Advances in Brief

Kinetics of Plasma Epstein-Barr Virus DNA during Radiation Therapy for Nasopharyngeal Carcinoma[1]

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Abstract

We studied the kinetics of circulating EBV DNA in the plasma of nasopharyngeal carcinoma (NPC) patients. Serial weekly sampling of 10 NPC patients revealed a rapid decline in plasma EBV DNA concentration after treatment. In two subjects, an initial rise in the circulating EBV DNA level was observed immediately after treatment initiation. Plasma EBV DNA levels were monitored daily during the first treatment week in a second cohort of five patients, and the results indicated that an initial rise in plasma EBV DNA concentration could be observed in all subjects during the first treatment week. This observation is consistent with the liberation of EBV DNA after therapy-induced cancer cell death. After this initial rise, plasma EBV DNA concentration was found to decay with a median half-life of 3.8 days (interquartile range, 2.4–4.4 days). Kinetic analysis of circulating tumor-derived DNA during treatment may be a powerful tool for evaluating the *in vivo* response of NPC and other tumors to antineoplastic treatment and may improve our understanding of the biology of plasma nucleic acids.

Introduction

NPC[3] is an important cancer in Southern China and Southeast Asia (1). Radiation therapy is the main treatment for this cancer (2). In Hong Kong, nearly all NPC cases are undifferentiated or poorly differentiated squamous cell carcinoma and harbor EBV in tumor tissues (1). The recent interest in circulating DNA in the plasma and serum of cancer patients (3, 4) has prompted the discovery of circulating cell-free EBV DNA in the plasma and serum of NPC patients (5, 6). Current data indicate that circulating cell-free EBV DNA is a promising marker for the detection and monitoring of NPC (6, 7). However, little information is available regarding the kinetics of tumor-derived DNA during cancer treatment. In this study, we perform a high time-resolution serial analysis of circulating cell-free EBV DNA during the course of radiation therapy. Such information might have important biological and clinical implications for NPC and for the general understanding of the biology of circulating tumor DNA in cancer patients.

Materials and Methods

Patients. Patients with histologically confirmed NPC under the care of the Department of Clinical Oncology at the Prince of Wales Hospital were re-cruited and gave informed consent. The study was approved by the Ethics Committee of The Chinese University of Hong Kong. All patients were investigated uniformly with an endoscopic examination of the nasopharynx and computed tomography of the nasopharynx and neck. None of the patients had any clinical evidence of distant metastasis on presentation. All recruited patients were treated with a uniform radiotherapy protocol (2). The radiation portals encompassed the nasopharynx and adjacent regions at risk of local tumor spread and both sides of the neck. Radiation therapy was delivered daily from Monday to Friday during each treatment week, with a total dose of at least 66 Gy given over a 6.6–8.6-week period. For the initial study, 3 ml of peripheral venous blood were collected into an EDTA tube on the first day of each weekly course of radiation therapy. The first blood sample was collected before any treatment. For the second phase of the study, in addition to the weekly blood sampling schedule, blood samples were also taken on each of the five treatment days during the first week. Great effort was expended in ensuring the collection of the scheduled specimens; however, in a small number of cases, not all specimens were collected because of patient or technical problems.

DNA Extraction from Plasma Samples. Plasma samples were harvested from the patients according to protocols described previously (6). The plasma 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 (3). A total of 400–800 μl of the plasma samples was used for DNA extraction per column. The exact amount was documented for calculation of the target DNA concentration. A final elution volume of 50 μl was used.

Real-Time Quantitative EBV DNA PCR. Plasma EBV DNA concentrations were measured using a real-time quantitative PCR system for the *Bam*HI-W fragment region of the EBV genome (6). The principles of real-time quantitative PCR and reaction set-up procedures were as described previously (6). All plasma DNA samples were also subjected to real-time PCR analysis for the *β-globin* gene (6), which served as a control for the amplifiability of plasma DNA. Both the EBV and *β-globin* PCRs were carried out 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 diploid EBV-positive cell line Namalwa (CRL-1432; American Type Culture Collection) containing two integrated viral genomes/cell as a standard (8). A conversion factor of 6.6 pg DNA/diploid cell was used for copy number calculation (6). Results were expressed as copies of EBV genomes/ml plasma.

Amplification data were collected using an ABI Prism 7700 Sequence Detector and analyzed using the Sequence Detection System software developed by Perkin-Elmer Biosystems. The mean quantity of each duplicate was used for further concentration calculation. The concentration (expressed in copies/ml) was calculated using the following equation (6):

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

where C = target concentration in plasma (copies/ml), Q = target quantity (copies) determined by a sequence detector in a PCR, V_{DNA} = total volume of DNA obtained after extraction (typically 50 μl/Qiagen extraction), V_{PCR} = volume of DNA solution used for PCR (typically 5 μl), and V_{ext} = volume of plasma extracted (typically 0.4–0.8 ml).

Fig. 1. Variations in plasma EBV DNA concentration in NPC patients treated with radiation therapy and sampled weekly during the course of treatment. The variation in plasma EBV DNA levels for each patient over time is plotted in a separate graph. The scale of the Y axis has been optimized for the concentration range for each case. X axis, time from the start of blood sampling, with the first day of radiotherapy treatment as day 0. Y axis, the plasma EBV DNA level (in copies/ml).
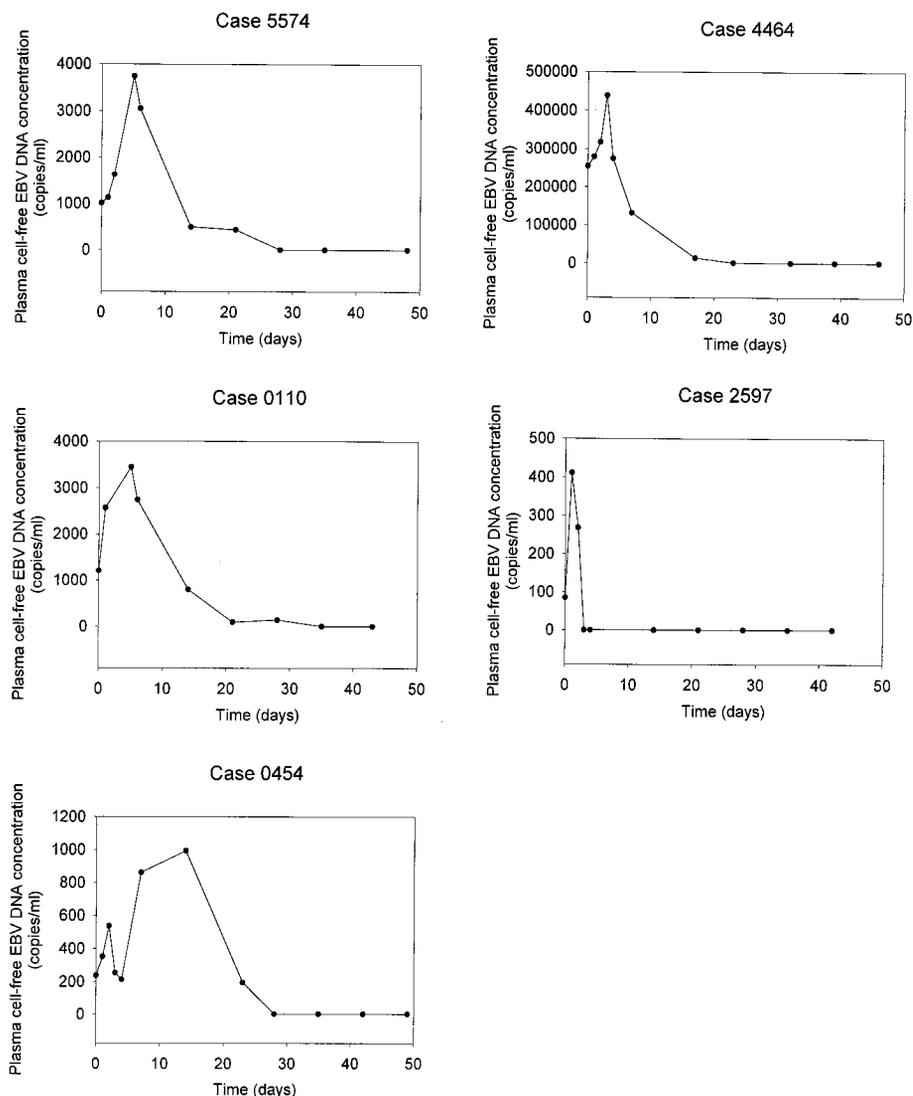
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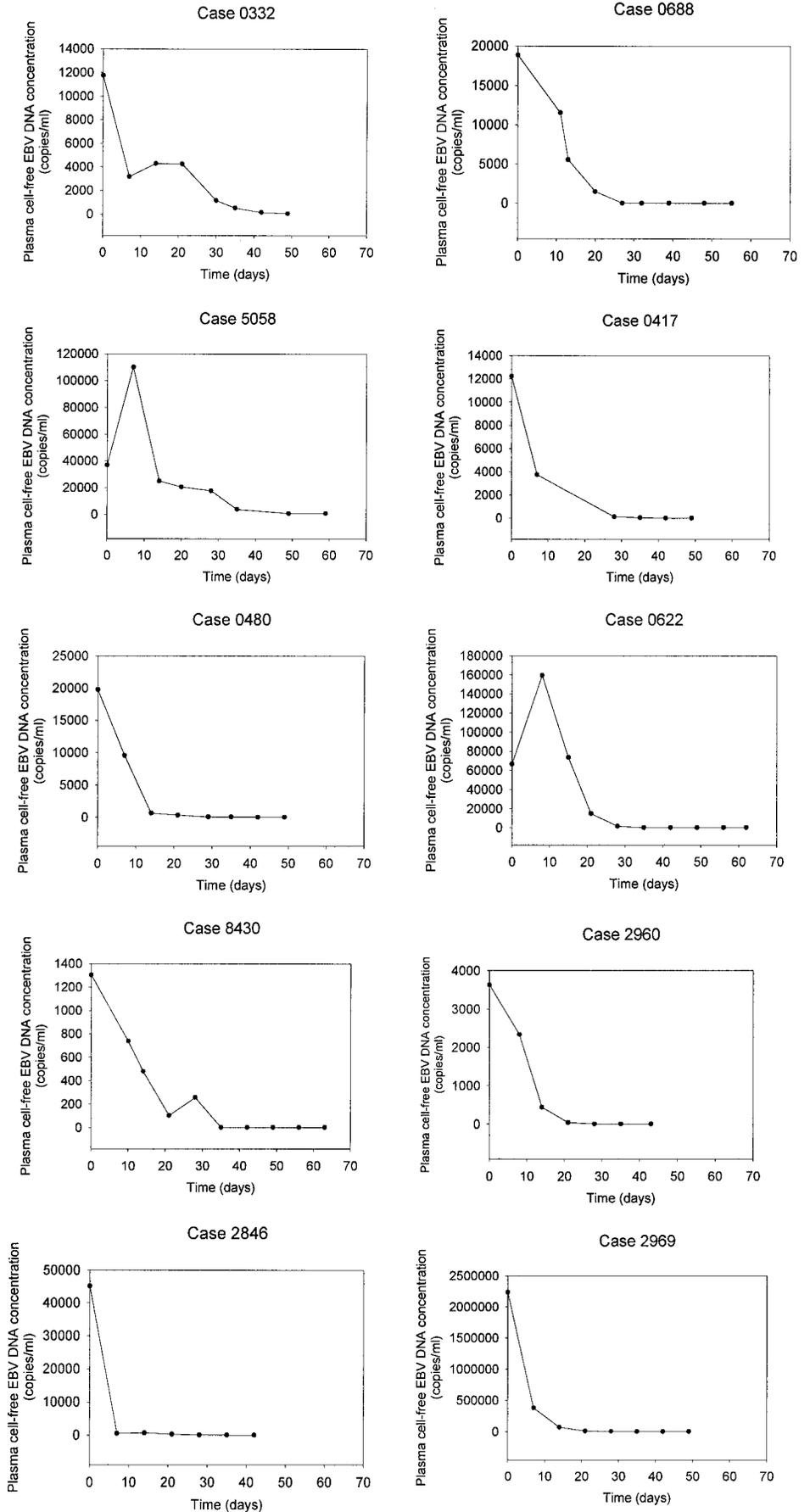
Fig. 2. Variations in plasma EBV DNA concentration in NPC patients treated with radiation therapy and sampled daily during the first treatment week and then weekly during the subsequent treatment course. The variation in plasma EBV DNA levels for each patient over time is plotted in a separate graph. The scale of the Y axis has been optimized for the concentration range for each case. X axis, time from the start of blood sampling, with the first day of radiotherapy treatment as day 0. Y axis, the plasma EBV DNA level (in copies/ml).

Calculation of the Half-Life of EBV DNA Decay. Assuming an exponential decay model, when the natural logarithm of the plasma EBV DNA concentration was plotted against time, a straight line with a slope of $-k$ would be seen. The half-life was determined using the equation below (9).

$$\text{Half-Life} = \frac{0.693}{k} \qquad (2)$$

For this study, the progressively decaying plasma EBV DNA concentrations between the third and seventh weeks (within the period from 14–48 days) after the initiation of radiation therapy were transformed into the corresponding natural logarithms. Because of the use of logarithmic transformation, analysis was carried out up to the last non-zero value for patients whose plasma EBV DNA concentration fell to zero during this period. The reason for the omission of the first two weeks of treatment in half-life analysis is presented in "Results." The slope of the resulting plot of the natural logarithms against time was calculated by linear regression using SigmaStat 2.0 software. The half-life was then computed using Eq. 2.

Results

The first phase of the study involved plasma samples obtained serially from 10 patients at weekly intervals during radiation therapy. The variations in the plasma EBV DNA concentrations in these patients are plotted in Fig. 1. In 8 of the 10 patients, plasma EBV DNA concentrations fell rapidly after the initiation of radiation ther-

apy. However, in the remaining two patients (patients 0622 and 5058), plasma EBV DNA concentrations rose to 2.4× (on day 8) and 3.0× (on day 7) the pretreatment levels, respectively, before falling.

The initial rise in plasma EBV DNA concentrations in patients 0622 and 5058 prompted us to recruit a second cohort of patients in whom daily sampling was performed during the first week of radiation therapy to investigate whether this rise could be detected in all cases with a more intensive sampling protocol. The variations in the plasma EBV DNA concentrations in this second cohort of five patients are plotted in Fig. 2, and the results indicate that in every case examined, an initial rise in EBV DNA concentration could be observed during the first treatment week after the commencement of radiation therapy. In addition to a peak in the first week, patient 0454 also exhibited a second peak on day 14 (Fig. 2). The median time to the peak plasma EBV DNA concentration during the first week was 3.0 days (interquartile range, 1.8–5.0 days). The median value of the ratio between the peak plasma EBV DNA concentration during the first week and the pretreatment level was 2.9 (interquartile range, 2.1–4.0). For patient 0454, the ratio of the second peak on day 14 to the pretreatment plasma EBV DNA concentration was 4.2.

Due to the transient rise in plasma EBV DNA concentration after the initiation of radiation therapy, the half-life of plasma EBV DNA decay was only determined after the first 2 weeks. Fourteen of the 15
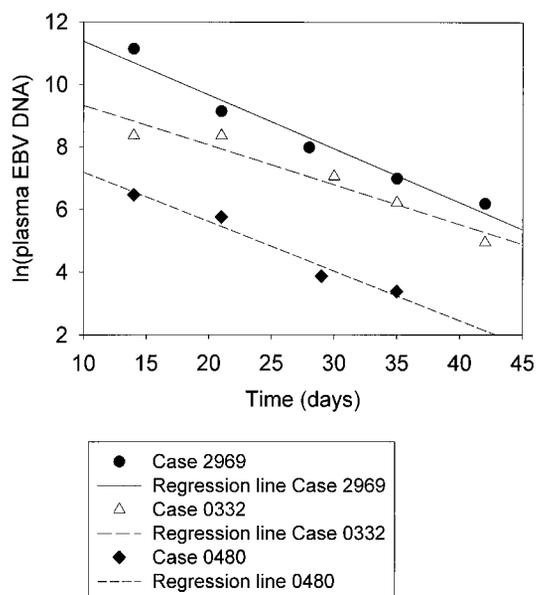
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Fig. 3. Half-life analysis for plasma EBV DNA decay. The natural logarithm of the plasma EBV DNA concentration (Y axis) is plotted against time (X axis) for the period between the third and seventh weeks of radiation therapy. For patient 2969, the coefficient of correlation (r^2) was 0.965, and the slope was -0.172, resulting in a half-life of 4.0 days. For case 0332, r^2 was 0.933, and the slope was -0.127, resulting in a half-life of 5.5 days. For case 0480, r^2 was 0.966, and the slope was -0.158, resulting in a half-life of 4.4 days.

cases studied were included in this analysis. One case (patient 2597; Fig. 2) was excluded from half-life analysis because the circulating EBV DNA fell to undetectable levels before the beginning of the third week. In each of the 14 included cases, the natural logarithm of the plasma EBV DNA concentration was plotted against time. Representative data are shown in Fig. 3. The linearity of the plots supports the exponential decay model. The median half-life of plasma EBV DNA decay in the period between the third and seventh week was determined to be 3.8 days (interquartile range, 2.4–4.4 days).

Discussion

Radiation therapy has remained the main treatment modality for NPC. To allow a more detailed understanding of the effects of radiation on NPC tumor cells, an effective monitoring system is needed. Invasive methods such as biopsy are obviously impractical for this purpose. The recent interest in circulating tumor-derived DNA in the plasma of cancer patients (3, 4), in particular, cell-free EBV DNA in the peripheral blood of NPC patients (5, 6, 7), has opened up exciting possibilities for understanding the *in vivo* effects of radiation on a molecular level.

Our finding that an initial rise in plasma EBV DNA concentration could be observed in all studied cases during the first week of radiation therapy resolves a long-standing paradox in the field of circulating tumor-derived DNA. The paradox is that whereas many investigators have postulated a link between circulating DNA and cell death (10), several studies on circulating DNA in cancer patients have failed to observe an initial rise in circulating DNA levels after treatment (11, 12). Our data suggest that provided sampling is done frequently enough, the initial rise in circulating tumor-derived DNA could be observed in all treated NPC patients. These results are consistent with the hypothesis that cell death is indeed the origin of the circulating tumor-associated EBV DNA. Along the same line of thought, Mutirangura *et al.* (5) have demonstrated a correlation between apoptosis in tumor tissues and the presence of EBV DNA in the serum of NPC patients. The phenomenon of the initial rise in tumor-

associated DNA after antineoplastic treatment is also consistent with the analogous and well-established phenomena of the tumor lysis syndrome, in which large amounts of intracellular products are liberated after the initiation of antineoplastic treatment (13), and the so-called tumor marker "surges," in which proteinaceous tumor markers such as α-fetoprotein and human chorionic gonadotropin have been observed to exhibit a transient rise in cancer patients undergoing induction chemotherapy (9).

Theoretically, two other possible reasons could be postulated to explain this rise in circulating EBV DNA during the first treatment week: (*a*) accelerated tumor cell growth in response to radiation treatment; and (*b*) altered circulating DNA clearance during the first treatment week. With regard to explanation (*a*), there is extensive experimental and clinical evidence that the so-called "accelerated repopulation" of tumor cells may occur after radiation therapy (14). However, this phenomenon is generally observed after a period of approximately 3–4 weeks after the initiation of treatment (15) and thus is not consistent with the observed circulating EBV DNA rise during the first treatment week. For explanation (*b*) to be considered a serious possibility, one has to postulate that the nasopharynx or adjacent regions are important organ systems involved in circulating DNA clearance. Because previous work involving animal models has implicated the liver, spleen (16), and kidney (17) to be the primary organs involved in the clearance of circulating DNA, we consider explanation (*b*) to be less probable. Based on these considerations, the most likely explanation for the plasma EBV DNA rise during the first treatment week is cancer cell death after treatment.

In addition to the rise in plasma EBV DNA concentration during the first treatment week, our data also indicated that in approximately 20% of cases, such as patients 5058 and 0622 in the first cohort (Fig. 1) and patient 0454 in the second cohort (Fig. 2), a more delayed peak during the second week and the beginning of the third week could be seen. The mechanisms underlying this more delayed elevation are unclear. One possible mechanism may be the presence of tumor cell populations with different radiosensitivity in these NPC patients. Additional prospective studies are needed to elucidate the potential clinical implications of this phenomenon.

We have determined that the median half-life of plasma EBV DNA decay between the third and seventh weeks after the start of radiation treatment was 3.8 days. Mechanistically, this half-life can be regarded as consisting of two parts: (*a*) the decay in tumor cell population responsible for liberating EBV DNA; and (*b*) the clearance rate of cell-free EBV DNA once it has been released into the plasma. Recently, circulating cell-free EBV DNA in another EBV-associated malignancy, Hodgkin's disease, has been determined to be present as "naked" DNA rather than free virions (18). If this observation can be extended to NPC, then it is reasonable to assume that, once liberated, the clearance rate of circulating EBV DNA from the plasma will be very fast, with a half-life in the order of minutes, as determined previously for other circulating DNA species (17, 19). Such considerations imply that the rate of decay in the tumor cell population responsible for liberating EBV DNA is likely to be the major determinant of the observed median half-life of 3.8 days. This figure could be regarded as a reflection of the radiosensitivity of the tumor cells and of the cell kill characteristic of a particular treatment regime.

The relative contribution of factors (*a*) and (*b*) in determining the observed half-life is expected to be different for different clinical scenarios. For example, if kinetic analysis of circulating tumor DNA is performed for other cancers that are treated by surgery in which the tumor bulk is removed within a relatively short period of time, the observed half-life of circulating tumor DNA decay can then be expected to be determined mainly by the clearance rate of the liberated tumor DNA [*i.e.*, factor (*b*)] and would be expected to be of a much shorter duration (*e.g.*, on the order of minutes). The extension of such

analysis to multiple tumors and different treatment modalities would yield valuable information on the biology of circulating tumor-derived nucleic acids.

Apart from its obvious biological interest, understanding of circulating tumor-derived DNA kinetics after treatment may also have prognostic implications. For example, it is possible that the radiosensitivity of different tumors may be determined early on during treatment and that additional therapeutic modalities, such as adjuvant chemotherapy, may be considered for tumors that are less radiosensitive. Conversely, for tumors that are highly radiosensitive, it is possible that a less aggressive treatment regime might result in a similar therapeutic effect while reducing the side effects. The realization of these possibilities would require future prospective trials designed with these questions in mind.

In addition to NPC, circulating EBV DNA kinetics could also be determined during the treatment of other EBV-associated neoplasms, such as Hodgkin's disease (18). This type of study may yield valuable information regarding the biological behavior of different EBV-associated malignancies. Such kinetic analysis of circulating tumor-associated viral DNA can also be readily applied to other virally associated cancers, *e.g.*, human papillomavirus and cervical cancer. The availability of other quantitative systems for measuring tumor-associated molecular changes, such as aberrant promoter methylation of tumor suppressor genes (20), may allow this type of analysis to be performed for nonvirally associated malignancies. It is hoped that such future studies will ultimately improve our understanding of the *in vivo* response of multiple tumor types to different treatment modalities and allow the development of more efficacious therapeutic regimes.

Acknowledgments

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