Quantitative Analysis of the Transrenal Excretion of Circulating EBV DNA in Nasopharyngeal Carcinoma Patients

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Abstract

Purpose: The existence of transrenal clearance of circulating cell-free DNA is controversial. In this study, we used NPC as a model to investigate if circulating EBV DNA can be excreted into urine and to quantify the contribution of renal excretion to the clearance of plasma EBV DNA. Experimental Design: Quantitative analysis of urine EBV DNA was done for 74 NPC patients using real-time PCR with two different amplicon sizes. The urine concentration of EBV DNA was expressed as copies per millimole of creatinine (copies/mmol Cr) to minimize the effects of interindividual variations in hydration status. Results: EBV DNA was detectable in the urine of 56% NPC patients using a 59-bp real-time PCR assay. The median urine EBV DNA concentrations measured by the 59- and 76-bp assays were 7,040 and 290 copies/mmol Cr, respectively. Patients with detectable urine EBV DNA had significantly higher plasma concentrations, with a positive correlation between the plasma and urine concentrations of EBV DNA. The fraction of plasma EBV DNA excreted into the urine was 0.0026% of that for creatinine. Conclusions: We have shown that circulating EBV DNA can be excreted transrenally into urine in NPC patient and the fraction of excretion is negatively associated with the size of the DNA molecules. Because there is a positive correlation between plasma and urine EBV DNA concentration, urine EBV DNA analysis may potentially be applicable as an ultra-noninvasive test for the monitoring and prognostication of NPC patients.

Tumor-derived DNA sequences have been detected in the circulation of patients suffering from a wide variety of cancers (1). These tumoral DNA sequences have been further shown to be rapidly eliminated from the plasma or serum of cancer patients after tumor resection or other cancer treatments (2, 3). However, the precise mechanism involved in the clearance of circulating DNA remains unclear. One possible mechanism is the transrenal excretion of circulating DNA into urine. In this regard, previous studies on the detection of transrenally excreted DNA have produced contradictory results (4–7). The phenomenon of transrenal excretion of circulating DNA in human subjects was first described by Botetatu et al. (4) who showed the presence of Y-chromosome sequences in the urine of pregnant women carrying male fetuses and the presence of KRAS mutations in the urine of patients suffering from pancreatic and colorectal cancers. However, several other groups subsequently reported that Y-chromosome sequences were not detectable in the urine of any pregnant women carrying male fetuses, even under conditions known to increase kidney permeability (5, 7). For the detection of tumoral DNA in urine, most other reports focused on urologic cancers (8, 9), which involve the direct release of tumoral DNA into urine instead of addressing the possible transrenal excretion of circulating DNA. Interestingly, in a previous report, KRAS mutation was detected in the urine of some colorectal cancer patients who did not exhibit the corresponding mutation in plasma (10). This observation has raised the possibility of urine contamination by fecal DNA instead of the phenomenon of transrenal DNA excretion.

In this study, we quantitatively analyzed the plasma and urine samples of nasopharyngeal carcinoma (NPC) patients for EBV DNA. For the demonstration of the phenomenon of transrenal excretion of circulating DNA, the present model has several advantages over the models used in previous studies (4, 5). First, EBV DNA sequences are present at relatively high concentrations in the plasma of NPC patients, which would make the detection in urine more robust. In contrast, fetal DNA and mutations or aberrant methylation of tumor suppressor genes are present at much lower concentrations in the plasma of pregnant women and cancer patients, respectively (11, 12), which make the detection of these sequences in urine much more technically challenging. Moreover, the high concentration of circulating EBV DNA allows the quantitative analysis of the contribution of renal excretion on the clearance of circulating DNA. Furthermore, because circulating EBV DNA is predominantly produced by the tumor tissues in NPC patients (13), its analysis is less susceptible to contamination from nonrenal sources. On the
**Translational Relevance**

Nasopharyngeal carcinoma (NPC) is one of the commonest cancers in Southern China. The gold standard for diagnosing NPC is by endoscopic examination of the nasopharynx and histologic examination of suspicious lesions. However, this approach is costly and would lead to discomfort and anxiety to the patients. Therefore, biomarkers have been developed for the detection and monitoring of NPC. Among different NPC biomarkers, plasma EBV DNA analysis has been shown to be clinically useful in all aspects of NPC management, including detection, monitoring, and prognostication of NPC. In the present study, we have shown that EBV DNA is also detectable in the urine of NPC patients. Furthermore, the level of urine EBV DNA is shown to be positively correlated to its plasma level, which has previously been shown to reflect tumor load in NPC patients. Therefore, urine EBV DNA analysis represents an ultra-noninvasive approach for the detection and monitoring of NPC. Because of its noninvasive nature, it is particularly useful for close monitoring of treatment progress. Biologically, this study has confirmed the phenomenon of transrenal excretion of circulating tumoral DNA and has paved the development of other urine tumor markers.

**Materials and Methods**

**Study subjects.** Seventy-four newly diagnosed NPC patients were recruited with informed consent from the Department of Clinical Oncology, Prince of Wales Hospital, Hong Kong. Five milliliters of peripheral venous blood and 30 mL of urine were collected from each patient before treatment. Seventy healthy volunteers were recruited as controls. These latter individuals were free of nasal symptoms and were not taking any steroid or immunosuppressive agents. The median ages of the NPC patients and control subjects were 49 y (interquartile range, 42-57 y) and 44 y (interquartile range, 30-52 y), respectively. Sixty (81%) NPC patients and 53 (76%) control subjects were male. Nineteen (26%) NPC patients had early-stage disease (stages I and II) and 55 (74%) had advanced stage disease (stages III and IV).

**Plasma samples processing.** DNA was extracted from 800 μL of freshly prepared plasma sample using the QIAamp mini kit (Qiagen) after centrifugation and eluted with 50 μL of H2O (13, 15).

**Urine samples processing.** Six hundred milliliters of EDTA were added to 30-mL freshly collected urine. The urine sample was then centrifuged at 6,000 × g for 10 min at 4°C and filtered through a 0.45-μm filter (Millipore). Fifteen milliliters of guanidine thiocyanate were mixed with 10-mL urine. DNA was then extracted from the mixture using the Wizard Plus MiniPreps DNA Purification System (Promega) and eluted with 100-μL H2O. An aliquot of each untreated urine sample was sent for analysis of creatinine concentration, microscopy examination, and culture. For all the NPC patients and healthy control subjects, the urine microscopy examination did not reveal any abnormality. All urine culture results were negative.

**Real-time quantification of EBV DNA.** To investigate the effect of DNA size on its transrenal excretion efficiency, two real-time PCR assays with different amplicon sizes were used in this study. Both assays targeted the W fragment of the EBV genome and the amplicon sizes of the two assays were 59 and 76 bp. PCR reactions were set up in a reaction volume of 50 μL with reagents (except the probes and primers) supplied in a TaqMan PCR Core Reagent Kit (Applied Biosystems). The two assays shared the same forward primer (5′-CTTTAGGACCTTGCCAGGG-3′) and fluorescent probe (5′-FAM-CAGACACTACACACACC-CACCCGCTCT-MGB-3′). FAM and MGB represent 5-methylfluorescein and the proprietary MGB quencher (Applied Biosystems), respectively. The reverse primers for the 59- and 76-bp assays were 5′-CCCAGGCA-CACACTACACACA-3′ and 5′-CCCACACATCCCACAC-3′, respectively. The reaction conditions and thermal profiles of the two assays were identical. Each reaction contained 5 μL of 10× TaqMan Buffer A; 4 mmol/L MgCl2; 200 μmol/L each of dATP, dCTP, and dGTP; 400 μmol/L of dUTP; 300 μmol/L of each primer; 50 μmol/L of TaqMan probe; 1.25 units of AmpliTaq Gold DNA polymerase; and 0.5 unit of AmpErase UNG. Five microliters of plasma DNA or 10 μL of urine DNA were used as template. The thermal profile was 50°C for 2 min and 95°C for 10 min, 40 cycles of 95°C for 30 s, and 56°C for 1 min (16). Serially diluted Namalwa DNA from 10,000 copies to 10 copies per reaction was used as calibration standards. All analyses were done in duplicate and the means were used for further analysis.

**Calculation of urine EBV DNA concentrations and fractional excretion of EBV DNA.** Because the absolute concentration of EBV DNA in a urine sample is dependent on the hydration status of the patient, the urine EBV DNA concentration was corrected with the urine concentration of creatinine and was expressed as copies per millimole of creatinine (copies/mmol Cr). Creatinine is produced at a constant rate by muscle cells and all creatinine filtered through glomeruli is excreted in urine. Therefore, the expression of urine EBV DNA concentration as per millimole of creatinine would minimize the variation of urine EBV DNA concentration arising from the difference in hydration status of the patients.

**Fig. 1.** Urine concentrations of EBV DNA measured by the 59- and 76-bp assays. The 59-bp assay was more sensitive than the 76-bp assay for the detection of urine EBV DNA, and the urine EBV DNA concentrations measured by the 59-bp assay were significantly higher than those measured by the 76-bp assay (P < 0.001, Wilcoxon test).
The fraction of plasma EBV DNA excreted \( F_{\text{EBV}} \) was expressed as a percentage of the clearance rate of creatinine and was calculated according to the following formula:

\[
F_{\text{EBV}} = \left( \frac{U_{\text{EBV}} \times P_{\text{Cr}}}{P_{\text{EBV}} \times U_{\text{Cr}}} \right) \times 100\%
\]

where \( U_{\text{EBV}} \) is the urine concentration of EBV DNA; \( P_{\text{EBV}} \) is the plasma concentration of EBV DNA; \( U_{\text{Cr}} \) is the urine concentration of creatinine; and \( P_{\text{Cr}} \) is the plasma concentration of creatinine.

Because creatinine is freely filtered with plasma at the glomerular membrane and is not reabsorbed at the kidney tubules, the clearance rate of creatinine would reflect the glomerular filtration rate. The fractional excretion of plasma EBV DNA in relation to creatinine would reflect the percentage of plasma EBV DNA being filtered through the glomerular membrane and passed into urine. A lower fraction than 1 indicates that either the EBV DNA is less permeable to the glomerular membrane or the filtered EBV DNA is reabsorbed in the renal tubules.

**Statistical analysis.** Statistical tests were done using the SigmaStat 3.0 (SPSS, Inc.) and MedCalc (MedCalc) software packages.

**Results**

**Detection of EBV DNA in the urine of NPC patients.** EBV DNA was detectable in the urine of 42 (57%) and 21 (28%) patients using the 59- and 76-bp assays, respectively. All the 21 patients who showed positive results with the 76-bp assay also had detectable urine EBV DNA using the 59-bp assay. In these 21 patients, the median urine EBV DNA concentrations measured by the 59- and 76-bp assays were 7,040 and 290 copies/mL, respectively. The median urine EBV DNA concentrations for all patients measured by the two assays were 7,040 and 290 copies/mL, respectively.

**Plasma EBV DNA concentrations in NPC patients.** EBV DNA was detectable in the plasma of 74 (99%) and 72 (97%) of the 74 NPC patients using the 59- and 76-bp PCR assays, respectively. The median plasma EBV DNA concentrations of all patients measured by the two assays were 5,560 and 2,220 copies/mL, respectively. Because the detection rate of the 59-bp assay was superior for urine and plasma EBV DNA, the results of this assay were used for the analyses on the relationship between the plasma and urine concentrations of EBV DNA. Using the 59-bp assay, the median plasma EBV DNA concentrations for patients with and without detectable urine EBV DNA were 12,800 and 2,350 copies/mL, respectively. Patients with detectable urine EBV DNA had significantly higher plasma levels \( (P < 0.001, \text{Mann-Whitney test; Fig. 2}) \).

**For the 42 NPC patients with detectable urine EBV DNA using the 59-bp assay, there was a significant positive correlation between their plasma and urine EBV DNA concentrations \( (R = 0.49, P = 0.001, \text{Spearman correlation; Fig. 3}) \).**

**Ratio of large and small EBV DNA in plasma and urine.** For each of the 21 patients who had detectable urine EBV DNA by both the 76- and 59-bp assays, the ratios of the concentrations measured by these two assays (59 bp/76 bp) were calculated for the urine and plasma samples. The 59 bp/76 bp ratios were significantly higher for the urine than for the plasma samples (median, 16 versus 2.0; \( P < 0.01, \text{Wilcoxon test; Fig. 4} \)).

**Fractional excretion of EBV DNA.** The fraction of plasma EBV DNA excreted transrenal was calculated for all patients who have detectable EBV DNA in the urine. Using the 59-bp assay, the median fraction of plasma EBV DNA excreted transrenally was 0.0026% (interquartile range, 0.00057-0.0058%) of the clearance of creatinine. Using the 76-bp assay, the median fraction of plasma EBV DNA excreted transrenally was 0.00018% (interquartile range, 0.000049-0.00028%) of the clearance of creatinine.

**Plasma and urine EBV DNA detection in healthy control subjects.** EBV DNA was detectable in the urine of 3 (4%) of the 70 healthy control subjects. Their urine EBV DNA concentrations were 1,650, 30,800, and 113 \( \times 10^6 \) copies/mmol Cr using the 59-bp assay. The concentrations measured by the 76-bp assay were 1,100, 34,400, and 96.4 \( \times 10^6 \) copies/mmol Cr, respectively. The 59 bp/76 bp ratios for these three urine samples were 1.50, 0.89, and 1.17, respectively. Interestingly, EBV DNA was not detectable in the plasma of these three subjects. EBV DNA was detectable in the plasma of another 3 (4%) control subjects and their plasma EBV DNA concentrations were 56, 57, and 73 copies/mL. EBV DNA was not detectable in their urine.

**Fig. 2.** Plasma EBV DNA concentrations for patients with detectable and undetectable urine EBV DNA. The results were measured by the 59-bp assay. Patients with detectable urine EBV DNA had significantly higher plasma EBV DNA concentration than those with undetectable urine EBV DNA.

**Fig. 3.** Correlation between the plasma and urine concentrations of EBV DNA in the 42 patients with detectable EBV DNA in urine. The results were measured by the 59-bp assay. There was a significant positive correlation between the plasma and urine EBV DNA levels.
In other words, the urine EBV DNA molecules were more fragmented. This indicates that a higher proportion of the EBV DNA in the urine were <76 bp. Significantly higher for the urine samples when compared with the plasma samples. The 59 bp/76 bp ratios for the urine and plasma samples were 16 and 2, respectively, indicating that a much higher proportion of urine DNA was <76 bp when compared with plasma DNA. These observations are consistent with the previous reports that the size of a molecule is the most important parameter to determine its permeability at the kidney barrier (19) and smaller molecules are more likely to be filtered through the glomeruli (19). These observations may explain why the previous studies using PCR assays with longer amplicons were unable to detect the target DNA sequences in urine (5, 7).

Diagnostically, the detection of urine EBV DNA can potentially be applied for monitoring the treatment progress of NPC patients. Plasma levels of EBV DNA before and after radiotherapy have previously been shown to be independent prognostic factors for disease-free survival in NPC patients (20, 21). In this study, we showed that patients with detectable urine EBV DNA had significantly higher plasma levels and there was a positive correlation between the plasma and urine concentrations. Therefore, it is logical to investigate if urine EBV DNA analysis can also be used for the monitoring and prognostication of NPC patients. Although the detection of EBV DNA in urine is not as sensitive as plasma EBV DNA analysis, urine analysis represents an ultra-noninvasive approach that can be useful for the frequent monitoring of treatment response. Moreover, the sensitivity of urine EBV DNA analysis may potentially be enhanced by using assays with extremely short amplicons.

Interestingly, EBV DNA was detectable in the urine of 3 (4%) healthy subjects. Comparing with the NPC patients, the urine EBV DNA in these three healthy subjects were more intact. Their urine 59 bp/76 bp ratios ranged from 0.89 to 1.5, in contrast to the median of 16 for the NPC patients. As their plasma EBV DNA were all negative, it is likely that the urine EBV DNA was produced by viral replication in the renal system. Considering the rapid clearance of circulating EBV DNA after tumor resection, with a half-life of ~2 hours (2), renal excretion of plasma EBV DNA should only play a minor role in its clearance.

Technically, this study has revealed the importance of the amplicon size of a PCR assay on the detection of transrenally excreted DNA. The detection rates of urine EBV DNA were 57% and 28% using the 59- and 76-bp assays, respectively. The better sensitivity for the assay with shorter amplicons reflects the fragmented nature of urine DNA. In previous studies, plasma DNA molecules derived from tumor and normal tissues have been shown to be short DNA fragments (17, 18). Here, we showed that the urine EBV DNA molecules were even shorter than those in the plasma samples. The median 59 bp/76 bp ratios for the urine and plasma samples were 16 and 2, respectively, indicating that a much higher proportion of urine DNA was <76 bp when compared with plasma DNA. These observations are consistent with the previous reports that the size of a molecule is the most important parameter to determine its permeability at the kidney barrier (19) and smaller molecules are more likely to be filtered through the glomeruli (19). These observations may explain why the previous studies using PCR assays with longer amplicons were unable to detect the target DNA sequences in urine (5, 7).

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In this study, we have conclusively shown the phenomenon of transrenal excretion of circulating DNA using the model of NPC. We have further shown that small DNA fragments are more likely to pass through the kidney barrier and be detectable in the urine. This information is important for the future development of urine-based diagnostic methods.

Discussion

The clearance of plasma cell-free DNA has been a long-standing question in the field of molecular biomarker research. Transrenal excretion of circulating DNA has been proposed as one possible mechanism. However, previous studies have failed to provide conclusive evidence to support the presence of this phenomenon. In this study, we use the detection of urine EBV DNA in NPC patients as a model to show the transrenal excretion of circulating cell-free DNA. Using the 59-bp PCR assay, EBV DNA was detectable in the urine of 56% NPC patients, and all of these patients were positive for plasma EBV DNA. The urine EBV DNA concentration measured by the 76-bp assay was 16-fold lower than the concentration measured by the 59-bp assay, suggesting that most of the urine EBV DNA molecules in the NPC patients were short DNA fragments and, hence, local production of virions through viral replication in the renal system is unlikely. Comparing with the previous studies that detect fetal-derived Y-chromosome sequences and KRAS mutations from gastrointestinal tract tumors, the detection of EBV DNA is less susceptible to contamination from nonrenal sources. Therefore, our results are strongly suggestive of the presence of transrenal excretion of circulating DNA. Furthermore, owing to the high plasma concentration of EBV DNA in some NPC patients, this study has provided, for the first time, quantitative information about the contribution of transrenal excretion on the clearance of circulating EBV DNA. We have shown that, using the 59-bp assay for measurement, the median fractional excretion of plasma EBV DNA was only 0.0026% of the clearance of creatinine. This indicates that only a small fraction of plasma EBV DNA was filtered through the glomerular membrane or EBV DNA is reabsorbed at the renal tubules. Because cell-free DNA has been shown to be stable for at least 4 hours in urine (10), the small amount of urine EBV DNA is unlikely to be a result of rapid degradation of these molecules in the renal system. Considering the rapid clearance of circulating EBV DNA after tumor resection, with a half-life of ~2 hours (2), renal excretion of plasma EBV DNA should only play a minor role in its clearance.

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Disclosure of Potential Conflicts of Interest

A. Chan and D. Lo have filed a patent application for technology related to this study.
References


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