Quantitative and Temporal Correlation between Circulating Cell-Free Epstein-Barr Virus DNA and Tumor Recurrence in Nasopharyngeal Carcinoma

Y. M. Dennis Lo, Lisa Y. S. Chan, Anthony T. C. Chan, Sing-Fai Leung, Kwok-Wai Lo, Jun Zhang, Joseph C. K. Lee, N. Magnus Hjelm, Philip J. Johnson, and Dolly P. Huang


Abstract

Recently, cell-free EBV DNA has been detected in the plasma and serum of patients with nasopharyngeal carcinoma (NPC). We studied the relationship between plasma/serum EBV DNA and tumor recurrence. Using real-time quantitative PCR, the median plasma EBV DNA concentration in 10 patients with tumor recurrence was determined to be 32,350 copies/ml, whereas in 15 patients in continuous remission for a mean period of 2 years was 0 copy/ml. Longitudinal follow-up of 17 NPC patients revealed 6 individuals with tumor recurrence and 11 patients who remained in remission. Significant elevations in serum EBV DNA, sometimes up to 6 months before detectable clinical deterioration, were observed in the patients who subsequently developed tumor recurrence. Continuously low or undetectable levels of serum EBV DNA were observed in the patients who remained in remission. These results suggest that plasma/serum cell-free EBV DNA may be a valuable tool for the monitoring of NPC patients for the early detection of tumor recurrence.

Introduction

NPC is an important cancer in Southern China and South East Asia. In Hong Kong, nearly all NPC cases are undifferentiated or poorly differentiated squamous cell carcinoma, and harbor EBV in tumor tissues. Prompted by recent reports that tumor-derived DNA is present in plasma and serum of cancer patients, Mutirangura et al. have demonstrated that cell-free EBV DNA is detectable in the plasma/serum of NPC patients. Because most of the reported data on tumor-derived DNA in the plasma and serum of cancer patients are qualitative in nature, there is little information regarding the temporal variation in the concentration of circulating tumor-derived DNA in treated cancer patients. Particularly, it is not known whether there is any difference in the patterns of variation in the levels of circulating tumor-derived DNA in patients who subsequently develop tumor recurrence and those who remain in continuous clinical remission. The recent development of a real-time quantitative PCR assay for measuring tumor-derived EBV DNA in the circulation of NPC patients has provided us with the necessary tool to address these important questions.

Materials and Methods

Patients. Patients with histologically confirmed NPCs attending the Department of Clinical Oncology at the Prince of Wales Hospital were recruited with informed consent. The study was approved by the Ethics Committee of The Chinese University of Hong Kong. All were investigated uniformly with endoscopic examination of the nasopharynx and computed tomography of the nasopharynx and neck. All patients had no clinical evidence of distant metastasis on presentation. In patients with nodally advanced disease (stage N3b, according to the American Joint Committee on Cancer/International Union Against Cancer Stage Classification; Ref. 6), abnormal liver function tests or raised serum alkaline phosphatase, thoracic computed tomography scan, liver ultrasound, and bone scintigram were also performed. All recruited patients were treated with a uniform radiotherapy protocol. The patients were assessed by nasopharyngoscopy and clinical examination at 4–6 weeks after radiotherapy, and subsequently the patients were followed-up every 8–12 weeks. Patients who developed symptoms and signs suspicious of local recurrence or metastasis would be subjected to confirmatory investigations including nasopharyngeal biopsy and imaging.

For the cross-sectional study, 10 previously treated NPC patients with an initial disease-free period, but who subsequently developed evidence of tumor recurrence, were recruited. As controls, 15 previously treated NPC patients who remained in remission for a mean duration of 2 years (range, 5 months to 5 years) were also studied. Blood samples for EBV DNA analysis were taken from each of these patients. For the longitudinal study, 17 NPC patients were recruited and followed up. Blood samples were taken immediately prior to radiotherapy and at multiple occasions after treatment. The first day of radiotherapy treatment was taken at day 0.

DNA Extraction from Plasma and Serum Samples. Plasma and serum samples were harvested from the patients in the cross-sectional and longitudinal groups, respectively, according to protocols described previously (5, 8). The plasma and serum samples were stored at −20°C until further processing. DNA from plasma/serum 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 (400–800 μl) were used for DNA extraction per column. The exact amount was documented for the calculation of the target DNA concentration. A final elution volume of 50 μl was used.

Real-Time Quantitative EBV DNA PCR. Plasma/serum EBV DNA concentrations were measured using a real-time quantitative PCR system toward the BamHI-W fragment region of the EBV genome (5). The principles of real-time quantitative PCR and detailed reaction set-up procedures were as described previously (5, 8, 9). All plasma/serum DNA samples were also subjected to real time PCR analysis for the β-globin gene (8), which served as a control for the amplifiability of plasma/serum 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 an EBV-positive cell line Namalwa (American Type Culture Collection no. CRL-1432; Ref. 10) as a standard. Namalwa was a diploid cell line (11) containing two integrated viral genomes/cell (12). A conversion factor of 6.6 pg of DNA per diploid cell was used for copy number calculation (13). Results were expressed as copies of EBV genomes/ml of plasma/serum.

Amplication data were collected using an ABI Prism 7700 Sequence Detector and were analyzed using the Sequence Detection System software developed by PE Applied Biosystems. The mean quantity of each duplicate
CIRCULATING EBV DNA AND NPC RECURRENCE

was used for further concentration calculation. The concentration expressed in copies/ml was calculated using the following equation (5):

\[ C = Q \times \frac{V_{\text{DNA}}}{V_{\text{PCR}}} \times \frac{1}{V_{\text{ex}}}, \]

where \( C \) = target concentration in plasma/serum (copies/ml), \( Q \) = target quantity (copies) determined by sequence detector in a PCR; \( V_{\text{DNA}} \) = total volume of DNA obtained following extraction, typically 50 \( \mu \)l/Qiagen extraction; \( V_{\text{PCR}} \) = volume of DNA solution used for PCR, typically 5 \( \mu \)l; \( V_{\text{ex}} \) = volume of plasma/serum extracted, typically 0.4–0.8 ml.

**Results**

**Cross-Sectional Study of NPC Patients with Tumor Recurrence and Those in Continuous Remission.** Plasma DNA from 10 NPC patients who had tumor recurrence and 15 who had remained in continuous clinical remission after radiotherapy were assayed for cell-free EBV DNA using the BamHI-W region PCR. The median EBV DNA concentration in the relapsed patients was 32,350 copies/ml (interquartile range, 0–67,420 copies/ml), whereas that in the patients in remission was 0 copy/ml (interquartile range, 0–45 copies/ml). These results are plotted in Fig. 1. The difference in EBV DNA concentrations between the individuals with tumor recurrence and those in continuous remission is statistically significant (Mann-Whitney Rank Sum Test, \( P = 0.01 \)). All plasma DNA samples were amplifiable using the \( \beta \)-globin PCR.

**Longitudinal Follow-Up of NPC Patients.** Seventeen NPC patients were recruited for longitudinal follow-up. The mean duration of follow-up was 398 days (range, 151–528 days). Serum EBV DNA was detectable in each of the 17 cases prior to radiotherapy. The median concentration of serum EBV DNA was 25,856 copies/ml (interquartile range, 3,155–77,407 copies/ml). All except one case (case 3138) showed a reduction in serum EBV DNA concentration after radiotherapy (Fig. 2). Upon follow-up, 6 patients developed evidence of tumor recurrence (Fig. 2A), whereas the remaining 11 cases stayed in continuous clinical remission (Fig. 2B). Significant elevations in serum EBV were observed in the patients who subsequently developed tumor recurrence (Fig. 2A). Continuously low or undetectable levels of serum EBV DNA were observed in the patients who remained in remission (Fig. 2B).

Correlation between serum EBV DNA levels and clinical events in the patients who developed tumor recurrence demonstrates a number of interesting features (Fig. 2A).

**Case 2808.** After radiotherapy, at day 92, the patient was in clinical remission. However, the serum EBV DNA level was still high at 1372 copies/ml. Follow-up imaging at day 180 showed bone metastasis. The serum EBV DNA level at that time was 51,894 copies/ml. By day 343, when the serum EBV DNA was 856,324 copies/ml, definite clinical signs of bone and hepatic metastases were found, with evidence of spinal cord compression. Radiotherapy was given to alleviate the cord compression, which resulted in a transient but dramatic reduction in serum EBV DNA level to 1966 copies/ml. The patient subsequently deteriorated with disseminated disease, with a serum EBV DNA level of over 11 million copies/ml at day 454.

**Case 0039.** After radiotherapy, at day 101, the patient was in clinical remission, with undetectable serum EBV DNA. The patient was found to have bone metastasis at day 216, paralleled by an increase in serum EBV DNA level to 8266 copies/ml. The patient then developed pulmonary metastasis, paralleled by an increase in serum EBV DNA level to 60,083 copies/ml at day 465.

**Case 0495.** After radiotherapy, at day 87, the patient was clinically in remission, although the serum EBV DNA level was still relatively high at 730 copies/ml. The patient remained clinically free of disease at day 151, when the serum EBV DNA level increased further to 8919 copies/ml. Subsequent clinical follow-up at day 270 revealed evidence of pulmonary and hepatic metastases.

**Case 0433.** After radiotherapy, at day 101, the patient showed dramatic reduction in serum EBV DNA level from a pretreatment level of 78,996 copies/ml to a posttreatment level of 97 copies/ml. Clinically, the patient was in remission up to day 293. However, the serum EBV DNA level gradually increased, reaching levels of 230 copies/ml at day 252 and 2196 copies/ml at day 293. By day 386, the serum EBV DNA level was 43,951 copies/ml, when the patient was found to have local recurrence.

**Case 2892.** After radiotherapy, the serum EBV DNA level reduced from a pretreatment level of 75,636 copies/ml to a lower but still high level of 2,306 copies/ml. Clinically, the patient was in remission at day 184, when the serum EBV DNA continued to increase to 9,750 copies/ml. By day 230, when the serum EBV DNA level was 22,940 copies/ml, the patient was found to have local recurrence.

**Case 3138.** After radiotherapy, the serum EBV DNA continued to increase from a pretreatment level of 102,860 copies/ml to a posttreatment level of 489,790 copies/ml. Clinically, the nasopharynx was free of disease, although a small residual cervical lymph node was palpable. Serum EBV DNA levels were persistently high at days 135 and 161. By day 209, when the serum EBV DNA level was 1,005,872 copies/ml, the patient had developed bone and hepatic metastases and had evidence of spinal cord compression.

**Discussion**

The presence of tumor-derived DNA in the plasma and serum of cancer patients opens up new possibilities for the detection and monitoring of cancer (2, 3). However, most of the published data are qualitative in nature, which do not allow detailed analysis to be made regarding the temporal relationship between the level of circulating tumoral DNA and clinical events. The recent development of real-time quantitative PCR (9) allows us to develop an accurate system for measuring tumor-derived DNA in the plasma of cancer patients, using NPC as a model system (5). Unlike conventional PCR analysis, real-time PCR does not require any postamplification manipulation and thus is more rapid and less likely to introduce PCR product contamination into the laboratory environment. For quantitative anal-
Fig. 2. Longitudinal follow-up of serum EBV DNA in NPC patients with tumor recurrence and those in continuous clinical remission. A, NPC patients who developed tumor recurrence on follow-up. The variation in serum EBV DNA levels for each patient over time is plotted in a separate graph. Key clinical events are indicated in the respective graphs. The scale of the Y axis has been optimized for the concentration range for each case. Linear scales are used throughout except for the Y axis of case 2808, which is in a common logarithmic scale to accommodate the large variations in serum EBV DNA levels. ○, serum EBV DNA level of 0 copy/ml; ●, detectable serum EBV DNA. RT, radiotherapy; CR, clinical remission; mets, metastasis. B, composite serum EBV DNA levels from 11 NPC patients who remained in continuous clinical remission. X axis, time from follow-up, with the first day of radiotherapy treatment as day 0. Y axis, serum EBV DNA level in copies/ml.
analysis, real-time PCR also has the advantages of having a high reproducibility and a large dynamic range (5, 8, 9). The availability of this powerful tool allows us to undertake a study aimed at obtaining data on the quantitative and temporal correlation between tumor-derived DNA and clinical progression in NPC.

In the first part of this study, we investigated the levels of plasma/serum EBV DNA in NPC patients with tumor recurrence and those who remained in continuous clinical remission. This cross-sectional study revealed high levels of circulating cell-free EBV DNA in many patients with tumor recurrence, with levels comparable with those observed at presentation (5). These results suggest that plasma/serum EBV DNA analysis may have potential clinical application in the detection of tumor recurrence.

The encouraging data from the cross-sectional study prompted us to proceed to study serial samples collected from a cohort of NPC patients at follow-up. For this cohort of 17 NPC patients, the median pretreatment serum EBV DNA level was 25,856 copies/ml, which was consistent with our previous data on circulating EBV DNA levels at NPC presentation (5). All, except one (case 3138), of these NPC patients showed an initial reduction in serum EBV DNA level after radiotherapy (Fig. 2). Longitudinal follow-up revealed that there was a gradual increase in serum EBV DNA concentrations in individuals who developed tumor recurrence. For individuals who remained in remission, continuously low or undetectable levels of serum EBV DNA were observed. These results were therefore entirely consistent with those obtained from the cross-sectional study.

The detailed correlation between serum EBV DNA levels and clinical events revealed that in many cases, progressive or persistent elevation in serum EBV DNA levels preceded the development of clinically detectable signs of disease recurrence or progression. For example, in case 2808, the high serum EBV DNA level at the first blood sampling after radiotherapy (day 92) preceded the imaging diagnosis of bone metastasis (day 180) by ~3 months (Fig. 2A). Similarly, in cases 4095 and 2892, the high levels of serum EBV DNA after radiotherapy were detectable 4–6 months before the development of clinical evidence of tumor recurrence (Fig. 2A). In case 0433, the gradual rise in serum EBV DNA level could be discerned at least 3 months prior to clinical evidence of local recurrence (Fig. 2A).

The mechanisms leading to the liberation of tumor-derived DNA into the circulation of cancer patients are unclear at present. Our data show that increased concentrations of EBV DNA are found during disease progression in NPC (Fig. 2A) and thus suggest that the concentration of circulating EBV DNA is related to the tumor burden. Further support for this hypothesis can be found in the sharp reduction in serum EBV DNA in most NPC patients after radiotherapy (Fig. 2).

Our study indicates that the regular assessment of plasma/serum EBV DNA levels in NPC patients after radiotherapy may contribute to an earlier detection of tumor recurrence. Future work will determine whether adjuvant treatment at the time when plasma/serum EBV DNA level is elevated in the absence of clinical evidence of disease recurrence would improve survival from NPC.

It is likely that our data may also be relevant for the monitoring of recurrence in other EBV-associated malignancies. In this regard, it is interesting to note that cell-free EBV DNA has recently been reported to be present in patients suffering from Hodgkin’s disease (14). For non-EBV-related neoplasms, the development of quantitative detection systems to other tumor-associated targets would potentially generate new tools for the early detection of tumor recurrence.

References

Quantitative and Temporal Correlation between Circulating Cell-Free Epstein-Barr Virus DNA and Tumor Recurrence in Nasopharyngeal Carcinoma

Y. M. Dennis Lo, Lisa Y. S. Chan, Anthony T. C. Chan, et al.