Rapid Clearance of Plasma Epstein-Barr Virus DNA After Surgical Treatment of Nasopharyngeal Carcinoma[1]

Edward W. H. To, K. C. Allen Chan, Sing-Fai Leung, Lisa Y. S. Chan, Ka-Fai To, Anthony T. C. Chan, Philip J. Johnson, and Y. M. Dennis Lo[2]

Departments of Surgery [E. W. H. T.], Chemical Pathology [K. C. A. C., L. Y. S. C., Y. M. D. L.], Clinical Oncology [S-F. L., A. T. C. C., P. J. J.], and Anatomical and Cellular Pathology [K-F. T.], The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China

ABSTRACT

Purpose: **Circulating EBV DNA analysis has been shown to be valuable in the detection, prognostication, and monitoring of nasopharyngeal carcinoma (NPC) patients. A previous study has shown that, after radiotherapy, plasma EBV DNA levels of NPC patients would decline exponentially with a median half-life of 3.8 days. We postulate that this decline in plasma EBV DNA reflects the decrease in cancer cell population and, therefore, the rate of decline reflects the radiosensitivity of the tumor. However, this postulation would hold true only if EBV DNA is rapidly eliminated from the circulation. In this study, we determined the *in vivo* elimination rate of plasma EBV DNA in NPC patients.**

Experimental Design: **We monitored the level of plasma EBV DNA in NPC patients during and after surgical resection of NPC. The half-life of plasma EBV DNA was then calculated by plotting the natural logarithm of EBV DNA concentrations against time.**

Results: **The median half-life of plasma EBV DNA after surgical resection of NPC was 139 min. After a median follow-up of 6.7 days, EBV DNA was undetectable in 8 of 11 patients. One of 8 patients with undectable EBV DNA and all of the patients with detectable EBV DNA developed clinical relapse.**

Conclusions: **The *in vivo* elimination of EBV DNA is very rapid after surgical resection of NPC. The failure of complete and rapid elimination of EBV DNA from the circulation predicts disease recurrence.**

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[2] To whom requests for reprints should be addressed, at Department of Chemical Pathology, Room 38023, 1/F, Clinical Sciences Building, Prince of Wales Hospital, 30–32 Ngan Shing Street, Shatin, New Territories, Hong Kong Special Administrative Region, China. Phone: 852-2632-2563; Fax: 852-2194-6171; E-mail: loym@cuhk.edu.hk.

INTRODUCTION

NPC[3] is an important cancer in Southern China and Southeast Asia. In Hong Kong, nearly all of the NPC cases are undifferentiated or poorly differentiated squamous cell carcinoma and harbor EBV in tumor tissues (1). Circulating cell-free EBV DNA has been shown recently to hold promise in the detection (2, 3), prognostication (4), and monitoring (5) of NPC patients. We have shown previously that, after radiotherapy, the plasma EBV DNA concentrations of NPC patients would rise transiently, followed by an exponential decline with a median half-life of 3.8 days (6). We postulate that the decline of plasma EBV DNA level can reflect the population of tumor cells. However, several factors may govern the concentration of circulating EBV DNA after radiotherapy, which include the release of nuclear contents from dying tumor cells, the decrease in tumor cell population, and the *in vivo* elimination rate of EBV DNA, which has already been released into the circulation. The radiotherapy model is also complicated by the fact that normal organ tissues adjacent to the tumor, such as normal oropharyngeal mucosa and salivary glands, and even circulating blood lymphocytes, are also incidentally irradiated. Cellular subpopulations of these tissues/organs have been shown to harbor EBV (7). In this study, we have investigated the elimination kinetics of circulating EBV DNA in the plasma of patients who underwent surgical removal of recurrent or persistent tumor in the nasopharynx or neck nodes. As the tumor cells have been removed by a surgical procedure, the decline in circulating EBV DNA concentrations should reflect the *in vivo* elimination rate of circulating EBV DNA molecules, which have already been released before the removal of the tumor.

MATERIALS AND METHODS

Patients. Twenty-one patients with postradiotherapy persistent or recurrent NPC referred to the Department of Surgery at the Prince of Wales Hospital, Hong Kong, for salvage surgery were recruited over a 12-month period. Persistent disease was defined as endoscopic evidence of incomplete regression of tumor in the nasopharynx, which was histologically positive for malignancy at >6 weeks after radiotherapy. Recurrent disease was defined as emergence, after prior complete regression, of histologically/cytologically proven disease at the nasopharynx and/or neck nodes at >2 months after radiotherapy. All of the

[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; EBER, EBV-encoded RNA.

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patients had received curative-intent radiotherapy as the initial treatment of NPC. Distant metastasis was excluded by skeletal, thoracic, and upper abdominal imaging before surgery. Nasopharyngectomy and/or radical neck dissection was performed. The first blood samples were collected before the operation, and the intraoperative samples were taken from an indwelling vascular line. Peripheral venous blood was taken during the postoperative period. All of the blood samples were collected into EDTA tubes. The study was approved by the local Ethics Committee, and written informed consent was obtained from all of the patients in the study.

***In Situ* Hybridization Analysis.** Microscopic examination of paraffin-embedded tissue sections of the biopsies and/or resected tumors of the NPC was carried out. The presence of EBV in tumor cells was assessed by *in situ* hybridization using an oligonucleotide probe for small EBERs (Novocastra, Newcastle, United Kingdom), as described previously (8).

DNA Extraction from Plasma Samples. Plasma samples were harvested from the patients according to protocols described previously (3). The plasma samples were stored at −20°C until additional 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. A total of 400–800 μl of the plasma samples was used for DNA extraction per Qiagen 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 (3). The principles of real-time quantitative PCR and reaction set-up procedures were as described previously (3). All of the plasma DNA samples were also subjected to real-time PCR analysis for the β-*globin* gene (3), 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 per cell as a standard (9). A conversion factor of 6.6 pg DNA per diploid cell was used for copy number calculation. Results were expressed as copies of EBV genomes per ml plasma.

Amplification data were collected using an ABI Prism 7700 Sequence Detector and analyzed using the Sequence Detection System software developed by Applied 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 (3):

$$C = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}} \qquad (A)$$

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).

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 (6).

$$\text{half-life} = \frac{0.693}{k} \qquad (B)$$

In most of the patients, the decay followed a transient elevation during the initial phase of the operation. The half-lives were calculated when the EBV DNA concentrations reached a maximum value until the first nonzero trough level. The rationale of the calculation is presented in "Results." The slope of the resulting plot of the natural logarithms against time was calculated by linear regression using the SigmaStat 2.0 software (SPSS, Chicago, IL). The half-life was then computed using Equation B (6).

RESULTS

Twenty-one NPC patients were recruited for the study, of whom 17 were diagnosed to have recurrent disease and 4 were diagnosed to have disease persistence. In the 17 patients who were diagnosed to have recurrent disease, 10 had local recurrence, 2 had both local and neck node disease, and 5 of them had neck node disease only. Sixteen of the 17 patients with recurrent disease showed detectable EBV DNA in the preoperative plasma samples, and their median EBV DNA concentration was 458 copies/ml, whereas only 1 of the 4 patients with persistent disease had detectable but very low level of plasma EBV DNA of 3.5 copies/ml. Interestingly, in 3 of the 4 patients with persistent disease, as confirmed by preoperative biopsies, no tumor cells could be detected on histological examination of the resected nasopharyngeal tissues. The time intervals between the last preoperative biopsy and the operation in these 3 cases ranged from 2 to 8 weeks. In the remaining case, only one focus of tumor could be found on the histological sections of the resected nasopharynx. The preoperative tumor biopsy tissues and resected tumor tissues from 17 patients were also sent for *in situ* hybridization analysis for *EBER* transcripts in addition to histological examination. All of the specimens were positive for *EBER* expression. One patient developed a second local recurrence 4 months after nasopharygectomy, and a second nasopharyngectomy with radical neck dissection was performed. On both occasions, EBV DNA could be detected in the plasma of this patient before the operation.

Serial monitoring of plasma EBV DNA concentrations had been performed in 11 patients with elevated preoperative plasma EBV DNA (Fig. 1). In the patient who had undergone two operations for local recurrences, plasma EBV DNA concentrations were monitored serially in both episodes (Fig. 1, case 9 and case 13). In 8 of the 12 episodes (in 11 patients), the plasma EBV DNA concentrations showed a transient increase and reached a maximum soon after the start of the operation. The median time to reach the peak plasma EBV DNA concentrations was 15 min (interquartile range: 15–60 min). The median value of the ratio between the peak concentration and the preoperative

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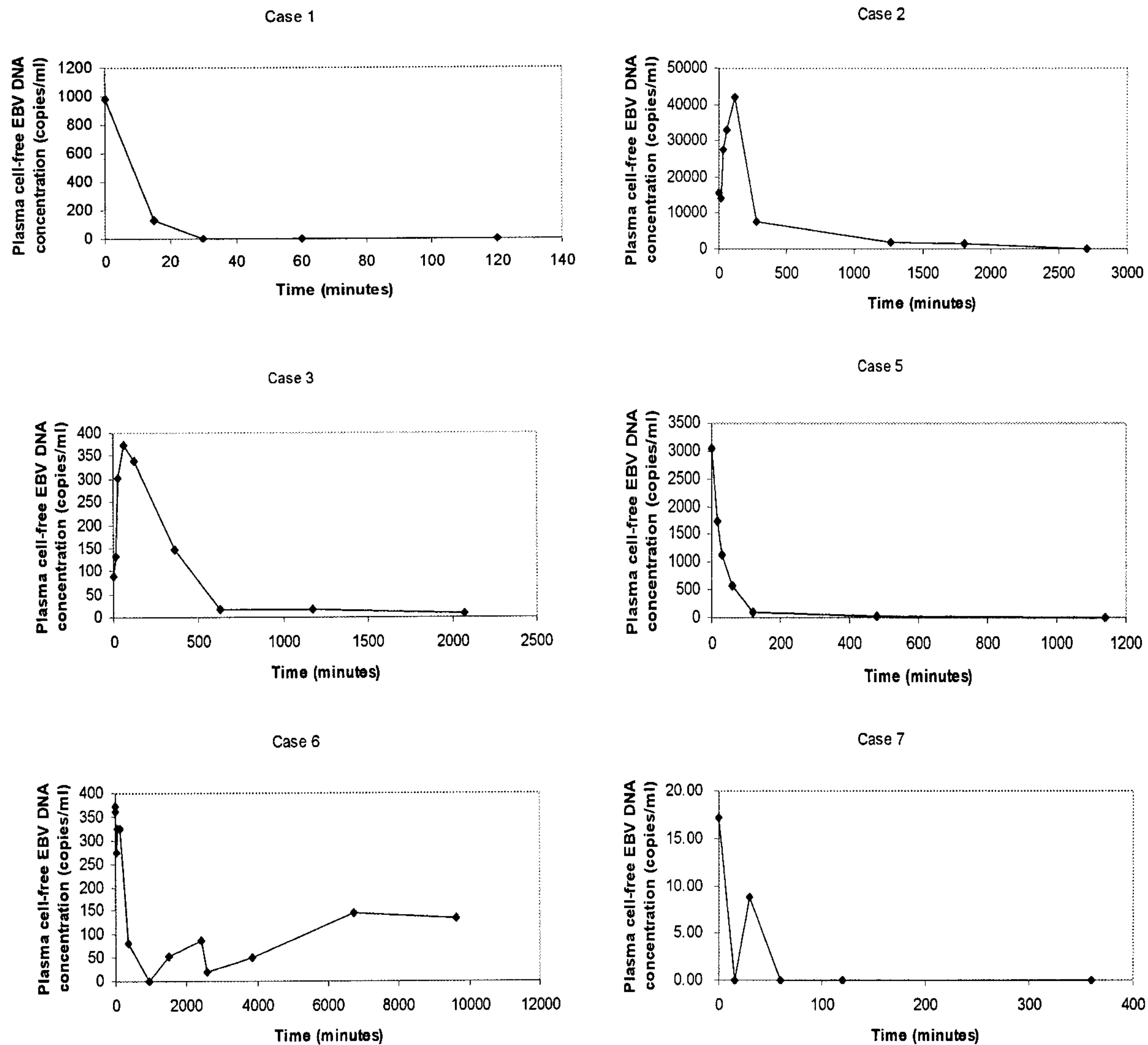

Fig. 1 Variation in plasma EBV DNA concentration in NPC patients during nasopharyngectomy and in the postoperative period. The variation in plasma EBV DNA levels for each operation over time is plotted in a separate graph. Case 9 and case 13 represent the first and second operations of the same patient. The scale of the *Y* axis has been optimized for the concentration range for each case. *X* axis, time from the beginning of the operation, with the time of the first incision as time 0 min. The scale of the *X* axis has been optimized to show most of the nonzero values. *Y* axis, the plasma EBV DNA level (in copies/ml).

EBV DNA concentration was 2.67 (interquartile range: 2.09–3.66).

Because of the transient rise in plasma EBV DNA concentration after the initiation of the operation, the half-life of plasma EBV DNA decay was determined after the peak concentration had been reached. Ten episodes (9 patients) were included in the analysis. Case 7 and case 12 were excluded from the half-life analysis because their plasma EBV DNA concentrations dropped to an undetectable level within 15 min after the peak level had been reached. In each of the 10 included episodes, the natural logarithm of the plasma EBV DNA concentration was plotted against time. Representative data are shown in Fig. 2. The linearity of the plots supports the exponential decay model. The median half-life of plasma EBV DNA decay was determined to be 139 min (interquartile range: 108–285 min).

The median duration for the serial monitoring of plasma EBV DNA concentrations in the 12 operations of the 11 patients is 6.7 days (9675 min). Plasma EBV DNA concentration was undetectable at the end of the monitoring period in 8 of these 12 cases. Only 1 of these 8 patients subsequently developed a local recurrence 18 months after the operation. Among the 3 patients (4 episodes) with detectable plasma EBV DNA at the end of the monitoring period, 2 patients were diagnosed to have either local recurrence or distant metastases within 4 months. In the patient who had received two operations, the plasma EBV DNA was 1399 copies/ml 6.7 days after the first operation (Fig. 1, case 9). This patient was diagnosed to have local recurrence 4 months later. After the second operation (Fig. 1, case 13), the plasma EBV DNA concentration dropped from 9441 copies/ml to 50 copies/ml 6 days after the operation, and the patient

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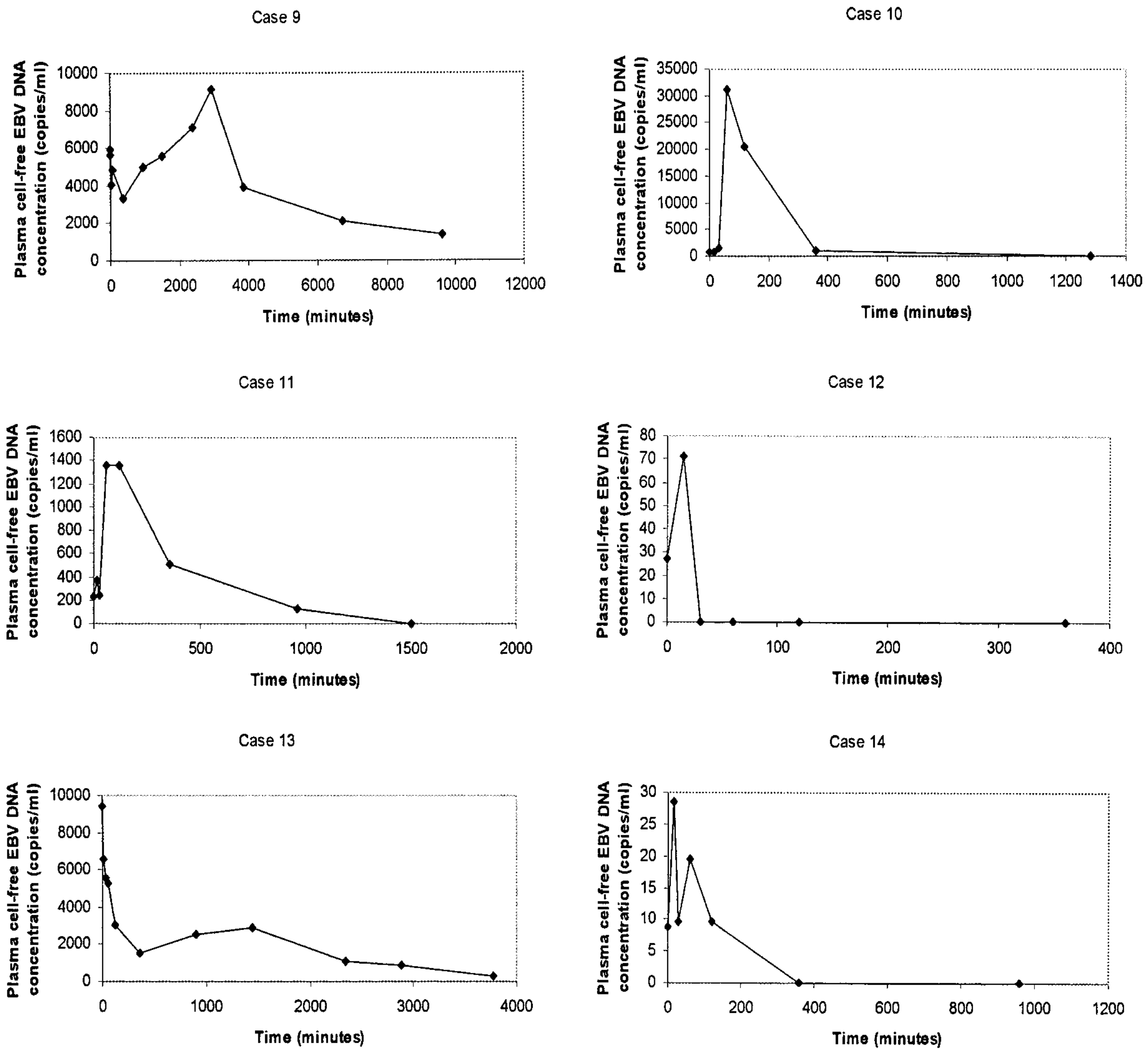

Fig. 1. *Continued.*

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remained in clinical remission 1 year later. In case 5, the patient plasma EBV DNA concentration dropped from 3045 copies/ml to an undetectable level in 28 h, but rebounded at 43 h and fluctuated between 8 and 70 copies/ml until the end of the study. This patient's plasma EBV DNA concentration was 11 copies/ml 69 days after the operation. In case 6, the plasma EBV DNA concentration reached an undetectable level in 16 h, rebounded at 25 h and showed a gradual increase afterward. This patient was diagnosed to have bone metastases 2 months after the operation.

DISCUSSION

The detection of cell-free EBV DNA in the plasma of NPC patients has opened up new possibilities for the noninvasive diagnosis and monitoring of NPC (3). We have shown previously that the plasma EBV DNA concentrations in NPC patients would decline exponentially during radiotherapy with a half-life of 3.8 days (6). This information is useful for the understanding of the *in vivo* effects of radiation on tumor cells. However, because radiotherapy is given in multiple daily fractions over several weeks, the decline of plasma EBV DNA during radiotherapy is not only affected by tumor cell death but also by the *in vivo* elimination rate of the EBV DNA after its release into the plasma. In other words, in the radiotherapy model reported previously, the plasma EBV DNA concentrations can reflect the tumor cell population only if the circulating EBV DNA molecules are rapidly removed from the circulation. Information regarding the latter parameter could be obtained by studying the clearance kinetics of circulating EBV DNA in patients undergoing surgical treatment of NPC. Because surgery is not the standard primary treatment modality for NPC, the opportunity to observe the effect of surgery needs to come from patients who developed locally recurrent tumors postradiotherapy, with or without neck node metastases, that are suitable for curative resection.

In this study, we have also observed that most of the

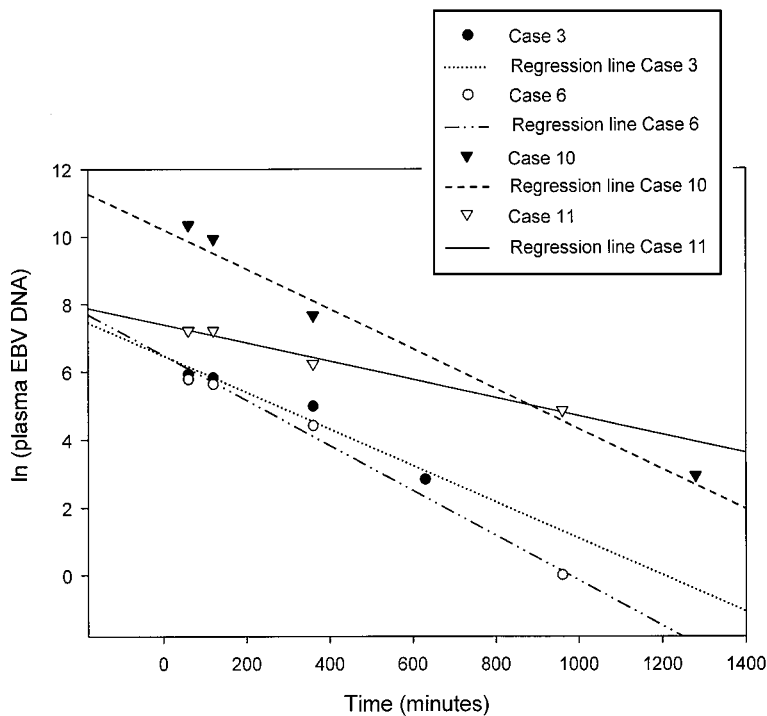

Fig. 2 Half-life analysis for plasma EBV DNA decay. The natural logarithm of the plasma EBV DNA concentration (*Y* axis) is plotted against time (*X* axis) for the period between the peak plasma EBV DNA concentration and the first nonzero trough. For case 3, the coefficient of correlation (r^2) was 0.949, and the slope was −0.00539, resulting in a half-life of 128 min. For case 6, r^2 was 0.990, and the slope was −0.00664, resulting in a half-life of 104 min. For case 10, r^2 was 0.946, and the slope was −0.00590, resulting in a half-life of 117 min. For case 11, r^2 was 0.916, and the slope was −0.00462, resulting in a half-life of 150 min.

patients with postradiotherapy persistent disease had either undetectable or low concentrations of plasma EBV DNA levels, compared with detectable plasma EBV DNA in 16 of 17 patients with local recurrence of NPC. It is also noted that, in 3 of the 4 patients with positive preresection biopsies, no residual disease could be detected through the histological examination of the resected nasopharynx, and only a single focus of tumor cells could be detected in the remaining patient. This suggests that, at least in some patients, biopsy-positive disease in the early postradiotherapy period may regress completely with time, and plasma EBV DNA may be an indicator of such. It is possible that through the monitoring of the plasma EBV DNA concentrations in patients with persistent disease in the early postradiotherapy period, some patients could be spared from surgery if their plasma EBV DNA levels are undetectable. Among the 17 patients operated for recurrent disease, only 1 had undetectable plasma EBV DNA preoperatively. This patient had received radiotherapy twice for recurrence of NPC before the operation. Thus, the absence of detectable plasma EBV DNA in this patient could be related to severe fibrosis and scarring of the nasopharynx after repeated radiotherapy, which might impede the release of tumor-derived DNA into the circulation.

We have observed a rise in plasma EBV DNA concentrations in 8 of 12 episodes (11 patients) during the operation, and the median time interval to reach the peak EBV DNA concentration is 15 min. As surgical resection is a localized treatment, the rise in EBV DNA is likely to be due to the release of cellular contents into the circulation during surgical manipulation of the tumor tissues. This observation is in line with our previous hypothesis that circulating EBV DNA in NPC patients is tumor-derived. The linearity of the plot of the logarithm of EBV DNA concentration against time supports a first-order kinetics model of EBV DNA elimination. This is also consistent with our previous finding of the clearance of fetal DNA from the maternal circulation after delivery (10) and the clearance of injected DNA in an animal model (11). We have determined that the median half-life of plasma EBV DNA decay after the peak concentration is 139 min. This figure indicates that the elimination of existing circulating DNA is extremely fast (of the order of hours) when compared with the decay of EBV DNA during a 6–8-week course of fractionated radiotherapy (of the order of days; Ref. 6). Therefore, the decline of circulating EBV DNA concentration during a fractionated course of radiotherapy is likely to reflect the change in tumor cell population.

An elevated EBV DNA at the end of the monitoring period (median, 6.7 days) appears to be associated with a higher chance of subsequent tumor recurrence. Only 1 of 8 cases with undetectable plasma EBV DNA recurred subsequently, whereas 2 of 3 cases with elevated EBV DNA levels recurred. A larger sample size may help to substantiate such observations. On the other hand, the clinical significance of the persistence of low concentrations of EBV DNA in the plasma after tumor resection remains unclear, as 7% of apparently healthy subjects would also exhibit a low concentration of EBV DNA in their plasma (3). In case 6, the gradual increase in plasma EBV DNA soon after the operation might signify the incomplete removal of EBV DNA-producing tissues, and the patient was diagnosed to have bone metastases 2 months later. Concerning the prognostic significance of tumor-derived DNA, several studies have also demonstrated that in patients suffering from non-EBV-related cancers, the reappearance of tumor-derived DNA in their

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serum after surgical resection of tumor could predict relapse (12, 13).

This study provides, for the first time, high time resolution information concerning the elimination kinetics of tumor-derived DNA in cancer patients after surgical removal of tumors. We have shown that the *in vivo* elimination of EBV DNA from the circulation is very fast, and the half-life is in the order of hundreds of minutes. This rapid elimination kinetics implies that the measured plasma EBV DNA level reflects tumor burden and makes circulating EBV DNA an ideal marker for studying the *in vivo* behavior of NPC. Whether this phenomenon can be generalized to other circulating tumoral DNA species warrants additional study. We believe that a better understanding of tumoral DNA kinetics would be of paramount importance toward the rational use of this new class of tumor markers.

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