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Epstein-Barr Virus-associated Post-transplant Non-Hodgkin's Lymphoma: Establishment and Characterization of a New Cell Line

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A new human lymphoma cell line derived from pulmonary non-Hodgkin's lymphoma that developed in a renal transplant recipient was established from the patient's pleural effusion and designated PTLC-1. PTLC-1 grew aggressively in suspension, forming very loose clumps with a doubling time of about 18.9 h. The morphological, chromosomal, and immunophenotypic characteristics of the patient's tumor cells and PTLC-1 cells were very similar. PTLC-1 showed a monoclonal rearrangement of IgH gene and was highly tumorigenic in athymic nude mice. *In situ* hybridization, Southern blot hybridization and polymerase chain reaction demonstrated the presence of Epstein-Barr virus (EBV) genome in the patient's tumor and PTLC-1. PTLC-1 has been maintained in culture for over 60 months. Since EBV has been implicated in the pathogenesis of post-transplant lymphoma, this new cell line should serve as a useful experimental model for studying the etiology and biology of lymphoma developing in organ transplant recipients.

Key words: Non-Hodgkin's lymphoma — Post-transplant lymphoma — Cell line — Transplantation — Epstein-Barr virus

An increased incidence of *de novo* neoplasms developing in organ transplant recipients has attracted much attention.[1-4] Immunosuppressive therapy is commonly indicated for the recipients, and it is generally held that such therapy places the recipients at increased risk for *de novo* neoplasms.[1] More than 30% of post-transplant neoplasms are malignant lymphomas.[4] The clinical and pathological features of the post-transplant lymphomas have been well described[5] and it has been reported that these lymphomas are associated with Epstein-Barr virus (EBV).[1] However, the pathogenetic role of EBV in the development of post-transplant lymphomas has not been determined precisely. The availability of a well-characterized experimental model for post-trnasplant lymphoma would provide an important tool for studies of the biology and therapeutics of the lymphoma.

We have established a cell line, designated PTLC-1, derived from pulmonary non-Hodgkin's lymphoma developed in a renal transplant recipient. Clinical and pathological manifestations in the original tumor were consistent with those commonly observed in post-transplant lymphoma and tumor cells carrying EBV. We describe here various properties of this cell line, including its morphologic, chromosomal, immunologic and biologic characteristics.

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MATERIALS AND METHODS

Patient and tumor cells A 31-year-old man who had received a renal transplant presented pulmonary nodules and pleural effusion 3 years after transplantation. The patient died with tumor progression shortly after carcinostatic chemotherapy. The histopathologic diagnosis was diffuse large cell lymphoma of B cell phenotype arising in the lung. The details of the clinical features, course, and postmortem examination were reported previously.[6] The patient's lung tumor obtained by autopsy was snap-frozen and stored at −70°C until analysis.

Establishment of cell line Lymphoma cells for culture were obtained from the patient's pleural effusion by needle aspiration. The mononuclear cells were then separated using lymphocyte separation medium (Ficoll-Conray, Sigma Chemical Company, St. Louis, MO, USA). The cells were washed and resuspended in RPMI-1640 medium (Life Technologies Inc., Gibco Products, Gaithersburg, MD, USA) containing 20% fetal bovine serum (ICN Biomedicals Inc., Costa Mesa, CA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). Five million cells/flask were cultured in the above medium in 25 ml cell culture flasks (Costar Scientific Co., Cambridge, MA, USA) at 37°C in 5% CO_2. The culture medium was replenished twice weekly by partial replacement with fresh medium. The cells have been maintained in culture for over 60 months and have been designated PTLC-1.

Cytomorphology PTLC-1 cells in culture were examined in cytocentrifuge preparations. The preparations were fixed with May-Gruenwald solution, stained with Giemsa solution, and examined by light microscopy.

Heterotransplantation Six-week-old BALB/c nude mice (Charles River Laboratories, Wilmington, MA, USA) were used for the examination of tumorigenicity. PTLC-1 cells (5×10^6 cells) were injected subcutaneously into the rear flank of mice. Tumor growth was checked daily by palpation. Tumor-bearing mice were killed 3, 6 and 9 weeks after the injection of PTLC-1 cells. A portion of the tumor was processed by DNase-collagenase digestion to obtain free cells for flow cytometric analysis and the remaining portion was fixed in 10% neutral buffered formalin for histological examination. The organs of the tumor-bearing mice were also removed and fixed for histological examination.

Surface marker analysis The fresh lymphoma cells from the patient's pleural effusion, PTLC-1 cells in culture and tumor cells from athymic nude mice were analyzed for cell-surface phenotype by using flow cytometry. The cells (2×10^3 cells) were incubated on ice with 40 μl of the following fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for 30 min; CD1 (OKT6), CD2 (NU-T1), CD3 (T3), CD4 (NU-Th/i), CD5 (Leu1), CD7 (Leu9), CD8 (NU-Ts/c), CD10 (J5), CD13 (MCS-2), CD14 (My4), CD19 (B4), CD20 (B1), CD21 (OKB7), CD23 (B6), CD25 (IL-2R1), CD33 (My9), CD44 (Leu44), HLA-DR, Sm-IgG, IgA, IgM, IgD, κ and λ. The cells were washed with phosphate-buffered saline (PBS) containing 0.1% sodium azide and the percentage of positive cells was determined by using a laser flow cytometer (JASCO Co., Tokyo).

Chromosome analysis The lymphoma cells from the patient's pleural effusion and PTLC-1 cells were analyzed for cytogenetic abnormalities by the trypsin-Giemsa banding method.[7] The cells were incubated with colcemid (Gibco) for 17 h under 5% CO_2 at 37°C, then treated with hypotonic medium and fixed with methanol/acetic acid (3:1). Fixed chromosome spreads were treated briefly with trypsin, stained with Giemsa, and examined using light microscopy.

DNA analysis DNA was extracted from lymphoma cells obtained from the patient's frozen lung tumor and PTLC-1, and used for detection of gene rearrangements according to the method described by Southern.[8] The DNA digested with *Eco*RI restriction enzyme was hybridized with a human immunoglobulin heavy-chain (J_H) probe, and the DNA digested with *Bgl*II restriction enzyme was hybridized with a T cell receptor (TCR)-β-chain ($J_{\beta2}$) probe. The placental DNA was used as a negative control.

EBV analysis: immunofluorescence The expression of EBV-associated nuclear antigen (EBNA) in PTLC-1 cells was determined by the indirect immunofluorescence technique.[9] A human lymphoma cell line in which the presence of EBV had been confirmed by electron microscopy was used as a positive control, and a human lymphoma cell line presenting no EBV was used as a negative control.

***In situ* hybridization** To determine the location of EBV genomes, *in situ* RNA hybridization was performed using a 30-base oligonucleotide antisense RNA probe, complementary to a portion of the EBER-1 gene, according to the method described by Weiss *et al.*[10] and Nagasato *et al.*[11] Paraffin-embedded tissues obtained from the patient's lung tumor and heterotransplanted tumor growing in an athymic nude mouse were cut into 5 μm thick sections and placed on glass slides. The specimens were deparaffinized, dehydrated, predigested with pronase, prehybridized, and hybridized overnight with digoxygenin(Dig)-labeled probe (0.5 ng/μl). After washing, the specimens were incubated with alkaline phosphatase-conjugated anti-Dig antibody (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 h. They were washed, developed by adding the substrate for alkaline phosphatase, and then counterstained with methyl green. As a control for the antisense probe, the sense oligonucleotide probe was used at the same concentration and in the same way.

Southern blot hybridization For analysis of EBV genomes in lymphoma cells, a *Bam*HI "W" fragment of EBV genome (kindly provided by Dr T. Takenouchi, First Department of Pathology, Chiba University Medical School, Chiba) was used for Southern blot hybridization.[8] DNA extracted from lymphoma cells of the patient's frozen lung tumor and PTLC-1 cells was digested with *Bam*HI restriction enzyme. A Hodgkin's lymphoma cell line in which the presence of EBV had been confirmed by electron microscopy was used as a positive control. A non-EBV-infected T cell leukemia cell line "Molt-4" was used as a negative control.

Polymerase chain reaction (PCR) DNA was extracted from lymphoma cells of the patient's frozen lung tumor and PTLC-1. The PCR was performed according to the method described by Saiki[12] using oligonucleotide primers[13] (primer 1: GGCGCACCTGGAGGTGGTCC, primer 2: TTTCCAGCAGAGTCGCTAGG) specific for the EBV lymphocyte-determined membrane antigen (LYDMA) gene, which is composed of variable numbers of tandem repeats. The size of this heterogeneous region is characteristic for a given EBV isolate and can vary between EBV isolates.[14] Temperatures during the 35 amplification cycles were 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. An EBV-immortalized Burkitt's lymphoma cell line "Raji" was used as a positive control.

RESULTS

Establishment and growth characteristics of the cell line PTLC-1 grew aggressively in suspension, forming very loose clumps, and was passaged at least twice a week. Fig. 1 shows the cytomorphology of PTLC-1 cells. The cells had a lymphoblast-like morphology with large nuclei, prominent nucleoli and acidophilic cytoplasma. The population doubling time was about 18.9 h.

Heterotransplantation PTLC-1 cells were implanted into 5 athymic nude mice and produced tumors at the site of inoculation in all the mice within 3 weeks. These tumors grew into walnut-sized masses after 9 weeks. Autopsy revealed that tumors were localized in the subcutaneous tissue and metastases or invasion to surrounding tissues were not found after 3 weeks. Metastases were found in the bilateral axillary lymph nodes after 6 weeks. After 9 weeks, metastases were found in the liver, but not in the lungs. Histologically, these tumors revealed the same cytologic and histologic characteristics as the patient's lung tumor (Fig. 2).

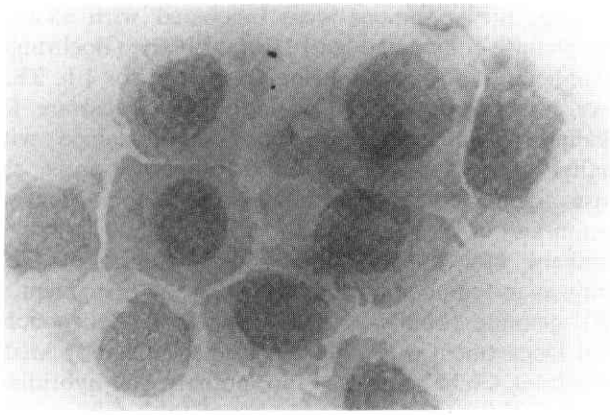

Fig. 1. Microscopic examination of smeared PTLC-1 cells, showing lymphoblast-like cells with large nuclei and prominent nucleoli. May-Gruenwald-Giemsa stain. Original magnification ×1,000.

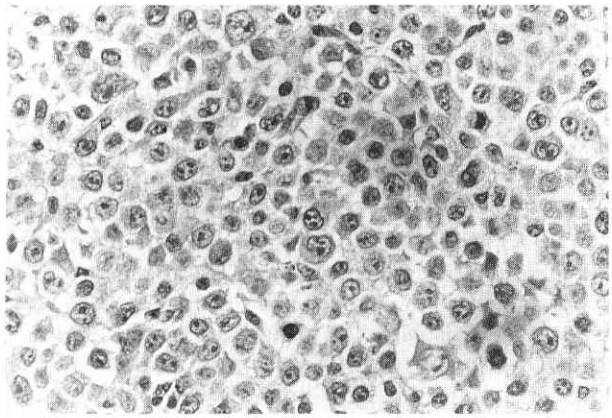

Fig. 2. Histological section of the heterotransplanted tumor, showing a diffuse large cell lymphoma. Hematoxylin-eosin stain. Original magnification ×400.

Surface markers Table I shows the results of surface marker analyses of fresh lymphoma cells from the patient's pleural effusion, PTLC-1 cells in culture and tumor cells from athymic nude mice. They had similar characteristics in that more than 75% of the cells expressed CD20 and HLA-DR antigens, demonstrating their B-cell nature. However, they lacked detectable cell surface immunoglobulin. Small percentages of PTLC-1 cells and tumor cells from athymic nude mice expressed CD21 (EBV receptor) antigen.

Chromosome abnormalities Fig. 3 shows the results of karyotype analysis of the lymphoma cells from the patient's pleural effusion and PTLC-1. This analysis revealed that the lymphoma cells had chromosomal abnormalities of 46,XY,5p+,16p+,22p+ in all 13 metaphase cells and that PTLC-1 had chromosomal abnormalities

Table I. Surface Marker Analysis of the Tumor Cells

Surface marker	Fresh tumor cells from pleural effusion (%)	Cells growing in	
		culture (%)	nude mice (%)
CD1 (OKT6)	15	0	1
CD2 (NU-T1)	17	0	0
CD3 (T3)	20	0	0
CD4 (NU-Th/i)	20	0	0
CD5 (Leu1)	20	0	1
CD7 (Leu9)	20	0	2
CD8 (NU-Ts/c)	24	0	1
CD10 (J5)	22	47	2
CD13 (MCS-2)	22	4	2
CD14 (My4)	20	0	1
CD19 (B4)	82	94	54
CD20 (B1)	78	93	82
CD21 (OKB7)	ND	10	3
CD23 (B6)	ND	82	3
CD25 (IL-2R1)	16	0	2
CD33 (My9)	26	59	1
CD44 (Leu44)	ND	30	ND
HLA-DR	96	100	98
Surface IgG	0	0	0
Surface IgA	0	0	0
Surface IgM	0	0	0
Surface IgD	0	0	0
κ	0	0	0
λ	0	0	0

Each value was determined by indirect immunofluorescence and flow cytometry. Numbers indicate percentage of positive cells. ND: not done.

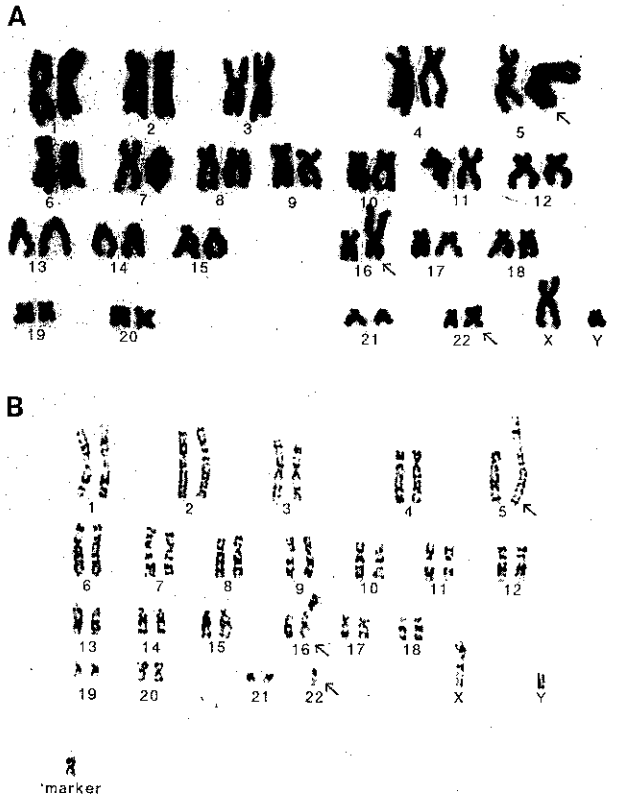

Fig. 3. G-Banded karyotype analysis. These karyotypes showed very similar chromosomal abnormalities. (A) Lymphoma cells from the patient's pleural effusion, showing a chromosomal abnormality of 46,XY,5p+,16p+,22p+. (B) PTLC-1, showing a chromosomal abnormality of 46,XY, −22,5p+,16p+,+mar.

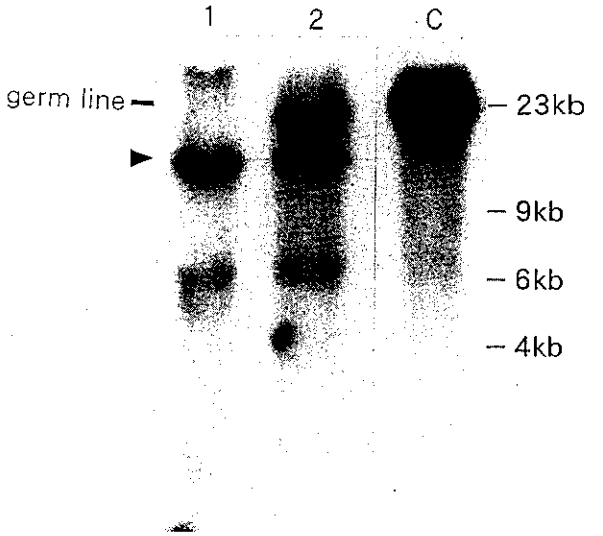

Fig. 4. Southern blot analysis of IgH (J_H) gene. The horizontal line shows the germ line. The arrowhead indicates a rearranged band. PTLC-1 and the patient's tumor showed an identical monoclonal rearrangement. Lane 1, PTLC-1; 2, patient's tumor; C, placental DNA.

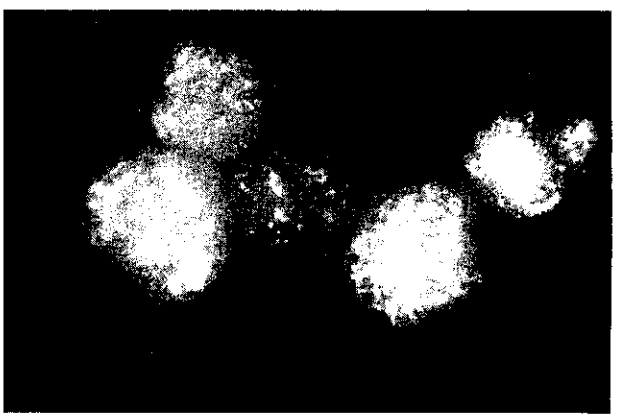

Fig. 5. Expression of EBNA on PTLC-1 cells detected by indirect immunofluorescence. Original magnification ×1,000.

of 46,XY,−22,5p+,16p+,+mar in all 20 metaphase cells.

DNA analyses Fig. 4 shows the result of Southern blot analyses in which the immunoglobulin heavy-chain gene rearrangement in the patient's tumor was compared to that in PTLC-1. The DNA extracted from the patient's tumor showed a single rearranged band and germ line configuration. In contrast, the DNA extracted from PTLC-1 showed a single rearranged band and the size of the rearranged band was identical to that of the patient's tumor. No rearrangement of the TCR-β gene was detected in the patient's tumor or PTLC-1. These results indicate that PTLC-1 is a monoclonal B-cell line derived from the patient's monoclonal tumor.

EBV analyses Fig. 5 shows positive EBNA expression in PTLC-1 cells as detected by the immunofluorescence technique. *In situ* hybridization technique revealed EBV mRNA expression in a majority of the patient's tumor cells and the heterotransplanted tumor cells (Fig. 6). Southern blot analysis further confirmed the presence of EBV genome in the patient's tumor and PTLC-1 (Fig. 7). The LYDMA PCR products from the patient's tumor and PTLC-1 presented a single band of identical size on agarose-gel electrophoresis (Fig. 8).

DISCUSSION

As exemplified by this patient, post-transplant lymphoma is characterized by the predominance of extra-

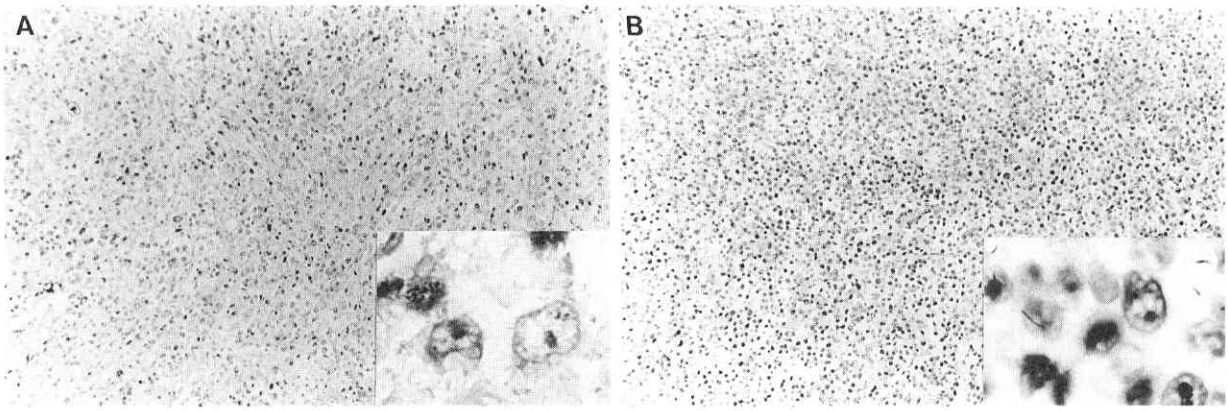

Fig. 6. *In situ* RNA hybridization using the Dig-labeled antisense EBER-1 probe. Original magnification ×100, inset ×1,000. Many lymphoma cells expressed EBER-1. (A) The patient's lung tumor; (B) the heterotransplanted tumor.

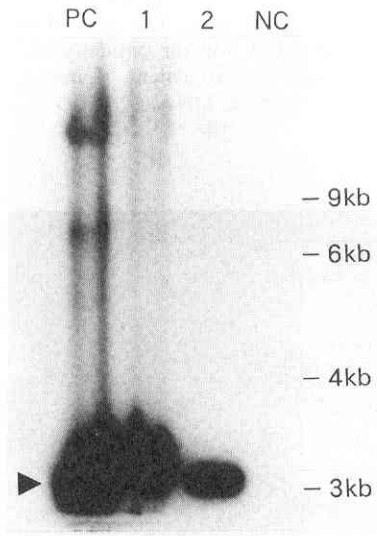

Fig. 7. Southern blot analysis of *Bam*HI "W" gene. PTLC-1 and the patient's tumor were EBV genome-positive. Lane 1, patient's tumor; 2, PTLC-1; PC, EBV-positive Hodgkin's lymphoma cell line; NC, Molt-4.

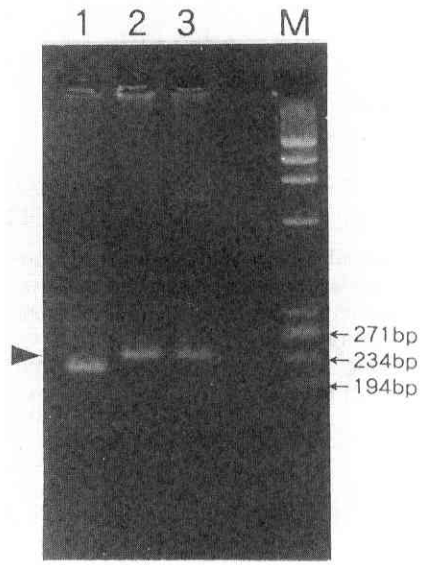

Fig. 8. PCR products amplified from a region of the EBV LYDMA gene. PTLC-1 and the patient's tumor showed an identical band. Lane 1, Raji; 2, PTLC-1; 3, patient's tumor; M, size markers ($\phi \times 174/HaeIII$).

nodal origin,[1–5] intermediate-grade or high-grade histology[15] when translated into NCI working formulation nomenclature,[16] B cell phenotype,[1] and poor prognosis after cytotoxic chemotherapy.[17] However, the mechanism of the tumor development is still under study, and a clear-cut conclusion has yet to be drawn.

Post-transplant lymphoma is often composed of a polyclonal population of B lymphocytes, and clonal progression has been demonstrated to occur in polyclonal and oligoclonal infiltrates in some patients.[18, 19] In addition, the demonstration of EBV genome or its products in the majority of the tumors has led to the widely accepted hypothesis that post-transplant lymphoma can be manifested as T lymphocyte function is inhibited under immunosuppression, allowing easy infection with EBV; this results in the development of B cell hyperplasia, providing a basis for transformation into a tumor.[20–22]

PTLC-1 showed several characteristics similar to those of the patient's tumor. These include morphology, expression of cell surface antigens, rearrangement of the immunoglobulin heavy-chain gene and chromosomal

abnormalities. PTLC-1 was successfully transplanted into athymic nude mice, indicating its malignant nature and the heterotransplanted tumor was morphologically closely similar to the patient's tumor. These findings indicate that PTLC-1 is a monoclonal B cell line that inherited the patient's non-Hodgkin's lymphoma.

The EBV analyses demonstrated the presence of EBV genomes in a majority of the patient's tumor cells and PTLC-1 cells. Furthermore, amplification of the polymorphic LYDMA gene sequence of the patient's tumor and PTLC-1 produced an identical-sized single band, indicating that EBV detected in PTLC-1 was also derived from the patient's tumor cells.[23]

Yoshizawa *et al.* recently reported a lymphoma cell line established from a liver transplant recipient.[24] No other cell lines derived from a post-transplant neoplasm have been reported. Our new cell line, designated PTLC-1, derived from a non-Hodgkin's lymphoma, has some typical characteristics of the post-transplant lymphoma and should serve as a useful experimental model for studying the etiology and biology of this kind of lymphoma.

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