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IMMUNOPHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF NASAL LYMPHOMA WITH POLYMORPHIC RETICULOSIS MORPHOLOGY

Masahiko OHSAWA[1], Shin-ichi NAKATSUKA[1], Hiroyuki KANNO[1], Hideaki MIWA[1], Shizuo KOJYA[2], Yasuaki HARABUCHI[3], Woo-Ick YANG[4] and Katsuyuki AOZASA[1]*

[1]*Department of Pathology, Osaka University Medical School, Osaka, Japan*
[2]*Department of Otorhinolaryngology, University of Ryukyus, Faculty of Medicine, Okinawa, Japan*
[3]*Department of Otorhinolaryngology, Sapporo Medical University, Sapporo, Japan*
[4]*Department of Pathology, Yonsei University College of Medicine, Seoul, Republic of Korea*

Nasal lymphoma with polymorphic reticulosis (PR) morphology is now categorized as T/natural killer (T/NK) cell lymphoma. In this study, immunophenotypes and genotypes of proliferating cells in 21 cases with PR were examined. The patients included 13 men and 8 women ranging in age from 20 to 74 (median 37) years. All patients presented with lesions in the upper respiratory tract, mostly in the nasal cavity. Histological specimens obtained from the primary lesions (19 cases) and metastatic cervical lymph nodes (2 cases) were used for analyses. Histologically, polymorphous proliferation was found in 20 cases, and these were thus diagnosed as PR. A monomorphous pattern was found in the remaining last case. Immunohistochemical analysis revealed that the proliferating cells were CD56 (123C3)$^+$ and/or CD16 (2H7)$^+$, TIA-1$^+$ and frequently stained CD3 ϵ^+. Tumor cells were frequently stained positively with monoclonal antibodies (mAbs) for T lymphocytes, but were negative for T-cell receptor (TCR) β and δ chain expression. *In situ* hybridization analysis using an Epstein-Barr virus-encoded early RNA 1 (EBER-1) probe revealed positive signals in 13 of the 15 cases examined. Southern blotting analysis for clonality of the Epstein-Barr virus (EBV) genome in 12 positive cases confirmed the presence of monoclonal proliferation in 7 cases. The pattern of TCR γ chain gene rearrangement was examined by PCR analysis of DNA from tumor tissues by the denaturing gradient gel electrophoresis method. The results demonstrated no clonal rearrangement in any of the 21 cases examined, including 7 cases with proven clonal proliferation of EBV-infected cells, indicating the absence of T-cell clones. Our findings strongly suggested that nasal T-cell lymphoma is in fact a NK cell lymphoma. *Int. J. Cancer* 81:865–870, 1999.
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Nasal lymphoma frequently exhibits a polymorphous pattern of proliferation consisting of large atypical cells with mono- or multinucleus, small lymphocytes, plasma cells, benign-appearing macrophages, neutrophils and much less frequently eosinophils. For this, the term polymorphic reticulosis (PR) was proposed (Kassel *et al.,* 1969). PR constitutes a clinical condition of lethal midline granuloma (LMG) which is characterized by necrotic and granulomatous lesions affecting the upper respiratory tract, especially the nasal cavity. Malignant lymphoma with monomorphous proliferation and Wegener's granulomatosis also show a condition similar to LMG.

Ishii *et al.* (1982) demonstrated that the proliferating cells in PR exhibit the T-cell immunophenotype by immunohistochemical analysis using polyclonal anti-T-cell antibody. Therefore, they proposed the term nasal T-cell lymphoma (NTL). However, monoclonal rearrangement of the T-cell receptor (TCR) genes has seldom been found in cases with NTL. Later, Ng *et al.* (1987) reported that tumor cells in this type of lymphoma showed positive immunoreactivity for the natural killer (NK) cell marker CD56. Immunophenotypic and genotypic studies of NTL have yielded contradictory results regarding the nature of tumor cells, *i.e.*, NK cells (CD56$^+$ and germline TCR β, γ, δ genes) (Emile *et al.*, 1996; Petrella *et al.*, 1996), NK-like T cells (CD56$^+$ and rearrangement of TCR genes) (Chiang *et al.*, 1996) or T cells (CD56$^-$ and rearrangement of TCR) (Harabuchi *et al.*, 1996). These discrepancies may have been due to the small number of cases examined, limitations of the methods used or the diseases themselves being heterogeneous. A workshop on the nasal and related extranodal T/NK cell lymphomas recommended the provisional terminology ''nasal and nasal-type T/NK cell lymphoma'' since the precise lineage of the neoplastic cells has not been demonstrated (Jaffe, 1996).

PCR-based strategies for analysis of gene rearrangement have become widespread (Hodges *et al.*, 1994). Denaturing gradient gel electrophoresis (DGGE), originally developed to detect point mutations, can separate PCR products according to their unique DNA sequences and not their length (Bourgium *et al.*, 1990; Theodorou *et al.*, 1996). Therefore, this method has a greater degree of specificity and 5- to 10-fold higher sensitivity than Southern blotting analysis (Bourgium *et al.*, 1990). In our study, the DGGE method was employed to examine the rearrangement of TCR γ chain genes in NTL. Rearrangement of TCR γ and δ chain genes precedes that of the β gene; thus, detection of rearrangement of the γ chain gene is more sensitive than that of the β chain (Theodorou *et al.*, 1996).

We examined the immunophenotypes and genotypes of proliferating cells in 21 cases with NTL, one of the Epstein-Barr virus (EBV)-associated lymphomas (Harabuchi *et al.*, 1990; Tomita *et al.*, 1995). Circularization of the EBV genome produces EBV episomes that have variable numbers of terminal repeats (TR) that differ between individual clones, and the presence of a single predominant band containing TR represents the clonal cellular proliferation of EBV-infected cells (Raab-Traub *et al.*, 1986). Thus, we examined the presence of clonal proliferation by determining patterns of fused TR of EBV.

PATIENTS AND METHODS

Patients

Review of our consultation files during the period from 1986 to 1997 revealed 17 cases (14 Japanese and 3 Chinese) of nasal lymphoproliferative diseases with histological features of PR and/or positive immunoreactivity of the proliferating cells for T-cell markers. Fresh frozen and paraffin-embedded specimens were available for 13 patients. Fresh frozen specimens and hematoxylin-eosin (H&E)-stained sections were available for 1 Japanese and 3 Chinese patients. In addition, fresh frozen and paraffin-embedded specimens from 4 Korean patients with PR were available; these patients had been admitted to Yonsei University Hospital, Seoul, in 1997. All these patients had the clinical

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*Correspondence to: Department of Pathology (C3), Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: (81) 6-879-3713. E-mail: aozasa@molpath.med.osaka-u.ac.jp

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features of LMG, *i.e.,* presence of necrotic granulomatous lesions in the upper respiratory tract. A total of 21 cases were included in our study: age at first admission ranged from 20 to 74 (median 37) years, and the subjects comprised 13 men and 8 women. The primary lesion was found in the nasal cavity in 18 patients, and in the epipharynx, nasopharynx and soft palate in 1 case each. Histological specimens, obtained from the primary lesions (19 cases) and metastatic cervical lymph nodes (2 cases), were fixed in 10% formalin and routinely processed for paraffin-embedding. Fresh specimens biopsied from the primary lesion were snap frozen in liquid nitrogen and stored at −80°C until use. All histological specimens were reviewed by one of the authors (K.A.), and a diagnosis of PR was made in 20 cases and of diffuse mixed cell type non-Hodgkin's lymphoma (NHL) in 1 case.

Immunohistochemistry

Staining by the avidin-biotin-peroxidase complex method was carried out on paraffin-embedded specimens in 16 cases and on fresh frozen specimen in 2 cases (1 and 2). The monoclonal antibodies (mAbs) and polyclonal antisera used for staining paraffin sections, their reactivity and suppliers are listed in Table I. Sections were treated with 0.1% trypsin solution (Sigma, St. Louis, MO) at 37°C for 30 min or 40 min before reaction with β F1 or anti-CD3, respectively. When 4C7, C8/144B, 2H7, 123C3, Leu7, CS1-4 and PE2 were used as primary antibody, sections were treated in a microwave oven for 15 min in 0.01% citrate buffer (pH 6) for antigen retrieval. Sections were microwaved for 15 min in 1 mM EDTA (pH 8.0) before incubation with 1F6. Cryocut sections in cases 1 and 2 were stained with anti-CD2 and TCRδ antibodies together with the antibodies used on paraffin sections. Five cases of nodal lymphoma of proven B- and T-cell immunophenotypes were included as controls.

In situ *hybridization for EBV*

EBV RNA *in situ* hybridization was performed as previously described (Weiss *et al.,* 1991) with some modifications. Briefly, 30-base sense and antisense oligonucleotide probes corresponding to a portion of the EBV-encoded early RNA 1 (*EBER-1*) gene, a region of the EBV genome that is actively transcribed in latently infected cells, were synthesized using a DNA synthesizer. As a positive control, the Raji cell line was used. As negative controls, hybridization was performed with a sense probe (a) and an antisense probe (b) after RNAse treatment. All controls for each case were run in parallel in each experiment.

DGGE method

Fresh frozen specimens obtained from 21 patients were available for DGGE analysis. To analyze the TCR γ gene configuration, we used a modification of the method of Theodorou *et al.* (1996). Briefly, we used 4 sets of 5′ primers designed for the V_γ families and 4 sets of 3′ primers designed for the different J genes; the sequences of these primers were described previously (Theodorou *et al.,* 1996). A 40-nucleotide GC clamp was attached at the 5′ end of each V_γ primer. All primers were purified by high performance liquid chromatography. For assessment of clonality, 1 of 4 V_γ primers and a mixture of 4 J primers were used in a single reaction (hemimultiplex PCR). When a rearranged band was found in hemimultiplex PCR, pairwise analysis (monoplex PCR) was performed with all VJ primer combinations to identify the V and J genes involved in rearranged alleles. DNA was prepared by proteinase K digestion, followed by phenol/chloroform extraction. The reaction mixture containing 250 ng of genomic DNA, 5 µl of 10× reaction buffer, 200 µM dNTPs, 0.8 or 1.2 µM of the selected V_γ primer and 0.8 µM of the 4 J_γ primers was dissolved in a final volume of 50 µl and overlaid with 1 drop of mineral oil. After initial denaturation at 94°C for 5 min, PCR was hot-started by adding 1.5 U of Taq polymerase (Wako, Osaka, Japan). PCR was performed with 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min. A final extension at 72°C was performed for 6 min. Before DGGE, aliquots of 10 µl of PCR products were screened by agarose gel electrophoresis in 1.5% agarose gels with TBE buffer (100 mM Tris base, 90 mM boric acid, 1 mM EDTA, pH 8.3) to confirm desirable amplification of DNA fragments with the expected sizes. For DGGE analysis, aliquots of 40 µl of PCR products were run on a 6.5% polyacrylamide gel containing a linear 10–60% denaturing gradient (100%

TABLE I – ANTIBODY PANEL[1]

Antibody	CD number	Reactivity	Dilution rate	Source
Paraffin and cryostat sections				
Mx-pan B	CD20	Pan B cell	1:50	Kyowa Medex (Tokyo, Japan)
MB-1	−	Pan B cell	1:50	Bioscience (Emmenbrücke, Switzerland)
MT-1	CD43	Pan T cell	1:50	Bioscience
UCHL-1	CD45RO	Pan T cell	1:100	Dakopatts (Glostrup, Denmark)
Anti-CD3 ϵ	CD3 ϵ	Pan T cell	1:100	Dakopatts
1F6	CD4	Helper/inducer T cell	1:200	Novocastra (Newcastle Upon Tyne, UK)
4C7	CD5	T cell, mantle zone B cell	1:200	Novocastra
C8/144B	CD8	Cytotoxic/suppressor T cell	1:200	Dakopatts
LeuM1	CD15	Granulocyte, macrophage	1:50	Becton-Dickinson (San Jose, CA)
2H7	CD16	Nk cell	1:200	Novocastra
123C3	CD56	NK cell	1:40	Zymed (South San Francisco, CA)
Leu7	CD57	NK cell	1:20	Becton-Dickinson
β F1	−	TCR β chain	1:50	T Cell Diagnostics (Woburn, MA)
CS1–4	−	Latent membrane protein^{-1}	1:20	Dakopatts
PE2	−	EBNA2	1:20	Dakopatts
TIA-1	−	Cytotoxic granules	1:500	Coulter (Hialeah, FL)
Cryostat sections				
Leu 5b	CD2	Pan T cell, NK cell	1:50	Becton-Dickinson
TCR δ 1	−	TCR δ chain	1:50	T Cell Diagnostics

[1]CD number, cluster of differentiation number; EBNA2, EBV nuclear antigen 2; NK cell, natural killer cell.

denaturant = 7 M urea and 40% volume of solute per volume of solution formamide) in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA, pH 7.4). The dimensions of gels were 16 cm × 16 cm × 1 mm. PCR products were precipitated in a solution containing 0.3 M sodium acetate and 70% ethanol at −20°C, and resuspended in 20 µl of loading buffer (20% Ficoll/10 mM Tris, pH 7.8/1 mM EDTA/0.5% bromphenol blue), incubated at 95°C for 5 min, rapidly cooled and held at 60°C for 1 hr prior to loading onto the gels. Gels were run at 150 V for 5 hr in TAE buffer at 60°C. After electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination.

Clonal rearrangement of the TCRγ gene results in 1 (monoallelic) or 2 (biallelic) bands on DGGE, whereas polyclonal rearrangement results in a smear. DNA extracted from 6 T-cell lines (Jurkat, Molt-4, Molt-14, HSB2, RPMI8402, Peer) with well-characterized TCR γ gene configurations and from a lymph nodal lesion in 1 case of peripheral T-cell lymphoma were used to establish the efficacy of the primers in DGGE procedures. HSB2 and 3 T-cell lines including Molt-14, RPMI8402 and Peer were gifts from the Human Science Research Resource Bank, Hayashibara Biochemical Laboratories, Inc., and Fujisaki Cell Center. DNA extracted from lymphocytes in peripheral blood of normal individuals (4 cases) and reactive lymphadenitis (1 case) with no monoclonal TCR γ gene rearrangement were used as negative controls. When DNA from a clonal T-cell line was serially diluted with DNA extracted from lymphocytes from a normal individual, the sensitivity of this system was estimated to be lower than 5% in all hemimultiplex primer combinations. Usage of monoplex PCR increases the sensitivity to 0.1%. The experiment was repeated twice for each sample.

Southern blotting analysis for EBV genome clonality

Aliquots of 10 or 20 µg of DNA were digested with Bam HI (GIBCO-BRL, Gaithersburg, MD), electrophoresed in 0.6% agarose gels and subjected to Southern blotting. The plasmid containing the joined termini of EBV genome, pCB 281, was kindly provided by Dr. E. Kieff (Boston, MA). The 1.9 kb Xho-I fragment adjacent to TR was used as a template and the probe was labeled with α-[^{32}P]-dCTP by random priming.

Southern blotted filters were prehybridized for 4 hr at 42°C in 6× SSPE (0.9 M NaCl, 0.06 M Na phosphate, 6 mM EDTA), 5× Denhardt's solution, 0.1% SDS, 200 µg/ml denatured salmon testes DNA (Sigma) and 50% formamide, and hybridized in a solution of the same composition containing the probe at 2×10^6 cpm/ml. Filters were hybridized for 20 hr at 42°C, washed twice for 30 min at room temperature with 1× SSPE, 0.1% SDS, and twice for 20 min at 70°C with 0.1× SSPE, 0.1% SDS, and exposed at −80°C.

RESULTS

Histological findings

The primary lesions showed varying degrees of necrosis. Diffuse proliferation of large atypical mono- or multinucleated cells intermingling with various numbers of small lymphocytes, plasma cells and macrophages was observed, giving a more or less polymorphous appearance in all but 1 case (Fig. 1). Angiodestructive pattern of proliferation was found in 5 of these cases. In general, mitotic figures were frequent. One case (21) showed monomorphous proliferation of medium- to large-sized lymphoid cells. This case was thus diagnosed as NHL of diffuse mixed cell type.

Immunohistochemical study

In the control cases, tumor cells in all 5 of the B-cell lymphomas showed positive reactivity for CD20. Small lymphocytes intermingling with large lymphoma cells were positive for T-cell markers including CD43, CD45RO, CD3 ϵ, CD4, CD5 and CD8. These small lymphocytes also reacted with β F1. The mantle zone lymphocytes in the preserved lymphoid follicles were positive for CD5. All of 5 T-cell lymphoma cases expressed pan T-cell markers and 4 were positive for β F1 with $CD4^+$, $CD8^-$, and 1 case being $CD4^-$, $CD8^+$. Small lymphocytes intermingled with lymphoma cells were positive for NK cell markers including CD16, CD56 and CD57.

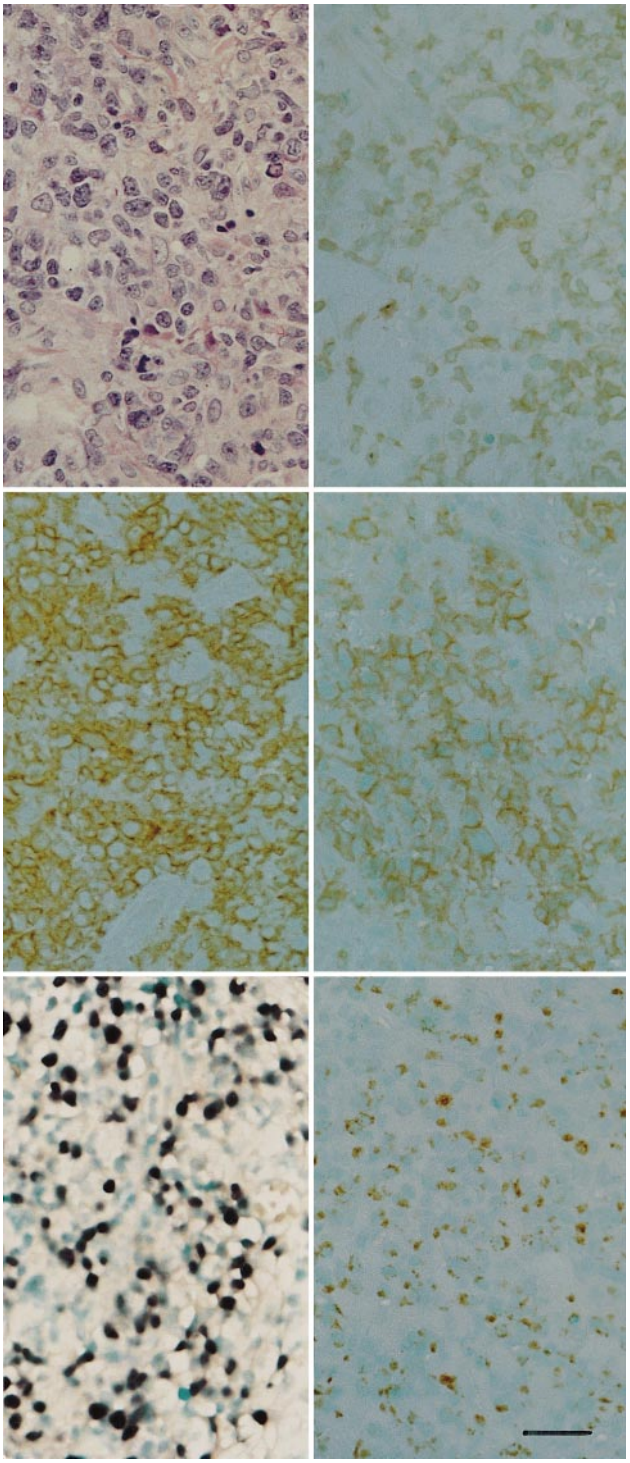

FIGURE 1 – Upper left: Diffuse proliferation of large cells with rich clear cytoplasm intermingling with small lymphocytes. H&E. Proliferating cells were positive for CD3 ϵ (upper right), CD16 (middle left), CD56 (middle right) and TIA-1 (lower right). Lower left: *In situ* hybridization using EBER-1 probe showed positive signals in the nucleus of these cells. Scale bar: 30 µm.

As summarized in Table II, none of the 18 cases examined showed a B-cell immunophenotype or CD15 positivity. All of the PR cases were positive for CD16 and/or CD56, together with various T-cell markers including CD3 ϵ, CD4, CD5, CD43 and CD45RO but negative for β F1 (Fig. 1). Two cases (1 and 2) examined as frozen specimens were $CD2^+$, $CD56^+$, β $F1^-$, TCR δ 1^-. Thus, all of the PR cases were tentatively considered to be NK cell or NK-like T-cell lymphoma. Twelve of these cases tested for TIA-1, known to react with cytotoxic granules of cytotoxic T cells or NK cells, showed positive granular staining in the cytoplasm. One case (21) that was diagnosed as diffuse mixed cell type was $CD43^+$, $CD45RO^+$, $CD56^-$. This case was tentatively categorized as T-cell lymphoma.

In situ *hybridization for EBV*

Among the 16 cases examined, positive signals were detected in the nuclei of large atypical cells in 13 (87%) of 15 PR cases but not in 1 case with ordinary NHL. Three (23%) of these 13 PR cases with EBV genome were positive for LMP-1 expression in the cytoplasm.

Southern blotting analysis for EBV genome clonality

A monoclonal band was observed in 7 of 12 cases on Southern blotting analysis of Bam HI-digested DNA samples with an EBV-DNA probe corresponding to the sequence adjacent to TR (Fig. 2): 2 cases (4 and 21) with application of 10 μg and 5 cases (1, 2, 7, 14 and 20) with 20 μg of genomic DNA from the lesions. The frequency of detection of the monoclonal band increased with application of larger amounts of DNA. Samples of 20 μg of genomic DNA were not available for the remaining 5 cases, which were negative on analysis of 10 μg of DNA. Therefore, clonality of EBV might have been detected in these 5 cases if larger amounts of DNA would have been available.

DGGE method

DGGE analysis did not show clonally rearranged bands in any of the present cases, including 7 with monoclonal proliferation of EBV-infected cells.

Based on the results of histological and immunohistochemical analyses, all of the 21 cases diagnosed as PR were judged to be NK cell lymphomas. Case 21 with diffuse mixed cell type of NHL showed the T-cell immunophenotype but germline configuration of the TCR γ chain gene.

DISCUSSION

The patients discussed here presented with necrotic, granulomatous lesions in the upper respiratory tract, especially the nasal cavity. The predominance of male patients and rather young age at the onset of disease, together with polymorphous histological pattern of cellular proliferation, were identical with those in PR or NTL (Kassel *et al.,* 1969; Tomita *et al.,* 1995; Harabuchi *et al.,* 1996). One case showed a rather monomorphous pattern of proliferation and was thus classified as NHL of diffuse mixed cell type. Such a monomorphous pattern of proliferation is occasional at the early stage and is relatively frequent at later stage of PR (Aozasa *et al.,* 1995).

Immunohistochemical analysis was performed in 17 cases, 10 of which were found to be positive for CD43, CD45RO and CD8. Another 3 cases were $CD8^+$ without positive immunoreactivity for CD43, CD45RO. These findings were roughly in agreement with those of previous immunohistochemical studies of cases with NTL (Ishii *et al.,* 1982; Tomita *et al.,* 1995; Harabuchi *et al.,* 1996). Proliferating cells in 4 of 12 cases (33%) were shown to be positive for CD5 using mAb 4C7. Previous studies usually with Leu 1 on frozen sections for detection of CD5 showed similarly positive rates, *i.e.,* 20–43% (van Gorp *et al.,* 1994; Harabuchi *et al.,* 1996). None of the present cases showed TCR β (β F1) or δ chain (TCR δ 1) expression, which questions the T-cell nature of NTL.

CD2 is an antigenic phenotype of pan T cells and NK cells. Two cases (1 and 2) from which frozen sections were examined were $CD2^+$, which is consistent with previous reports on nasal lymphomas of NK and T-cell types (Petrella *et al.,* 1996; Chiang *et al.,* 1996; Jaffe *et al.,* 1996). Cytoplasmic CD3 ϵ positivity is now regarded as a supporting finding for NK cell nature of the proliferating cells in NTL (Jaffe *et al.,* 1996). In the current series, more than 60% of cases showed intracytoplasmic expression of CD3 ϵ. Anti-CD56 antibody is known to recognize neuronal cell adhesion molecule (N-CAM) (Jaffe *et al.,* 1996) and to show positive immunoreactivity with NK cell neoplasms (Ng *et al.,* 1987; Jaffe *et al.,* 1996). More than 80% of the cases in the present series were positive for CD56, further supporting the NK cell

TABLE II – SUMMARY OF IMMUNOPHENOTYPE, EBV STUDY AND DGGE METHOD IN 21 PATIENTS WITH PR[1]

Patient number	Immunophenotype														EBV			TCR γ gene rearrangement	Histology
	CD20	CD45RA	CD43	CD45RO	CD3 ϵ	CD4	CD5	CD8	CD15	CD16	CD56	CD57	TIA-1	β F1	CS1–4	PE2	EBER		
1	−	nd	nd	nd	+	−	−	+	−	+	+	−	+	−	−	−	nd	G	PR
2	−	−	+	+	+	−	−	−	−	−	+	−	+	−	−	−	nd	G	PR
3	−	−	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	G	PR
4	−	−	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	G	PR
5	−	−	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	G	PR
6	−	−	+	−	+	−	+	+	−	+	+	−	+	−	−	−	+	G	PR
7	−	nd	+	+	+	nd	nd	nd	nd	nd	+	nd	nd	nd	+	nd	+	G	PR
8	−	nd	+	−	+	nd	nd	nd	nd	nd	+	nd	nd	nd	−	nd	+	G	PR
9	−	−	+	−	−	+	+	−	−	+	+	−	+	−	+	−	+	G	PR
10	−	nd	+	+	−	nd	nd	nd	nd	nd	+	nd	nd	nd	+	nd	+	G	PR
11	−	−	−	−	−	−	+	+	−	+	+	−	+	−	−	−	−	G	PR
12	−	−	−	−	−	−	+	+	−	+	+	−	+	−	−	−	−	G	PR
13	−	nd	+	+	nd	nd	nd	nd	nd	nd	+	nd	nd	nd	−	nd	+	G	PR
14	−	nd	+	−	nd	nd	nd	nd	nd	nd	+	nd	nd	nd	−	nd	+	G	PR
15	−	−	+	−	+	−	−	−	−	+	−	−	+	−	−	−	+	G	PR
16	−	−	−	−	+	−	−	−	−	+	−	−	+	−	−	−	+	G	PR
17	−	−	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	G	PR
18	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	G	PR
19	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	G	PR
20	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	G	PR
21	−	nd	+	+	−	nd	nd	nd	nd	nd	−	nd	nd	nd	−	nd	−	G	Dmix

[1]Cases 1 and 2 from which frozen material was available were positive for CD2 and negative for TCR δ 1. G, germline; R, rearrangement; Dmix, diffuse mixed; nd, not done; EBER, EBV-encoded early RNA.

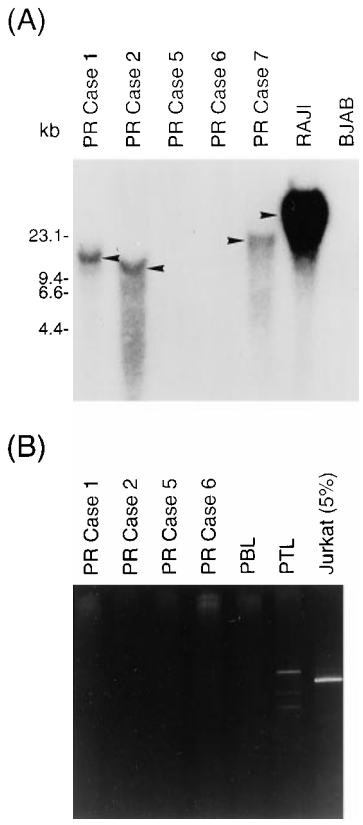

FIGURE 2 – *(a)* Southern blotting analysis for clonality of the EBV genome. Twenty micrograms of DNA was digested with Bam HI, electrophoresed in agarose gels, Southern blotted and hybridized with ^{32}P-labeled EBV-DNA probe. DNA size markers are indicated on the left. Five-day exposure at −80°C. PR biopsy specimens and Raji cells contained a predominant TR of EBV (arrowheads). Raji, EBV-positive Burkitt's lymphoma cell line; BJAB, EBV-negative Burkitt's line. *(b)* DGGE patterns of PCR products amplified with V_γ primer and mixed J primers in a single reaction. Representative PR cases and peripheral blood leukocytes (PBL) from normal individual showed diffuse smears without predominant bands, representing polyclonal T-cell populations. Peripheral T-cell lymphoma (PTL) showed 3 discrete bands, representing a predominant clonal T-cell population. This pattern results from biallelic rearrangement with heteroduplex formation. DNA from Jurkat cell line diluted to 5% in DNA from PBL also showed 1 discrete band.

nature of the proliferating cells. However, it has become evident that CD56 does not have lineage or disease specificity for NK cells (Jaffe *et al.,* 1996). CD57 is also a marker for NK cells, but none of our cases showed a positive immunoreactivity for this molecule, which is also in agreement with the results of previous studies on NTL (Ng *et al.,* 1987) or nasal and nasal type T/NK cell lymphomas (Jaffe *et al.,* 1996). CD16 is the Fc receptor through which NK cells mediate antibody-dependent cytotoxicity (Raulet *et al.,* 1998). Previously, the Leu 11b mAb was used on frozen sections to detect CD16 and the results showed that about half of the NTL cases were CD16-positive (Ng *et al.,* 1987; Harabuchi *et al.,* 1996). In the present study, mAb 2H7, which can be used to stain routinely processed paraffin-embedded specimens, was used. Positive staining was observed in proliferating cells in 11 of 12 cases. 2H7 might be a sensitive marker for NK cell tumors. All of the present cases were positive for CD56 and/or CD16.

TIA is a 15 kDa protein structurally related to the necrotic factor receptor family, which induces apoptotic cell death when introduced into permeabilized target cells (Kawakami *et al.,* 1992). Antibodies to TIA-1 have been shown to be highly specific for the cytolytic granules of NK cells and cytotoxic T lymphocytes (CTLs) (Felgar *et al.,* 1997). Thus, such antibodies could be useful in identification of specific subsets of lymphoid neoplasms derived from CTLs or NK cells. From the results of light and electron microscopic observations of leukemic cells in the peripheral blood of 1 case, a rare event in NTL, we showed that tumor cells had large granules in the cytoplasm, *i.e.,* were large granular lymphocytes (LGL) (Aozasa *et al.,* 1995). It is well known that NK cells and CTLs have the morphology of LGL (Timonen *et al.,* 1981). Indeed, all of the current cases examined for TIA-1 showed positive immunoreactivity in the cytoplasm of tumor cells.

NTL is considered to be a lymphoid neoplasm. However, methods to confirm the clonality of proliferating cells in NTL have been quite limited. NTL has been reported to be one of the EBV-associated malignancies (Harabuchi *et al.,* 1990; Tomita *et al.,* 1995). The presence of clonal cellular proliferation of EBV-infected progenitor cells has been confirmed in NTL by examining the fused pattern of EBV terminal repeats (Raab-Traub *et al.,* 1986). Indeed, *in situ* hybridization revealed positive signals for the EBV genome in all but 2 of the present cases. EBV genome clonality analysis confirmed the presence of monoclonal proliferation in 7 of these 12 cases. Clonal bands were initially found in 2 cases when analyzing 10 µg of DNA, but they were also found in an additional 5 cases when 20 µg of DNA was used. There seem to be problems in the sensitivity of the technique, and thus monoclonal bands might have been found in the remaining 5 cases if larger amounts of DNA had been available for examination. Only a small amount of DNA is usually available from nasal lesions accompanying necrotic changes.

Southern blotting analysis has been used for analysis of TCR gene rearrangement in NTL and has shown negative results in the vast majority of cases (Weiss *et al.,* 1988; Emile *et al.,* 1996; Jaffe *et al.,* 1996). This procedure, however, has some disadvantages including limitation of sensitivity for clonal cell detection and misinterpretation of results due to artifacts such as non-specific degeneration of DNA prior to or during extraction from the tissues. Moreover, electrophoretic separation is based principally on the difference in length of DNA fragments digested with restriction enzymes, and thus the presence of monoclonal rearrangement could be confirmed only when mobility shifts were found relative to the germline configuration. If germline DNA and monoclonally rearranged DNA have the same size, the results would be interpreted as negative. The PCR-DGGE method does not suffer from these disadvantages of Southern blotting analysis and could be more sensitive and specific for detection of TCR γ chain gene rearrangement (Bourgium *et al.,* 1990). There have been few studies of TCR γ chain gene rearrangement using PCR-DGGE in NTL cases: Petrella *et al.* (1996) reported that 4 cases with nasopharyngeal lymphomas showed the germline configuration. However, the presence of the monoclonal proliferation of the EBV genome-containing cells was not examined in these cases. As mentioned, samples from nasal lesions of NTL frequently contain necrotic tissues and small amounts or even no vivid tumor tissue. Therefore, confirmation of the presence of clonal cell proliferation in the lesional tissues from the nasal cavity is essential before application of the PCR-DGGE method. Clonally rearranged bands of the TCR γ chain gene were not found in any of the present 21 cases with NTL including 7 cases with proven clonal proliferation of EBV genome-containing cells. These findings strongly suggest the absence of T-cell clones in NTL.

In conclusion, NTL cases were shown to be $CD56^+$ and/or $CD16^+$, $TIA\text{-}1^+$, $CD2^+$ and frequently CD3 ϵ^+ by immunohistochemistry. Tumor cells were frequently stained positively with mAbs for T lymphocytes but were negative for TCR β and δ chain expression. Taken together with the absence of T-cell clones defined by sensitive genotyping, this strongly suggests that NTL is, in fact, a NK cell lymphoma.

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