Molecular Characterization of Circulating EBV DNA in the Plasma of Nasopharyngeal Carcinoma and Lymphoma Patients

K.C. Allen Chan, Jun Zhang, Anthony T.C. Chan, Kenny I.K. Lei, Sing-Fai Leung, Lisa Y.S. Chan, Katherine C.K. Chow, and Y.M. Dennis Lo

Abstract

Despite the increasing clinical applications of circulating EBV DNA analysis as a tumor marker, the molecular nature of these EBV DNA molecules remains unclear. We subjected plasma/serum samples of nasopharyngeal carcinoma and lymphoma patients to DNase digestion and ultracentrifugation and showed that circulating EBV DNA molecules are “naked” DNA fragments instead of being contained inside virions. We further showed that these EBV DNA fragments were relatively short, and 87% of them were shorter than 181 bp. These results provide fundamental information that may improve our understanding of the release of tumor-derived nucleic acids into the blood of cancer patients.

Introduction

EBV has been associated with the development of a variety of cancers, including NPC, Burkitt’s lymphoma, HD, and NK cell lymphoma. EBV DNA could be detected not only in tumor tissues but also in the plasma/serum of NPC patients (1). Quantitative analysis of plasma/serum EBV DNA levels has been shown to be clinically important in the diagnosis (2) and monitoring of NPC (3) and EBV-associated lymphoid malignancy patients (4, 5). This approach has also been found to be useful for evaluating the efficacy of cancer treatment and the prognosis of NPC patients (6). However, little information is available regarding the molecular characteristics of circulating cell-free EBV DNA. Regarding this issue, two groups have demonstrated that circulating cell-free EBV DNA in HD patients and NPC patients had different susceptibility to DNase digestion (7, 8). Their findings have suggested that circulating EBV DNA may exist in different forms in different clinical contexts. However, these studies are qualitative in nature. There are also reports suggesting the presence of active viral replication in the tumor tissues of NPC (9, 10). These results have prompted us to further investigate whether such circulating EBV DNA molecules exist as EBV DNA fragments and/or intact virions in different types of cancers by the use of real-time quantitative PCR assay. Moreover, we have also studied the size distribution of circulating EBV DNA using qualitative and quantitative PCR assays.

Materials and Methods

Patients. Forty-nine patients with NPC and 24 patients with HD or NK cell lymphoma, all histologically confirmed, were recruited for this study with informed consent before commencement of oncological treatment. Approval of the study was obtained from the Clinical Research Ethics Committee of the authors’ affiliated institution.

Collection and Processing of Blood Samples. Peripheral venous blood (5–10 ml) was taken from the patients into an EDTA-containing tube for the isolation of plasma and a plain tube for the isolation of serum. All blood samples were centrifuged at 1,600 × g for 10 min. For clotted blood samples, serum samples were then collected and used for further processing. For EDTA/blood, plasma samples were then microcentrifuged at 16,000 × g for 10 min and filtered through a 0.22-µm filter (Milllex-GV; Millipore) to ensure the complete removal of cells.

Ultracentrifugation Analysis of Plasma EBV DNA. To examine whether plasma EBV DNA was pelletable, the filtered plasma samples (300–1000 µl) from 21 NPC and 18 NK cell lymphoma/HD patients were divided into two portions. One portion was ultracentrifuged at 70,000 × g for 2 h (11). Supernatant (200–800 µl) was used for DNA extraction. The pellet was resuspended in 300-1000 µl of PBS, followed by ultracentrifugation at 70,000 × g for 2 h. The supernatant was then discarded, and the pellet was resuspended in 300-1000 µl of PBS for DNA extraction. Another portion (200–800 µl) was used directly for DNA extraction.

As controls for the ultracentrifugation analysis, EBV particles (American Type Culture Collection no. VR1492) and EBV DNA extracted from the plasma of NPC patients were spiked into umbilical cord blood plasma. The cord blood plasma samples with virions and extracted EBV DNA molecules were subjected to ultracentrifugation as described above. Under such centrifugation condition, virus particles should be pelleted down, whereas EBV DNA molecules should remain in the supernatant. The resulting supernatant and pellet were used for DNA extraction.

DNase Treatment. To further distinguish cell-free circulating EBV DNA fragments from EBV particles, 400 µl of serum samples from 18 NPC patients and 10 NK cell lymphoma/HD patients were digested by DNase I (Invitrogen) at 37°C for 12 h. As controls, the supernatant of the B95–8 cell line was harvested and subjected to ultracentrifugation to separate the resulting supernatant and pellet. Afterward, DNase I was added separately into the resulting supernatant and pellet and then incubated at 37°C for 12 h. The pellet was expected to be resistant to DNase I digestion because it contained virions, whereas the resulting supernatant with only cell-free EBV DNA molecules should be digested by DNase I (7). All digested and undigested samples were stored at −20°C until DNA extraction.

DNA Extraction. DNA from plasma, serum, and other forms of samples was extracted using a QIAamp Blood Kit (Qiagen) as described previously (2).

Real-time Quantitative EBV DNA PCR. DNA samples resulted from the above experiments were then quantified for EBV DNA using a real-time quantitative PCR system toward the BamHI-W fragment region of the EBV genome as described previously (2). Different portions of DNA samples were also subjected to real-time PCR analysis for the β-globin gene, which served as a control for the amplifiability of DNA. The compositions and conditions of the PCR assay were as described previously (2).

Size Determination by Conventional PCR Analysis. For the determination of the size of EBV DNA fragments in the plasma of cancer patients, 10 PCR assays were developed for the amplification of different-sized amplicons...
in the region coding for EBERs. These PCR systems consisted of 10 different forward primers and 1 common reverse primer. The sequences of the primers and corresponding amplicon sizes are listed in Table 1. PCR amplifications were set up in a total volume of 50 μl by the use of reagents supplied in a GeneAmp DNA Amplification Kit (Applied Biosystems) with the AmpliTaq Gold polymerase. Each reaction contained 5 μl of plasma DNA sample, 5 μl of 10 mM buffer II, 1.5 mM MgCl₂, 200 μM each deoxynucleoside triphosphate, 1 μM each primer, and 2 units of AmpliTaq Gold polymerase. The amplification was carried out in a thermocycler (PTC-200 DNA Engine Thermal Cycler; MJ Research).

The thermal profile consisted of an initial denaturation step of 95°C for 12 min followed by 50 repetitive cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 10 min. DNA extracted from umbilical cord blood and the Namalwa cell line was used as negative and positive controls, respectively. Ten microliters of each PCR product were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. To evaluate the sensitivities of these PCR assays, serial 10-fold dilutions of DNA extracted from the Namalwa cell line, ranging from 100 to 0.001 ng, were used for amplification. DNA samples from the plasma of 5 lymphoma and 10 NPC patients were tested using these PCR assays.

Quantitative Size Analysis of Plasma EBV DNA Fragments. In later experiments, multiple real-time quantitative PCR assays were used to determine the amounts of different-sized plasma EBV DNA fragments. In addition to the primers mentioned above, we designed one common TaqMan minor-groove-binding probe used for these PCR assays. The sequence of the probe is listed in Table 1. PCR reactions were also set up in a reaction volume of 50 μl by the use of components (except the TaqMan probe and amplification primers) supplied in a TaqMan PCR Core Reagent Kit (Applied Biosystems). Each reaction contained 5 μl of 10 mM buffer II, 4 mM MgCl₂, 200 μM each of dATP, dCTP, and dGTP; 400 μM dUTP; 1 μM each primer; 500 μM TaqMan probe; 2 units of AmpliTaq Gold polymerase; 0.5 unit of AmplErase uracil-N-glycosylase; and 5% DMSO. Five microliters of plasma DNA were used for amplification. Each of the real-time quantitative PCR was performed in duplicate in an Applied Biosystems 7700 Sequencer Detector (Applied Biosystems). Amplification data were collected and analyzed by the Sequence Detection System Software (Ver. 1.9; Applied Biosystems).

An identical thermal profile was used for all PCR systems. After initial incubation at 50°C for 2 min and 95°C for 10 min, 50 cycles of thermal cycling at 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min were carried out. A standard curve composed of serial 10-fold dilutions of Namalwa DNA (12–14)–1 copies was run for each assay and in duplicate. Multiple negative water blanks were included in every analysis. The plasma concentrations of the different-sized EBV DNA fragments were expressed as copies/milliliter. The amounts of different-sized EBV DNA fragments in the plasma of 31 NPC and 8 NK cell lymphoma/HD patients were measured using these quantitative PCR assays.

Results

**DNase I Digestion.** EBV DNA could be detected in all plasma samples of the NPC and NK cell lymphoma/HD patients before DNase digestion. The median concentration of plasma EBV DNA of NPC and lymphoma patients was 721 copies/ml (interquartile range: 199–4476 copies/ml) and 75 copies/ml (interquartile range: 40–724 copies/ml), respectively. After digestion with DNase I for 12 h, EBV DNA, as well as the β-globin gene, could not be detected in any of the 18 NPC patients’ serum samples, 10 lymphoma patients’ serum sample, or the supernatant of the B95-8 cell suspension. As a control, EBV DNA could be amplified from the pellet of the B95-8 cell suspension.

**Ultracentrifugation.** The effect of ultracentrifugation on the plasma EBV DNA in NPC and NK cell lymphoma/HD patients is presented in Fig. 1. The relative concentration is calculated by dividing the actual EBV DNA concentration of either the supernatant or pellet by that of the uncentrifuged sample of the corresponding patient. Spiking of intact EBV virions and extracted EBV DNA into cord plasma was performed as controls. After ultracentrifugation, most of the intact virions spiked could be pelleted down, whereas the spiked EBV DNA remained in the supernatant. The median relative concentrations of EBV DNA in the pellet were 0.86 (interquartile range: 0.76–1.05) and 0.78 (interquartile range: 0.67–0.93) for NPC and NK cell lymphoma/HD patients, respectively. The median relative concentrations of EBV DNA in the supernatant were 0.01 (interquartile range: 0.00–0.01) and 0.01 (interquartile range: 0.00–0.05) for NPC and NK cell lymphoma/HD patients, respectively.

**Size Determination by Conventional PCR.** PCR systems with different sets of primers were designed for the amplification of different-sized PCR products in the region encoding EBERs. DNA extracted from the plasma of NPC and NK cell lymphoma/HD patients was subjected to PCR for EBV DNA. The PCR reactions were optimized such that they were able to detect as little as 30 copies of EBV DNA. Results from representative cases are shown on Fig. 2. A progressive decrease in band intensity was noticed with the increase in sizes of the ampiclons. In most of the cases, no PCR products could be visualized when the ampiclon sizes were >500 bp.

**Quantitative PCR for Different-sized Amplicons.** Real-time PCR assays with amplicons sized 82–382 bp were able to detect one copy of EBV DNA, whereas those with amplicons sized 493–1000 bp were able to detect 10 copies of EBV DNA. As the EBV DNA concentrations differed by up to six orders of magnitude in different patients, the relative concentration for each amplicon size was calculated such that the size distribution of EBV DNA in the patients can be compared and summarized. The relative concentration was calculated by dividing the absolute concentration of EBV DNA determined by a PCR system of a particular amplicon size by the absolute concentration of EBV DNA determined in the PCR system with the 82-bp amplicon (the shortest amplicon used). The size distribution of plasma EBV DNA of NPC patients is shown in Fig. 3A. As the

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Forward/reverse probe</th>
<th>Amplicon size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
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<td>EBERS2F</td>
<td>5'-GAGGAGTTCCCGCCCTAGA-3'</td>
<td>Forward</td>
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<td>58</td>
</tr>
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<td>5'-TACACCAAAAAGAGACGCGGT-3'</td>
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<td>58</td>
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<td>EBERS294F</td>
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<td>Forward</td>
<td>294</td>
<td>58</td>
</tr>
<tr>
<td>EBERS385F</td>
<td>5'-GAATTTGTTCAAGCCGCTCCT-3'</td>
<td>Forward</td>
<td>385</td>
<td>58</td>
</tr>
<tr>
<td>EBERS493F</td>
<td>5'-TACAGCAGCCTTATGAGAAACCC-3'</td>
<td>Forward</td>
<td>493</td>
<td>58</td>
</tr>
<tr>
<td>EBERS584F</td>
<td>5'-ACAGGAGCTGATGAGAAAGG-3'</td>
<td>Forward</td>
<td>584</td>
<td>58</td>
</tr>
<tr>
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<tr>
<td>EBERS1000F</td>
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<td>Forward</td>
<td>1000</td>
<td>64</td>
</tr>
<tr>
<td>EBER-R</td>
<td>5'-AAATAGGCGAGCAGCGGAAAAT-3'</td>
<td>Reverse</td>
<td>Probes</td>
<td>Probes</td>
</tr>
<tr>
<td>EBERU3T</td>
<td>5'- (FAM) TCTCGAGAGGATTAGA (MGBNFQ) -3'</td>
<td>Reverse</td>
<td>Probes</td>
<td>Probes</td>
</tr>
</tbody>
</table>

* A The annealing temperature of the reverse primer and probe corresponds to the annealing temperature of the forward primer used.

**Table 1** List of primers, probes, and their corresponding annealing temperatures for both conventional and real-time quantitative PCR.

FAM, 6-carboxyfluorescein; MGBNFQ, minor groove binding nonfluorescent quencher.
sensitivities of assays with different amplicon sizes were different, the calculation of the median relative concentration was based on those cases with detectable EBV DNA only. The reason of adopting this approach is presented in the “Discussion” section. The median relative concentrations were 13, 9.6, 2.8, and 0.9% using primers with amplicon sizes 181, 294, 385, and 493 bp, respectively. The size distribution of plasma EBV DNA of each NK cell lymphoma/HD patient is plotted in Fig. 3B. The median relative concentrations using primers with amplicon size 181 bp was 17.1%. Although <3 patients showed detectable levels of EBV DNA when the amplicon size was increased to 294 bp, the median relative concentrations with amplicon sizes longer than 294 bp were not calculated.

Discussion

Circulating EBV DNA has been shown to be a valuable diagnostic and prognostic marker for several EBV-associated malignancies, in particular, NPC and lymphoma (2, 3, 5, 6, 15). However, the precise molecular nature of plasma EBV DNA remains unclear. Several groups have suggested that active viral replication may occur in cancers associated with EBV infection, e.g., Feng et al. (9) have shown that mRNA of the immediate early lytic gene, BRLF1, could be detected in NPC biopsies. Furthermore, Trumper et al. (10) have reported that infectious EBV particles could be isolated from the tumor cells of NPC. Furthermore, antiviral agents have been shown to retard NPC tumor growth in animal studies (16). Thus, it would be logical to investigate if plasma EBV DNA might exist, at least in part, as virions.

To investigate the nature of circulating EBV DNA, Shotelersuk et al. (8) and Gallagher et al. (7) have subjected the serum of NPC and HD patients, respectively, to DNase digestion and showed that EBV DNA could still be detected in the serum of half of the NPC patients but in none of the NK cell lymphoma/HD patients’ sera. Their results have suggested that circulating EBV DNA molecules in the serum of NPC patients are contained inside virions, whereas those present in the serum of lymphoma patients exist as DNA fragments. However, their discrepancies might be attributable to the fact that EBV DNA concentrations in lymphoma patients were much lower than those of NPC patients. Thus, in this study, we determined the serum EBV DNA concentrations for each patient before and after DNase digestion and incorporated controls of viral DNA fragments and intact virions. The concentrations of EBV DNA in these controls were comparable with EBV DNA concentrations of the NPC patients’ sera. We have shown that serum EBV DNA could be completely digested by DNase in all NPC patients. Our observations suggested that circulating EBV DNA molecules were “naked” DNA fragments and not protected by viral protein coat, which would be expected to protect such particles. To confirm these data, we further subjected the plasma of NPC and lymphoma patients to ultracentrifugation. After ultracentrifugation, a
median of 86 and 78% EBV DNA remained in the supernatant for NPC and lymphoma patients, respectively, whereas a median of 0 and 1% of EBV DNA was pelleted down. This observation was identical to results obtained when extracted EBV DNA was spiked into cord plasma and then subjected to ultracentrifugation. In contrast, virions spiked into cord plasma could be pelleted down after ultracentrifugation. Our observations support the hypothesis that circulating EBV DNA in NPC, as well as lymphoma, patients are DNA fragments instead of being contained inside intact viral particles.

We further determined the size distribution of these circulating EBV DNA fragments. Firstly, we performed preliminary analysis and showed that when the lengths of the amplicons were increased, the band intensities of PCR products, using DNA extracted from the plasma of NPC and lymphoma patients as template, decreased. On the other hand, the signal intensities using EBV DNA extracted from the Namalwa cell line as template were relatively constant. This observation suggests that the plasma EBV DNA mainly consists of short DNA fragments. We have therefore proceeded to perform quantitative analysis for plasma EBV DNA with different-sized amplicons and shown that the concentration of detectable EBV DNA dropped by a median of 87% when the amplicon size was increased from 82 to 181 bp. Moreover, <1% (median value) of the EBV DNA molecules could be detected when the amplicon size was increased to beyond 493 bp. When determining the median relative concentrations, we only included those cases with detectable EBV DNA. This approach could avoid potentially biasing the median proportion toward zero when the concentrations of cases with low EBV DNA concentrations fell below the detection limit, e.g., assuming that 10% of the DNA molecules are longer than 500 bp, if the absolute concentration of the DNA fell below the detection limit of the assay for 500 bp, the relative concentration calculated would be 0%, instead of 10%. Furthermore, we generated independent calibration curves for the quantification of the EBV DNA for different-sized amplicons. This approach could eliminate the potential errors in estimating the EBV DNA concentrations caused by the difference in the efficiencies between different PCR assays. Therefore, our observations should indicate that most of the circulating EBV DNA molecules are 180 bp in length, and long EBV DNA fragments are scarce in the plasma of NPC and NK cell lymphoma/HD patients. Our observations are also in line with the findings of Mutirangura et al. (1) and Jahr et al. (17), who have demonstrated previously an apparent relationship between circulating DNA and apoptosis of tumor cells, because, during apoptosis, DNA is fragmented by caspase-activated DNase and, thus, resulting in DNA fragment lengths in multiples of nucleosomal DNA (18), which is ~180 bp. However, whether EBV DNA would be degraded in the same pattern as human nuclear DNA during apoptosis has not been
by using an amplicon sized 82 bp. The expressed as the percentage of the concentration of the corresponding patients determined size. A marked decrease in EBV DNA concentrations was observed when the amplicon concentrations of plasma EBV DNA in NK cell lymphoma/HD patients against amplicon the 75, 50, and 25 percentiles of the fractional concentrations of the NPC patients.

Y

bp);

Solid circles,

bottom bars

DNA concentration is listed below the box plot of each amplicon size. The top, middle, and bottom bars of each box represent the 75, 50, and 25 percentiles of the fractional concentrations of plasma EBV DNA in NPC patients.

B

NK cell lymphoma/HD patients

Fractional concentration of EBV DNA (relative to the 82 bp amplicon)

Fractional concentration of EBV DNA (relative to the 82 bp amplicon)

Fig. 3. A. plot of fractional concentrations of plasma EBV DNA in NPC patients against amplicon size. The most significant drop in EBV DNA concentrations was noticed when the amplicon size was increased from 82 to 181 bp. The median fractional EBV DNA concentration is listed below the box plot of each amplicon size. The top, middle, and bottom bars of each box represent the 75, 50, and 25 percentiles of the fractional concentrations of the NPC patients. Solid circles, outliers; X-axis, size of amplicons (in bp); Y-axis, fractional concentration of plasma EBV DNA. B. plot of the fractional concentrations of plasma EBV DNA in NK cell lymphoma/HD patients against amplicon size. A marked decrease in EBV DNA concentrations was observed when the amplicon size was increased from 82 to 181 bp. X-axis, size of amplicons (in bp); Y-axis, fractional concentration of EBV DNA. The fractional concentration for each amplicon size was expressed as the percentage of the concentration of the corresponding patients determined by using an amplicon sized 82 bp. The top, middle, and bottom bars of each box represent the 75, 50, and 25 percentiles of the fractional concentrations of the NPC patients.

addressed. However, it has been shown previously that EBV DNA is organized in nucleosomes in latently infected cells (19, 20), and therefore, it is logical to deduce that EBV DNA would be degraded by caspase-activated DNase in a similar pattern as genomic DNA during apoptosis. Taking all together, our observation of the presence of short EBV DNA fragments in the plasma of NPC and NK cell lymphoma/HD patients supports the hypothesis that these EBV DNA molecules are released into the circulation by apoptosis of cancer cells instead of active viral replication. However, other mechanisms that may account for the short fragment size of circulating EBV DNA are still theoretically possible, e.g., DNase and endogenous free radicals present in the plasma may damage or shorten the EBV DNA fragments present in the plasma. A systematic evaluation of these mechanisms would be an interesting avenue of further research.

Acknowledgments

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References


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