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Original Article

Clonal Analysis of Bilateral, Recurrent, or Systemically Multifocal Ocular Adnexal Lymphoma

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The purpose of this study is to determine the same or different clonality between bilateral or recurrent lesions, or between or among ocular adnexal lesions and systemically multifocal lesions in 10 consecutive patients with ocular adnexal lymphoma : 8 had extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) and 2 had mantle cell lymphoma, observed from 1995 to 2008 at Okayama University Hospital. A discrete DNA fragment was generated by polymerase chain reaction amplification of the *immunoglobulin heavy chain* gene from all samples except for two samples of bilateral orbital mantle cell lymphoma lesions and one sample of the scalp skin MALT lymphoma lesion. The size of DNA fragments were the same between bilateral orbital or conjunctival lesions of 5 patients, between original and recurrent conjunctival lesions of one patient, between the orbital lesion and the oral cavity lesion in one patient, and among bilateral orbital lesions, buccal, rectum, and stomach lesions of one patient. Sequencing of the DNA fragments showed the same sequence between bilateral or recurrent or multifocal lesions in 8 patients except for one : the bilateral orbital lesions, rectum, and stomach lesions shared the same sequence while the buccal lesion had one nucleotide difference compared to the sequence shared by the other 4 lesions. In conclusion, bilateral, recurrent, or systemically multifocal lesions of ocular adnexal lymphoma shared the clonality between or among the lesions. 〔*J Clin Exp Hematopathol 50(1) : 27-38, 2010*〕

Keywords: clonal (clonality), ocular adnexal lymphoma, immunoglobulin heavy chain gene, fluorodeoxygluocose (FDG) positron emission tomography (PET), conjunctival and orbital lymphoma

INTRODUCTION

Ocular adnexa is a term that comprises tissues which support the eye globe in the orbit, and represents the eyelids, the lacrimal gland and sac, extraocular muscles, and the conjunctiva. The ocular adnexa is one of the main sites involved with extranodal lymphoma, following the stomach, tonsils, skin, and intestine.[1] The extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) is the most frequent histopathological type in ocular adnexal lymphoma, followed by rarer types of diffuse large B-cell lymphoma and mantle cell lymphoma.[2-4]

The ocular adnexal lymphoma frequently occurs bilaterally on both sides of the orbits and conjunctivas.[3-6] The sequence of events on both sides is varied in the time course. In the long term, ocular adnexal lymphoma is sometimes preceded or followed by lymphoma in other areas of the body.[3] Until now, multiple lesions of lymphoma have been shown either to share the same clonality or to originate from different clones,[7-13] but such clonal analyses have not been done for bilateral ocular adnexal lymphoma. We raised questions whether bilateral ocular adnexal lymphoma lesions share a clone or whether ocular adnexal lymphoma shares a clone with preceding lymphoma in the other sites of the body. In this study, we analyzed the clonality of lymphoma cells by polymerase chain reaction amplification of the *immunoglobulin heavy chain* gene.[14-19] In addition, we examined the role of whole-body 2-[^{18}F] fluoro-2-deoxy-D-glucose (FDG) positron emission tomography fused with computed tomography (PET/CT) (FDG-PET/CT) after the pathological and clonal diagnoses of ocular adnexal lymphomas.

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PATIENTS AND METHODS

Patients

This study involved 10 consecutive patients with ocular adnexal lymphoma, seen from 1995 to 2008 at Okayama University Hospital, who showed bilateral ocular adnexal lesions or recurrent ocular adnexal lesions or other systemic foci of lymphoma lesions in addition to ocular adnexal lesions. The histopathological and immunohistochemical diagnoses were reviewed, the stored paraffin sections or paraffin blocks were used for polymerase chain reaction of the *immunoglobulin heavy chain* gene.

Histopathology and immunohistochemistry

Tissues were fixed for 3 hr with formaldehyde and embedded in paraffin. Paraffin sections were deparaffinized and stained with hematoxylin-eosin and also by immunohistochemistry. In brief, sections were incubated with 3% hydrogen peroxide for 5 min to inactivate endogenous peroxidase, and blocked with 10% normal goat serum for 10 min. The sections were then incubated with primary antibodies overnight at 4°C, washed with 0.05% Tween 20-containing phosphate buffered saline three times, incubated with the secondary antibody at room temperature for 30-60 min, and washed. The color was developed with diaminobenzidine, and the nuclei were counterstained with hematoxylin.

Immunoglobulin heavy chain *gene rearrangement*

Immunoglobulin heavy chain gene rearrangement was detected by polymerase chain reaction (PCR).[2,11,19,20] Briefly, unstained, formaldehyde-fixed, paraffin sections placed on slide glasses were deparaffinized with xylene and graded ethanol series, and samples for DNA isolation were cut out from at least two different areas of the deparaffinized section. The amplification of *immunoglobulin heavy chain* genes was performed by semi-nested PCR, using primers directed to the framework 2 region (FR2A : 5'-TGGRTCCGMCAGSCYYCNGG-3' for both the first and the second PCR) and to the joining region (LJH : 5'-TGAGGAGACGGTGACC-3' for the first PCR, and VLJH : 5'-GTGACCAGGGTNCCTTGGCCCCAG-3' for the second PCR). At least two DNA samples from each paraffin sections were separately subjected to PCR with TAKARA Ex Taq (Takara Bio Inc., Otsu, Japan). The amplified products from each patient were electrophoresed in parallel on a 3% agarose gel. The determination of 'clonal' was made only when a single or dominant discrete band was consistently reproduced from different specimens.[2,11]

For the sequencing of clonal bands, the PCR products were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and used as a template for direct sequencing with the ABI 310 Genetic Analyzer (Perkin-Elmer, Foster, CA, USA) using the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) and either of the two primers (FR2A and VLJH) to sequence in both directions. At least two PCR products from different samples derived from the same tissue were sequenced in both directions. Nucleotide changes were defined as those which repeatedly occurred between the two different tissues.

RESULTS

Clinical features

Conjunctival MALT lymphoma developed simultaneously in 3 patients (Case 1, 5, and 6) while conjunctival MALT lymphoma recurred in the same left eye of one patient with a 3-year interval (Case 2, Fig. 1). Orbital MALT lymphoma developed first on the left side and then on the right side with a 3-year interval in one patient (Case 4, Fig. 2) while orbital MALT lymphoma developed simultaneously on both sides in 2 patients (Case 3 and 8) : one patient (Case 8, Fig. 3 and Fig. 4) had preceding MALT lymphoma in the left buccal skin, rectum, and stomach in a 2-year interval. Orbital mantle cell lymphoma developed simultaneously on both sides of one patient (Case 7). Another patient (Case 9, Fig. 5 and Fig. 6) developed unilateral orbital mantle cell lymphoma more than 2 years after mantle cell lymphoma in the oral cavity on the same right side. One patient (Case 10, Fig. 7) developed orbital MALT lymphoma more than 3 years after scalp skin MALT lymphoma on the same right side.

Fluorodeoxyglucose positron emission tomography/computed tomography (PET/CT)

PET/CT was only recently available and was performed in 9 patients (Table 1) : for the purpose of late follow-up in the initial 6 patients years after the initial diagnosis and treatment, and for the purpose of assessing systemic involvement in 3 recent patients one month after excisional biopsy. The late follow-up in the 6 patients showed no abnormal uptake of fluorodeoxyglucose, indicating no recurrence. For the purpose of staging lymphoma, two patients (Case 8 and Case 10, Fig. 3 and Fig. 7, respectively) showed no abnormal uptake systemically while one patient (Case 9, Fig. 5) with mantle cell lymphoma showed systemic foci of abnormal uptake in addition to the uptake in the original orbital lesion.

Immunoglobulin heavy chain *gene rearrangement*

A single or dominant discrete DNA fragment (Fig. 8 top) was generated by polymerase chain reaction amplification of the *immunoglobulin heavy chain* gene from all samples except for two samples of bilateral orbital mantle cell lymphoma lesions (Case 7) and one sample of the scalp skin lesion (Case

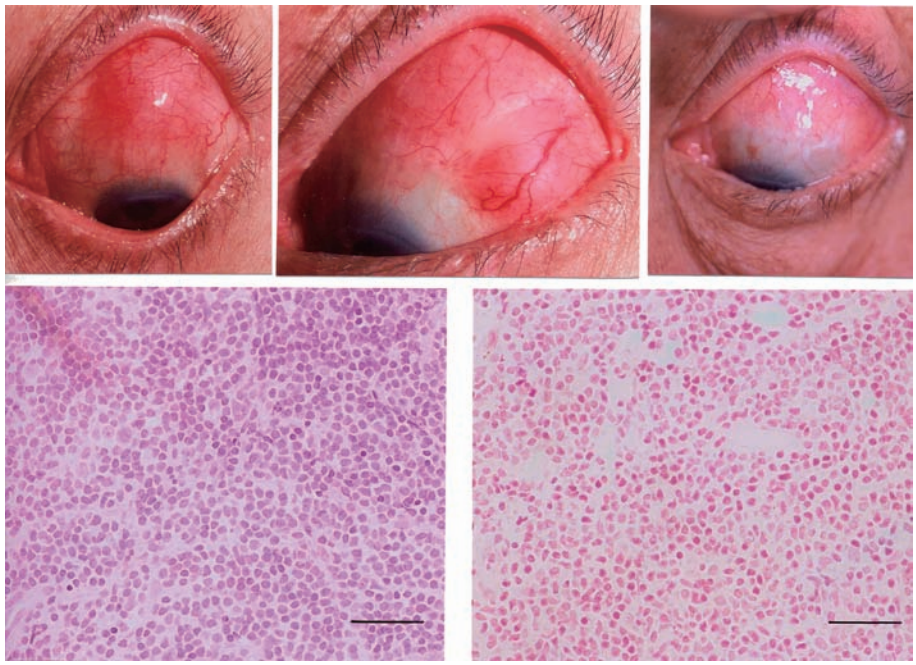

Fig. 1. Case 2. Conjunctival marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue in the left eye. The first biopsy in 1995 (***top left***), the recurrence and second biopsy in 1998 (***top middle***), and the spontaneous remission in 2003 (***top right***). Histopathology in 1995 (***bottom left***) and in 1998 (***bottom right***). Hematoxylin-eosin stain. ***Bar*** = 50 μm.

10). The size of DNA fragments was the same between bilateral orbital or conjunctival lesions of 5 patients, between original and recurrent conjunctival lesions of one patient (Case 2), between the orbital lesion and the oral cavity lesion in one patient (Case 9), and among bilateral orbital, buccal, rectum, and stomach lesions in one patient (Case 8).

Sequencing of the DNA fragments showed exactly the same sequence between the bilateral lesions or recurrent lesions or multifocal lesions in 8 patients except for one (Case 8) : the bilateral orbital, rectum, and stomach lesions shared exactly the same sequence while the buccal lesion had one nucleotide difference, as compared to the sequence shared by the other 4 lesions (Fig. 8 bottom).

DISCUSSION

The goal of this study was to examine the same or different clonality of lymphoma cells between multifocal lesions which manifested mainly as ocular adnexal lesions. The multifocal lesions in this study represented bilateral involvement of the ocular adnexa or the combination of ocular adnexal lymphoma with lymphoma in the neighboring buccal or oral areas or in the scalp skin or in the gastrointestinal tracts. The determination of clonality of lymphoma cells is accomplished by the well-established method of polymerase chain reaction amplification of the *immunoglobulin heavy chain* gene.[14-19] This method of clonal analysis was widely used to differentiate reactive lymphoid hyperplasia from malignant lymphoma, especially in ocular adnexal lymphoid lesions which comprises both orbital pseudotumor and malignant lymphoma.[14,15,17,20,21]

In this study, a single or dominant discrete DNA fragment indicating clonal nature of lymphoid cells was generated from all lymphoma tissues except for 3 samples : bilateral orbital lesions of mantle cell lymphoma in one patient (Case 7) and a scalp skin MALT lymphoma lesion in another patient (Case 10). The bilateral orbital lesions of mantle cell lymphoma in Case 7 did not give rise to amplified DNA fragments, in contrast with discrete DNA fragments of the same size which were generated from the orbital and oral cavity lesions of mantle cell lymphoma in Case 9. These discrepant results would be explained by a lower rate of DNA fragment amplification from the *immunoglobulin heavy chain* gene in mantle cell lymphoma, compared with MALT lymphoma.[21,22] No amplification from the scalp skin lesion of MALT lymphoma in Case 10 might be attributed to the rather old paraffin-embedded tissue.

As far as 8 of the 10 patients in this study were concerned, DNA fragments generated from different lesions in the same patient were the same in size, supporting the same clonality between and among the different lesions. We further sequenced DNA fragments to examine nucleotide changes. The

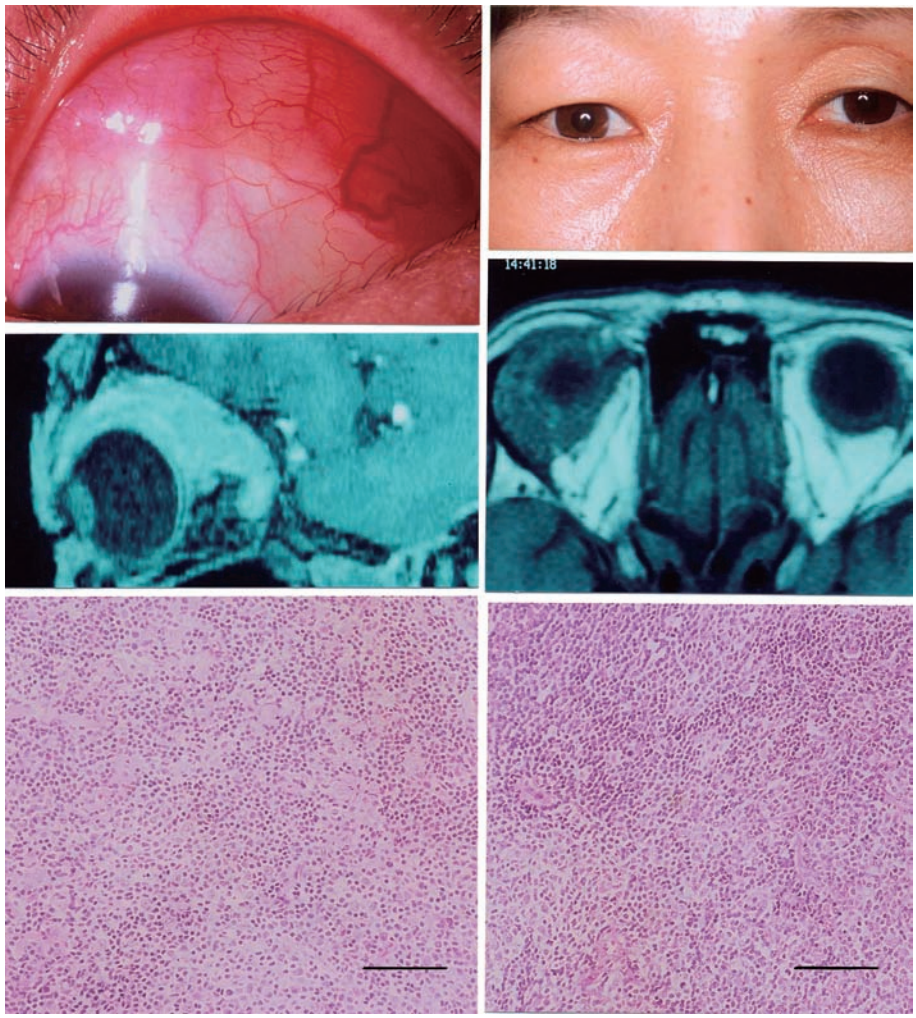

Fig. 2. Case 4. Bilateral orbital marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue arising with a 3-year interval. Clinical appearance (***top row***), magnetic resonance imaging (***middle row***), and pathology (***bottom row***, hematoxylin-eosin stain, ***bar*** = 100 μm). (***Left column***) Orbital tumor on the left side visible through the upper bulbar conjunctiva in 2000. (***Right column***) Lid swelling caused by orbital tumor on the right side in 2003.

sequences of the DNA fragments were proven to be exactly the same between the different lesions in the same patient except for Case 8. In Case 8, the sequence of the DNA fragment generated from the buccal MALT lymphoma lesion had at least one nucleotide change compared with the sequence shared by the DNA fragments from the other 4 lesions of MALT lymphoma involving the bilateral orbits, rectum, and stomach. The 5 different lesions in Case 8 are interpreted to arise from the same clone since a few nucleotide changes are known to occur during the expansion of the same clone of lymphoma cells.[11,18]

Whole-body FDG-PET/CT is a new method for staging of lymphoma after pathological diagnosis,[23,24] and has been applied for the staging of ocular adnexal lymphoma.[25,26] Until now, FDG-PET/CT has been recognized as superior to gallium-67 scintigraphy and whole-body CT for the staging and follow-up of nodal and extranodal malignant lymphomas in general.[23,24] However, the real power of FDG-PET/CT, specifically for the detection of ocular adnexal lymphoma, remains to be determined. We currently use PET/CT solely for the staging and follow-up of lymphoma and have abolished gallium scans and whole-body CT for these purposes. In this study, 3 recent patients underwent PET/CT to search for systemic involvement of lymphoma one month after excisional biopsy of the orbital lesions (Table 1).[12] Complete removal of bilateral orbital lesions in Case 8 resulted in no abnormal uptake of FDG on both sides of the orbits. In contrast, abnormal uptake of FDG only in the original orbital

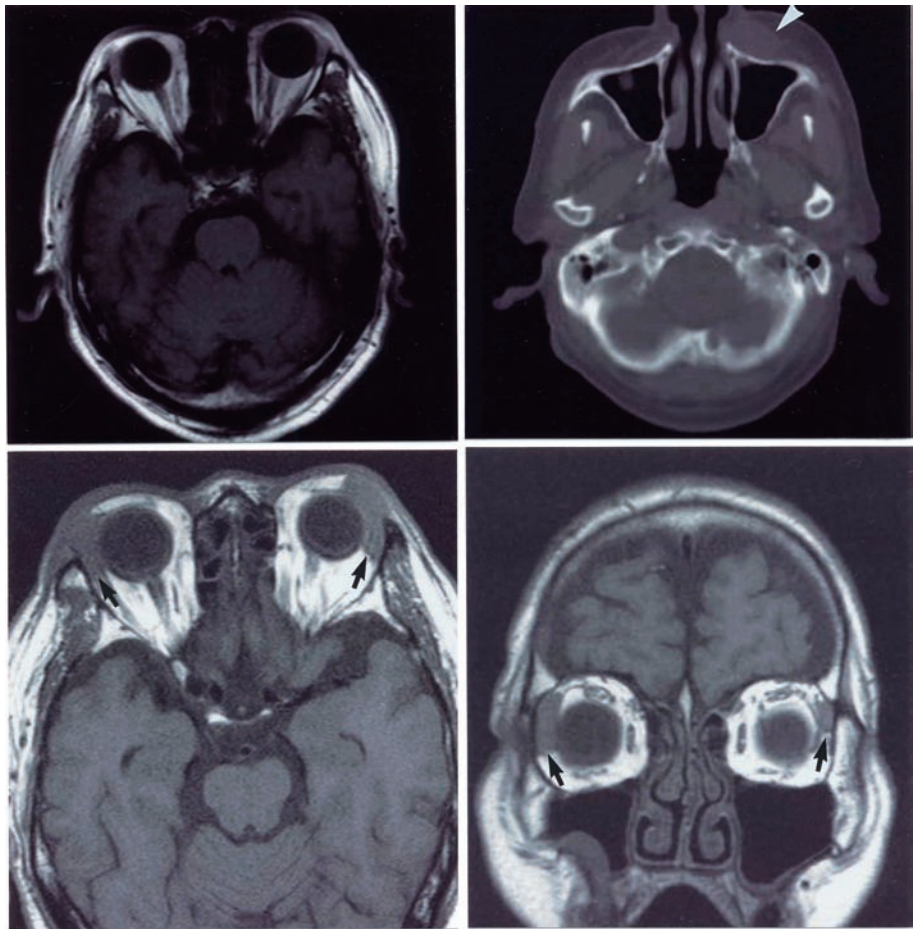

Fig. 3. Case 8. Buccal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue on the left side in 2005 ***(top row***) and bilateral orbital marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue in 2007 (***bottom row***). At the time of the buccal lesion shown by computed tomography (*arrowhead* in ***top right***), the bilateral lacrimal glands are somewhat enlarged on magnetic resonance imaging (***top left***). Magnetic resonance imaging in 2007 (*arrows* in ***bottom***) shows apparent bilateral orbital lesions involving the lacrimal glands.

lesion in Case 10 directed the treatment option to radiation of the orbital lesion, which led to remission. The presence of systemic foci of abnormal uptake in addition to the original orbital lesion in Case 9 with mantle cell lymphoma led to the treatment with rituximab and finally with umbilical cord blood stem cell transplantation. Thus, PET/CT provides a rationale to choose the treatment options.

In addition, the other 6 patients showed no abnormal uptake on the whole-body FDG-PET/CT in the long term of follow-up after the initial diagnosis and treatment for ocular adnexal lymphoma (Table 1). For conjunctival MALT lymphoma, we did not perform additional treatments including radiation and chemotherapy after resection of the conjunctival lesions on condition that no systemic involvement was detected.[5,6] No uptake on PET/CT in the long-term follow-up of 4 patients of conjunctival MALT lymphoma supports observation as a treatment option after the resection of MALT lymphoma of conjunctival origin. We chose radiation of orbital lymphoma, either MALT lymphoma or mantle cell lymphoma, when no systemic involvement was found. Orbital mantle cell lymphoma should be carefully followed for systemic involvement as two patients in the present series showed evident systemic involvement.

In conclusion, this study is the first to address the nature of clonality of bilateral ocular adnexal lymphoma lesions. The bilateral, recurrent, or systemically multifocal lesions of ocular adnexal lymphoma in the present series of patients shared clonality between the two lesions or among the multiple lesions. The expansion of the same clone of lymphoma cells in bilateral ocular adnexa and the movement among different areas of the body including ocular adnexa may provide insight for understanding the pathogenesis of bilateral

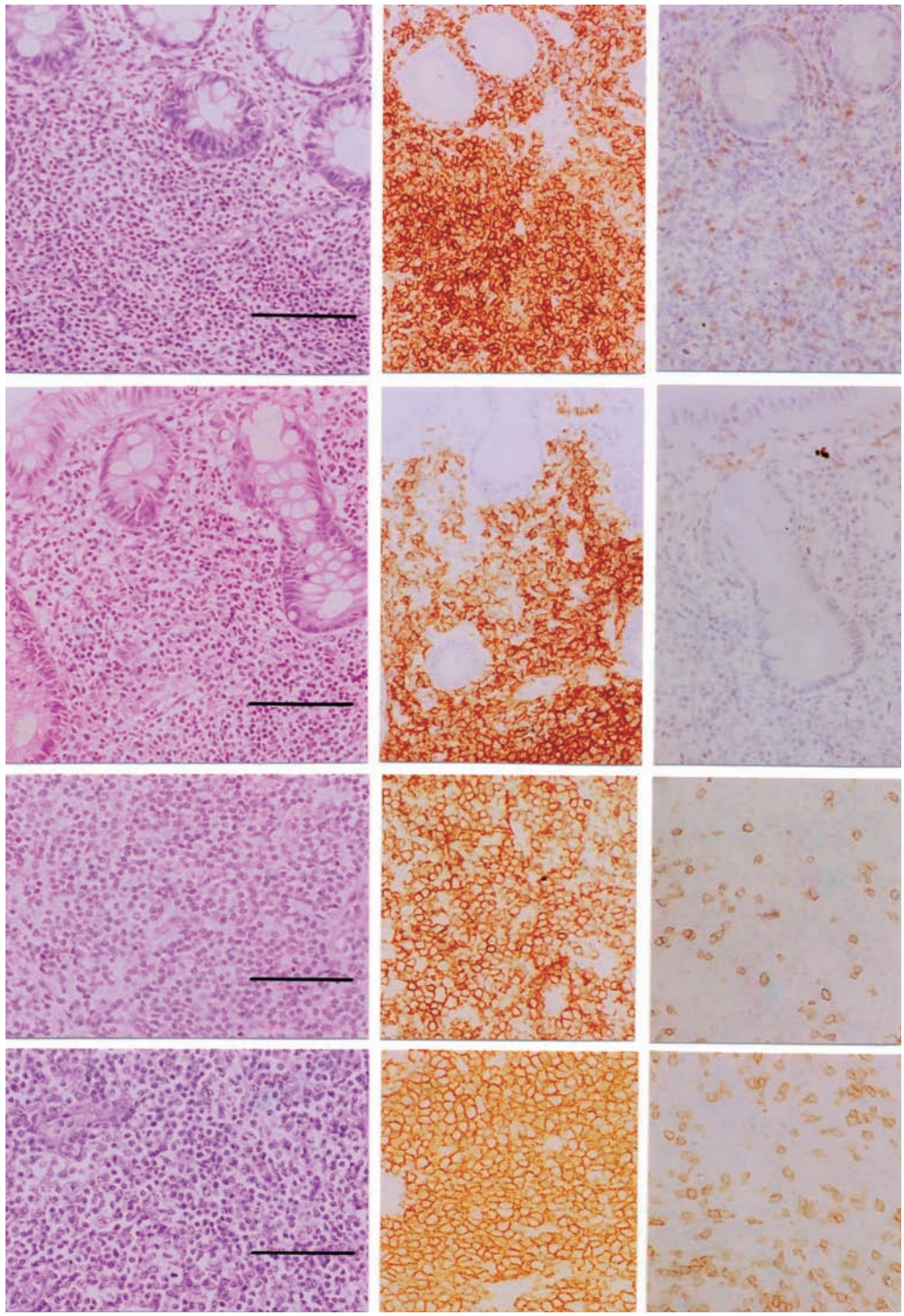

Fig. 4. Case 8. Histopathology and immunohistochemistry of extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue lesions in the stomach in 2005 (***top row***), rectum in 2006 (***second row***), bucca in 2005 (***third row***), and right orbit in 2007 (***bottom row***). Hematoxylin-eosin stain (***left column***), CD20 (***middle column***), and CD3 (***right column***). Lymphoma cells are positive for CD20, but mostly negative for CD3. *Bar* = 100 μm.

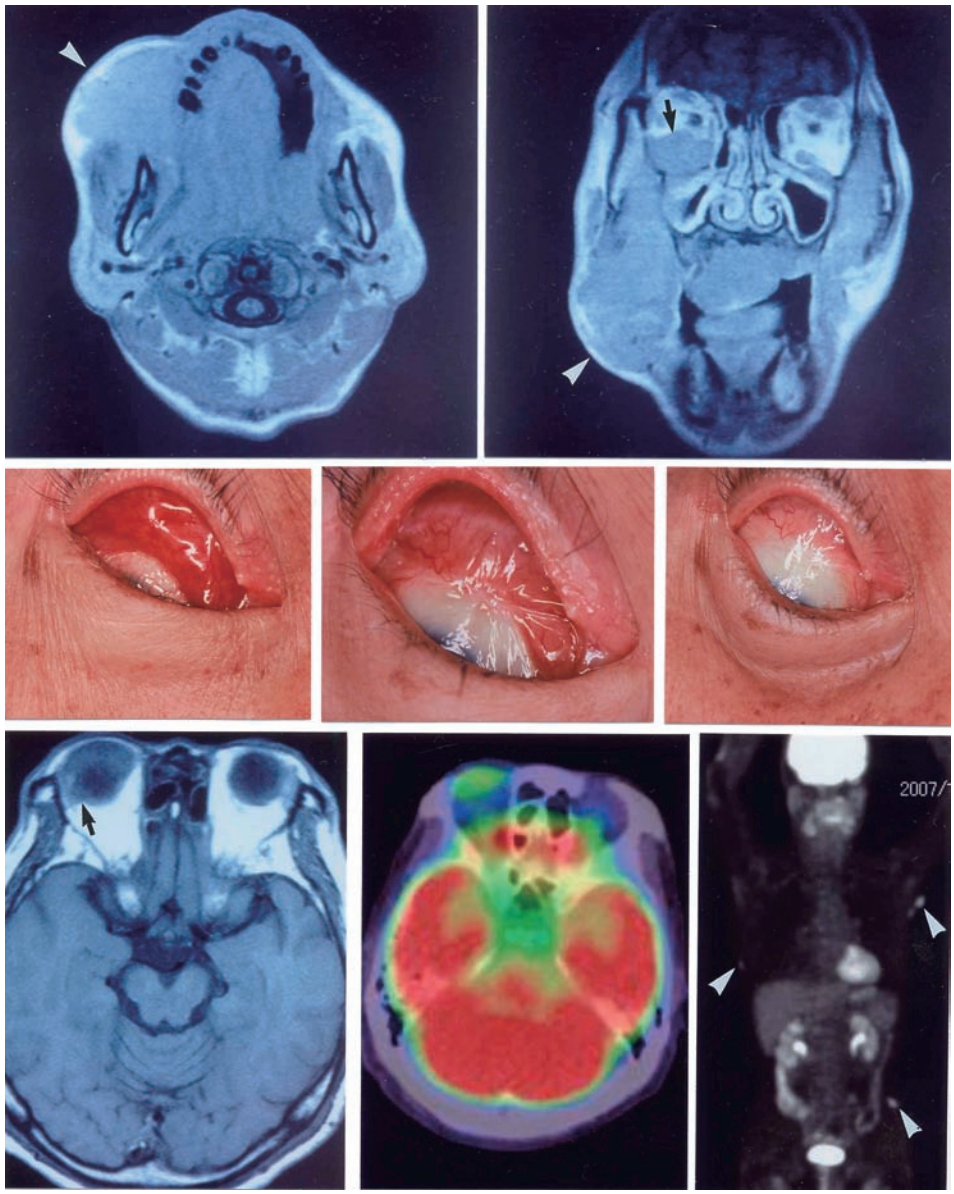

Fig. 5. Case 9. Mantle cell lymphoma in oral cavity on the right side (*arrowheads*) extending to the lower part of the orbit (*arrow*) in 2004 (magnetic resonance imaging in ***top row***). Mantle cell lymphoma recurs as an orbital lesion observed from the upper bulbar conjunctiva in 2007 (***middle row***). The conjunctival lesion before biopsy (***middle row left***) and after biopsy (***middle***) has diminished in 3 months with rituximab treatment (***right***). The orbital lesion (*arrow*) shown by magnetic resonance imaging (***bottom left***) has abnormal uptake (SUVmax = 3.5) on PET/CT (***bottom middle***). Lymphadenopathy (*arrowheads*) is also revealed by PET/CT (***bottom right***).

ocular adnexal or systemically multifocal lymphoma. Furthermore, the clonality determination followed by whole-body FDG-PET/CT evaluation might serve as a basis for clinical decisions and thus aid future treatment options.

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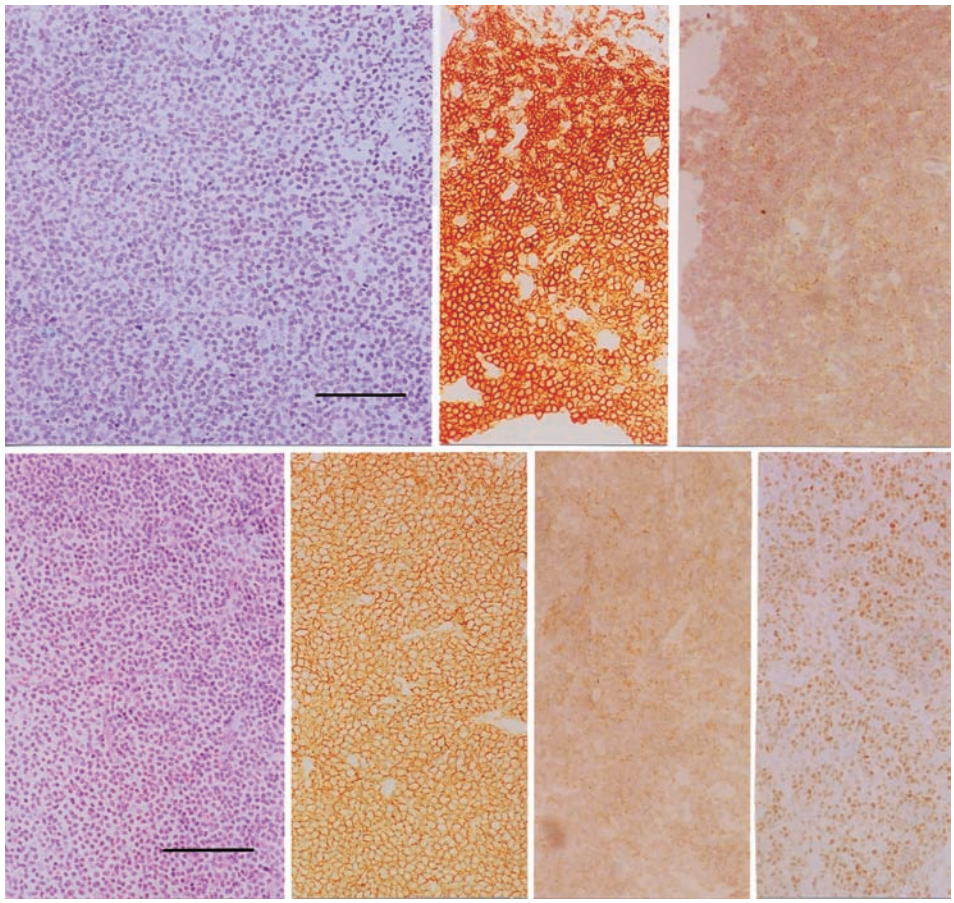

Fig. 6. Case 9. Histopathology and immunohistochemistry of mantle cell lymphoma lesions in the oral cavity in 2004 (***top row***) and in the right orbit in 2007 (***bottom row***). (***Top row***) (oral cavity) : hematoxylin-eosin stain (*left*), CD79a (*middle*), and CD10 (*right*). (***Bottom row***) (right orbit) : hematoxylin-eosin stain (*leftmost*), CD20 (*middle left*), CD10 (*middle right*), and cyclin D1 (*rightmost*). Lymphoma cells are positive for CD79a and CD20, also positive for CD10 and cyclin D1. *Bar* = 100 μm.

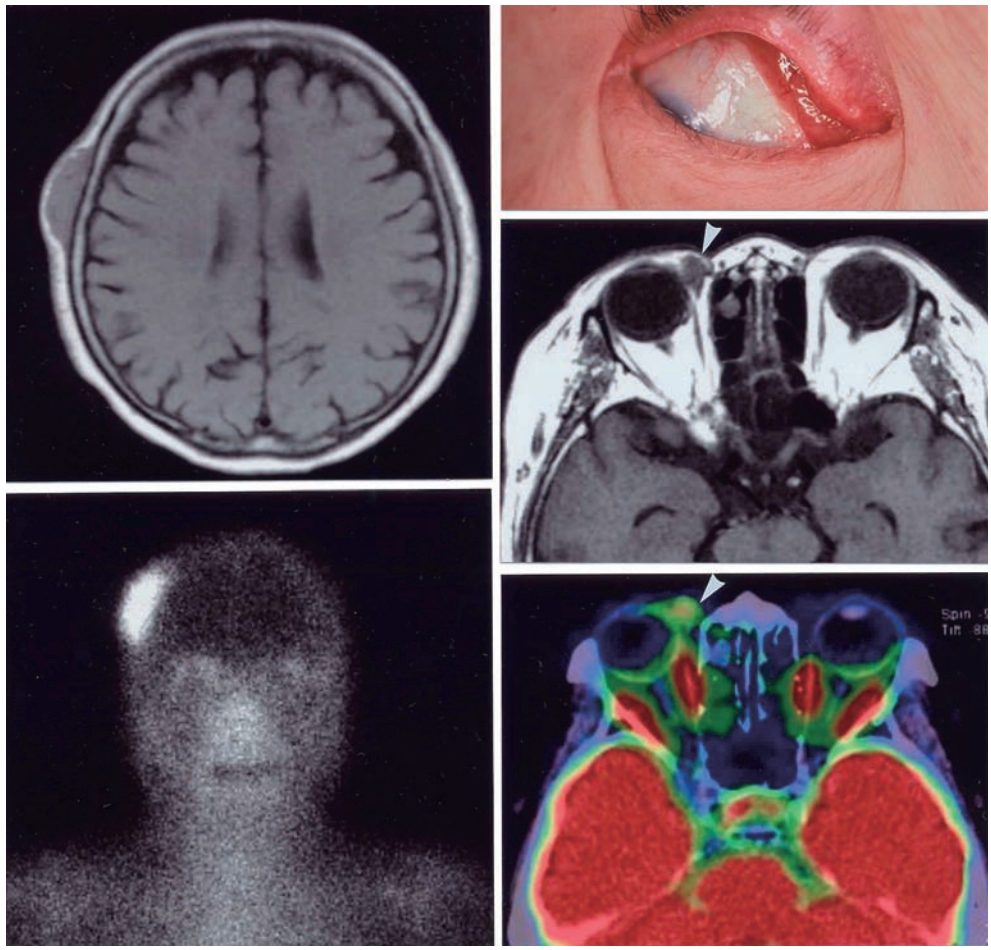

Fig. 7. Case 10. Scalp skin marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue in 2003 (***left column***) and right orbital marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue in 2008 (***right column***). The scalp skin lesion on the right side on magnetic resonance imaging (***top left***) shows high uptake on gallium scintigraphy (***bottom left***) in 2003. The right orbital lesion (*arrowhead*) on the nasal side by magnetic resonance imaging (***middle right***) is observed from the nasosuperior bulbar conjunctiva (***top right***) and shows high uptake (SUVmax = 3.9) on PET/CT (*arrowhead* in ***bottom right***).

Table 1. Clinical features, pathological diagnoses, and polymerase chain reaction amplification of the *immunoglobulin heavy chain* (*IgH*) gene in 10 patients with ocular adnexal lymphoma

Case No. /Sex/Age*	Month and Year at biopsy	Laterality	Location	Onset of ocular adnexal lesions	Pathological diagnosis	*IgH* gene PCR		Treatment	Whole-body fluorodeoxyglucose PET/CT
						Single band	Sequence		
1/Female/56	Jan, 1995	Right eye	Conjunctiva	Simultaneous	MALT	Yes	Same	Bilateral resection and observation	Not done
		Left eye	Conjunctiva		MALT	Yes			
2/Male/68	Oct, 1995	Left eye	Conjunctiva		MALT	Yes ?	Same	Resection and observation	No uptake in Feb, 2008
	Oct, 1998	Left eye	Conjunctiva	Recurrent	MALT	Yes		Resection and observation	
3/Male/54	June, 2001	Right side	Lacrimal gland	Simultaneous	MALT	Yes	Same	Bilateral complete extirpation and observation	No uptake in June, 2007
		Left side	Lacrimal gland		MALT	Yes			
4/Female/48	Oct, 2000	Left side	Orbit		MALT	Yes	Same	Radiation 30Gy	No uptake in June, 2007
	July, 2003	Right side	Orbit		MALT	Yes		Radiation 30Gy	
5/Male/72	June, 2004	Right eye	Conjunctiva	Simultaneous	MALT	Yes	Same	Bilateral resection and observation	No uptake in May, 2007
		Left eye	Conjunctiva		MALT	Yes			
6/Female/66	July, 2004	Right eye	Conjunctiva	Simultaneous	MALT	Yes ?	Same	Bilateral resection and observation	No uptake in June, 2007
		Left eye	Conjunctiva		MALT	Yes			
7/Female/63	Aug, 2004	Right side	Orbit	Simultaneous	Mantle cell	No	Not applicable	Bilateral radiation 40Gy and later chemotherapy due to bone marrow involvement	No uptake in Apr, 2008
		Left side	Orbit		Mantle cell	No			
8/Male/69	Nov, 2007	Right side	Orbit	Simultaneous	MALT	Yes	Same except for one nucleotide change in bucca	Bilateral complete extirpation and observation	No uptake in Nov, 2007#
		Left side	Orbit		MALT	Yes			
	June, 2005	Left side	Bucca		MALT	Yes		Radiation 36Gy	
	Aug, 2005		Stomach		MALT	Yes		Diagnosed[13] as gastric and colonic MALT lymphoma and chemotherapy in 1996	
	Jan, 2006		Rectum		MALT	Yes		Sigmoid colon resection in 1997	
								Stomach radiation 30Gy in 2002	
9/Male/66	Nov, 2007	Right side	Orbit		Mantle cell	Yes	Same	Rituximab and chemotherapy	Uptake in right orbit (SUVmax = 3.5)
								Umbilical cord blood stem cell transplantation	Uptakes in lymph nodes in Nov, 2007#
	May, 2004	Right side	Oral cavity		Mantle cell	Yes		Rituximab	
10/Female/74	June, 2008	Right side	Orbit		MALT	Yes	Not applicable	Radiation 30Gy	Uptake in right orbit (SUVmax = 3.9) in July, 2008#
	Dec, 2003	Right side	Scalp skin		MALT	No		Radiation 20Gy	

*; Age at presentation to Ophthalmology Department, #; PET/CT done one month after excisional biopsy, Yes ?; indicating a weak band, PCR; polymerase chain reaction, MALT; extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue, Mantle cell; mantle cell lymphoma, PET/CT; positron emission tomography fused with computed tomography, SUVmax; the maximum of standardized uptake value

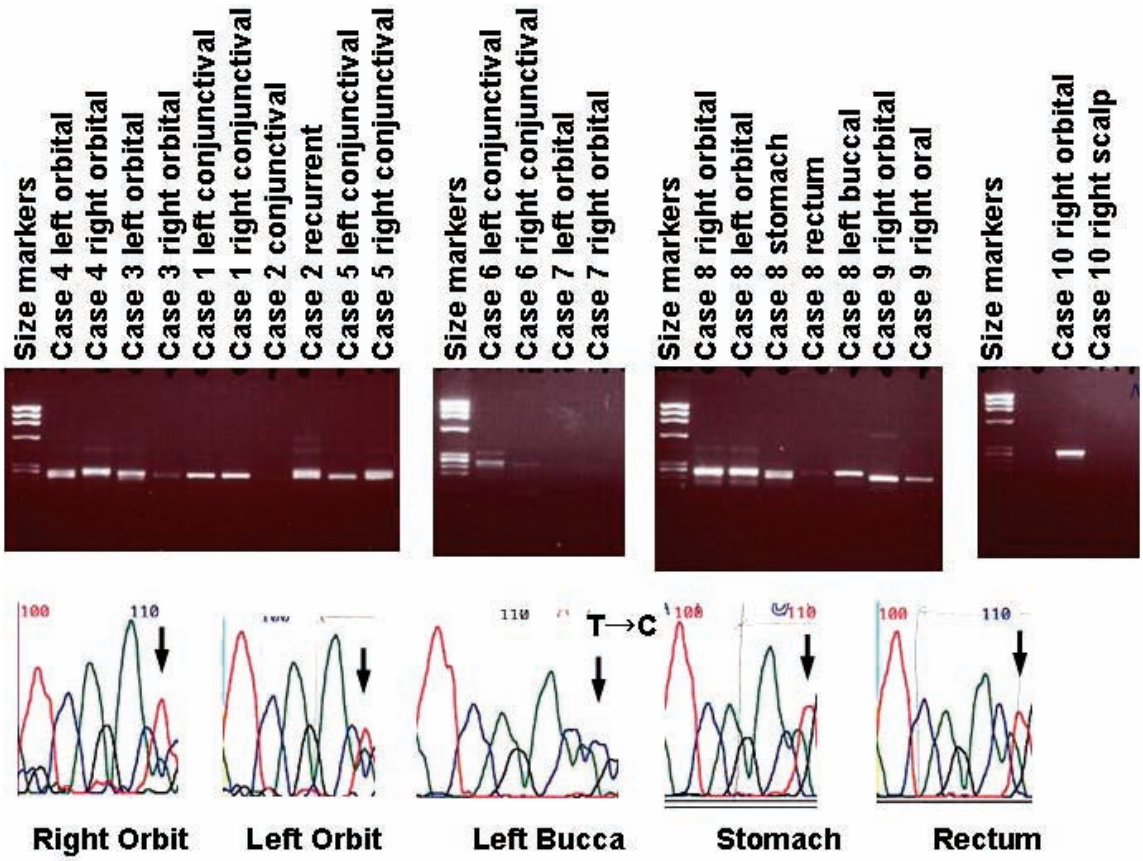

Fig. 8. Clonal analysis of lymphoma lesions. (***Top***) Polymerase chain reaction amplification of the *immunoglobulin heavy chain* gene from lymphoma lesions. A single discrete DNA fragment, indicating clonality of cells, is generated from all lesions except for bilateral orbital lesions of mantle cell lymphoma in Case 7 and scalp skin lesion of extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue in Case 10. Size markers : phage X174 DNA Hae III digest (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 base pairs). (***Bottom***) Sequencing of DNA fragments in the same size generated from extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue lesions in the right and left orbits, left bucca, stomach, and rectum of Case 8. The sequences of the DNA fragments are the same except for at least one nucleotide change (*arrows*) in the fragment from the buccal lesion.

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