Evaluation of plasma Epstein-Barr virus DNA load as a prognostic marker for nasopharyngeal carcinoma

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ABSTRACT

Introduction: Nasopharyngeal carcinoma (NPC) is an important cancer in Malaysia and is one of the major causes of cancer mortality in this country. This study evaluates the diagnostic and prognostic values in the quantitative relationship between the cell-free Epstein-Barr virus (EBV) deoxyribonucleic acid (DNA) load and the tumour burden.

Methods: Blood plasma from 18 untreated NPC patients, 20 NPC patients who had been treated with radiotherapy, and 12 healthy individuals were evaluated. EBV copy number was determined following DNA extraction using real-time quantitative polymerase chain reaction.

Results: The cell-free EBV DNA load was shown to be proportionately related to the presence of malignant disease. While the EBV copy number in untreated NPC patients had a median of 2,043 copies/ml, viral load in plasma of healthy controls was significantly lower (median of 0 copy/ml). A significant decrease in EBV load was observed in patients who had undergone radiotherapy while a high viral load indicated in one patient correlated to tumour relapse and presence of distant metastasis upon clinical investigation.

Conclusion: The blood plasma EBV DNA load was shown to be proportionately related to the presence of malignant disease. This preliminary study underscores the prognostic value of cell-free EBV DNA quantification.

Keywords: DNA, Epstein-Barr virus, human herpes virus, nasopharyngeal carcinoma

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) represents an important cancer among populations in Southern China and Southeast Asia, including Malaysia[1,2]. In the year 2003, NPC was the second major cause of cancer mortality among males and ranked 12th among females. The frequencies of NPC in males and females were 8.8% and 2.5%, respectively, of the total cases of cancers reported[3]. NPC is an epithelial neoplasm arising from the fossa of Rosenmuller of the postnasal space. Epstein-Barr virus (EBV) has long been implicated as an important aetiological factor of NPC with histological evidence, indicating the consistent presence of the viral deoxyribonucleic acid (DNA) and proteins in malignant tissue[4]. The importance of EBV in NPC was further demonstrated by the elevated IgA antibody titres against the viral capsid antigen (VCA) in most NPC patients.

More recently, it was shown that cell-free EBV DNA could be detected in the serum of patients with NPC[5]. This discovery raises the possibility of a new strategy for noninvasive cancer detection and monitoring. Furthermore, serum/plasma EBV DNA was reported to be a promising marker that is useful for the management of NPC[6]. Data from Hong Kong, where NPC is endemic, showed that 96% of NPC patients had detectable serum/plasma EBV DNA, and the incidence of recurrence corresponded tightly with the cell-free viral DNA load after radiotherapy[7]. Similar findings were also reported by Lin et al[8]. In analysing a cohort of NPC patients with advanced disease, the authors found that disease recurrence was especially prevalent in patients who had a significantly higher plasma EBV DNA concentration before treatment than those who did not have a relapse. The authors thus concluded that plasma EBV DNA load is useful for monitoring patients with NPC and for predicting the outcome of treatment, and herein lies the prognostic value of the cell-free viral DNA load.

It is of prime interest to know if the plasma viral DNA load is also elevated in NPC cases in Malaysia, and if this method is to be established as an alternative indicator for NPC in the near future. In this present

study, the quantities of cell-free EBV DNA in the plasma of untreated and treated NPC patients, as well as in healthy individuals, were evaluated.

METHODS

The Namalwa cell line was established from the tumour mass of an African child with Burkitt's lymphoma in 1967[9]. The cells were cultured in RPMI-1640 supplemented with 10% (v/v) foetal calf serum (FCS), 300 mg/L L-glutamine. Each cell contained two integrated copies of the EBV genome. DNA extracted from the Namalwa line was used as quantitative standards in the real-time polymerase chain reaction (PCR) assay. As each cell was estimated to contain 6.6 pg of DNA, a conversion factor of two EBV copies per 6.6 pg DNA was used.

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) to facilitate the subsequent separation of plasma. Blood samples were collected from 38 patients with undifferentiated NPC (WHO Type II), comprising 18 untreated and 20 treated patients who sought treatment at the ENT Clinic of the University of Malaya Medical Centre after informed consent was obtained. Of the 18 untreated patients, three presented with Stage I tumour, three with Stage IIA, two with Stage IIB, six with Stage III and four with Stage IVA. Of the 20 treated patients, five were presented with Stage IIA, two with Stage IIB, six with Stage IIIB, four with Stage IVA and three with Stage IVB.

Blood samples were collected from the untreated NPC patients when they first presented at the hospital for clinical examination and their samples were only included in the study after they had been histologically confirmed to have NPC by the pathologist. The treated patients consisted of NPC patients who had received radiotherapy and whose primary tumours were under remission. Blood samples were collected from this group of patients during their follow-up visits to the hospital. The controls comprised 12 blood samples from healthy individuals. Plasma isolation was performed for each sample immediately upon receipt in the laboratory, aliquoted and stored at -20ºC until use.

DNA was extracted from plasma samples and the Namalwa cells using the QIAamp® DNA minikit (Qiagen, Hamburg GmBH, Germany) following the manufacturer's instructions. A 200 μL aliquot of plasma from each sample or suspension containing 10^7 Namalwa cells was used for DNA extraction. From this point onwards, all samples were processed according to the manufacturer's instructions. The extracted DNA was quantified and checked for purity using a spectrophotometer (Shimadzu, Kyoto, Japan).

Quantification of EBV DNA copies in plasma-derived DNA was performed using the iCycler iQ™ Real Time PCR system (Bio-Rad, Hercules, CA, USA). The quality of purified DNA from plasma samples was validated by conventional PCR amplification of the human β-globin gene using gene-specific primers (βF: 5'-AGGAGTGGTGGCTCATGTCT-3' and βR: 5'-CTCAAGGGATCCTCCCATTT-3'). Primers flanking the *Bam*H1W region (EBV coordinate: 14649-14724) of the EBV genome and TaqMan® probe (Applied Biosystems, Foster City, CA, USA) directed within this flanked region (EBV coordinate: 14672-14698) were reported by Lo et al[7], and were custom-made (Applied Biosystems, Foster City, CA, USA).

An aliquot of 5 μL of purified DNA isolated from the plasma was used for amplification in a total reaction volume of 50 μL, which contained the following components: 300 nM of each primer, 25 nM of TaqMan® probe and TaqMan® PCR reagents consisting of 4mM $MgCl_2$; 200 μM each of dATP, dCTP and dGTP; 400 μM of dUTP; 0.5 U AmpErase uracil N-glycosylase (UNG) and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Amplification reaction for each sample and standard was performed in duplicate. The standard curve correlating the viral DNA copy to threshold cycle was constructed by amplifying 5 μL aliquots of serially diluted DNA isolated from Namalwa cells that contained 45, 450, 45,000, 100,000 and 450,000 EBV DNA copies per ml.

To assess the inter-assay reproducibility of our RTQ-PCR assay, the Namalwa DNA standards in five logs of concentration as mentioned were amplified in triplicates on four separate days. Amplification cycling was initiated by incubation at 50ºC for two minutes for UNG activation and initial denaturation at 95ºC for eight minutes, followed by 40 cycles of 95ºC for 30 seconds and 56ºC for one minute. A standard curve was only accepted if the correlation coefficient is 0.996 or higher and its slope ranged between -3.74 and -3.32, which correlated to amplification efficiency of between 85 and 100%, respectively.

The fluorescence detection threshold value was set at 10× the mean standard deviation of fluorescence in all reactions. EBV DNA load, expressed as viral copy number per ml of plasma, was determined using the equation below.

EBV DNA copy/ml = Q (VE/VA) * 1/VP

where Q: DNA copy determined from standard curve; VE: volume of DNA eluent (50 μL); VA:

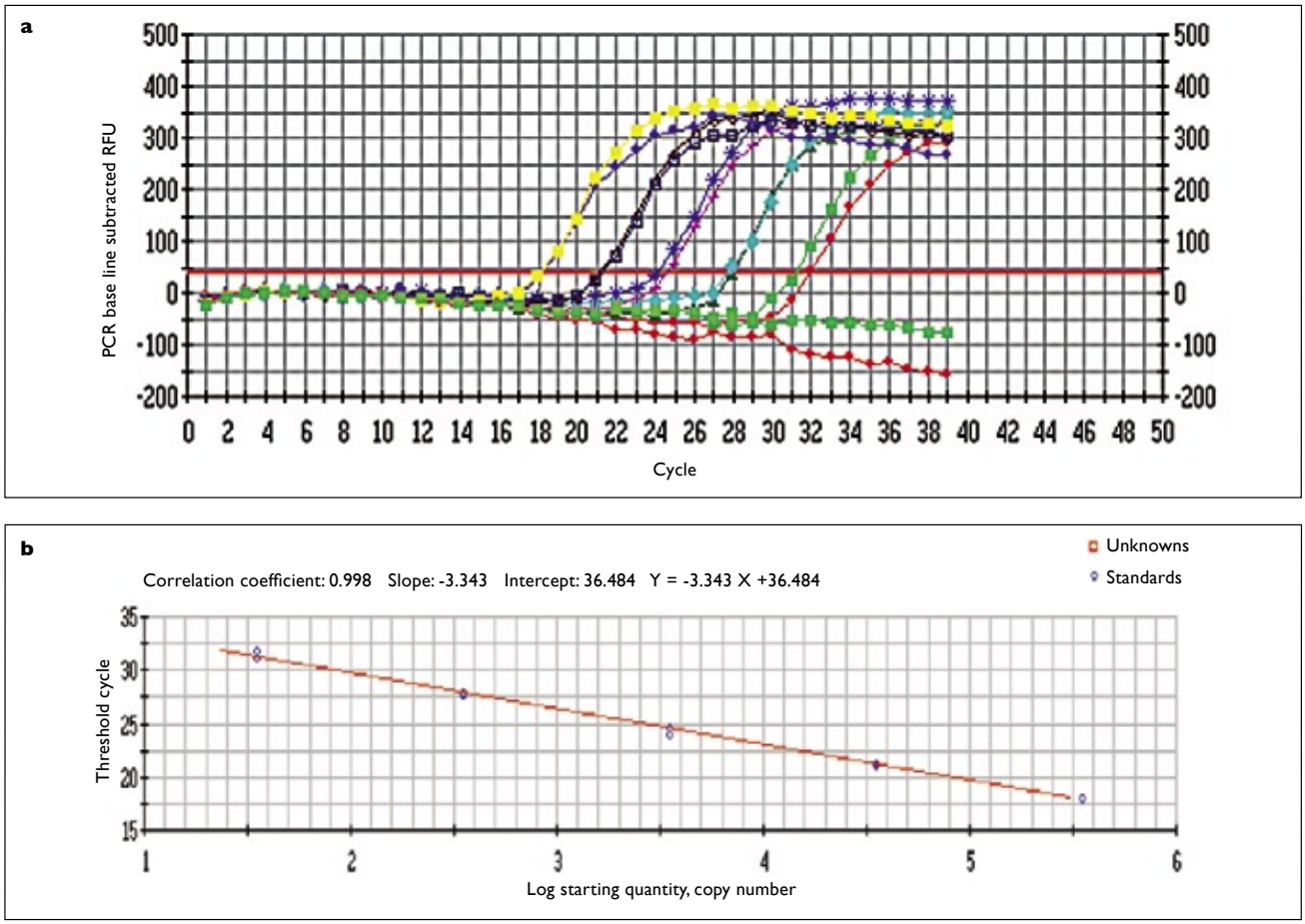

Fig. 1 (a) Representative amplification plots for reactions with starting EBV DNA copy number ranging from 45 to 450,000 copies per ml. Real-time quantitative PCR amplification was performed using Namalwa DNA harbouring two integrated copies of the EBV genome per cell. A duplicate was performed for each copy number. Cycle number is plotted versus change in normalised reported signal (ΔR_n). (b) Standard curve relating the log starting copy number of five orders of magnitude against their respective threshold cycles (TC).

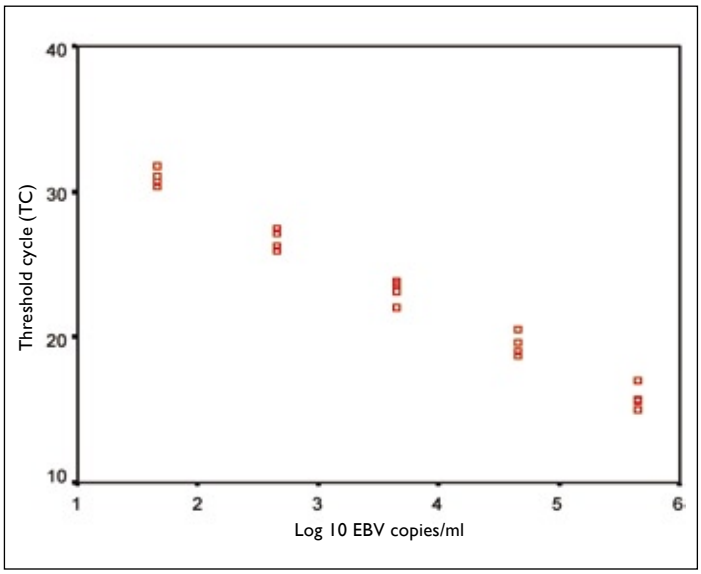

Fig. 2 Scatter plot of inter-assay comparisons between starting EBV quantity and threshold cycles (TC). The TC of each starting EBV copy number ranging from 45 to 450,000 copies per ml in ten-fold increments was determined in a total of four assays performed on four different days. Each assay was carried out in triplicate. The error variances among the TCs at each level of EBV copies were equal (Levene's test) and the differences in mean TCs for each starting EBV quantity were significant at the 0.05 level (one-way ANOVA). The correlation between TC and starting EBV quantity and TC were close to unity [R(14)=0.984, p<0.001; Pearson correlations].

volume of DNA template amplified (5 μL); VP: volume of plasma used for DNA extraction (200 μL).

All statistical analyses were preformed using the Statistical Package for Social Sciences (SPSS) version 10 (Chicago, IL, USA). The means of EBV load in plasma derived from untreated NPC patients, treated NPC patients and healthy controls were analysed using one-way analysis of variance (ANOVA). The ANOVA *F* test was used to evaluate whether the group means on the dependent variable differ significantly from each other. Levene's Test was used to determine the equality of the error variances among the threshold cycles at each concentration of EBV DNA. Pearson correlation was used in determining the correlation between the threshold cycles and starting EBV quantities in the standard curve.

RESULTS

The real-time quantitative PCR method performed on the iCycler Real-time PCR System (Bio-Rad, Hercules, CA, USA) was tested for its reliability

and experimental reproducibility before being implemented for testing unknown plasma samples. Quantitative assays using DNA from Namalwa cells in five logs of concentrations ranging from 45 to 450,000 EBV copies per ml in ten-fold increments were compared within and between assays (Fig. 1a). The standard curve (Fig. 1b) indicated the broad dynamic range of the assay with amplification efficiency close to 100% for each cycle (slope -3.343) and a strong correlation coefficient of 0.998. This method was also shown to be highly reproducible between assays with standard deviations of the mean threshold cycle for each EBV copy number being less than one cycle (Fig. 2).

The EBV copy number was quantified in the plasma from 18 untreated NPC patients, 20 NPC patients who had been treated by radiotherapy and 12 healthy controls. The β-globin gene was amplified and detected in all plasma DNA samples (Fig. 3). This control step was needed to ensure the presence of DNA before the subsequent determination of EBV load. A high viral DNA load was observed in untreated NPC patients with EBV copy number in range of thousands (mean=11,553 copies/ml, median=2,043 copies/ml), and was greatly reduced in patients who had been treated with radiotherapy (mean=2,000 copies/ml, median=0 copy/ml) (Fig. 4). This compared to a mean of 133 copies/ml (median=0 copy/ml) reported in the healthy controls.

The differences in mean EBV DNA load between untreated and treated patients, and between untreated patients and healthy controls, were statistically significant at p=0.05 level. However, the difference in cell-free EBV loads between treated patients and controls was insignificant suggesting a significant reduction in viral DNA load following radiotherapy. A high viral load was detected in the plasma of one treated NPC patient (3.25×10^5 copies/ml) that was significantly higher than the viral load established in the other 19 treated patients. The said patient was called back for follow-up examination at the clinic and tumour recurrence at the primary site with distant metastasis was indicated. This finding underscored the value of plasma EBV load in the prognostication of NPC recurrence and metastasis.

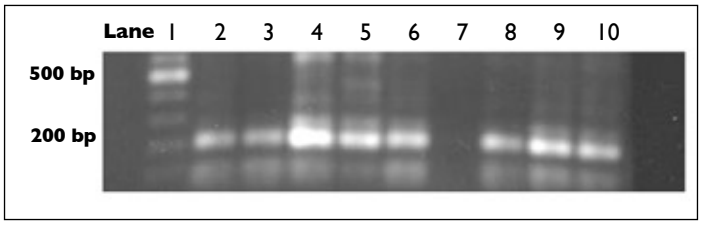

Fig. 3 Amplification of the 200-bp β-globin amplimer from DNA extracted from a selected number of plasma samples from untreated NPC patients (Lanes 2-4), treated patients (Lanes 5-7) and healthy controls (Lanes 8-10). Only samples with detectable β-globin amplimers were used in subsequent EBV real-time quantitative PCR. The sample in Lane 7 had no detectable β-globin amplimer and was not considered for subsequent analysis. Lane 1: 100-bp DNA molecular weight marker.

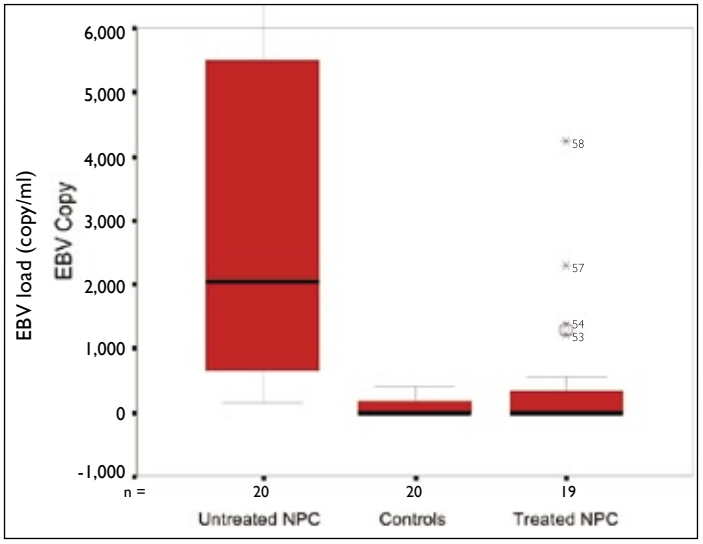

Fig. 4 Box plots represent the median (middle solid intersecting lines), interquartile ranges, maximum and minimum EBV loads (in copy number per ml plasma) for each group. Untreated NPC patients had a median of 2,043 EBV copies/ml (mean = 11,553 copies/ml), and a maximum of 121,500 copies/ml was detected in one patient from this group. Both the control and treated patients had a median of zero copy/ml and mean EBV load of 133 and 2,000 copies/ml, respectively. The mean EBV load between untreated NPC patients with both the controls and treated patients was significant (one-way ANOVA analysis, p=0.05).

DISCUSSION

This study demonstrated the positive correlation between the increase in cell-free EBV DNA and tumour burden. Clonality assay based on the number of terminal repeats in the EBV genome indicated that these cell-free viral DNA were of monoclonal origin, thus suggesting their tumour origin. The real-time quantitative method developed in this study was shown to be robust and highly reproducible in four different assays conducted on four different days, with standard deviations of the mean threshold cycle for each EBV copy number being less than one cycle (Fig. 2). This attests to both the sensitivity and specificity of the EBV-specific TaqMan probe.

Untreated NPC patients had a mean viral DNA load that was significantly higher than the healthy subjects and a decrease in serum viral load was detected in patients after successful treatment by radiotherapy and the primary tumour was under control (Fig. 4). This observation concurred with

recent reports postulating that cell-free EBV DNA load in the serum/plasma was an accurate prognostication for the presence of NPC and of the relapse of the disease[7,8,10]. Interestingly, the plasma of one patient who had been treated with radiotherapy was found to contain an unusually high viral DNA load that was significantly greater than the average detected in the other treated patients. Subsequent clinical examinations revealed the relapse of primary tumour and the presence of distant metastasis. This case represents a prominent demonstration of the disease prognostic value associated with the quantification of plasma EBV DNA load.

It is of clinical importance and interest to evaluate the period between the elevation in cell-free EBV load and the onset or recurrence of primary tumour in future investigations. Most importantly, the data from this study agreed with that reported from Hong Kong, which is an indication that the quantitative relationship between cell-free EBV load and tumour burden is not a geographically isolated phenomenon. This finding is important if the quantitative assay for plasma EBV load is to be developed as an alternative indicator of NPC that is widely applicable in all regions of the world where NPC is prevalent. The levels of plasma EBV DNA load in our untreated NPC patients spanned a wide spectrum but were usually in the order of thousands of copies per ml (Fig. 4).

Because the controls and the treated patients had a median viral load of zero copy/ml, a threshold or cut-off value of 1,000 copies/ml is discriminative between the presence or absence of tumour burden. This threshold value compares favourably with the suggested values reported in other similar studies[7,8,10]. Further investigations should also attempt to investigate the combined prognostic capabilities of serum/plasma EBV DNA load and the EBV-specific IgA-VCA serological test in view of developing an enhanced, minimally invasive and low-cost test that is of significant benefit to patients with NPC.

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