Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Epstein-Barr Virus DNA Load in Nasopharyngeal Brushings and Whole Blood in Nasopharyngeal Carcinoma Patients before and after Treatment

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Abstract

Purpose: Nasopharyngeal carcinoma (NPC) is consistently associated with Epstein-Barr virus (EBV) and highly prevalent in Indonesia. EBV-DNA load can be used for early diagnosis and may have prognostic value. In this study, EBV-DNA load was evaluated in minimal invasive nasopharyngeal (NP) brushings and whole blood for initial diagnosis and therapy assessment against the standard-of-care diagnosis by biopsy with EBV-RISH and standard EBV-IgA serology.

Experimental Design: NP brushings and blood samples were collected from 289 consecutive ENT patients suspected of NPCs and 53 local healthy controls. EBV-DNA load was quantified by real-time PCR and serology by peptide-based EBV-IgA ELISA. Tissue biopsies were examined by routine histochemistry and by EBER RNA *in situ* hybridization.

Results: Repeated NP brushing was well tolerated by patients and revealed high viral load in the 228 NPC cases at diagnosis than 61 non-NPC cancer cases and healthy controls ($P < 0.001$). The diagnostic value of EBV-DNA load in blood and EBV-IgA serology was inferior to the NP brush results. The level of EBV-DNA load in brushes of patients with NPC was not related to T, N, or M stage, whereas elevated EBV-DNA load in blood correlated with N and M stage. EBV-DNA levels in brushings and whole blood showed a significant reduction at 2 months after treatment ($P = 0.001$ and $P = 0.005$, respectively), which was not reflected in EBV-IgA serology.

Conclusions: NP brush sampling combined with EBV-DNA load analysis is a minimal invasive and well-tolerated diagnostic procedure, suited for initial diagnosis and follow-up monitoring of NPCs. *Clin Cancer Res; 19(8); 2175–86. ©2013 AACR.*

Introduction

Nasopharyngeal carcinoma (NPC) is a distinct head and neck cancer, occurring at high frequency in Southeast-Asian, North-African, and Inuit populations (1). In Indonesia, with an ethnically diverse population of 225 million people, NPC is the most common head and neck cancer with high prevalence among native populations and an overall incidence estimated at 6.2 per 100,000 (2). In the Dr. Cipto Mangunkusumo Hospital (Jakarta, Indonesia), NPC is the fifth most frequent cancer overall after cervical carcinoma, breast cancer, colon, and skin cancer with an incidence of 6.6% (cervical cancer 16.1%, breast cancer 14.5%, colorectal cancer 9.9%). NPC is the most common tumor in the head and neck, constituting 23.8% of all head and neck cancer cases (3).

Because NPC is highly radiosensitive the mainstay treatment is radiotherapy (RT), which can result in a 5-year overall survival rate of 90% for early stage I disease and in late-stage disease (stages III and IV), the treatment outcome has a cure rate of less than 58% (4). Thus, diagnosis at early stage of NPC is a clear medical need. Unfortunately more than 85% of patients with NPCs in Indonesia present in the clinic with advanced stage of disease and treatment outcome is poor (3).

NPC has a close association with Epstein-Barr virus (EBV), a ubiquitous human herpesvirus infecting more than 90% of the world population and viral gene products are expressed in all tumor cells. EBV is present in almost 100% of undifferentiated NPC cases (UCNT WHO type III),

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Translational Relevance

Diagnosis and posttreatment monitoring of Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC) is complicated and requires repeated painful biopsies and pathologic examination. Early tumor detection and timely initiation of treatment are important for patient survival. The results from this study in 228 patients with NPCs reveal that simple noninvasive nasopharyngeal brushing plus EBV-DNA load as tumor marker gives excellent diagnostic and prognostic results compared with the biopsy. The nasopharyngeal brush approach proved better than EBV-DNA load assessment in blood and EBV-IgA serology. The data suggest that nasopharyngeal brush sampling may provide a useful instrument for direct *in situ* NPC tumor detection in populations with symptoms suspected of NPCs and may replace repeated biopsies during follow-up. The nasopharyngeal brush is not perceived as painful by patients, is suited for remote sampling in regional hospitals, and allows parallel assessment of additional tumor markers. The nasopharyngeal brush appears well suited for use in NPC screening in high incidence regions like Indonesia.

whereas its association with squamous cell carcinoma (WHO type I) and nonkeratinizing carcinoma (WHO type II) is variable. In NPC endemic regions, WHO type I and II tumors are also frequently associated with EBV (5), but in nonendemic regions, these often result from tobacco and alcohol abuse (6). Undifferentiated NPC represents 85% of all NPC cases in endemic regions and is a major cause of cancer morbidity and mortality imposing a significant socioeconomic burden to families and the population in general (7).

Currently, diagnosis of NPC requires a biopsy from the suspected tumor site with histopathologic assessment and demonstration of EBV involvement by *in situ* hybridization for EBER1/2 RNA or immunohistochemistry for EBNA1 or LMP1 protein. The detection of EBER transcripts by *in situ* hybridization remains the standard of care for identifying latent EBV infection. A biopsy from the postnasal space is an invasive and painful procedure that may lead to extensive bleeding and cannot be repeated easily without compromising the patient (8). At early stage, NPC often presents with minimal or nonspecific local symptoms and the nasopharynx is difficult to access for (repeated) routine examination making early diagnosis challenging. Thus, the biopsy is crucial for defining NPCs as cause of symptoms and subsequent medical handling. In addition, it is important to obtain biopsies of adequate depth as nasopharyngeal carcinoma may spread submucosally and are easily missed by endoscopic examination, even in patients with an obvious exophytic tumor, due to slough, necrotic tissue, and inflammatory tissue overlying the tumor. Therefore, biopsy with a small endoscopic forceps may result in a high false negative rate. A representative biopsy can be difficult to obtain and requires the use of flexible and rigid endoscopes to allow good visualization of the nasopharynx. Local anesthesia permits biopsies to be taken under direct vision and therefore anesthesia is recommended to avoid missing small or submucosal lesions yielding sensitivity of 95.1% and 95.6% respectively (9, 10). When no obvious tumor is present, a biopsy from the lateral pharyngeal recess can be conducted because this is the most common site for early disease (11).

There is a clear need for more simple noninvasive diagnostic assays for early NPC detection, in particular in endemic regions, which can also be used in monitoring therapy requiring repeated sampling. Previous studies revealed nasopharyngeal brushing as a simple procedure with minor discomfort, being well tolerated and reflecting carcinoma-specific EBV involvement at the anatomical site of tumor development, thereby reducing the need for invasive biopsies (12–14). This procedure has promise as confirmation test in serological NPC screening programs and has potential as prognostic tool for therapy assessment and follow-up monitoring. Furthermore, aberrant tumor-associated DNA methylation patterns can be analyzed in the same brush specimen (15, 16). In addition to viral load in nasopharyngeal brushings, measuring the level of EBV-DNA in whole blood, plasma, or serum of patients with NPCs before and after treatment may be valuable for assessment of disease progression (17), as levels of EBV-DNA in the circulation of patients with NPCs with recurrence were shown to be much higher than EBV-DNA levels of those who remain in continuous clinical remission (18, 19). These studies indicated that monitoring EBV-DNA load may provide useful diagnostic information for NPC diagnosis and posttreatment management.

The present study evaluates the diagnostic and posttreatment value of viral DNA load measurement in minimal invasive nasopharyngeal brushings and in parallel in whole blood samples collected at diagnosis and 2 months after start of therapy in 228 patients with advanced NPCs. The viral load was compared with standardized peptide-based EBV-IgA serology and clinical treatment response.

Material and Methods

Patients and controls

Two hundred and eighty-nine consecutive patients presenting to the ENT clinic of Dr. Cipto Mangunkusumo Hospital, Universitas Indonesia (Jakarta, Indonesia) with suspected NPCs during 2006–2009 were enrolled into this study. About 20% of the patients were referred by regional health centers where initial diagnosis was conducted. Medical ethical approval for this study was obtained and all patients and controls signed for informed consent. TNM staging was done for all patients using the 2002 American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) staging system. Assessment for diagnosis included medical history, particularly on NPC-related symptoms, physical examination for enlarged neck node and examination of the suspected nasopharyngeal lesion by fiber optic nasopharyngoscopy with photography and computed tomographic (CT) scans. In all patients, a

Table 1. Numbers of patients and controls used for validation of viral load by nasopharyngeal brush

Patient description		
NPC		228
EBV-related malignancy		19
Non–Hodgkin lymphoma	8	
T/NK cell lymphoma	10	
Burkitt's lymphoma	1	
Non-NPC head and neck carcinoma		25
Other ENT disorder		17
Healthy control		53

nasopharyngoscopy-guided nasopharyngeal brushing was conducted first, followed by biopsy from the same area of the suspected NPCs. Endoscopic findings were classified as normal (no tumor), suspicious tumor, or clearly abnormal. Of the 289 patients at intake, 228 had biopsy-proven NPCs and 61 were proven to have a variety of malignant and nonmalignant head and neck diseases and served as clinical controls in this study, as specified in Table 1. Unfortunately due to problems inherent to the Indonesian health care system (lack of medical facilities, low socioeconomic status, insufficient insurance coverage, and the often remote areas where patients are living), detailed follow-up proved difficult. A total of 202 brushings, 149 whole blood, and 174 serum samples at diagnosis and from follow-up 69 brushings, 65 whole blood, and 68 serum samples were available for analysis (Table 2). Clinical characteristics and NPC stage information is given in Table 3. Diagnosis was based on routine pathologic assessment of paraffin-embedded tumor biopsy specimens and WHO typing of NPC was assessed by 2 independent pathologists. The presence of EBV was confirmed by EBER-RISH using the commercial PNA-Based Hybridization Kit (Dakocytomation) in 116 of 228 patients from whom an adequate biopsy specimen was available.

Treatment

In NPC cases, radiotherapy was uniformly administered to the primary tumor and neck region. The total dose delivered was 66 to 70 Gy during 6 to 8 weeks by conventional fractionation or hyperfractionation-accelerated radiotherapy. Neoadjuvant/adjuvant chemotherapy consisted of 5-fluorouracil (FU; 1,000 mg/m^2 days 1–5) and cisplatin (100 mg/m^2 day 1) in 3 cycles every 3 weeks. Concurrent chemotherapy was delivered with cisplatin at 40 mg/m^2 weekly during radiotherapy courses. Because of undercapacity of radiotherapy and the poor financial situation of most patients, optimal treatment, that is, full chemoradiation, was not always feasible and different treatment protocols had to be implemented.

Table 2. Numbers of samples of patients with NPCs at diagnosis and 2 months follow-up

Patients with NPC	Nasopharyngeal brushing	Whole blood	Serology
At diagnosis	208	149	174
After 2 months follow-up	69	65	68

Sampling procedures

Nasopharyngeal brushing was conducted under rigid or flexible endoscopic guidance by experienced ENT specialists and ENT resident trainees. Endoscope-guided nasopharyngeal brushings were conducted under local anesthesia (1% Lidocaine spray, Astra Zeneca). An endoscope was used to evaluate the entire nasopharynx and photographs were taken routinely from the site of tumor involvement. Localization and appearance of the tumor was defined and graded into 3 groups (none, suspicious, and clear abnormal). A Cytobrush Plus (Medscand) was used in combination with a plastic catheter covering the entire brush to prevent contamination by cells from non-nasopharyngeal sites. The catheter covering the cytobrush was inserted via the nose until the nasopharyngeal cavity was reached. Subsequently, the brush was released from the catheter and the cytobrush was rotated several times over the nasopharyngeal epithelium at the site of the suspected lesion, returned into the catheter, and removed. Immediately after sampling, the brush tip (1.5 cm) was cut and placed in 4 mL of NucliSens Lysis buffer (LB; BioMerieux) mixed well and stored in 1-mL aliquots at −80°C until use (14, 20). In all NPC-suspected patients, nasopharyngeal brushings were obtained from the site of suspected tumor involvement before taking the biopsy at the same site. In 20 patients, both sides of the nasopharyngeal wall were brushed at diagnosis or during follow-up under endoscopic guidance (twenty-five 2-sided samples were collected). To compare the level of discomfort between the brushing procedure and the biopsy, 57 patients at random answered a questioner form based on visual analog scale 1 to 10. Furthermore, we conducted standard nasopharyngoscopy and brushings with informed consent in 53 healthy regional controls. At the same time, 5 mL whole blood was taken, of which 4.5 mL was used to make serum for serology and 0.5 mL was added to 4.5 mL LB for measuring EBV-DNA load, exactly as described before (21, 22). Frozen samples were shipped on dry ice and analyzed blindly to the NPC status for EBV-DNA load at the department of Pathology, VU University Medical Centre, Amsterdam, the Netherlands.

Quantification of EBV-DNA load and cellular DNA by LightCycler-based real-time PCR assays

DNA was isolated from 1 mL nasopharyngeal brush samples in LB by silica-based nucleic acid extraction and eluted in 100 μL H_2O, exactly as described before (14, 22). Reagents for the isolation procedure were obtained from BioMerieux. EBV real-time PCR described for nasopharyngeal brush samples in this study was based on amplification of well-conserved 213-bp region of the BKRF1 gene

Table 3. Characteristics of patients with NPCs ($N = 228$)

	Number (%)
Sex	
Male	164 (71.9)
Female	64 (28.1)
Histopathology	
WHO 1	28 (12.3)
WHO 2	5 (2.2)
WHO 3	195 (85.5)
Age, y	
<10	5 (2.2)
10–20	19 (8.3)
21–40	81 (35.5)
≥41	123 (53.9)
T stage	
T1	18 (6.4)
T2a	16 (5.7)
T2b	69 (24.6)
T3	53 (18.9)
T4	72 (25.6)
N stage	
N0	25 (11)
N1	61 (26.8)
N2	48 (21.1)
N3a	77 (33.8)
N3b	17 (7.5)
N stage	
N0	25 (11)
N+	203 (89)
M stage	
M0	210 (74.7)
M+	18 (6.4)
Stage AJCC-UICC	
Stage I	2 (0.9)
Stage IIA	1 (0.4)
Stage IIB	25 (11)
Stage III	55 (24.1)
Stage IVA	42 (18.4)
Stage IVB	85 (37.3)
Stage IVC	18 (7.9)
Stage summary	
Early	3 (1.3)
Advanced	225 (98.7)
Type of treatment	
Neoajuvant + RT	81 (35.5)
Neoajuvant + HPF	40 (17.5)
Neoajuvant + CRT	9 (3.9)
Concurrent CRT	87 (38.2)
Radiotherapy	1 (0.4)
Chemotherapy full dose	9 (3.9)
No treatment	1 (0.4)

(Continued on the following column)

Table 3. Characteristics of patients with NPCs ($N = 228$) (Cont'd)

	Number (%)
Clinical response treatment at 2 mo posttreatment	
Complete response	52 (22.8)
Partial responses	30 (13.2)
Progressive disease	2 (0.9)
Death	7 (3.1)
Loss to FU	137 (0.9)

Abbreviations: CRT, chemoradiation; FU: follow-up; HPF, hyperfractionation; RT, radiotherapy.

encoding Epstein-Barr nuclear antigen-1 (EBNA-1), a single-copy gene of EBV, and blood samples were analyzed by PCR using a 99-bp region from the same EBNA1 region to reliably detect fragmented EBV-DNA, as described before (21, 22). Most brush samples were analyzed by both PCR assays, yielding no significant different result (Supplementary Fig. S1). Primers, probes, and PCR conditions have been described in detail previously (14, 22). Cutoff value (COV) for EBV-DNA load in nasopharyngeal brushings was defined at 2,300 copies per brush, being the mean $+3\times$SD of brush EBV-DNA load in non-NPC case–controls as previously defined (14) and confirmed in the current group of healthy Jakarta EBV carriers, excluding 4 individuals with elevated EBV-DNA load also having aberrant EBV serology, possibly relating to stress-induced EBV reactivation. The COV for EBV-DNA in blood was defined at 2,000 copies/mL, based on prior studies (21, 22). These COVs were validated and confirmed in the healthy control group in this study used to determine sensitivity and specificity, positive and negative predictive values (PPV and NPV).

The amount of human diploid genome equivalent in nasopharyngeal brushing specimens was determined by quantitative LightCycler-based polymerase chain reaction (LC-PCR) targeting a 197-bp fragment of the human β-globin gene (23).

EBV serology

Serum samples from patients with NPCs, control patients, and healthy controls (Table 2) were analyzed for IgA antibodies to EBV-specific immunodominant epitopes of VCA-p18 and EBNA1 using individual synthetic peptide–based ELISA assays for each marker exactly as described previously (24).

Statistics

One-way ANOVA was used for comparison of EBV-DNA load and EBV IgA antibody levels between NPC and non-NPC groups. In addition, one-way ANOVA was used for comparing EBV-DNA load and antibody levels to TNM stage of NPCs at intake. $P < 0.05$ was considered to be significant.

Mann–Whitney test: $P < 0.001$ used for subjective evaluation for visual analog scale (VAS) between brushing

and biopsy procedures to examine the median difference between 2 groups (procedures) and for analyzing the level of comfort of the conducting a nasopharyngeal brush or biopsy.

The evaluation of viral DNA load of bilateral side nasopharyngeal brushing was conducted by a Mann–Whitney test. Testing the viral DNA load decreases in nasopharyngeal brush and whole blood at diagnosis and after treatment of the paired samples was conducted by a Wilcoxon test.

Results

Patient characteristics

For this study, 289 consecutive patients with suspected NPCs were enrolled. In 228 cases, NPC diagnosis was confirmed by pathological examination of the biopsy using routine histochemistry. Patient characteristics are summarized in Table 3. The non-NPC group consisted of patients diagnosed with EBV-related malignancy, EBV-negative non-NPC head and neck cancer, nonmalignant ENT disorders and 53 healthy individuals (Table 1).

In the NPC group, male-female ratio was 3:1 and 85% were classified as WHO type 3. Although the age of the majority of patients with NPCs (54%) was above 40 years, 11% was of juvenile (5–20 years) age. At presentation, 99% of patients had advanced stage of disease, with 85 patients (37%) in stage IVB (AJCC-UICC staging system) and 18 patients (8%) had distant metastasis. The treatment of choice for these patients is a combination of chemotherapy and radiotherapy (Table 1). The patients with distant metastasis were treated with palliative chemotherapy. For this study, 208 nasopharyngeal brushes, 149 whole blood, and 174 serology samples could be evaluated at diagnosis. Posttreatment nasopharyngeal brush samples of 69 patients were analyzed as well as 65 parallel whole blood (WB) and 68 serology samples.

Viral DNA load in nasopharyngeal brushings at diagnosis

An accurate well-validated real-time PCR procedure for EBV-DNA quantification, detecting a conserved region of the single copy EBNA1 (BKRF1) gene, was used for analyzing the EBV-DNA load in nasopharyngeal brushings taken at diagnosis. COV for viral DNA load in nasopharyngeal brushings was previously defined at 2,300 EBV-DNA copies/nasopharyngeal brushing in healthy EBV seropositive individuals and non-NPC patients with various head and neck complaints (14) and was here confirmed in the healthy controls in the Jakarta population (Fig. 1A). This COV was used to determine sensitivity, specificity, PPV, and NPV of 94%, 90%, 84%, and 80% respectively, as indicated in Table 4.

Figure 1A shows that NP brushings from patients with NPCs showed significant higher levels of viral load than controls (median NPC, 1.0×10^6; range 0×10^8 to 1.9×10^8 vs. median, 4.0×10^3; range, 0×10^5 to 1.2×10^5; $P < 0.0001$).

EBV-DNA was detected above COV in 95% of NPC cases, confirming NPC tumor cell presence. The higher EBV-DNA values in nasopharyngeal brushings in the "other" tumor group compared with the EBV-related tumors may be explained by the location of the tumor in the ENT region that might associate with reactivating EBV. The non-NPC EBV-associated tumors are not located in the ENT region. Frequently extreme EBV-DNA levels were reached in NPC cases, up to 100 million copies of EBV-DNA per brush. Ten cases (5%) had an EBV-DNA level below COV. Viral DNA load at diagnosis was not related to age or sex of patients with NPCs (data not shown).

Erroneous sampling was excluded by quantifying the cellular β-globin DNA, which showed similar host genomic levels (3–10 million copies/brush), indicating that brush sampling itself was done appropriately (14). However, absence of EBV load may be caused by sampling outside the tumor field. Brush viral DNA load in NPC cases was higher than in patients with non-NPC head and neck cancers ($P = 0.059$), other EBV-related malignancies ($P = 0.001$), and nonmalignant ENT complaints ($P < 0.001$). However, EBV-DNA load in nasopharyngeal brushings of these patients with mainly advanced-stage NPCs did not correlate with T, N, or M substage of the tumor at diagnosis, as shown in Fig. 2A, C, and E ($P = 0.60$, 0.071, and 0.092, respectively, as determined by one-way ANOVA). Some control individuals having no detectable NPC tumor mass did show elevated EBV-DNA levels. In these cases EBV IgA serology was also elevated indicating EBV reactivation (data not shown), as recently found in defined NPC risk groups in Indonesia (25).

Viral DNA load in whole blood at diagnosis

The whole blood EBV-DNA load of patients with NPCs at diagnosis was significantly higher than the clinical COV of 2,000 copies/mL whole blood (21) compared with the control groups and even compared with that in other EBV-related malignancies (Fig. 1B). However and importantly, a high number of NPC cases had low (<COV) or undetectable EBV-DNA levels in blood which was even observed in some patients with bulky disease (stage IVA and IVB), confirming our previous independent findings (21). No correlation was found between EBV-DNA load in whole blood and T stage of the tumor at presentation as shown in Fig. 2B ($P = 0.25$). However, considering the positive samples only, a correlation was found between whole blood EBV-DNA load and N and M stage ($P < 0.001$ and $P = 0.010$, respectively; Fig. 2D and F).

Serology IgA VCA-p18 and IgA-EBNA1

IgA VCA-p18 serology, reflecting viral replication at the mucosal surface, showed higher values in sera from patients with NPCs at primary intake (median, 2.3; range, 0.29–30) than in healthy controls ($P = 0.001$). Sera form 79.8% of the patients with NPC had IgA VCA-p18 values above the COV level. The sera obtained from all other patient groups including the EBV-related malignancies and non-NPC head and neck cancer had lower antibody levels against VCA-p18 compared with patients with NPCs and did not reveal

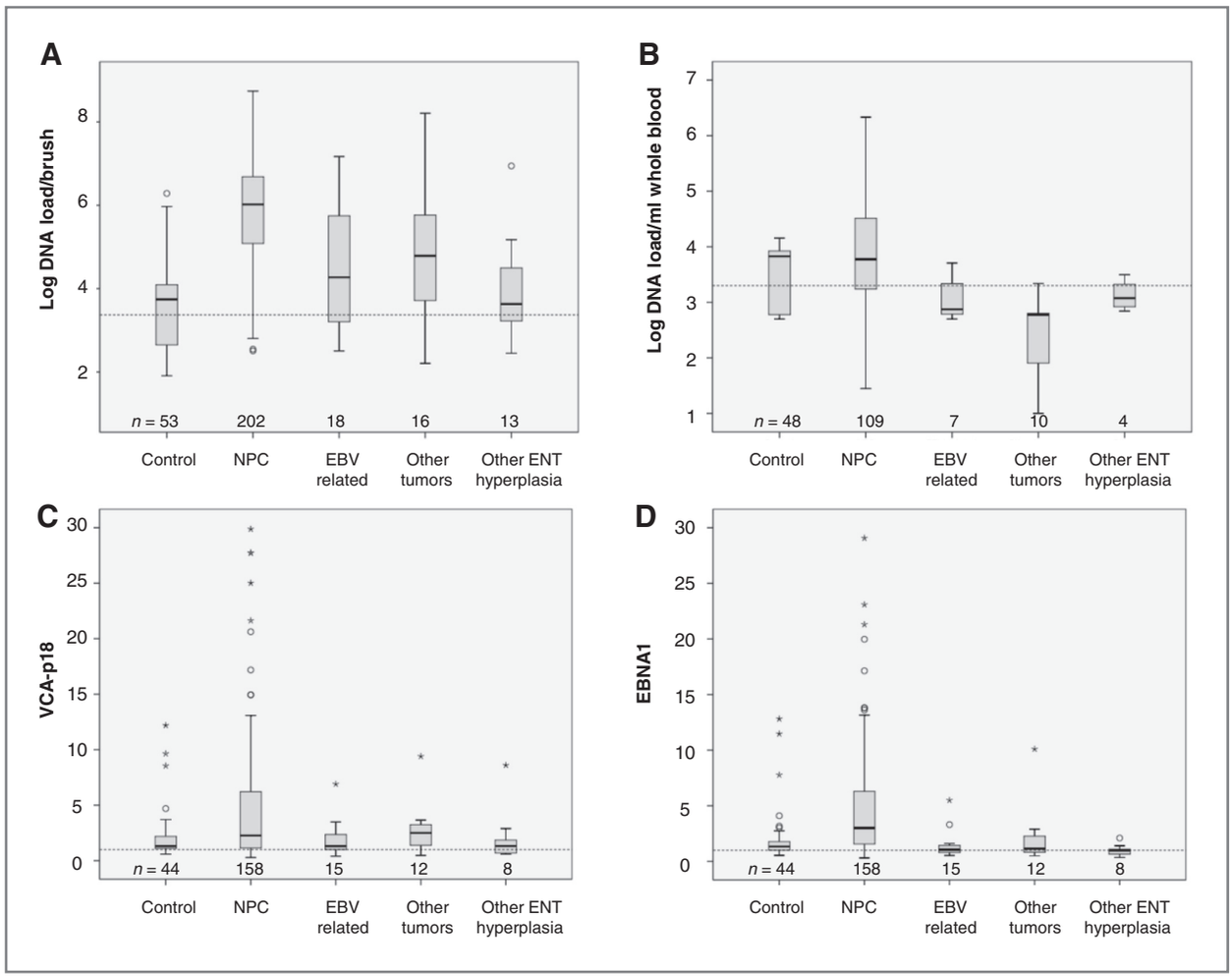

Figure 1. EBV parameters at diagnosis. A, viral DNA load in nasopharyngeal brushings (log scale). The EBV-DNA levels observed were significantly different between NPCs and healthy controls, EBV-related malignancies ($P < 0.001$), and other ENT disorders ($P < 0.001$), whereas a near significant difference was found between NPCs and non-NPC head and neck cancer ($P = 0.059$). B, viral DNA load in whole blood (ilog scale). The mean EBV-DNA load in blood was not significantly different between NPCs and healthy controls ($P = 0.601$), EBV-related malignancies ($P = 0.109$), and other ENT disorders ($P = 0.401$), whereas NPCs and non-NPC head and neck cancer did show a significance difference ($P < 0.001$). C, IgA VCA-p18 serology. EBV-specific VCA-p18 IgA serology was significantly higher in NPCs versus healthy controls ($P = 0.011$) but not between NPCs and EBV-related malignancy ($P = 0.21$), non-NPC head and neck cancer ($P = 0.75$), and other ENT disorders ($P = 0.57$). D, EBNA1-IgA serology. EBNA1-IgA serology was significantly higher in NPC versus healthy controls ($P < 0.001$), and EBV-related malignancies ($P = 0.018$) and was close to significance for NPC versus non-NPC head and neck cancer ($P = 0.20$) and other ENT disorders ($P = 0.054$). The dotted line in each graph represents the cutoff value for each assay, as defined in the methods section.

further statistically significant differences between the groups. (Fig. 1C)

IgA EBNA1 serology, reflecting latent (tumor) antigen expression, revealed significant higher values in NPC cases than in other groups resulting in 85.6% of the patients having IgA EBNA1 responses above the COV. Patients with NPCs have higher median value (median, 3.0; range, 0.3–29) than EBV-related malignancy (median, 1.1; range, 0.53–5.5), other malignant conditions (3.05; range, 0.3–29), healthy controls (median, 1.1; range, 0.5–4.1), and other ENT disorders (median, 1.1; range, 0.5–10; $P < 0.05$; Fig. 1D). No correlation was found between VCA-IgA or EBNA1-IgA antibody levels in ELISA and TNM staging of the NPC tumor at intake (data not shown).

Diagnosis by biopsy versus brushing

Biopsy was conducted as standard-of-care diagnosis in all 228 patients with NPCs. We obtained information on the level of discomfort experienced during brushing and biopsy procedures in 57 patients, which were quantified by VAS. The brush procedure was characterized by a median VAS score of 5 (range, 3–6), which is significantly less compared with the biopsy with a median VAS of 9 (range, 4–10; Kolmogorov–Smirnov: $P < 0.001$). Only 1 patient stated the biopsy was less painful than brushing.

In 11 patients, repeated biopsies were required to obtain the diagnosis. One patient needed even 3 subsequent biopsies to obtain diagnostic evidence explaining the mass observed by CT scan. In all 11 cases, the viral DNA

Table 4. Sensitivity, specificity, PPV, and NPV of EBV markers

	Brush	Whole blood	IgA VCA P18	IgA EBNA 1
Sensitivity	94.3	71.1	65.4	74.3
Specificity	90.0	50.0	60.0	72.0
PPV	84.4	85.7	84.7	89.8
NPV	80.0	20.0	33.3	44.6

load in the initial brush was above COV allowing direct diagnosis.

In a selected group of 25 patients giving separate informed consent, we collected nasopharyngeal brushings from both sides of the nasopharyngeal cavity, that is, at and opposite to the suspected tumor site (defined by location of neck node in most cases). EBV-DNA load values in parallel brushings were higher at the tumor site (72% >COV; median, 16.700 c/brush; mean, 188.782 c/brush; range, 414×10^6 to 4.7×10^6 copies/brush) than in the opposite site (48% >COV; median, 2.400 c/brush; mean, 43.258 copies/brush; range, 0×10^6 to 1.1×10^6 copies/brush; Fig. 3). These differences were not statistically different ($P = 0.13$). However, in NPC cases, both the median and mean EBV-DNA level in brushings taken from the nonlesional side of the nasopharynx were still significantly higher than the EBV-DNA load observed in non-NPC tumors and ENT hyperplasia ($P < 0.001$)

Comparison of viral DNA load at diagnosis and 2 months posttreatment

In 69 patients, the effect of the therapy on the viral load was analyzed by comparing the viral DNA load in nasopharyngeal brush and whole blood at diagnosis and 2 months posttreatment. The median EBV-DNA load in nasopharyngeal brushing at diagnosis was 9×10^5 copies/nasopharyngeal brush and decreased after 2 months posttreatment to a median of 3×10^3 copies/nasopharyngeal brush indicating a 300-fold reduction (Fig. 4). Initially, 96% of patients had a viral load > COV level in the nasopharyngeal brush, but after treatment, this dramatically reduced to 39.4%. Similarly, the level of EBV-DNA in whole blood was significantly lower posttreatment with a reduction of 27-fold ($P < 0.001$ for both), and the percentage of patients with a viral load > COV in the circulation dropped from 51% to 8.8%. Although the fold reduction in viral DNA load in both nasopharyngeal brush and blood samples reflected the treatment response, irrespective of the regimen used (see below), the level of EBV-DNA at diagnosis did not have any predictive value for treatment outcome.

Patients treated with neoadjuvant plus hyperfractionated radiotherapy had a median value of 8.9×10^6 copies/brush at diagnosis that decreased after therapy to 1.7×10^5 copies/brush ($P = 0.006$). Patients treated with concurrent chemoradiation had a median DNA viral load in brushings of 1.9×10^7 copies/brush at diagnosis decreasing to 5.8×10^4 copies/brush after therapy ($P = 0.049$). Only one patient with neoadjuvant and radiotherapy had increased DNA viral load brush posttreatment (9.5×10^5 copies/brush) and one patient had increased viral load in whole blood at 2 months posttreatment, both linked to progressive disease.

On the basis of response to treatment, 41 patients had a complete response at 2 months posttreatment as judged by clinical examination plus a negative CT scan and negative biopsy. These patients had a posttreatment median viral DNA load in the nasopharyngeal brushing of 3.0×10^3 copies/brush, a significant difference compared with the pretreatment value of 1.7×10^6 copies/brush in this group ($P = 0.013$; Fig. 5). In 22 patients with partial response, the median EBV-DNA load in nasopharyngeal brush pretreatment was 1.3×10^6 and posttreatment 3.2×10^3 copies/brush ($P = 0.14$). For whole blood samples, most cases with an initial positive DNA load, the EBV-DNA load became undetectable after 2 months after starting treatment for both complete and partial responses. Two of 3 patients with progressive disease posttreatment showed a median of EBV-DNA load in brush being below COV, whereas the posttreatment median level in blood was above COV in all 3. Two patients died within 2 months posttreatment and their DNA viral load brush was above COV, whereas the viral load in the whole blood was negative. No significant difference was observed for EBV-IgA serology levels at diagnosis compared with 2 months after treatment, neither for VCA-p18 nor for EBNA1 antibody levels individually, nor for different treatment regimens (data not shown).

Discussion

Pathologic examination for diagnosis of NPCs requires an invasive biopsy that is painful and cannot be repeated easily. A less invasive diagnostic procedure by using nasopharyngeal brush sampling would be preferred, also for assessment of posttreatment tumor activity. This nasopharyngeal brush procedure may also be combined with detection of aberrant EBV-IgA serology in screening approaches of patients at (family) risk or having symptoms suggestive of early-stage NPCs (25, 26). In this study, we evaluated minimal-invasive nasopharyngeal brushing with quantification of EBV-DNA load for primary NPC diagnosis and assessment of treatment response relative to the standard biopsy taken in parallel (14). We also measured EBV-DNA load in whole blood and VCA-p18 and EBNA1-specific EBV-IgA serology in simultaneous venous blood samples (21). We showed that measuring EBV-DNA load in nasopharyngeal brushings provides a highly specific tool for primary NPC diagnosis with minimal patient discomfort, giving better sensitivity/specificity compared with EBV-IgA serology and EBV-DNA load in blood, as detailed in Table 4. Because most patients in this study presented with advanced-stage NPCs, the use of nasopharyngeal brushing for detecting early-stage NPCs remains to be defined. In ongoing studies in patients with persistent head and neck complaints, nonresponsive to antibiotic or anti-allergy

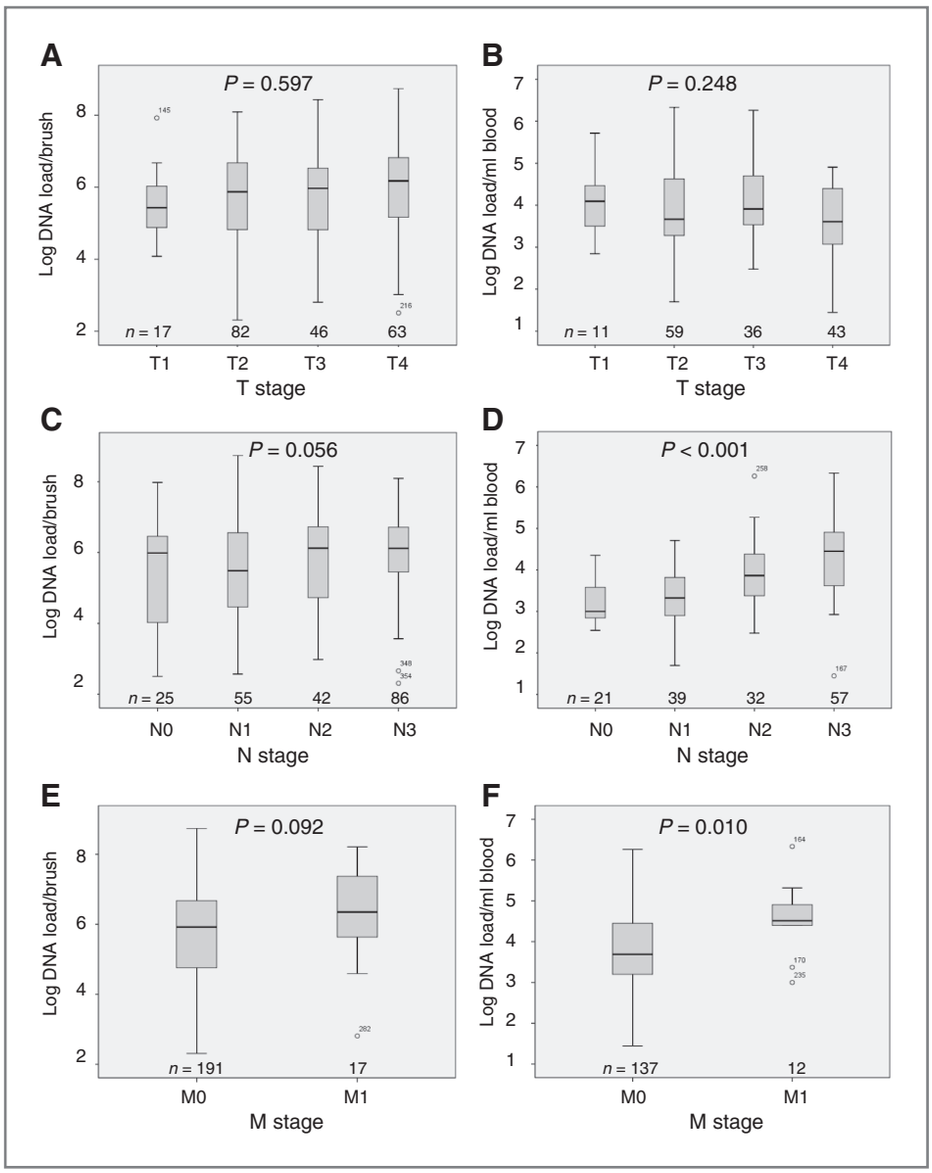

Figure 2. EBV markers in relation to tumor characteristics at intake. A, correlation of EBV-DNA load in brush with T stage (DNA copies in log scale), showing no relation using ANOVA ($P = 0.597$). B, correlation of EBV-DNA load in whole blood (WB) with T stage ($P = 0.248$), C, correlation of EBV-DNA load in brush with N stage ($P = 0.056$), D, correlation of EBV-DNA load in WB with N stage ($P < 0.001$), E, correlation of EBV-DNA load in brush with M stage ($P = 0.092$), F, correlation of EBV-DNA load in WB with M stage ($P = 0.010$).

therapy, we are currently validating this method for identification of early-stage NPCs. The diagnostic use of nasopharyngeal brush may be further increased by assessing a combination of molecular carcinoma markers in the same brush material, including tumor-specific EBV-RNA transcripts (14), host genomic methylation patterns (15, 16), and other genetic abnormalities linked to NPCs.

Although detection of NPCs at early stage is important for the patient outcome, diagnosis is often difficult because of the nonspecific nature of the clinical symptoms and difficulty in visualizing the nasopharynx (4). Only 12.2% of our patients presented with early TI–IIa stage, whereas 24.6% presented with TIIb with tumor already invading into the parapharyngeal area giving worse prognosis compared with localized disease limited to the nasopharynx. The majority (89%) of patients, however, already had parallel enlargement of the regional lymph node indicative of advanced (late) stage (Table 1), which is typical for most endemic regions (3). This situation reflects the need for novel diagnostic procedures for regular testing of NPC risk populations, such as family members of patients with NPCs and patients with chronic head and neck complaints suggestive for early-stage NPCs (25, 26, 27).

Nasopharyngeal brushings from patients with NPCs frequently contain extremely high levels of EBV-DNA compared with other clinical conditions, including EBV-related non-NPC head and neck cancers (Fig. 1), confirming previous studies (28, 29). More than 95% of our patients with NPCs had a brush containing viral loads above COV. A negative result (5%) of EBV-DNA viral load brush might be caused by absence of cancer cells or obscured by blood, by

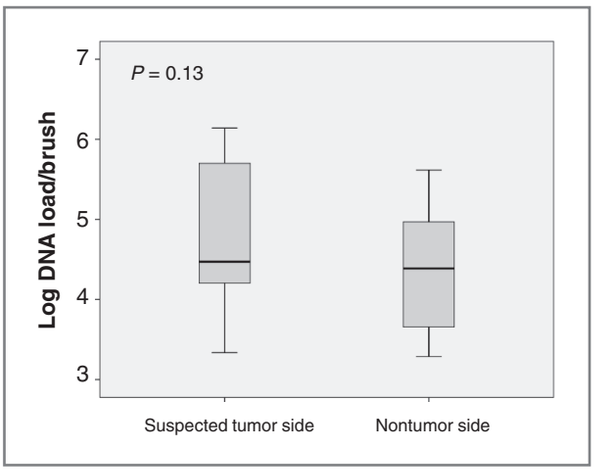

Figure 3. Viral DNA load of bilateral side nasopharyngeal brushing. Bilateral brushing ($n = 20$) was conducted at the site of suspected tumor location and the nontumor site. The mean viral DNA load was 3.4×10^5 versus 7.1×10^4 copies per brush, respectively ($P = 0.13$), which indicates that single brushing at the site of enlarged neck node may be more representative for detecting NPC presence.

tumor detritus, or due to improper sampling. Both primary and recurrent cancers may be located deep under the overlying mucosa and early lesions not invading the nasopharyngeal surface can be difficult to detect when biopsy or brushing is done too superficially (30). Contrary, NPC tumor–derived EBV-DNA from submucosal locations may reach the surface (shedding) leading to detectable aberrant levels in the brush. Our data indicate that nasopharyngeal brushing combined with quantitative real-time PCR directly reflects carcinoma-specific EBV involvement at the anatomical site of tumor development. The nasopharyngeal brush may greatly reduce the number of invasive nasopharyngeal biopsies required when applied for diagnosis and follow-up monitoring. As bilateral brushing might be necessary for EBV tumor detection, Tune and colleagues originally recommend bilateral brushing as a routine to avoid missing small, localized tumors (13). We conducted brush and biopsy sampling under endoscopic guidance for all patients, which may be a preferred procedure for accuracy of sampling. Our data on bilateral brushing (Fig. 3) indicate that random brushing of the nasopharyngeal cavity may be adequate, supporting the general applicability of the brush technique for NPC diagnosis, without the need for endoscope guidance. Blind brushing may be done in the nasopharyngeal area on the side of the neck node at the lateral pharyngeal recess because this is the most common site for early disease. However, this needs to be further evaluated.

The level of discomfort and pain was analyzed between brushing and biopsy procedure in 57 suspected patients with NPCs. The nasopharyngeal brushing procedure was well-tolerated and none of the patients or controls complained of negative effects like pain or bleeding, etc. In contrast, the biopsy procedure frequently associated with excessive bleeding and pain. In 11 patients, repeated biopsies were needed to pathologically verify the presence of tumor cells, whereas EBV-DNA load in the parallel brush was above COV at the first sample with 2 patients having very high viral loads. Overall, nasopharyngeal brushing proved to be a specific and minimal invasive diagnostic tool for NPC diagnosis. However, the possibility remains that a deeply located tumor is missed by the nasopharyngeal bushing procedure, whereas a deep biopsy may be able to yield sufficient number of tumor cells for making a diagnosis (30). This can only be confirmed in more extensive studies.

The sensitivity, specificity, PPV, and NPV for detecting EBV-DNA load above the predetermined clinical cutoff level in whole blood, being 71%, 50%, 86%, and 20%, respectively (Table 2), were low compared with the nasopharyngeal brush values. This confirms a previous independent study showing that many patients have only minimal (50%) or even negative (25%) EBV-DNA levels in blood (21). Circulating EBV-DNA does not reflect intact circulating tumor cells because EBV-RNA transcripts from BART, LMP2 or BARF1 reading frames were not detectable in the whole blood samples. EBV-DNA in blood reflects apoptotic release of DNA fragments with an average size of 150 bp or less, which are rapidly cleared from the circulation (18, 28, 29). High EBV-DNA blood levels therefore may reflect ongoing tumor apoptosis and necrosis rather than a growing tumor mass (21). Our quantitative data on

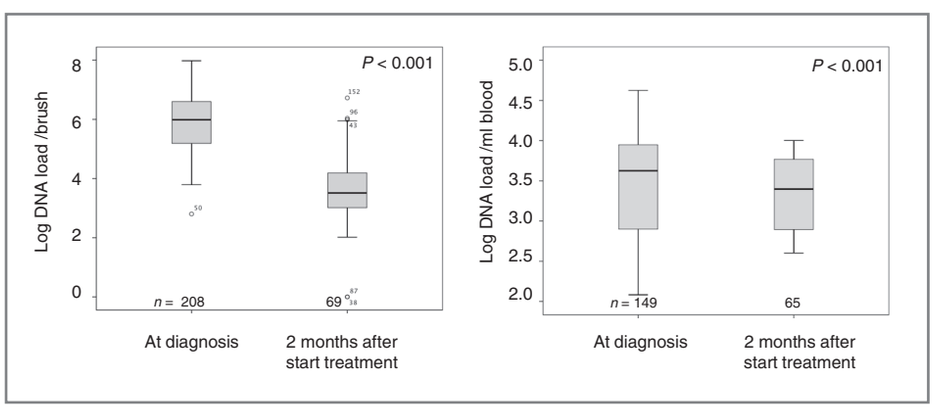

Figure 4. Viral DNA load in nasopharyngeal brush and whole blood at diagnosis and after treatment. A, EBV-DNA load in nasopharyngeal brushings (copies/brush). B, EBV-DNA load in whole blood. There was a significant decrease in EBV-DNA load in both nasopharyngeal brushings and whole blood at diagnosis compared to 2 months posttreatment for samples paired before and after treatment ($P < 0.001$).

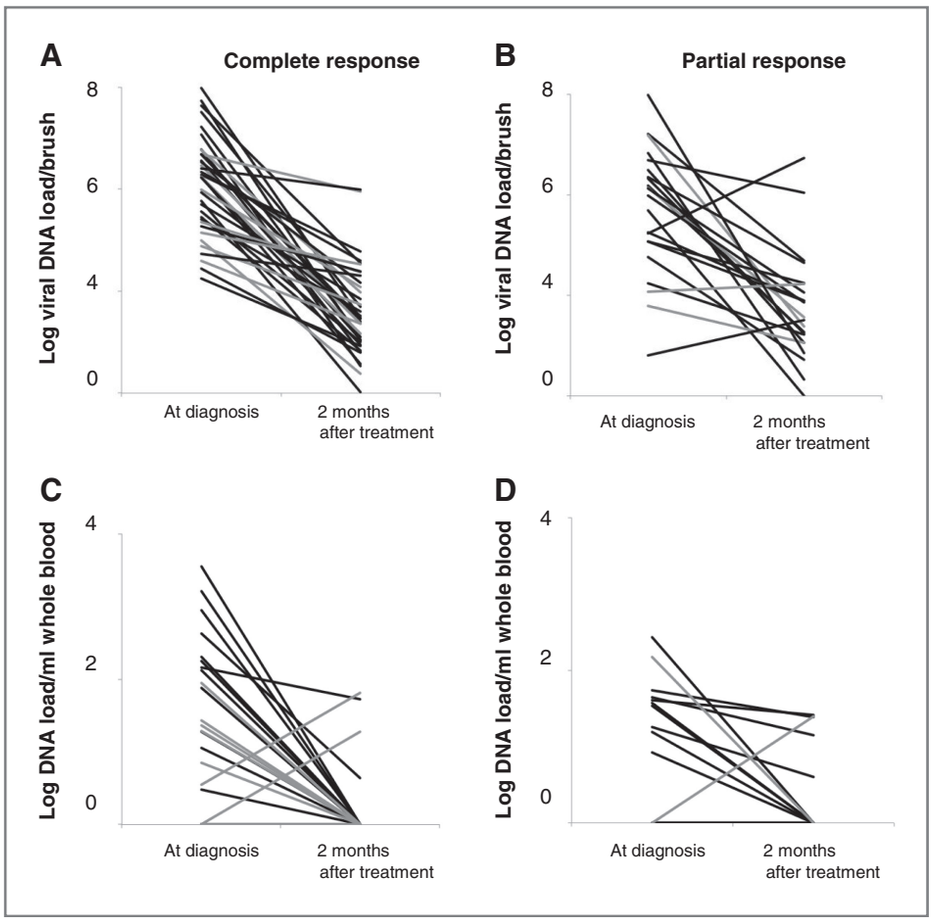

Figure 5. Viral DNA load of NPC patient samples before and 2 months posttreatment in relation with treatment response. Patients with NPCs treated with concurrent therapy are given in black and patients receiving neoadjuvant therapy are presented in gray. Presented is EBV-DNA load in (A) brushes of patients with NPCs with complete response; (B) brushes of patients with NPCs with partial response; (C) whole blood of patients with NPCs with complete response; (D) whole blood of patients with NPCs with partial response. Complete response is defined as complete disappearance of locoregional disease by physical examination or X-ray and CT scan and endoscopic examination and a negative biopsy at 2 months posttreatment; partial response is defined by reduction of disease by 30% or more based on clinical examination or X-ray and CT scan. If the disease shows a slight increase in size or extends after treatment, it is defined as progressive disease.

circulating EBV-DNA load differ from the initial studies by Lo and colleagues in Hong Kong (17, 18), as detailed elsewhere (22). Pretreatment level of circulating EBV-DNA is considered to be a prognostic factor for NPCs (19, 28, 29, 31). Others showed that circulating EBV-DNA levels may correlate with stage of disease (18), which was not observed in this study. However, percentage of patients with NPCs with elevated EBV-DNA levels in blood or plasma differ between studies and procedures are not well-standardized. In this study, elevated EBV-DNA load in blood above the clinical COV of 2000 copies/mL was detected in only 50% of the patients with NPCs. Some patients with extensive clinical disease (stage IVB) completely lacked circulating EBV-DNA, despite having high EBV-DNA levels in the nasopharyngeal brush collected at the same time. These observations confirm prior findings that EBV-DNA load in blood may not provide strong diagnostic information (21). Tong and colleagues found that T1 tumors had a significantly lower EBV-DNA level than cases with locally more advanced disease (32). In this study, only a tendency of increasing DNA viral load between early and advanced tumor stage was observed (Fig. 2). In addition, we found no correlation between the level of EBV-DNA in blood or nasopharyngeal brushing at diagnosis and the clinical response at 2 months posttreatment. Therefore, the initial EBV-DNA load values may not be taken as a prognostic marker.

At 2 months after treatment, the level of EBV-DNA load in brush and whole blood showed a significant decrease in most cases, being clinically relevant and reflecting reduced tumor activity. For viral DNA load in nasopharyngeal brushings, there is a substantial reduction (43-fold), similar to whole blood (27-fold reduction). We did not find any correlation between type of treatment, treatment response, and the fold reduction of viral DNA load. Two patients died before treatment was finished both having an initial high EBV-DNA load in whole blood and distant metastasis pointing to an initial poor prognosis. Posttreatment EBV-DNA levels have proven to be a strong predictor for relapse and survival in larger studies (17, 19, 31, 33–35). The time point of 2 months follow-up chosen for this study may be too short to permit complete disappearance of treatment-induced tumor-related EBV activity in complete responders. More long-term follow-up is needed to define the clinical relevance of persisting EBV-DNA levels in nasopharyngeal brush samples.

In summary, this study shows that EBV-DNA quantification in nasopharyngeal brushings is a promising approach for NPC diagnosis and posttreatment monitoring and may

reduce the number of invasive nasopharyngeal biopsies required. Although pathologic examination for definite NPC diagnosis remains needed, molecular testing of nasopharyngeal brush material provides a promising and minimally invasive alternative requiring further validation. Nasopharyngeal brush sampling is suitable for follow-up monitoring to measure EBV-DNA load dynamics during and after treatment aiming at detection of progressive or recurrent disease without significant discomfort for the patient.

Disclosure of Potential Conflicts of Interest

J.M. Middeldorp holds proprietary rights to the EBV peptides used in this study. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: M. Adham, A.E. Greijer, A. Roezin, S. Gondhowiardjo, B. Hermani, I.B. Tan, J.M. Middeldorp
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Adham, L. Rachmadi, A. Kurniawan, S. Gondhowiardjo, D. Atmakusumah
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.E. Greijer, S.A.W.M. Verkuijlen, S. Fleig, O. Malik, S.J. Stevens, J.M. Middeldorp
Writing, review, and/or revision of the manuscript: M. Adham, A.E. Greijer, S. Gondhowiardjo, B. Hermani, I.B. Tan, J.M. Middeldorp
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Adham, A.E. Greijer, S.A.W.M. Verkuijlen, H. Juwana, S. Fleig, L. Rachmadi, O. Malik, J.M. Middeldorp
Study supervision: A. Roezin, S. Gondhowiardjo, D. Atmakusumah, B. Hermani, I.B. Tan, J.M. Middeldorp

M. Adham did all the job of treatment's patients since diagnosis until 2 years follow-up; took biopsy, brush, blood, and some laboratory experiments such as ELISA, DNA isolation, PCR; managed the data; writing, collect, and organizing all data and samples from other department besides ENT, such as radiotherapy, pediatrics, medical oncology, histopathology; made review from senior author. He loves this study and knows it is useful for him as a clinician by combined clinical and laboratory expertise.

L. Rachmadi made diagnosis of NPCs with paraffin block and evaluated of expression EBER-ISH and also supervised the procedure of staining.

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