Pyothorax-associated Lymphoma: Loss of Epstein-Barr Virus Nuclear Antigen-3B Protein Expression as a Result of Mismatch Repair Phenotypes

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Abstracts: Pyothorax-associated lymphoma (PAL) is an EBV-associated lymphoma, which develops in the pleural cavity of immunocompetent patients with an over-20-year history of pyothorax. PAL tumor cells taken from a 72-year-old man revealed having mismatch repair (MMR) phenotype and expression of all Epstein-Barr virus latent genes except EBNA-3B. EBNA-3B gene in the derived cell line had frameshift mutation in mononucleotide repeats, indicating that EBNA-3B was the target for the MMR phenotype. Because EBNA-3B works as target antigens for host cytotoxic T-lymphocyte (CTL) responses via two HLA-A11-restricted epitopes, loss of EBNA-3B expression may contribute for PAL cells to escape from host immune surveillance in the present case.

Key words: Pyothorax-associated lymphoma, Epstein-Barr virus, EBNA-3B, Mismatch repair phenotypes

Introduction

Malignant lymphoma frequently develops in the pleural cavity of patients with over 20-year history of pyothorax. This tumor stand as a distinct clinicopathologic entity, thus the term pyothorax-associated lymphoma (PAL) has been proposed for this type of tumor. PAL is now listed as a distinct disease entity in the recent World Health Organization Classification of tumors. PAL is non-Hodgkin lymphoma, mostly diffuse large B-cell lymphoma (DLBCL), which is strongly associated with Epstein-Barr virus (EBV) infection.

Previous study on a relatively small number of PAL cases reported the Latency (Lat) III pattern of EBV infection at protein level, i.e. the tumor cells express all 9 latent genes. Whereas, we recently demonstrated that the EBV latency pattern in PAL was various among cases. Latent infection antigens of EBV, including EBV nuclear antigens (EBNAs) and latent membrane proteins, are expressed in latently infected and immortalized B cells but they work as target antigens for host cytotoxic T-lymphocyte (CTL) responses in a HLA class I-restricted manner. Among these latent antigens, the immunodominant CTL epitopes in EBNA-3B (ERNA-3B 399-408 and EBNA-3B 416-424) are well characterized. The major cytolytic activity of the host CTL is induced via two HLA-A11-restricted epitopes in the viral EBNA-3B antigen.

Usually, there are no clinical findings suggesting the presence of immune dysfunction in patients with PAL. Therefore some mechanism for PAL cells to escape from host immune surveillance must be present. Production of immunosuppressive cytokine IL-10 by PAL cells, decreased expression of HLA class I molecules in PAL cells, or mutation of CTL-epitope in EBNA3B might be involved in the escape of PAL cells from CTL.

We have established several cell lines from PAL tissues; among them one line (OPL-3) with Lat III pattern of latent gene expression did not express EBNA-3B protein. Sequencing analysis revealed frameshift mutations in mononucleotide repeats within EBNA-3B gene, which generates premature stop codon, thus abolish EBNA-3B protein expression. In addition to reduction of HLA class I molecules, loss of the EBNA-3B expression may contribute to PAL cells to escape from host immune surveillance. The host tumor cells were mismatch repair (MMR) phenotype, which may cause in mutations of EBNA-3B genes.

Case report

A 72-year-old man was admitted to National Kinki-Chou Hospital with left chest pain. He had suffered from pulmonary tuberculosis in the left lung and received an artificial pneumothorax when he was 22 years old. Computed tomographic (CT) scan on admission of this time revealed the masses in the left pleural cavity and diaphragm,
Aspiration biopsy from the pleural cavity revealed a monomorphic proliferation of large lymphoid cells in the collagen and fat tissues (HE). Immunohistochemistry revealed that these cells were positive for CD79a and negative for CD3, thus diagnosed as diffuse large lymphoma of B-cell type. Few intermingling small lymphocytes were observed.

which were affected by pyothorax. Aspiration biopsy from the pleura revealed monomorphic proliferation of large lymphoid cells in the collagen and fat tissues (Fig. 1). Immunohistochemistry revealed that most proliferating cells were positive for CD20 and CD79a, and negative for CD3, thus diagnosed as diffuse large lymphoma of B-cell type. As for EBV gene expression, both LMP-1 and EBNA-2 were positive in immunohistochemistry. A cell line was established from a part of the sample with informed consent in accordance with the Declaration of Helsinki\(^1\). HLA-A allele of the patient was A2/A11 (data not shown). Flow-cytometric analyses of the cell line revealed slightly reduced, but definite expression of HLA class I molecule (Fig. 2). Radiographic examination including abdominal CT, brain magnetic resonance imaging, and Gallium scintigraphy together with bone marrow biopsy revealed metastasis in the spleen. Combined two courses of chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisolone) and 50 Gy of radiation therapy were started, but he died of disease 30 months after admission. Autopsy was not performed.

**Materials and Methods**

1. **HLA-A allotyping and flow-cytometric analysis for HLA class I**

Low-resolution HLA-A locus-specific PCR typing of the patient was performed using HLA-A locus sequence-specific primers combinations in PCR amplification of genomic DNA as described previously\(^9\). Flow-cytometric analysis for expression of HLA class I molecule in the cell lines was done.

2. **RT-PCR analysis for detection of EBV latent gene expression**

Total RNA was extracted from the samples with the TRIzol reagent (Invitrogen, Inc., Rockville, MD) according to the manufacturer’s instructions. Five \(\mu\)g of total RNA were reverse-transcribed by random hexamer priming using the Superscript first strand synthesis system (Invitrogen). Primer pairs were designed to distinguish different EBNA-1 promoter usage from each other, and to detect EBNA-2 and
EBNA-3B transcripts as described previously\textsuperscript{5,12}.

3. Detection of mutations

Long-distance PCR was performed to amplify the whole EBNA-3B gene. After electrophoresis on 1.0\% agarose gel, DNA fragments were excised and purified using the Wizard SV gel extraction kit (Promega Co., Madison, WI). The purified DNA was used as template for PCR, and the products were directly sequenced with appropriate primers.

4. Western blotting

Western blotting was carried out as described previously\textsuperscript{13}. Briefly, whole cells were lysed in 1 x sample buffer, separated with 10\% SDS-PAGE, and then blotted to polyvinylidene difluoride membrane using a wet-blotting apparatus. Anti-LMP-1 and -EBNA-2 antibodies were purchased from DAKO (Glostrup, Denmark), and anti-EBNA-3B antibody from Exalpha Biologicals (Boston, MA). Anti-actin antibody (Sigma, St. Louis, MO) was used as control. Signals were visualized with ECL plus Chemiluminescent reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Results

The cell line (OPL-3) established from the patient was an immunophenotype of B cell and similar to that of the original biopsy specimen (i.e. positive for CD20, CD30 and CD79a, and negative for CD3, CD4, CD5, CD7, and CD8), which were confirmed with three different subclones. Terminal repeat of fusion of EBV in lymphoma cells was monoclonal pattern. Karyotype was complex with numerous structural and numerical abnormalities. Disruption of the MMR system, as revealed by Microsatellite Instability (MSI) was found (Fig. 3\textsuperscript{11}). According to the International Workshop on MSI and RER phenotypes in Cancer Detection and Familial Predisposition\textsuperscript{14}, four of five microsatellite repeats were positive. MMR target genes such as \textit{IGFIIR} and \textit{hMSH6} were also mutated. Promoter usage of EBNA-1 was Lat III pattern in OPL-3 (Fig. 4). LMP-1, EBNA-2, and EBNA-3B were also expressed as expected. LMP-1 and EBNA-2 proteins were expressed as well, whereas, EBNA-3B protein was not detected by Western Blotting analysis (Fig. 5).

Sequencing analysis of EBNA-3B in OPL-3 and peripheral blood leukocytes from the patient was performed. Compared with EBV genome in the strain B95-8, (Gene Acc V01555.2), more than 10 polymorphisms were observed in EBNA-3B from both OPL-3 and
Fig. 5  Latent gene protein expression in PAL cell lines by Western blotting. Expression of EBNA-3B was not detected in OPL-3.

Fig. 6  Sequencing of EBNA-3B gene containing at mononucleotide tracts from nucleotide 96007 to 96013 in OPL-3, which generates premature stop codon, was observed in OPL-3 but not in the peripheral blood leukocytes (Fig. 6).

Change in mononucleotide repeat lengths was not observed in other EBV latent genes including, LMP-1 (poly C7; nt number 5804-5810 in B95-8), EBNA-2 (poly C8; nt number 48696-48703), EBNA-3A (poly C7; nt number 94177-94183, nt number 94607-94703), EBNA-3B (poly A7; nt number 95837-95893, poly G7; nt number 95387-95393, nt number 95999-96005, number 98075-98081), EBNA-3C (poly C7; nt number 100738-100744), EBNA-1 (poly G7; nt number 107026-107032) as well as OriP regions (poly A8; nt number 8339-8346, poly C8; nt number 9439-9446) (data not shown).

Discussion

PAL is an EBV-associated lymphoma, which develops in patients with over 20 year-history of chronic pyothorax and with no evidence of immune dysfunction. Because most PAL expresses full range of EBV latent genes, escape mechanisms from host CTL should work in PAL. Indeed, OPL-3 showed the Lat III pattern of EBV latent gene expression and expressed HLA-A11. However, histologic examination of biopsy sample did not reveal the
findings suggestive of CTL response against the proliferating PAL cells, i.e., infiltration of T lymphocytes.

EBNA-3B protein was not expressed in OPL-3. The sequencing analysis revealed the frameshift mutations within EBNA-3B gene, which might result in loss of the EBNA-3B protein. The mutations were insertions within mononucleotide repeats, which are frequently observed in cells with MMR phenotypes. In the previous report(11), we demonstrated that OPL-3 had MMR phenotypes and MMR-target genes such as IGFIIR and hMSH6 was mutated. Because EBV replication is synchronized with host cell division using the host replication system, the simple repeat sequences in EBV genome might also be the target in the host with MMR phenotype. EBV genome contains many mononucleotide repeats. In the present study the insertion G was observed in only EBNA-3B gene in OPL-3. Further in vitro and in vivo study may be necessary whether loss of EBNA-3B may be growth advantage or not.

MMR type is rare in non-Hodgkin lymphoma except for immunodeficiency-related lymphomas such as HIV infection-related lymphomas and posttransplant lymphoproliferative disorders(19). Immunotherapy employing antigen-specific T cells for treatment of human malignancies is becoming popular. EBNA-3B epitope is one of the candidates for the virus-specific CTL therapy in fatal lymphoproliferative diseases. Gottschalk et al reported a case in which CTL therapy to fatal lymphoproliferative disorders resulted in the selection of clone with EBNA-3B deletion(20). It is possible that EBNA-3B deletion clone might be selected in such lymphomas as same mechanism as observed in the present case.

The present case suggests that not only host genome but also infected EBV genome could be targets for mutations in cells with MMR phenotype. Resultant loss of latent gene EBNA-3B expression provides an advantage for tumor growth via escape from host CTL responses. This seems to be one of the mechanisms how PAL cells avoid from CTL response. It is interesting to know whether other EBV-related lymphomas have deficiency in EBNA-3B expression.

**References**

ミスマッチ修復異常によって EBNA-3B 蛋白の発現消失を来したと考えられる膿胸関連リンパ腫の1例

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要約

膿胸関連リンパ腫（PAL）は Epstein-Barr ウイルス（EBV）関連リンパ腫の一つで、免疫能低下などのみられない慢性膿胸の患者において、20年以上の慢性炎症を経て胸腔に発症する。72歳の男性症例から採取された同リンパ腫において EBV の潜伏遺伝子の発現は EBNA-3B 遺伝子を除いて発現が認められた。EBNA-3B 蛋白は宿主の CTL 応答の標的として働くので、その発現消失は、免疫監視機構を回避し腫瘍増殖に有利に働いた可能性がある。EBNA-3B 遺伝子の塩基配列を検討すると単塩基重複配列部分に塩基の欠失がみられた。このような欠失はミスマッチ修復異常（MMR type）により生じることが知られ、同腫瘍細胞も MMR type であった。宿主の MMR type が感染した EBV ゲノム変異に影響を及ぼした点は大変興味深い。