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Immunohistochemical and Immunogenetic Analyses of Ocular Adnexal Lymphoid Proliferation

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Purpose: To compare polymerase chain reaction (PCR) results with histological, immunohistochemical, and clinical findings to understand the nature of ocular adnexal lymphoid proliferation.

Methods: We examined 20 cases (21 specimens) of ocular adnexal lymphoid proliferation, using histological, immunohistochemical, and molecular genetic methods. The latter two types of experiments were performed to examine the light chain restriction of immunoglobulin using the peroxidase-antiperoxidase method, and the clonality of immunoglobulin heavy chains using the PCR method.

Results: Although in 8 cases it could not be determined morphologically whether the tumors were neoplastic or not, clonality was revealed in 1 case by immunohistochemistry and in 4 cases by PCR. Two cases showed disparate results between immunohistochemistry and PCR, probably due to somatic mutation of the framework region of the immunoglobulin heavy chain gene.

Conclusion: Examination using these methods contributes to a better understanding of the nature of the ocular adnexal lymphoid proliferation. Furthermore, the immunoglobulin gene PCR method is very useful in practice for examination of specimens, as it can be used with formalin-fixed and paraffin-embedded specimens. **Jpn J Ophthalmol 2000;44:368–373** © 2000 Japanese Ophthalmological Society

Key Words: Clonality, immunohistochemistry, malignant lymphoma, ocular adnexal lymphoid proliferation, polyermase chain reaction.

Introduction

Ocular adnexal lymphoid proliferation is classified broadly as benign lymphoid proliferation, which includes reactive lymphoid hyperplasia and inflammatory pseudotumor or malignant lymphoma. Small lymphocytic proliferation includes reactive lymphoid hyperplasia, atypical lymphoid hyperplasia and low-grade malignant lymphoma, which account for a high proportion of ocular adnexal lymphoid proliferation, but in some cases these various forms cannot be differentiated histologically. When the differential diagnosis is between malignant lymphoma and reactive lymphoid hyperplasia, immunohistochemistry is valuable.[1–4] Immunologic studies of cell suspensions have shown that ocular adnexal lymphomas generally consist of one type of B-cell proliferation. Therefore, ocular adnexal lymphoma reveals light chain restriction (B-lymphocytes containing expression of only κ or λ light chain). In contrast, reactive lymphoid hyperplasia does not reveal light chain restriction (instead, it consists of B-lymphocytes, containing a mixture of κ and λ light chain). It has been reported that light chain restriction was present in 70%[1,2] of ocular adnexal lymphoid proliferation cases. Despite the usefulness of this distinction, however, it has not completely resolved the diagnostic dilemma. Therefore, in this study we examined the hemi-nested polymerase chain reaction (PCR) technique for the variable, diversity, and joining (V-D-J)

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regions of the immunoglobulin heavy chain gene to examine the clonality of ocular adnexal lymphoid proliferation. This method is sensitive and works well, even if only paraffin-embedded tissues are available. We compared the PCR results with clinical, histological, and immunohistochemical findings.

Materials and Methods

Patients

We examined 20 patients who underwent open biopsy and were diagnosed as having ocular adnexal lymphoid proliferation. Patients with lymphoid proliferation in the choroid, retina, or vitreous body and patients with a history of prior extraocular lymphoma were excluded.

Histology and Immunohistochemistry

Twenty-two biopsy specimens were obtained from 20 patients and embedded in paraffin. We examined the histology of the 22 biopsy specimens and the immunohistochemistry of 21 of the 22 biopsy specimens. Malignant lymphoma was classified according to the Working Formulation.[5] Benign lymphoid proliferation was classified histologically according to the previously described criteria.[6] Biopsy specimens were classified according to their expression of the B cell-associated antigen CD 20 (DAKO, Copenhagen, Denmark). Staining for cytoplasmic immunoglobulins (IgA, IgG, IgM, κ and λ anti-human rabbit polyclonal antibodies) (DAKO) was performed by the peroxidase-antiperoxidase method.[7]

Molecular Genetic Analysis

The principle of clonal identification is shown in Figure 1. DNA was extracted from formalin-fixed, paraffin-embedded specimens. A DNA template was made as follows. Ten-micrometer-thick sections were cut from each paraffin block and placed in 1.5-mL Eppendorf tubes. Each section was deparaffinized with xylene and ethanol, followed by vacuum centrifugation to remove all traces of ethanol, and then digested with 10 μg/mL proteinase K overnight. Proteinase K was then inactivated by boiling. Oligonucleotide primers designed to amplify the V-D-J region were made based on previously described sequences.[8] The V-D-J region was amplified by hemi-nested PCR. The hemi-nested amplification step consisted of 10 cycles of DNA denaturation at 94°C, annealing at 50°C, and synthesis at 72°C, followed by 25 cycles of DNA denaturation at 94°C, annealing at 62°C and synthesis at 72°C. A negative control amplification using DNA from placental cells and a positive control from the "Raji"' B-cell line was performed for each experiment. Products were separated on 4% high-sensitivity agarose gels (Nusieve GTC agarose gels; Takara, Kyoto), and a broad band was taken to indicate a polyclonal B cell population, while a discrete band was taken to indicate a clonal B cell population.

Results

Clinical Features

The age, sex, anatomic site, form of therapeutic intervention, and subsequent clinical course of the 20 patients are summarized in Table 1. There were 7 men and 13 women, with a mean age of 65. The orbit was involved in 14 patients, the conjunctiva in 5 patients, and the eyelid in 1 patient. No patient had Sjögren's syndrome. The treatment regimens and the number of patients treated with each regimen were as follows: chemotherapy (cyclophosphamide, daunorubicin hydrochloride, vincristine sulfate, prednisolone), 3 cases (1, 7, and 11); chemotherapy and radiotherapy, 2 cases (3 and 15); radiotherapy, 6 cases (5, 6, 8, 9, 12, and 17); oral corticosteroids only, 1 case (16); and periodic observation only, 8 cases (2, 4, 10, 13, 14, 18, 19, and 20).

Histopathology

Ocular adnexal lymphoid proliferation was classified as malignant lymphoma in 13 cases (1–12 and 15); atypical lymphoid hyperplasia in 3 cases (12–14); reactive lymphoid hyperplasia in 5 cases (15–19); and inflammatory pseudotumor in 1 case (20). Malignant lymphoma was characterized as diffuse small lymphocytic (including plasmacytoid) in 9 cases (4–12); follicular, mixed in 2 cases (2 and 3); diffuse, large in 1 case (1); and follicular, large in 1 case (15). Two biopsy specimens were obtained from cases 12 and 15. In case 12, the first biopsy specimen was diagnosed as atypical lymphoid hyperplasia, and the second as small lymphocytic type. In case 15, the first biopsy specimen was diagnosed as reactive lymphoid hyperplasia, and the second as follicular lymphoma, large cell type.

Hemi-Nested PCR and Immunohistochemistry

The results of histopathology, immunohistochemistry and PCR are summarized in Table 2. Twelve of the 13 lymphomas (cases 1–12 and 15) revealed both light chain restriction and immunogenetic clonality. One exceptional case (case 5) did not reveal clonality using PCR. Of 7 cases diagnosed as reactive lymphoid hyperplasia and atypical lymphoid hyperplasia (cases 12–14, and 16–19), one case (case 13) revealed light chain restriction only, and three cases (cases 12, 16, and

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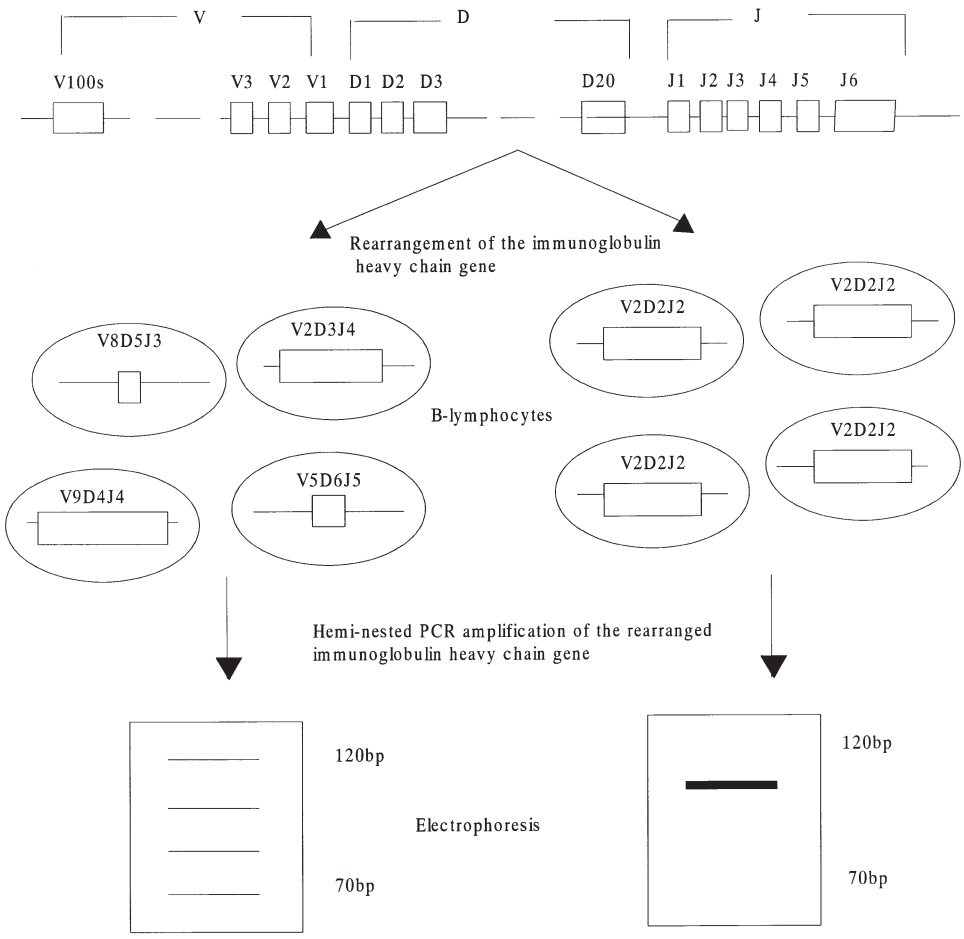

Figure 1. Principle of clonal identification. B-lymphocytes recognize a specific antigen, and one gene is selected from each of the variable, diversity, and joining (V-D-J) regions of an immunoglobulin heavy chain gene to form rearranged target genes. In reactive lymphoid lesions, lymphocytes recognize many antigens, and when various rearranged heavy chain genes are amplified by hemi-nested polymerase chain reaction (PCR), polyclonal population shows a range of product sizes. In contrast, because lymphomas are clonal proliferations, when one rearranged heavy chain gene is amplified by hemi-nested PCR, a clonal population is indicated by the presence of a product of a discrete size. The appearance of smears is due to amplification of polyclonal reactive B cells present in the samples.

17) had immunogenetic clonality only. Inflammatory pseudo-tumor revealed neither light chain restriction nor immunogenetic clonality. Two biopsy specimens were obtained from patient 12: the first biopsy specimen was diagnosed as atypical lymphoid hyperplasia and the second as malignant lymphoma (Figure 2). The first biopsy specimen revealed immunogenetic clonality (Figure 4), while the second biopsy specimen revealed light chain restriction (Figure 3) and immunogenetic clonality.

Outcome

During the observation period, 2 patients (cases 8 and 12) metastasized to the contralateral orbit, 1 patient (case 4) to the lung, 1 patient (case 2) to the submandibular gland and 1 patient (case 15) to the kidney. The clinical outcomes of the 13 patients diagnosed histologically with malignant lymphoma were as follows. Six patients (cases 1, 3, 5, 7, 9, and 11) achieved a complete remission with no recurrence of lymphoma. Three patients (cases 6, 10, and 12) were alive with evidence of lymphoma. Two patients (cases 4 and 15) died with clinical evidence of disseminated lymphoma. Two patients (cases 2 and 8) died of secondary unrelated causes. On the other hand, 7 patients (cases 13, 14, and 16–20) diagnosed with benign lymphoid proliferation were alive, except for 2 patients (cases 13 and 16) who died of other diseases.

Table 1. Clinical Findings in 20 Patients with OcularAdnexal Lymphoid Proliferation

Case No./ Age (y)/Sex	Site	Treatment	Clinical Course	Outcome (Months)
1/67/F	L, orbit	Chemotherapy	CR	NED (22)
2/81/F	B, orbit	None	submandibular relapse	Died NED (7)
3/70/M	R, orbit	Chemotherapy + Radiation	CR	NED (26)
4/84/F	L, orbit	None	lung relapse	DWD (60)
5/59/M	R, conjunctiva	Radiation	CR	NED (35)
6/29/F	R, conjunctiva	Radiation	PR	AWD (45)
7/88/F	R, orbit	Chemotherapy	CR	NED (15)
8/63/M	L, orbit	Radiation	R, orbital relapse	Died NED (8)
9/72/F	L, orbit	Radiation	CR	NED (39)
10/50/F	L, conjunctiva	None	Asymptomatic	AWD (17)
11/52/F	R, conjunctiva	Chemotherapy	CR	NED (35)
12/81/F	L, orbit	Radiation	R, orbital relapse	AWD (38)
13/84/F	R, orbit	None	Asymptomatic	Died NED (22)
14/66/F	R, conjunctiva	None	Asymptomatic	AWD (39)
15/77/M	L, orbit	Chemotherapy + Radiation	Kidney relapse	DWD (37)
16/81/F	R, orbit	Steroid	PR	Died NED (6)
17/49/M	L, orbit	Radiation	CR	NED (26)
18/48/M	R, orbit	None	SR	NED (123)
19/51/F	L, eyelid	None	SR	NED (26)
20/49/M	R, orbit	None	SR	NED (48)

F: Female; M: male; R: right; L: left; CR: complete remission; PR: partial remission; SR: spontaneous remission; NED: no evidence of disease; DWD: died with disease; AWD: alive with disease; Died NED: died with no evidence of disease.

Table 2. Analyses of Biopsy Specimens

Patient No.	Histopathology	Immunohistochemistry	PCR
1	DL	λ	Clonal
2	FM	λ	Clonal
3	FM	κ	Clonal
4	SL	λ	Clonal
5	SL	κ	Polyclonal
6	SL	λ	Clonal
7	SL	λ	Clonal
8	SL	κ	Clonal
9	SL	κ	Clonal
10	SL	κ	Clonal
11	SL	κ	Clonal
12	ALH	Polyclonal	Clonal
	SL	λ	Clonal
13	ALH	κ	Polyclonal
14	ALH	Polyclonal	Clonal
15	RLH	ND	ND
	FL	λ	Clonal
16	RLH	Polyclonal	Clonal
17	RLH	Polyclonal	Clonal
18	RLH	Polyclonal	Polyclonal
19	RLH	Polyclonal	Polyclonal
20	IPH	Polyclonal	Polyclonal

PCR: polymerase chain reaction; DL: diffuse, large; FM: follicular, mixed small and large cells; SL: small lymphocytic (plasmacytoid); ALH: atypical lymphoid hyperplasia; RLH: Reactive lymphoid hyperplasia; FL: follicular large; IPH: Inflammatory pseudotumor; ND: not detected.

Discussion

In the present study, the incidence of reactive lymphoid hyperplasia and inflammatory pseudotumor (24%), atypical lymphoid hyperplasia (14%), and malignant lymphoma (62%) was similar to those reported by Knowles et al.[5] It was difficult to diagnose 3 cases (atypical lymphoid hyperplasia) histologically, because the majority of ocular adnexal lymphoid proliferations were small lymphocytic proliferations which were difficult to diagnose histologically. In our study, of 21 biopsy specimens, 17 (81%) were small lymphocytic proliferations. In these cases, it was useful to examine the light chain restriction. Of our 3 atypical lymphoid proliferations, 1 case revealed light chain restriction; malignant lymphoma was diagnosed in this case.

Of the 7 specimens not diagnosed as malignant lymphoma by histopathology and immunohistochemistry in the present study, hemi-nested PCR identified clonality in 4 specimens. We speculate that these 4 ocular adnexal lymphoid proliferations were malignant lymphoid proliferations that escaped detection by morphologic and immunohistochemical analyses. Concerning the cases of conflicting results of hemi-nested PCR and immunohistochemical analyses, some possible explanations include the follow-

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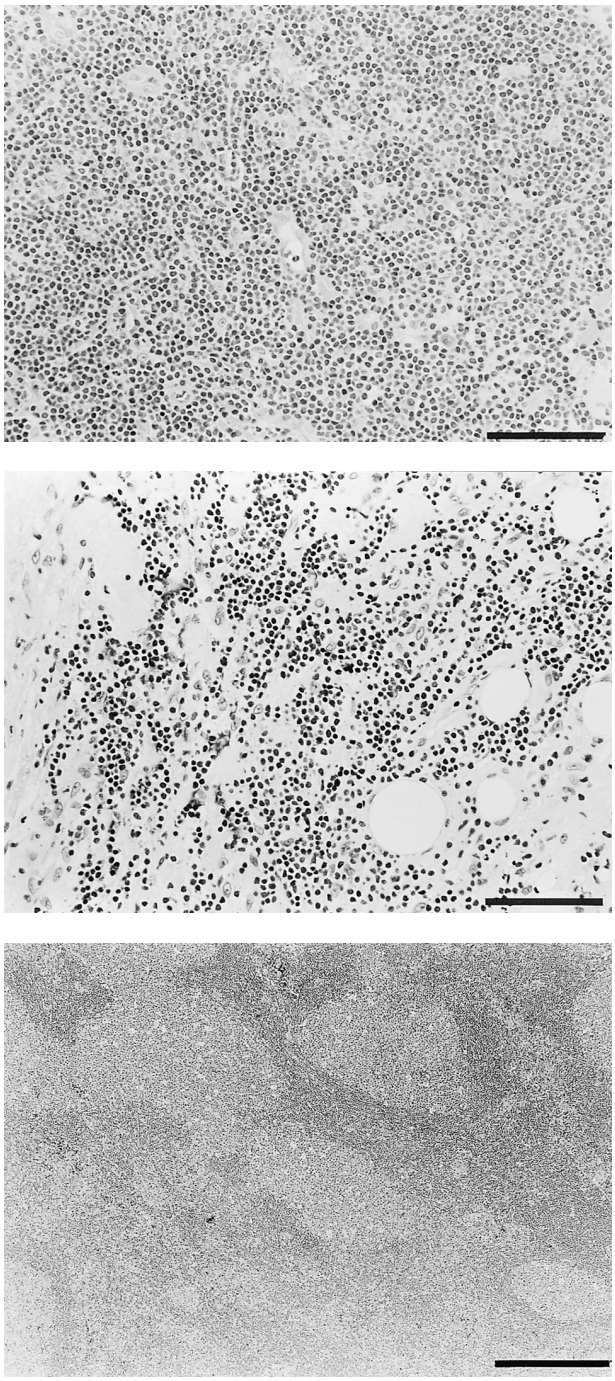

Figure 2. Histopathology (hematoxylin-eosin) (Top) Malignant lymphoma, diffuse small cell type. Figure reveals dense monomorphous proliferation of small lymphoid cells (patient 12, second biopsy specimen. Bar = 200 μm). (Middle) Atypical lymphoid hyperplasia. Figure reveals small number of sparse lymphoid cells with few inflammatory cells (patient 12, first biopsy specimen. Bar= 200 μm). (Bottom): Follicular reactive lymphoid hyperplasia. Several well-developed germinal centers are observed (case 19). (Bar = 500 μm).

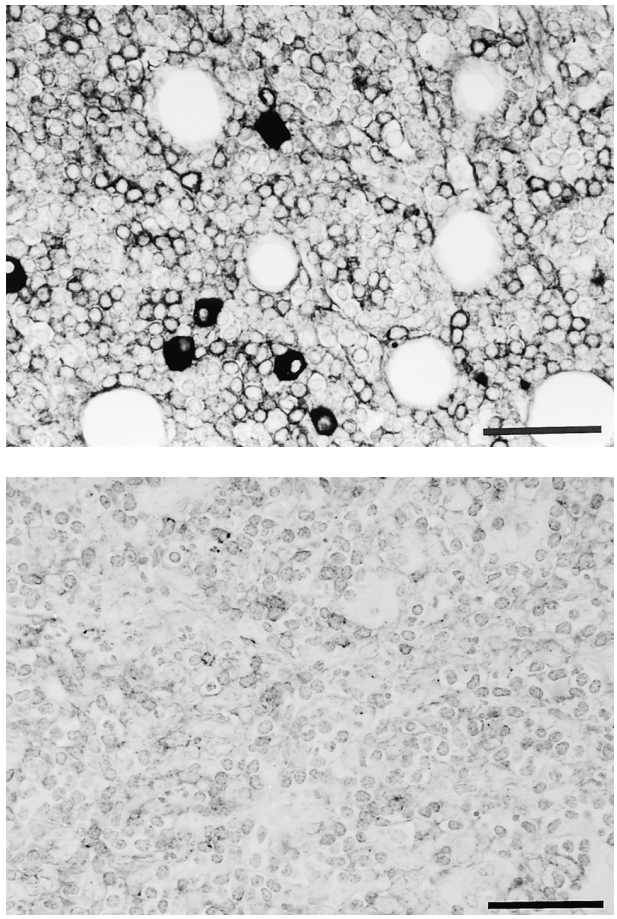

Figure 3. Results of immunoglobulin κ and λ staining (patient 12, second biopsy specimen). Immunoglobulin staining revealed positive reactivity for λ-chain (Top), but negative for κ-chain (Bottom). Bar = 100 μm.

ing. The first possibility is that because it is often difficult to extract biopsy tissues from ocular adnexal lymphoid proliferations, sample sizes insufficient to fully examine the tumors histologically and immunohistochemically may have been used. The discrepancy between the results of immunohistochemical and hemi-nested PCR examinations may thus have been due to the fact that the number of cells was not always sufficient to reveal light-chain restriction immunohistochemically. It is considered, for example, that the first specimen of case 13 contained an inadequate amount and quality of sample to carry out histological and immunohistochemical examinations. In terms of immunogenetic analyses, the accuracy of Southern blot analysis using fresh or frozen tissues of malignant lymphoma for diagnosis is reportedly 99%.[9] However, it is often difficult in practice to obtain both formalin-fixed, paraffin-embedded tissues

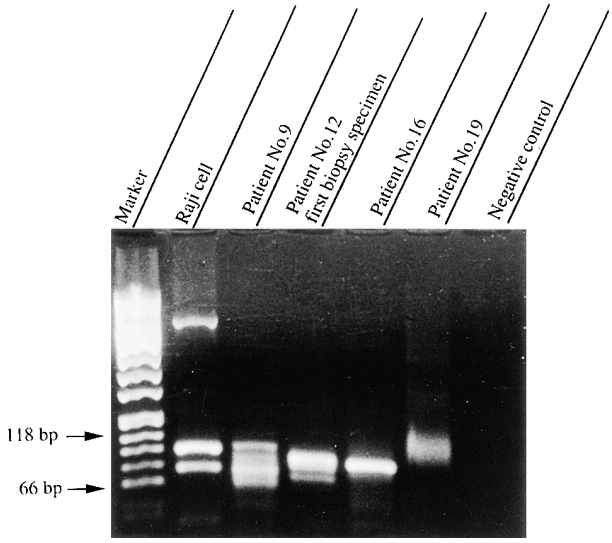

Figure 4. Polyacrylamide gels showing hemi-nested polymerase chain reaction (PCR) products. Product of patient 12 showed one discrete band. Product of patient 9 showed one discrete smeared band. This result was suggestive of the existence of minimal lymphoma in reactive lymphoid proliferations. Product of patient 19 showed smeared pattern. This result indicated reactive lymphoid proliferation.

and fresh or frozen tissues. Hemi-nested PCR is useful when only formalin-fixed, paraffin-embedded materials are available. Moreover, compared with Southern blot analysis, hemi-nested PCR is not only simpler and faster, but it requires much less DNA. A second possibility is that reactive lymphoid lesions may contain minimal lymphomas. In minimal specimens, before identifying a neoplasm by histopathology and immunohistochemistry, detection of the neoplasm is carried out by molecular genetic analysis. The same phenomenon has been reported also in examinations of lymphoma.[10] In cases 16 and 17, in spite of not having carried out histopathological and immunohistochemical analyses, we could identify the neoplasm by hemi-nested PCR. This result was suggestive of the existence of minimal lymphoma. On the other hand, of the 14 specimens diagnosed as malignant lymphoma by histopathology and immunohistochemistry in the present study, hemi-nested PCR did not identify clonality in 2 specimens (cases 5 and 13). This result probably indicated that PCR could not identify clonality due to the fact that the lymphocytes contained a somatic mutation of the framework region of the immunoglobulin heavy chain gene. This somatic mutation was observed even in lymphoma. In our two cases, it is suggested that this somatic mutation prevented annealing. Overall, the present study shows that combined use of immunohistochemistry and hemi-nested PCR is useful for the assessment of ocular adnexal lymphoid proliferation.

In conclusion, we studied the histopathology, immunohistochemistry, and immunogenetics of ocular adnexal lymphoid proliferation in 20 patients and examined the tumors for clonality. We found that combined immunohistochemistry and hemi-nested PCR are useful for detection of clonality in specimens of ocular adnexal lymphoid proliferation. Of the 20 cases, 17 cases were confirmed to display clonality by immunohistochemical analysis and/or hemi-nested PCR. Of these 17 cases, 3 were confirmed to display clonality by hemi-nested PCR only. We conclude that hemi-nested PCR is thus useful for the detection of clonality.

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