CD3 and EBER double positive cells in bone marrow are a diagnostic aid for EBV-positive T-cell lymphoproliferative disorders of childhood

Yuri Yamada · Ayako Kamiunten · Tomonori Hidaka · Masafumi Sekine ·
Kousuke Marutsuka · Keiko Katayose · Yoko Kubuki · Hisanori Iwakiri ·
Satoru Hasuike · Mai Tsuchimochi · Kenichi Nakamura · Shojiro Yamamoto ·
Yoshinori Otsubo · Norihisa Kusumoto · Toshiharu Eto · Kenji Nagata ·
Akira Kitanaka · Kazuya Shimoda

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Abstract We describe a case of Epstein–Barr virus (EBV)-positive T-cell lymphoproliferative disorders (EBV+ T-cell LPD) of childhood. The patient presented with high fever, jaundice, hepatosplenomegaly, pancytopenia, and disseminated intravascular coagulation (DIC). On admission, liver biopsy could not be conducted due to DIC. The small but definitive number of abnormal lymphocytes in bone marrow (BM) was indicative of malignant lymphoma or related diseases. The diagnosis of EBV+ T-cell LPD of childhood was tentatively made on the basis of the presence of CD3 and EBER double positive cells in BM. We started chemotherapy, which promptly resolved the symptoms and DIC. Later the diagnosis was confirmed by the T-cell receptor rearrangement and a single band for the EBV terminal repeat region by Southern blot analysis of BM cells. The detection of EBER-positive CD3 cells in BM is a diagnostic aid for EBV+ T-cell LPD of childhood.

Keywords EBV · Hemophagocytosis · Southern blotting

Introduction

Epstein–Barr virus (EBV)-positive T-cell lymphoproliferative disorders of childhood (EBV+ T-cell LPD of childhood) is a life-threatening illness of children and young adults characterized by a clonal proliferation of EBV-infected T cells [1]. It has a rapid progression with multiple organ failure usually from days to weeks. Patients develop hepatosplenomegaly and liver failure, usually accompanied by hemophagocytic syndrome and coagulopathy (disseminated intravascular coagulation, DIC) [2]. As most cases have a fulminant clinical course resulting in death, prompt diagnosis is required. Diagnosis is based on the clonal proliferation of EBV+ T cells in liver, lymph nodes, and bone marrow (BM). They are usually small and lack significant cytologic atypia [3]. The typical phenotype of neoplastic cells is CD2+ CD3+ CD56–, and either CD4+ for chronic active EBV infection (CAEBV) or CD8+ for secondary to acute primary EBV infection. EBV-encoded RNA (EBER) is positive. T-cell receptor (TCR) genes are monoclonally rearranged in neoplastic cells.

We present a case of EBV+ T-cell LPD of childhood, the diagnosis of which was tentatively made on the basis of the presence of CD3 and EBER double positive cells in BM.

Case report

A 20-year-old Japanese woman was admitted with general fatigue, high fever, and jaundice. On examination, she was listless. The temperature 38.7°C, and the pulse 74 beats per minute. The conjunctivae were not anemic and icteric. The liver edge extended 5.5 cm below the costal margin, and the spleen edge extended 1.0 cm below the left costal

Y. Yamada · A. Kamiunten · T. Hidaka · M. Sekine ·
K. Katayose · Y. Kubuki · H. Iwakiri · S. Hasuike ·
M. Tsuchimochi · K. Nakamura · S. Yamamoto · Y. Otsubo ·
N. Kusumoto · T. Eto · K. Nagata · A. Kitanaka ·
K. Shimoda (✉)
Departments of Gastroenterology and Hematology,
Faculty of Medicine, Miyazaki University, 5200 Kihara,
Kiyotake, Miyazaki 889-1692, Japan
e-mail: kshimoda@med.miyazaki-u.ac.jp

K. Marutsuka
Department of Pathology, Faculty of Medicine,
Miyazaki University, 5200 Kihara, Kiyotake,
Miyazaki 889-1692, Japan

margin. There were some purpura on extremities. The remainder of the examination including tonsil and lymph nodes was normal. Abdominal echogram showed huge hepatosplenomegaly, but absence of lymphadenopathy.

Laboratory examination showed pancytopenia (Hb 10.4 g/dL, WBC $2.9 \times 10^9/L$, Plt $21 \times 10^9/L$) with coagulopathy (PT 14.1 s; control 10.5–12.5 s, APTT 53.4 s; control 24.0–36.0 s, D-dimer 48 ng/mL; normal range <1, fibrinogen 199 mg/dL; normal range 200–400). Other abnormal laboratory findings were as follows: AST 366 U/L (normal range 13–33), ALT 252 U/L (6–27), total bilirubin 5.5 mg/dL (0.2–1.2), direct bilirubin 3.7 mg/dL (0.1–0.7), LDH 1,195 U/L (119–229), ferritin 8,420 ng/mL (2.7–141.1), triglycerides 176 mg/dL (37–161). CRP was 6.95 mg/dL (<0.3). Renal function was normal.

The serum titers for EBV-related antibodies showed anti-viral capsid antigen (VCA) IgG $<16 \times 10$ (<10), anti-VCA IgM <10 (<10), and EB nuclear antigen (EBNA) <10 (<10). Quantitative polymerase chain reaction (qPCR) for plasma EBV DNA showed 2.0×10^4 copies/mL (<100).

The BM aspirate showed a small number of phagocytic cells and pleomorphic medium- to large-sized lymphoid cells with irregular nuclei and frequent mitosis (Fig. 1a). They had higher forward scatter, and were positive for CD3, CD8, and TCR $\alpha\beta$ by flow cytometry analysis. BM aspiration clots were employed for histological examination, immunohistochemistry (IHC), and in situ hybridization (ISH) of EBV-encoded small RNA (EBER). The samples were fixed in 10% buffered formalin, dehydrated in alcohol, and embedded in paraffin. Paraffin blocks were sectioned at 2- μ m thickness. Routine hematoxylin- and

eosin (H&E)-stained sections were made for histological examination. For double staining with EBER and either CD3 or CD20, ISH using DAB chromogen was performed first, followed by IHC for CD3 or CD20 using Fast Red chromogen. Immunostaining was performed on an automated immunostainer (BOND-MAX, Leica Microsystems Japan, Tokyo) according to the company's protocols (Bond Polymer Refine RNA ISH protocol and Bond Polymer Refine Red IHC protocol, Leica Microsystems). The antibody panel included L26 (CD20, DakoCytomation, Glostrup, Denmark) and anti-CD3 (DakoCytomation). Pleomorphic medium- to large-sized lymphoid cells with irregular nuclei were observed (Fig. 1b). Most of the EBER positive cells were positive for CD3, but not for CD20, indicating that the EBV+ cells were of T-cell origin (Fig. 1c, d).

A tentative diagnosis of EBV+ T-cell LPD of childhood was made. She got worse on the second day. We started chemotherapy with PSL, cyclophosphamide, vincristine, and etoposide, which promptly resolved the fever, jaundice, hepatosplenomegaly, pancytopenia, and DIC. On day 19, after the recovery of chemotherapy-induced BM suppression, the liver biopsy was done. A small number of lymphocytes invaded the portal area (Fig. 2a, b). Double staining of liver tissue for ISH for EBER and IHC for CD3 or CD20 were performed according to the methods described above. Most of the EBER-positive lymphocytes that invaded the portal area were positive for CD3 (Fig. 2c, d). Southern blot analysis of BM cells revealed a thin but definitive additional single band for the TCR C β 1 and a single band for the EBV terminal repeat region (Fig. 3).

Fig. 1 Microscopic appearance of BM smear and tissue. **a** May–Giemsa staining, $\times 1,000$; **b** H&E staining, $\times 400$; **c** the double staining of EBER-ISH (brown) and CD3-IHC (red), $\times 400$; **d** the double staining of EBER-ISH (brown) and CD20-IHC (red), $\times 400$

Fig. 2 Microscopic appearance of liver tissue after chemotherapy. **a** H&E staining, $\times 200$; **b** H&E staining, $\times 400$; **c** the double staining of EBER-ISH (brown) and CD3-IHC (red), $\times 400$; **d** the double staining of EBER-ISH (brown) and CD20-IHC (red), $\times 400$

Fig. 3 Southern blotting analysis of high molecular DNA obtained from BM cells. **a** Probed with TCR C β 1, **b** probed with EBV-terminal repeated (TR) sequence. *Bam*HI DNA samples digested with *Bam*HI, *Eco*RV DNA samples digested with *Eco*RV, *Hind*III DNA samples digested with *Hind*III, **b** DNA samples digested with *Bam*HI. Arrow a clonal band

Discussion

This case presented with hepatosplenomegaly, abnormal liver function tests, hemophagocytosis, and DIC at the initial visit. The BM aspiration biopsy specimen showed a small but definite number of abnormal cells. They are pleomorphic medium- to large-sized lymphoid cells with irregular nuclei and frequent mitosis. EBV serology was

both anti-VCA IgM and anti-VCA IgG negative. A differential diagnosis list was malignant lymphoma or EBV+ T-cell LPD of childhood. The main affected organs were the liver and spleen, and a small number of abnormal cells infiltrated the BM. At this point, a liver biopsy was perceived as dangerous due to the DIC. To establish an accurate diagnosis, we examined TCR rearrangement in BM cells by Southern blotting. It requires several weeks in Japan to obtain the result from commercial laboratories performing such tests. The patient got worse on the second day. To get a prompt and reliable diagnosis, we performed double staining of BM cells for ISH for EBER and IHC for CD3, which is the most commonly used method [4, 5]. As shown in Fig. 1c and d, most of the EBER positive cells were also positive for CD3, but not for CD20. We tentatively diagnosed EBV+ T-cell LPD of childhood, although the monoclonal proliferation of infiltrated T cells was not proven conclusively at this point. The negative status for anti-VCA IgG probably indicated that she was affected with acute primary EBV infection. Chemotherapy with PSL, cyclophosphamide, vincristine, and etoposide promptly resolved the fever, jaundice, hepatosplenomegaly, pancytopenia, and DIC. The liver biopsy specimens, which were obtained on day 19, showed residual lymphocytes positive for CD3 and EBER in the portal area. After 4 weeks, her diagnosis was confirmed by having monotonally rearranged TCR genes in BM cells.

More than 90% of adults have been infected with EBV. Primary infection often occurs in children and adolescents and may manifest as an acute infectious mononucleosis (IM). EBV enters CD21+ B cells and EBV-specific

cytotoxic T cells evoked to eliminate infected B cells [5]. EBV is known primarily for its ability to infect B cells, but it can also infect other cells [6–8]. EBV+ T-cell LPD of childhood is a systemic disease characterized by a clonal expansion of EBV-infected T cells with an activated cytotoxic phenotype [1]. Systemic EBV+ T-cell LPD of childhood is most prevalent in Asia, primarily in Japan and Taiwan [9, 10]. The details of how clonal EBV+ T cells are allowed to proliferate in patients have not been clarified. Some genetic defect in the host immune response to EBV is strongly speculated to be involved because of its association with primary EBV infection, and racial predisposition. The end-to-end length of fused terminal repeat may drive the clonal selection of EBV-infected cells as an oncogenic component during primary infection [11]. The most commonly involved sites are the liver and spleen followed by lymph nodes, BM, skin, and lung. The diagnosis of systemic EBV+ T-cell LPD of childhood is made by the presence of a clonal proliferation of EBV-infected T cells with an activated cytotoxic phenotype. In clinical practice, the infiltration of T cells which have immunophenotypic and genetic monoclonality into affected organs is a direct clue. In the present case, we could not perform a liver biopsy due to DIC on admission. We tentatively diagnosed EBV+ T-cell LPD of childhood on the basis of the infiltration of CD3 and EBER double positive cells in BM. Double staining of BM aspiration smears with CD3 and EBER takes only a day, and the presence of double positive cells indicates the infiltration of abnormal cells in BM, although monoclonal expansion of them was not proven. The detection of EBER-positive CD3 cells in BM is a diagnostic aid for EBV+ T-cell LPD of childhood.

Conflict of interest We declare that we have no conflict of interest.

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