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Quantitative Detection of Plasma Cell-free Epstein-Barr Virus DNA in Nasopharyngeal Carcinoma Patients by Real-time PCR

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Abstract **Nasopharyngeal carcinoma (NPC) is obviously a serious cancer in southern China, Taiwan, and Southeast Asia including Thailand. The Epstein-Barr virus (EBV) is a human oncogenic virus that is strongly associated with NPC development. Recently, plasma cell-free EBV DNA levels detected by the real-time polymerase chain reaction (R-PCR) have been reported to be a useful marker for rapid diagnosis and monitoring of NPC patients. The objectives of this study are to measure the amount of plasma cell-free EBV DNA in NPC patients and healthy controls by using the R-PCR and to determine the relationship between plasma cell-free EBV DNA levels and NPC status in Thai population. The concentrations of plasma cell-free EBV DNA of 60 NPC patients and 60 healthy controls were determined by using the R-PCR assay with the LightCycler® EBV quantification kit. Plasma cell-free EBV DNA levels in NPC patients [4679.88 ± 1381.99 copies/ml (mean ± standard error, SE)] were significantly higher than healthy controls (3.93 ± 2.32 copies/ml) ($p < 0.001$). The median concentration of EBV DNA in NPC and healthy control groups was 1135.00 and 0.00 copies/ml, respectively. The cutoff value of plasma cell-free EBV DNA in Thais is 50 copies/ml (mean+2standard deviation, SD). Fifty-nine NPC patients (98%) and 3 healthy controls (5%) had plasma cell-free EBV DNA levels over the cutoff value (50 copies/ml). However, there were no significant differences between the levels of plasma cell-free EBV DNA and histological types, and TNM staging of NPC. The results of this study suggest that plasma cell-free EBV DNA may be a useful molecular marker for diagnosis of EBV-associated NPC in Thailand. Further investigation on the relationship of plasma cell-free EBV DNA levels and the clinical outcome needs to be explored.** *(Thai Cancer J 2008;28:13-23.)*

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บทคัดย่อ **การตรวจหาปริมาณ Cell-free DNA ของเอปสไตน์-บาร์ ไวรัสในพลาสม่าของผู้ป่วยโรคมะเร็งโพรงหลังจมูกโดยวิธี Real-time Polymerase Chain Reaction**

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จากการตรวจหาปริมาณของ cell-free EBV DNA ในพลาสม่าของผู้ป่วยโรคมะเร็งโพรงหลังจมูก 60 ราย และคนสุขภาพปกติ 60 คน ด้วยวิธี real-time polymerase chain reaction (R-PCR)โดยใช้ LightCycler® EBV quantification kit พบว่า ปริมาณ cell-free EBV DNA ในพลาสม่าของกลุ่มผู้ป่วยโรคมะเร็งโพรงหลังจมูก [4679.88 ± 1381.99 สำเนา/มล. (ค่าเฉลี่ย ± ค่าความคลาดเคลื่อนมาตรฐาน)] มีค่าสูงกว่าของกลุ่มคนสุขภาพปกติ (3.93 ± 2.32 สำเนา/มล.) อย่างมีนัยสำคัญทางสถิติ ($p < 0.001$) ซึ่งในกลุ่มของผู้ป่วยโรคมะเร็งโพรงหลังจมูกนั้นมีค่ามัธยฐานของปริมาณ cell-free EBV DNA = 1135.00 สำเนา/มล. ในขณะที่ในกลุ่มของคนสุขภาพปกติมีค่า = 0.00 สำเนา/มล. และพบว่าในคนไทยมีค่าปกติของปริมาณ cell-free EBV DNA = 50 สำเนา/มล. (ค่าเฉลี่ย +2 เท่าของค่าความเบี่ยงเบนมาตรฐาน) นอกจากนี้ยังพบว่าผู้ป่วยโรคมะเร็งโพรงหลังจมูก 59 ราย (98%) และคนสุขภาพปกติ 3 ราย (5%) มีปริมาณ cell-free EBV DNA สูงกว่าค่าปกติ (50 สำเนา/มล.) แต่อย่างไรก็ตามไม่พบความแตกต่างระหว่างปริมาณของ cell-free EBV DNA ในพลาสม่าในกลุ่มของชนิดของเซลล์มะเร็ง ขั้นของโรค ระยะของมะเร็ง ระยะของต่อมน้ำเหลือง การแพร่กระจายของโรค (ค่า $p > 0.05$) ผลของการศึกษาครั้งนี้แสดงว่าการตรวจหาปริมาณของ cell-free EBV DNA ในพลาสม่าน่าจะมีประโยชน์ใช้เป็น molecular marker ช่วยในการวินิจฉัยโรคมะเร็งโพรงหลังจมูกในคนไทยได้ อย่างไรก็ตามควรมีการศึกษาความสัมพันธ์ของ cell-free EBV DNAในพลาสม่ากับ ผลของการรักษาในผู้ป่วยโรคมะเร็งโพรงหลังจมูกต่อไป *(วารสารโรคมะเร็ง 2551;28:13-23.)*

Introduction

Nasopharyngeal carcinoma (NPC) is a cancer of the epithelial cells that line and cover the surface of the nasopharynx. Epidemiologically, NPC is specifically endemic in southern China (age-standardized incidence rate, ASR = 30-50/10^5/year) and Southeast Asia (ASR = 9-12/10^5/year) but it is rare in the West (ASR <1/10^5/year). NPC is a serious public health problem in Thailand. The ASR of NPC in Thais is 3-10/10^5/year and the ratio of males:females is 2.8:1. The age distribution of NPC in Thailand shows incidence beginning among young adults and then a gradual increase is observed up to the age of 65 years with the peak age at 40-50 years. NPC is one of the ten leading cancers in Thailand and has caused vast yearly losses in terms of human resources and economic status[1-5]. Etiologically, NPC is a multi-factorial disease caused by any number of combinations of Epstein-Barr virus (EBV) infection[6-10], environmental carcinogen[11] and genetic susceptibility[12,13]. However, the mechanism of NPC development induced by these factors is still unclear. EBV infection is widely recognized as the risk factor for NPC by the detection of high titers of specific serum IgA and IgG antibodies to EBV early and viral capsid antigens in NPC patients[14-21] and by identification of EBV genome in NPC epithelial tumor cells[22]. NPC is considered as a treatable disease. Radiotherapy is the first choice of treatment for NPC since patients respond well to radiation treatment with overall 5 years survival rates of 70-80% for stage I disease and 20-30% for stage IV disease, respectively[23,24]. However, most of NPC patients were diagnosed at late stages. The follow up treatment of NPC is needed since the recurrences of the disease are always occurred

within the first 2 years after treatment and can detect loco-regionally or systemically, or both[25].

Actually, NPC patients are mostly diagnosed at the late stages with a poor prognosis and are not respond well to the treatment. These patients would be died within one year if the treatment was insufficient. However, the early stages (stage I and II) of NPC are treatable by radiotherapy whereas in the late stages (stage III and IV) the combination treatment of radiotherapy and chemotherapy increased the 3-5 years survival rate[26-29]. Thus, the detection of a high-risk group who will develop the early stages of NPC in Thais followed by intensive follow-up and immediate treatment when NPC was identified is urgent to fight against NPC.

Recently, it is suggest that the detection of NPC by the measurement of anti-EBV IgA and IgG antibodies against early and viral capsid antigen (EA and VCA)[14-21] as well as the measurement of cell-free EBV DNA levels in plasma and serum[30-35] followed by the effective treatment is an important strategy for NPC control. Among the EBV detection methods, the real-time PCR (R-PCR) is proposed to be the most sensitive and specific method. Several investigators have been demonstrated that serum/plasma cell-free EBV DNA levels as detected by R-PCR are associated with NPC. The levels of plasma cell-free EBV DNA in NPC patient is significantly higher than healthy control, increased with the stages of the diseases and decreased after successful treatment with radiation and surgery. However, plasma cell-free EBV DNA levels were increased again when the recurrent of NPC was detected in patients[36]. Therefore, it is suggested that the detection of plasma cell-free EBV DNA may be a useful tool for detection of NPC in Thai patient.

Since NPC is a harmful cancer in Thailand, but the data on association between plasma cell-free EBV DNA and NPC development by using real-time PCR are limited. In addition, the early detection of NPC in Thai population is extremely needed. Therefore, the specific aims of this study were to examine association of plasma cell-free EBV DNA levels and NPC development and to evaluate the relationship of plasma cell-free EBV DNA levels with the stages of NPC.

Materials and Methods

Sample Collection

Plasma samples from 60 pre-treatment Thai NPC patients with pathological proven [used WHO criteria and the 1997 American Joint Committee on Cancer Staging (AJCC) criteria], age between 30-80 years-old, that admitted at the Thai-NCI were collected and informed consent was obtained. There were 40 undifferentiated carcinoma and 20 poorly differentiated squamous cell carcinoma patients. Nine patients were classified as NPC stages I & II whereas other 51 patients were stages III & IV. At the same time, the plasma samples from 60 age-match healthy control that visited the Thai-NCI for general check-up were also collected as a control group. The EDTA whole blood was kept at room temperature for 30 min followed by centrifugation for the plasma separation at 2000 x g for 10 min. The plasma samples were collected and kept at -20 °C until examined.

Extraction of EBV DNA

Cell-free EBV DNA from all plasma samples

was extracted by using a High Pure Viral Nucleic Acid Kit (Roche) according to the user manual and kept at −20 °C until used. Briefly, a 200 µl plasma sample was added to 257.5 µl working solution (200 µl binding buffer supplemented with 5 µl poly (A), 50 µl proteinase K and 2.5 µl internal control) in a 1.5 ml microcentrifuge tube, mixed immediately and incubated at 72 °C for 10 min with 100 µl binding buffer. Then, the samples were applied to the upper reservoir of the High Pure spin column (High Pure filter tube which was on the top of the collection tube) and centrifuged at 8,000 x g (Heraeus, Germany) for 1 min followed by discarding the flowthrough and collection tube. Added 500 µl inhibitor removal buffer and centrifuged at 8,000 x g for 1 min, then discard the flowthrough and collection tube. The membrane-bound EBV DNA was washed twice with wash buffer by centrifuged at 8,000 x g for 1 minute for the first wash, and at 8,000 x g for 3 min plus at 13,000 x g for 10 sec in the second wash. Finally, EBV DNA was eluted from the membrane with 50 µl of elution buffer by centrifuged at 8,000 x g for 1 minute. The final volume of EBV DNA obtained from each sample was 50 µl.

Detection of Plasma Cell-free EBV DNA

The LightCycler® EBV quantification kit contained a specific pair of fluorescence resonance energy transfer (FRET) hybridization probes was used for detection of the EBV DNA fragment in the plasma. The master mix reagent included 2 µl of LightCycler® EBV reaction mix (including FastStart Taq DNA polymerase, reaction buffer, and deoxynucleoside triphosphates), 2 µl of LightCycler® EBV detection mix (the hybridization probe mixture) and 11 µl PCR-grade water. Then, 5 µl of sample DNA was added to make a final reaction volume of 20 µl. EBV DNA standards were included in each run. The protocol of the LightCycler® R-PCR was programmed as suggested by the user manual. Briefly, thermal cycling was initiated with a 10-minute incubation at 95 °C for DNA denature , followed by 45 cycles of 95 °C for 10 sec (denature of DNA template), 55 °C for 15 sec (primer annealing) and 72 °C for 15 sec (extension of the annealed primers).

A color compensation file was used for the data analysis. A melting curve analysis program was run immediately after the R-PCR. The lower detection limit of the assay was ≤10 copies per reaction as specified in the user manual. All purified DNA samples were not inhibited the amplification of the internal control. The five concentrations of standard EBV DNA at 102 to 106 copies per reaction were run in parallel in every analysis and the standard curve is shown in Figure 1. Finally, the cell-free EBV DNA concentration was calculated into copies/ml of plasma according to the following equation:

$$\text{copies/ml} = \frac{\text{number of detected EBV DNA copy (X)} \times \text{DNA volume after extraction}}{\text{DNA volume used in R - PCR} \times \text{plasma volume for DNA extraction}} \times 1000$$

$$\text{copies/ml} = \frac{X\ 50}{5 \times 200} \times 1000 = 50x$$

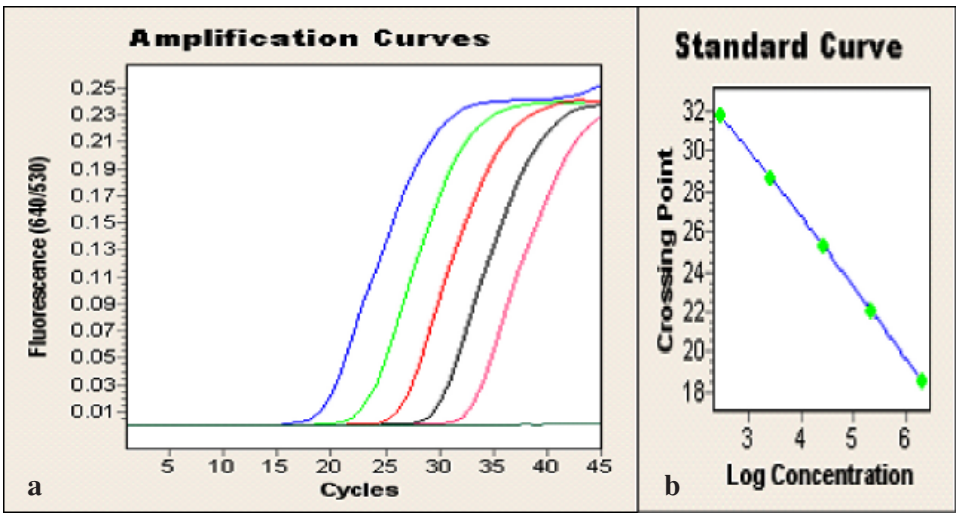

Figure 1 The R-PCR amplification plot of standard control of EBV DNA, the point that the fluorescence signal increased above baseline is the threshold cycle (a) and its standard curve (b).

Statistical Analysis

The difference between the mean levels of cell-free EBV DNA of NPC and healthy controls groups was compared in order to evaluate the association between pretreatment plasma cell-free EBV DNA levels and the NPC development in Thais by using the Chi-square (χ^2) test. The relationship of cell-free EBV DNA levels and clinical variables in NPC patients such as tumor histology, overall disease stage and TNM staging was also determined by using the χ^2 test.

Results

The plasma cell-free EBV DNA was successfully detected in 3 out of 60 (5%) healthy controls with the mean level of 3.9 ± 2.3 copies/ml. In contrast, cell-free EBV DNA was detected in 59 out of 60 (98%) NPC patients with the mean level of 4679.9 ± 1381.9 copies/ml. The mean level of plasma cell-free EBV DNA in NPC patients was significantly higher than healthy controls at $p < 0.001$. The median concentration of plasma cell-free EBV DNA in NPC group was 1135.0 copies/ml whereas in healthy controls group was 0.0 copies/ml. The cutoff value (mean + 2SD) of plasma cell-free EBV DNA levels in this study was 50.00 copies/ml (13.90+36.10 copies/ml) (Table 1 and Figure 2).

The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the assay by using LightCycler® EBV quantification kit were 95.00%, 98.00%, 97.00%, 98.00% and 95.00%, respectively.

In the group of NPC patients, the mean levels of cell-free EBV DNA of 40 undifferentiated carcinoma (UD) cases (67%) and of 20 poorly differentiated squamous cell carcinoma (PDSCC) cases (33%) were 3978.2 ± 1645.9 and 6083.3 ± 2553.7 copies/ml, respectively. However, there was no significant difference in the mean levels of plasma cell-free EBV DNA between these two different histological types.

Table 1 The plasma cell-free EBV-DNA levels in healthy controls and NPC Patients

Group	Age (mean)	Number	Plasma cell-free EBV-DNA			
			mean ± SE	median	+ve cases	-ve cases
Healthy controls	55	60	3.9 ± 2.3	0.0	3	57
NPC Patients	56	60	4679.9 ± 1381.9***	1135.0	59	1
- UD	57	40	3978.2 ± 1645.9	10418.7	40	0
- PDSCC	52	20	6083.3 ± 2553.7	11414.8	19	1
- Stages I and II	51	9	3687.2 ± 496.0	4188.1	9	1
- Stages III and IV	56	51	4855.1 ± 1465.0	10462.2	50	0

****, p< 0.001*

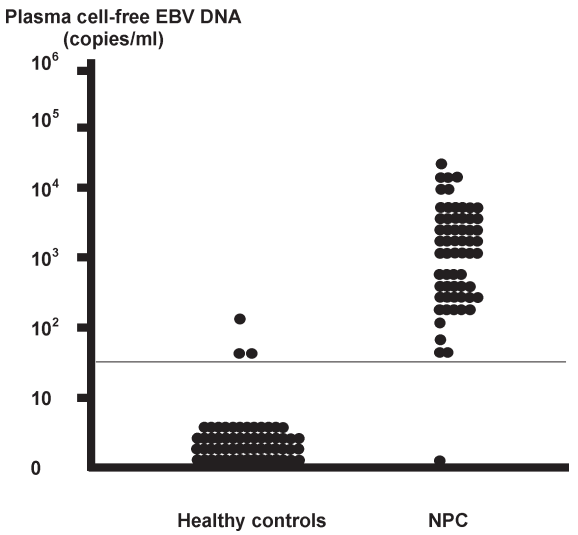

Figure 2 The distribution of plasma cell-free EBV DNA levels in healthy controls and NPC patients

With respect to NPC staging, there was no significant difference between the mean levels of cell-free EBV DNA of stage I&II (3687.2 ± 496.0) and stage III&IV (4855.1 ± 1465.0) ($p > 0.05$). In addition, there was also no significant difference between the mean levels of cell-free EBV DNA between the T (tumor), N (node) and M (metastasis) staging of NPC (Table 1 and 2).

Discussion

NPC is one of the major causes of dead in Thai population, particularly in males. Actually, the early stage of NPC is difficult to diagnose since its symptoms are non-specific, thus, most of NPC patients are diagnosed at the late stages of the disease with the common feature of a neck mass[37]. The identification of an effective marker that could detect these patients would be useful. It could lead to early diagnosis and more efficient therapy.

EBV is a human herpesvirus included in the *Gammaherpesvirinae* subfamily[38] that infects over 90% of the population worldwide[39]. EBV infections are generally silent and asymptomatic, however, the virus has been reported as the causative agent of infectious mononucleosis[40] and is associated with the development of NPC[41], oral hairy leukoplakia[42], Burkitt's lymphoma, Hodgkin's disease and T-cell non-Hodgkin's lymphoma[43].

The relationship of NPC and EBV was evidenced by the seroepidemiologic findings and the identification of viral genome in the epithelial NPC cells. Since the use of anti-EBV antibodies as the diagnostic and prognostic tools for monitoring of NPC was inapplicable to all populations[44], thus, the R-PCR assay that could reduce costs, time-

Table 2 Concentrations of plasma cell-free EBV-DNA in NPC Patients

Group	Age (mean)	Number	Plasma cell-free EBV-DNA	
			mean ± SE	median
NPC Patients	56	60	4679.9 ± 1381.9	10710.5
- T1	52	10	3258.5 ± 1167.7	3692.7
- T2	54	14	5911.9 ± 4610.5	17251.1
- T3	57	13	2219.4 ± 685.9	2473.4
- T4	58	23	5938.7 ± 2242.1	10758.9
- N0	49	11	2441.1 ± 732.4	2428.9
- N1	50	15	4333.2 ± 2616.1	9896.3
- N2	59	15	2074.7 ± 850.7	3218.1
- N3	57	19	8306.5 ± 3740.3	16303.4
- M0	51	56	3284.8 ± 1404.8	1097.5
- M1	56	4	24211.3 ± 8102.6	13700.0

consuming, carcinogenic exposure and the risk of amplicon contamination was developed as an alternative method for detection of EBV DNA that released from tumor cells into the plasma and serum of NPC patients[45]. Recently, plasma and serum EBV DNA load as detected by the R-PCR assay is reported to be a sensitive marker for diagnosis, prognosis and monitoring of NPC recurrence[31,32,36].

After the development of R-PCR in the year 1990s, several assay formats and applications have been established and various types of R-PCR machines are increasing. Many reports describe the development of R-PCR protocols for EBV DNA detection by using resonance energy transfer (FRET) probes[46-48] and TaqMan probes[34,49]. Recently, two commercial kits for quantitative detection of cell-free EBV DNA levels are available: the LightCycler EBV quantification kit (Roche Diagnostics, Mannheim, Germany) and the RealArt EBV LC PCR kit (Artus, Hamburg, Germany). In this study we selected the LightCycler EBV quantification kit since it had a wider dispersal in results but produced substantially more-accurate melting temperature profile curves than the RealArt EBV LC PCR kit[50]

In the present study, we found that the plasma cell-free EBV DNA levels were detected over the cutoff value in 59 (98%) of 60 NPC patients and in 3 (5%) of 60 healthy controls, respectively. In addition, the levels of plasma cell-free EBV DNA in the NPC patients were significantly higher than those in controls (Figure 2), indicating that quantitative plasma EBV DNA analysis may be a useful marker for detection of NPC. Our data suggested that the LightCycler EBV quantification kit may be a feasible assay for diagnosis of NPC in Thai patient with a rapid, sensitive and accurate result.

The detection rate for plasma cell-free EBV DNA at 98% in the present study was similar to those in the study of Lo et al.[31] who reported that plasma cell-free EBV DNA was detected in 55 (96%) of 57 NPC patients. However, it was higher than in the previous study of Pholampaisathit

et al.[35] who showed that serum cell-free EBV DNA was detected in 61 (66%) of 92 NPC patients. This disagreement might be due to the differences in the source of the cell-free EBV DNA and the histological types of NPC patients included in the study. In Lo et al.[31] and our studies, plasma was used as the source of the cell-free EBV DNA, while that of Pholampaisathit et al.[35] used serum samples. There are some studies have been demonstrated that plasma contains more copies of the cell-free EBV DNA than serum[31], but the whole blood (WB) and peripheral blood mononuclear cell (PBMC) are more sensitive than plasma for the quantification of the EBV DNA in transplant patients[51-53]. Thus, it was of interest to investigate the usefulness of WB and PBMC as the source of EBV DNA for the clinical diagnosis of NPC in the future. In addition, the levels of plasma EBV DNA in the NPC patients were much higher than those in controls, including the three control subjects with detectable EBV DNA (Figure 2). These data suggest that quantitative plasma EBV DNA analysis may be useful as a screening method for NPC in high-risk populations. The quantitative R-PCR may be suitable for this application due to its rapidity and accuracy.

Interestingly, one of NPC patient showed cell-free EBV DNA level = 0.0 copies/ml with unclear reason. Since this case was UD patient with stage I (T1N0M0), thus we suggested that the tumor cell death (source of EBV DNA in the blood circulation) may be very low or probably may not occurred resulting in a very low number or non of the cell-free EBV DNA released into the plasma. In contrast, three healthy subjects had shown the cell-free EBV DNA levels above cutoff value. We suggested that these three persons may be developed an early stage of NPC without any detectable symptoms. All of these individuals were planed to carrying out long-term follow-up to monitor the clinical symptoms of NPC and the alterations in their plasma cell-free EBV DNA levels.

The median level of cell-free EBV DNA of NPC patients in our study was 18 times lower than the study of Lo et al.[32] (1135 versus 21058 copies). This disagreement may be caused by the differences of primer/probe set that designed for detection of the specific regions of EBV DNA. It was reported that in the R-PCR assay method that used a primer/probe set that span the BamHI-W region which its sequence occurs with multiple repeats could be detect the EBV DNA level higher than those used a primer/probe set that span the *Pol*-1 and *LMP2* region[32,54,55]. To date, two PCR methods for detection of EBV load during infection by using a *Pol*-1 (polymerase-1 gene) and *Lmp2* (latent membrane protein-2 gene) primer/probe set, both of which are single-copy genes[34], were well accepted. Unfortunately, the LightCycler® EBV quantification kit that used in this study did not mention about the region of EBV DNA that the primer/probe set was span for, thus, it was difficult to compare our data with other previous studies directly.

With respect to the cutoff value of plasma cell-free EBV DNA, we found that it was 50.0 copies/ml in our study, while it was 0.0 copies/ml in other previous studies[31,35]. The reason for this disagreement is unknown, but we suggested that it might be resulted from the differences in the type of samples that selected for the individual studies. Stevens SJC et al.[56,57] have been used a cutoff value of 2,000 EBV DNA copies/ml blood (using

unfractionated whole blood for EBV DNA extraction) to identified EBV-positive posttransplant lymphoproliferative disease patients from EBV-seropositive healthy carriers, in whom the loads were invariably below this value.

The results of our study showed no correlation between plasma cell-free EBV DNA levels and the stages of NPC in Thai patients. Our results were correlated with the results of Mutirangura et al.[30] and Pholampaisathit et al.[35], but not Lo et al.[31] In the study of Lo et al., they could distinguish early stage patients from advanced-stage patients by the amount of cell-free EBV DNA level in plasma. They suggested that EBV was more strongly associated with NPC in areas of higher prevalence and in those patients whose tumors were WHO type II and III than in less aggressive types and in areas of lower incidence[4,5].

In conclusion, the results of our study suggest that plasma cell-free EBV DNA is a highly specific marker for a screening and diagnosis of EBV-associated NPC. The feasibility of using quantitative analysis to determine the possible relationship between the clinical outcome and the level of plasma cell-free EBV DNA are warranted.

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