Epstein-Barr Virus DNA in Serum/Plasma as a Tumor Marker for Nasopharyngeal Cancer

Kanjana Shotelersuk,1 Chonlakiat Khorprasert,2 Sairoong Sakdikul, Wichai Pornthanakasem, Narin Voravud, and Apiwat Mutirangura3

Radiotherapy Section, Department of Radiology [K. S., C. K.], Genetics Unit, Department of Anatomy [S. S., A. M.], Department of Microbiology [W. P.], and Medical Oncology Unit, Department of Medicine [N. V.], Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT

Nasopharyngeal cancer (NPC) constitutes a type of carcinoma encountered frequently in Southern China, among Eskimos of the Arctic region, and to a lesser extent in Southeast Asia. Because EBV DNA present in plasma or serum of NPC patients has proven to represent a promising noninvasive tumor marker, the present study was designed to determine the incidence of serum/plasma EBV DNA by nested PCR during various disease management stages. By this method, we could detect EBV DNA in plasma/serum of 98 of 167 NPC patients prior to treatment, compared with 10 of 77 samples derived from healthy blood donors serving as controls, with a similar prevalence observed in plasma versus serum. Investigation of 13 patients subjected to radiotherapy revealed plasma EBV DNA to persist in the plasma of one case, whereas among the remaining patients, it had vanished during the early phase of treatment. Finally, with 52 samples derived from 37 NPC patients during follow-up, we established 100% specificity and 0% false-positive rate for plasma DNA detection by nested PCR. Moreover, we subjected 24 known EBV DNA-positive serum samples to DNase digestion prior to DNA extraction and amplification to differentiate between free and encapsulated viral DNA, which demonstrated complete absence of the human β-globin genomic DNA in contrast to EBV DNA detectable in 14 samples. In conclusion, applying this noninvasive method, serum/plasma EBV DNA constitutes a reliable tumor marker prior to, during, and after treatment of NPC.

INTRODUCTION

The discovery of tumor-derived DNA in the circulation of cancer patients raises the possibility of a new strategy for noninvasive cancer detection and monitoring (1–3). In NPC,4 we have previously demonstrated that EBV DNA is not only detectable in tumor tissue but is also detectable in the patients’ cell-free sera (4). In addition, comparison between EBV typing of primary tumors and their sera showed identical results, suggesting that serum EBV DNA represents tumor DNA. In a recent study, Lo et al. (5) have further explored this issue with respect to sensitivity and quantity of the plasma EBV DNA derived from NPC patients. A very high incidence of plasma EBV DNA was shown prior to treatment. Furthermore, the presence of plasma EBV DNA 1 month after completion of radiotherapy was associated with the disease persisting, either because of partial response or distant metastasis. Hence, serum/plasma EBV DNA represents a promising tumor marker for noninvasive cancer detection and monitoring of NPC.

NPC constitutes an important cancer in Asia encountered frequently in Southern China and among Eskimos of the Arctic region. An intermediate incidence is observed in Southeast Asia (6–8). Therefore, the attempt at understanding how serum/plasma EBV DNA could be used for diagnosis and monitoring of NPC is crucial. In the present study, we applied nested PCR to analyze the incidence of serum/plasma EBV DNA during several phases of NPC, prior to as well as in the course of treatment and during follow-up. In addition, we determined whether the viral DNA was encapsulated. The data presented here not only demonstrate sensitivity and specificity of serum/plasma EBV DNA in each phase of the disease but also assist in an increasing comprehension as to its biological significance.

MATERIALS AND METHODS

Sample Collection. Upon informing the patients about the purpose of the study and obtaining their consent, primary NPC tissues were collected before onset of treatment at Chulalongkorn University Hospital. The tissues were divided into two parts. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen for future DNA extraction and PCR analysis for the presence of the EBV genome. All tumors were histologically ascertained to be undifferentiated NPC according to the WHO classification.

Blood samples were obtained by venipuncture from several groups of patients selected on the grounds that the EBV genome was present in their tumor tissues. The first group, 146 serum and 21 plasma samples, comprised patients prior to treatment. The second group included plasma samples obtained from 13

Received 10/21/99; revised 12/13/99; accepted 12/14/99.

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1Supported by the Molecular Biology Project, Faculty of Medicine, Chulalongkorn University, and the Thailand Research Fund.
2The first two authors contributed equally to this work.
3To whom requests for reprints should be addressed, at Genetics Unit, Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: mapiwat@chula.ac.th.

4The abbreviations used are: NPC, nasopharyngeal cancer; EBNA, EBV nuclear antigen.
patients at the weekly complete blood count evaluation in the course of radiotherapy. The last group constituted 52 plasma samples from 37 patients, who after completion of treatment came to the hospital every 3 months for follow-up. The DNA samples extracted from the sera of healthy blood donors serving as controls in a previous study were used again for the same purpose (1). To obtain cell-free sera and plasma, clotted and EDTA blood specimens were centrifuged at low speed for 5 min within 1 h after venipuncture. Both sera and plasma samples were stored at \(-20^\circ C\) until further analysis.

**DNA Isolation and DNase Treatment.** NPC tissue was incubated in Tris/HCl buffer containing SDS and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA (9). As for serum or plasma, 200 μl were purified for DNA extraction on Qiagen columns (Qiamp blood kit; Qiagen, Basel Switzerland) according to the "blood and body fluid protocol." Ten sera and 10 plasma samples, respectively, were reanalyzed for the presence of the EBV genome to compare the efficiency of the Qiamp blood kit with that of reextracting the nucleic acid with the QIAamp viral RNA mini kit (Qiagen). One-tenth of the DNA extracted was then used for nested PCR analysis.

To distinguish free EBV DNA molecules from virions, 400 μl of 24 known positive EBV DNA serum samples were divided into two parts. The first part was twice digested extensively with DNase I (37°C for 1 h; Ref. 10). Both parts were then subjected to DNA extraction and nested EBV DNA PCR analysis. Semi-nested PCR for amplification of β-globin DNA was used to determine whether free DNA had been digested completely.

**EBV Detection by Nested PCR.** For the detection and typing of EBV DNA in tumor tissue and serum/plasma samples, nested PCR protocols were used, modifying those described previously for amplification of the EBNA-2 (11, 12). DNA extracted from the cell line B958, EBV-transformed lymphocytes (American Type Culture Collection), was used as positive control and double-distilled water as a negative control.

The first PCR amplified the EBNA-2, generating a DNA fragment of 237 bp for EBV type A and of 253 bp for EBV type B, respectively. With nested primers, the PCR product comprised 168 bp for EBV type A and 184 bp for EBV type B.

**Table 1 Incidence of serum/plasma EBV DNA during various NPC management stages**

<table>
<thead>
<tr>
<th>Diagnosis Status</th>
<th>Sample Description</th>
<th>Sample Positive</th>
<th>Total (case)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC Before treatment</td>
<td>Serum/plasma</td>
<td>98</td>
<td>167</td>
<td>58.7</td>
</tr>
<tr>
<td>NPC Serum</td>
<td>83</td>
<td>146</td>
<td>56.9</td>
<td></td>
</tr>
<tr>
<td>NPC Plasma</td>
<td>15</td>
<td>21</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td>Known positive EBV DNA in serum During radiotherapy Plasma treated with DNase</td>
<td>14</td>
<td>24</td>
<td>58.3</td>
<td></td>
</tr>
<tr>
<td>Known positive EBV DNA in serum During radiotherapy Plasma (before Rx, 1, 2, 3–6 wks)*</td>
<td>9, 6, 3, 1</td>
<td>13</td>
<td>(69, 46, 23, 7)</td>
<td></td>
</tr>
<tr>
<td>Follow-up Plasma</td>
<td>3</td>
<td>37</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>Recurrence or partial response Plasma</td>
<td>3</td>
<td>5</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Complete remission Plasma</td>
<td>0</td>
<td>32</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Normal control Serum</td>
<td>10</td>
<td>77</td>
<td>13.0</td>
<td></td>
</tr>
</tbody>
</table>

* (before Rx, 1, 2, 3–6 wks) incidence of plasma EBV DNA before treatment at first, second, and third to sixth weeks during radiotherapy.
respectively. The nucleotide sequences for these first PCR primers were 5'-GCGGGTGGAGGGAAAGG-3' (E3–44mer) and 5'-GTCAGCCAAGGGACGCG-3' (E5–25mer). The nested PCR primers were E3 and E5 primers for EBNA2 (11, 12).

Amplification of the $\beta$-globin gene by seminested PCR was used to determine the presence of amplifiable human DNA in all samples tested for EBV DNA. The primers GH20 and PCO4 were used for the first-round primary PCR, generating a DNA fragment of 260 bp (13). The $\beta$-globin-specific seminested primers were 5'-ACCTCACCCTGTGGAGCCA-3' ($\beta$-globin 62028) and PCO4, generating a 231-bp PCR product. The sequences of the primers used for EBNA-2 and $\beta$-globin PCRs were identical to those reported previously (11–13).

The first-round PCR reactions were performed in a total volume of 20 µl using one-tenth of the extracted DNA in a reaction mixture containing 200 µmol of each deoxynucleotide triphosphate, 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.5 unit of *Thermus aquaticus* DNA polymerase, and 0.2 µM for EBV or 0.5 µM for $\beta$-globin primers. The PCR amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, with an extension at 72°C for 1 min, and a final extension at 72°C for 7 min. One µl of each PCR product was used as the template for nested and seminested PCR, respectively. The 35 cycles of second-round PCR reactions were performed in a manner identical to that applied for the first-round PCR, except for using different sets of primers, 0.5 µM for EBV or 1 µM for $\beta$-globin primers, and adjusting the annealing temperature to 50°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide upon preparation.

**Statistical Analysis.** Data regarding histology, tumor staging, EBV detection, and response to treatment were collected in a double-blind fashion until analyzed. The $\chi^2$ test was used to compare the results obtained from serum/plasma analysis with clinical and tumor parameters.

### RESULTS

#### Sensitivity and Specificity of Nested PCR to Detect EBV DNA in Serum and Plasma.

In this study, nested PCR was applied to improve the sensitivity for identifying EBV DNA. For the control cell line, nested PCR for *EBNA-2* still yields a positive result with 10 fg DNA (Fig. 1). This suggests a much higher sensitivity of nested PCR. We then reevaluated sensitivity and specificity of EBV DNA detection in sera and plasma of NPC patients, as well as of normal blood donors (Table 1). Whereas higher sensitivity, 98 of 167 cases (58.68%), was demonstrated, fewer cases of normal blood donors, 10 of 77 cases (13%), showed positive results. The positive EBV DNA incidence from plasma samples, 71.4%, was slightly higher than from serum, 56.85%. Twenty previously analyzed samples, 10 serum and 10 plasma samples, were reextracted for nucleic acid by QIAamp viral RNA mini kit. With these samples, testing for the EBV genome was repeated by nested PCR and yielded identical results. There is no significant statistical correlation between serum EBV DNA and clinical parameters, staging, histological classification, or response to treatment at 3 and 6 months.

To evaluate whether the EBV DNA detected in serum was present as a free nucleic acid or within intact virions, 24 EBV DNA-positive sera from different patients were subjected to DNase I treatment prior to DNA extraction. After extensive DNase I treatment, nested PCR did not yield any detectable product of the control $\beta$-globin DNA, whereas nested PCR for EBV DNA remained positive for 14 cases (Fig. 2; Table 1).

![Fig. 2 Nested and seminested PCR from known EBV DNA-positive serum samples after DNase treatment. DNA. A 100-bp ladder, distilled H$_2$O (dH$_2$O), and DNA from B958 cell lines were used as size standard, negative control, and positive control, respectively. Lanes with (+) and without (−) DNase treatment are shown. A, seminested PCR for human $\beta$-globin. B, nested PCR for EBV DNA. All of these cases showed complete DNase treatment. Whereas cases 38 and 46 demonstrated resistant of EBV DNA from DNase, sera from cases 33, 47, and 48 were digested.](image-url)
Plasma EBV DNA during Radiotherapy. Plasma samples were collected from 13 cases, and the EBV DNA status was determined on a weekly basis in the course of radiotherapy (Fig. 3; Table 1). Five of these cases showed no serum/plasma EBV DNA prior to treatment and remained negative during treatment. With the others, serum/plasma EBV DNA was detectable prior to treatment. In seven of them, plasma EBV DNA disappeared between the first and third week of radiotherapy. One case showed persistence of plasma EBV DNA throughout the course of radiotherapy.

Sensitivity and Specificity of Plasma EBV DNA for Patients’ Follow-Up. Fifty-two blood samples of 37 patients after treatment of between 3 and 42 months duration were evaluated at follow-up for the presence of the EBV genome in plasma (Fig. 4; Table 1). Using WHO criteria for response, five cases presented with evidence of NPC, three with recurrence, and two with partial response, ≥50% decrease in total tumor size of the lesions and no appearance of new lesions or progression of any lesion. Plasma EBV DNA was detectable in three cases, one partial response, patient 365, and two recurrence cases, patients 72 and 106. Interestingly, whereas the plasma of both recurrence cases, patients 72 and 106, were positive for EBV DNA in their serum EBV DNA absent prior to treatment. Furthermore, repeated evaluation of the plasma for the EBV DNA status prior to further treatment twice in patient 106 and four times in patient 240, respectively, still showed identical results. The other 32 cases were in complete remission at the time of evaluation. Case 106R was patient 106 after the second course of radiotherapy. In the 32 plasma samples tested from all complete remission cases, some of which were examined more than once, no plasma EBV DNA was detectable. This suggested 100% specificity and 0% false positive rate, respectively, for serum/plasma EBV DNA to be used as an NPC tumor marker for follow-up after completion of treatment.

DISCUSSION

The discovery of tumor-derived DNA in the circulation of cancer patients implies the possibility of a new strategy for noninvasive cancer detection and monitoring (1–3). For NPC, using EBV DNA for this purpose should prove advantageous because virtually all cases of NPC have been found associated with EBV (14, 15). Because a comparison between two studies has shown much divergence as to the prevalence of EBV DNA in serum/plasma samples, we improved the sensitivity for further analysis by using nested PCR (4, 5). This technique should prove to be the one with the highest sensitivity. Several positive cases (13%) were identified among normal blood donors infected previously with EBV, compared with not a single case identified previously by one-round PCR or with 7% established by the quantitative PCR study. The present study revealed serum/plasma EBV DNA to be present in 58.68% of NPC cases. Although plasma samples demonstrated a slightly higher prevalence of EBV DNA, there was no significant difference of DNA identification between plasma and serum samples or between two different DNA extraction methods, Qiamp blood kit and Qiamp viral RNA mini kit. The continuous presence or absence of EBV DNA in serum/plasma was confirmed by repeatedly evaluating the plasma for EBV DNA status in two patients. The difference in prevalence of the tumor DNA in the circulation of NPC patients was similar to that reported for other types of cancer. For example, the frequency of identifying serum/plasma colorectal cancer DNA can vary between studies.
from 40 to 90% (16–18). Although differences in serum/plasma EBV DNA between EBV DNA prevalence reported by real time PCR and nested PCR may be attributable to different PCR primers and detection system. Nevertheless, as yet unsuspected biochemical and/or environmental mechanisms may be responsible for this finding.

Serum/plasma EBV DNA has been studied in several conditions, such as infectious mononucleosis, acute lymphoproliferative disorder, and EBV-associated lymphoma (19–24). In addition to the association with EBV-associated cancers, serum/plasma EBV DNA was found to be an indicator for active infection rather than latent virus (19, 21–24). The purpose of the DNase digestion experiment was to establish whether the presence of serum EBV DNA was attributable to lytic replication or release of latent episomal DNA. The results suggest that there might be two simultaneously present populations of serum EBV DNA, one encapsulated in the viral particle and the other the free nucleic acid probably released from NPC as episomal DNA. The presence of virions in the circulation of NPC patients is surprising because the majority of EBV in NPC cells should be in the latent phase, as shown by EBV clonal studies (25, 26). Consequently, serum/plasma EBV DNA should all be present as free nucleic acids. Nevertheless, our data invite the hypothesis that some EBV in NPC should enter lytic replication. This could explain why antibody titers to lytic cycle antigens, such as VCA and ZEBRA, rise in NPC patients (27, 28). In addition, expression of the immediate early BZLF1 and BHIF1 genes or ZEBRA protein is frequently detectable in NPC (29, 30). Because there may be only few cells entering lytic viral replication, studying the clonal progression of EBV by analyzing terminal repeat lengths by Southern blot and hybridization may not be sensitive enough to commonly detect the lytic replication or might be interpreted as impurities and hence ignored. Definite proof, such as electron microscopic analysis, is required to identify virions in NPC circulation.

Studying plasma EBV DNA during radiotherapy not only suggests a direction to further explore the behavior of EBV DNA present in the circulation of patients receiving radiotherapy but may also lead to clinical implications. EBV DNA positive in serum/plasma before treatment disappeared early in the course of radiotherapy, whereas plasma EBV DNA initially negative remained negative. This suggests two important findings: (a) serum/plasma EBV DNA is short lived; and (b) cell death as a consequence of radiation does not prevent the presence of EBV DNA in the patients’ circulation. On the contrary, because EBV DNA could disappear from plasma as early as during the first week of treatment, radiation may in addition to its ability to decrease the number of NPC cells use a specific mechanism that prevents the tumor from releasing EBV DNA. The biological effect of ionizing radiation at the cellular and molecular level appears to be DNA double strand break and a subsequent activation of DNA-dependent protein kinase (31, 32). It will be interesting to further explore whether DNA-dependent protein kinase plays any role in the release of EBV DNA from NPC into the patients’ circulation. Whereas most cases of previously positive serum/plasma EBV DNA disappeared during radiation, one case showed persistence of plasma EBV DNA. This NPC case may not respond to the radiation induced inhibition of virus release, or it may indicate microme-tastases. This persistence of serum/plasma EBV DNA during radiotherapy may be crucial for future clinical treatment modalities.

The other important clinical application of serum/plasma EBV DNA with NPC suggested here is as a tumor marker for patients’ follow-up. NPC is a form of cancer with a high success rate of radiochemotherapy (33). However, many cases may recur, even after very long periods of latency (34). Consequently, most NPC patients require very consistent and long follow-up studies. Some of them may require expensive investigations, such as computed tomography scan or invasive methods, especially punch biopsy. Analyzing serum/plasma EBV DNA is an inexpensive and noninvasive technique suitable for clinical application. Upon using plasma EBV DNA as a marker for follow-up, the sensitivity (60%) shown in this study was similar to the prevalence detected prior to treatment. In addition, whereas only patients with evidence of disease showed plasma EBV DNA, the DNA was not detectable in any of the cases with complete remission. This suggested 100% specificity and a 0% rate of false positives. Interestingly, whereas we detected 13% of serum EBV DNA in 77 healthy individuals, no EBV DNA was identified in 42 tests of 32 NPC cases with complete remission. The usefulness of serum/plasma EBV DNA as a molecular marker for NPC patient monitoring was emphasized recently by Lo et al. (35). They demonstrated a close relationship between plasma/serum EBV DNA quantity and tumor recurrence. In addition, significant elevations in serum EBV DNA were observed in the patients who subsequently developed tumor recurrence.

In conclusion, this study has shown how frequently serum/plasma EBV DNA can be discovered in the course of NPC prior, during, and after treatment. Approximately 59% of NPCs prior to treatment were positive for serum/plasma EBV DNA. The presence and/or absence of serum/plasma EBV DNA is likely to depend on each individual and remain persistent as long as there is no change in the tumor status. Radiotherapy cannot induce but rather prevents NPC from releasing EBV DNA into the patients’ circulation. Finally, investigating serum/plasma EBV DNA after treatment suggested its potential as a tumor marker.

ACKNOWLEDGMENTS

We are deeply indebted to the staff of the Department of Otolaryngology and the Radiotherapy Section, Department of Radiology, Chulalongkorn University Hospital and National Blood Center for the recruitment of patients and collection of materials. We also thank Petra Hirsch for critical review of the manuscript.

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