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Preferential Expansion of Vγ9-JγP/Vδ2-Jδ3 γδ T Cells in Nasal T-Cell Lymphoma and Chronic Active Epstein-Barr Virus Infection

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We recently established an Epstein-Barr virus (EBV)-positive γδ T-cell line from a nasal T/natural killer (NK)-cell lymphoma (Nagata H, Konno A, Kimura N, Zhang Y, Kimura M, Demachi A, Sekine T, Yamamoto K, Shimizu N: Characterization of novel natural killer (NK)-cell and γδ T-cell lines established from primary lesions of nasal T/NK-cell lymphomas associated with the Epstein-Barr virus. Blood 2001, 97:708–713). Subsequently, we established two novel EBV-positive γδ T-cell lines from the peripheral blood of patients with chronic active EBV infection. Analysis of the terminal repeat of EBV showed that the three cell lines consisted of monoclonal populations, and flow cytometry showed that they had a common phenotype of γδ T cells: $CD3^+$ $CD4^-$ $CD8^-$ $CD16^-$ $CD19^-$ $CD56^+$ $CD57^-$ HLA-DR^+ T-cell receptor (TCR) $\alpha\beta^-$ TCR $\gamma\delta^+$. Analysis for the expression of TCR by flow cytometry showed that all three cell lines were $V\gamma9^+/V\delta2^+$, but negative for VγI, Vδ1, or Vδ3 TCR. Southern blot analysis for TCR genes showed that the three cell lines had a common rearrangement of Vγ9-JγP and Jδ3 genes. Polymerase chain reaction and sequence analysis of the junction between Vδ and Jδ genes revealed that the Jδ3 genes were rearranged with the Vδ2 genes. In contrast, none of the EBV-negative γδ T-cell lines, Molt-14, Peer, or Loucy, which were analyzed for controls, had Vγ9 or Vδ2 TCR, or a rearrangement of Jδ3 genes. These results indicated that $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ γδ T cells were preferentially affected by EBV and expanded in patients with nasal γδ T-cell lymphoma and chronic active EBV infection. $J\delta3^+$ γδ T cells are known to be a very minor population in γδ T cells of peripheral blood, whereas $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta1^+$ cells are the major population. The close association of EBV with this particular γδ T-cell population may provide a key to the etiology of EBV-positive lymphoproliferative diseases. *(Am J Pathol 2003, 162:1629–1638)*

γδ T cells comprise only a small proportion (1 to 5%) of the lymphocytes in the peripheral blood and organs, but are far more widespread within epithelial-rich tissues such as the skin, gut, and reproductive tract, where they comprise up to 50% of T cells.[1] Postthymic peripheral T-cell lymphomas of the γδ T-cell subset are rare, and most present as hepatosplenic γδ T-cell lymphomas.[2,3] Otherwise, they arise in the skin, nose, larynx, lung, or gastrointestinal tract.[4] They are believed to include some different disease entities based on their clinical and morphological features. Hepatosplenic γδ T-cell lymphomas show a specific sinusoidal localization of malignant cells in the spleen, liver, and bone marrow. Other nonhepatosplenic γδ T-cell lymphomas appear to be regarded as activated cytotoxic lymphomas arising in the mucosae and skin. Among these, nasal γδ T-cell lymphomas in particular show a strong association with Epstein-Barr virus (EBV), and together with natural killer (NK)-cell lymphomas, constitute the distinct disease category nasal T/NK-cell lymphoma.[4–7]

The relationship between EBV and peripheral T/NK-cell neoplasms has been studied intensely since 1988, when Kikuta and associates[8] detected EBV in T lymphocytes in a boy with chronic active EBV infection (CAEBV),

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Table 1. Clinical Profile of Patients

Patient no.	Age/sex	Diagnosis	VCA IgG	Anti-EBV titers EA-DR IgG	EBNA	Peripheral blood findings	Cell line	Origin of cell line
1*	48/F	Nasal T/NK-cell lymphoma	640	10	20	WBC count 4400 (3% γδ T cells in total lymphocytes)	SNT-8	Primary lesion in the nasal cavity
2	13/F	CAEBV	1280	320~640	20~40	WBC count 3500 to 6000 γδ T-lymphocytosis (50 to 70% in total lymphocytes)	SNT-13	Peripheral Blood
3	15/F	CAEBV	1280	160	10	WBC count 1950 γδ T-lymphocytosis (23% in total lymphocytes)	SNT-15	Peripheral Blood

CAEBV, chronic active Epstein-Barr virus infection; VCA, viral capsid antigen; EA-DR, early antigens-diffuse and restricted; EBNA, Epstein-Barr nuclear antigen; WBC, white blood cell.
*Case 1 was previously reported.[7]

and Jones and colleagues[9] identified EBV in T-cell lymphomas arising in patients with CAEBV. As a result, it is now recognized that nasal T/NK-cell lymphoma has the strongest association with EBV among various kinds of peripheral T/NK-cell lymphomas.[2,3,10] However, studies on this disease have been limited because of the rarity of the disease and the tendency of the tumor to cause necrosis. To investigate this disease, we recently established two EBV-positive NK- and γδ T-cell lines, SNK-6 and SNT-8, from independent patients with nasal T/NK-cell lymphomas, showing that there are at least two phenotypes in this tumor, NK and γδ T cells.[7]

Another important lymphoproliferative disease inevitably associated with EBV is CAEBV. In most CAEBV cases, the T and NK cells become positive for EBV,[8,9,11–25] and such EBV-positive T or NK cells showed monoclonal or oligoclonal expansion in many cases.[11,12,17,19,20,24,25] In addition, in some severe CAEBV cases, the establishment of cell lines of T- and NK-cell lineage has been reported,[16,21] suggesting that the clonal expansion of T/NK cells in CAEBV is in part because of an acquired great proliferative capacity brought by EBV. In an effort to understand the role of EBV in lymphoproliferative diseases of T/NK-cell lineage, we have been trying to establish cell lines from patients with CAEBV. Fortunately, we have to date successfully established six EBV-positive cell lines from the peripheral blood of patients with CAEBV. These include two NK−cell lines, two αβ T-cell lines, and two γδ T-cell lines.

In the present study, to clarify the role of EBV in the lymphoproliferative diseases of γδ T cells, we determined to analyze and compare the three EBV-positive γδ T-cell lines established. One was the previously reported SNT-8, and the other two were newly established lines from patients with CAEBV, SNT-13 and SNT-15. We aimed to analyze the phenotypes of the cell surface markers, the expression of T-cell receptors (TCRs) and the rearrangement of TCR genes in the cell lines. In addition, EBV-negative γδ T-cell lines, Molt-14, Peer, and Loucy were used for controls. We determined that Vγ9-JγP/Vδ2-Jδ3 TCR was commonly expressed in the three EBV-positive cell lines, but was not observed in the EBV-negative cell lines. The present study suggests that EBV probably affects γδ T cells expressing specific TCR to develop lymphoproliferative diseases.

Materials and Methods

Patients and Cell Lines

Three cell lines, designated as SNT-8, SNT-13, and SNT-15, established from patients with nasal T-cell lymphoma or CAEBV were used in this study. The clinical features of the patients are summarized in Table 1. SNT-8 is a γδ T-cell line established from the primary lesion of a nasal T-cell lymphoma. Some of the characteristics of the cell line, as well as the case history, have been reported previously.[7] The other two cell lines were newly established from the peripheral blood of two unrelated patients with CAEBV. Both patients suffered from chronic mononucleosis-like symptoms, but showed no immunological abnormality or infection other than EBV infection; patient 2 had repeated fever and aphthae, and patient 3 had hepatosplenomegaly. They showed elevated titers for IgG to the Epstein-Barr viral capsid antigen (VCA-IgG) and diffuse and restricted components of early antigens (EA-DR IgG). They were thus diagnosed as having CAEBV in accordance with the criteria described by Straus.[26] They had increased γδ T-cell numbers among peripheral blood lymphocytes, and their peripheral blood was strongly positive for the EBV genome by polymerase chain reaction (PCR).

For controls, three EBV-negative γδ T-cell lines, Molt-14, Peer, and Loucy, were analyzed in parallel. Molt-14 and Peer were obtained from Fujisaki Cell Center (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan), and Loucy was kindly provided by Dr. Hannah Ben-Bassat (Hadassah University Hospital, Jerusalem, Israel). The origin of these EBV-negative cell lines has been described elsewhere.[27–29]

Establishment of EBV-Positive γδ T-Cell Lines

The method used for establishing SNT-8 was described in our previous report.[7] Essentially the same method was used for the establishment of the SNT-13 and SNT-15 cell lines. Briefly, peripheral blood mononuclear cells were isolated from 10 ml of peripheral blood of the patients with CAEBV by the Ficoll-Hypaque method, and suspended in a 10-ml RPMI 1640 medium supplemented with 10% heat-inactivated human serum and 700 U/ml of

interleukin (IL)-2. Then, $CD4^+$ and $CD8^+$ lymphocytes were removed using anti-CD4 and anti-CD8 monoclonal antibody-conjugated magnetic beads (Dynal, Oslo, Norway). The remaining $CD4^-$ $CD8^-$ cell fraction was seeded in a 96-well plate at 1×10^4 cells/well and cultured in a humidified atmosphere at 37°C with 5% CO_2. Two days later, rapid cell growth was observed in all wells of the plate. After 1 week of culture, the growing cells were collected together. These cultured cells have been maintained in the presence of IL-2 for more than 40 months and 34 months, respectively, and were designated SNT-13 and SNT-15.

Flow Cytometric Analysis

The cell lines were analyzed by two-color immunofluorescence with a flow cytometer (EPICS XL; Beckman Coulter, Hialeah, FL) for the expression of surface markers. The following antibodies conjugated with fluorescein isothiocyanate or phycoerythrin (Becton Dickinson, San Jose, CA) were used: anti-CD3, -CD4, -CD8, -CD16, -CD19, -CD21, -CD56, -CD57, -HLA-DR, -TCR α/β, and -TCR γ/δ.

Flow cytometry was also performed to analyze variable regions of γδ TCR expressed on the cells. Antibodies used were anti-Vγl and anti-Vδ1 monoclonal antibodies (Beckman Coulter), which required secondary antibodies to be detected: $F(ab')_2$ goat anti-mouse IgG conjugated with fluorescein isothiocyanate was used. Other antibodies used were fluorescein isothiocyanate-labeled anti-Vγ9, anti-Vδ2, and anti-Vδ3 monoclonal antibodies (Beckman Coulter).

Assessment of the Number of EBV Terminal Repeats

To determine the clonality of the EBV-positive cell lines, the number of EBV terminal repeats (EBV-TR) was assessed by Southern blot analysis. DNA extracted from the cell lines was digested with *Bam*HI, separated on a 0.7% agarose gel, and transferred to a nylon membrane. A 1.9-kb *Xho*I subfragment of *Bam*HI-Dhet derived from EBV termini was used as a probe for EBV-TR DNA.[30] Hybridization was visualized using a Fluorescein Gene Images System (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. Raji and B95-8 cells were used as controls for monoclonal cell expansion with a single EBV-TR and polyclonal cell proliferation with EBV replication, respectively.

Southern Blot Analysis for TCR and Immunoglobulin Genes

The cell lines were analyzed for rearrangements of the TCR β-, γ-, and δ-chain and the immunoglobulin heavy-chain genes by Southern blotting. Three μg of DNA extracted from each of the cell lines was digested with a restriction endonuclease *Bam*HI, *Eco*RI, *Hind*III, or *Kpn*I (Takara, Kyoto, Japan), electrophoresed through a 0.7% agarose gel, and transferred onto a nylon membrane. Human placental DNA was used as a germline control. The membrane was exposed to a fluorescein-labeled Cβ1, Jγ1, Jδ1, or Jδ3 probe for the TCR genes, or a J_H probe for the immunoglobulin heavy-chain gene.[31–35] The Jγ1 probe, a 700-bp *Hind*III-*Eco*RI fragment, includes the Jγ1 segment and cross-hybridizes with Jγ2 but not with the additional Jγ segments. The Jδ1 probe consists of a 1-kb *Pst*I-*Eco*RI segment. The Jδ3 probe was a 1.5-kb *Xba*I genomic fragment of the Jδ3 region. The organization of the human TCRγ and TCRδ loci is shown in Figure 1. Hybridization was visualized by the same methods used for the Southern blot analysis of the EBV-TR.

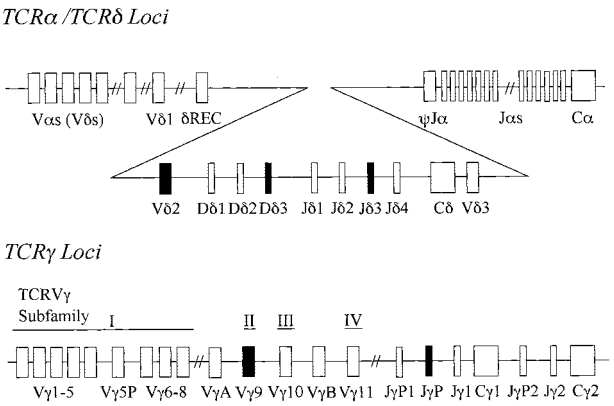

Figure 1. Schematic organization of the human TCRδ and TCRγ loci. The TCRδ locus is located entirely within the TCRα locus between the TCRVα and the TCRJα gene segments. TCRVγ genes are divided into four subfamilies (I to IV) based on sequence similarity. Each Vγ gene is assigned to its subgroup as indicated. The **solid boxes** are gene segments used in SNT cell lines.

PCR Analysis for Combinations of Vδ and Jδ Genes

To define Vδ-Jδ gene rearrangements in the cell lines, PCR analysis of the Vδ1, Vδ2, or Vδ3 gene and the Jδ1 or Jδ3 gene was performed. The sequences of primers used are listed in Table 2.

Fifty ng of DNA extracted from each cell line was amplified with a pair of each combination of L-Vδ1, L-Vδ2, or L-Vδ3 and 3′-Jδ1 or Jδ3 primers. The PCR was performed with reagents composed of 500 nmol/L of primers, 200 μmol/L of dNTPs, 20 mmol/L of Tris-HCl, 1.2 mmol/L of $MgCl_2$, and 2.5 U of *Taq*DNA polymerase (Takara) in a 100-μl reaction solution. The program was

Table 2. Primers for PCR Analysis of TCR Rearrangement

	TCR δ gene	Primer	Nucleotide sequence (5′ to 3′)
Sense	V δ 1	L-V δ 1	GTGTGTATTTGTGGCCTTCA
	V δ 2	L-V δ 2	TCATCCATCTCTCTCTCTTC
		V δ 2	GCACCATCAGAGAGAGATGA
	V δ 3	L-V δ 3	TCTACAGGGGCACGCTGTGT
Anti-sense	J δ 1	3′-J δ 1	AAATGCTAGCTATTTCACCCA
	J δ 3	J δ 3	GAGTTTGATGCCAGTTCCGAA
	Cδ	Cδ	TAACTTGGCAGTCAAGAGAAA

Table 3. Immunophenotype of SNT Cells

Cell line	CD3	CD4	CD8	CD16	CD19	CD21	CD56	CD57	HLA-DR	TCR$\alpha\beta$	TCR$\gamma\delta$
SNT-8	+	−	−	−	−	−	+	−	+	−	+
SNT-13	+	−	−	−	−	−	+	−	+	−	+
SNT-15	+	−	−	−	−	−	+	−	+	−	+
Molt-14	+	−	−	−	−	+	−	−	−	−	+
Peer	+	+	−	−	−	−	−	−	−	−	+
Loucy	+	+	−	−	−	−	−	−	−	−	+

Antibody reactivity: +, ≥75% of the cells were positive; −, <15% of the cells were positive.

94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 40 cycles. The products were analyzed by electrophoresis on a 2% agarose gel after staining with ethidium bromide.

Reverse Transcriptase (RT)-PCR and Direct Sequencing for Junction of Vδ-Jδ Genes

Total RNA was extracted from each of the cell lines using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD), and 0.5 μg was reverse-transcribed into cDNA using a Thermoscript RT-PCR System (Life Technologies, Inc.). An oligo$(dT)_{20}$ primer was used for the generation of cDNAs. After incubation at 60°C for 60 minutes, the samples were heated for 5 minutes at 85°C to terminate the reactions. PCR amplification of TCR δ-chain cDNA was accomplished by use of a Cδ anti-sense primer in combination with a Vδ2 sense primer. A human β-actin primer pair was obtained from Takara. The PCR cycling conditions were 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 40 cycles. Amplified products were identified by 2% agarose gel electrophoresis followed by staining with ethidium bromide. Amplification products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and direct sequencing was performed using a BigDye Terminator Reaction kit version 2.0 and an ABI PRISM 377 genetic analyzer (Applied Biosystems, Foster City, CA), according to the manufacturer's protocols.

Results

Phenotypes and Clonality of the Cell Lines

Flow cytometry showed that the SNT-8, SNT-13, and SNT-15 cells had an identical phenotype: $CD3^+$ $CD4^-$ $CD8^-$ $CD16^-$ $CD19^-$ $CD21^-$ $CD56^+$ $CD57^-$ HLA-DR^+ TCR α/β^- TCR γ/δ^+ (Table 3). Although they were positive for the NK-cell marker CD56, the expression of CD3 and $\gamma\delta$ TCR indicated that they were derived from $\gamma\delta$ T cells and distinct from NK-cell lines. We previously reported phenotypes of two NK-cell lines SNK-1 and SNK-6 as $CD3^-$ $CD4^-$ $CD8^-$ $CD19^-$ $CD25^+$ $CD56^+$ $CD57^-$ TCR α/β^- TCR γ/δ^-.[7,25]

Because the established EBV-positive cell lines proved to be of the $\gamma\delta$ T-cell subset, we analyzed the expression of variable regions of $\gamma\delta$ TCR by flow cytometry. We found the three EBV-positive cell lines to be positive for the anti-Vγ9 and anti-Vδ2 antibodies and negative for the anti-VγI, anti-Vδ1, and anti-Vδ3 antibodies (Table 4). By contrast, the EBV-negative cell lines Molt-14, Peer, and Loucy were negative for the anti-Vδ2 antibodies, and the former two were negative for the anti-Vγ9 antibody as well; the last cell line weakly reacted with the anti-Vγ9 antibody (Table 4).

Regarding the clonality of the EBV-positive cell lines, the Southern blot analysis for the number of EBV-TR showed a single band in each of the SNT-8, SNT-13, and SNT-15 cells (Figure 2). The results indicated that these cell lines consisted of respective monoclonal cell popu-

Table 4. Expression of TCR-Vγ and -Vδ Proteins by $\gamma\delta$ T-Cell Lines

	Antibodies				
Cell line	VγI	Vγ9	Vδ1	Vδ2	Vδ3
SNT-8	−	+++	−	+++	−
SNT-13	−	+++	−	+++	−
SNT-15	−	+++	−	+++	−
Molt-14	+	−	+++	−	−
Peer	−	−	++	−	−
Loucy	+	+	−	−	−

Antibody reactivity: +++, >90% of the cells were positive; ++, 50 to 90% of the cells were positive; +, 15 to 50% of the cells were positive; −, <15% of the cells were positive.

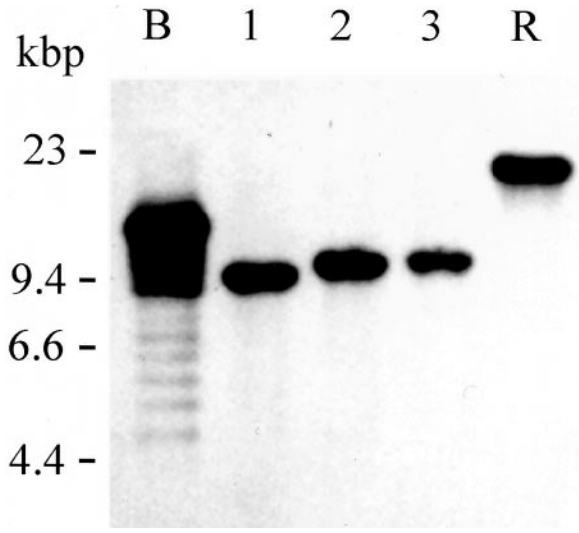

Figure 2. Clonality of cells assessed using EBV terminal repeats. Southern blot analysis of the number of EBV-TRs as evidence for monoclonal expansion of EBV-positive cells in SNT cell lines. **Lane 1**, SNT-8; **lane 2**, SNT-13, and **lane 3**, SNT-15 cells. B95-8 cells (B) and Raji cells (R) were used as controls for cells with productive EBV expansion and monoclonal EBV replication, respectively.

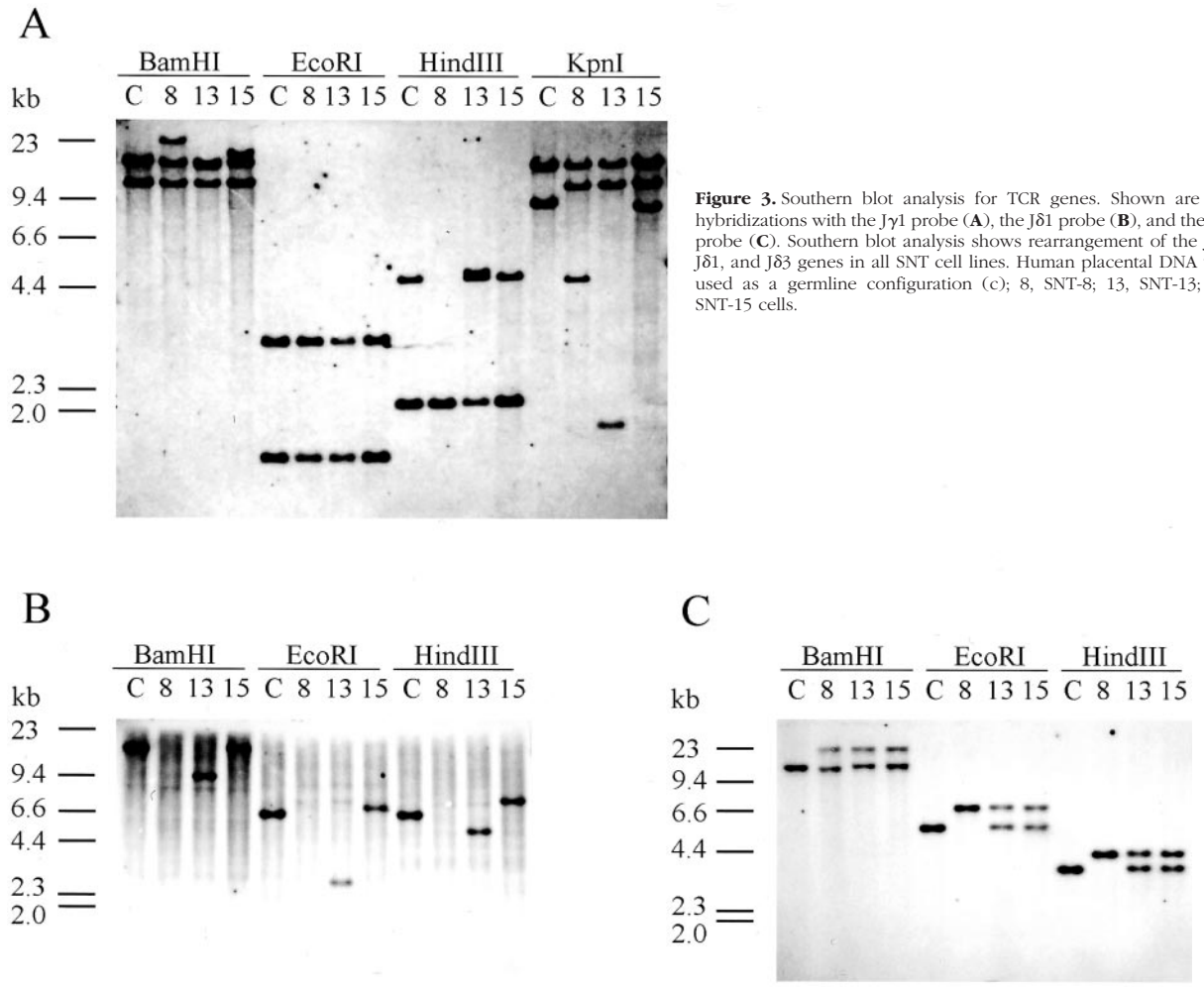

Figure 3. Southern blot analysis for TCR genes. Shown are the hybridizations with the Jγ1 probe (**A**), the Jδ1 probe (**B**), and the Jδ3 probe (**C**). Southern blot analysis shows rearrangement of the Jγ1, Jδ1, and Jδ3 genes in all SNT cell lines. Human placental DNA was used as a germline configuration (c); 8, SNT-8; 13, SNT-13; 15, SNT-15 cells.

lations and were Vγ9/Vδ2$^+$ γδ T cells. In addition, we previously reported that the clonality of SNT-8 was identical to that of the original tumor.[7] As to SNT-13, we could confirm that the clonality was the same as that detected in the peripheral blood mononuclear cells of the patient (data not shown).

Southern Blot Analysis for TCR and Immunoglobulin Genes

TCR γ-Chain Genes

Southern blot hybridization of the *Kpn*I digests of DNA with the Jγ1 probe detected rearrangements of TCR γ-chain genes in the three EBV-positive cell lines (Figure 3A). The EBV-positive cell lines showed three bands in the *Kpn*I digests, whereas the germline control showed two bands of 16 kb and 9 kb. The sizes of bands detected in the cell lines were as follows; 16 kb, 12 kb, and 4.7 kb in SNT-8; 16 kb, 12 kb, and 1.8 kb in SNT-13; 16 kb, 12 kb, and 8.5 kb in SNT-15. Thus, the three cell lines commonly showed 12-kb bands, which are known to be generated by rearrangement of the Vγ9 gene to the JγP gene. Rearranged genes were deduced from the sizes of *Kpn*I digests, because it was reported that the Vγ9-JγP rearrangement frequently observed in rearranging γ-loci can be detected with the *Kpn*I digest, but the *Bam*HI-rearranged bands are virtually the same size as the germline bands and the *Eco*RI and *Hind*III bands are in a germline configuration in this rearrangement for their restriction sites.[36,37] The results, thus, showed that the three cell lines had the Vγ9-JγP rearrangement in one allele. Additionally, the other bands were supposed to be generated by the following rearrangement of the γ-chain genes in the other alleles: the 4.7-kb band in SNT-8 was generated by rearrangement of the Vγl or VγIII gene to the JγP2 gene, the 1.8-kb band in SNT-13 was made by rearrangement of the Vγl or VγIII gene to the Jγ2 gene, and the 8.5-kb band in SNT-15 was derived from rearrangement of the Vγl or VγIII gene to the JγP1 gene. Although the three cell lines showed the rearrangement of γ-chain genes in both alleles, it was suggested that the Vγ9-JγP chain was the expressed allele because flow cytometry showed that the three cell lines expressed Vγ9-encoded receptors.

The EBV-negative cell lines Molt-14, Peer, and Loucy were analyzed in the same manner, but none showed the 12-kb band in *Kpn*I digests, a hallmark of the Vγ9-JγP rearrangement. Molt-14 showed rearrangements in both alleles: Vγl or VγIII to JγP1, and Vγl or VγIII to Jγ2. Peer

and Loucy had the same rearrangement, Vγ9 to Jγ2 in one allele and Vγl or VγIII to Jγ2 in the other (data not shown). The results of the Southern blot analyses are summarized in Table 6.

TCR δ-Chain Genes

Using the Jδ1 probe, we detected no band in SNT-8 and a rearranged band in each digest of DNA of SNT-13 and SNT-15 (Figure 3B). The sizes of the bands were 9.4 kb in the *Bam*HI digest, 2.8 kb in the *Eco*RI digest, and 4.8 kb in the *Hind*III digest in SNT-13, and 19.5 kb in the *Bam*HI digest, 6.9 kb in the *Eco*RI digest, and 7.1 kb in the *Hind*III digest in SNT-15. The deduced rearrangement was an incomplete rearrangement of Dδ2-Jδ1 in SNT-13 and Vδ2-Dδ3 in SNT-15.[38,39] Therefore, a complete Jδ1 rearrangement to be expressed was not detected in the three cell lines.

Hybridization with the Jδ3 probe revealed that the three cell lines showed rearranged bands of an identical size in each of the *Bam*HI, *Eco*RI, and *Hind*III digests (Figure 3C). The results suggest that the three cell lines have a common rearrangement of Jδ3 genes. Because of the lack of a precise map for restriction enzymes in this region, the Southern blot analysis could not determine how Jδ3 genes were rearranged with Vδ genes.

SNT-8 showed only rearranged bands in the *Bam*HI, *Eco*RI, and *Hind*III digests, and these results suggested that SNT-8 had the rearrangement of Jδ3 genes in both alleles, or the rearrangement of a Jδ3 gene in one allele with a deletion of Jδ genes in the other. In addition, the SNT-8 cells were found to express γδTCR by flow cytometry, indicating that deletion of the Jδ1 gene was a consequence of the usage of the Jδ3 gene at the same δ locus.

Both SNT-13 and SNT-15 showed the rearranged band and a germline band in each digest obtained with the Jδ3 probe. These results suggested that SNT-13 and SNT-15 had a rearrangement of Jδ3 genes, which was common to that in SNT-8, in one allele, and incomplete rearrangements in the other allele upstream of the Jδ3 segment as described above.

The three EBV-negative cell lines Molt-14, Peer, and Loucy showed rearranged bands with the Jδ1 probe, but not with the Jδ3 probe (data not shown). The deduced rearrangement in these cell lines is summarized in Table 6.

TCR β-Chain and Immunoglobulin Genes

All of the EBV-positive and -negative cell lines showed rearrangements of the Cβ genes, whereas none showed a rearrangement of the immunoglobulin heavy-chain genes (data not shown). It is known that rearrangements of the β-chain gene are commonly found in peripheral γδ T-cell lymphomas.[40,41]

PCR for Combinations of Vδ and Jδ Genes

To elucidate the rearrangement of Vδ and Jδ genes, PCR analysis was performed for each combination of the Vδ1, Vδ2, or Vδ3 gene and the Jδ1 or Jδ3 genes. It was found that, only PCR using the L-Vδ2 and Jδ3 primers amplified fragments with an expected size of ~500 bp in the three EBV-positive cell lines, indicating that the cell lines had the Vδ2-Jδ3 rearrangement, but not the Vδ1-Jδ1, Vδ1-Jδ3, Vδ2-Jδ1, Vδ3-Jδ1, and Vδ3-Jδ3 rearrangements (Figure 4). In terms of the EBV-negative cell lines, the DNA of Molt-14 and Peer was amplified with the L-Vδ1 and 3′-Jδ1 primers. This result indicated that these two cell lines had the Vδ1-Jδ1 rearrangement (data not shown).

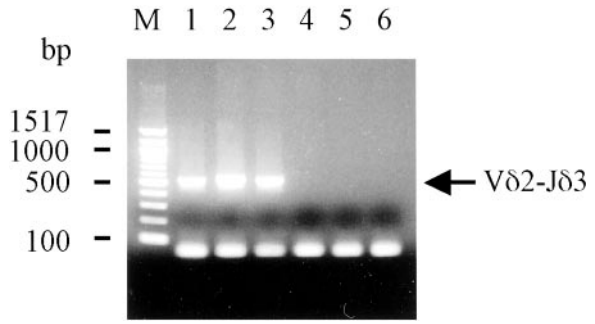

Figure 4. PCR analysis for the junction of Vδ and Jδ genes. PCR analysis shows the presence of the Vδ2-Jδ3 TCR gene rearrangement in the three SNT cell lines. Only the combination of a L-Vδ2 sense primer and a Jδ3 anti-sense primer amplified ~500-bp PCR products identified as Vδ2-Jδ3. **Lane 1**, SNT-8; **lane 2**, SNT-13; **lane 3**, SNT-15 cells. No signal was detected in EBV-negative γδ T-cell lines; **lane 4**, Molt-14; **lane 5**, Peer; **lane 6**, Loucy.

Analysis of TCR δ-Chain Transcripts

RT-PCR and direct sequencing were performed to confirm the results of flow cytometry, Southern blotting and PCR analysis showing that the three cell lines had the Vδ2-Jδ3 rearrangement. RT-PCR with the Vδ2 and Cδ primers commonly amplified ~550-bp single bands in the three cell lines (data not shown), and β-actin transcripts were amplified in all of the samples and served as a control for amplifiable mRNA. Direct sequencing of the amplified RT-PCR products clearly showed that the three cell lines had sequences for the Vδ2-Dδ3-Jδ3 rearrangement (Table 5). The results demonstrate that the Vδ2-Jδ3 rearrangement took place, and mRNA for Vδ2-Jδ3-Cδ receptor was then transcribed in the SNT cell lines. In addition, the same RT-PCR and sequence analysis was performed on peripheral blood of the patient from which SNT-13 was derived at primary diagnosis. The patient's blood had completely the same sequence as that of SNT-13 (data not shown).

The results of the flow cytometric, Southern blot, PCR, RT-PCR, and direct sequencing analyses are summarized in Table 6. The table shows that the results are consistent. Overall, they demonstrate that the three EBV-positive cell lines always express Vγ9-JγP/Vδ2-Jδ3 TCR, a phenomenon not observed in the EBV-negative γδ T-cell lines.

Discussion

In this study, we reported characteristics of three EBV-positive cell lines of γδ T cells. One of these was the

Table 5. Nucleotide Sequence of Vδ2-Jδ3 Transcripts Expressed by γδ T-Cell Lines

	Vδ2	N/P	Dδ3	N/P	Jδ3
Germline	GCCTGTGACACC		ACTGGGGGATACG		CTCCTGGGACACCC
SNT-8	GCCTGTGA	TCT	ACTGG	CGGG	CTCCTGGGACACCC
SNT-13	GCCTGTGACACC	GTGA	ACTGGGGGATACG	CGG	CTCCTGGGACACCC
SNT-15	GCCTGTGACAC	GT	ACTGGGGGA	G	CTCCTGGGACACCC

The germline sequences of the 3′ end of Vδ2, the Dδ3, and the 5′ end of Jδ3 gene segments are at the top. The sequences of TCR δ-chain transcripts of the γδ T-cell lines are aligned with the germline sequences. N/P; template-independent "N" nucleotide insertions or palindromic "P" synthesis.

previously-reported γδ T-cell line SNT-8, which was established from the primary lesion of a nasal T/NK-cell lymphoma,[7] whereas the other two, SNT-13 and SNT-15, were newly established lines from the peripheral blood of patients with CAEBV. In the previous study, SNT-8 was demonstrated to have developed from the lymphoma cells in the patient, because SNT-8 had the same immunophenotype and monoclonal EBV clones as the original lesion.[7] In patient 2, SNT-13 had the same EBV clone and TCR δ-chain as the peripheral blood mononuclear cells, indicating that SNT-13 had developed from cells predominantly expanding in the patient and responsible for her disease. These data, thus, suggest that it is the cells proliferating in patients with nasal T/NK-cell lymphoma and CAEBV that exclusively grow in our culture system.

The three cell lines showed a phenotype typical of γδ T cells, and were associated with a consistent rearrangement of the TCR genes Vγ9-JγP/Vδ2-Jδ3. To the best of our knowledge, these are the only γδ T-cell lines that have been found to be positive for EBV. Moreover, this study presented the first evidence of the existence of cases of CAEBV of the γδ T-cell subset.

Not only SNT-8 cells but also SNT-13 and SNT-15 cells proliferated *in vitro*, and an analysis of the number of EBV-TR showed that every cell line consisted of monoclonal cells. The monoclonal expansion of the cells suggested that not only SNT-8 cells but also SNT-13 and SNT-15 cells were transformed to a certain degree and acquired a greater proliferative capacity compared with normal T cells. Many studies have documented monoclonal and oligoclonal proliferation of EBV-positive T cells and NK cells in patients with CAEBV.[11,12,17,19,20,24,25] In addition, the development of malignant lymphomas of T/NK-cell lineage from patients with CAEBV has been reported.[9,14,18,19,23,25,42] Furthermore, the establishment of cell lines of αβ T-cell[16] and NK-cell lineage[21] has been reported in some severe CAEBV cases, and the present study described the establishment of γδ T-cell lines. Therefore, the present study, together with those previous reports, suggests that most cases of CAEBV are considered as lymphoproliferative diseases of NK cells, αβ T cells, or γδ T cells, rather than as infectious diseases.

The most interesting finding in the present study was that the EBV-positive γδ T-cell lines SNT-8, SNT-13, and SNT-15 expressed exactly the same TCR, Vγ9-JγP/Vδ2-Jδ3. This was, on the other hand, not observed in the EBV-negative γδ T-cell lines, which were established from T-lymphoblastic leukemia patients and expressed Vδ1 protein with the Vδ1-Jδ1 gene rearrangement. Consistent with our observation, Arnulf and colleagues[4] have reported that five EBV-positive cases (three nasal, one laryngeal, and one intestinal lymphomas) in a series of nonhepatosplenic γδ T-cell lymphomas were invariably $Vδ2^+$, whereas five EBV-negative or -weakly positive cases expressed one of the Vδ1 (one case in the lung),

Table 6. Summary of Analysis for γδ TCR

		Southern blot analysis				PCR, sequencing, and Southern blot analysis	Flow cytometry
Cell line	EBV	TCR Jγ	TCR Jδ1	TCR Jδ3	TCR γ-chain gene rearrangement	TCR δ-chain gene rearrangement	γδ TCR-protein expression
SNT-8	+	R	D	R	Vγ9-JγP Vγl/III-JγP2	Vδ2-Jδ3	Vγ9/Vδ2
SNT-13	+	R	R	R	Vγ9-JγP Vγl/III-Jγ2	Vδ2-Jδ3 Dδ2-Jδ1	Vγ9/Vδ2
SNT-15	+	R	R	R	Vγ9-JγP Vγl/III-JγP1	Vδ2-Jδ3 Vδ2-Dδ3	Vγ9/Vδ2
Molt-14	−	R	R	G	Vγl/III-JγP1 Vγl/III-Jγ2	Vδ1-Jδ1 Unknown R	Vγl/Vδ1
Peer	−	R	R	G	Vγ9-Jγ2 Vγl/III-Jγ2	Vδ1-Jδ1	Unknown/Vδ1*
Loucy	−	R	R	G	Vγ9-Jγ2 Vγl/III-Jγ2 Unknown R	Dδ2-Jδ1 Unknown R	ND†

G, germline band; R, rearrangement band; D, deletion of germline band (s); ND, not determined.
*Vγ protein was not determined. Peer was negative for anti-Vγ9 and -Vγl antibodies, although it was positive for anti-TCR γ/δ antibody.
†Not determined, but Vγ9/Vδ2 TCR was not detected.

Vδ2 (two cases in the skin), or Vδ3 (two cases in the gastrointestinal tract) chain. Thus, it was suggested that EBV has a close affinity to $V\delta2^+$ cells in the lymphoproliferative diseases, especially to $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells.

It is of interest when and where EBV affected the $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells in our patients. It is known that the usage of TCRs in human γδ T cells has limited diversity, and the type of cells that constitute the majority of changes during fetal and postnatal life in individual organs.[1] For example, in the postnatal thymus and other epithelial-rich tissues such as the intestine and spleen, $V\delta1\text{-}J\delta1^+$ cells are the major population.[1,43,44] It is thus reasonable that cases of γδ T-cell acute lymphoblastic leukemia, cells of which are supposed to be the malignant counterpart of thymocytes, expressed Vδ1-Jδ1 TCR.[45] Likewise, most hepatosplenic γδ T-cell lymphomas were reported to express Vδ1 proteins and/or rearranged Vδ1 genes.[40,41,46,47] In contrast, $V\delta2^+$ cells are known to be the major population in the peripheral blood during adulthood.[1,43,44] In the peripheral blood, $V\delta1^+$ cells constitute the majority at birth, and $V\delta2^+$ cells increase from a mean of 20% in the cord blood to ~60 to 70% before the age of 10.[44] In addition, $V\gamma9\text{-}J\gamma P/V\delta2^+$ cells, which are capable of recognizing mycobacterium and Daudi Burkitt's lymphoma cells,[48,49] are believed to be selected and expand in number in the periphery.[1] With regard to the nose, the major population among the nasal intraepithelial γδ T cells was reported to express Vγ1/Vδ1 genes.[50] Thus, it is likely that EBV affected the $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells in the peripheral blood in our patients with nasal T-cell lymphoma and CAEBV. Nonetheless, although $V\delta2\text{-}J\delta3^+$ cells constitute the major population in cord blood, $V\delta2\text{-}J\delta1^+$ cells comprise the largest population, ~50 to 98% of γδ T cells, in the peripheral blood of healthy adults.[1,43] Therefore, $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells are a minor population in the peripheral blood in adulthood in the context of Jδ3 expression, although probably more common in infants and youth. Thus, it may be reasonable that EBV affected these specific γδ T cells in the peripheral blood of our two patients with CAEBV because they were young people. It is unknown, however, why EBV affected these specific γδ T cells in SNT-8 because the patient was 48-years-old. It may be that EBV has particular affinity to the Jδ3 TCR. Otherwise, EBV might affect $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells when the patient is young, and the cells transform later to form the nasal T-cell lymphoma.

It is thus an intriguing question how EBV affected the $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells in our patients. A possible explanation for the close affinity between EBV and this specific cell population is that there is a specific receptor for EBV other than CD21 on the $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells because the three EBV^+ cell lines were negative for the CD21 antigen, a known receptor for EBV.[51,52] One approach to assessing such a possibility is to study whether isolated $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells can be infected with EBV.

Alternatively, the relationship between EBV and $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells may be explained as follows: the population of these cells expanded in response to a certain antigen and infiltrated a tissue to then be infected with resident EBV. Indeed, $V\gamma9\text{-}J\gamma P\text{-}C\gamma1/V\delta2^+$ cells were reported to have a great ability to respond to Daudi cells or mycobacterial antigens and comprise a large proportion of the γδ T cells in the peripheral blood.[49] This also suggests that they are highly responsive to antigens structurally identical or related to Daudi or mycobacterial antigens. Alternatively, $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells might expand in number during EBV infection because γδ T cells were reported to increase in acute and convalescent phases of infectious mononucleosis.[53,54] Therefore, it seems to be worth investigating how $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cells react with Daudi antigens, mycobacterial antigens, or EBV.

Another possible mechanism for the expansion of the $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3^+$ cell population in our three patients is that EBV affected immature T-lymphocytes and drove them to differentiate into this population. Indeed, it has been reported that EBV infects immature T lymphocytes. Imai and co-workers[16] established four EBV-positive T-cell lines from patients with CAEBV, and one of the four had an immature T-cell phenotype without TCR gene rearrangement. It has also been reported that immature thymocytes and an immature T-cell line HPB-ALL expressed CD21 and could be infected with EBV.[55,56] Moreover, it has been shown that infections of EBV in HPB-ALL altered the expression of T-cell surface molecules involved in antigen recognition. It has thus been suggested that EBV infection of T cells at early stage of differentiation may lead to a failure in the development of the normal T-cell repertoire.[56] Therefore, we believe that the effect of EBV infection of immature thymocytes on the development of the T-cell repertoire is an important issue to be addressed.

The present study raised another important question as to why the infection by EBV of an identical γδ T-cell subset resulted in different diseases, ie, nasal T/NK-cell lymphoma and CAEBV; the former is a malignant lymphoma and the latter a nonmalignant lymphoproliferative disease. We recently reported a patient with CAEBV of NK-cell lineage who subsequently developed nasal NK-cell lymphoma.[25] There were three NK-cell clones in the patient, and it was suggested that one of the three was involved in the formation of the malignant lymphoma. We concluded that the type and/or degree of transformation of EBV-positive NK cells could be variable even in a single patient. Similar conditions showing variability in the transformation of EBV-positive cells have been reported in the posttransplantation lymphoproliferative disorders (PT-LPD) of B lymphocytes.[57–61] These reports hypothesized that the development of PT-LPD is a multistep process, initiates as polyclonal expansions of EBV-carrying B cells on the basis of immunosuppression, and may progress to lymphomas. The present study together with these previous reports, suggests that transformation of cells with EBV only is not sufficient for the development of a malignant lymphoma, and that an additional abnormality such as genetic mutation is likely to be involved in the malignant transformation of EBV-affected cells.

In conclusion, this study demonstrated that the three EBV-positive γδ T-cell lines expressed a common TCR, the $V\gamma9\text{-}J\gamma P/V\delta2\text{-}J\delta3$ TCR, and shed light on the etiology of nasal T-cell lymphoma and CAEBV of the γδ T-cell

subset. The establishment of the cell lines allowed us to focus on these diseases. Thus, we believe that the results warrant the use of these cell lines in future studies, and necessitate further efforts to develop more cell lines from lymphoproliferative diseases caused by EBV.

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