



Epstein-Barr virus genome level, T-cell clonality and the prognosis of angioimmunoblastic T-cell lymphoma

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Background and Objectives. Angioimmunoblastic T-cell lymphoma (AILT) is a peripheral T-cell tumor of unknown etiology with variable biological and clinical presentations. Previous clonality studies have shown heterogeneous clonal restrictions of B- and T-cell populations in this tumor. AILT is characterized by the presence of increased numbers of Epstein-Barr virus (EBV) infected cells. The aim of this study was to clarify the correlation between clonality, EBV and prognosis.

Design and Methods. Frozen material from 59 cases of AILT was used for DNA isolation and gene analysis by Southern blotting. A real-time polymerase chain reaction was used to quantify the amount of EBV-DNA in the tissue. Survival data were retrieved from clinical records.

Results. Clonal T cells were found in 15/50 and clonal B-cells in 2/50 tumors, using Southern blot analysis. Bands of EBV-W were found in 10/50 tumors. Survival rate did not correlate with either T-cell clonality ($p=0.84$), or presence of EBV-infected cells ($p=0.84$). The EBV-DNA copy number in EBV-infected tissue did not correlate with disease progression ($p=0.87$). The survival rate and clinical status according to the international prognostic index (IPI) did not correlate with T-cell clonality status or EBV infection.

Interpretations and Conclusions. AILT remains a heterogeneous disease with clinical behavior that varies irrespective of the genomic parameters investigated.

Key words: AILT, TCR, EBV infection, disease progression.

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Angioimmunoblastic T-cell lymphoma (AILT), originally described by Frizzera *et al.* in 1974, is a systemic disease of uncertain etiology, in which the major pathological findings are noted in the lymph nodes.^{1,2} Node biopsy specimens share a common appearance, with normal tissue effaced by a mixed cellular infiltrate in which immunoblasts and plasma cells are prominent. This cellular infiltrate is accompanied by a striking arborizing proliferation of blood vessels and by an amorphous deposition of acidophilic material between the cells.¹⁻³

The disease has generally been considered to be an abnormal hyperimmune reaction. Immunophenotypic studies have identified the majority of proliferating cells as T cells of either CD4 (majority) or CD8 (minority) type.⁴ DNA clonal analysis studies have shown heterogeneous T-cell receptor (TCR) and immunoglobulin heavy chain (IgH) gene rearrangements. The majority of cases of AILT exhibit monoclonal rearrangements of TCR genes, although a small number of cases show polyclonal

rearrangements. Some cases show rearrangements of heavy and light chain immunoglobulin genes, and co-existing rearrangements of TCR and immunoglobulin genes.⁵⁻⁸ Furthermore, clonal rearrangements have been shown to regress or appear during the course of the disease.⁹

In 1976, Bornkamm *et al.*¹⁰ found evidence of Epstein-Barr virus (EBV)-DNA in one case of AILT using nucleic acid reassociation kinetics. Subsequently, there have been similar sporadic publications based on Southern blot analysis.¹¹ In more recent studies using the more sensitive polymerase chain reaction (PCR) technique, EBV-DNA was demonstrated in 7 of 13 cases¹¹ and 5 of 8 cases.¹² Weiss *et al.*¹³ demonstrated the presence of EBV-DNA in 96% of cases of AILT using PCR and a highly sensitive RNA *in situ* hybridization technique. Recently, a novel real-time quantitative PCR method was developed.¹⁴ This method measures the accumulation of PCR products with a fluorogenic probe and real-time laser scanning in a 96-well plate. Since this assay does not

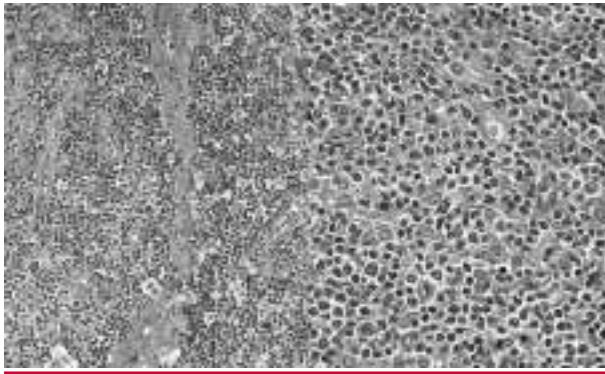


Figure 1. Low power magnification shows architectural effacement of the lymph node by a polymorphous lymphoid infiltrate. Prominent arborizing blood vessels are evident. The infiltrate is composed of medium to large sized lymphoid cells with abundant clear cytoplasm.

require post-sample handling, much faster assays are possible. The assay can detect a very large dynamic range of target molecules because the real-time measurement of the PCR product enables quantification of the amplified products in the log phase of the reaction.¹⁴

EBV infection is related to AILT. However, it is uncertain whether there is any relationship between EBV infection, clonal TCR rearrangement, and disease progression. The present retrospective molecular study of 50 archival cases of AILT was designed to assess the following: (i) the incidence of clonal/oligoclonal TCR, (ii) the incidence of EBV infection, (iii) the levels of EBV-DNA in lymph node specimens using real-time PCR, and (iv) the prognostic significance of T-cell clonality and EBV presence in AILT.

Design and Methods

Tissue specimens filed in the Department of Pathology, Fukuoka University, Japan were used in the present study. The lymph nodes obtained were fixed in buffered formalin or B5 solution, embedded in paraffin wax, and stained with hematoxylin-eosin. Immunostaining was performed using CD20 for B-cells (Dakopatts; Glostrup, Denmark), and CD3 and UCHL-1 for T cells (Dakopatts). The sections were graded by two histopathologists (MK & KO). The histological diagnosis of AILT was based on the criteria in the World Health Organisation lymphoma classification (Figure 1). Part of the frozen material was used for DNA isolation and gene analysis by Southern blotting. The details of the examination methods have been reported previously.¹⁰ The T-cell receptor gene C β 1, the immunoglobulin heavy chain (JH) gene, and the EBV gene (BamHI W region) (Enzo; Hudson, NY, USA) were used as probes. DNA was digested with restriction

enzymes EcoRI, HindIII, or BamHI. We then performed real-time quantitative PCR with a fluorogenic probe. The PCR primers for this assay were selected in the BALF5 gene encoding the viral DNA polymerase.¹⁵ The upstream and downstream primer sequences were 5'-CGGAAGCCCTCTGGACTTC-3' and 5'-CCCTGTTTATCCGATGGAATG-3', respectively. A fluorogenic probe (5'-TGTACACGCACGAGAAATGCGCC-3') with a sequence located between the PCR primers was synthesized by PE Applied Biosystems (Foster City, CA USA). The PCR reaction was performed using the TaqMan PCR kit (PE Applied Biosystems) as described previously.¹⁶ Briefly, DNA from each lymph node was added to a PCR mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM MgCl₂, 100 μ M dATP, dCTP, dGTP, dTTP, 0.2 μ M of each primer, 0.1 μ M fluorogenic probe, and 1.25 U of AmpliTaq Gold (PE Applied Biosystems). Following activation of the AmpliTaq Gold for 10 min at 95°C, 45 to 50 cycles of 15 sec at 95°C and 1 min at 62°C were carried out in a model 7700 Sequence Detector (PE Applied Biosystems).

Real-time fluorescence measurements were taken, and the threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline).¹⁴ For a positive control, a plasmid that contained the BALF5 gene was constructed from pGEM-T vector (Promega, Madison, WI, USA) and termed pGEM-BALF5. A standard graph of the C_T values obtained from serially diluted pGEM-BALF5 was constructed. The C_T values from clinical samples were plotted on the standard curve, and the copy number was calculated automatically by Sequence Detector version 1.6 (PE Applied Biosystems), a software package for data analysis. Each sample was tested in duplicate, and the mean of the two values was taken as the copy number of the sample. Samples were defined as negative if the C_T values exceeded 50 cycles. We divided cases into two groups based on the amount of EBV-DNA genome. We initially divided the group into three patterns because the amount of EBV-genome varied from case to case: pattern 1, greater or less than 100 copies; pattern 2, greater or less than 1,000 copies; and pattern 3, greater or less than 10,000 copies. We evaluated the relationship between the amount of EBV-genome and prognosis, but did not find definitive differences for any pattern.

We, therefore, counted the copies of EBV-DNA genome and defined cases as negative (<10³ copies/ μ g of DNA) or positive (>10³ copies/ μ g of DNA).

Statistical analysis

Survival curves were calculated using the Kaplan-Meier method, and differences in survival rates were

tested for significance using the generalized Wilcoxon test. Differences were considered statistically significant if the p value was <0.05 .

Results

Clinical data

Fifty-nine patients diagnosed with AILT at the Fukuoka University Hospital between 1988 and 2003 were included in the study. At the time of diagnosis, the patients were aged between 42 and 81 years (median age= 63 years). The 5-year survival rate was 45% (Figure 2A).

Histopathological and immunohistochemical examination

The pathognomonic histological feature was the total disappearance of normal lymph node architecture, with prominent proliferation of venules and polymorphic infiltrates of CD3⁺ lymphocytes, plasma cells and eosinophils. Proliferation of dendritic reticular cells was also evident. Clear cell infiltrates were noted around the vessels, and CD20⁺ cells were present in the subcapsular region. The EBV-infected cells were B cells. This was demonstrated by double-labeling immunohistochemical/*in situ* studies of the samples. The majority of the cells positive for EBV RNA were labeled with the B-lineage marker, CD20, and were negative for the T-cell marker, CD45RO (*data not shown*). This result has been previously reported.¹⁷

TCR and IgH genes

Clonal rearrangements of the TCR genes were detected in the lymph nodes of 15 out of 50 of the patients with AILT. Clonal IgH rearrangements were identified in two cases, and oligoclonal bands were observed in one case.

EBV gene rearrangement in Southern blot analysis and real-time PCR

Southern blot analysis revealed EBV-W bands in lymph nodes of 10 out of 51 patients. The lymph nodes of 33 of the 49 patients studied by real-time PCR analysis were positive ($>10^3$ copies/ μ g of DNA) for EBV-DNA genes.

Survival analysis based on TCR clonality status and EBV infection

There was no correlation between survival and TCR clonality ($p=0.84$, Figure 2B) or EBV-W bands ($p=0.84$, Figure 2C). We also used a real-time PCR assay to quantify the amount of EBV-DNA in the tissue. EBV-DNA copy numbers in the tissue from patients with EBV infections did not correlate with

disease progression (negative: $<10^3$ copies, positive: $>10^3$ copies) ($p=0.872$, Figure 2D).

The clinical status, as defined by the international prognostic index (IPI), did not correlate with TCR-clonality, EBV-W bands or EBV-DNA copy numbers as assessed by real-time PCR.

Discussion

AILT is a systemic disease of uncertain etiology, originally defined as a non-neoplastic lymphoproliferative process.^{1,2} The reactive features indicate that this lymphoma may originate from a hyperplastic/dysplastic state of T cells.² Scattered EBV-infected lymphocytes have been documented in up to 97% of AILT cases.⁷ Double-labeling techniques have revealed the unexpected presence of EBV mainly in B cells, rather than in the T-cells which constitute the major neoplastic population in AILT.¹⁹ This suggests that a small population of cells is infected with EBV or, more likely, EBV is reactivated in these cells as a consequence of immunosuppression in a primarily T-cell disorder. It is well known that patients with AILT have a profound immunologic deficiency and that intercurrent infections are frequent and a common cause of death. It is possible that abnormal immunoregulation leads to polyclonal and oligoclonal proliferation of EBV-positive cells, which are mostly B cells. EBV-infected B cells may have a growth advantage and proliferate into polyclonal or oligoclonal populations under these conditions. Abnormal immunoregulation may also explain the development of clonal rearrangements of Ig genes observed in a subset of AILT patients,¹⁸ and may similarly explain the presence of Ig gene rearrangements that appear and disappear over time as reported in a previous study.⁸

However, in another study¹⁷ no association was found between the amount of EBV and Ig gene rearrangement. This was probably due to the presence of a polyclonal EBV-infected B-cell population. However, it was uncertain whether there is a correlation between the clinical course of AILT and EBV infection. We initially predicted that the prognosis and immunodeficient state of AILT lymphoma would be correlated with the EBV infection. However, the data obtained from real-time quantitative PCR and Southern blotting analysis showed no relationship between the prognosis of patients with AILT and EBV infection.

Clonal rearrangements of TCR and IgH genes are useful molecular markers of clonality in T- and B-cell lymphomas. Frizzera *et al.*²⁰ reviewed data from previous studies that used Southern blotting and found that rearrangement of the TCR gene had been detected in 85% of cases of AILT. In addition, 8% had rearrange-

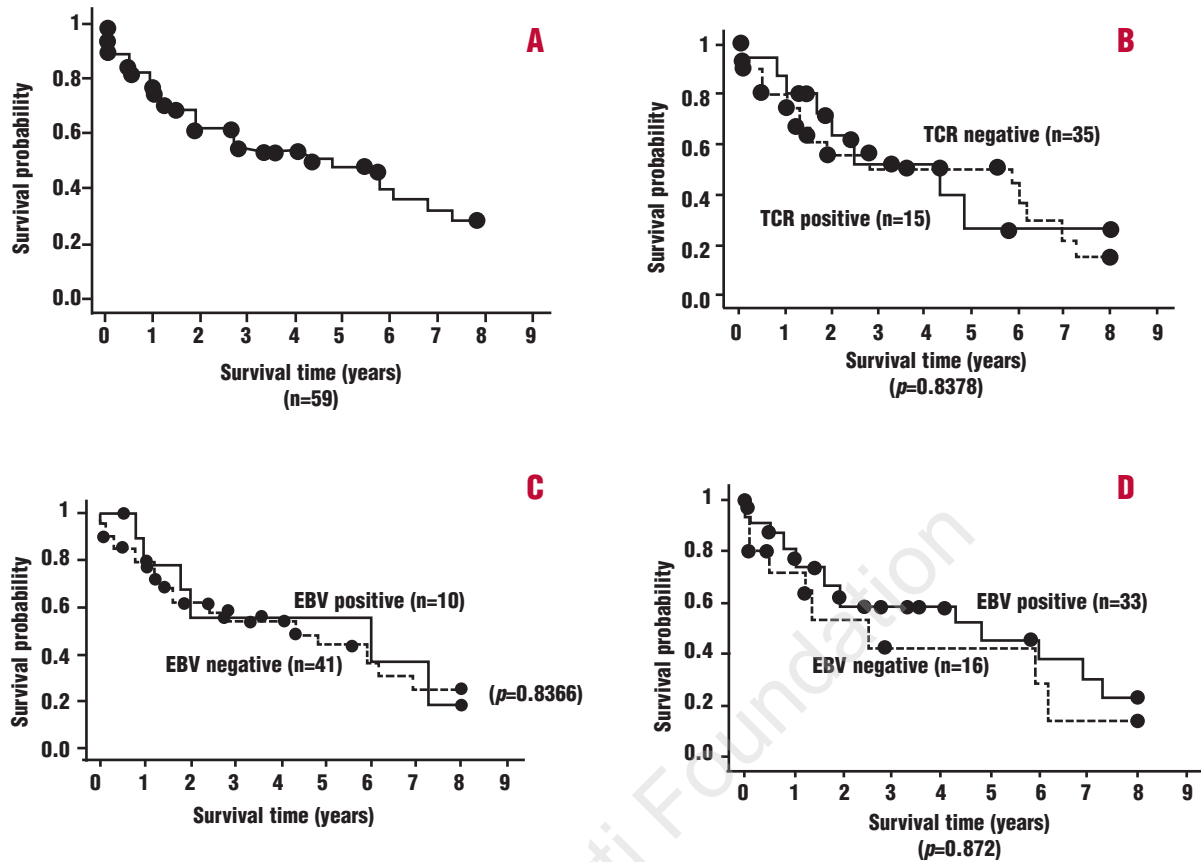


Figure 2. Survival rate of all participating patients with AILT (A), and according to TCR clonality (B), bands of EBV-W (C) and EBV-DNA by real-time PCR (D).

ments of both the Ig heavy chain and one light chain gene, or of the lambda chain gene only, indicating the presence of B-cell clones. Furthermore, 7% had co-existing rearrangements of the TCR and Ig heavy or light chain genes, indicating the presence of either two different clonal populations or one clone of indefinite lineage, carrying both TCR and Ig gene rearrangements. It is significant that in AILT, as in other atypical lymphoproliferative disorders,²¹ the rearrangement bands are multiple and of significantly lighter intensity than normal bands.^{7,8}

In addition, one study found that multiple different clones appeared and disappeared over time in the same patient.⁸ Weiss *et al.*⁷ provided evidence that a *prelymphomatous* lesion may exist and one patient, whose initial lymph node biopsy specimen was interpreted as AILT, showed no evidence of immunoglobulin or TCR gene rearrangement. The present study also confirmed the heterogeneity of TCR and Ig gene rearrangements in AILT. Clonal rearrangements were observed to regress or appear during the course of the disease (*data not shown*).

O'Connor *et al.*⁶ proposed two hypotheses concern-

ing the etiology of AILT. Firstly, if a monoclonal proliferation of T-cells is always present, the pronounced polyclonal proliferation of T and B cells characteristic of this disorder may merely reflect a reaction to the underlying monoclonal population. However, an alternative hypothesis is that AILT is initially a polyclonal hyper-reactive disorder affecting both T and B cells, and that T-cell clones arise as a secondary event. These hypotheses are based primarily on the gene rearrangements seen in Southern blot analysis, which cannot detect a monoclonal population of either B or T cells when they constitute less than 5% of the cells present in tissue samples.^{22,23} However, PCR can detect smaller monoclonal populations of under 2%.²⁴

Our previous PCR data²⁵ support the hypothesis of selective proliferation of one clone of T cells among several clones, rather than an initial monoclonal proliferation of T cells. In view of these findings, we consider that disease progression in AILT patients is associated with clonal T-cell populations. It should be mentioned that Lee²⁶ and Willenblock²⁷ suggested from a single cell study that only a minority of T cells belong to the tumor cell population. Therefore, the failure to

detect clonal populations may be purely technical.

The TCR clonality status had no influence on survival in AILT patients. Cytokines play an important role in infectious diseases, and are important in the pathogenesis of lymphomas via autocrine or paracrine mechanisms, or both.²⁸ AILT is a lymphoproliferative disease characterized by general lymphadenopathy, fever, weight loss, and skin rashes.¹² AILT patients may develop hypergammaglobulinemia or raised levels of cytokines, including interleukin. We also reported an increase in the number of cytokine-producing lymphoma cells and EBV genomes in AILT. Considered together, the immunodeficiency and hypercytokinemia of AILT do not seem to exclude the role of EBV-infected cells in AILT.

In conclusion, TCR clonality status and EBV infection had no influence on the survival of AILT patients. It is possible that AILT is a disease of immunodeficiency with a clinical behavior that varies irrespective of these parameters.

RK, SW, JS, MK: substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; KO: drafting the article or revising it critically for important intellectual content; KT; final approval of the version to be submitted. The authors declare that they have no potential conflict of interest.

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