Quantitative Analysis of Cell-Free Epstein-Barr Virus DNA in Plasma of Patients with Nonnasopharyngeal Head and Neck Carcinomas

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ABSTRACT

Purpose: **We investigated the detectability of EBV DNA in the plasma of patients with non-nasopharyngeal head and neck carcinomas (NNHNC). Previous studies have shown that EBV is present in the tumor tissue of some NNHNC.**

Experimental Design: **We recruited 101 patients with NNHNC and 48 healthy controls. Blood samples were taken from controls and patients before treatment. Tumor tissue samples were tested for the presence of EBV in the first 69 patients by *in situ* hybridization for small EBV-encoded RNA (EBER). Plasma EBV DNA was measured by real-time quantitative PCR in patients and controls.**

Results: **Squamous cell carcinoma (SCC) was the commonest histology (78 patients) followed by lymphoepithelial carcinoma (8 patients). EBER was detected in tumor cells in 7 of 69 patients tested. All of the EBER-positive tumors were lymphoepithelial carcinoma. Two controls (2 of 48; 4.2%) had detectable plasma EBV DNA. Plasma EBV DNA was detected in all of the patients with EBER-positive tumors, and in 23 of 94 (24.5%) patients with tumors of EBER-negative or unknown status. The proportion of plasma EBV DNA-positive cases in either group was significantly higher than that in the control group ($P < 0.0027$). Plasma EBV DNA concentrations in patients with EBER-positive tumors (median, 3827 copies/ml) were significantly higher than those in the controls (median, 0 copy/ml; $P = 0.0001$). Of patients with SCC, 21 (26.9%) had detectable plasma EBV DNA (median concentration, 34 copies/ml). Plasma EBV DNA concentrations in the whole group of patients with SCC (median, 0 copy/ml; interquartile range, 0–4 copies/ml) were also significantly higher than those in the controls ($P = 0.001$).**

Conclusions: **Our data indicate that plasma EBV DNA reflects tumoral EBER status, and it may be of use as a tumor marker for EBER-positive NNHNC. The biological and clinical significance of low levels of circulating EBV DNA in the minority of patients with EBER-negative tumors remain to be elucidated.**

INTRODUCTION

Head and neck carcinomas comprise ~5% of all malignancies diagnosed annually. They are a heterogeneous group of malignancies and can be classified into different subgroups according to primary tumor site and histological type. Squamous cell carcinoma (SCC) is the commonest histology in mucosal sites of the upper aerodigestive tract. Nasopharyngeal carcinoma (NPC), with a unique geographical and ethnic distribution and high radio- and chemosensitivity, is usually considered separately from other head and neck carcinomas. The undifferentiated subtype of NPC has a strong association with EBV (1–3).

EBV is a herpesvirus that has been linked to several human malignancies (4). It is present in the tumor tissue of nasopharyngeal undifferentiated carcinoma and various lymphoid malignancies (1–5). The virus is also present in the tumor cells of a number of NNHNC (6–15). In particular, it is strongly associated with lymphoepithelial carcinoma (LECA) of the salivary glands in Eskimos and Asian patients (1, 12, 13, 16, 17).

Tumor-derived cell-free EBV DNA has been detected in the blood of patients with NPC (18, 19), EBV-associated lymphoid malignancies (5), and EBV-positive gastric carcinoma (20). Real-time quantitative PCR assay for EBV DNA has a 96% sensitivity for detecting NPC (18), and data show that circulating cell-free EBV DNA is useful for early detection of tumor recurrence (21) and predicting prognosis (22, 23). Although plasma EBV DNA is a useful tumor marker for NPC, there is little information on whether EBV DNA is present in the plasma of patients with NNHNC. Currently, there are no tumor markers that are used in routine clinical practice to monitor treatment response or predict prognosis of SCC of head and neck sites. In view of the possible association of EBV with subgroups of NNHNC, we conducted a prospective study to determine the proportion of patients who had cell-free EBV DNA detectable in the plasma, and to correlate histological type and primary site with plasma of EBV DNA concentrations.

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PATIENTS AND METHODS

Patient Selection, Evaluation, and Treatment. Patients attending the Department of Clinical Oncology and Division of Ear Nose and Throat/Head and Neck Surgery, Prince of Wales Hospital, Hong Kong SAR, China were eligible for the study if they had a newly diagnosed or relapsed biopsy-proven carcinoma of any head and neck primary site except the nasopharynx. Patients with synchronous malignancy and those with metastatic cervical nodes from unknown primary were excluded. Healthy volunteers were recruited as controls. Written informed consent was obtained from controls and eligible patients. The study was approved by the Ethics Committee of the Faculty of Medicine of the Chinese University of Hong Kong.

All of the patients were evaluated by clinical history and physical examination. Patients underwent computed tomography and/or magnetic resonance imaging and ultrasonogram of the head and neck. Examination under anesthesia, endoscopic examination of the upper aerodigestive tract including the trachea, and esophagogastroduodenoscopy were performed in patients with oropharyngeal, hypopharyngeal, and laryngeal carcinomas. Esophagogastroduodenoscopy was also performed in patients with other primary sites if they had symptoms referable to the upper gastrointestinal tract. Biopsy was obtained from the primary tumor and any synchronous tumors for histological diagnosis and from any gastric lesions for ruling out gastritis. Fine needle aspiration of suspicious neck lymph nodes was performed for cytology as indicated. Investigations for systemic disease included chest radiograph and ultrasonogram of the abdomen and, in selected patients, computed tomography of the thorax and abdomen. In patients with LECA (17) of salivary gland, nasopharyngoscopy and multiple nasopharyngeal biopsies were performed to rule out NPC.

Tumors were staged according to the 1997 American Joint Committee on Cancer/International Union Against Cancer stage classification (24). Definitive treatment was planned after multidisciplinary consultation and discussion with the patient. In general, patients amenable to curative treatment were treated by radical radiotherapy or chemoradiotherapy for organ preservation. Patients with salivary gland or thyroid carcinomas were treated with surgery and postoperative radiotherapy as indicated. Patients not amenable to curative treatment were given palliative radiotherapy, chemotherapy, or supportive care.

Test for EBV in Tumor Samples. Tumor tissue was tested for the presence of EBV in tumor cells and tumor-infiltrating lymphocytes (20) in the first 69 patients by *in situ* hybridization on paraffin-embedded tissue sections using a fluorescein-conjugated oligonucleotide probe for EBER (Novocastra, United Kingdom), as described previously (25).

Plasma EBV DNA Measurement

DNA Extraction from Plasma Samples. Peripheral venous blood (2.5 ml) was drawn from each recruited control and patient before treatment. DNA was extracted from plasma samples, and cell-free EBV DNA concentration was measured with real-time quantitative PCR as described previously (18). Briefly, plasma samples were stored at −20°C until DNA extraction. DNA from plasma samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) after the "blood and body fluid protocol" as recommended by the manufacturer (26). Four hundred to 800 μl of the plasma 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 DNA from the extraction column.

Real-Time Quantitative EBV DNA PCR. Circulating EBV DNA concentrations were measured using a real-time quantitative PCR system that amplified a DNA segment in the *Bam*HI-W fragment region of the EBV genome (18). The principles of real-time quantitative PCR and reaction set-up procedures were as described previously (18). Data were collected using an ABI Prism 7700 Sequence Detector and analyzed using the Sequence Detection System software (version 1.6.3) developed by PE Biosystems. Results were expressed as copies of EBV genome per milliliter of plasma.

All of the plasma DNA samples were also subjected to real-time PCR analysis for the β-*globin* gene (18), which gave a positive signal on all of the tested samples, thus demonstrating the quality of the extracted DNA. Multiple negative water blanks were included in every analysis.

Determination of the Integrity of Plasma EBV DNA. The integrity of the plasma EBV DNA was determined as described (27). Briefly, the concentration of plasma EBV DNA was measured by three real-time PCR systems targeting the *EBER* gene. The three systems shared the same reverse primer and probe. The position of the forward primers was varied to give amplicon sizes of 82 bp, 181 bp, and 385 bp. The quantity of DNA measured by the latter two systems was expressed as a fractional concentration of the system with shortest amplicon. The median fractional concentration was then calculated for all of the cases with detectable EBV DNA as described (27).

Statistical Analysis. The primary objective of the study was to determine the proportion of patients who had plasma EBV DNA concentrations >0 copy/ml. Comparison of proportions between control and patient groups was performed with the χ^2 test or Fisher's exact test as appropriate. Comparison of plasma EBV DNA concentrations between control and patient groups was performed using the Wilcoxon rank sum test. A $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Characteristics of Patients and Controls. One hundred and one patients and 48 controls were recruited in the study. The male to female ratio was 80:21 for patients and 16:32 for controls. The mean age of patients was 64 years (range, 32–87 years), and the mean age of controls was 42 years (range, 23–74 years). Twenty-four male patients (24 of 80) and 6 female patients (6 of 21) had a plasma EBV DNA concentration >0 copy/ml; the proportions were not significantly different ($P = 0.90$). The plasma EBV DNA concentrations of male patients (median 0 copy/ml; interquartile range, 0–11.75 copies/ml) were significantly higher than those of male controls (median, 0 copy/ml; interquartile range, 0–0 copy/ml; $P = 0.013$). Similarly, the plasma EBV DNA concentrations of female patients (median, 0 copy/ml; interquartile range, 0–13.9 copies/ml) were significantly higher than those of female controls (median, 0 copy/ml; interquartile range, 0–0 copy/ml; $P = 0.019$). Seven-

Table 1 Characteristics of tumors

Features	No. (n = 101) (%)
Primary site	
Larynx	40 (39.6)
Hypopharynx	16 (15.8)
Oropharynx	14 (13.9)
Oral cavity	9 (8.9)
Salivary gland	8 (7.9)
Thyroid	5 (4.9)
Others	9 (8.9)
Nasal cavity	4 (4.0)
Paranasal sinus	4 (4.0)
Cervical esophagus	1 (1.0)
Histology	
Squamous cell carcinoma	78 (77.2)
Lymphoepithelial carcinoma	8 (7.9)
Papillary carcinoma	3 (3.0)
Adenocarcinoma	1 (1.0)
Follicular carcinoma	1 (1.0)
Others	10 (9.9)
Adenoid cystic carcinoma	6 (5.9)
Poorly differentiated carcinoma	2 (2.0)
Mucoepidermoid carcinoma	1 (1.0)
Anaplastic carcinoma	1 (1.0)
T-stage	
T0[a]	13 (12.9)
T1	18 (17.8)
T2	33 (32.7)
T3	18 (17.8)
T4	19 (18.8)
N-stage	
N0	72 (71.3)
N1	3 (3.0)
N2	20 (19.8)
N3	6 (5.9)
M-stage	
M0	90 (89.1)
M1	11 (10.9)
Stage	
I	17 (16.8)
II	27 (26.7)
III	9 (8.9)
IV	48 (47.5)

[a] These patients presented with recurrent disease in cervical lymph nodes or distant sites with previous resection of the primary tumors.

teen of 52 patients of age ≤64 years and 13 of 49 patients of age >64 years had detectable plasma EBV DNA; the proportions were not significantly different (P = 0.50).

Histology and Tumor Cell EBER-Positivity. Characteristics of the tumors are summarized in Table 1. SCC was the commonest histological type (78 patients) and LECA (8 patients) was the second commonest.

EBER was detected in tumor cells in 7 of 69 patients tested. All of the EBER-positive tumors were LECA and in all cases >80% of tumor cells were EBER-positive. Thus, 7 of 8 LECA tumors were EBER-positive, whereas none of the 61 tumors of other histology were EBER-positive ($P < 0.0001$). Thirty-two tumors were not tested for EBER, none of which being LECA. Only 2 patients had EBER-positive tumor-infiltrating lymphocytes shown in the tumor specimen. One patient had an adenocarcinoma of the hypopharynx in which <5% of tumor-infiltrating lymphocytes were EBER-positive; he was plasma EBV DNA-negative. The other patient had an LECA of the parotid in which the tumor cells were EBER-positive and 1% of tumor-infiltrating lymphocytes were EBER-positive.

Plasma EBV DNA Concentration by Tumor Cell EBER Positivity and Histological Type. Plasma EBV DNA concentrations according to tumor cell EBER-positivity and histological types are shown in Table 2 and Fig. 1.

Thirty patients (29.7%) and 2 controls (4.2%) had detectable plasma EBV DNA. The difference in proportion was statistically significant (P = 0.0004). Plasma EBV DNA was detected in all of the patients with EBER-positive tumors, and in 23 of 94 (24.5%) patients with tumors of EBER-negative or unknown status. The proportion of plasma EBV DNA-positive cases in either group was significantly higher than that in the controls ($P < 0.0027$; Table 2).

Plasma EBV DNA concentrations in patients with EBER-positive tumors (median, 3,827 copies/ml; interquartile range, 1,890–142,521 copies/ml) were significantly higher than those in the controls (median, 0 copy/ml; interquartile range, 0–0 copy/ml; P = 0.0001; Table 2).

Plasma EBV DNA concentrations in patients with LECA (median, 3,006 copies/ml; interquartile range, 1,739–73,472 copies/ml) were significantly higher than those in the controls (P = 0.0001) and also significantly higher than those in patients with SCC (median, 0 copy/ml; interquartile range, 0–4 copies/ml; P = 0.001). Of patients with SCC, 21 (26.9%) had detectable plasma EBV DNA (median concentration, 34 copies/ml; range, 2–908 copies/ml). Plasma EBV DNA concentrations in the whole group of patients with SCC were also significantly higher than those in the controls (P = 0.001).

Plasma EBV DNA Concentration by Primary Tumor Site. Table 3 and Fig. 2 show the plasma EBV DNA concentrations in the controls and in patients with different primary sites. Larynx (40 patients) was the commonest primary site, followed by hypopharynx (16 patients) and oropharynx (14 patients); plasma EBV DNA was detectable in 20.0%, 43.8%, and 28.6% of patients for those three primary sites, respectively. Except for salivary gland, the proportion of subjects with detectable plasma EBV DNA in each primary site was significantly higher than the corresponding proportion in the control group ($P < 0.05$; Table 3). Plasma EBV DNA concentrations for each of the primary tumor sites were statistically higher than those for the controls (Table 3).

Plasma EBV DNA Concentrations by Tumor Stage, T-Stage, and Presence of Gastritis. Table 4 depicts the plasma EBV DNA concentrations in patients with SCC of different stages and T-stages. The EBV DNA concentrations did not vary significantly with these parameters.

For all of the histological types except LECA, plasma EBV DNA concentrations in patients with gastritis (n = 20; median 0 copy/ml; interquartile range, 0–7 copies/ml) were not significantly different from those in patients without gastritis (n = 73; median 0 copy/ml; interquartile range, 0–0 copy/ml; P = 0.39). Plasma EBV DNA concentrations in patients with gastritis (P = 0.0022) or without gastritis (P = 0.0035) were significantly higher than those in the controls.

Integrity of Plasma EBV ENA. The integrity of EBA DNA was determined for all of the patients with detectable EBV DNA in their plasma. As the EBER system was less sensitive than measuring the repetitive W-fragment, EBV DNA was only

Table 2 Plasma EBV DNA concentrations according to tumor cell EBER[a]-positivity and histology

Characteristic	No. of subjects	Percentage of subjects with EBV DNA concentration >0 copy/ml (95% confidence limits)	Comparison with controls, *P* (χ^2 test or Fisher exact test)	Median EBV DNA concentration (interquartile range) (copies/ml)	Comparison with controls, *P* (Wilcoxon rank sum test)
Controls	48	4.2 (0–9.8)		0 (0–0)	
Tumor cell EBER-positivity					
Positive	7	100.0	0.0001	3827 (1890–142521)	0.0001
Negative or unknown	94	24.5 (0–33.2)	0.0027	0 (0–0)	0.002
Histology					
Squamous cell carcinoma	78	26.9 (17.1–36.8)	0.0001	0 (0–4)	0.001
Lymphoepithelial carcinoma	8	87.5 (64.6–110.4)	<0.0001	3006 (1739–73472)	0.0001
Papillary carcinoma	3	33.3 (0–86.7)	0.169	0 (0–23)	0.0348
Adenocarcinoma	1	0/1	NA	NA	NA
Follicular carcinoma	1	0/1	NA	NA	NA
Others	10	10.0 (0–28.6)	0.439	0 (0–0)	0.0436

[a] EBER, EBV-encoded RNA; NA, not calculated because of small patient number.

detectable by the EBER system in 11 patients. The median fractional concentrations for amplicon sizes of 181 bp and 385 bp were 27% and 9%, respectively.

DISCUSSION

Cell-free EBV DNA concentration was markedly elevated (median, 3827 copies/ml) in the plasma of all our patients with EBER-positive NNHNC. Histologically, all of the EBER-positive tumors were LECA, which were morphologically identical with nasopharyngeal undifferentiated carcinoma (17). Conversely, the great majority of LECA were EBER-positive. These observations are consistent with the fact that 96% of patients with nasopharyngeal undifferentiated carcinoma have circulating tumor-derived EBV DNA (18, 28). The plasma EBV DNA concentrations of our patients with EBER-positive tumors were comparable with those of patients with NPC, EBER-positive gastric carcinomas (20), and EBV-associated lymphoid malignancies (5, 23, 29).

EBER-positive carcinomas are relatively uncommon in non-nasopharyngeal sites, and often present in a subtle way in sites that are difficult to examine clinically, such as the nasal cavity, paranasal sinuses, and the base of the tongue. Plasma EBV DNA may be a useful noninvasive laboratory test to prompt diagnosis of new cases and monitor treatment response. Furthermore, plasma EBV DNA level may carry prognostic value in patients with EBER-positive NNHNC, as it does in NPC patients (22), and should be investigated in a larger study. Our data show that the most of the LECA are EBER-positive tumors with markedly elevated plasma EBV DNA. Therefore, routine histological examination is useful in guiding selection of patients for plasma EBV DNA testing.

Most of our patients had SCC, which were not EBER-positive. The finding that 20.0–43.8% of patients with SCC of common primary sites, including larynx, hypopharynx, and oropharynx, had low levels of plasma EBV DNA (median concentration 34 copies/ml) has not been reported previously. One

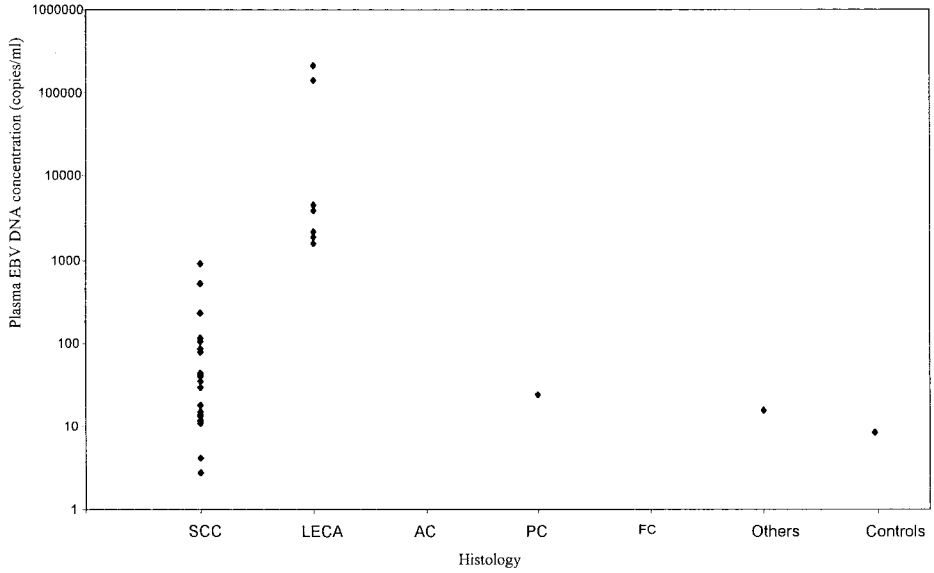

Fig. 1 Plasma EBV DNA concentrations according to histology. All plasma EBV DNA-positive lymphoepithelial carcinoma (*LECA*) were EBER-positive. Only cases with plasma EBV DNA concentrations >0 copy/ml are plotted. The *Y*-axis is in the common logarithmic scale. *SCC*, squamous cell carcinoma; *AC*, adenocarcinoma; *PC*, papillary carcinoma of thyroid; *FC*, follicular carcinoma of thyroid.

Table 3 Plasma EBV DNA concentrations according to primary tumor site

Characteristic	No. of subjects	Percentage of subjects with EBV DNA concentration >0 copy/ml (95% confidence limits)	Comparison with controls, *P* (χ^2 or Fisher exact test)	Median EBV DNA concentration (interquartile range) (copies/ml)	Comparison with controls, *P* (Wilcoxon rank sum test)
Controls	48	4.2 (0–9.8)		0 (0–0)	
Primary site					
Larynx	40	20.0 (7.6–32.4)	0.039	0 (0–0)	0.0153
Hypopharynx	16	43.8 (19.4–68.1)	0.0001	0 (0–34)	0.0001
Oropharynx	14	28.6 (4.9–52.2)	0.02	0 (0–13)	0.005
Oral cavity	9	33.3 (2.5–64.1)	0.024	0 (0–2)	0.0046
Salivary gland	8	25.0 (0–55.0)	0.94	0 (0–945)	0.0295
Thyroid	5	40.0 (0–82.9)	0.04	0 (0–15)	0.0031
Others	9	44.4 (12.0–76.9)	0.0001	0 (0–1588)	0.0002

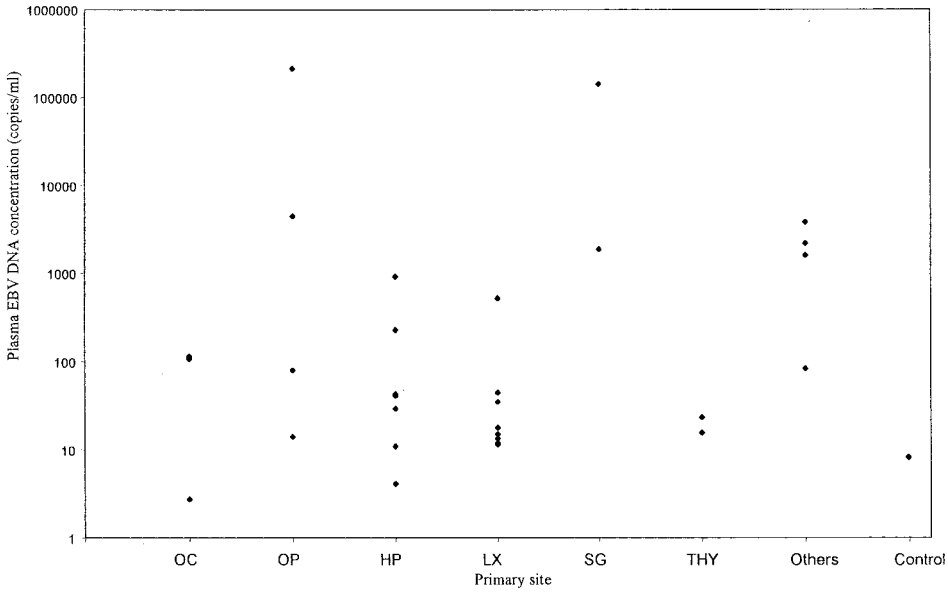

Fig. 2 Plasma EBV DNA concentrations in control subjects and in patients with different primary sites. Only cases with plasma EBV DNA concentrations greater than 0 copy/ml are plotted. The *Y*-axis is in the common logarithmic scale. *OC*, oral cavity; *OP*, oropharynx; *HP*, hypopharynx; *LX*, larynx; *SG*, salivary gland; *THY*, thyroid.

possible explanation for the finding is that EBV DNA is present in tumor cells in some cases, although they are EBER-negative (9). If this is the case, however, plasma EBV DNA concentrations are expected to be much higher, comparable with those observed in EBER-positive tumors. The lack of correlation of plasma EBV DNA concentration with stage in our patients with SCC additionally suggests that the plasma EBV DNA was not derived directly from the tumor cells. More likely, our observation is similar to that reported in patients with EBER-negative gastric carcinoma and EBER-positive infiltrating lymphocytes who had detectable levels of EBV DNA in the serum (median concentration 50 copies/ml; Ref. 20). EBV-infected B lymphocytes at the primary tumor site may have been the origin of the low levels of plasma EBV DNA in our patients. Possible mechanisms of release of cell-free EBV DNA include active release of DNA (30) and the activation of lytic EBV infection in a proportion of the lymphocytes. As we have shown that the plasma EBV DNA are short fragments as in the plasma of NPC patients (27), it is unlikely that lytic EBV infection is the main mechanism leading to the presence of EBV DNA in the plasma of these patients. However, as most patients underwent small biopsies before organ-preservation treatment, only scanty lymphoid cells were available to evaluate EBER-positivity.

It is known that almost all adults have been infected with EBV, and the virus remains in B-lymphocytes in pharyngeal and

Table 4 Plasma EBV DNA concentrations in patients with squamous cell carcinoma according to stage and T-stage

Characteristic	No. of subjects	Median EBV DNA concentration (interquartile range) (copies/ml)	*P* (Wilcoxon rank sum test)
Stage			
I–II	37	0 (0–0)	0.37
III–IV	41	0 (0–10)	
T-stage			
0–2	50	0 (0–0)	0.18
3–4	28	0 (0–22)	

other mucosal sites with a B-lymphocyte infiltrate (28). Normally, the proportion of B lymphocytes in pharyngeal lymphoid tissue that carry EBV is very low, but it is increased in such circumstances as parasitic infection, which provides a chronic antigenic stimulus to the B-cell system (28). It has been found that cytokines important in proinflammatory responses are detectable in tumor specimens and serum from patients with head and neck SCC (31). These include interleukin-6 and granulocyte-macrophage colony-stimulating factor, which promote proliferation of B lymphocytes (32). Thus, it is possible that the low levels of plasma EBV DNA in some of our patients with SCC were due to tumor production of proinflammatory cytokines, which cause proliferation of EBV-infected B lymphocytes. Similarly, benign inflammatory conditions (such as gastritis) or infections may also increase plasma EBV DNA concentrations through the production of cytokines (20) and may account for the low detectable plasma EBV DNA in two of the apparently healthy controls. In EBV-negative tumors, assuming cytokine-induced proliferation of EBV-positive lymphocytes is the mechanism of increased release of EBV DNA into the circulation, tumor is likely to be more important than intercurrent benign inflammatory conditions in the production of cytokines, because a higher proportion of patients with tumors had detectable plasma EBV DNA and the concentrations were higher, compared with controls.

In the current study, an increased incidence of gastritis, a known factor associated with elevated plasma EBV DNA (20), in the patient group was unlikely to be the cause for the higher level of plasma EBV DNA in patients with EBER-negative tumors compared with controls. Our data showed that the plasma EBV DNA concentrations in patients without gastritis were significantly higher than those in control subjects. Also, there was no significant difference in plasma EBV DNA concentration for patients with gastritis and those without.

In conclusion, plasma EBV DNA is markedly elevated in EBV-positive NNHNC and slightly increased in a significant proportion of patients in EBV-negative SCC. If tumor-derived cytokines are the major contributory factor to the increase in plasma EBV DNA concentrations in a proportion of patients with EBV-negative carcinomas, measurement of plasma EBV DNA in those patients may be used for early detection of residual tumors cells after cancer therapy. Additional studies are warranted to investigate the mechanism of the increase in plasma EBV DNA in EBV-negative tumors and whether this has any value in monitoring disease progress or predicting prognosis.

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