

Quantitative Analysis of Cell-free Epstein-Barr Virus DNA in Plasma of Patients with Nasopharyngeal Carcinoma¹

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Abstract

Using real-time quantitative PCR, cell-free EBV DNA was detectable in the plasma of 96% (55 of 57) of nasopharyngeal carcinoma (NPC) patients (median concentration, 21058 copies/ml) and 7% (3 of 43) of controls (median concentration, 0 copies/ml). Advanced-stage NPC patients had higher plasma EBV DNA levels than those with early-stage disease. At 1 month after completion of radiotherapy, plasma EBV DNA was undetectable in 7 of 15 subjects (47%) but remained high in the remaining 8 subjects (53%). Clinical examination revealed that all of the former seven subjects had complete tumor regression, whereas six of the eight latter subjects exhibited evidence of disease persistence or had developed distant metastases. These results suggest that quantitative analysis of plasma EBV DNA may be a useful clinical and research tool in the screening and monitoring of NPC patients.

Introduction

NPC³ is an important cancer in Southern China and Southeast Asia (1). In Hong Kong, nearly all NPC cases are undifferentiated or poorly differentiated squamous cell carcinoma and harbor EBV in tumor tissues (1). The demonstration that tumor-derived DNA is detectable in the plasma and serum of cancer patients (2, 3) raises the possibility that noninvasive detection and monitoring of NPC may be feasible by EBV DNA PCR analysis of plasma or serum samples. This possibility was realized by Mutirangura *et al.* (4), who reported that EBV DNA is detectable in the serum of 31% of NPC patients. However, little quantitative information is available regarding the levels of tumor-derived DNA in cancer patients in general and the levels of plasma EBV DNA in NPC patients in particular. To further understand the biological, temporal, and clinical parameters governing the liberation of EBV DNA into the circulation of NPC patients, we developed a real-time quantitative PCR assay (5, 6) for EBV DNA and used this tool to analyze plasma samples from NPC patients.

Materials and Methods

Patients. Fifty-seven patients with histologically confirmed NPC under the care of the Department of Clinical Oncology at the Prince of Wales Hospital were recruited, and informed consent was obtained. All were investigated uniformly with an endoscopic examination of the nasopharynx and computed tomography and staged according to the American Joint Committee on Cancer Staging criteria (7). Blood samples for EBV DNA analysis were taken before the commencement of radiotherapy. An additional blood sample taken 1 month

after the completion of radical radiotherapy (≥ 66 Gy over a 6.6–8.6-week period) was available in 15 of these patients. The mean time interval between blood sampling at presentation and after radiotherapy was 3.5 months. At the time of the second blood sampling, clinical and fiber-optic nasopharyngoscopic examinations and nasopharyngeal biopsies were carried out. The control group consisted of 43 subjects attending a screening clinic for relatives of patients with NPC. These subjects had all undergone examination of the nasopharynx and neck and had been followed-up for a minimum of 1 year with no evidence of NPC development. The study was approved by the Ethics Committee of The Chinese University of Hong Kong.

DNA Extraction from Plasma Samples. Peripheral blood (5 ml) was collected from each subject into an EDTA tube for the isolation of plasma. Blood samples were centrifuged at $1600 \times g$, and plasma was carefully removed from the EDTA-containing tubes and transferred into plain polypropylene tubes. The samples were stored at -20°C until further processing. DNA from plasma samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the blood and body fluid protocol as recommended by the manufacturer (2). Plasma samples (130–800 μl /column) were used for DNA extraction. The exact amount was documented for the calculation of the target DNA concentration. A final elution volume of 50 μl was used.

DNA Extraction from Nasopharyngeal Biopsy Samples. Pre- and post-radiotherapy nasopharyngeal biopsy specimens from two NPC patients (TM6 and TM42) were studied. Fifteen paraffin-embedded sections, each of which was 5- μm thick, were made from each biopsy specimen. DNA was extracted with a QIAamp Tissue Kit (Qiagen) using a protocol recommended by the manufacturer. A final elution volume of 50 μl was used.

Real-Time Quantitative PCR. Real-time quantitative PCR is based on the continuous optical monitoring of the progress of a fluorogenic PCR reaction (5, 6). In this system, in addition to the two amplification primers used in conventional PCR, a dual-labeled fluorogenic hybridization probe is also included (8). One fluorescent dye serves as a reporter (FAM), and its emission spectra is quenched by a second fluorescent dye (TAMRA). During the extension phase of PCR, the 5' to 3' exonuclease activity of *Taq* DNA polymerase (9) cleaves the reporter from the probe, thus releasing it from the quencher and resulting in an increase in fluorescence emission at 518 nm.

Two real-time quantitative PCR systems were developed for EBV DNA detection: (a) one toward the *Bam*HI-W region; and (b) the other toward the *EBNA-1* region (10). The *Bam*HI-W system consisted of the amplification primers W-44F (5'-CCCAACTCCACCACACC-3') and W-119R (5'-TCTT AGGAGCTGTCCGAGG-3') and the dual-labeled fluorescent probe W-67T [5'-(FAM)CACACACTACACACACCCACCCGCTCTC(TAMRA)-3']. The *EBNA-1* system consisted of the amplification primers EBNA-1162F (5'-TCATCATCATCCGGGTCTCC-3') and EBNA-1229R (5'-CCTACAGGGT-GGAAAAATGGC-3') and the dual-labeled fluorescent probe EBNA-1186T [5'-(FAM)CGCAGGCCCTCCAGGTAGAA(TAMRA)-3']. The fluorescent probes contained a 3'-blocking phosphate group to prevent probe extension during PCR. Primer/probe combinations were designed using Primer Express software (Perkin-Elmer Corp., Foster City, CA). Sequence data for the EBV genome were obtained from the GenBank Sequence Database (accession number V01555). Real-time quantitative PCR for the β -globin gene consisted of primers and probe, as described previously (6), and was used as a control for the amplifiability of plasma DNA. For DNA extracted from paraffin-embedded nasopharyngeal biopsy samples, the β -globin gene quantitative PCR results were used to normalize the EBV DNA quantity.

Fluorogenic PCR reactions were set up in a reaction volume of 50 μl using

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³ The abbreviations used are: NPC, nasopharyngeal carcinoma; FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

components (except for the fluorescent probes and amplification primers) supplied in a TaqMan PCR Core Reagent Kit (Perkin-Elmer Corp.). Fluorescent probes were custom-synthesized by Perkin-Elmer Applied Biosystems. PCR primers were synthesized by Life Technologies, Inc. (Gaithersburg, MD). Each reaction contained 5 μ l of 10 \times buffer A; 300 nM of each of the amplification primers; 25 nM (for the EBV probes) or 100 nM (for the β -globin probe) of the corresponding fluorescent probe; 4 mM MgCl₂; 200 μ M each of dATP, dCTP, and dGTP; 400 μ M dUTP; 1.25 units of AmpliTaq Gold; and 0.5 unit of AmpErase uracil N-glycosylase. Extracted plasma DNA or nasopharyngeal tissue DNA (5 μ l) was used for amplification. DNA amplifications were carried out in a 96-well reaction plate format in a Perkin-Elmer Applied Biosystems 7700 Sequence Detector. Each sample was analyzed in duplicate. Multiple negative water blanks were included in every analysis.

A calibration curve was run in parallel and in duplicate with each analysis, using DNA extracted from the EBV-positive cell line Namalwa (American Type Culture Collection CRL-1432; Ref. 11) as a standard. Namalwa is a diploid cell line (12) that contains two integrated viral genomes/cell (13). A conversion factor of 6.6 pg of DNA/diploid cell was used for copy number calculation (14). Concentrations of circulating cell-free EBV DNA were expressed as copies of EBV genome/ml plasma. For nasopharyngeal biopsies, results were expressed as a ratio of the copy number of the EBV genome:the copy number of the β -globin gene.

An identical thermal profile was used for the EBV *Bam*HI-W and *EBNA*-I PCR systems. Thermal cycling was initiated with a 2-min incubation at 50°C for the uracil N-glycosylase to act, followed by an initial denaturation step of 10 min at 95°C, and then 40 cycles of 95°C for 15 s and 56°C for 1 min were carried out. Conditions for the human β -globin PCR system were as described previously (6).

Amplification data collected by the 7700 Sequence Detector and stored in a Macintosh computer (Apple Computer, Cupertino, CA) were then analyzed using the Sequence Detection System software developed by Perkin-Elmer Applied Biosystems. The mean quantity of each duplicate was used for further concentration calculation. The plasma concentration of EBV DNA or the β -globin gene (expressed in copies/ml) was calculated using the following equation:

$$C = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}}$$

in which *C* represents the target concentration in plasma (copies/ml), *Q* represents the target quantity (copies) determined by a sequence detector in a PCR, *V*_{DNA} represents the total volume of DNA obtained after extraction (typically 50 μ l/Qiagen extraction), *V*_{PCR} represents the volume of DNA

solution used for PCR (typically 5 μ l), and *V*_{ext} represents the volume of plasma/serum extracted (typically 0.13–0.80 ml).

Results

Development of Real-Time Quantitative PCR. To determine the dynamic range of real-time quantitative PCR, serial dilutions of Namalwa DNA were made and subjected to analysis by the two EBV real-time quantitative PCR systems. Fig. 1A shows the results obtained using the *Bam*HI-W region PCR system. The amplification curve shifted to the right as the input target quantity was reduced. This was expected 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 5 copies of EBV DNA. Similar results and the detection limit were obtained using the *EBNA*-I PCR system (data not shown).

Fig. 1B shows a plot of the threshold cycle (*C*_T) of the EBV *Bam*HI-W region PCR against the input target quantity, with the latter plotted on a common logarithmic scale. The *C*_T was set at 10 SDs above the mean baseline fluorescence calculated from cycles 1–15 and was proportional to the starting target copy number (logarithmic scale) used for amplification (5). The linearity of the graph demonstrates the large dynamic range and the accuracy of real-time quantitative PCR. Similar results were obtained using the *EBNA*-I system (data not shown).

The reproducibility of DNA extraction from plasma and EBV quantitative PCR systems was tested by performing 10 replicate extractions from pooled plasma samples from NPC individuals. These replicate extractions were then subjected to real-time quantitative PCR using the EBV PCR systems. The coefficients of variation of *C*_T values of these replicate analyses were 0.83% for the EBV *Bam*HI-W region PCR and 0.55% for the *EBNA*-I PCR.

Quantitative Analysis of Cell-free EBV DNA in NPC and Control Subjects. Plasma DNA samples from 57 NPC patients on presentation and 43 control subjects were analyzed using the two EBV real-time quantitative PCR systems. The *Bam*HI-W region PCR measured median plasma EBV DNA concentrations of 21,058 copies/ml (interquartile range, 4,279–93,589 copies/ml) in NPC subjects and 0 copies/ml (interquartile range, 0–0 copies/ml) in control subjects

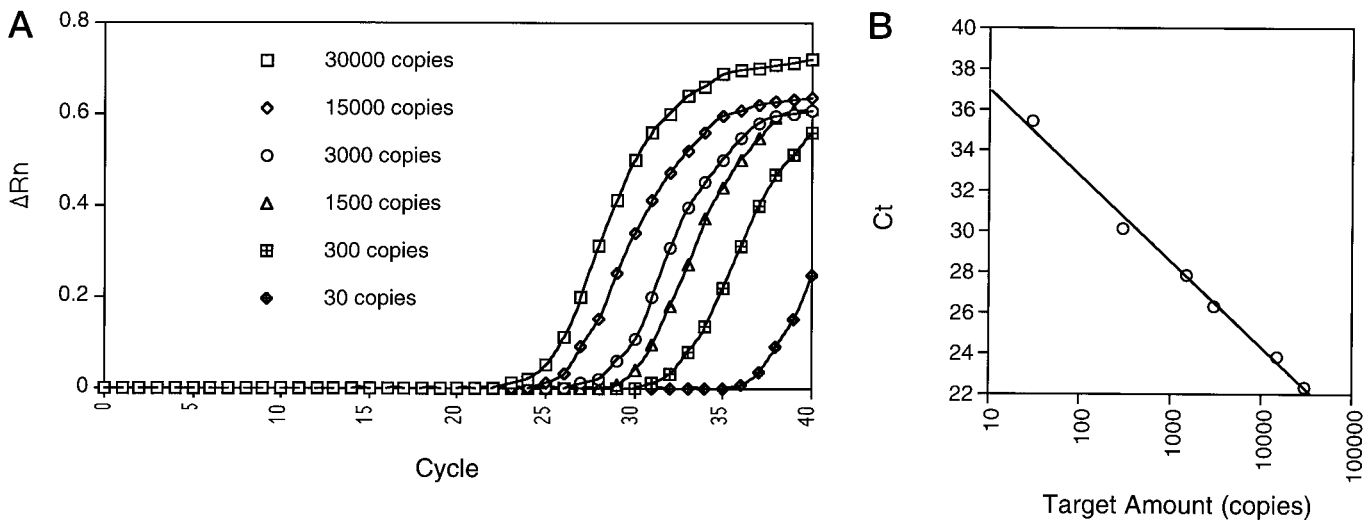


Fig. 1. Detection of EBV DNA by real-time quantitative PCR for the *Bam*HI-W region. A, amplification plot of fluorescence intensity against the PCR cycle. Each plot corresponds to a particular input target quantity marked by a corresponding symbol. The X axis denotes the cycle number of a quantitative PCR reaction. The Y axis denotes the ΔRn , which is the fluorescence intensity over the background (5). B, plot of the threshold cycle (*C*_T) against the input target quantity (common log scale). The input target quantity was expressed as copies of EBV DNA. The correlation coefficient is 0.994.

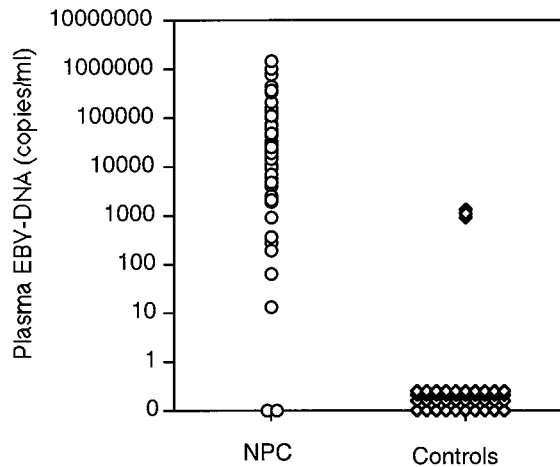


Fig. 2. Comparison of plasma cell-free EBV DNA in NPC and control subjects. The categories (NPC and control subjects) are plotted on the X axis. Results from NPC and control subjects are indicated by \circ and \diamond , respectively. The Y axis denotes the concentration of cell-free EBV DNA (copies of EBV DNA/ml of plasma) detected by the *Bam*HI-W region PCR system.

(Fig. 2). Similar results were obtained using the *EBNA-1* PCR that showed a strong correlation with the *Bam*HI-W region PCR data (Spearman rank order correlation, correlation coefficient = 0.918; $P < 0.0005$). The differences in EBV DNA levels between the NPC and control groups using both the *Bam*HI-W region PCR (Mann-Whitney rank-sum test, $P < 0.001$) and the *EBNA-1* PCR (Mann-Whitney rank-sum test, $P < 0.001$) were highly significant. All plasma DNA samples (NPC and controls) were amplifiable using β -globin PCR, thus confirming the quality of the plasma-extracted DNA.

Correlation of Plasma Cell-free EBV DNA Levels with Staging in NPC Patients. Of the 57 NPC cases, 22 were classified as early-stage disease (stages I and II) and 35 were classified as advanced-stage disease (stages III and IV). Plasma cell-free EBV DNA levels in advanced NPC cases (median, 47,047 copies/ml; interquartile range, 17,314–133,766 copies/ml) were significantly higher than those in early-stage NPC cases (median, 5,918 copies/ml; interquartile range, 279–20,452 copies/ml; Mann-Whitney rank-sum test, $P < 0.001$).

Change in Plasma EBV DNA Levels after Radiotherapy. Fifteen NPC patients with no clinical evidence of distant metastases on presentation were further studied at 1 month after the completion of radiotherapy. All 15 patients had detectable plasma cell-free EBV DNA at presentation (Fig. 3). After the completion of radiotherapy, 47% (7 of 15) of the patients had no detectable plasma EBV DNA by both EBV PCR assays. The remaining eight subjects (53%) continued to have detectable plasma EBV DNA (Fig. 3). The change in the proportion of individuals with detectable cell-free EBV DNA after radiotherapy is statistically significant (Fisher's exact test, $P = 0.006$). As a control for the quality of plasma DNA, all pre- and postradiotherapy samples were shown to be amplifiable using the β -globin PCR.

At the time of postradiotherapy blood sampling, clinical evaluation showed that all seven patients with undetectable EBV DNA had complete regression of the tumor, whereas six of the eight patients with detectable EBV DNA levels had incomplete regression of the tumor or had developed distant metastases. The difference in the proportions of cases with these clinical outcomes is statistically significant (Fisher's exact test, $P = 0.007$).

The pre- and postradiotherapy nasopharyngeal biopsies from the two subjects (TM6 and TM42; Fig. 3) without clinical evidence of tumor persistence who had detectable plasma EBV DNA after radio-

therapy were analyzed by real-time quantitative PCR. For subject TM6, the copy number ratios of EBV DNA: β -globin gene pre- and postradiotherapy were 14.3 and 0.18, as determined by the *Bam*HI-W fragment quantitative PCR system. For subject TM42, the corresponding figures were 33.6 and 0.16, respectively. These results represent a reduction of the EBV DNA level in the nasopharyngeal tissue samples of 79.4-fold (subject TM6) and 210-fold (subject TM42) after radiotherapy. The results obtained using the *EBNA-1* quantitative PCR system were highly concordant and demonstrated a reduction in EBV DNA level in the nasopharyngeal tissue samples of 118.6-fold (subject TM6) and 275-fold (subject TM42) after radiotherapy.

Discussion

In this study, we have developed real-time quantitative PCR assays for two regions of the EBV genome, the *Bam*HI-W and the *EBNA-1* regions. When these assays were used to analyze plasma DNA extracted from NPC patients, circulating cell-free EBV DNA was detected in 55 of 57 patients (96%). Plasma EBV DNA was detected in 3 of 43 controls (7%). In addition, the levels of plasma EBV DNA in the NPC patients were much higher than those in controls, including the three control subjects with detectable EBV DNA (Fig. 2). These data suggest that quantitative plasma EBV DNA analysis may be useful as a screening method for NPC in high-risk areas. Real-time quantitative PCR is highly suitable for this application due to its rapidity and accuracy.

The clinical significance of the presence of plasma EBV DNA in the three control subjects without clinical evidence of NPC is unclear at present. We are carrying out long-term follow-up of these individuals to monitor any clinical signs of NPC and any change in their plasma EBV DNA levels.

We have taken special precautions to maximize the sensitivity of our system, including the use of prospectively collected samples that were promptly processed after venesection. We chose to use plasma instead of serum because previous work has indicated that DNA is liberated during the clotting process, which results in an increase in

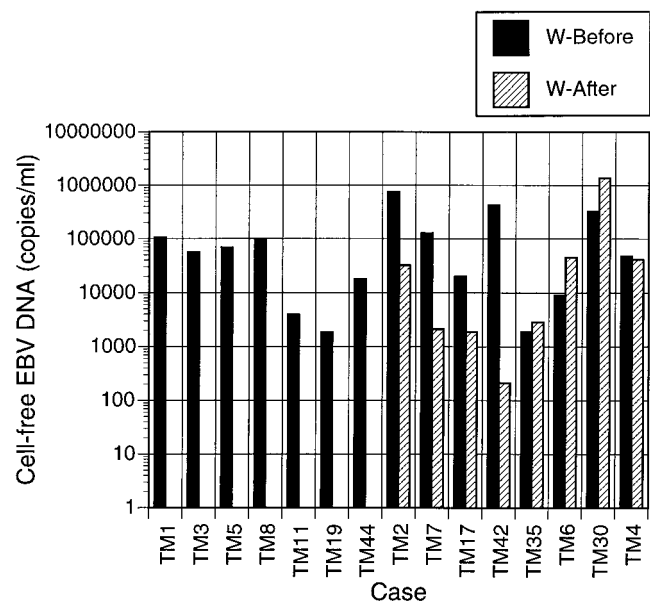


Fig. 3. Plasma cell-free EBV DNA in NPC subjects before and after radiotherapy. The X axis marks each of the 15 subjects studied. The Y axis denotes the amount of cell-free EBV DNA (expressed as copies of EBV DNA/ml of plasma) detected by the *Bam*HI-W region PCR system. Data before and after radiotherapy are plotted as \blacksquare and \hatched , respectively. For visual clarity, the seven cases with no detectable EBV DNA after radiotherapy are grouped together on the left of the graph. These cases are followed by the eight cases with detectable levels of EBV DNA after radiotherapy.

human DNA background when serum DNA is used (6). Our use of the real-time PCR method using multiple primer/probe combinations also contributed to the sensitivity and robustness of detection. We believe that these technical improvements have combined to result in a much greater sensitivity in detecting plasma EBV DNA in NPC subjects than the previously reported figure of 31% (4).

Our quantitative approach allowed us to correlate the levels of plasma cell-free EBV DNA with disease staging. Our data indicate that the plasma EBV DNA levels were approximately eight times higher in advanced NPC (stages III and IV) than in early-stage disease (stages I and II). One explanation for these results is that the liberation of EBV DNA into the blood may be related to the tumor load. This contention is supported by our findings on follow-up of patients after radiotherapy.

By analyzing plasma samples from NPC patients before and 1 month after radiotherapy, we demonstrated that 47% (7 of 15) of NPC patients did not have detectable EBV DNA in the plasma by 1 month postradiotherapy. These seven patients had complete regression of the tumor. In contrast, among the eight patients with detectable plasma EBV DNA after radiotherapy, six had incomplete regression of the tumor or had developed distant metastases. These results suggest that plasma EBV DNA measurement may be useful in identifying patients at increased risk of disease persistence after radiotherapy who may benefit from more aggressive treatment such as combined chemotherapy and radiotherapy (15).

To obtain more information on the two patients (subjects TM6 and TM42) without clinical evidence of tumor persistence who still had detectable plasma EBV DNA after radiotherapy, we analyzed the pre- and postradiotherapy nasopharyngeal biopsies from these individuals. The preradiotherapy nasopharyngeal biopsies from these subjects showed histological evidence of NPC and demonstrated high levels of EBV DNA by quantitative PCR analysis. Large reductions in tissue EBV DNA levels were observed in the postradiotherapy biopsies of both of these individuals. In subject TM42, this reduction in the tissue level of EBV DNA was paralleled by a reduction in plasma EBV DNA levels (Fig. 3). On the contrary, in subject TM6, the plasma EBV DNA level showed an additional increase after radiotherapy (Fig. 3), suggesting that the nasopharyngeal biopsy might have missed residual foci of disease or that occult lymph node or distant metastases might have occurred. We are currently following-up these and other individuals with NPC in an attempt to further understand the relationship between plasma EBV DNA levels and patient survival.

Our data raise the possibility that quantitative measurement of tumor-derived DNA in other cancers may also have clinical and biological value. The most direct extension of our findings would be for the analysis of other viral-associated cancers such as hepatocellular carcinoma and cervical cancer, in which associations with hepatitis B virus (16) and human papillomavirus have been found (17). Real-time quantitative PCR can be readily adapted for quantifying hepatitis B virus and human papillomavirus DNA in plasma DNA.

The mechanism of liberation of cell-free EBV DNA into the plasma of NPC patients is currently unclear. Fournie *et al.* (18) suggested that plasma DNA may be a marker of cell death. In this regard, apoptosis

in tumor tissues has been demonstrated to correlate with the presence of EBV DNA in the serum of NPC patients (4). Our quantitative EBV assays may allow investigation of the mechanism of EBV DNA release by studying viral DNA levels under different clinical conditions, *e.g.*, immediately after radiotherapy or biopsy. Apart from improving our understanding of the biology of NPC, these future studies may also increase our knowledge regarding the biology of plasma tumoral DNA in general.

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