Advances in Brief

Circulating Epstein-Barr Virus DNA in the Serum of Patients with Gastric Carcinoma[1]

Y. M. Dennis Lo,[2] Wing Y. Chan, Enders K. W. Ng, Lisa Y. S. Chan, Paul B. S. Lai, John S. Tam, and S. C. Sydney Chung

Departments of Chemical Pathology [Y. M. D. L., L. Y. S. C.], Anatomical and Cellular Pathology [W. Y. C.], Surgery [E. K. W. N., P. B. S. L., S. C. S. C.], and Microbiology [J. S. T.], Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, People's Republic of China

Abstract

Purpose: **We investigated the detectability of EBV DNA in the serum of gastric carcinoma patients in Hong Kong. Previous data have shown that approximately 10% of gastric carcinomas in Hong Kong are associated with EBV.**

Experimental Design: **We recruited 51 patients with gastric carcinoma, 30 patients with gastritis, and 197 apparently healthy controls. For gastric carcinoma patients, blood samples were obtained before surgery. After surgery, the resected tumor samples from the cancer cases were subjected to *in situ* hybridization for small EBV-encoded RNA (EBER). Serum EBV DNA in all cases was measured by real-time quantitative PCR.**

Results: **Serum EBV DNA was detectable in 5 of 5 (100%) EBER-positive gastric carcinoma cases (median concentration, 1063 copies/ml), in 13 of 14 (93%) EBER-negative gastric carcinoma cases with EBER-positive infiltrating lymphocytes (median concentration, 50 copies/ml), and in 0 of 32 (0%) EBER-negative cases. In the nontumor controls, serum EBV DNA was detectable in 7 of 30 (23%) gastritis cases (median concentration, 0 copies/ml) and in 7 of 197 (3.6%) apparently healthy individuals (median concentration, 0 copy/ml).**

Conclusions: **Our data indicate that serum EBV DNA reflects tumoral EBER status and opens up the possibility that circulating EBV DNA may be used as a tumor marker for the EBER-positive gastric carcinomas. The biological and clinical significance of the presence of low levels of circulating EBV DNA in the minority of gastritis patients and healthy individuals remains to be elucidated.**

Introduction

Recently, much interest has been focused on the presence of tumor-derived DNA in the plasma and serum of cancer patients (1, 2). For virally associated cancers, cell-free tumor-associated viral DNA has been detected in the plasma and serum of patients (3–5). One important virus which has been associated with many types of malignancy is EBV (6). In this regard, circulating EBV DNA has been detected in the plasma and serum of patients with NPC[3] (3, 4) and certain lymphoid malignancies (7–9).

EBV infection has also been reported to be associated with a proportion of gastric carcinomas (10). In Hong Kong, ~10% of gastric carcinoma cases have been found to be associated with EBV infection (11). In this report, we studied whether EBV DNA could also be detected in the sera of patients with gastric carcinoma.

Materials and Methods

Subjects. Fifty-one patients with gastric carcinoma were recruited with informed consent from the Prince of Wales Hospital, Hong Kong, People's Republic of China. Blood samples were taken before surgical resection of the tumor. After surgery, sections of the tumor were taken for *in situ* hybridization analysis for EBER. Blood samples were also taken from 30 individuals with gastritis without evidence of carcinoma and from 197 apparently healthy control subjects.

DNA Extraction from Serum Samples. Serum samples were stored at −20°C until DNA extraction. DNA from serum samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) following the "blood and body fluid protocol" as recommended by the manufacturer (1). Four hundred to 800 μl of the serum samples 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 for eluting the DNA from the extraction column.

Real-Time Quantitative EBV DNA PCR. Circulating EBV DNA concentrations were measured using a real-time quantitative PCR system toward the *Bam*HI-W fragment region of the EBV genome (4). The *Bam*HI-W PCR system consisted of the following amplification primers: W-44F, 5′-CCC AAC ACT CCA CCA CAC C-3′; W-119R, 5′-TCT TAG GAG CTG TCC GAG GG-3′; and a dual-labeled fluorescent probe W-67T, 5′-(FAM)CAC ACA CTA CAC ACA CCC ACC CGT CTC(TAMRA)-3′. FAM and TAMRA were the fluorescent reporter and quencher, as described before (4). The principles of

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[1] Y. M. D. L. is a member of the Hong Kong Cancer Genetics Research Group, which is supported by the Kadoorie Charitable Foundation.

[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. Phone: 852-2632-2563; Fax: 852-2194-6171; E-mail: loym@cuhk.edu.hk.

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

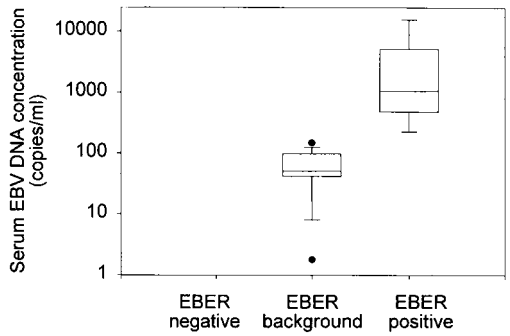

Fig. 1 Serum EBV DNA concentrations among the three gastric cancer patient groups. The *Y* axis is in the common logarithmic scale. *Lines* inside the boxes denote the medians. *Boxes* mark the interval between the 25th and 75th percentiles. *Whiskers* (*short horizontal lines*) denote the interval between the 10th and 90th percentiles. ●, the 5th and 95th percentiles.

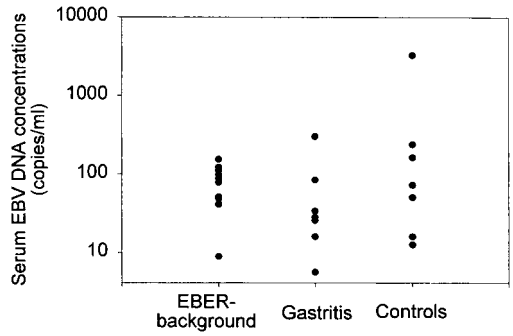

Fig. 2 Serum EBV DNA concentrations in gastric carcinoma cases with background EBER-positivity, gastritis cases, and healthy controls. Only cases with non-zero serum EBV DNA concentration are plotted. The *Y* axis is in the common logarithmic scale.

real time quantitative PCR and reaction set-up procedures were as described previously (4). Data were collected using an ABI Prism 7700 Sequence Detector and analyzed using the Sequence Detection System software (Version 1.6.3) developed by Applied Biosystems. Results were expressed as copies of EBV genomes/ml of serum.

All serum DNA samples were also subjected to real-time PCR analysis for the β-*globin* gene (4). The β-*globin* PCR system consisted of the following amplification primers: β-globin-354F, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′; β-globin-455R, 5′-CCT TGA TAC CAA CCT GCC CAG-3′; and a dual-labeled fluorescent probe β-globin-402T, 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG-(TAMRA)-3′. The β-*globin* PCR gave a positive signal on all tested samples, thus demonstrating the quality of the extracted DNA. Multiple negative water blanks were included in every analysis.

EBER Analysis of Tumor Samples. The presence of EBV in tumor cells was assessed by *in situ* hybridization on paraffin-embedded tissue sections using a fluorescein-conjugated oligonucleotide probe for EBER (Novocastra, Newcastle upon Tyne, United Kingdom) as described previously (12).

Results

Circulating EBV DNA in Patients with Gastric Carcinoma. A total of 51 gastric carcinoma patients were recruited. All extracted serum DNA was amplifiable using the β-*globin* PCR system, yielding a median serum DNA concentration of 5989 genome-equivalents/ml (interquartile range, 2452–20210 genome-equivalents/ml). Using a conversion factor of 6.6 pg of DNA/diploid cell, these values translated to a median serum DNA concentration of 40 ng/ml (interquartile range, 16–133 ng/ml).

In this cohort, five gastric carcinomas were EBER-positive, and 32 cases were negative. In the remaining 14 cases, the tumor cells were EBER-negative, but there were occasional infiltrating lymphocytes that were EBER-positive. These 14 cases were classified as having "background" positivity. Fig. 1 illustrates the difference in the levels of circulating EBV DNA among these three patient groups. Serum EBV DNA was detected in every one of the EBER-positive cases (median serum EBV DNA concentration, 1063 copies/ml; interquartile range, 485-5141 copies/ml). No serum EBV DNA was detected in any of the 32 negative cases (Fig. 1). Thirteen of 14 cases (93%) demonstrating background EBER positivity had detectable serum EBV DNA. These cases had an intermediate median serum EBV DNA concentration of 50 copies/ml (interquartile range, 42–98 copies/ml). The difference between these three groups is statistically significant ($P < 0.001$; Kruskal-Wallis test). Pairwise multiple comparison analysis indicates a significant difference between the EBER-positive and EBER-negative groups ($P < 0.05$; Dunn's method) and between the EBER-background and EBER-negative groups ($P < 0.05$; Dunn's method).

Circulating EBV DNA in Patients with Gastritis and Healthy Controls. EBV DNA was detectable in the serum of 7 of 30 gastritis patients (23%) and 7 of 197 healthy controls (3.6%). The proportions of serum EBV DNA-positive cases between these groups are significantly different (χ^2 test; $P = 0.028$). Even in the cases with detectable circulating EBV DNA, the actual serum EBV DNA concentrations were generally lower than those in the EBER-positive gastric carcinoma cases. Thus, the median serum EBV DNA concentration in the gastritis cases was 0 copies/ml (interquartile range, 0–0 copies/ml). Similarly, the median serum EBV DNA concentration in the healthy controls was 0 copies/ml (interquartile range, 0–0 copies/ml).

Comparison of the Serum EBV DNA-positive Cases in the EBER-Background Gastric Carcinoma, Gastritis, and Control Groups. A comparison was made for the cases with detectable serum EBV DNA in the gastric carcinoma cases with background EBER-positivity, gastritis cases, and control subjects. The serum EBV DNA concentrations of these three groups are plotted in Fig. 2. There is no statistically significant difference in circulating EBV DNA levels among these three groups (Kruskal-Wallis test; $P = 0.296$).

Discussion

We have shown that cell-free EBV DNA could be detected in serum samples obtained from a proportion of gastric carcinoma patients. In addition, these data demonstrate an interesting

correlation between the detectability of serum EBV DNA and tumoral EBER status. Thus, EBER-positive gastric carcinoma cases were associated with high levels of serum EBV DNA; gastric carcinoma cases with background EBER-positivity were associated with intermediate levels; and no serum EBV DNA was seen in EBER-negative cases. This observation lends additional support to the suggestion that plasma and serum represent noninvasive sources of materials for monitoring cancer (13).

Clinically, circulating EBV DNA may have application in the diagnosis and monitoring of the portion of gastric carcinoma patients who have EBER-positive tumors, similarly to what has been achieved for NPC (4, 14) and certain lymphomas (7–9). The recent demonstration of the prognostic significance of circulating EBV DNA in NPC (15) suggests that EBV DNA measurement may also have prognostic importance for gastric carcinoma.

The detection of circulating EBV DNA in gastric carcinomas demonstrating background EBER-positivity is interesting. One interpretation of these data is that the EBER-positive lymphocytes infiltrating the tumor tissues are the origin of the low levels of serum EBV DNA that are detectable in these cases. If this hypothesis is correct, then additional work would be needed to elucidate the mechanism of EBV liberation by these EBER-positive lymphocytes. Possible mechanisms include the active release of DNA (16) and the activation of lytic EBV infection in a proportion of these cells.

The association between circulating EBV DNA and EBER-positive infiltrating lymphocytes prompts us to investigate whether low levels of circulating EBV DNA may also be seen in non-neoplastic inflammatory conditions. This line of thought led us to demonstrate the presence of circulating EBV DNA in gastritis patients. As controls, serum EBV DNA was also detected in 3.6% of apparently healthy control individuals. Previously, the presence of low levels of circulating EBV DNA in apparently healthy subjects has been reported by other studies (4, 17). In those gastritis and healthy control cases with detectable serum EBV DNA, the concentrations of viral DNA were very similar to those in the gastric carcinoma cases with background EBER-positivity (Fig. 2). This observation suggests the possibility that circulating EBV DNA may also be released by EBV-positive lymphocytes in the gastritis and healthy controls, as has been postulated above for EBER-background gastric carcinoma. With regard to the levels of circulating EBV DNA, it is important to note that among these 27 cases with detectable serum EBV DNA, 26 had levels <500 copies/ml. In contrast, 4 of 5 (80%) EBER-positive gastric carcinomas and, in a previous study (4), 48 of 57 (84%) of nasopharyngeal carcinoma cases had circulating EBV DNA levels >500 copies/ml. This analysis suggests that a plasma/serum EBV DNA concentration of 500 copies/ml may be a practical diagnostic cutoff for identifying patients with these carcinomas with high specificity. It is expected that additional refinement of this diagnostic cutoff value will be forthcoming with large-scale clinical studies.

The long-term significance of the presence of low levels of circulating EBV DNA in the blood of apparently healthy individuals remains to be elucidated. Importantly, future studies should address the possibility that these individuals might be at increased risk of developing EBV-associated diseases. This issue would be of tremendous public-health and biological importance.

Biologically, the detection of circulating EBV DNA in patients with EBER-positive gastric carcinoma might help us answer important questions regarding the clearance rate of tumor-derived DNA from the plasma. This remains a poorly understood issue because previous work on the kinetics of tumor-derived DNA clearance has been performed in patients undergoing radiation therapy (18) and chemotherapy (7). The kinetic parameters established from these previous studies represent a composite of: (*a*) tumor cell death attributable to the treatment regime; and *(*b) the clearance of tumor-derived DNA from the plasma. As gastric carcinoma is treated predominantly by surgical resection, this new model system might allow one to study the clearance of tumor-derived DNA after cancer removal at a single time point, *e.g.*, at surgery. Such a study would be expected to enhance our understanding of plasma DNA clearance *in vivo*, just as has already been done for fetal DNA clearance from maternal plasma (19). Indeed, preliminary data obtained for the clearance of mutant DNA in the circulation of colorectal cancer patients have demonstrated the intriguing possibility that plasma DNA clearance may be impaired in cancer patients (20).

Our data also suggest that circulating EBV DNA should be sought in many other cancer types that have been reported to be associated with EBV. Examples include breast cancer (21) and hepatocellular carcinoma (22). Because the proposed association between some of these tumor types with EBV is still controversial, the possible detection of EBV DNA in the plasma of patients with these neoplasms might contribute toward resolving these issues.

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