Evaluation of plasma Epstein–Barr virus DNA as a biomarker for Epstein–Barr virus–associated Hodgkin lymphoma

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Abstract
Objectives: Epstein–Barr virus is a tumorigenic virus and has been extensively studied as a causative agent for Hodgkin lymphoma. Although immunostaining of the tumor biopsy is the standard method for diagnosis of Epstein–Barr virus–driven Hodgkin lymphoma, the invasiveness of the procedure renders it difficult and less desirable for the patients. Therefore, we designed this study to evaluate the efficiency of plasma Epstein–Barr virus DNA detection as an alternative diagnostic and prognostic method for Epstein–Barr virus–associated Hodgkin lymphoma.

Methods: This analytical cross-sectional study was conducted during March 2017 to December 2018 including 43 Hodgkin lymphoma patients diagnosed histopathologically followed by the latent membrane protein-1 immunohistochemistry to determine their Epstein–Barr virus association. Plasma Epstein–Barr virus DNA in these samples was measured using quantitative polymerase chain reaction (qPCR).

Results: Of total, 29 (67.44%) patients tested positive for plasma Epstein–Barr virus DNA. On comparing results of latent membrane protein-1 immunohistochemistry (IHC) with plasma Epstein–Barr virus DNA, plasma Epstein–Barr virus DNA was found in 25 of 30 patients with latent membrane protein-1 expression and 4 of 13 patients without latent membrane protein-1 expression. The sensitivity and the specificity of plasma Epstein–Barr virus DNA detection with respect to latent membrane protein-1 IHC were found to be 83.33% and 69.23%, respectively (p = 0.0014).

Conclusion: Determination of plasma Epstein–Barr virus DNA was found to be highly sensitive and specific in characterizing Epstein–Barr virus–associated Hodgkin lymphoma, suggesting that this diagnostic method holds promise as an alternative and more convenient method of diagnosis compared with tissue biopsy.

Keywords
Epstein–Barr virus, Hodgkin lymphoma, real-time polymerase chain reaction, immunohistochemistry, diagnostic performance

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Introduction
Hodgkin lymphoma (HL) is a monoclonal lymphoid neoplasm that originates from B lymphocytes and is characterized by a rare population of Hodgkin Reed–Sternberg (HRS) cells surrounded by a massive inflammatory infiltrate.1,2 Infectious etiology has been suspected of HL, and it has been found that Epstein–Barr virus (EBV) is the only candidate infectious agent causing HL. EBV is a ubiquitous oncopogenic virus belonging to the Herpesviridae family. About 90% of the global population is latently infected with EBV, and it preferentially infects human B cells, mainly persisting as a harmless passenger. However, EBV can transform lymphocytes and is associated with a variety of human malignancies,

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such as Burkitt’s lymphoma, HL and non-HL. The contribution of EBV to HL etiology differs according to the immune status, age group and geographic origin of the infected individual. The EBV association rate varies with the geographical location and ranges between 30% and 50% in developed countries, with much higher rates reported in the developing countries. EBV-associated diseases can be diagnosed in several ways. The most used diagnostic method is principally based on the biopsy of the primary tumor. Detection of viral RNAs, referred to as EBV-encoded RNAs (EBERs), by in situ hybridization and immunohistochemical detection of latent membrane protein-1 (LMP-1) are other widely used methods in the diagnostic laboratories. LMP-1 immunohistochemistry (IHC) is a protein-based assay, which can define HL cases as EBV-related by localization of LMP-1 protein of EBV in neoplastic HRS cells. Confirmation of HL using a biopsy can be challenging because of the poor clinical status of the patients or difficulties in accessing the tumor due to the absence of the required facilities. Therefore, various studies have been conducted globally to explore the efficacy of determining the EBV DNA load as a non-invasive and convenient marker for the diagnosis of HL. This EBV DNA measurement can be utilized as a convenient biomarker to monitor the disease progression. Keeping the merits of this diagnostic method in consideration, this study aimed to evaluate the efficacy of plasma EBV DNA as an alternative marker to determine EBV-associated HL.

Methods

Patients

This analytical cross-sectional study was conducted between March 2017 and December 2018 at the outpatients department of Bangabandhu Sheikh Mujib Medical University (BSMMU) including the clinically suspected cases of lymphoma presented with fever and/or night sweat, weight loss, generalized weakness with the presence of regional or generalized lymphadenopathy over the body, especially on neck, axilla, groin and so on. A minimum sample size of 31 was calculated using two-sided power analysis considering the type I error-5%, power-90% with prevalence rate of 3.9%. During the study period, a total of 115 clinically suspected cases were selected for histopathological diagnosis of HL using lymph node biopsied samples, and finally, 43 (37%) histologically proven HL cases were selected for the further study and rest of the samples other than HL were excluded from this study.

In the selected samples, EBV association with the disease was determined by LMP-1 IHC at the Department of Pathology of BSMMU. For the molecular analysis, 5 mL blood sample was aseptically collected from all the recruited patients and plasma EBV DNA determination was performed using quantitative polymerase chain reaction (qPCR) at the Department of Microbiology of the University of Dhaka. In addition, blood samples from 20 healthy volunteers were collected for a procedural quality control assessment of the molecular technique.

Plasma EBV DNA quantification

DNA was extracted from 200 µL of the plasma samples using the QIAamp DNA Blood Mini Kit (QIAGEN, Germany) following the manufacturer’s instructions. Following DNA extraction, EBV DNA was quantified using qPCR (Applied Biosystems™ StepOne™ Real-Time PCR, USA). For the molecular detection of EBV, conserved portions of the Epstein–Barr nuclear antigen 1 (EBNA 1) gene (78 bp) were targeted (forward primer: 5’TACAGGACCTGGAAATGGCC 3’ and reverse primer: 5’TCTTTGAGGTCGACTGCGG 3’). Briefly, each 20 µL reaction contained 1× Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix, 0.2 µmol/L each of forward and reverse primers, and 1 µL of template DNA. Reaction conditions were as follows: 50°C for 2 min to activate the uracil-N-glycosylase enzyme, followed by Dual-Lock™ DNA polymerase activation step at 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and final extension at 72°C for 60 s. A melt curve analysis was performed post-PCR to verify the specificity and identity of the PCR products. In addition, to the melt curve analysis, PCR products were resolved on a 2.5% agarose gel to verify the specificity. For EBV DNA load determination, a standard curve of five-fold serial dilutions from 3125 to 5 copies per µL of EBV DNA positive control (AMPLIRUN® Epstein–Barr Virus DNA Control, Vircell, Spain) (NC_007605.1) was generated. Viral load was determined by comparing the threshold cycle values of the tested samples with a standard curve and presented as the number of copies per mL of plasma.

Statistical analysis

Descriptive parameters of all the study participants have been expressed as frequency (n) and percentage (%). The Mann–Whitney U test was used to compare the case and control groups. Gender, stage, age and histological types of HL of the cases were analyzed using Fisher’s exact test, Mann–Whitney U test and chi-square test, respectively. In addition, Fisher’s exact test was used to calculate the sensitivity and specificity of plasma EBV with respect to LMP-1 IHC. All p-values were two-tailed, with a cut-off value of less than 0.05 as significant, and all the analyses were conducted using the GraphPad Prism software version 8.

Ethical consideration

During sample collection, a pre-designed written questionnaire was utilized to collect the patients’ demographics and clinical profiles and written informed consent from all the participants or from legally authorized representatives of the
minor was collected before the procedure. The study was conducted as per the Declaration of Helsinki, 1975. This study was approved by the Institutional Review Board (BSMMU) before the commencement of the study (BSMMU/2017/9012, date: 29 August 2017).

**Results**

In this study, 29 HL cases (67.44%) out of 43 were found to be EBV positive, and all the healthy volunteers were confirmed to be EBV negative through qPCR. HL was found to be more prevalent in the male population and mixed cellularity HL subtypes. According to Ann Arbor staging of HL, 40 of the 43 HL cases were belong to the early stage (I and II) and the rest 3 were in the advanced stage (III and IV) of the disease. There were no statistically significant association \((p = 0.977)\) was observed between the stages of the disease with the EBV DNA status. The clinical characteristics, staging, histological types and molecular analyses of the study population have been shown in Table 1.

Comparing the observation of LMP-1 IHC in the tissue samples with plasma EBV DNA in blood samples of the patients, it was found that 25 out of 30 LMP-1-positive HL patients and 4 out of 13 patients with LMP-1-negative HL tested positive for the plasma EBV DNA (Figure 1). The median viral load was higher in EBV-associated HL (8430 copies/mL, range = 0–291,167.5) as compared to EBV-negative HL (0 copy/mL, range = 0–50,499). The Mann–Whitney \(U\) test showed a significant difference in the presence of EBV DNA between the EBV-associated and EBV-non-associated HL groups \((p = 0.0190)\).

In this study, the discordant results between the EBV DNA positivity with LMP-1 IHC were observed in 9 (20.9%) cases. Five LMP-1 IHC positive samples were found to be negative for the EBV DNA. However, four LMP-1 IHC negative samples had detectable EBV DNA. Still, we did not find any notable variation as per the age, gender, stage and histological subtype of HL among the cases. The details characteristics of these cases have been given in Table 2.

| Parameter                        | Total \((n = 43)\) | EBV DNA positive \((n = 29)\) | EBV DNA negative \((n = 14)\) | \(p\)-value |
|---------------------------------|------------------|-------------------------------|-------------------------------|------------|
| Gender (male/female)            | 34/9             | 23/6                          | 11/3                          | >0.999     |
| Age (median, range), years      | 25 (4–61)        | 25 (4.5–65)                   | 30 (4–61)                     | 0.24       |
| Ann Arbor stage                 |                  |                               |                               |            |
| Early stage (I and II)          | 40               | 27                            | 13                            | 0.997      |
| Advanced stage (III and IV)     | 3                | 2                             | 1                             | 1          |
| Histologic type                 |                  |                               |                               |            |
| Mixed cellularity               | 33               | 25                            | 8                             | 0.07       |
| Nodular sclerosis               | 4                | 3                             | 1                             |            |
| Lymphocyte rich                 | 5                | 1                             | 4                             |            |
| Lymphocyte predominant          | 1                | 0                             | 1                             |            |

**EBV**: Epstein-Barr virus.

**Table 1.** Characteristics of patients with HL according to their plasma EBV DNA status.

**Figure 1.** Diagrammatic comparisons of EBV-associated HL between LMP-1 IHC and plasma EBV DNA estimation.

Exploration of the diagnostic performance of the plasma EBV DNA detection over LMP-1 IHC for diagnosing EBV-associated HL revealed that the sensitivity and the specificity of plasma EBV DNA detection method were 83.33% and 69.23%, respectively.

**Discussion**

This study evaluated the rapidity and accuracy of the qPCR in detecting and quantifying the EBV DNA in HL patients.\(^{11,16–19}\) This study revealed that 67.44% of the diagnosed HL cases were EBV-positive based on the plasma EBV DNA quantification, which is much higher than that reported in developed nations. Similar studies from North America, Europe, and Australia revealed 15%–20% EBV association, while a study in Brazil
showed 43% EBV association with HL through plasma EBV DNA estimation.8,13,20,21 In this study, plasma EBV DNA was detected in 83% of the EBV-associated HL and 30% of the non-EBV-associated HL samples. In an earlier study conducted results showed 91% of EBV-associated HL samples had EBV DNA and 16% of cases were without any association with EBV.13 Similar findings in line to this study have been reported in other previous studies.6,22 Here in, we observed a high plasma EBV DNA load in EBV-associated HL, while a few cases with low levels of EBV-DNA were observed in EBV-negative HL patients (p = 0.019).

The discordant results of the LMP-1 IHC positivity with plasma EBV DNA detection in this study remain to be elucidated. The molecular quantifications in the qPCR techniques usually vary as per the sensitivity of the target gene, sample DNA load, efficiency during the sample handling and processing steps. Undetectable EBV DNA in the EBV-associated HL cases in this study could be due to the low DNA load and/or due to the early stage of disease in the patients or any procedural errors mentioned earlier.23 Detection of EBV DNA in the non-EBV-associated HL may be due to the release of EBV DNA from the scattered lymphocytes harboring the EBV or from cells undergoing viral lytic replications.18,24 Moreover, the detectable circulating EBV DNA in some non-EBV-associated HL may be explained by the “hit and run” mechanisms.25 In these particular cases, detection of EBV-encoded viral and host miRNAs could be indicative to be performed in highly clinical susceptible cases.26

Hence, considering LMP-1 IHC as a standard diagnostic method for the EBV-associated HL and comparing it with the results obtained from DNA detection method, this study showed that the quantitative detection of plasma EBV DNA was very sensitive and specific. Therefore, it could serve as a promising and convenient detection tool for the EBV-associated HL cases.27,28

**Study limitations**

This study has some limitations. This was performed in a relatively small number of population due to the limited availability of the study cases. Most of HL cases in this study were recruited during their initial diagnostic evaluation which impeded to predict the EBV DNA status with the different stages of HL. Serial measurements of EBV DNA of HL patients to monitor the long-term prognosis and response to therapy could not be done during this period.

**Conclusion**

This study was the first of its kind to the best of our knowledge that investigated the EBV DNA association among the HL patients from Bangladesh. Our results suggested that developing a plasma EBV DNA-based biomarkers will surely help to establish a new way of monitoring disease activity and response to the treatment among the patients with EBV-associated HL. So, further longitudinal studies on this EBV DNA positive HL group are needed to establish it as a convenient diagnostic tool during the course of management of these patients.

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**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

**Ethical approval**

Ethical approval for this study was obtained from the Institutional Review Board, Bangabandhu Sheikh Mujib Medical University (BSMMU/2017/9012, date: 29 August 2017).

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### Table 2. Cases of discordant EBV-associated HL in terms of LMP-1 IHC and EBV DNA results.

| Serial number | Cases | Gender | Age | Ann Arbor stage | Lymphadenopathy | Histological subtype | LMP-1 IHC | EBV DNA (copies/mL plasma) |
|---------------|-------|--------|-----|-----------------|-----------------|----------------------|-----------|--------------------------|
| 1             | 09    | Female | 60  | III             | Cervical        | Lymphocyte rich      | +         | –                        |
| 2             | 14    | Male   | 50  | I               | Cervical        | Mixed cellularity    | –         | 49,200                   |
| 3             | 19    | Female | 35  | I               | Cervical        | Lymphocyte rich      | +         | –                        |
| 4             | 21    | Male   | 35  | I               | Abdominal       | Mixed cellularity    | –         | 9602.5                   |
| 5             | 23    | Male   | 13  | I               | Cervical        | Mixed cellularity    | +         | –                        |
| 6             | 24    | Male   | 51  | I               | Cervical        | Mixed cellularity    | +         | –                        |
| 7             | 45    | Male   | 14  | I               | Inguinal        | Mixed cellularity    | –         | 50,499                   |
| 8             | 49    | Male   | 07  | I               | Cervical        | Mixed cellularity    | +         | –                        |
| 9             | 50    | Male   | 28  | I               | Cervical        | Nodular sclerosis    | –         | 8680                     |

LMP: latent membrane protein; IHC: immunohistochemistry; EBV: Epstein–Barr virus.
Informed consent
Written informed consent was obtained from all subjects or legally authorized representatives of the minor before the study.

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