

Epstein-Barr DNA in advanced pediatric nasopharyngeal cancer

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Abstract

Background Studies suggest that the most common type of nasopharyngeal carcinoma (NPC) is WHO-3, which is strongly associated with Epstein-Barr virus (EBV).

Objective To assess NPC patient characteristics in a national general referral hospital in Indonesia, with regards to EBV DNA load and treatment response.

Methods Twenty-three pediatric patients diagnosed with NPC were included in the study. Data collected were history, physical examination, tissue biopsy, CT scan, staging and EBV DNA load from nasopharyngeal (NP) brushing as well as blood specimens. The NP brushing, blood specimens and CT scan evaluations were done two months post-treatment.

Results Pediatric patients with symptoms such as blood tinged secretion, lymph node enlargement, and nasal congestion were more likely to have higher EBV DNA loads in their NP brushings ($P < 0.05$) (including T3 and higher). Despite significant reduction of EBV DNA load in NP brushing post-treatment, it was not associated with treatment response, as evaluated by CT scan.

Conclusion Higher DNA load from NP brushings is associated with a higher tumor stage. Larger sample size and follow-up data are needed to assess the usefulness of EBV DNA load assessment in pediatric patients. [Paediatr Indones. 2021;61:261-70 ; DOI: 10.14238/pi61.5.2021.261-70].

Keywords: EBV; pediatrics; nasopharyngeal carcinoma; Indonesia

Nasopharyngeal carcinoma (NPC) is a rare type of cancer with a global estimated incidence of 1.5 per 100,000. In Asia, the number was higher at 2.1 per 100,000, due to its endemicity in China and some Southeast Asian countries.¹ The age distribution of NPC patients most likely peaks at adulthood. However, in low risk countries such as North America, India, and Japan, the peak is during adolescence and later in the elderly (ages 65-79).² The incidence of NPC in pediatric patients varies among areas. Non-endemic countries, such as Germany and the United Kingdom, have lower incidences of 0.1 and 0.4, respectively, whereas in China, the numbers are as high as 2.8.^{3,4} In Indonesia, the incidence in the year 2000 for all NPC cases was 5.66.⁵ Data suggests that much like in adults, NPC in children is also found in areas with lower education and income.⁶

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Most pediatric patients are diagnosed at more advanced stages than adults, but findings suggest that their mortality is much lower, by 60%.⁶ Common symptoms in pediatric patients are neck masses, blocked nose, and hearing problems.⁷⁻⁹ These symptoms are sometimes regarded as other diseases and, therefore, misdiagnosed. Owing to their locoregional mass extension, some patients present with cranial nerve palsies causing diplopia, trismus, as well as facial numbness and pain.⁷ Apart from histological analysis and MRI/CT scan, diagnosis of NPC in children also includes EBV load from blood or the tumor itself. EBV is mostly associated with NPC WHO types 2 and 3.¹⁰ Type 3 is the most common type of pediatric NPC.^{3,11} It is believed that environmental factors cause a loss of heterozygosity of the host alleles, leading to increased susceptibility to latent EBV infection. The proposed alleles are located on chromosome 3p and 9p causing inactivation of RASSF1A and CDKN2A.¹⁰ This infection progresses to dysregulation and overexpression of certain genes and enzymes, increasing the tumor growth and decreasing survival of the patient.¹⁰

Quantification of EBV in whole blood or nasopharyngeal brushing are possible methods for screening. In Indonesia, the cut-off value to distinguish between non-NPC and NPC patients is 2,312 copies/brush.¹² However, in children, where most patients are diagnosed at advanced stages, the usefulness of the quantification method is unknown because of the invasiveness of the nasopharyngeal swab procedure, leading to difficulty obtaining control population.¹³

Treatment for NPC in children includes radiation and chemotherapy. Recommendations by the *Society of Pediatric Oncology and Hematology Study Group* for stage 1 patients are radiotherapy followed by 6-month interferon beta treatment, and radiochemotherapy with cisplatin and 5-fluorouracil followed by 6-month interferon beta treatment. However, in patients with metastasis, treatment starts with chemotherapy followed by radiotherapy on the main tumor site, lymph nodes, and metastatic sites.⁴ The outcomes of treatment itself may also be affected by multiple factors.

Studies conducted on pediatric NPC in Indonesia have been limited due to the rarity of disease. We aimed to document patient characteristics, as well as assess for possible relationships to EBV DNA load in nasopharyngeal brushing and blood with treatment outcomes at a national general referral hospital in

Jakarta, Indonesia.

Methods

In this cross-sectional study, patients diagnosed with NPC were interviewed for their medical history and examined by otorhinolaryngology (ENT) residents in Dr. Cipto Mangunkusumo Hospital (RSCM), a tertiary referral hospital located in Jakarta, Indonesia.

Ethnicity was divided into four major categories based on the result from a previous study conducted in RSCM for NPC.⁵ Staging for NPC was categorized according to the *American Joint Committee on Cancer* (AJCC) system and tumor histopathology was classified according to the WHO classification.^{14,15} Both results were obtained from biopsies taken from the lesions. Tumor stage was classified as T2 (extension to parapharyngeal space), T3 (infiltration to bony structure), or T4 (intracranial/hypopharynx/orbital/parotid gland extension), while nodal stage was defined as N1 (unilateral cervical/unilateral or bilateral retropharyngeal lymph node) or N2 (bilateral cervical lymph node <6 cm), N3 (size > 6 cm or extension below cricoid cartilage). Metastasis was considered as M0 (no metastasis) or M1 (metastasis). The AJCC stage consisted of stage III (T0-1N2M0/T2N2M0/T3N0-2M0), stage IV A (T4N0-2M0), stage IV B (anyTN3M0), and stage IV C (anyT any N M1).

Concurrent conformal radiotherapy (CRT) was defined as using chemotherapy concurrently with radiation acting as radiosensitizer, aiding the destruction of radio resistant clones or as organ-preserving intent compared with surgical resection. its systematic action potentially able to prevent distant metastases.¹⁶

The EBV DNA load was categorized into three groups consisting of below cut-off value, high, and extremely high, based on a previous study.¹²

Evaluation for response in these patients was done 2 months after the last treatment cycle using CT Scan based on RECIST criteria. Complete response was defined as the disappearance of all target lesions on CT scan for a period of at least one month. Partial response were defined as a 30% decrease in the sum of the longest diameter of lesions measurement (with known baseline). Progressive disease was defined as a 20% increase in the longest diameter of target lesions or additional lesions.¹⁷

Patients diagnosed with NPC, aged 20 and under, were enrolled. This age cut-off was chosen based on a previous study and to increase the sample size.⁸ Data collected through interviews included identity, risk factors, signs, and symptoms. Such data were archived in patient medical records. Parent of patients provided written informed consent for their data to be analyzed in this study.

The patients went through additional examinations including nasopharyngeal (NP) brushing, followed by biopsy. The NP brushing was guided by nasoendoscopy and prepared using 1% lidocaine nasal spray. A cytobrush was placed completely inside a plastic catheter then inserted through the nostril. Upon reaching the suspected lesion, the cytobrush was pushed from the tube and brushed over the epithelium in a rotating manner, then reinserted into the tube. After brushing was complete, the brush tip was mixed in a *NucliSens Lysis Buffer* (LB) (*BioMerieux*, Marcy l'Etoile, France) and stored in 1 mL aliquots at -80°C. A biopsy was taken from the same lesion, embedded in paraffin, and assessed by two independent pathologists.

In addition, 5 mL blood specimens were evaluated for EBV DNA load. Both blood and NP brushing specimens were analyzed using PCR assay to detect regions of the BKRF-1 gene (a 99-bp region for blood and a 213-bp region for NP brushing). The cut-off values for EBV-DNA load were 2,312 copies/brush for NP brushing and 2,000 copies/mL for whole blood. Any number below the cut off value are categorized into below, number above the cut off value are categorized into high and extremely high range from 1.08×10^2 - 4.88×10^7 . More detailed specimen procedures were published in previous studies.^{12,18}

Pediatric treatment was the same as that given to adults. Treatment consisted of radiotherapy administered to the primary tumor region with dosage of 66-70 Gy in 6-8 weeks with neoadjuvant or adjuvant chemotherapy of 5-FU (1000 mg/m² days 1-5) and cisplatin (100mg/m² day 1) in 3 cycles every 3 weeks. The concurrent chemotherapy (dosage was cisplatin 40 mg/m² weekly. Follow-up and additional data were collected two months post-treatment. The two-month mark was set considering post-radiation inflammatory response.

Using *SPSS ver. 21 for Mac*, EBV load from NP brushing and whole blood was transformed using log₁₀ to normalize the distribution, which was evaluated

using Shapiro-Wilks test. We analyzed for possible correlations between EBV DNA load of brushing at diagnosis to tumor/node/metastasis (TNM) stage, nodal involvement, and AJCC stage, as well as between EBV DNA load in whole blood at diagnosis to TNM stage, nodal involvement, and AJCC stage using one-way ANOVA test. Independent T-test was used to evaluate for relationships between EBV DNA in blood and brush specimens to clinical features at diagnosis. Finally, Fisher's exact test was used to evaluate for relationships between clinical features and staging to treatment outcome.

Results

Of 228 NPC patients diagnosed and treated between 2006-2009, 23 were pediatric patients (10.5%). Their characteristics are displayed in **Table 1**. Subjects' median age was 13 (range 3-20) years. Most patients were male and of Sundanese ethnicity. All patients were in advanced stages, mostly stage 4B, with histological pattern WHO-3. Clinical characteristics and distributions among the stages are shown in **Table 2** and **Figure 1**, respectively. All 23 patients underwent either chemotherapy or a combination of chemotherapy and radiotherapy, as shown in **Figure 2**. At diagnosis, 21 NP brushing and 13 blood specimens were collected. Out of the initial 21 available data of EBV DNA load by brushing, only 7 patients completed follow-ups of NP brushing, and only 1 patient had a follow-up EBV DNA load measurement in whole blood.

The EBV virus remains latent in tumor cells, and in our study, much higher DNA load was seen in patients with T2 tumors compared to those with T3 (**Table 1**). From **Table 2** some symptoms are related to EBV DNA count on brushings (lymph node involvement, nasal congestion, and blood tinged secretion) and whole blood DNA (lymph node involvement). Further explanation regarding this findings will be discussed in the next part.

There was a significant difference in EBV DNA load between T2 and T3, but not with T4 ($P=0.049$). Patients who experienced symptoms such as lymph node enlargement, blood-tinged secretion, and nasal congestion were more likely to have a higher load of DNA in their NP brushing.

Twenty-one out of 23 patients had EBV DNA

Table 1. Patient characteristics

Characteristics	(N=23)	P value		
		Treatment response	EBV DNA brush (n=21)	EBV DNA WB (n=13)
Gender, n				
Female	8	0.131		
Male	15			
Ethnicity, n				
Javanese	5			
Sundanese	9			
Sumatran	6			
Others	3			
WHO histopathological pattern, n				
WHO-1	1			
WHO-2	0			
WHO-3	22			
Tumor stage, n				
T2	6	1.00	0.049	0.91
T3	6			
T4	11			
Nodal stage, n				
N1	2	0.34	0.21	0.224
N2	8			
N3	13			
Metastasis, n				
M0	20	0.53	0.2	0.26
M1	3			
AJCC stage, n				
III	6	1.00	0.86	0.45
IVA	4			
IVB	10			
IVC	3			
Treatment, n				
Neoadjuvant + radiotherapy	18	0.53		
Concurrent CRT	4			
Chemotherapy	1			

CRT=conformal radiotherapy; WB=whole blood

Table 2. Clinical features of NPC patients

Clinical features at diagnosis	Yes	No	P value		
			Treatment response	EBV DNA brush (n=21)	EBV DNA WB (n=13)
Lymph node involvement, n	20	3	0.53	0.011	0.08
Unilateral	7				
Bilateral	13				
None	3				
Nasal congestion, n	14	9	0.34	0.04	0.91
Blood secretion, n	13	10	0.34	0.017	0.28
Diplopia, n	4	19	0.27	0.94	0.52
Ear complaints, n	14	9	1.00	0.21	0.73
Tinnitus, n	11	12	0.069	0.33	0.28
Cephalgia, n	16	7	0.124	0.5	0.76

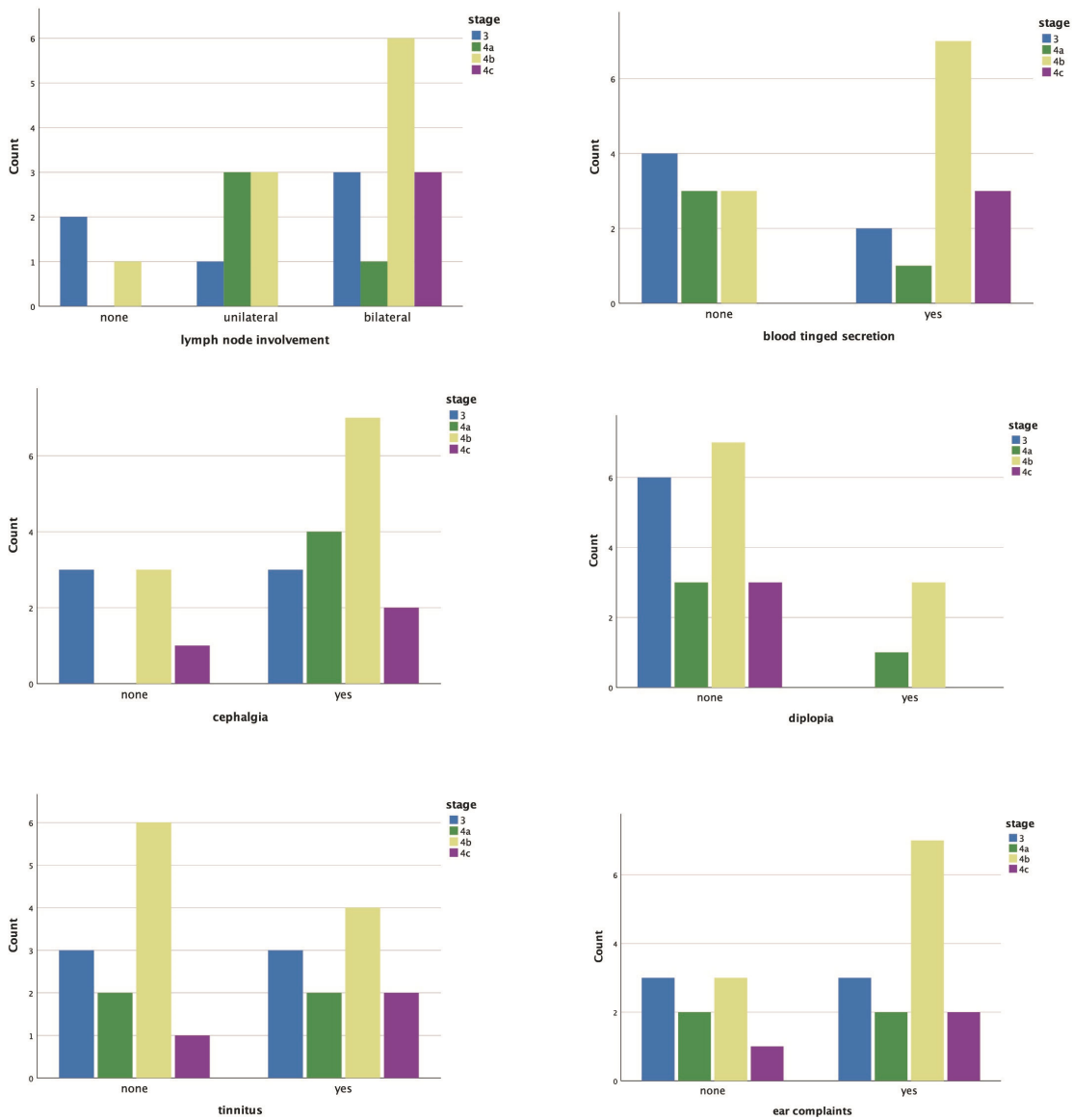


Figure 1. Patient clinical feature distribution according to stage

Stage / therapy	III	IV A	IV B	IV C
NA + RT	≤ 3	≤ 3	≥ 7	≤ 3
CRT	≤ 3	≤ 3	≤ 3	≤ 3
CT full dose	≤ 3	≤ 3	≤ 3	≤ 3

NA=neoadjuvant chemotherapy; RT=radiotherapy; CT=chemotherapy

Figure 2. Distribution of therapy according to stage

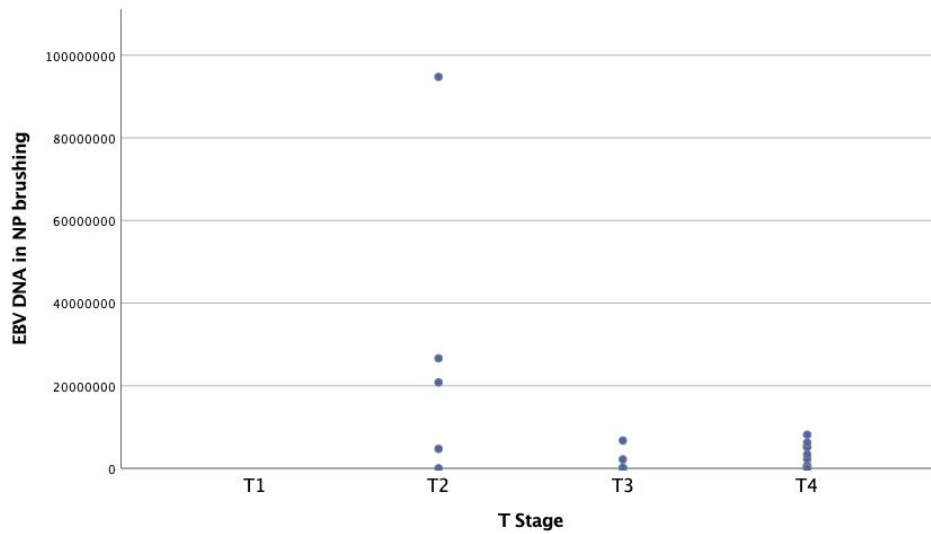


Figure 3. EBV-DNA load in NP brushing according to stage

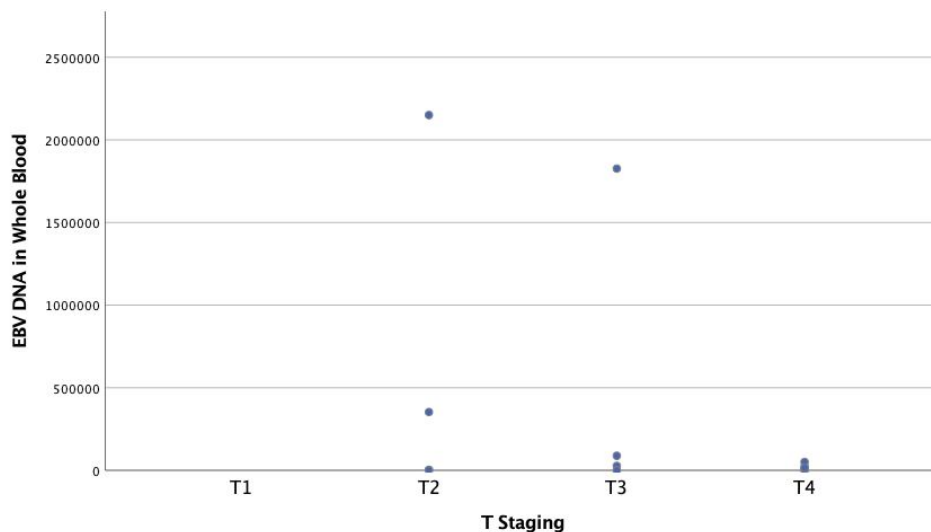


Figure 4. EBV-DNA load in whole blood

brush data at treatment initiation. Their patterns are displayed in **Figure 3**, with median 2.24×10^6 copies/brush and mean 8.94×10^6 copies/brush. All but three patient loads were above the 2,312 copies/brush cut-off value (COV). Patients with EBV DNA above the COV were further categorized into high DNA load and extremely high DNA load. There was no difference in treatment response between the high and extremely high load groups ($P=1.00$). Thirteen of 23 patients had their whole blood EBV DNA load tested. **Figure 4** shows that 11/13 patients had EBV DNA above the COV at 2,000 copies/mL.

From available cases, EBV DNA loads at diagnosis were above COV, with mean 16.31×10^6 copies/brush. Mean EBV DNA 2 months post-treatment was 1.83×10^4 copies/brush. Despite the significant reduction in EBV DNA, there was no connection between load reduction and treatment response ($P=0.185$). Most patients showed load reductions, but still above the COV; only one patient with a partial response showed EBV load below the COV. Detailed reductions per patient are shown in **Figure 5** and **Table 3**.

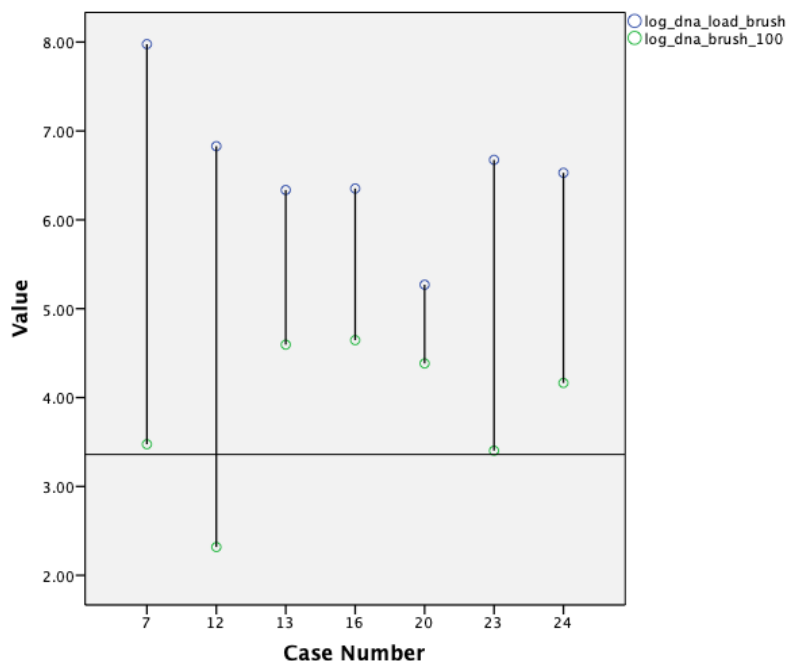


Figure 5. Logarithmic EBV DNA brush load reduction at diagnosis and two months post-treatment

Table 3. Load reduction in 7 pediatric cases

Case #	EBV load at diagnosis	EBV load 2 months post-radiation	Load reduction, %	Load reduction	Treatment response (2 months, CT*)	P value of load response to treatment
7	94,760,000	2,984	99.997	31,000 x	Partial	0.185
12	6,735,600	208	99.997	32,000 x	Partial	
13	2166,400	39,384	98.7	55x	Complete	
16	2,249,600	44,240	98.04	50x	Partial	
20	186,000	24,200	87	7x	Complete	
23	4,728,000	2,526	99.95	1,800x	Complete	
24	3,379,600	14,600	56.79	231x	Complete	

*CT: treatment response was evaluated by CT scan; Late response treatment=difference between EBV DNA load at diagnosis and at 2 month post radiation.

Discussion

Nasopharyngeal carcinoma in children is not a prevalent disease, with only 1% of overall malignancies in children. We aimed to describe the characteristics of pediatric NPC patients in Dr. Cipto Mangunkusumo Hospital, Jakarta. Of 286 NPC patients, 23 were children (10.5%), similar to percentages in American and Chinese studies with much larger sample sizes.^{6,7} The gender distribution was also consistent with studies from China, Egypt, and Morocco. Gender may influence overall survival, with higher percentage

of 5-year overall survival and disease free survival, but we must note that epidemiologically NPC affect boys more than girls with ratio up to 3:1.^{6,7,19} we found no difference in treatment response between boys and girls two months after chemotherapy and radiotherapy. This observation might have been due to an insufficient number of subjects.

Previous studies have reported that NPC prevalence is high among certain ethnicities. According to the *Singapore Cancer Registry*, NPC was equally distributed between people of Chinese and Malay descent, but much lower in the Indian ethnic

group.²⁰ Other than acting as a risk factor, ethnicity showed no significant difference in patient survival.²¹ In Indonesia, there was a higher percentage among Sundanese and Javanese ethnicities, with only 1.9% from Chinese descent.⁵ Similarly, most of our subjects were Javanese and Sundanese.

Clinical presentation of NPC comprised mainly nasal and ear symptoms, as well as symptoms caused by extension of nasopharyngeal and neck masses. In the overall Indonesian population, ear problems were the most common presenting symptom, but in the pediatric population, most patients presented with lymph node involvement, either unilateral or bilateral, and advanced stages.⁵ Ear symptoms were the second most common in our study, followed by headache as third. Neck mass was the most prevalent impetus to seek treatment, as shown in previous studies.^{7,9,19} A study in China reported that neck mass affected up to 40.9% of pediatric patients and cranial nerve involvement in 3% patients.⁷ This percentage was lower than in our study (Table 2), but neck mass was the most common symptom. WHO-3 was the most common histopathology in Indonesia as a part of an endemic area, and more sensitive to chemoradiation compared to types WHO-1 and -2, but with higher recurrence.

Cephalgia is not a pathognomonic sign of NPC, with high rate of misdiagnosis up to 84%, leading to delay in treatment.²² Cephalgia indicates skull base lesions or intracranial invasion.²³ Most RSCM patients with complaints of cephalgia already had multiple symptoms preceding or coinciding with the cephalgia complaints. Delays in diagnosis may be related to patients' parents being insensitive to complaints or the children themselves unaware of changes or progression of symptoms, or primary care physicians who are unfamiliar with non-specific symptoms of early stage NPC.^{5,7} Because of the non-specific symptoms, patients seek treatment based on the complaints, for example, diplopia to ophthalmologists, cephalgia to neurologists or neurosurgeons, neck mass to surgical oncologists, or nasal congestion and ear fullness to otorhinolaryngologists. Hence, delayed diagnoses can be found at such times.

Treatment given to pediatric patients were a combination of chemotherapy and radiation, but one patient was given a full chemotherapy dose due to the patient's histopathological type (WHO-1). Of three

patients with metastasis, two received chemoradiation with neoadjuvant and concurrent chemoradiotherapy, while the other one was treated with full dose chemotherapy. Only 6 of 23 patients showed complete response, while the remainder had partial response. The treatment responses were not connected with the stage or clinical symptoms at the time of diagnosis. A study reported that despite having advanced stage disease, mortality risk was lower compared to those in adults, due to fewer comorbidities in pediatric patients.⁶ We were unable to determine the overall survival in pediatric patients because of small sample size and high rate of patients lost to follow up.

The EBV DNA load for pediatric patients at diagnosis were high and extremely high values, with 3 patients under the COV, possibly due to hard to be reached due to anatomic location, or limited visible tumor area by endoscopy. Children can be difficult to examine or to perform biopsies under local anaesthesia. Similarly, a previous study reported that advanced stages (T3/T4) had a significantly higher EBV load compared to early stages (T1/T2).²⁴ But further a study in Indonesia find no correlation between tumor extensiveness and EBV DNA load. Which may explain the result of our study which show T2 to have a much higher load compared to T3 and T4.²⁵ Reduction of EBV DNA load in brush did not significantly differ between those who had complete vs. partial response to treatment. Other patients had complete responses, despite having small reduction in EBV DNA load.

While EBV DNA load in brushing was significantly higher in subjects with lymph node involvement than those without, EBV DNA load was not significantly different in whole blood. The EBV DNA in whole blood at the beginning of treatment may reflect circulating DNA because viral particles are mostly released at the apoptotic stage, then DNA fragments are rapidly cleared.²⁶ In contrast, previous studies reported that plasma EBV (pEBV) DNA had strong correlations with all factors related to lymph nodes.^{24,27} Our observation may have been to our small study population, which included only pediatric patients and only 12 whole blood specimens.²¹ The considerable reduction in EBV DNA brushing specimens was not reflected by the treatment response as evaluated by CT scan. Since pEBV (plasma) DNA is released by nasopharyngeal carcinoma cells, further

studies are needed to determine the usefulness of EBV DNA for detecting disease progression by a less invasive method.

Conflicts of Interest

JM 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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