal stage — no. (%) [†]	
N0 or N1	2 (2)
N2	64 (65)
N3	33 (33)
Overall stage — no. (%)	
III	25 (25)
IV	74 (75)

* WHO denotes World Health Organization.

† The staging system used was that of the 1997 American Joint Committee on Cancer.¹⁶

nasopharyngeal carcinoma. The median concentration of EBV DNA in plasma from the patients with nasopharyngeal carcinoma was 1461 copies per milliliter (interquartile range, 302 to 4390). One of five patients with no copies of EBV DNA in plasma had a tumor consisting of keratinizing squamous-cell carcinoma.

RELATION OF PLASMA EBV DNA CONCENTRATIONS TO CLINICAL STAGE

Of the 99 patients, 25 had stage III and 74 had stage IV disease. For comparison, blood samples were collected from another 19 patients who had distant metastasis (M1) before treatment. The median concentrations of plasma EBV DNA in patients with stage III, stage IV, and stage M1 disease were 681 copies per milliliter (interquartile range, 134 to 1555), 1703 copies per milliliter (interquartile range, 345 to 4454), and 291,940 copies per milliliter (in-

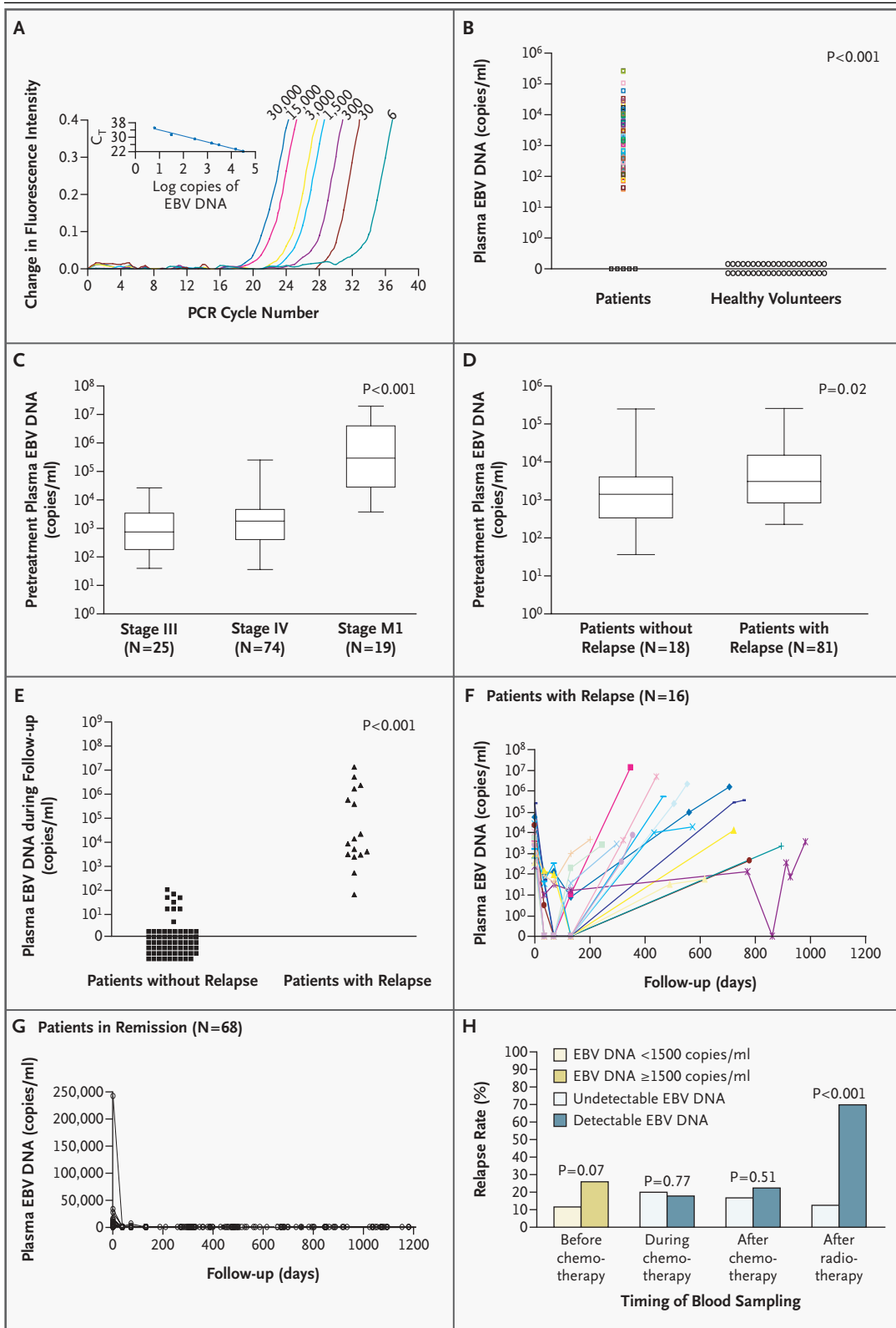
Figure 1 (facing page). Real-Time Quantitative PCR Assay Targeting the *Bam*HI-W Region of EBV DNA and Clinical Analyses of Plasma EBV DNA Concentrations in Patients with Nasopharyngeal Carcinoma and Controls.

Panel A shows the results of studies in which serial dilutions containing known quantities of DNA of the EBV-positive Namalwa cell line were subjected to real-time quantitative PCR analysis of the *Bam*HI-W region. The amplification curve shifted to the right as the quantity of DNA was reduced, because reactions with fewer target molecules required more amplification cycles to produce a certain quantity of reporter molecules than reactions with more target molecules. The system was sensitive enough to detect as few as six copies of EBV DNA. For samples with an extraordinarily high concentration of EBV DNA, outside the reliable range of the real-time quantitative PCR, the assay was repeated after dilution of the samples. The inset shows a plot of the threshold cycle (C_T) against the target quantity, with the latter plotted on a common logarithmic scale. The C_T was set at 10 SD above the mean baseline fluorescence calculated from cycles 1 to 15 and was proportional to the starting target copy number (on a logarithmic scale) used for amplification. The linearity of the graph demonstrates the large dynamic range and the accuracy of the real-time quantitative PCR assay. The EBV DNA content of each sample was derived by interpolation of the mean C_T value of the triplicate assays with the standard curve. If the difference in the C_T value between triplicate assays was too large (>1), we repeated the assay. Panel B shows the plasma EBV DNA concentrations in patients with nasopharyngeal carcinoma and in healthy volunteers. Panel C shows the pretreatment plasma EBV DNA concentration according to the stage of disease, and Panel D the pretreatment plasma EBV DNA concentration according to the presence or absence of relapse. The pretreatment plasma EBV DNA concentration in patients with nasopharyngeal carcinoma was correlated with the clinical stage of disease ($P<0.001$) and with the occurrence of relapse ($P=0.02$). In each panel, the lines inside the boxes denote the medians, the boxes denote the interquartile ranges, and the I bars indicate the 10th and 90th percentiles. Panel E shows plasma EBV DNA concentrations measured during follow-up in patients with and those without a relapse ($P<0.001$). Panels F and G show the results of longitudinal monitoring of the change in plasma EBV DNA concentrations in 16 patients with relapse for whom blood samples were available and 68 patients in continuous remission, respectively. Panel H shows the effect of measurement of plasma EBV DNA at various points on the prediction of subsequent relapse.

terquartile range, 27,190 to 4,105,000), respectively ($P<0.001$) (Fig. 1C).

PLASMA EBV DNA CONCENTRATIONS AND RELAPSE

The median plasma EBV DNA concentrations at the time of the initial presentation were 3035 cop-



ies per milliliter (interquartile range, 806 to 16,130) among the 18 patients who had a relapse and 1202 copies per milliliter (interquartile range, 271 to 3280) among the 81 patients who did not have a relapse ($P=0.02$) (Fig. 1D). These results were confirmed by univariate binary logistic-regression analysis ($P=0.01$). The relative risk of relapse for each increase by a factor of 10 in the plasma EBV DNA concentration was 2.3 (95 percent confidence interval, 1.9 to 10.4).

The pretreatment plasma EBV DNA concentrations were higher in patients with distant relapse (median, 4253 copies per milliliter; range, 226 to 249,900) than in those with local or regional relapse (median, 1311 copies per milliliter; range, 353 to 21,920), but the difference was not significant ($P=0.37$). Of the 18 patients with recurrent disease, blood samples obtained at the time of recurrence were available in 16. These 16 patients had elevations of plasma EBV DNA up to a median of 10,020 copies per milliliter (interquartile range, 2686 to 1,052,000) at the time of recurrence (Fig. 1E). The elevation of plasma EBV DNA in 8 of the 16 patients with relapse occurred six months before the recurrence was detected clinically (Fig. 1F). No association was observed between the site of relapse and the concentration of EBV DNA during treatment, at the end of chemotherapy, or one week after radiotherapy, and a shorter time to relapse was not associated with higher EBV DNA concentrations (data not shown).

FOLLOW-UP STUDY

The median EBV DNA concentration decreased to 0 copies per milliliter (interquartile range, 0 to 0) one week after the completion of radiotherapy. Only 10 of 99 patients had a detectable concentration of EBV DNA in their plasma at that time (median, 121 copies per milliliter; range, 8 to 5066). Seven of these 10 patients subsequently had a relapse: 6 had distant metastases alone, and 1 had a distant metastasis plus a recurrence in the neck. There was no relation between the EBV DNA concentration one week after the completion of radiotherapy and the risk of relapse in these 10 patients.

A total of 130 blood samples were obtained from 68 patients who were in continuous remission during the follow-up period (Fig. 1G). The highest plasma EBV DNA value was selected in the case of patients who had more than one measurement during the follow-up period. The median plasma EBV DNA

concentration in these 130 samples was 0 copies per milliliter (interquartile range, 0 to 0). The difference in EBV DNA concentrations during the follow-up period between patients who had a relapse and those who did not was significant ($P<0.001$) (Fig. 1E). When we evaluated the timing of blood sampling (Fig. 1H), we found that the EBV DNA concentrations in samples obtained one week after the completion of radiotherapy were the best predictors of the likelihood of relapse ($P<0.001$).

TYPING OF EBV DNA FROM PLASMA AND PRIMARY TUMOR

On gel electrophoresis, qualitative PCR showed that the sizes of the PCR products for *LMP-1* (Fig. 2A) and *EBNA-3C* were identical in paired samples of the primary tumor and plasma. Direct sequencing of the PCR products demonstrated complete homology of *EBNA-3C* in all 11 patients for whom samples were available and of *LMP-1* for 7 of the 11 patients (Fig. 2B). The remaining four patients had a difference of only one base between samples.

SURVIVAL

Kaplan–Meier estimates showed that pretreatment EBV DNA concentrations and the presence or absence of EBV DNA in plasma after radiotherapy correlated significantly with overall and relapse-free survival (Fig. 3). In addition, the response to chemotherapy had a significant influence on overall survival ($P=0.04$ for the comparison between patients with a complete response and those with a partial response) but not on relapse-free survival ($P=0.12$). Age, sex, pathological type, Karnofsky performance status, plasma EBV DNA status during and at the end of chemotherapy, T stage, N stage, and overall stage had no significant effect on overall or relapse-free survival. Overall survival at two years was 100 percent among patients with pretreatment plasma EBV DNA concentrations of less than 1500 copies per milliliter, and 83.4 percent among those with pretreatment plasma EBV DNA concentrations of at least 1500 copies per milliliter ($P<0.001$) (Fig. 3A). The two-year relapse-free survival rates were 88.8 percent among patients with pretreatment plasma EBV DNA concentrations of less than 1500 copies per milliliter and 66.4 percent among patients with concentrations of at least 1500 copies per milliliter ($P=0.02$) (Fig. 3B). The two-year overall survival rates were 56.3 percent among patients with persistently detectable plasma EBV DNA after radiother-

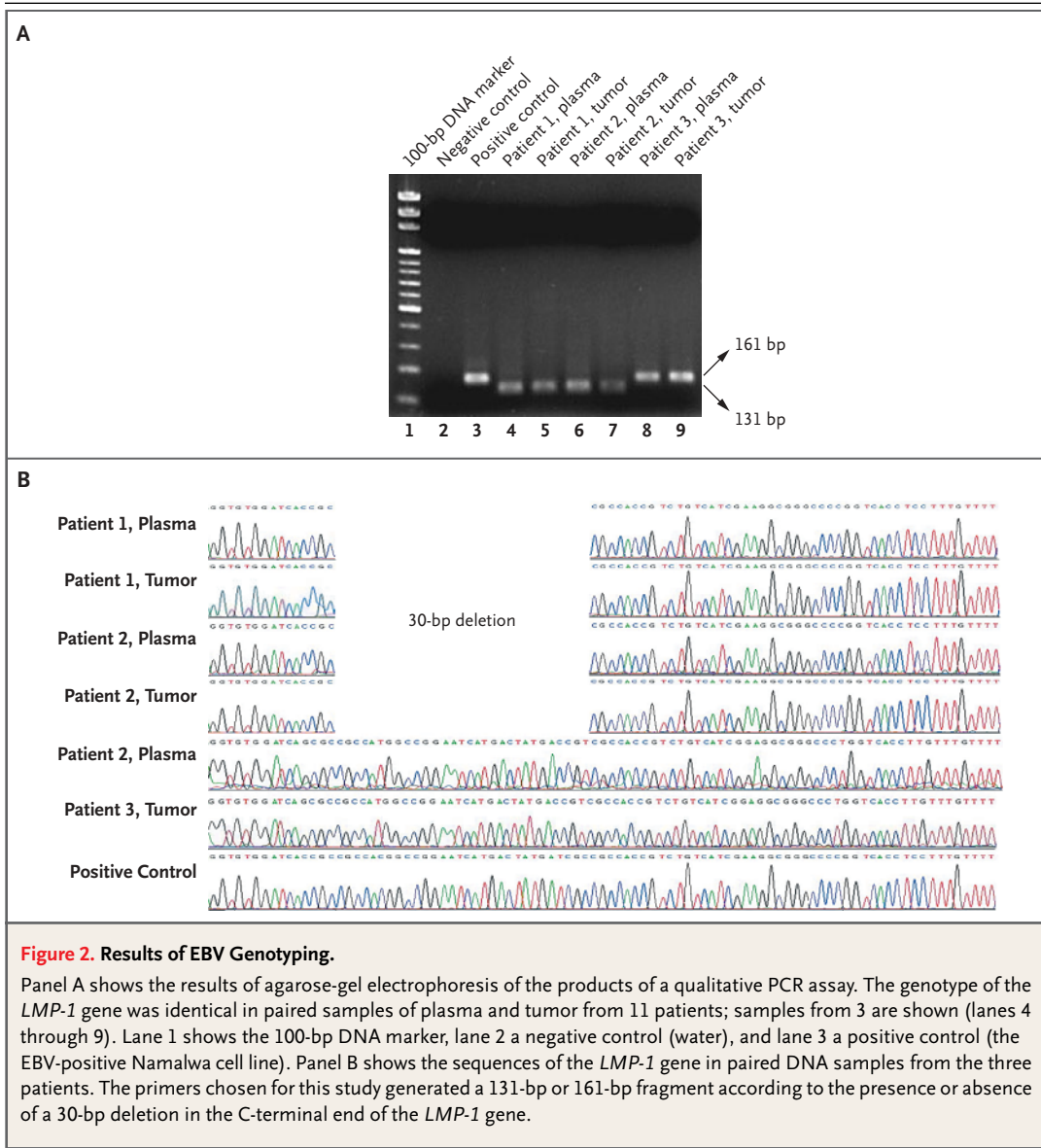


Figure 2. Results of EBV Genotyping.

Panel A shows the results of agarose-gel electrophoresis of the products of a qualitative PCR assay. The genotype of the *LMP-1* gene was identical in paired samples of plasma and tumor from 11 patients; samples from 3 are shown (lanes 4 through 9). Lane 1 shows the 100-bp DNA marker, lane 2 a negative control (water), and lane 3 a positive control (the EBV-positive Namalwa cell line). Panel B shows the sequences of the *LMP-1* gene in paired DNA samples from the three patients. The primers chosen for this study generated a 131-bp or 161-bp fragment according to the presence or absence of a 30-bp deletion in the C-terminal end of the *LMP-1* gene.

apy and 96.7 percent among those with undetectable EBV DNA after radiotherapy ($P < 0.001$) (Fig. 3C). The relapse-free survival rates at two years were 28.6 percent among patients with persistently detectable plasma EBV DNA after radiotherapy and 84.2 percent among those with undetectable EBV DNA after radiotherapy ($P < 0.001$) (Fig. 3D). The overall survival rates at two years were 100 percent among patients with stage III disease and 92.3 percent among patients with stage IV disease ($P = 0.10$), and the rates of relapse-free survival were 90.3 percent and 75.1 percent, respectively ($P = 0.05$) (Fig. 3E and 3F).

MULTIVARIATE COX ANALYSIS

When the pretreatment plasma EBV DNA concentration and clinical features (age, sex, Karnofsky performance status, pathological type, response to chemotherapy, T stage, N stage, and overall stage) were entered into a multivariate analysis, only the pretreatment plasma EBV DNA concentration was significantly related to relapse-free survival ($P = 0.03$; hazard ratio for relapse with a pretreatment EBV DNA concentration of at least 1500 copies per milliliter as compared with less than 1500 copies per milliliter, 3.2; 95 percent confidence interval, 1.1 to 9.0). The hazard ratio for death could not be calcu-

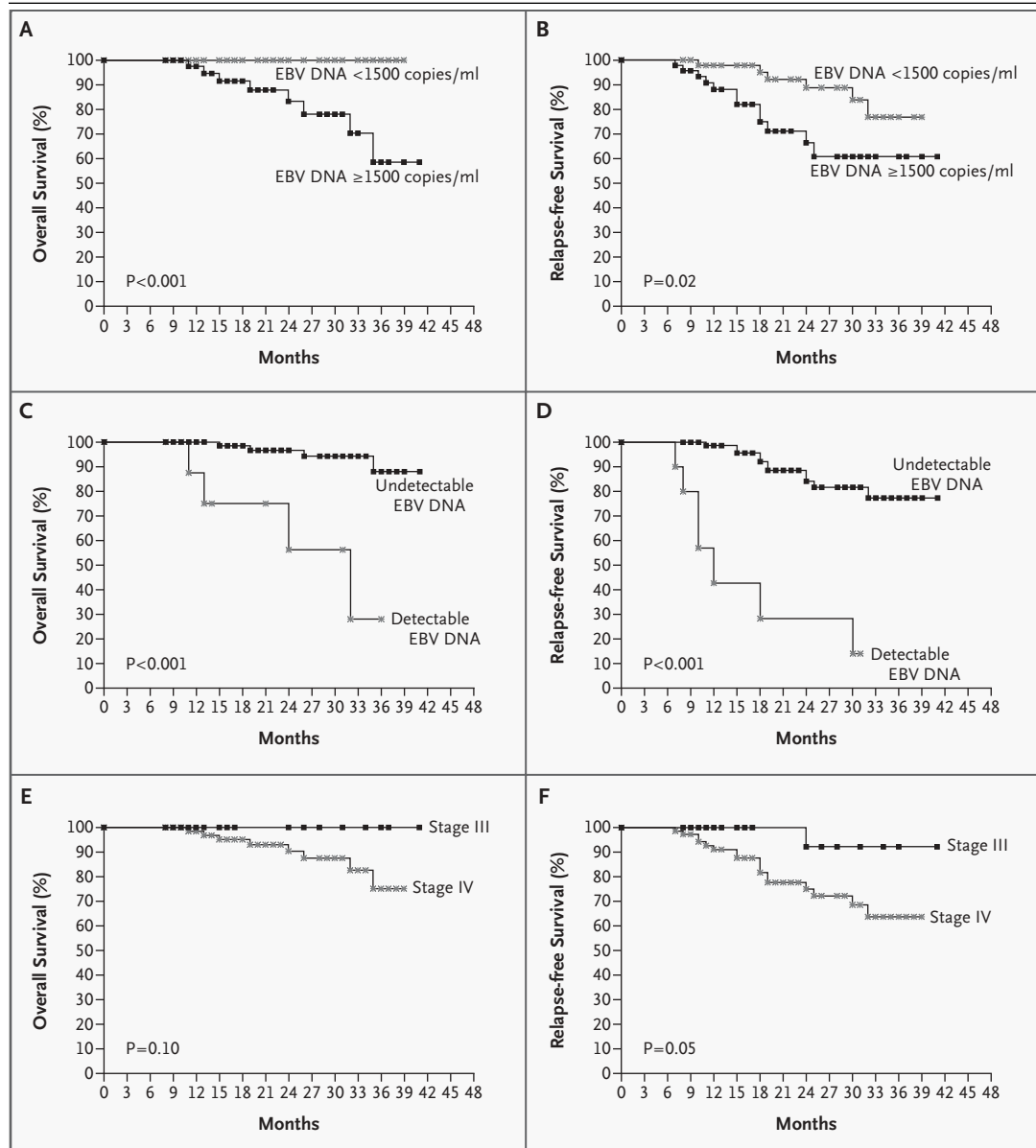


Figure 3. Kaplan–Meier Estimates of Overall and Relapse-free Survival, According to the Plasma EBV DNA Concentration or the Clinical Stage.

Panels A and B show overall survival and relapse-free survival, respectively, according to the pretreatment plasma EBV DNA concentration. Panels C and D show overall survival and relapse-free survival, respectively, according to the plasma EBV DNA status one week after the completion of radiotherapy. Panels E and F show overall survival and relapse-free survival, respectively, according to the clinical stage. The log-rank test was used to calculate P values.

lated because no patients with EBV DNA concentrations of less than 1500 copies per milliliter died. If both pretreatment and post-treatment EBV DNA concentrations were included with clinical features in a Cox analysis (Table 2), a persistently detectable concentration of EBV DNA in plasma after radio-

therapy was the most important prognostic factor in terms of both overall survival ($P=0.002$; hazard ratio for death, 22.9; 95 percent confidence interval, 3.0 to 173.5) and relapse-free survival ($P<0.001$; hazard ratio for relapse, 34.5; 95 percent confidence interval, 7.4 to 162.1) after adjustment for other var-

Table 2. Results of Multivariate Cox Proportional-Hazards Analysis.*

Variable	Hazard Ratio for Death (95% CI)	P Value	Hazard Ratio for Relapse (95% CI)	P Value
Age \geq 45 yr (vs. <45 yr)	1.6 (0.2–12.4)	0.65	0.6 (0.2–2.0)	0.43
Male sex (vs. female sex)	4.5 (0.4–46.7)	0.21	2.3 (0.5–10.8)	0.27
Karnofsky performance status score \geq 90 (vs. <90)	0.8 (0.1–5.9)	0.81	1.5 (0.4–6.3)	0.58
WHO pathological type I (vs. II or III)	—	—	1.4 (0.1–15.1)	0.79
Partial response (vs. complete response)	2.4 (0.4–15.5)	0.37	1.6 (0.5–4.8)	0.41
Tumor stage T3 or T4 (vs. T1 or T2)	2.1 (0.2–27.4)	0.57	6.1 (1.0–37.4)	0.05
Nodal stage N3 (vs. N0, N1, or N2)	1.8 (0.2–20.1)	0.63	0.7 (0.1–3.8)	0.64
Overall stage IV (vs. III)	—	—	6.4 (0.6–64.4)	0.12
Pretreatment plasma EBV DNA \geq 1500 copies/ml (vs. <1500 copies/ml)	—	—	2.7 (0.9–8.1)	0.07
Detectable plasma EBV DNA after treatment (vs. undetectable)	22.9 (3.0–173.5)	0.002	34.5 (7.4–162.1)	<0.001

* The Wald test was used to calculate P values. Dashes indicate variables that were removed from the full model because of the absence of patients who died in one of the subgroups. CI denotes confidence interval, WHO World Health Organization, and EBV Epstein–Barr virus.

iables. Tumor stage and pretreatment plasma EBV DNA concentration showed borderline effects on relapse-free survival ($P=0.05$ and $P=0.07$, respectively).

DISCUSSION

A close association between EBV and nasopharyngeal carcinoma has been established on the basis of the presence of DNA, RNA, and proteins of EBV in almost all cancer cells of primary sites and various metastatic sites^{8–15}; the origin of the tumor in a single EBV-infected cell¹⁸; and the presence of high concentrations of antibodies against EBV proteins in healthy people in whom nasopharyngeal carcinoma later developed.¹⁹ Furthermore, EBV has been detected in premalignant nasopharyngeal lesions, including carcinoma in situ and dysplasia.²⁰ Latent EBV infection does not occur in normal nasopharyngeal epithelial cells, however.²¹

In a previous study of EBV in patients with nasopharyngeal carcinoma, we demonstrated that the presence of *EBNA-1* DNA in cells in the peripheral blood predicted a high risk of distant metastases and a low risk of survival.²² The evidence we obtained indicates that the source of *EBNA-1* DNA in these circulating cells is disseminated nasopharyngeal-carcinoma cells.²³

EBV DNA has been detected by qualitative PCR in plasma or serum samples from patients with na-

sopharyngeal carcinoma.^{24–26} But the low sensitivity and substantial false positive rate limited the value of this approach. Lo et al. detected cell-free EBV DNA in the plasma of 55 of 57 patients with nasopharyngeal carcinoma (96 percent) and 3 of 43 control subjects (7 percent).¹⁷ They demonstrated that circulating EBV DNA concentrations correlated with the tumor stage,²⁷ the likelihood of recurrence,²⁸ the likelihood of survival,²⁹ and the presence of residual disease³⁰ in patients with nasopharyngeal carcinoma who received radiotherapy. They found that circulating EBV DNA molecules are naked DNA fragments, most of which are shorter than 181 bp.³¹ Although the sensitivity of the PCR assay may vary with different segments of viral DNA, similar results were obtained with the use of the *Bam*HI-W region or the *EBNA-1* gene.¹⁷ In a small group of patients, Ngan et al. showed that serum EBV DNA could be detected a mean of 17.4 weeks before recurrence was clinically apparent.³² After the completion of salvage chemotherapy, serum EBV DNA profiles concordant with the clinical outcomes were observed in most patients.

Chan et al.³⁰ reported results similar to ours, but they used a pretreatment cutoff value of 4000 copies per milliliter and a post-treatment cutoff value of 500 copies per milliliter, whereas we used values of 1500 and 0 copies per milliliter, respectively. Taken together, the data show that plasma EBV DNA concentrations can be a useful molecular marker for

screening, monitoring, and prediction of relapse in patients with nasopharyngeal carcinoma. Our results and those of previous studies^{17,30} cannot yet be considered definitive, however, because relapse can occur 3 to 10 years after the completion of initial therapy.

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