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Detection of Epstein–Barr virus DNA in peripheral blood of paediatric patients with Hodgkin's disease by real-time polymerase chain reaction

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Abstract

Hodgkin's disease (HD) is commonly associated with latent Epstein–Barr virus (EBV) infection. The aim of our study was a detailed molecular analysis of the EBV status in the peripheral blood of paediatric patients with HD. Blood samples from HD patients were examined before ($n=28$) and after treatment ($n=12$). The control group consisted of 20 healthy children and 10 immunosuppressed children with primary EBV infection. EBV load in plasma and peripheral blood mononuclear cells (PBMC) were determined by real time quantitative polymerase chain reaction (RQ-PCR) as recently described. Before treatment, EBV DNA was detected in the plasma of 13/24 EBV-seropositive HD patients, whereas in plasma of healthy controls no EBV DNA was detectable ($P<0.001$). After treatment, no EBV genomes were found in the plasma of 6 HD patients in stable and complete remission. In contrast, 2/5 HD patients with relapse of disease were positive for EBV DNA in the plasma. In PBMCs, no differences were found in EBV load measured in HD patients before or after treatment and healthy controls. A high EBV load was found in both the plasma and PBMCs of all immunosuppressed patients with primary EBV infection. Thus, EBV DNA detection in the plasma of paediatric HD patients might be of value for non-invasive diagnostic, prognostic and follow-up tests for HD.

Keywords: Hodgkin's disease; Epstein–Barr virus; Plasma; Real-time polymerase chain reaction

1. Introduction

The epidemiological and clinicopathological features of Hodgkin's disease (HD) suggest that an infectious agent is involved in its aetiology. Epstein–Barr virus (EBV), a lymphotrophic herpesvirus that is widespread in the human population, is associated with approximately 40% of non-randomly distributed HD cases. A mixed cellularity histological subtype is more likely to occur than nodular sclerosis, subtype patients are more likely to be in early childhood and older age groups than young adults and the patients are more likely to originate in developing countries than industrialised countries. Latent EBV infection is found in 20% to greater than 80% of Hodgkin's and Sternberg–Reed cells [1]. In these cases, monoclonal EBV genomes are present in the tumour cells indicating that infection occurs prior to the clonal expansion of the transformed cells. Viral gene products such as Epstein–Barr virus nuclear antigen 1 (EBNA1), latent membrane protein 1 (LMP1) and Epstein–Barr virus encoded RNA 1 and 2 (EBER1 and 2) are expressed of which at least LMP1 has oncogenic potential [2]. Recently, it was shown that EBV association provides a survival advantage to HD patients treated with standard chemotherapy and radiotherapy [3].

Small amounts of free DNA circulate in the plasma of both healthy individuals and patients. The concentration of DNA is 10 times higher in cancer patients compared with healthy individuals because tumour necrosis, apoptosis or actively-released DNA may contribute to the higher amounts of DNA in plasma. Hence, analysis of serum/plasma DNA may be of value for both diagnosis and follow-up of cancer patients [4]. In a HD

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patient, the identical *immunoglobulin* gene rearrangement was demonstrated in both biopsy and serum samples, indicating that in HD a release of DNA into the sera by Hodgkin's and Sternberg–Reed cells is indeed the case [5].

The aim of our study was a detailed molecular analysis of the EBV status in the peripheral blood of children with HD prior to and after treatment. We especially focused on the question of whether EBV DNA is detectable in the plasma of patients during the course of disease.

2. Patients and methods

2.1. Patients

40 paediatric HD patients from the study GPOH-HD95 supported by the German Society of Pediatric Oncology and Hematology (GPOH) were enrolled (21 male, 19 female; median age 14 years, range 5–19 years). 28 patients (14 male, 14 female) were examined before and 12 patients (7 male, 5 female) after treatment. Of the 12 patients examined after termination of treatment, 7 were in stable and complete remission and 5 patients showed a histologically proven relapse of disease. The control group consisted of 20 healthy non-immunosuppressed children with the same age and gender distribution, and 10 immunosuppressed renal transplanted children with primary EBV infection. Ethylene diamine tetraacetic acid (EDTA)-anticoagulated blood samples were taken from patients and controls after informed consent. Plasma samples and peripheral blood mononuclear cells (PBMC) were processed in parallel for all patients and controls apart from 2 patients in complete and stable remission of whom only a single plasma sample or a single PBMC preparation were available. PBMCs were obtained from EDTA-anticoagulated blood by standard density centrifugation (Ficoll Separation Solution, Biochrom, Berlin, Germany).

2.2. EBV serology

EBV serology was analysed by enzyme-linked immunosorbent assays (ELISA), detecting IgG-, IgM-antibodies against the early antigen (EA) of EBV, as well as IgG against EBNA 1 (Biotest, Dreieich, Germany). Assays were performed according to the instructions of the manufacturer. Primary EBV infection was serologically confirmed by positivity of anti-EA-IgM and/or anti-EA-IgG in the absence of anti-EBNA1 antibodies, whereas latent or past EBV infection was characterised by positivity of anti-EBNA1-IgG. Reactivation of latent EBV infection was indicated in EBNA1-IgG-positive individuals by additional elevated anti-EA-IgM (optical density (OD)-values >0.5) or additional positive anti-EA-IgG and anti-EA-IgM regardless of the OD value. EBV-seronegative individuals were negative for all three parameters [6].

2.3. Real-time quantitative polymerase chain reaction (RQ-PCR)

DNA from plasma samples and PBMCs was extracted using the QIAamp Blood Kit (Qiagen, Hilden, Germany). An equivalent of 100 μl plasma and 500 μg PBMC DNA were analysed for each patient. Detection and quantification of EBV-specific sequences was performed by real time quantitative polymerase chain reaction (RQ-PCR) assay (TaqMan technology) using the ABI PRISM 7700 Sequence Detection System (SDS) (PE Applied Biosystems, Foster City, CA, USA). RQ-PCR is a reliable, rapid and accurate quantitative technique [7] based on the continuous optical monitoring of the progress of a fluorogenic hybridisation reaction. In RQ-PCR, a dual-labelled flourogenic hybridisation probe is included in the reaction besides the two amplification primers used in conventional PCR. One fluorescent dye serves as a reporter of which emission spectra is quenched by a second fluorescent dye. During the extension phase of the PCR, the 5′-3′ exonuclease activity of *Taq* polymerase cleaves the reporter from the probe releasing it from the quencher. The resulting exponential increase in fluorescence emission during PCR is monitored automatically and used for quantitative measurement of templates [7].

For EBV-specific RQ-PCR, primers and probes detecting the *Bam HI* W region of EBV were employed. Whenever possible ($n=45$), PCR results were confirmed with a different set of EBV-specific primers and probes detecting a region within the *Bam HI* K region of EBV (*EBNA1* gene). For quantification, DNA of the EBV-positive Burkitt's lymphoma cell line, Namalwa (American type culture collection CRL-1432), was used as a standard. Namalwa cells contain two integrated EBV-copies per cell and therefore provide equivalent amounts of genomic and viral copies. Details of the protocols used, as well as primers and probes are described elsewhere [8].

2.4. Statistical analysis

For statistical comparisons of viral load between patients and controls, the Mann–Whitney U test was used. A P value of <0.05 was regarded as statistically significant. For analysis of correlations, the Spearman rank test was employed. Calculations were performed using the software Statistical Package for the Social Services (SPSS) 9.0 for Windows (SPSS Inc., Richmond, CA, USA).

3. Results

24/28 (86%) of HD patients prior to treatment and 11/12 (92%) patients after treatment were EBV-seropositive. 3/24 (13%) seropositive HD patients prior to treatment and 1/5 (20%) patients with relapse of disease had serological signs of EBV reactivation. No serological signs of primary EBV-infection were found in any of the HD patients.

As depicted in Fig. 1, 13/24 (54%) EBV-seropositive HD patients prior to treatment had EBV DNA detectable in the plasma (median 1.2 EBV copies/100 μl plasma, range 0–348), which was in contrast to healthy individuals that were always negative for plasma EBV DNA ($P<0.001$). In patients with stable complete remission following termination of treatment ($n=6$), no EBV-related sequences were found. However, in 2/5 (40%) patients with relapse of disease, EBV DNA was detected in the plasma (median EBV 0 copies/100 μl plasma, range 0–106). In the DNA from the PBMCs, no differences were found between HD patients, regardless of the treatment status, and healthy controls (Fig. 2). When EB viral load detected in the plasma or in the PBMCs was compared with the corresponding anti-EA-IgG or anti-EA-IgM titres of the same individual, no correlation was found in the HD patients.

Although the EB viral load in the PBMCs was within the normal range. EBV-seropositive HD patients with a positive detection of EBV DNA in the plasma examined before initiation of treatment, had a significantly higher viral load in the corresponding PBMC compartment ($n=13$; median 26.7 EBV copies/μg PBMC DNA, range 0–4482) compared with HD patients with no detectable EBV-related sequences in plasma ($n=11$; median 0 EBV copies/μg PBMC DNA, range 0–60.5) ($P<0.05$). Accordingly, in HD patients with a positive detection of EBV DNA in the plasma, there was a significant correlation between EB viral load detected in the plasma and PBMCs ($P<0.05$; correlation coefficient $r=0.649$).

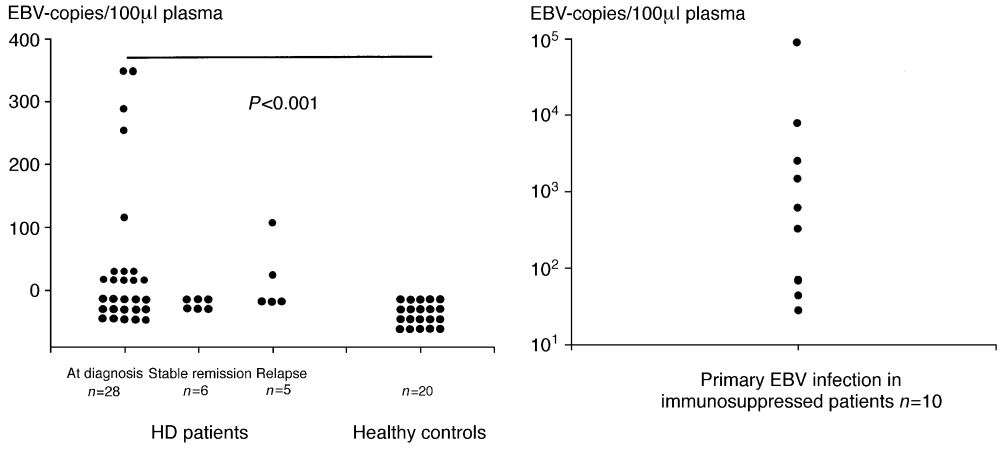

Fig. 1. Epstein–Barr virus (EBV) load in plasma samples of Hodgkin's disease (HD) patients and healthy controls. For further comparison, immunosuppressed patients with primary EBV infection were analysed in parallel.

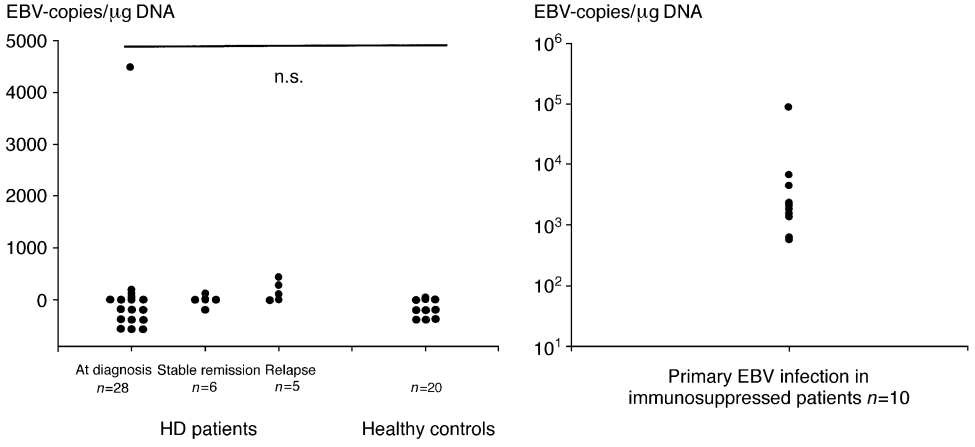

Fig. 2. Epstein–Barr virus (EBV) load in peripheral blood mononuclear cells (PBMCs) of Hodgkin's disease (HD) patients, healthy controls and immunosuppressed patients with primary EBV infection. n.s., non significant.

In the 5 EBV-seronegative HD patients, no EBV-related sequences were found in the plasma or PBMCs.

All patients with primary EBV infection had significantly elevated EBV loads both in plasma (median 465 EBV copies/100 μl plasma, range 30–88 235) and PBMCs (1940 EBV copies/μg PBMC DNA, range 565–87 410) in comparison to healthy controls ($P<0.0001$ for the plasma and PBMCs, respectively). In patients with primary EBV infection, there was a statistically significant correlation between the EB viral load measured in the plasma and PBMCs ($P<0.05$; correlation coefficient $r=0.99$).

Correlation between *Bam HI* W- and *Bam HI* K-specific RQ-PCR was 94.6% (for plasma: $n=23$, $r=0.91$; for PBMC: $n=22$; $r=0.97$). There was only one plasma sample in which a discrepancy arose in the plasma PCR detection, where a positive result was obtained with the *Bam HI* W-specific RQ-PCR (3 EBV copies/100 μl plasma) and a negative result was obtained for the *Bam HI* K-specific RQ-PCR.

4. Discussion

In the present study, we performed a detailed molecular analysis of the EBV status in the peripheral blood of paediatric patients with HD. Samples from these patients were analysed by RQ-PCR, with two different sets of EBV-specific primers and probes. There was a high correlation between the results obtained from each set of primers and probes, which is consistent with previously published studies [8,9]. EBV DNA was detected in the majority of plasma samples from EBV-seropositive HD patients prior to treatment, as well as in some patients with relapse of disease, whereas in healthy individuals or HD patients in stable and complete remission, no EBV-related sequences were found. In previous studies, EBV DNA was never detected in the serum or plasma of more than 355 healthy, non-immunosuppressed children and adults by PCR [8]. However, EBV DNA was frequently found in the plasma or serum preparations of patients with primary EBV infection [8,10,11] or with EBV-associated malignancies such as nasopharyngeal carcinoma [9] and post-transplant lymphoproliferative disorder [8,12,13]. These observations show that the positive detection of EBV DNA in the serum or plasma of patients is a specific indicator for EBV-related diseases. Thus, quantification of EBV viral load in the plasma or serum of patients seems to be a powerful parameter for diagnosing EBV-associated diseases.

Patients with primary EBV-infection harbour EBV-infected cells proliferating in the peripheral blood [14]; and in some of these infected cells, EBV undergoes lytic replication leading to release of free EB virions into plasma [9,15]. As a consequence, high levels of EBV DNA are detectable in both the plasma and PBMCs of all control patients with primary EBV infection examined in this study. Because EBV DNA detected in the plasma originates from active infection ongoing in the peripheral blood, there is a high correlation between viral load in the plasma and PBMCs of patients with primary EBV infection in our study.

The number of untreated EBV-seropositive HD patients that were positive for EBV DNA (13/24; 54%) in plasma in this study is in accordance with the observations by Gallagher and colleagues [16] and Drouet and of coworkers [17] who found EBV-related sequences in serum/plasma of 36/59 (61%) or of 15/30 (50%) HD patients, respectively. These previous studies [16,17] also determined that EBV DNA was more frequently detected in serum/plasma of HD patients with EBV-positive Hodgkin's and Sternberg–Reed cells (30/33 or 10/12, respectively) than in HD patients with EB-negative tumour cells (6/26 or 5/18, respectively). Furthermore, Gallagher and colleagues [16] showed that EBV DNA in positive serum samples from HD patients consisted of naked DNA released by cells and not from free EBV virions, which is the situation seen in primary EBV infection. Taken together, these observations suggest that the majority of EBV DNA detectable in the plasma of HD patients may originate from EBV-positive Hodgkin's and Sternberg–Reed tumour tissue cells.

We did not find an increased EBV viral load in the PBMCs of HD patients before or after treatment when compared with healthy controls. However, even though the results obtained lie within the normal range of EB viral load for PBMCs, we found that HD patients positive for EBV DNA in their plasma had a higher EB load in their PBMCs when compared with patients negative for EBV DNA in their plasma. These results are in contrast to Gallagher and colleagues [16] who did not find a correlation between EB load in the plasma and PBMCs. The difference between the two results is most likely due to the higher number of patients evaluated in our study ($n=24$) compared with their study ($n=13$).

In HD, the general T-cell function is impaired, as measured by mitogen or antigen-induced proliferation [2]. Moreover, in EBV-positive HD cases, tumour-specific factors may elicit a localised suppression of EBV-specific cellular immunity [18]. Thus, the diminished immune surveillance in HD patients may lead to an enhanced proliferation of EBV-infected cells and reactivation of EBV in non-neoplastic cells found within the peripheral blood, and consequently result in a relatively high EB load in the PBMCs of some patients. This could explain our observation of a relatively high EB load in PBMCs found in HD patients who were positive for EBV DNA in their plasma. Therefore, in addition to the malignant Hodgkin's and Sternberg–Reed cells, EBV released by non-neoplastic cells may also contribute to detectable EBV DNA levels in the plasma of those HD patients who have a relatively high EB viral load in their PBMCs.

In summary, EBV DNA is frequently found in the plasma of HD patients before treatment and at relapse of disease by RQ-PCR analysis. EBV DNA detectable in the plasma from HD patients may originate from HD tumour cells and for patients with relatively high EBV load in the PBMCs from non-neoplastic cells as well. Further longitudinal studies will demonstrate the diagnostic impact of this assay for monitoring treatment response and the remission status of HD patients.

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