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ORIGINAL ARTICLE

Association of Plasma Epstein-Barr Virus LMP1 and EBER1 with Circulating Tumor Cells and the Metastasis of Nasopharyngeal Carcinoma

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Abstract

Epstein-Barr virus (EBV) has been widely recognized to contribute to the development of nasopharyngeal carcinoma (NPC). The present study was to explore the association of plasma Epstein-Barr Virus LMP1 and EBER1 with circulating tumor cells (CTCs) and the metastasis of nasopharyngeal carcinoma. In the present study, we quantified the plasma levels of EBV DNA/RNAs, such as LMP1, LMP2, BART and EBER1 with real-time quantitative PCR, and CTCs with a CellSpotter Analyzer in NPC patients, with or without metastasis. Then the correlation of each biomarker with other biomarkers and tumor metastasis was analyzed. Our data indicated that the plasma levels of EBV LMP1, BART, EBER1, along with CTCs were significantly higher in metastatic NPC patients than in non-metastatic patients. Plasma LMP1 DNA and EBER1 discriminate metastatic NPC patients from non-metastatic patients, correlate with tumor stage and node stage for metastatic NPC patients. In summary, there were significantly higher plasma levels of Epstein-Barr Virus DNAs / RNAs in nasopharyngeal carcinoma patients. LMP1 DNA and EBER1 RNA correlated with the metastasis of nasopharyngeal carcinoma.

Keywords Epstein-Barr virus · LMP1 · BART · EBER1 · Metastasis · Nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) is an endemic and most aggressive head and neck squamous cell carcinomas (HNSCC). It is mainly prevalent in southern China, Southeast Asia, North Africa, Middle East and Alaska [1, 2], exactly where Epstein-Barr virus (EBV) is mostly prevalent [3]. Thus, NPC is believed to be closely associated with the latent EBV infection [4–6]. NPC was classified as group I carcinogen by the International Agency for Research on Cancer(IARC), particularly, the outcomes for NPC patients with distant metastases at diagnosis were poor [7], and almost all poorly-differentiated NPC cases were EBV positive [5, 6]. Post the first contact in childhood, EBV infection lasts for life as a way of latent infection [8], during which, the viral genome is maintained in host chromatin, and the viral expression is limited to few genes [9]. The EBV genomic deoxyribonucleic acid (DNA) is detectable in the plasma of NPC patients [10], and is positively correlating with the NPC tumor burden [10]. Therefore, the plasma EBV DNA is a reliable biomarker in screening, differential diagnosis, prognosis predicting and follow-up in NPC [11–13].

EBV is maintained in such epithelial cells as nasopharyngeal epithelial cells in the latent infection [14, 15]. Only occasionally producing virus progeny [16], due to the heavy methylation of viral episome [17, 18]. However, NPC tumor cells express abundantly the mRNAs of Epstein-Barr nuclear antigen 1(EBNA1), latent membrane protein 1 (LMP1), LMP2A, LMP2B and the non-coding small RNAs of EBV-encoded small RNAs (EBERs) and EBER2, and BART microRNAs [19, 20]. Recently, plasma EBV DNA levels has been used as a circulating biomarker for the diagnosis, risk stratification, monitoring, and predicting NPC prognosis [21, 22]. Oncogenic virus factors in NPC such as LMP1 [23, 24] and EBNA1, EBERs and BART [25]. Chromosomal integration of EBV genomes has been sporadically observed in NPC cells [26, 27]. However, little is known about the association of the

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EBV-encoding DNA / RNA with the distant metastasis of NPC. In addition, the importance of circulating tumor cells (CTCs) as a promising biomarker for tumors, including NPC has recently been emphasized [28, 29].

In the present study, we quantified the plasma levels of EBV DNA/RNAs, such as LMP1, LMP2, BART and EBER1 with real-time quantitative PCR, and CTCs with a CellSpotter Analyzer in NPC patients, with or without metastasis. Then the correlation of each biomarker with other biomarkers and tumor metastasis was analyzed. Our data indicate a significant correlation of plasma EBV LMP1, BART and EBER1 with CTCs and tumor metastasis in NPC patients.

Materials and Methods

Ethics Statement

This study was approved by the institutional ethics committees of the Department of Otolaryngology, Head & Neck Surgery, First Hospital of Jilin University (Changchun, PR China). Written informed consent was obtained from each participant.

NPC Patients and Sample Collection

Between Feb 2016 and Dec 2018, 136 metastatic NPC patients and 114 non-metastatic NPC patients were enrolled for the present study. The clinical stages of these patients were classified according to the 8th edition of Union for International Cancer Control stage classification. Diagnosis was performed by chest radiograph, abdominal sonography, nasopharyngeal and neck magnetic resonance imaging, fiberoptic nasopharyngoscopy, bone scan, and EBV serology.

Fig. 1 Difference between metastatic and non-metastatic nasopharyngeal Carcinoma (NPC) patients in the circulating tumor cell (CTC) and plasma levels of EBV DNA/RNA. Circulating tumor cells (**a**), plasma anti-LMP1 IgG (**b**), relative levels of Epstein–Barr virus (EBV) infection-associated latent membrane protein 1 (LMP1) DNA (**c**), LMP2 DNA (**d**), EBV-encoded small RNAs (EBERs) (**e**), EBV-encoded microRNA (BART) (**f**) were examine in 136 cases of metastatic and 114 cases of non-metastatic NPC patients. Circulating tumor cells were counted from 7.5 ml whole blood; relative levels of plasma EBV DNA/RNA was quantified with real-time quantitative PCR. The maximum value, 75%-, 50%- and 25%- quantile values and the minimum value were indicated as the top whisker, the top boarder, the middle line, the bottom boarder and the bottom whisker respectively; outliers were indicated as diamonds. Statistical significance was considered when a p value less than 0.05

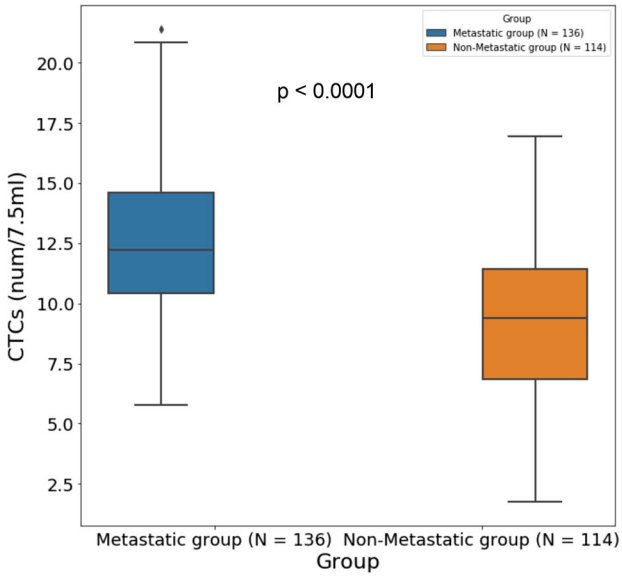
Peripheral venous blood (5 mL) was obtained before any treatment and was centrifuged at 1600 g for 15 min, and then the plasma sample was collected and was stored at −80 °C before use. Peripheral blood (7.5 ml) for CTC enumeration was obtained from each patient and placed in10 ml EDTA Vacutainer tubes (Becton Dickinson) to which a cell preservative was added. Samples were maintained at room temperature and processed within 72 h after collection.

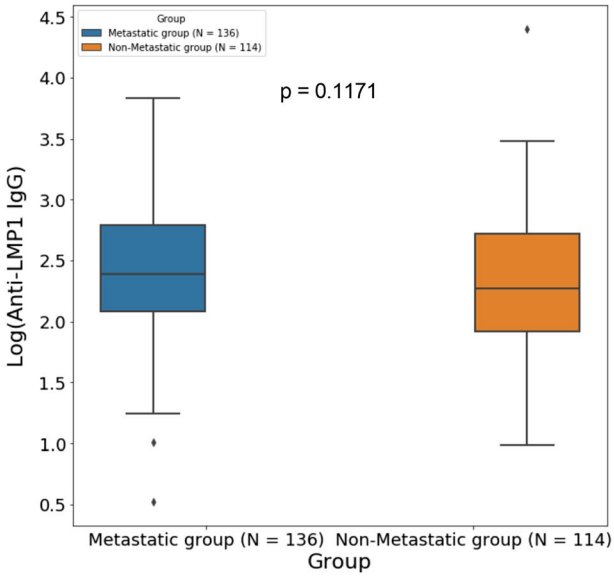
Extraction of EBV DNA/RNA and Real-Time Quantitative PCR (RT-qPCR)

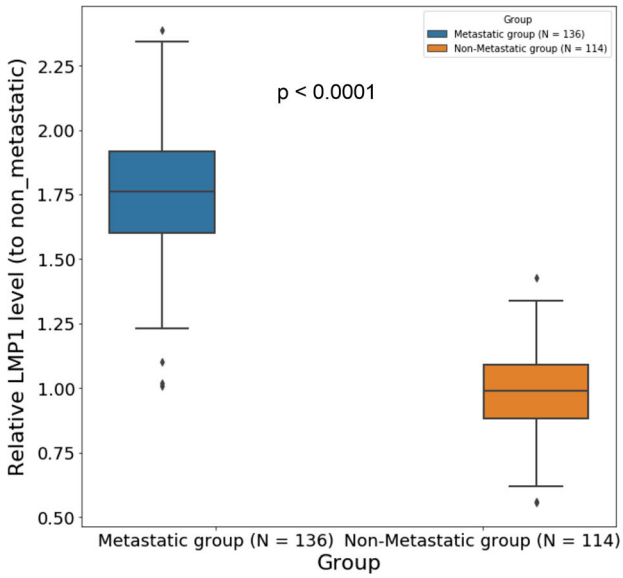
EBV DNA and RNA from NPC plasma were extracted simultaneously using silica-based extraction procedure exactly as described before [30], with the basic kit ingredients from BioMérieux (Boxtel, USA). Finally, DNA/RNA was eluted in 100 μl sterile ultra-pure water. DNA/RNA samples were stored at −80 °C before use. The relative EBV DNA of LMP1 and LMP2 (LMP2A) was determined on LightCycler 2.0 (LC, Roche) with RT-qPCR method targeting conserved region of

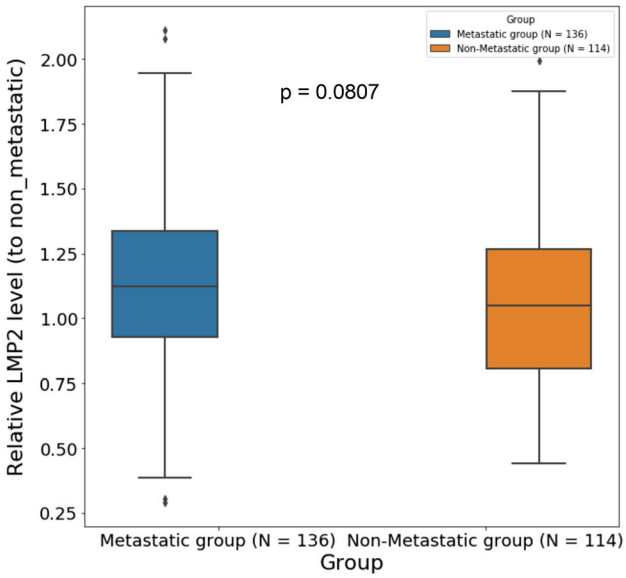
Table 1 Clinical characteristics of NPC patients

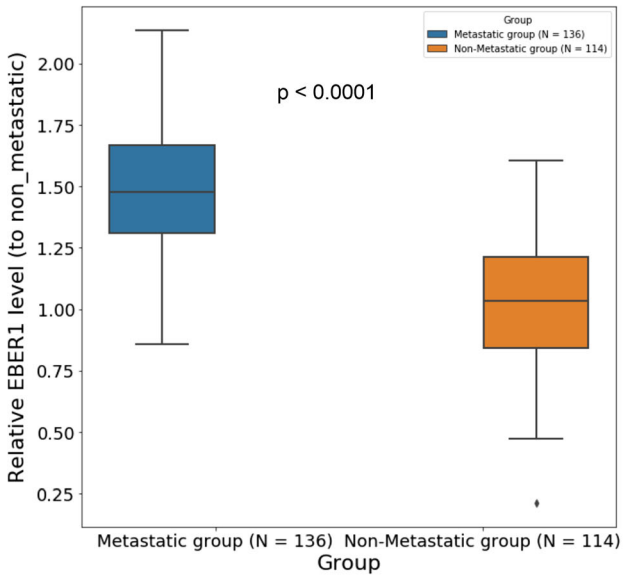
Items	Metastatic NPC ($N = 136$)	Non_metastatic NPC ($N = 114$)	p value
Age (years)	45.54 ± 4.52	44.63 ± 4.86	>0.05
Gender (Male, number (%))	106 (77.94)	89 (78.07)	>0.05
Smoking (number (%))	61 (44.85)	49 (42.98)	>0.05
Tumor stage (T1–4)			\
T1 (number (%))	9(6.41)		
T2 (number (%))	8(5.72)		
T3 (number (%))	60(44.37)		
T4 (number (%))	59(43.5)		
Node stage (N0–3)			\
N0 (number (%))	8(5.87)		
N1 (number (%))	44(32.32)		
N2 (number (%))	46(33.69)		
N3 (number (%))	38(28.12)		
Distant metastasis (number (%))	87(64.3)		\

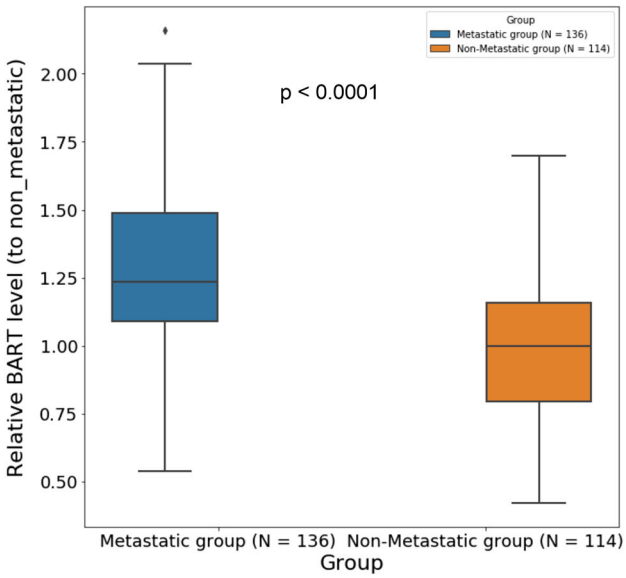

Group
Metastatic group (N = 136)
Non-Metastatic group (N = 114)
p < 0.0001
CTCs (num/7.5ml)
Metastatic group (N = 136) Non-Metastatic group (N = 114)
Group


Group
Metastatic group (N = 136)
Non-Metastatic group (N = 114)
p = 0.1171
Log(Anti-LMP1 IgG)
Metastatic group (N = 136) Non-Metastatic group (N = 114)
Group


Group
Metastatic group (N = 136)
Non-Metastatic group (N = 114)
p < 0.0001
Relative LMP1 level (to non_metastatic)
Metastatic group (N = 136) Non-Metastatic group (N = 114)
Group


Group
Metastatic group (N = 136)
Non-Metastatic group (N = 114)
p = 0.0807
Relative LMP2 level (to non_metastatic)
Metastatic group (N = 136) Non-Metastatic group (N = 114)
Group


Group
Metastatic group (N = 136)
Non-Metastatic group (N = 114)
p < 0.0001
Relative EBER1 level (to non_metastatic)
Metastatic group (N = 136) Non-Metastatic group (N = 114)
Group


Group
Metastatic group (N = 136)
Non-Metastatic group (N = 114)
p < 0.0001
Relative BART level (to non_metastatic)
Metastatic group (N = 136) Non-Metastatic group (N = 114)
Group

EBV LMP1 or LMP2A. The sequences of primers and probes used here were available upon a request. The relative level of each DNA was presented as a relative value to the value in non-metastatic group. EBV LMP1 and LMP2 (LMP2A) from EBV-positive cell line, C666.1 were utilized as internal control. The quantification of EBER1 and BART was performed with Real time LC-PCR reagents (Roche Diagnostics, Almere, USA). The level of U6 and beta-actin were taken as internal control for BART and EBER1 respectively.

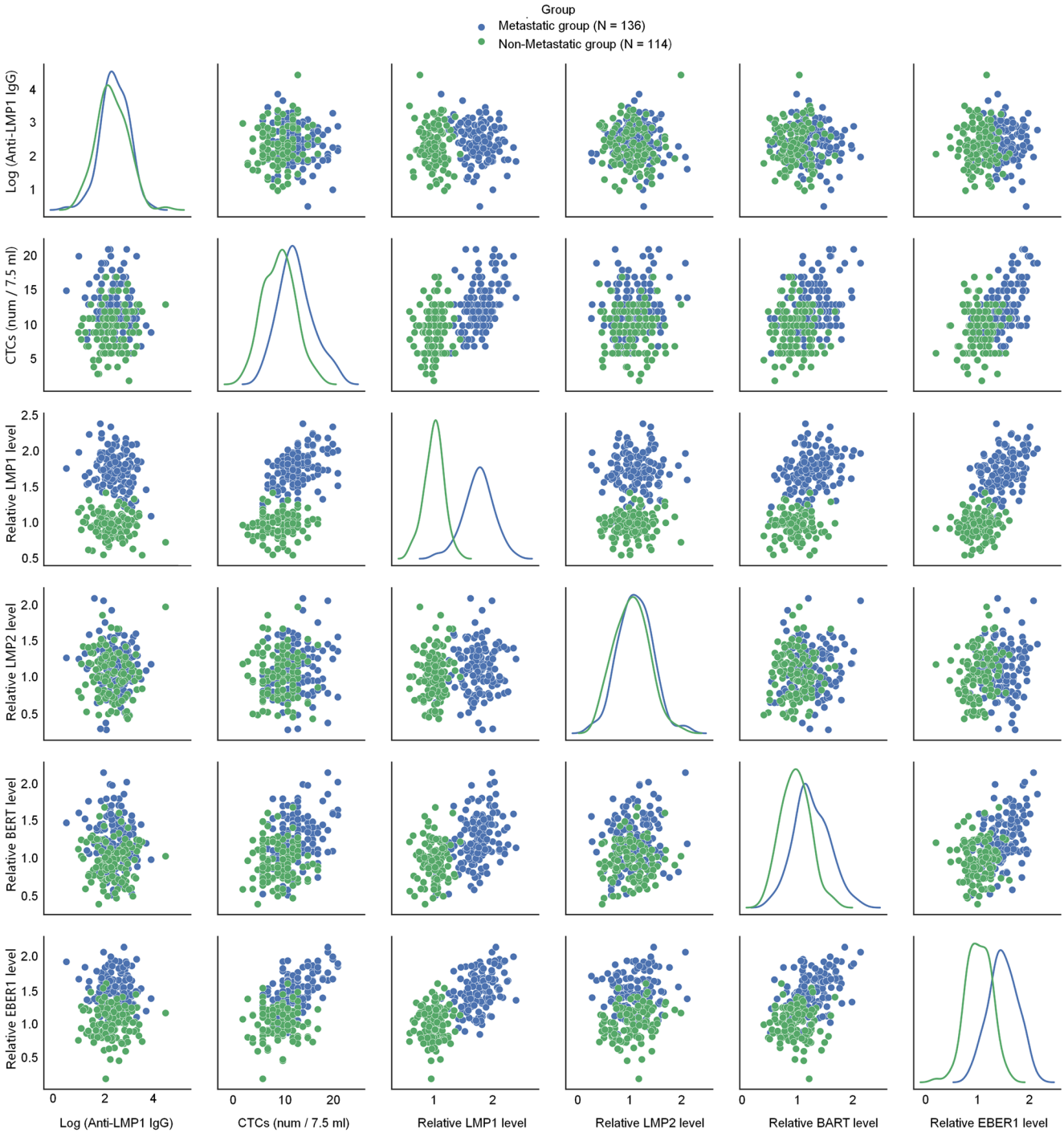


Fig. 2 Data distribution difference between metastatic and non-metastatic groups of circulating tumor cell (CTC) and plasma levels of EBV DNA/RNA. Each value of the CTC and the plasma levels of EBV DNA/RNA was plotted as a scatterplot, in which one variable in the same data row is matched with another variable's value. The color of blue and orange were set for metastatic and non-metastatic patients respectively. The sub-figure with both x and y axis as same item was curved as the value frequency respectively for both groups

Enumeration of CTCs and EBV DNA

The CellSearch System (Veridex) was used for the isolation and enumeration of CTCs in combination with a CellSearch Epithelial Cell Kit. Fluorescently-labeled monoclonal antibodies specific for leukocytes (CD45-allophycocyan) and epithelial cells (cytokeratin 8, 18, 19-phycoerythrin) were used to distinguish epithelial cells from leukocytes. The identification and enumeration of CTCs were performed using a CellSpotter Analyzer. CTCs were defined as nucleated cells lacking CD45 and expressing cytokeratin.

Statistical Analysis

Statistical analysis was executed by the SPSS version 16.0 (SPSS Inc.) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, California, USA). EBV DNA values between the patient and control groups and the positive rates of individual EBV gene transcripts were compared by using the Mann-Whitney test and Pearson correlation test. Linear regression was used to correlate the number of lytic transcripts with (a) the amount of EBV genome in NPC tumor biopsy and (b) level of EBV-IgA antibody response. A P value below 0.05 was considered to be significant.

Results

Clinical Characteristics of and CTCs in NPC Patients

Between Feb 2016 and Dec 2018, 136 metastatic NPC patients and 114 non-metastatic NPC patients were enrolled for

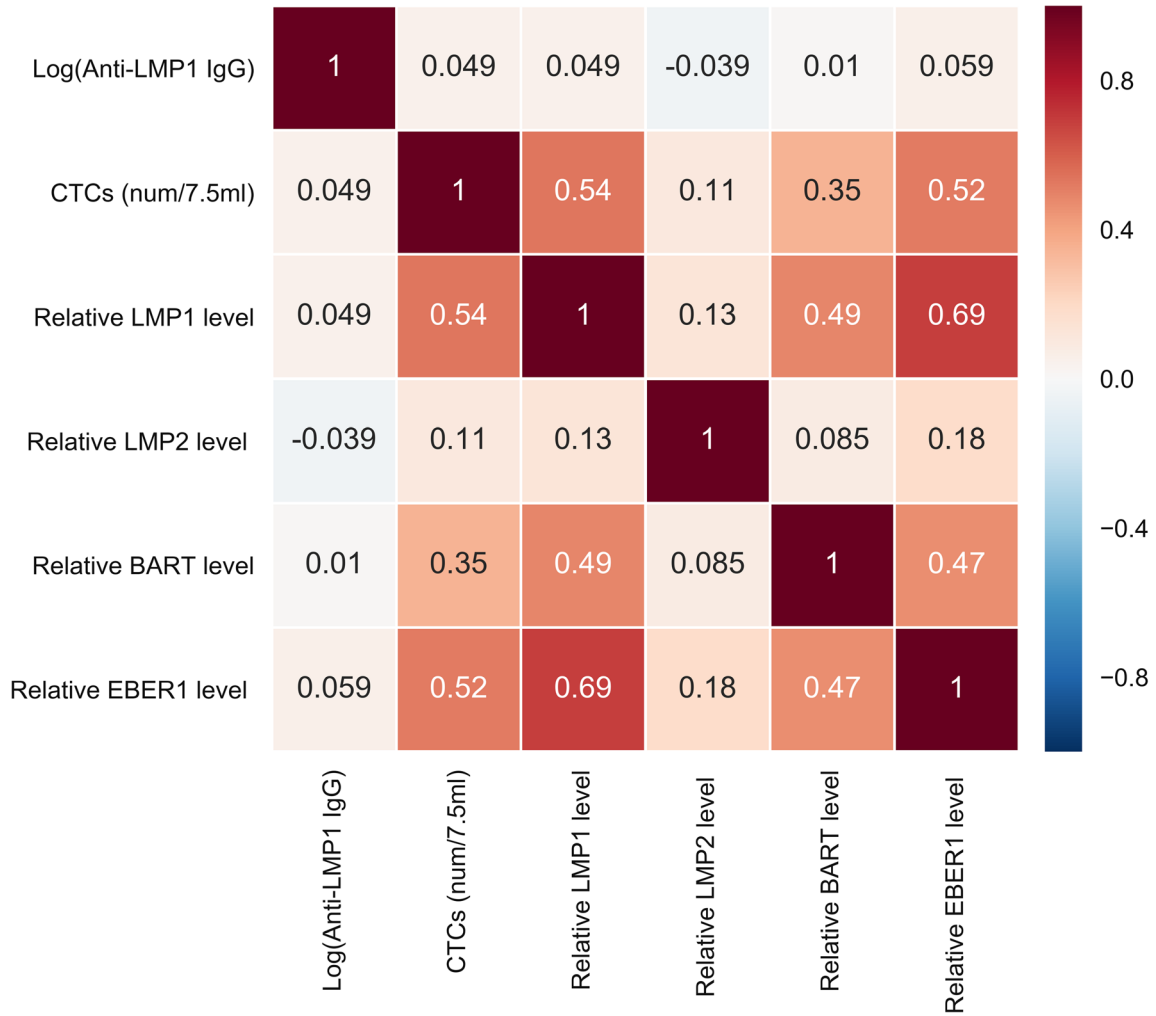


Fig. 3 Correlation analysis for the CTC and the plasma levels of EBV DNA/RNA in all (metastatic and non-metastatic) NPC patients. Spearman correlation was performed to analyze the correlation between every two items of the CTC and the plasma levels of EBV DNA/RNA in all NPC patients (N = 136 for metastatic patients and N = 114 for non-metastatic patients). 0.25 was set as a correlation threshold; Significant correlation between every two items was considered when $R^2 > 0.25$

the present study. The clinical characteristics of these patients were listed in Table 1. As indicated, average age of metastatic NPC patients was 45.54 ± 4.52 (years), not significantly different from 44.63 ± 4.86 (years) for non-metastatic patients. Either gender (Male, number (%), 106 (77.94%) vs. 89 (78.07%) for both groups, $p > 0.05$) or smoking (number (%), 61 (44.85%) vs. 49 (42.98%) for both groups, p > 0.05). CTCs in both groups were counted for 7.5 ml whole blood. It was shown in Fig. 1a that the mean CTC number in metastatic group, 12.5 ± 3.5, was significantly higher than 9.34 ± 3.0 in non-metastatic NPC patients ($p < 0.0001$). To examine EBV infection in all NPC patients, serum anti-LMP1 IgG was examined with ELISA and was presented as a log value. There was no statistical difference in anti-LMP1 IgG for metastatic and non-metastatic groups (2.42 ± 0.56 vs. 2.35 ± 0.57, $p = 1171$).

High Plasma Levels of EBV DNAs / RNAs in NPC Patients with Metastasis

To examined the release / expression of EBV DNA / RNA in NPC blood, we quantified the plasma levels of LMP1, LMP2, BART and EBER1 with real-time quantitative PCR. Figure 1 demonstrated that LMP1 DNA was markedly higher in metastatic group than in non-metastatic group (1.72 ± 0.26 vs. 1 ± 0.15, $p < 0.0001$, Fig. 1c), whereas the difference in the relative LMP2 DNA level (1.12 ± 0.33 vs. 1 ± 0.34, $p = 0.0807$, Fig. 1d) was not significant. Interestingly, the relative levels of both BART and EBER1 were also significantly higher in metastatic patients than in the non-metastatic patients (1.45 ± 0.29 vs. 1 ± 0.24 and 1.28 ± 0.31 vs. 1 ± 0.27, respectively for BART and EBER1, either $p < 0.0001$, Fig. 1e and f). Thus, the plasma levels of EBV DNAs / RNAs was significantly

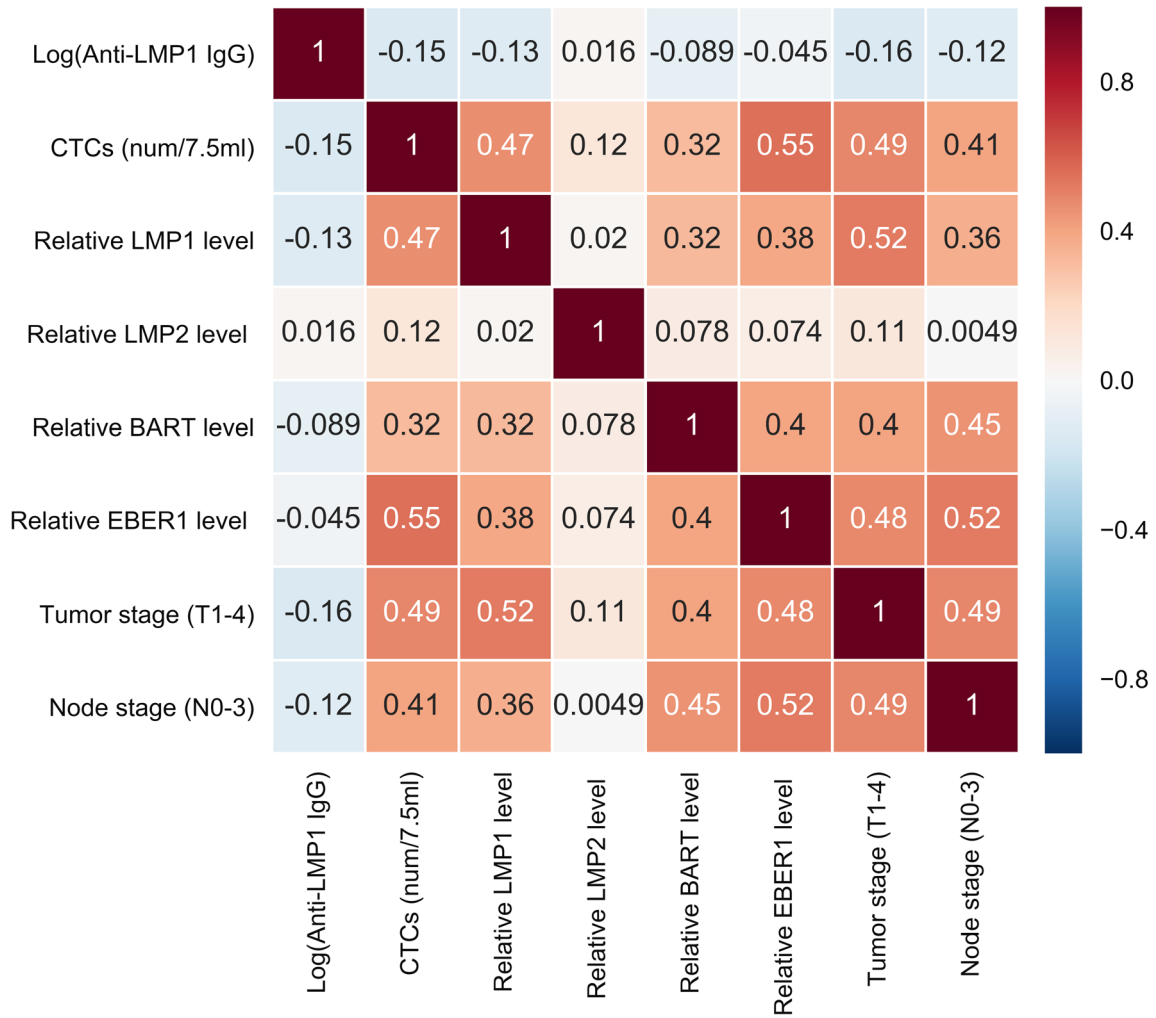


Fig. 4 Correlation of CTC and the plasma levels of EBV DNA/RNA with metastasis of NPC patients. Spearman correlation was performed to analyze the correlation of the CTC and the plasma levels of EBV DNA/RNA with tumor metastasis (tumor stage and node stage) for metastatic NPC patients ($N = 136$). 0.25 was set as a correlation threshold; Significant correlation was considered when $R^2 > 0.25$

higher in metastatic NPC patients than in non-metastatic patients.

Plasma LMP1 DNA and Non-coding Small RNA EBER1 Discriminates Metastatic NPC Patients from Non-metastatic Patients

To further analyze importance of each EBV DNA or RNA as a potential diagnostic biomarker for NPC and the relationship of each biomarker with others, we plotted CTC, anti-EBV IgG and the four types of EBV DNAs / RNAs as a scatterplot, in which every variable was taken as both x and y variable. It was demonstrated that the peak and the curve of either anti-EBV IgG (1st row and 1st column in Fig. 2) or LMP2 (4th row and 4th column in Fig. 2) was overlapped for the two groups. Given either anti-EBV IgG or LMP2 as a x variable, there was no obvious difference in the distribution of other biomarkers between the two groups. The peaks of LMP1 DNA and EBER1 demonstrated the highest difference between the two groups (3rd row and 3rd column, 6th row and 6th column in Fig. 2). Moreover, taken each of them as a category indicator, all the other four biomarkers were clearly discriminated in distribution. In addition, CTC and BART were also largely overlapped in distribution curve (2nd row and 2nd column, 5th row and 5th column in Fig. 2), and not markedly efficient in the discrimination of other biomarkers, though they were significantly different in average level.

To analyze the importance of each biomarker as a discriminator for NPC metastasis, we performed Spearman correlation for every two biomarkers. As Fig. 3 indicated, when all 250 samples (metastasis was not considered) were taken as one sample population, either anti-EBV IgG or relative LMP2 DNA level correlated any other biomarker ($R^2 < 0.25$). The relative level of either LMP1 DNA or EBER1 RNA correlated mostly with each other ($R^2 = 0.69$), and highly with CTC ($R^2 = 0.54$ or 0.52) or BART ($R^2 = 0.49$ or 0.47). Therefore, LMP1 DNA and EBER1 RNA mostly discriminated metastatic NPC patients from non-metastatic NPC patients.

Plasma LMP1 and EBER1 Correlate with Tumor Stage and Node Stage for Metastatic NPC Patients

Finally, to explore discriminative role for metastasis of these biomarkers, we analyzed the correlation of LMP1, EBER1, and other biomarkers with tumor stage and node stage for metastatic NPC patients. As shown in Fig. 4, CTC, relative level of LMP1 DNA, BART or EBER1 significantly correlated with both tumor stage and node stage ($R^2 > 0.25$ for each of the four biomarkers either with tumor stage or node stage). In particular, tumor stage mostly correlated with the relative LMP1 level ($R^2 = 0.52$), node stage mostly correlated with the relative EBER1 level ($R^2 = 0.52$). In addition, the distribution of each of the six biomarkers was scattered when tumor stage or node stage was taken as a category item. It was

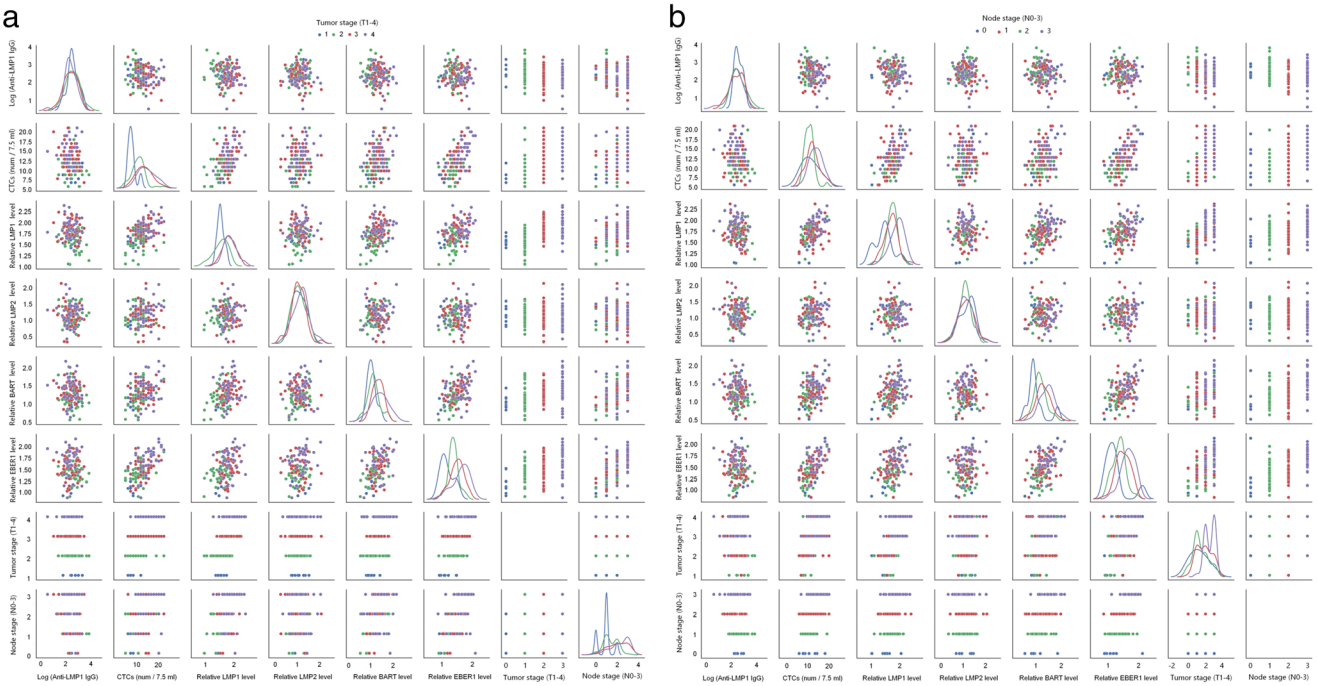


Fig. 5 Data distribution difference of circulating tumor cell (CTC) and plasma levels of EBV DNA/RNA in patients with various tumor stage and node stage. Each value of CTC, plasma levels of EBV DNA/RNA, tumor stage (T1–4) (**a**) and node stage (N0–3) (**b**) scattered for one variable in the same data row being matched with another variable's value. The color of blue, orange, green and red were set for T1, T2, T3 and T4 respectively for tumor stage or N0, N1, N2 and N3 respectively for node stage. The sub-figure with both x and y axis as same item was curved as the value frequency respectively for the four types of tumor stage or node stage

indicated in Fig. 5, the curve peak of CTC, relative level of LMP1, BART or EBER1 was discriminable when either tumor stage or node stage taken as category item.

Discussion

EBV-associated EBNA1, LMP1, LMP2A, LMP2B, non-coding small EBER RNA and BART RNAs are abundantly expressed in NPC patients [19, 20]. Detailed research indicated that the integrations of EBV genes into the introns decreased the expression of the inflammation-related genes in NPC tumors. Such integration is an additional mechanism mediating tumorigenesis in EBV associated malignancies [31]. These EBV-associated biomarkers and circulating tumor cells (CTCs) have been taken as promising biomarkers for NPC [28, 29]. In the present study, we focused on the relationship between CTCs and EBV-associated DNAs / RNAs, and on the importance of these biomarkers on the discrimination on NPC metastasis. Our results revealed the significant role of plasma LMP1 DNA and non-coding small RNA EBER1 in discriminating the metastasis in NPC patients. The prominent diagnostic role of EBV DNAs / RNAs was also indicated by other studies. Viral RNA profiling and DNA fragmentation of EBV in NPC brushings and parallel biopsies were indicated to reflect the tumor origin of NPC [32]. It implies that LMP1 DNA and EBER1 RNA might be potential diagnostic biomarker for NPC.

In the present study, the analysis of EBV DNAs / RNAs demonstrated that there were significantly higher plasma levels of LMP1, BART and EBER1 in the NPC patients with metastasis. Spearman correlation analysis for every two biomarkers indicated that LMP1 DNA and EBER1 RNA mostly discriminated metastatic NPC patients from non-metastatic NPC patients. Accumulating reports have indicated the promotive role of EBV infection to NPC metastasis. EBV-coded miR-BART promotes NPC cell growth and metastasis [33], induces epithelial-mesenchymal transition (EMT) and promotes metastasis [33] through activating NF-κB pathways. The EBV LMP1 also mediates EMT and metastasis of NPC cells, via activating Cadherin 6 [34]. EBV infection even drived NPC metastasis via inducing VEGF and GM-CSF, and then recruiting and activating macrophages [35]. In this study, the detailed Spearman correlation analysis of these biomarkers with the tumor stage and the node stage for metastatic NPC patients was also performed. We found that plasma LMP1 and EBER1 correlate with tumor stage and node stage for metastatic NPC patients.

Recently, the clinical significance of CTCs in malignant tumors, particularly in NPC has been recognized. CTCs in NPC patients were correlated with NPC clinical characteristics, in a relation with EBV DNA [36–38]. Interestingly, our results demonstrated that LMP1 DNA or EBER1 RNA correlated mostly with each other, and highly with CTCs or with BART. Therefore, We speculated that EBV-coded viral DNA and viral microRNAs were associated with NPC CTCs, LMP1 DNA and EBER1 RNA mostly discriminated metastatic NPC patients from non-metastatic NPC patients.

Conclusion

In summary, there were significantly higher plasma levels of Epstein-Barr Virus DNAs / RNAs in nasopharyngeal carcinoma patients. LMP1 DNA and EBER1 RNA correlated with the metastasis of nasopharyngeal carcinoma.

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Compliance with Ethical Standards

Conflict of Interests The Authors declare that they have no conflict of interests.

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