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Diagnostic Value of Measuring Epstein-Barr Virus (EBV) DNA Load and Carcinoma-Specific Viral mRNA in Relation to Anti-EBV Immunoglobulin A (IgA) and IgG Antibody Levels in Blood of Nasopharyngeal Carcinoma Patients from Indonesia

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Nasopharyngeal carcinoma (NPC) is a prevalent malignancy in Southeast Asia and is strongly associated with Epstein-Barr virus (EBV). We investigated the primary diagnostic value of circulating EBV DNA and anti-EBV immunoglobulin G (IgG) and IgA levels in Indonesian NPC patients ($n = 149$). By a 213-bp Epstein-Barr virus nuclear antigen 1 (EBNA1)-based real-time LightCycler PCR, 72.5% of patients were positive for EBV DNA in whole blood, with 29.5% having levels above a previously determined clinical cutoff value (COV) of 2,000 EBV DNA copies/ml, the upper level in healthy carriers. In a 99-bp LightCycler PCR, 85.9% of patients were positive and 60.4% had levels above the COV. This assay quantified a significantly higher EBV load than the 213-bp PCR assay ($P < 0.0001$), suggesting that circulating EBV DNA is fragmented. Using data from 11 different studies, we showed a significant inverse correlation between PCR amplicon size and the percentage of patients positive for circulating EBV DNA (Spearman's rho = −0.91; $P < 0.0001$). EBV DNA loads were unrelated to anti-EBV IgG or IgA levels, as measured by VCA-p18 and EBNA1-specific synthetic peptide-based enzyme-linked immunosorbent assays. The presence of circulating tumor cells was assessed by amplification of BamHI-A rightward frame 1 (BARF1) mRNA, a viral oncogene abundantly expressed in EBV-carrying carcinomas but virtually absent from EBV-associated lymphomas. Despite high EBV DNA loads and the presence of EBNA1 and human U1A small nuclear ribonucleoprotein mRNA, BARF1 mRNA was never detected in blood. We conclude that amplicon size significantly influences EBV DNA load measurement in NPC patients. The circulating EBV DNA load is independent of serological parameters and does not reflect intact tumor cells. The primary diagnostic value of the EBV DNA load for the detection of NPC is limited.

Nasopharyngeal carcinoma (NPC) is a rare tumor in most parts of the world; but it occurs endemically in southern China, Hong Kong, Singapore, and some other parts of Southeast Asia with high incidences (>15 cases/100,000 population/year) (17). NPC has a strong etiological link with Epstein-Barr virus (EBV), a ubiquitous herpesvirus that establishes a lifelong, mainly asymptomatic infection in >90% of the world's population (27, 58). EBV is clonally present in virtually 100% of undifferentiated NPC cases, and the virus may express a restricted number of latent genes. These include the small EBV-encoded nonpolyadenylated RNAs (EBER1 and EBER2), the Epstein-Barr virus nuclear antigen 1 (EBNA1), two latent membrane proteins (LMP1 and LMP2), the noncoding BamHI-A rightward transcripts (BARTs), and BamHI-A rightward frame 1 (BARF1) (27, 37, 40, 42). EBNA1 is essential for maintenance of the viral genome and its anchoring to host chromosomes. The LMP1, LMP2, and BARF1 proteins all have transforming properties in epithelial cells in vitro (26, 45, 46, 64, 65). Whereas EBNA1, LMP1, and LMP2 are expressed in both EBV-linked lymphomas and carcinomas, BARF1 is a viral oncogene that is almost exclusively transcribed in EBV-positive carcinomas and that is virtually absent from EBV-associated lymphomas (5, 12, 21, 69).

Carcinoma-associated EBV activity in NPC patients may be reflected in the circulation by a typical anti-EBV serological profile and increased viral DNA levels. In comparison to healthy EBV carriers, NPC patients generally show strong immunoglobulin G (IgG) and especially IgA reactivities to EBV early antigens, viral capsid antigens, and EBNA1 (22). Serodiagnostic assays based on defined EBV-derived epitopes may facilitate population-based screening aimed at the early identification of NPC patients. Monitoring of EBV DNA and RNA parameters in blood, if it is proven to be sensitive and specific, could be potentially useful for confirmation of initial serodiagnostic risk stratification in mass surveys.

Studies on monitoring of the EBV DNA load in the circulation of NPC patients mostly originate from Hong Kong (7, 30, 31, 34, 59); but elevated EBV DNA loads can also be found in the plasma of Thai (38, 48), Taiwanese (25), Chinese (47), and some Italian (41) NPC patients. The reported percentage of NPC patients positive for EBV DNA in peripheral blood,

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however, varies strongly from approximately 30 to 98%; and many studies report sensitivities below 90% and very low EBV DNA loads in a significant proportion of patients (9, 24, 25, 38, 48).

Therefore, the aim of this study was to determine the primary diagnostic value of circulating EBV DNA loads in a large cohort of Indonesian NPC patients ($n = 149$) from the Yogyakarta region, where NPC represents the number one malignancy in males and the number four tumor in females (50). We tried to find an explanation for the large variations in peripheral blood EBV DNA positivity in NPC patients reported thus far by using different DNA target sizes in PCR. We used unfractionated whole blood, a clinical specimen type previously shown to be diagnostically relevant in transplant recipients, human immunodeficiency virus-positive and AIDS patients, and Burkitt's lymphoma patients (51–57). In these populations a cutoff value of 2,000 EBV DNA copies/ml blood clearly demarcates patients with EBV-positive tumors from healthy EBV-seropositive carriers, in whom the loads were invariably below this value (54, 55). The use of whole blood might increase the sensitivity of detection by combining plasma and cellular fractions and, furthermore, enables the parallel detection of EBV mRNA. At present it is not clear whether NPC tumor cells can enter the circulation. In order to detect possible circulating NPC tumor cells, whole-blood specimens were studied for the presence of EBNA1 and BARF1 mRNA. In contrast to EBV DNA, which may be present at low levels in the circulation of healthy EBV-seropositive carriers, BARF1 RNA may directly reflect NPC-related EBV transcriptional activity, whereas EBNA1 mRNA may be found in both circulating latently EBV-infected B cells undergoing cell division (23) and intact NPC tumor cells. EBV mRNA detection in peripheral blood has hitherto not been studied as an NPC marker, but we recently showed its diagnostic potential in nasopharyngeal tissue biopsy specimens (5, 21) and nasopharyngeal brushings (S. J. C. Stevens et al., 11th Biennial Symp. Int. Assoc. Res. EBV Associated Dis., abstr. 13.02, 2004). Finally, we investigated the relationship between EBV DNA loads in blood and quantitative anti-EBV IgA and IgG serology in the Indonesian cohort. Previous studies predominantly compared the diagnostic performance of EBV DNA load measurement versus serological assays (47), but the putative quantitative relation between these two parameters has not been described.

MATERIALS AND METHODS

Patients. During the period from 2001 to 2003, 149 NPC patients were identified at the Department of Pathology, Gadjah Mada University, School of Medicine/Sardjito Academic Hospital (Yogyakarta, Indonesia). NPC diagnosis was based on pathological assessment of paraffin-embedded tumor biopsy specimens, EBER1/2 RNA in situ hybridization, and immunohistochemical staining for EBNA1 and LMP1 by using previously defined monoclonal antibodies (35, 36, 68). TNM staging for tumor size (T), lymph node involvement (N), and metastasis (M) was done by using the 1997 criteria of the Union International Contre le Cancer (49) for all patients by using clinical measurements and computer tomography scans as part of the routine patient workup. Approval of the local medical ethical committee was obtained.

Unfractionated whole-blood samples. Unfractionated EDTA-anticoagulated whole-blood samples were obtained from the NPC patients at the time of diagnosis and before any therapeutic intervention during their visit to the Sardjito Academic Hospital, Yogyakarta, Indonesia. Fresh EDTA-anticoagulated blood (0.5 ml) was directly lysed in 4.5 ml of NucliSens lysis buffer (BioMerieux, Boxtel, The Netherlands) within 1 h after donation, mixed thoroughly, and immediately stored at −80°C until it was used for nucleic acid isolation. This lysis buffer enables short-term stabilization of nucleic acids at room temperature and long-term cryopreservation at −80°C (6, 11).

EBV nucleic acid isolation. EBV DNA and RNA were simultaneously isolated from 1 ml of lysed whole blood by silica-based extraction (4). Nucleic acids were eluted in 100 μl, and the nucleic acid equivalent form 5 μl of whole blood was used in subsequent DNA and RNA amplification assays. All reagents for nucleic acid isolation were obtained from BioMerieux.

EBV DNA load quantification by quantitative LC-based real-time PCR. The EBV DNA load in whole blood was determined by a quantitative LightCycler (LC) real-time PCR that targets a highly conserved 213-bp region of EBNA1. The primers used in this assay were QP1 and QP2, and the fluorigenic internal hybridization probes were EBNA LCN and EBNA FLN (TIBMolBiol, Berlin, Germany), described in detail elsewhere (53, 54). In addition, a newly developed LC-based PCR that amplifies a 99-bp part of EBNA1 (located within the 213-bp QP1-QP2 amplicon) was used for quantification of small DNA fragments. The experimental conditions, reagents, and hybridization probes in this 99-bp LC-based PCR assay were identical to those used in the 213-bp LC-based PCR assay, except that different primers were used (forward primer QP3 [5′-CCACAATG TCGTCTTACACC-3′] and reverse primer QP4 [5′-ATAACAGACAATGGA CTCCCT-3′]). Real-time PCR reagents were obtained from Roche Diagnostics (Almere, The Netherlands). Tenfold dilutions of spectrophotometrically quantified plasmid DNA containing the EBNA1 target sequence were used to create a standard curve (53, 54). To check for putative inhibition of PCR, EBV DNA-negative samples were spiked with 1,000 copies of EBV plasmid DNA (53, 57). β-Globin PCR was performed with primers PCO3 (5′-ACACAACTGTGTTCA CTAGC-3′) and PCO5 (5′-GAAACCCAAGAGTCTTCTCT-3′), which generate a 209-bp PCR product. Reaction conditions were as follows: a volume of 50 μl with 50 mM of each primer, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 10 mM Tris (pH 8.5), and 1 U of *Taq* polymerase (Perkin-Elmer). Amplification was done for 40 cycles of 1 min at 95°C, 2 min at 55°C, and 1.5 min at 72°C. The first denaturation step and the last elongation step were extended for 4 min. PCR products were visualized by standard agarose gel electrophoresis.

NASBA for EBV RNA detection. Nucleic acid sequence-based amplification (NASBA) assays for BARF1 and EBNA1 mRNA and the low-copy-number human U1A small nuclear ribonucleoprotein (U1A snRNP) housekeeping mRNA were done as previously described by us (5, 19, 21). NASBA is an isothermal RNA amplification technique that enables highly specific RNA amplification in a DNA background, regardless of the splice patterns (13). NASBA reagents were obtained from BioMerieux (NucliSens basic kit). The primers for BARF1 and EBNA1 were located within the open reading frames of these genes, which enabled simultaneous amplification of all putative splice variants (5, 21). Both assays yield amplification products of 203 nucleotides. The marmoset lymphoblastoid cell line B95-8, which expresses both EBNA1 and BARF1 mRNA transcripts, was used as a positive control. The sensitivities of the BARF1 and EBNA1 NASBAs were previously determined to be 10 mRNA molecules or <1 EBV-positive RNA cell equivalent (5).

Several precautions described previously (29) were taken during the PCR and NASBA procedures to avoid false positivity. In all experiments appropriate negative and positive controls were included during nucleic acid isolation and amplification.

EBV serology. IgG seroreactivity against EBV was assessed by immunoblotting by using the nuclear fraction of HH514.c16 cells, which were chemically induced to express the viral capsid antigen (15). Immunoblots were scored semiquantitatively from 1 (weakest) to 4 (strongest), with reference to controls analyzed in parallel (15). IgA reactivity was quantitatively assessed by a synthetic peptide-based enzyme-linked immunosorbent assay (ELISA) for immunodominant epitopes derived from EBNA1 and VCA-p18 (BFRF3), as described previously (15).

RESULTS

EBV DNA load in whole-blood samples of NPC patients determined by 213-bp EBNA1 LC-based PCR. Initially, we used a quantitative LC-based real-time PCR assay that targets a 213-bp region of EBNA1 for determination of the EBV DNA loads in unfractionated blood samples. Of 149 fresh whole-blood samples obtained from 149 NPC patients, 108 (72.5%) were positive by this 213-bp LC-based PCR, with EBV

DNA loads ranging from 0 to 110,400 copies/ml blood (mean, 4,167 EBV DNA copies/ml blood; median, 1,050 EBV DNA copies/ml blood). Forty-four samples (29.5%) had loads above the 2,000-copy/ml cutoff value. This value of 2,000 copies/ml corresponds to 10 EBV DNA copies per PCR tube, based on a DNA input in PCR equivalent to 5 μl of unfractionated whole blood (54, 55). Ten copies per reaction is the lower limit of the EBV DNA load that can still be reliably quantified, albeit with a large standard deviation (54). Lower amounts of EBV DNA per reaction may provide a positive result but cannot be reliably quantified by real-time PCR.

All EBV DNA-negative samples were positive by the β-globin PCR. Spiking of EBV DNA-negative samples with small amounts of EBV plasmid DNA in quantitative LC-based PCR revealed that the LC-based PCR-negative samples were not inhibited for DNA amplification but were truly EBV DNA negative.

LC-based PCR target size significantly influences the EBV DNA load in blood of NPC patients. We reasoned that if the EBV DNA in whole blood was (partially) fragmented, e.g., due to apoptosis, which yields fragments of approximately 150 bp in length (39), the use of a smaller DNA target size in the real-time PCR would give a higher EBV DNA load. Therefore, we developed a novel 99-bp LC-based real-time PCR that targets EBNA1 with QP3 and QP4 primers located internally in the 213-bp QP1-QP2 amplicon (53, 54). This enabled the use of the same validated set of hybridization probes and the same standard dilution curve of EBNA1 plasmid DNA in both PCR assays. The 99-bp LC-based PCR had an amplification efficiency similar to that of the 213-bp LC-based PCR assay, based on the slopes of the amplification curves in the log-linear phase for the individual clinical samples and for the standard dilution samples consisting of EBNA1 plasmid DNA. The mean amplification efficiencies were calculated for both the 99-bp and the 213-bp LC-based PCRs by using the standard curve data for each run ($n = 10$ runs per LC-based PCR assay). The mean amplification efficiency in the 213-bp PCR was 96.2%, with a standard deviation of 2.6%. In the 99-bp LC-based PCR assay, the mean efficiency was 95.3%, with a standard deviation of 2.7%. There was no statistically significant difference between the amplification efficiencies in the two LC-based PCR assays (Mann-Whitney test, $P = 0.367$). Furthermore, both assays gave the same quantified DNA amount at different inputs of spiked EBNA1 plasmid DNA; these amounts ranged from 10 to 10^5 copies.

The number of whole-blood samples from NPC patients positive for EBV DNA by the 99-bp LC-based PCR assay was 128 of 149 (85.9%; compared to 72.5% in the 213-bp PCR). The range was 0 to 8,962,000 EBV DNA copies/ml blood (median, 4,048 EBV DNA copies/ml blood; mean, 86,960 EBV DNA copies/ml blood). Ninety of 149 samples (60.4%) had loads above the 2,000-copy/ml cutoff value (compared to 29.5% by the 213-bp PCR). Eight samples had loads >2,000 copies/ml by the 99-bp LC-based PCR but were negative by the 213-bp LC-based PCR. The 99-bp LC-based PCR assay quantified a statistically significantly higher EBV DNA load in whole-blood specimens than the 213-bp LC-based PCR assay (Wilcoxon's test, $P < 0.0001$) (Fig. 1). The mean difference was a 19.7 times higher EBV DNA load by the 99-bp LC-based PCR than by the 213-bp LC-based PCR assay (median difference, 4.9 times; range, 1.1 to 995.8 times).

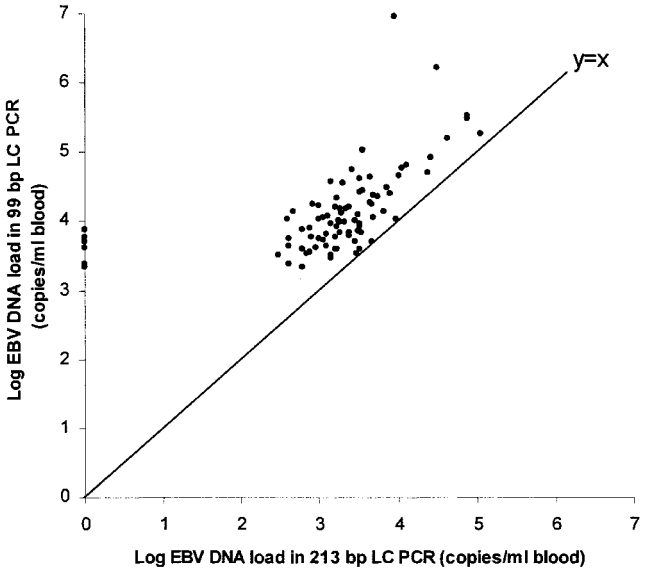

FIG. 1. Comparison of EBV DNA loads in blood of NPC patients as determined by the 213-bp and 99-bp EBV LC-based real-time PCR. All 90 samples with EBV DNA loads >2,000 EBV copies/ml by the 99-bp LC-based PCR are included (corresponding to >10 copies per LC-based PCR, the lower limit that can still be reproducibly quantified). The 99-bp LC-based PCR assay significantly quantifies a higher EBV DNA load than the 213-bp LC-based PCR assay (Wilcoxon's test, $P < 0.0001$). The line $y = x$ denotes equal amounts of EBV DNA in both assays.

The increase in the EBV DNA load by the 99-bp PCR compared to that determined by the 213-bp PCR was highly variable between individual whole-blood samples and was not related to the absolute EBV DNA amount in the sample in either assay (Fig. 1). Our results show that the EBV DNA load is highly dependent on the PCR target size and are consistent with the data obtained by Chan et al. (8), which indicated that the viral DNA circulating in NPC patients is fragmented.

Fourteen healthy EBV-seropositive donors were tested for EBV DNA positivity by the 99-bp LC-based PCR assay. All were negative, confirming previous studies showing low cell-associated EBV DNA loads in the blood of healthy EBV carriers, with approximately only 1 to 10 per 10^6 circulating B cells carrying the EBV genome (54, 55, 63, 67).

Relation between circulating EBV DNA positivity and PCR amplicon length. The proportion of NPC patients positive for EBV DNA in their circulation has been remarkably variable between studies published to date and ranges from 30% to 98% (9, 24, 25, 31, 38, 47). Based on the indications for fragmented EBV DNA (8; see above), we tried to find a possible explanation for this wide range of positivity and differences in clinical sensitivity. Therefore, we investigated the relationship between PCR amplicon length and circulating EBV DNA positivity in patients at the time of primary diagnosis of NPC using data from our present study and nine previous studies with sufficient information on the DNA target size used in PCR and the patient positivity rate. As is shown in Fig. 2, there is a statistically significant inverse correlation between the re-

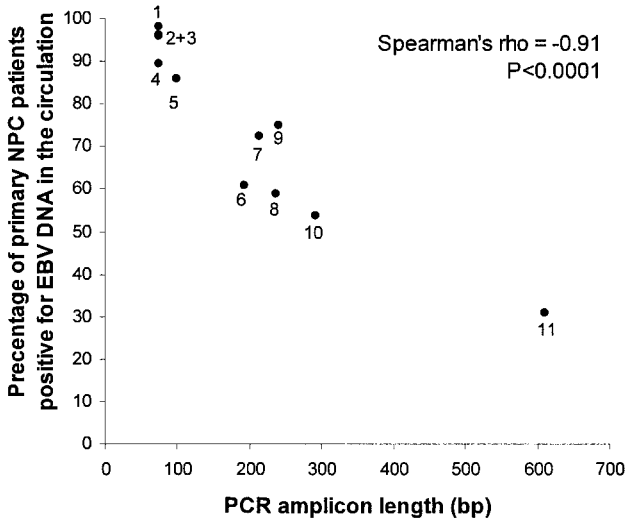

FIG. 2. Correlation between PCR amplicon lengths and percentage of patients with a primary diagnosis of NPC positive for EBV DNA in their circulation, using data from 11 different studies. The data for the following points are from the indicated references: 1 (31), 2 (47), 3 (34), 4 (7), 5 (this study), 6 (9), 7 (this study), 8 (48), 9 (25), 10 (24), and 11 (38).

ported number of patients positive for EBV DNA in their circulations and the length of the amplicon in PCR (Spearman's rho = −0.91; $P < 0.0001$), independent of the PCR technique, EBV target sequence, or clinical specimen type.

Relation of TNM staging and EBV DNA load in blood of NPC patients. We hypothesized that advanced NPC stages or larger tumors would be related to higher EBV DNA loads in blood. However, we did not find a relation between the TNM stage of the primary tumor and the presence or the level of EBV DNA in the circulation, as determined by either the 213-bp LC-based PCR (Kruskal-Wallis test, $P = 0.423$) or the 99-bp LC-based PCR ($P = 0.658$). Furthermore, the primary tumor size (T1 to T4) did not correlate with the circulating EBV DNA load by either the 213-bp LC-based PCR (Kruskal-Wallis test, $P = 0.260$) or the 99-bp LC-based PCR ($P = 0.218$).

Absence of BARF1 mRNA expression in the circulation of NPC patients indicates a virtual absence of intact circulating NPC cells. To investigate whether (part of the) EBV DNA in NPC patients was derived from intact circulating tumor cells and whether detection of the carcinoma-specific, EBV-encoded BARF1 mRNA in the circulation of NPC patients has diagnostic value, we performed NASBA for this oncogene using whole-blood RNA obtained from 19 NPC patients with the highest EBV DNA loads in both the 99-bp LC-based PCR (range, 4,048 to 8,962,000 EBV DNA copies/ml blood) and the 213-bp LC-based PCR (range, 2,600 to 75,200 EBV DNA copies/ml blood). All 19 samples were positive for low-copy-number human housekeeping gene U1A snRNP RNA, indicating amplifiable RNA quality. Seven of 19 samples (37%) were EBNA1 mRNA positive, but none was BARF1 mRNA positive. To exclude the possibility that the absence of BARF1 mRNA in whole blood was due to the absence of this transcript in the primary tumor itself, we investigated whether this mRNA was detectable in nasopharyngeal brush specimens obtained simultaneously with the whole-blood samples. Brush specimens were available from 13 of the 19 patients indicated above. In 12 of 13 cases, BARF1 mRNA was clearly detectable, and extremely elevated EBV DNA loads were found (>150,000 copies/brush sample) in all brush specimens except the BARF1 mRNA-negative brush sample, which had a load below 2,000 EBV DNA copies/brush sample (S. J. C. Stevens et al., unpublished data; Stevens et al., 11th Biennial Symp. Int. Assoc. Res. EBV Associated Dis.).

Correlation of EBV DNA loads in whole blood with EBV-specific IgG and IgA serology. The molecular diversity and intensity of serological responses against EBV proteins in the cohort of Indonesian NPC patients were recently described by us, and serological data were available for 139 of 149 patients (15). We correlated the semiquantitative immunoblot score (score of 1 to 4) to the whole-blood EBV DNA load, as determined by the 99-bp or the 213-bp LC-based PCR. As shown in Fig. 3A and B, no significant difference was observed between these four groups (Kruskal-Wallis test, $P = 0.124$ for the 99-bp LC-based PCR and $P = 0.091$ for the 213-bp assay). Furthermore, the EBV DNA load in blood did not correlate significantly with anti-EBNA1 IgA levels (Spearman's rho = −0.14 and −0.113 for the 213- and 99-bp LC-based PCRs, respectively) (Fig. 4A and B) or with anti-VCA p18 IgA levels (Spearman's rho = −0.102 and −0.099 for the 213-bp and 99-bp LC-based PCRs, respectively) (Fig. 4C and D). Finally, ELISA optical density (OD) values for anti-EBNA1 IgA did not differ significantly between EBV DNA-positive and -negative whole-blood samples by either the 99-bp or the 213-bp LC-based PCR (Mann-Whitney test, $P = 0.115$ and 0.955, respectively), nor did the OD values in the anti-VCA-p18 IgA ELISA differ ($P = 0.814$ and 0.103, respectively). Thus, circulating EBV DNA and EBV antibody responses are independent and quantitatively unrelated parameters in individual NPC patients.

DISCUSSION

This study is the first to investigate the primary diagnostic value of circulating EBV DNA load determination and its relation to quantitative EBV serology in Indonesian NPC patients. In general, the significantly higher whole-blood EBV DNA loads in these NPC patients compared to those in healthy EBV carriers (43, 55, 56, 63) confirm the findings for Chinese, Thai, Italian, and Taiwanese NPC patients (10, 38, 41, 47, 48, 60, 66). Still, about 15% of the whole-blood samples from our patients were EBV DNA negative, while the load in an even larger fraction (40%) was below the 2,000-copy/ml clinical cutoff value previously determined to be the upper limit in healthy EBV carriers (54, 55). This is in agreement with previous studies showing the absence of plasma or serum EBV DNA in a considerable proportion (15 to 50%) of NPC patients at the time of primary diagnosis and the corresponding low clinical sensitivities (<90%) of EBV DNA load monitoring (9, 24, 25, 38, 48). These observations question the general use of EBV DNA load monitoring for the primary or confirmatory diagnosis of NPC in future population-based screening studies. In contrast, studies in Hong Kong by Lo and coworkers, predominantly by a PCR that targeted the (vari-

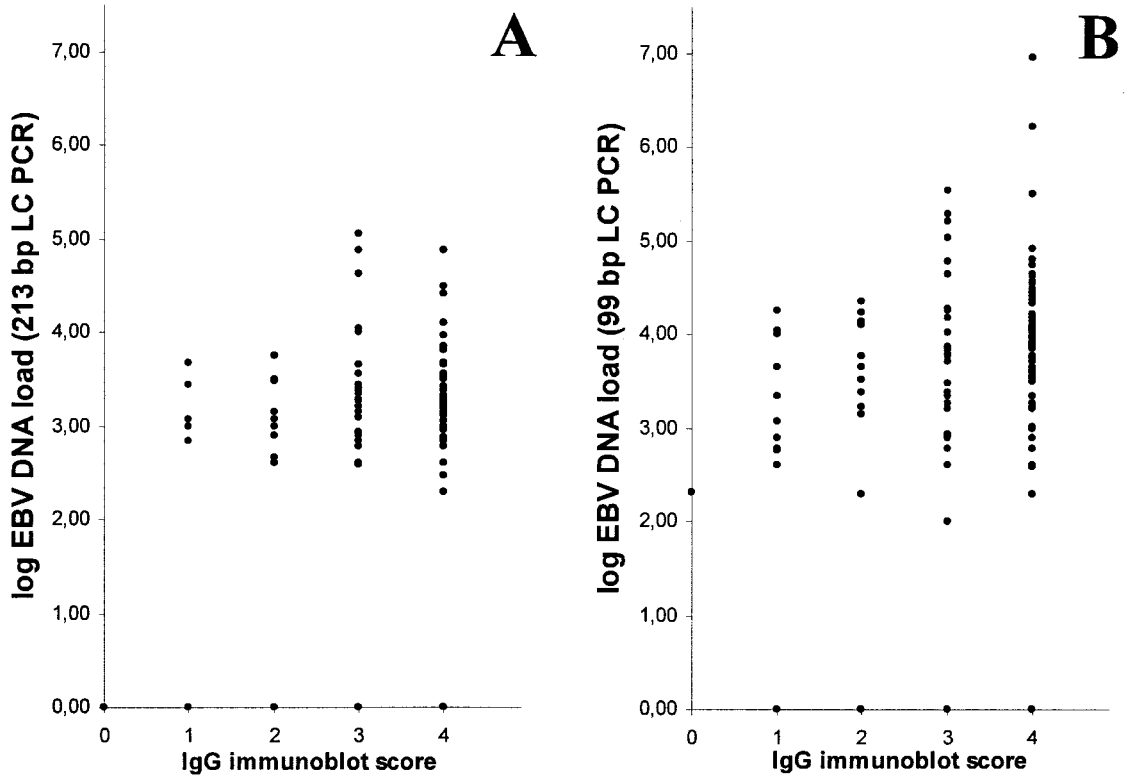

FIG. 3. EBV DNA load in blood of Indonesian NPC patients is not related to anti-EBV IgG reactivity, as assessed by immunoblotting. EBV-specific immunoblot data and scoring were previously described by us (15). The overall immunoblot intensity was scored semiquantitatively as negative (n = 1, group 0), 1 (n = 12, group 1), 2 (n = 16, group 2), 3 (n = 33, group 3), and 4 (n = 77, group 4). No significant difference in EBV DNA load was observed between the four groups for either the 213-bp LC-based PCR (A) (Kruskal-Wallis test, P = 0.091) or the 99-bp LC-based PCR (B) (P = 0.124).

able) BamHI-W repeat domain, invariably showed the high specificity of plasma EBV load quantification, although only trace amounts of EBV DNA (<10 EBV DNA copies/PCR) were found in some NPC patients, while healthy blood donors are also occasionally positive (7, 18, 30, 31).

Our study now explains the large variations in the diagnostic sensitivity of circulating EBV DNA load quantification reported in the literature. As the majority of EBV DNA may be fragmented, a small PCR target size will quantify a significantly higher EBV load (Fig. 1) and yield more EBV DNA-positive samples (Fig. 2), which we show is independent of the PCR technique, the amplicon, or the clinical specimen used. These findings also substantiate a recent, smaller study that found that the majority of plasma EBV DNA in NPC patients is highly fragmented (<180 bp) and is not virion associated (8). It probably originates from the release of the DNA from apoptosed tumor cells into the circulation (8). However, Shotelersuk et al. (48) found DNase-resistant circulating DNA in more than 50% of NPC patients tested, suggesting that EBV DNA is protected in virions. In contrast to those two studies (8, 48), which both used plasma for EBV DNA characterization, we used fresh whole blood that was directly lysed in a nucleic acid-stabilizing buffer within 1 h after collection. This approach enables parallel EBV DNA and RNA analyses; ensures a direct fixation of the in vivo EBV DNA and RNA status; and may increase the sensitivity by combining plasma and cellular blood compartments, which may both harbor EBV DNA (32, 41, 57). The use of whole blood omits cell separation techniques and delayed preparation of plasma, factors that may possibly affect viral DNA and RNA characteristics by inducing uncontrollable cell lysis, apoptosis, or lytic virus replication (52).

The absence of circulating BARF1 mRNA, despite high EBV DNA loads, further substantiates the fact that the circulating EBV DNA is probably not derived directly from intact circulating NPC cells. The undetectable presence of only small numbers of circulating NPC cells which have silenced their BARF1 RNA expression cannot be excluded. This then, however, would be selective for circulating cells because BARF1 mRNA can easily be detected in NPC tissue biopsy and brush specimens (5, 21). There is also a possibility of quick in vivo RNA degradation upon the immediate lysis of tumor cells entering the circulation.

The detection of EBNA1 mRNA in the circulation may reflect increasing numbers of EBV-positive B cells, as already suggested in a previous study that showed elevated EBV DNA loads in peripheral blood mononuclear cells of Taiwanese NPC patients (32). This phenomenon may relate to de novo B-cell infection due to local lytic viral replication in NPC (16, 61, 68), of which IgG and IgA reactivity to numerous early and late lytic viral antigens in NPC patients is a reflection (15). An enhanced replication frequency of circulating EBV-positive B

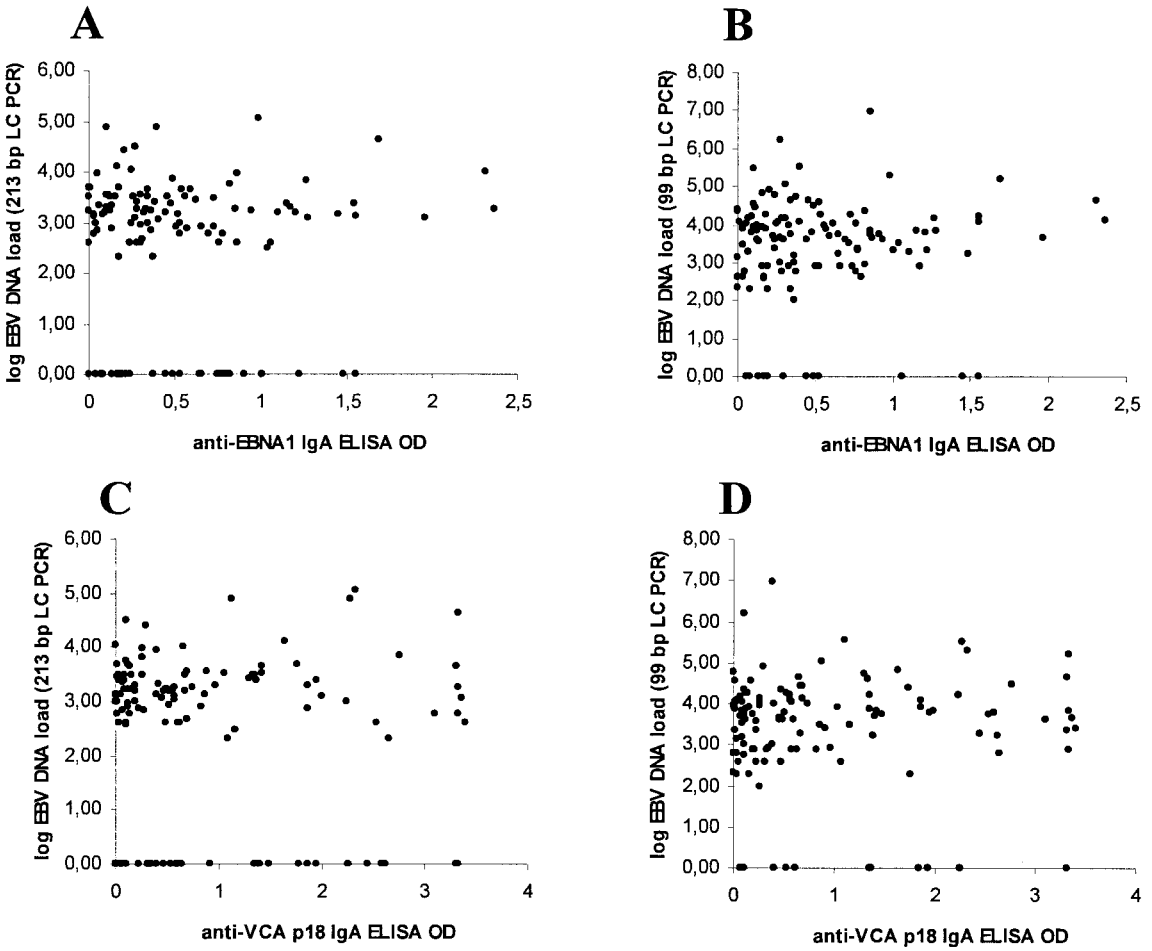

FIG. 4. Circulating EBV DNA loads in Indonesian NPC patients do not correlate with quantitative EBV IgA serology, as determined by synthetic peptide-based ELISA for immunodominant epitopes of EBNA1 and VCA-p18. (A and B) Relation between anti-EBNA1 IgA ELISA OD values (with the background subtracted) and EBV DNA loads in whole blood determined by the 213-bp or the 99-bp LC-based PCR (Spearman's rho = −0.14 and −0.113, respectively); (C and D) relation for anti-VCA-p18 ELISA OD values (Spearman's rho = −0.102 and −0.099 for 213-bp and 99-bp LC-based PCRs, respectively).

cells may lead to higher levels of EBNA1 mRNA expression (14, 23). Furthermore, EBNA1 transcription in blood indicates that not all EBV DNA is fragmented but that a part of the circulating viral DNA load is cell associated in the context of an intact and transcriptionally active EBV genome (23), in agreement with the findings of Lin et al. (32) and Shotelersuk et al. (48).

The indications for EBV DNA fragmentation may have profound diagnostic consequences. The PCR target size should preferably be as small as technically possible to increase detection rates and to assess the EBV load as accurately as possible. Still, a recent study that used a 72-bp PCR found a low sensitivity (0.85) similar to that found in our present study at a diagnostic cutoff of 7 copies/ml serum (28). We consider such a low value to be diagnostically irrelevant. Intrinsic to real-time PCR assays, small amounts of DNA (<100 copies/reaction) cannot be as accurately and reproducibly quantified as large amounts (54, 63).

The fragmentation of viral DNA was recently also reported for another herpesvirus, i.e., cytomegalovirus, in the blood of transplant recipients (3), a population in which monitoring of circulating EBV DNA loads is also widely applied. Discussions about the sources and characteristics of circulating EBV DNA and the preferred clinical specimen type in different patient populations are ongoing (20, 44, 56, 62) and should also quantitatively address the origin and the physical nature of the EBV DNA. In NPC patients this should be extended to defining a lymphoid versus an epithelial cell tropism of EBV. It may, however, be technically challenging to quantitatively detect and differentiate EBV DNA originating from lysed NPC cells, circulating intact tumor cells, latently infected B cells, and/or virions.

Despite the development of a short-fragment LC-based PCR and the fact that the majority of patients had NPC stage III or IV disease, circulating EBV DNA load quantification was not sensitive enough for primary diagnosis either in this study or in numerous other studies from regions where NPC is endemic (24, 28, 31, 38, 41, 48). Thus, EBV DNA load measurement may have limited value for the primary or confirmatory diagnosis of NPC in population screening in high-inci-

dence regions and is not recommended as the sole means of risk assessment. Finally, our results demonstrate that serology and the EBV DNA load in blood are independent parameters that are not quantitatively related to each other. This may explain why the combination of circulating EBV DNA detection and EBV serology increases the diagnostic sensitivity (9). By using follow-up sampling, we are studying whether monitoring of EBV DNA parameters in whole blood is useful for the prediction of therapeutic efficacy and NPC recurrence or persistent disease in patients with elevated viral DNA loads at the time of primary diagnosis (30, 33).

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