Comparison of Epstein-Barr Virus DNA Level in Plasma, Peripheral Blood Cell and Tumor Tissue in Nasopharyngeal Carcinoma

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Abstract. *Background: The plasma Epstein-Barr virus DNA (EBV-DNA) level has been found to be an indicator for staging and prognosis of nasopharyngeal carcinoma (NPC). Materials and Methods: The EBV-DNA level in plasma, peripheral blood cells (PBC) and neoplastic tissues was quantitatively analyzed and potential associations with clinical parameters of NPC were investigated. Results: The plasma EBV-DNA detecting rate and level in NPC (92%, 82,500 copies/ml) was significantly higher than that in NPC after treatment (19%, 0 copy/ml) and in controls (12%, 0 copy/ml) (p<0.001); while there was no significance of the PBC EBV-DNA detecting rate and EBV-DNA load in NPC before (24%, 0 copy/actin) and after treatment (14%, 0 copy/actin), and in controls (16%, 0 copy/actin). The plasma EBV-DNA level was not correlated to the PBC EBV-DNA load in NPC before (p=0.92) and after treatment (p=0.267), and in controls (p=0.735). The EBV-DNA level in NPC tumor (27.8 copies/actin) was significantly higher than that in nasopharyngitis and was positively correlated to the ratio of EBER1-positive cells on the NPC section (p=0.001). The plasma EBV-DNA level was significantly increased in TNM stages I, II, III and IV NPC, whereas there was no significant difference of PBC EBV-DNA load in different stage NPC. Conclusion: Our results indicate that plasma EBV-DNA is a more sensitive and reliable biomarker than PBC EBV-DNA for diagnosis, staging and therapeutic effect evaluation at a molecular level in NPC clinical practice. Plasma EBV-DNA may derive from the cancer cells and PBC EBV-DNA from circulating mononuclear cells in NPC patients.*

The Epstein-Barr virus (EBV) infects both epithelial cells and B lymphocytes *in vivo* and is associated with malignancies which arise in both cell types, including nasopharyngeal carcinoma (NPC), Burkitt's lymphoma and infectious mononucleosis (1,2). NPC is one of the most common cancers in southern China. EBV infection, genetic and environmental factors have been reported as being important etiological factors in the tumorigenesis of NPC (3-6). Recently, serum/plasma EBV-DNA was detected in high incidence in NPC patients, and it was regarded to be a reliable tumor marker for NPC (7,8). Quantitative analysis revealed that the pretreatment plasma EBV-DNA level was strongly associated with the overall survival of NPC patients and was a powerful prognostic factor of NPC (9,10). The persistence of serum/plasma EBV-DNA after completion of radiotherapy of NPC has been found to be a bad prognostic sign (9,11). PBC EBV-DNA was also reported to be a sensitive marker for prediction of survival and distant metastasis of NPC (12). However, little quantitative information is available regarding the associations of the plasma EBV-DNA level, PBC EBV-DNA load, tumor tissue EBV-DNA level and their correlations with clinical parameters of NPC.

In the present study, by using the RQ-PCR technique, we performed this investigation to compare the variation of the EBV-DNA level in plasma, PBC and tumor tissues in NPC and their correlations to each other before and after treatment. Moreover, we determined whether the plasma EBV-DNA level and PBC EBV-DNA load had any association with clinical parameters of NPC. The data presented here not only demonstrated the sensitivity and

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Table I. *Summary of clinical parameters of the 150 NPC patients.*

	Parameters	Cases.
Total		150
Gender	Male	41
	Female	109
TNM stage*	I	13
	II	50
	III	51
	IV	36

*The China 92 TNM stage system (13)

reliability of plasma EBV-DNA in therapeutic effect evaluation and staging at a molecular level in NPC practice, but also assist in better understanding the biological significance of the serum/plasma EBV-DNA in NPC.

Materials and Methods

Patients and tumor materials. Patients with histologically confirmed NPC, attending the Cancer Center, Sun Yat-Sen University (Guangzhou, China), were recruited for this study. Blood samples included 150 primary NPC and 75 healthy people. The clinical parameters of the NPC patients are summarized in Table I. Disease stage was determined in accordance with the China 92 TNM stage classification (13,14). Biopsy specimens included 49 tumor tissues corresponding to part of the NPC blood samples and 47 nasopharyngitis tissue samples. At the time of the second blood sampling of NPC after completion of treatment, clinical and fiber-optic nasopharyngoscopic examinations and tumor biopsies were carried out.

DNA extraction. Samples of peripheral blood (5ml) were collected in an EDTA tube from all subjects and were centrifuged at 1600g for 15 min for isolation of plasma and PBC. DNA from plasma and PBC were extracted using the QIAamp Blood Kit (Qiagen, Hilden, Germany) and the "blood and body fluid protocol", as recommended by the manufacturer. A total of 500 μl of each plasma sample was used for DNA extraction per column and the final elution volume was 50 μl from the extraction column. The DNA from the NPC tumor tissue section was extracted using the DNeasy Tissue Kit (Qiagen) as in "isolation of DNA from paraffin-embedded tissue" recommended by the manufacturer.

Real-time quantitative PCR. The principles of RQ-PCR were described previously (10,15-17). The RQ-PCR system for EBV-DNA (GenBank Sequence Database, accession number V01555) detection towards the BamHI-W region consisted of the amplification primers W-44F: 5'- AGTCT CTGCC TCCAG GCA - 3' and W-119R: 5' - ACAGA GGGCC TGTCC ACCG - 3', and the dual-labelled fluorescent probe W-67T: 5'- (FAM) CACTG TCTGT AAAGT CCAGC CTCC (TAMRA) - 3'. The RQ-PCR system for the β-actin gene consisted of forward primer F: 5' - ACAGG CACCA GGGCG TGATG G - 3'; reverse primer R: 5' - CTCCA TGTCG TCCCA GTTGG T - 3', and the dual-labelled fluorescent probe: 5' - (FAM) CATCC TCACC CTGAA GTACC CCATC (TAMRA) - 3'.

Duplicate amplification reactions were performed in a 96-well microplate. The real-time PCR protocol and the results analysis were described previously (15). The plasma level of EBV-DNA and the β-actin gene was expressed as copies/ml, calculated using the following equation (10): $C = Q \times V_{DNA}/V_{PCR} \times 1/V_{EXT}$ which C represents the target concentration in plasma (copies/ml), Q represents the target quantity (copies) determined by a sequence detector in a PCR, V_{DNA} represents the total volume of DNA obtained after extraction (typically 50 μl), V_{PCR} represents the volume of DNA solution used for PCR (typically 2 μl) and V_{EXT} represents the volume of plasma extracted (typically 0.5 ml).

The EBV-DNA in PBC and NPC tumor tissue was expressed as copy/actin by calculating the ratio of EBV-DNA copies to β-actin gene copies in the same tested samples, thus precisely indicating the relative EBV-DNA level in the tested samples.

Nested PCR amplification. The nested PCR to amplify the EBV-DNA W fragment was designed with two pairs of primers. The first round PCR primers were primer F: 5'- GCCAG AGGTA AGTGG ACTTT - 3' and primer R: 5'- TGGAG AGGTC AGGTT ACTTA - 3' from which a 240bp PCR product was generated. The second round PCR primers were primer F: 5'- TTCTG CTAAG CCCAA CACTC - 3' and primer R: 5' - TGAAG GTGAA CCGCT TA - 3', which generated a 192bp PCR product. Both rounds PCR reactions were set up in a volume of 25 μl, which contained 10 x buffer 2.5 μl, 10 mM dNTPs 0.5 μl, 1 mM MgCl$_2$, 1.5 U of Ampli Taq Gold, 10pmol or 50pmol (the second round) of each primer and 2 μl of DNA template. PCR reactions were carried out in a 9600 amplifier (Applied Biosystems Inc., USA). Thermal cycling was initiated with 10 min at 95˚C followed by 10 cycles at 95˚C for 45 sec, 55˚C for 1 min, then 30 cycles at 95˚C for 30 sec and 55˚C for 45 sec. Multiple blanks were set up as negative control. The nested PCR products of PBC are presented in Figure 1.

EBER1 in situ hybridization. EBER1 ISH was performed to calculate the ratio of tumor cells on the tissue section of NPC. A 30-base fluorescein-conjugated oligonucleotide probe, corresponding to the EBER1 gene, was used (EBV Probe ISH Kit; Novocastra Laboratories Ltd., Newcastle, UK). EBV-infected tonsil sections supplied with the kit were used as positive controls. The fluorescein-labelled random oligonucleotide cocktail probe supplied with the kit was used as a negative control probe. Briefly, paraffin sections were dewaxed, dehydrated, air-dried, predigested with proteinase K, hybridized overnight with FITC-labelled antisense probe, which was detected by an alkaline phosphatase-conjugate anti-FITC antibody, and visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase enzyme substrate (18). A strong nuclear positive signal was observed for EBER1-positive cells (Figure 2).

Statistical analysis. The plasma EBV-DNA level, PBC EBV-DNA load and NPC neoplastic tissue EBV-DNA level were compared using the Mann-Whitney rank-sum test. The plasma EBV-DNA level and PBC EBV-DNA load in NPC patients before and post treatment were analyzed using the Wilcoxon Signed Ranks Test. The Chi-square and Fisher's exact test were used to analyze the difference of proportions. The Pearson correlation coefficient was used to analyze the correlations between the plasma EBV-DNA level and PBC EBV-DNA load, as well as between the EBV-DNA level and EBER1-positive cell ratio in the neoplastic NPC tissue. A value that was equal to or less than 0.05 was considered statistically significant.
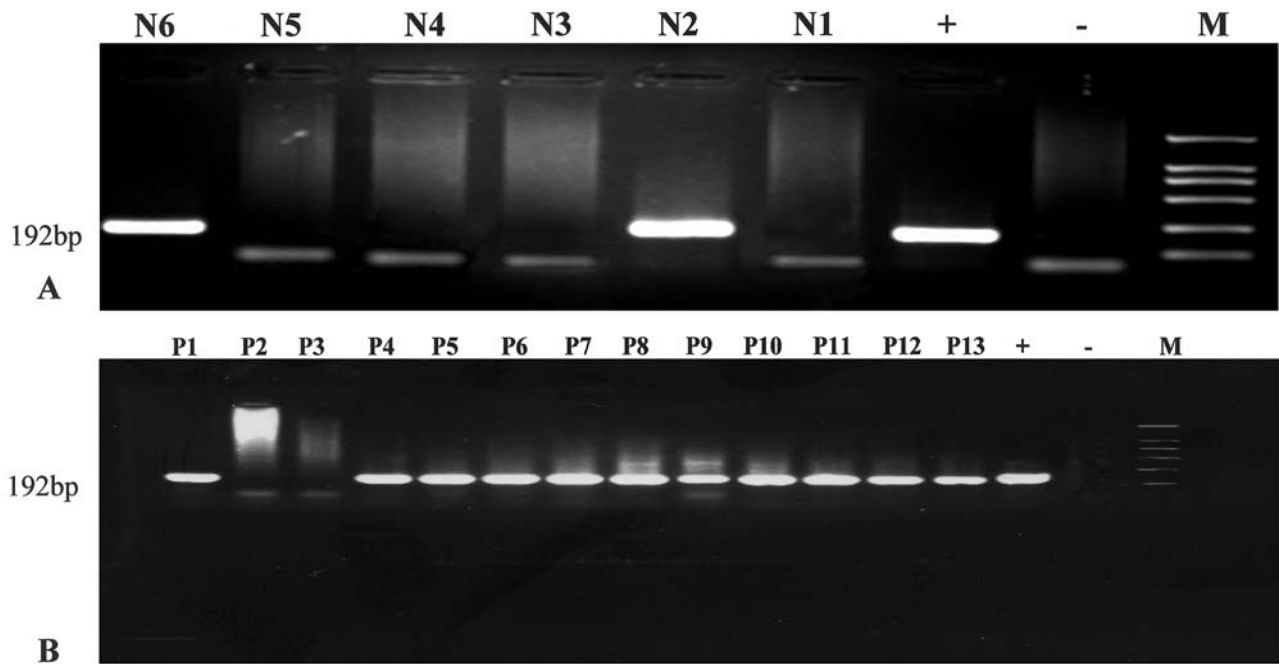
Figure 1. *Nested PCR amplification of PBC EBV-DNA from NPC patients. A: RQ-PCR- negative samples were positive with nested PCR detection (lanes N2 and N6). B: RQ-PCR-positive samples were positive with nested PCR detection (Lanes P1, P4 – P13). Marker (M) sizes were 100bp, 250bp, 500bp, 750bp, 1000bp and 2000bp DNA fragment. "+" denotes positive control and "–" denotes negative control.*

Results

Detection of plasma EBV-DNA level and PBC EBV-DNA load in NPC. By using RQ-PCR, plasma EBV-DNA was detected in 92% (138/150), 19% (26/138) and 12% (9/75) of primary NPC before treatment, in NPC after completion of treatment and in control subjects, respectively. The detection rate of plasma EBV-DNA in NPC prior to treatment was significantly higher than that in NPC after completion of treatment and in controls (Chi-square test, $p < 0.001$) (Figure 3A). The median plasma EBV-DNA level was 82,500 copies/ml (quartiles range 10,727-387,500 copies/ml) in NPC, which was significantly higher than that in controls (median, 0 copy/ml, quartiles range 0-0 copy/ml) (Mann-Whitney rank-sum test, $p = 0.0000$) and in NPC after completion of treatment (median, 0 copy/ml, quartiles range 0-0 copy/ml) (Wilcoxon test, $p = 0.0001$) (Figure 3B).

By using RQ-PCR, PBC EBV-DNA was detected in 24% (34/143), 14% (19/132) and 16% (12/75) in NPC before treatment, after completion of treatment and in controls, respectively. There was no significant difference in the detection rate in different subjects (Figure 3A). The median PBC EBV-DNA load was 0 copy/actin (quartiles range, 0-0 copy/actin) in NPC before and after treatment, as well as in controls, and there was no statistical significance between each subject (Figure 3B).

The plasma EBV-DNA level was not correlated to the corresponding PBC EBV-DNA load in NPC before (Spearman test, $p = 0.92$) and after treatment (Spearman test, $p = 0.267$), as well as in controls (Spearman test, $p = 0.735$).

For comparison of the sensitivity of the RQ-PCR and nested PCR technique, a nested PCR protocol was used to detect PBC EBV-DNA in a randomly selected 36 RQ-PCR-positive and 36 negative NPC. In total, PBC EBV-DNA was detected in 75% (64/72) of NPC. In 36 patients who were positive with RQ-PCR detection, 34 (94%) were positive with nested PCR; in 36 patients who were negative with RQ-PCR detection, 20 (55%) were positive with nested PCR.

Detection of EBV-DNA and EBER1 in neoplastic tissue of NPC. By using RQ-PCR, EBV-DNA was detected in 100% (49/49) of NPC tissues and 40% (19/47) of nasopharyngitis tissues. The detection rate was statistically significant ($p < 0.01$, Chi-square test). The EBV-DNA level in NPC tissues was 27.8 copies/actin (median, quartiles range 6.35-279.5 copies/actin), which was significantly higher than that in nasopharyngitis tissues (median, 0 copies/actin; quartiles range 0-0.000923 copies/actin). No correlation of EBV-DNA level between NPC tumor tissue and the corresponding plasma (Spearman test, $p = 0.247$) and PBC (Spearman test, $p = 0.673$) was observed.
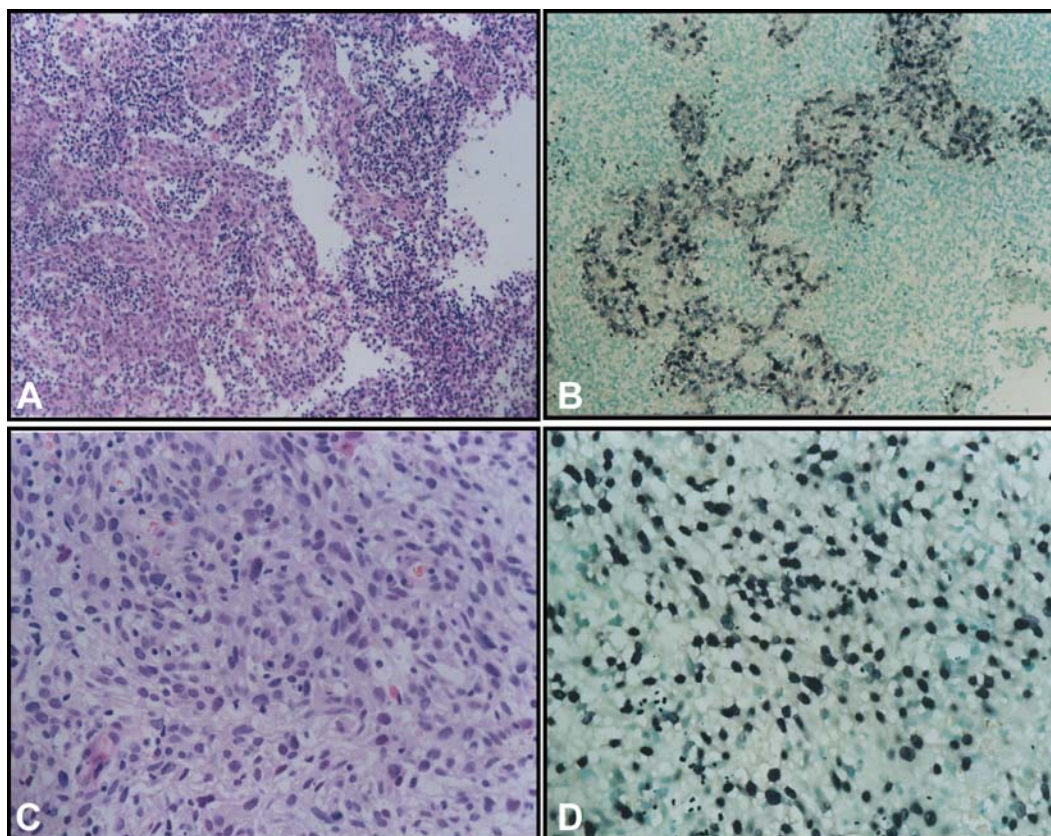
Figure 2. *EBER1 ISH results of different histological types of NPC. A: NPC with lymphocyte infiltration B: This case showed only parts of the tissue cells were NPC neoplastic cell and were EBER1-positive. C: Undifferentiated NPC showed no lymphocytes infiltration D: This case presented that most of the tissue cells were NPC neoplastic cell and were positive for EBER1.*

By ISH, EBER1 was detected in 100% (49/49) of NPC tumor, but was undetectable in all nasopharyngitis. By using the Computer Image Analysis technique, the median ratio of EBER1-positive cells to total cells on the NPC tumor section was 0.35 (quartiles range, 0.22-0.50), which was positively correlated with the EBV-DNA level in the corresponding NPC tissue (Spearman test, $p=0.001$) (Figure 4).

Correlation of plasma EBV-DNA level, PBC EBV-DNA load to TNM stage of NPC. By using RQ-PCR, plasma EBV-DNA was detected in 50% (2/4), 94%(32/34), 91%(59/65) and 94%(44/47) in TNM stages I, II, III and IV NPC before treatment, respectively. There was no significant difference of the detection rate in different stage NPC (Chi-square test, $p>0.05$) (Figure 5A). The median plasma EBV-DNA levels were 2,500 (quartiles range, 0-11,885), 32,590 (quartiles range, 8,875-122,900), 86,000 (quartiles range, 11,000-433,000) and 166,200 (quartiles range, 15,000-1,118,000) copies/ml in TNM stages I, II, III and IV NPC, respectively. The plasma EBV-DNA level was increased significantly from

stage I to stage IV, with statistical differences (Kruskal-Wallis test, $p=0.004$) (Figure 5B).

By RQ-PCR technique, PBC EBV-DNA was detected in 25% (1/4), 10% (3/30), 26.6% (17/64) and 28.9% (13/45) in TNM stages I, II, III and IV NPC, respectively. There was no significance of the detection rate in different TNM stage NPC (Chi-square test, $p=0.06$) (Figure 5A). The median PBC EBV-DNA load was 0 copies/actin (quartiles range, 0-0 copies/actin) in all different TNM stage NPC, and no significant difference was observed (Kruskal-Wallis test, $p=0.299$) (Figure 5B).

By using nested PCR, PBC EBV-DNA was detected in 84.3% (43/51) of late stage (stage III + IV) NPC, which was higher than that in stage I+II NPC (50%, 7/14); with a significant difference (Chi-square test, $p<0.05$). The median level of EBV-DNA in NPC tissues were 40 (2-78), 3.17 (3-35), 24.65 (11-352) and 93.9 (18-1126) copies/actin in TNM stages I, II, III and IV of NPC, respectively. There was no significant difference of the EBV-DNA level in different stage NPC.
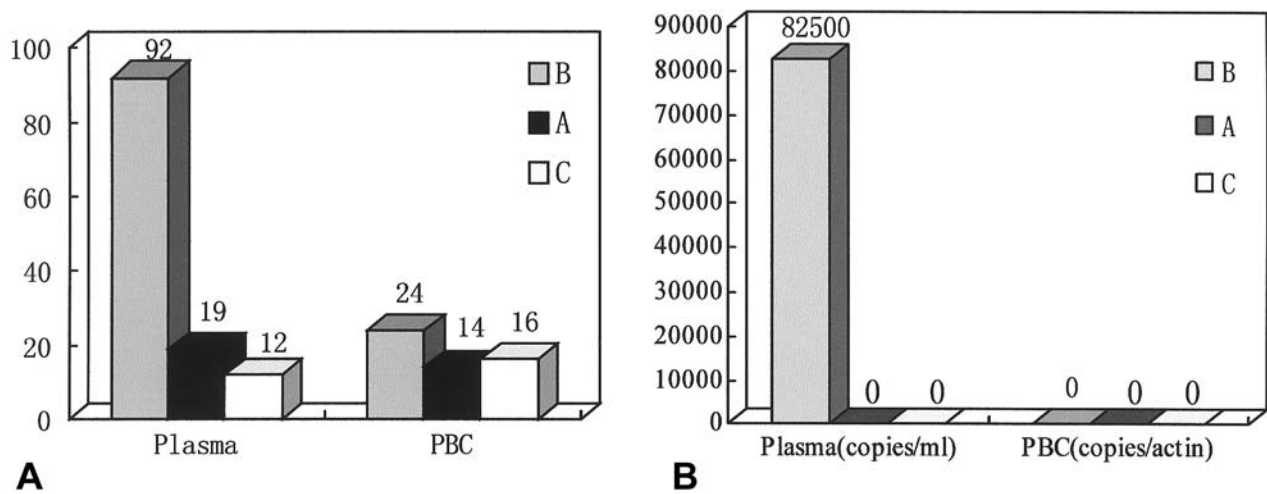
Figure 3. *Correlations of plasma EBV-DNA and PBC EBV-DNA with NPC. A: Primary NPC showed significantly higher detection rate than controls and NPC after treatment, while the PBC EBV-DNA detection rate was not significant in these different subjects. B: Primary NPC presented significantly higher plasma EBV-DNA level than controls and NPC after treatment, whereas the PBC EBV-DNA load was not significant in these subjects. Series B denotes NPC before treatment, Series A denotes NPC after completion of treatment, Series C denotes control subject.*

In NPC patients with N0M0 stage, the plasma EBV-DNA level was positively correlated with T stage. The plasma EBV-DNA level was 4,050 copies/ml (median, quartiles range 331-14,052 copies/ml) in early T stage (T1 + T2) NPC, which was significantly lower than that in late T stage (T3 + T4) NPC (median, 17,165 copies/ml, quartiles rang, 1,930-191,150 copies/ml) (Mann-Whitney rank-sum test, $p=0.03$) (Figure 5C). In NPC patients with N*M* stage, there was no significance of plasma EBV-DNA level between late T stage (T3+T4) NPC (median, 100,000 copies/ml, quartiles range, 10,660-218,000 copies/ml) and early stage (T1+T2) NPC (median, 420,000 copies/ml, quartiles ranges, 11,380-4,760,000 copies/ml) (Mann-Whitney rank-sum test, $p=0.09$) (Figure 5D).

Discussion

The demonstration of tumor-derived genetic alterations in the plasma and serum of cancer patients supported that part of the circulating DNA may be released by tumor cells (19-21). NPC has a very strong association with EBV infection, thus targeting EBV-DNA in the plasma and serum of NPC patients would be reasonable in NPC clinical practice. Previous studies have detected a high incidence of plasma/serum EBV-DNA in NPC patients, and have revealed that the plasma EBV-DNA level was a tumor marker that could be used in staging and prognosis of NPC (7-11,15,22,23). Besides plasma/serum EBV-DNA, PBC EBV-DNA (EBNA-1 fragment) was also detected with high
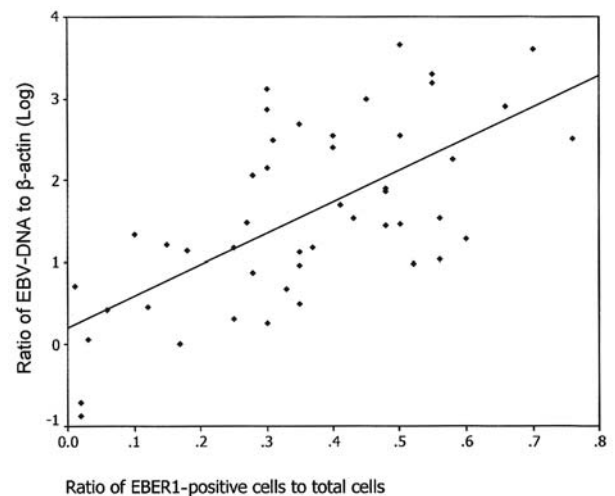

Figure 4. *Correlation of the EBV-DNA level with EBER1-positive cell ratio in NPC tumor tissue. The result showed that the EBV-DNA level was positively correlated to the EBER1 -positive cell ratio in NPC tumor tissue.*

incidence (71%) in NPC patients, which was regarded as being a risk factor for predicting distant metastasis and lower survival rate(12). In this study, we first compared the EBV-DNA status in plasma and PBC simultaneously in large samples of NPC by using the RQ-PCR technique. Plasma EBV-DNA was detected in 92% (138/150) of NPC, the detection rate being in accordance with previous studies (15,22) and significantly higher than that in controls (12%)
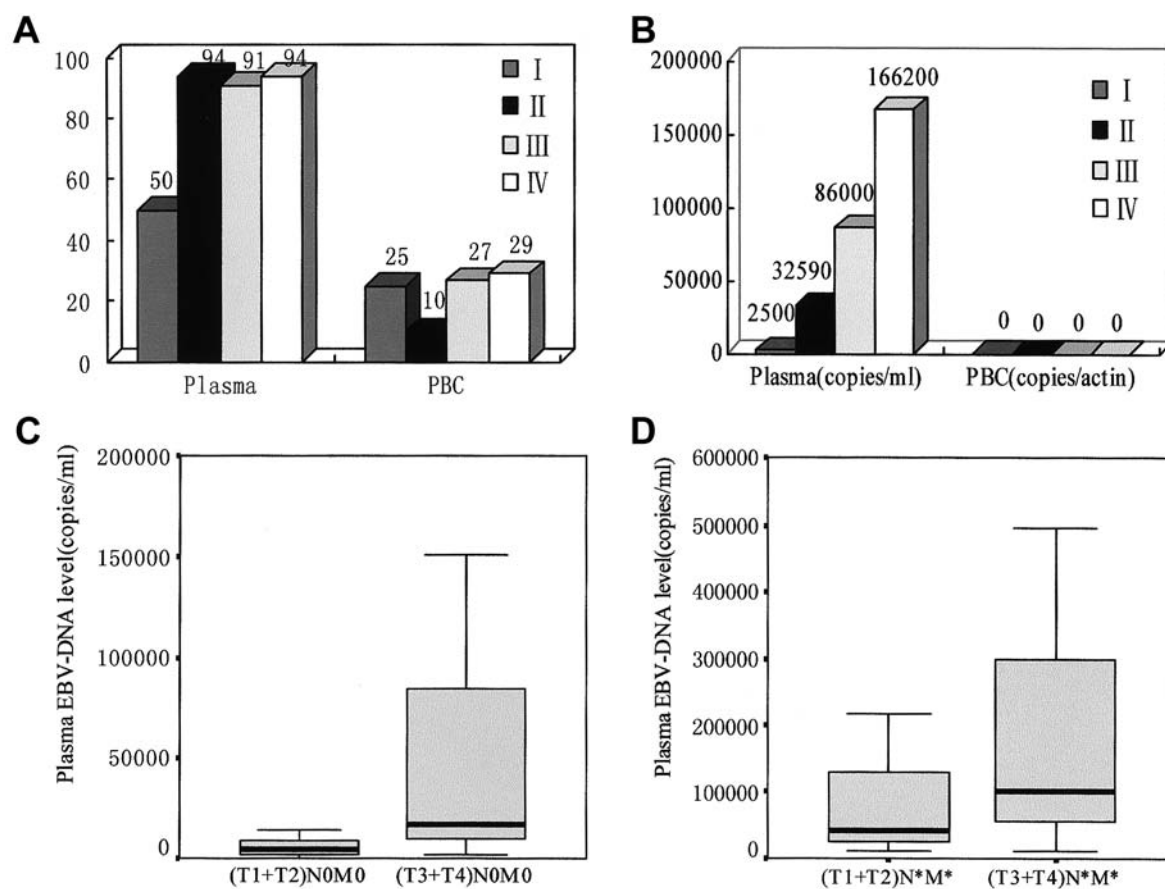
Figure 5. *Correlations of plasma EBV-DNA and PBC EBV-DNA with TNM stage of NPC. A: EBV-DNA detection rate in plasma was significantly higher than that in PBC in different stage NPC, but no significance was observed. B: Plasma EBV-DNA was closely correlated to TNM stage progression of NPC, whereas PBC EBV-DNA presented no significance in different stage NPC. C: Significance of plasma EBV-DNA level was observed between early T stage (T1+T2) and late T stage (T3+T4) NPC when the patients had no lymph node and/or organ metastasis (N0M0).D: No significance of plasma EBV-DNA was observed between early T stage (T1+T2) and late T stage (T3+T4) NPC when the patients had lymph node and organ metastasis (N*M*).*

and NPC after completion of treatment (19%). By the RQ-PCR technique, PBC EBV-DNA was detected in 24% of NPC, the detection rate being lower than that (71%) by using nested PCR, reported by Lin *et al.* (12), and significantly lower than that of plasma EBV-DNA in NPC. No significance of the PBC EBV-DNA detection rate was observed between NPC prior to and post treatment, as well as the control subjects. These results strongly suggest that plasma EBV-DNA is a sensitive biomarker for NPC clinical practice.

In this investigation, we first compared the variation of plasma EBV-DNA level and paired PBC EBV-DNA load simultaneously in a group of NPC patients, before and after completion of treatment. We found that the plasma EBV-DNA level decreased sharply from 42,000 copies/ml (median, before treatment) to 0 copy/ml (median), while the PBC EBV-DNA load presented no change in NPC patients after completion of treatment. In addition, there was no

correlation of plasma EBV-DNA level to corresponding PBC EBV-DNA load in NPC patients before (p=0.92) and after treatment (p=0.555), as well as in control subjects (p=0.735). These results proved that, unlike the plasma EBV-DNA level, the PBC EBV-DNA load remained stable in NPC patients before and after treatment, Plasma EBV-DNA may be released by the tumor cells, but not PBC in NPC patients, which is a more reliable marker for evaluating the therapeutic effect of NPC treatment.

A recent study has demonstrated that 85% of apparently healthy adults have serological evidence of prior EBV infection and carry about 1 viral genome/10^5 B cells (17). Theoretically, RQ-PCR can detect EBV-DNA at a sensitivity of 100 to 1000 copies per ml of plasma, whereas the nested PCR has a sensitivity of 1 copy viral genome /10^7 B cells, which is 10 to 100 times higher than the sensitivity of RQ-PCR (24,25). In the present study, PBC EBV-DNA could be

detected in 75% of NPC (in 94% of RQ-PCR-positive and in 55% of RQ-PCR-negative NPC) patients by nested PCR. The detection rates of PBC EBV-DNA by nested PCR were in accord with those previously reported in NPC patients (71%) and in healthy subjects (52%) (11,22). These results verified that the nested PCR was more sensitive than RQ-PCR for the detection of EBV-DNA in NPC patients. Since there was no difference in PBC EBV-DNA load in NPC patients before and after completion of treatment, as well as in controls, we suggest that PBC EBV-DNA in NPC patients originated from circulating mononuclear cells, rather than disseminated cancer cells.

Previous studies have supposed that the liberation of EBV-DNA into the blood may be related to the tumor load. We found evidence that the plasma EBV-DNA level is correlated with primary tumor invasiveness of NPC. In NPC patients with N0M0 stage (to eliminate metastatic tumor factors), the advanced T stage (T3 + T4) presented a higher plasma EBV-DNA level than early T stage (T1+T2) NPC patients. Whereas when the NPC cases included lymph node and organ metastasis (N*M* stage), there was no significance of plasma EBV-DNA level between advanced T stage (T3+T4) and early stage (T1+T2) NPC. Moreover, the difference of plasma EBV-DNA level among these four stages was statistically significant. There was no correlation between PBC EBV-DNA load and detection rate and disease stage of NPC. However, the plasma EBV-DNA level was strongly correlated with tumor stage of NPC, suggesting that the plasma EBV-DNA is more useful than the PBC EBV-DNA load for NPC staging. The circulating EBV-DNA level may be a reflection of tumor burden, which will be helpful in improving the traditional TNM staging system at the molecular level.

The RQ-PCR assay enables us to quantify the amplified products in the log-phase of the reaction. Moreover, this assay does not require post-sample handling and thus can avoid contamination because the technique is performed in completely sealed wells. This is a great improvement over the nested PCR assays, which have considerable risks of carryover contamination. With its rapidity, accuracy and ability to handle many samples, considering the readily obtainable serum and plasma as well as easy handling of DNA from them, we suggest that RQ-PCR is more applicable and useful than nested PCR for cell-free EBV-DNA detection in NPC.

The expression of the virus-encoded EBER1 has been accepted as a marker of EBV latency and was regarded as a valuable diagnostic criterion. EBER1 ISH in the tumor tissue section has been widely used diagnostically as evidence of EBV-positivity in tumor samples (18,26). In the present study, EBER1 and EBV-DNA (BamHI-W fragment) were detected in NPC tumor tissues by EBER1 ISH and RQ-PCR protocols. Our results are consistent with previous reports and support the previous view that all

histological types of NPC are, in reality, variants of EBV-infected neoplasia (27). Particularly, an expected positive correlation between these two markers has demonstrated that the higher the proportion of EBER1 ISH-positive cells in the tissue section, the higher the ratio of EBV-DNA/β-actin presented in the tumor tissue sample. Both the EBER1 ISH-positive cell proportion and EBV-DNA/β-actin ratio (RQ-PCR) in the tumor tissue showed no associations with the plasma EBV-DNA level, PBC EBV-DNA load and TNM stage of NPC patients. This result may due to the small size of the biopsy specimen obtained, which could not reflect the total tumor volumes.

In summary, our results demonstrated that plasma EBV-DNA is a more sensitive and reliable biomarker than PBC EBV-DNA for therapeutic effect evaluation, improving the TNM staging system at a molecular level in NPC clinical practice. Plasma EBV-DNA may be released by NPC cancer cells, being a reflection of the primary tumor burden. PBC EBV-DNA in NPC patients may originate from circulating mononuclear cells.

Acknowledgements

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