Multigene Methylation Analysis of Ocular Adnexal MALT Lymphoma and Their Relationship to *Chlamydophila psittaci* Infection and Clinical Characteristics in South Korea

Ho-Kyung Choung,[1] *Young A. Kim,*[2] *Min Joung Lee,*[3] *Namju Kim,*[4] *and Sang In Khwarg*[5]

Purpose. We investigated the aberrant promoter methylation status of known or suspected tumor suppressor genes in ocular adnexal lymphoma (OAL) and the possible association with clinical characteristics and *Chlamydophila psittaci* infection.

Methods. Thirty-five cases of ocular adnexal mucosa-associated lymphoid tissue (MALT) lymphoma cases were examined for the methylation status of nine genes using methylation-specific PCR and for the detection of *C. psittaci* DNA using PCR. The medical records were reviewed retrospectively. Patient demographics, clinical characteristics including the response of the lymphoma to the therapy, and *C. psittaci* infection status were evaluated for possible association with methylation frequencies.

Results. CpG island methylation in nine genes was variously found as follows; *DAPK* (94.3%), *ECAD* (77.1%), *MT1G* (48.6%), *THBS1* (37.1%), *RAR-β* (31.4%), *p16* (20%), *MGMT* (5.7%), *p14* (0%), and *RASSF1A* (0%). Methylation was not observed in any of 13 control cases. *C. psittaci* DNA was observed in 25 (75.8%) of 33 patients with available tumor tissues, and *ECAD* hypermethylation was significantly higher in *C. psittaci*-positive cases ($P = 0.041$). Promoter hypermethylation status was not correlated with clinical characteristics.

Conclusions. Aberrant CpG island methylation of tumor suppressor genes is a frequent event in ocular adnexal MALT lymphoma. In particular, high frequencies of *DAPK* and *ECAD* methylation may be strongly correlated with ocular adnexal MALT lymphomagenesis in South Korea. Furthermore, *ECAD* hypermethylation is closely associated with *C. psittaci* infection, which may shed light on the mechanisms of bacterium-induced oncogenesis. (*Invest Ophthalmol Vis Sci.* 2012;53:1928–1935) DOI:10.1167/iovs.11-7668

From the [1]Department of Ophthalmology, Seoul National University Boramae Hospital, Seoul, Republic of Korea; [2]Department of Pathology, Seoul National University Boramae Hospital, Seoul, Republic of Korea; [3]Department of Ophthalmology, Hallym University Sacred Heart Hospital, Anyang, Republic of Korea; [4]Department of Ophthalmology, Seoul National University Bundang Hospital, Seongnam, Republic of Korea; and [5]Department of Ophthalmology, Seoul National University Hospital, Seoul, Republic of Korea.

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Corresponding author: Sang In Khwarg, Department of Ophthalmology, Seoul National University Hospital, 101 Daehangno, Jongno-gu, Seoul 110–744, Republic of Korea; khwarg@snu.ac.kr.

Extranodal marginal zone lymphoma is the most frequent lymphoma subtype found in the orbit and ocular adnexa,[1–3] and the incidence of non-Hodgkin's lymphoma (NHL) of the ocular adnexa has increased steadily, especially in Asia-Pacific Islanders, according to the Surveillance, Epidemiology, and End Results Program.[4] Extranodal marginal zone B-cell lymphomas (also known as "mucosa-associated lymphoid tissue [MALT] lymphoma" when involving a mucosa or epithelium) constituted three quarters of the lymphoproliferative lesions of the ocular adnexa in South Korea,[5] a higher proportion than in Western countries.

The pathogenesis of MALT lymphoma is largely unknown. However, it is generally believed that both chronic antigenic stimulation and acquired genetic alterations are involved.[6,7] In gastric MALT lymphoma, *Helicobacter pylori* has been shown to be the causative agent in almost all cases,[8] and a possible connection between ocular adnexal MALT lymphoma and *Chlamydophila psittaci* in some countries has been suggested.[9] However, the results from various studies differ (0–87%), possibly because of geographical or genetic differences.[10–13]

Genetic abnormalities found in various cancers do not provide the complete picture of molecular mechanisms of cancers and malignancies. Epigenetic changes, mainly DNA methylation and histone modification, are additional mechanisms that contribute to the malignant phenotype.[14–16] Hypermethylation of CpG islands in the promoter region is an important mechanism of gene silencing for tumor suppressor genes (TSGs).[17] The aberrant methylation of the CpG islands has been correlated with loss of gene expression and provides an alternative pathway for gene deletion or mutation for the loss of TSG function.[17,18] Aberrant promoter methylation has been described in several kinds of malignant tumors, and each type of tumor may have its own distinct pattern of methylation.[18–20] In gastric MALT lymphoma, *H. pylori* infection causes the aberrant DNA hypermethylation of specific genes, such as *p16*, *MGMT,* and *MINT31,* which is an important mechanism for the development and progression of gastric MALT lymphoma.[21] Furthermore, *p16* methylation disappeared after the eradication of *H. pylori* in South Korean cases,[22] suggesting that aberrant DNA methylation may be closely associated with *H. pylori* infection.

To date, there have not been extensive studies about aberrant promoter methylation of TSGs in MALT lymphomas of ocular adnexa; recently, only one paper by Carugi et al. has analyzed *p16* promoter methylation.[23]

In this study, we explored both the prevalence of aberrant methylation in a selected panel of nine TSGs that are known or rarely known to exist in lymphomas, using methylation-specific PCR, and the possible association with *C. psittaci* infection. The selected nine TSGs are known to be involved in cell cycle regulation (*p14, p16,* and *RASSF1A*),[24,25] DNA

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repair (*MGMT*),[26] apoptosis (*DAPK* [death-associated protein kinase] and *RASSF1A*),[27,28] angiogenesis inhibition (*THBS1*),[29] cell proliferation (*MT1G* and *RAR-β*),[25,30] and invasion suppression (*ECAD* [E-cadherin, CDH1]).[31] We also investigated the relationship between the profile of promoter hypermethylation and clinical characteristics or *C. psittaci* infection.

Methods

Patients

Thirty five patients who were diagnosed with MALT lymphoma at the Seoul National University Hospital and Seoul National University Boramae Hospital between 2002 and 2009 were enrolled in this study. We retrospectively reviewed the medical records and collected patient demographics, details of treatment modality, and lymphoma response to the therapy. The protocol of this study was approved by the Institutional Review Board of Seoul National University Boramae Hospital. All subjects were treated in accordance with the Declaration of Helsinki. The diagnosis of MALT lymphoma was established by histopathologic examinations, including immunohistochemical analysis after incisional biopsy. All cases were classified according to World Health Organization (WHO) classification of lymphoma by a hematopathologist (Young A. Kim).

A staging workup was carried out based on physical examination, complete ophthalmologic examination, chest radiograph, magnetic resonance imaging (MRI) of the orbit, computed tomography (CT) of the chest and abdomen, and bone marrow aspiration and biopsy. All patients were staged according to the Ann Arbor classification and American Joint Committee on Cancer classification.[32]

CVP (cyclophosphamide, vincristine, and prednisolone) chemotherapy or radiotherapy was performed from 2002 through 2006, and doxycycline treatment has been performed since 2007. CVP chemotherapy consisted of cyclophosphamide (1000 mg/m^2 intravenously [IV] for >30 min) on day 1, vincristine (1.5 mg/m^2 [maximum 2 mg] IV bolus) on day 1, and oral prednisolone (40 mg/m^2) on days 1 to 10. Treatment cycles were repeated every 3 weeks in an outpatient clinic. All patients were scheduled to receive six cycles of CVP, assuming no disease progression or substantial toxicity. Doxycycline was given orally at a dose of 100 mg twice a day for 3 weeks or at the same dose for 3 weeks, followed by 3 weeks off, and repeated for a second 3 weeks. Double-course therapy was assigned to patients with residual eye-related symptoms who did not respond to single-course therapy. The objective lymphoma response to the therapy was assessed in all patients by biomicroscopic examination or orbital imaging study (CT or MRI) by experienced oculoplasty specialists. The objective response was defined according to the WHO criteria. Complete remission was defined as the disappearance of all clinical evidence of the disease, partial response was defined as a more than 50% reduction in size of all measurable lesions, stable disease was defined as regression of any measurable lesion by ≤50% (minimal response) or no change for the measurable lesions, and progressive disease was defined as the appearance of any new lesion or an increase in the size of a tumor of ≥25% at a previously involved site. Treatment failure was defined as conversion of treatment modality due to minimal response, local or systemic progression of lymphoma, or relapse (lymphoma recurrence after initial response).

Tumor Samples and DNA Preparation

The tumor samples were formalin-fixed, paraffin-embedded tissues derived from conjunctiva or orbit. The control samples included the DNAs obtained from excised pterygium and conjunctiva from conjunctivochalasis cases. The genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Bisulfite Modifications

DNAs were subjected to sodium bisulfite modifications as described previously.[31] In brief, 40 μL of DNA (2 μg) was denatured at 97°C for 6 min and then quickly centrifuged and chilled on ice. Ten microliters of 1 M NaOH was then added, and the mixture was stored at room temperature for 15 min. Five hundred fifty microliters of a mixture of 3.5 M sodium bisulfate and 1 mM hydroquinone was added to the denatured DNA, which was then stored at 55°C for 16 hours. The treated DNA was purified with a JETSORB gel extraction kit (Genomed, Bad Oeyhausen, Germany) and desulfonated with 0.3 M NaOH at room temperature for 10 min. After three volumes of 100% cold ethanol and a ⅔ volume of 7.5 M ammonium acetate were added and the mixture was stored at −20°C for 12 hours, the precipitated DNA was centrifuged. After being washed in 70% ethanol and dried, it was dissolved in 10 mM Tris buffer.

Methylation-Specific PCR (MSP)

A panel of nine genes was analyzed for methylation status using MSP. The primer sequences of each gene, the product size, the annealing temperature, and references are given in Table 1. All of the PCR amplifications were performed using bisulfate-modified DNA (30–50 ng), primers (10 pmol each), deoxynucleoside triphosphates (1 mM each), and 10× standard PCR buffer (Qiagen) in a volume of 20 μL. The reactions were hot started at 95°C for 5 min, followed by 35 cycles at 97°C (30 seconds per cycle), with the annealing temperature being specific for each reaction (30 seconds per cycle), and then 72°C (30 seconds per cycle), and a final extension step at 72°C for 10 minutes in the PTC200 thermal cycler (MJ research, Waltham, MA). The PCR products (5 μL) were electrophoresed on 2% agarose gels and visualized after staining with ethidium bromide. For each MSP reaction, we used normal lymphocyte DNA treated with Sss1 methyltransferase (New England Biolabs, Beverly, MA) and distilled water without template DNA as a positive and negative control, respectively.

Cloning of Chlamydophila DNA

Chlamydophila DNA was generously provided by Seung-Joon Lee of Kangwon National University, Korea. DNA was amplified by PCR and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For verification, the cloned DNA was sequenced in both directions with a BigDye terminator DNA sequencing kit (Applied Biosystems, Foster City, CA) and analyzed using an ABI 3730XL DNA analyzer (Applied Biosystems).

Detection of Chlamydophila DNA

For each extracted DNA sample, touchdown enzyme time release PCR for *C. psittaci* was performed as described previously but with some modification of the annealing temperature. The primer sequence is shown in Table 1.[33]

Ta-CLONED *Chlamydophila* DNA was used as a positive control. The annealing temperature was 54°C. The amplified DNA fragments were electrophoresed on 2% agarose gels, and they were visualized after staining with ethidium bromide. To exclude the possibility of contamination of the extracted DNA, The PCR products positive for *C. psittaci* DNA were sequenced.

Statistical Analysis

The frequencies of methylation for each TSG are analyzed. The methylation status was analyzed specifically according to the *C. psittaci* infection status. Statistical analysis of methylation frequencies of each gene between *C. psittaci*-positive and -negative groups was performed using the Mann-Whitney nonparametric *U* test. Correlations between methylation frequencies and clinical characteristics, *C. psittaci* infection status, and clinical characteristics were analyzed by the

Table 1. Primer Sequences and PCR Conditions of Tested TSGs and *C. psittaci* for MSP Analysis and Detection of *C. psittaci* DNA

Primer		Primer Sequence (5′–3′) Forward	Reverse	Product Size (bp)	Annealing Temperature (°C)	Reference
ECAD	m	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	97	53	31
	u	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	91	59	
DAPK	m	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60	27
	u	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	98	60	
MGMT	m	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	65	26
	u	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	59	
MT1G	m	TGCGAAAGGGGTCGTTTTGC	GCGATCCCGACCTAAACTATACG	93	59	30
	u	GTGAGTTGGTGTGAAAGGGGTT	CCACACCACCCACAATCCCA	113	59	
p16	m	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65	31
	u	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60	
RASSF1A	m	GGGTTTTGCGAGAGCGCG	GCTAACAAACGCGAACCG	169	64	28
	u	GGTTTTGTGAGAGTGTGTTTAG	CACTAACAAACACAAACCAAAC	169	59	
THBS1	m	TGCGAGCGTTTTTTTAAATGC	TAAACTCGCAAACCAACTCG	74	62	29
	u	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	115	62	
p14	m	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122	64	25
	u	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132	64	
RARβ	m	TCGAGAACGCGAGCGATTCG	GACCAATCCAACCGAAACGA	146	59	25
	u	TTGAGAATGTGAGTGATTTGA	AACCAATCCAACCAAAACAA	146	59	
C. psittaci		CCCAAGGTGAGGCTGATGAC	CAAACCGTCCTAAGACAGTTA		54	32

m, methylated sequence; u, unmethylated sequence.

Mann-Whitney *U* test, Fisher's exact test, and the Cox professional hazard model.

For all tests, a P value of <0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS software (SPSS for Windows Release, version 14.0, SPSS, Chicago, IL).

Results

This study included 35 patients. The mean age of our patients was 51 years with a male-to-female ratio of 16:19. Eight patients had bilateral disease. The median follow-up period was 23 months (range, 3–101). All patients had stage I_E disease by Ann Arbor classification, and no patient showed nodal or distant metastasis (N0M0). Twenty-one patients with lymphoma involving only the conjunctiva were classified as T1N0M0 stage, 13 patients with orbital lymphoma were T2N0M0 stage, and one patient who showed preseptal involvement was considered to be at T3N0M0 stage.

None of the nine genes had methylation detected in the 13 control samples. However, methylation for these genes was common in ocular adnexal MALT lymphomas. The detailed results for the methylation of nine genes and the presence of *C. psittaci* DNA in each lineage of MALT lymphoma are given in Table 2. Each of the nine genes showed methylation of CpG islands in the promoter region at frequencies of 0 to 94.3% (Fig. 1). In particular, two genes (*DAPK* and *ECAD*) were frequently methylated (>70%). Interestingly, there was no case showing *RASSF1A* and *p14* hypermethylation. All of the ocular adnexal MALT lymphoma cases had promoter hypermethylation in at least one of these genes. *C. psittaci* DNA was observed in 25 (75.8%) of 33 patients with available tumor tissues (Fig. 2). The methylation frequencies of each gene were not correlated with age group, clinical stage, or response to treatment, and the detailed P values are demonstrated in Table 3. The associations between sex and *RAR-β* and between laterality and *DAPK* were statistically significant in univariate analysis, but there was no significance in the Cox regression hazard model. In terms of *C. psittaci* infection, only *ECAD* showed statistical significance ($P = 0.04$, Fisher's exact test). In 28 patients who were followed up for more than 6 months, 18 patients were treated with doxycycline monotherapy, 8 patients with chemotherapy, and 1 patient with primary radiation therapy. One patient underwent no initial treatment based on patient preference. In 18 patients who received doxycycline monotherapy, the response rate to the treatment was 66.7% (12/18) with a median follow-up of 16 months (Fig. 3). Five patients received a 3-week doxycycline treatment and the other 13 patients a 6-week treatment, and the response rate was not statistically different between the two regimens (40.0% and 76.9%, respectively, $P = 0.26$). However, the response to doxycycline was not significantly correlated with the methylation frequencies of each gene (Table 3). In the chemotherapy group, the response rate was 62.5% (5/8) with a 59-month follow-up and was not significantly correlated with methylation frequencies of each gene (Table 3).

The *C. psittaci* infection status did not appear to be statistically correlated with promoter hypermethylation of the genes except for *ECAD* (Table 3). *C. psittaci* positivity was significantly more frequent in early-stage lymphoma ($P = 0.035$) (Table 4). Of 18 patients who received doxycycline monotherapy, *C. psittaci* DNA was observed in 15 patients. The response rate to doxycycline monotherapy was 66.7% in *C. psittaci*-positive cases and the same in *C. psittaci*-negative cases, and *C. psittaci* status was not correlated with treatment response ($P = 1.00$).

Discussion

In this study, we comprehensively investigated aberrant promoter methylation of multiple TSGs in ocular adnexal MALT lymphomas using MSP. Further studies using pyrosequencing or reverse transcriptase PCR are needed to confirm these findings and verify the role of hypermethylation in the pathogenesis of ocular adnexal MALT lymphoma. Of these nine genes included in our study, only one gene (*p16*) has been previously reported to be methylated in ocular adnexal MALT lymphomas.[23] To the best of our knowledge, the other eight genes (*p14, RASSF1A, MGMT, DAPK, THBS1, MT1G, RAR-β,*

Table 2. Summary of Methylation Analysis of Nine Genes (*DAPK, ECAD, MT1G, THBS1, RAR-β, p16, MGMT, RASSF1A,* and *p14*) and Detection of *C. psittaci* DNA in Ocular Adnexal MALT Lymphoma Samples

Case No.	Tumor Suppressor Gene									Total No. of Methylated Genes	*C. psittaci* Infection
	DAPK	*ECAD*	*MT1G*	*THBS1*	*RAR-β*	*p16*	*MGMT*	*RASSF1A*	*p14*		
1	+	+	+	+		+	+			6	+
2	+	+	+		+	+				5	+
3	+	+	+	+	+					5	
4	+	+	+		+	+				5	+
5	+	+	+	+		+				5	+
6	+	+	+	+			+			5	+
7	+	+	+		+					4	+
8		+	+		+	+				4	+
9	+	+	+			+				4	+
10	+	+	+		+					4	+
11	+	+	+			+				4	+
12	+	+	+	+						4	+
13	+		+	+	+					4	+
14	+	+		+						3	+
15	+	+		+						3	+
16	+	+		+						3	
17	+	+		+						3	+
18	+	+		+						3	+
19	+	+			+					3	+
20	+	+			+					3	
21	+	+	+							3	+
22	+	+			+					3	+
23	+		+		+					3	
24	+	+	+							3	+
25	+	+	+							3	+
26	+	+								2	+
27	+			+						2	
28	+			+						2	
29	+	+								2	N/A
30	+	+								2	+
31	+									1	
32	+									1	
33	+									1	N/A
34	+									1	+
35		+								1	+
No. of samples with methylation (%)	33 (94.2)	27 (77.1)	17 (48.6)	13 (37.1)	11 (31.4)	7 (20.0)	2 (5.7)	0 (0)	0 (0)		

A plus sign indicates the presence of methylation or *C. psittaci* DNA, and no plus sign indicates absence. N/A, tumor tissues were not available.

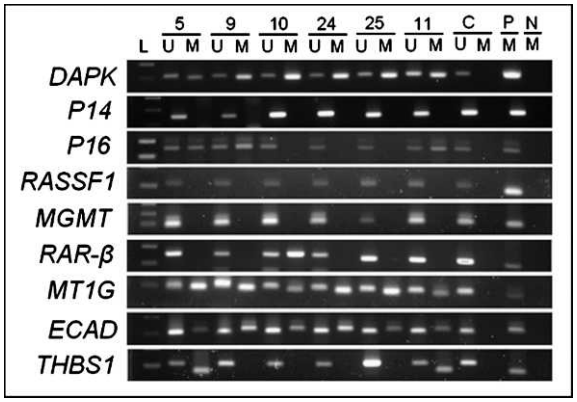

Figure 1. MSP results for eight genes in ocular adnexal MALT lymphomas. The PCR products in lane U indicate the presence of unmethylated alleles, and the products in lane M indicate the presence of methylated alleles. L, size marker (100-bp DNA ladder); C, control DNA; P, positive control; N, negative control.

and *ECAD*) we studied have not previously been investigated in detail in ocular adnexal MALT lymphomas.

In this study, seven of nine genes (*DAPK, ECAD, MT1G, THBS1, RAR-β, p16,* and *MGMT*) showed methylation of CpG islands in the promoter region at frequencies of 5.7 to 94.3%, and all of the ocular adnexal MALT lymphoma cases had promoter hypermethylation in at least one of these genes. *C. psittaci* DNA was observed in 25 (75.8%) of 33 patients with available tumor tissues, and *ECAD* hypermethylation was significantly greater in *C. psittaci*-positive cases.

DNA methylation is a normal process used by mammalian cells in maintaining a normal expression pattern; it is involved in the regulation of imprinted gene expression and X

Figure 2. Results of PCR using *C. psittaci*-specific primers. Thirteen of 14 ocular adnexal MALT lymphoma specimens show positive bands. P, positive control for *C. psittaci*; N, negative control.

Table 3. Statistical Differences between Methylation Frequencies of Nine Genes and Clinical Characteristics, Treatment Response, or *C. psittaci* Infection Status in Ocular Adnexal MALT Lymphoma

	No. of Cases	*P* value for Tumor Suppressor Gene						
		p16	*DAPK*	*ECAD*	*MGMT*	*MT1G*	*THBS1*	*RAR-β*
Age group*	35	0.20	0.51	0.69	0.55	0.09	1.00	0.14
Gender*	35	0.10	0.49	1.00	0.23	0.09	0.28	0.04†
Clinical stage*								
T1N0M0 vs. higher than T1N0M0	35	0.68	0.51	0.22	1.00	0.09	0.73	0.46
Laterality*								
Unilateral vs. bilateral	35	1.00	0.05†	0.65	1.00	0.23	0.41	0.69
Response to doxycycline*	18	0.31	0.50	0.53	0.39	1.00	0.14	1.00
Response to chemotherapy*	8	−‡	+§	0.46	−‡	−§	1.00	1.00
C. psittaci infection*	33	0.15	1.00	0.04	1.00	0.11	0.23	1.00

There was no case showing *RASSF1A* and *p14* hypermethylation.
* Fisher's exact test.
† Cox-regression hazard model showed no significant correlation between methylation of *DAPK* or *RAR-β*.
−‡ Methylation frequency of this gene was 0% in eight patients with chemotherapy.
+§ Methylation frequency of this gene was 100% in eight patients with chemotherapy.

chromosome inactivation and in the fine-tuning of specific differentiation of cells and development from stem cells.[34–37] However, aberrant promoter hypermethylation of the CpG islands leads to epigenetic silencing of multiple genes, including TSGs, and has been recognized as an important mechanism in carcinogenesis.[38–40] Furthermore, concordant promoter hypermethylation of multiple genes has been found in gastric and colorectal carcinomas.[41–43]

In this study, DNA methylation was found in seven of nine genes, indicating that methylation of these certain genes may play a significant role in the pathogenesis of ocular adnexal MALT lymphoma. In particular, two genes (*DAPK* and *ECAD*) were frequently methylated (94.3% and 77.1%, respectively). The frequency of methylation of these genes appears to be inconsistent with the previous studies of other types of lymphoma (Table 5). Because most previous reports on hypermethylation in NHLs were about gastric lymphoma or diffuse large B cell lymphomas (DLBCL), we analyzed the methylation frequencies of ocular adnexal MALT lymphomas compared to those of DLBCLs or gastric lymphomas. Of the nine genes tested, *ECAD* and *DAPK* were more frequently methylated in ocular adnexal MALT lymphomas than in other lymphomas. Several genes, such as *MGMT, p16, THBS1, RASSF1A, p14,* and *MT1G,* showed much lower frequencies of promoter methylation.[6,22,46] Thus, these different characteristics of promoter methylation suggest that other lymphomas and ocular adnexal MALT lymphomas are morphologically similar but there might be some different pathways related to the lymphomagenesis.

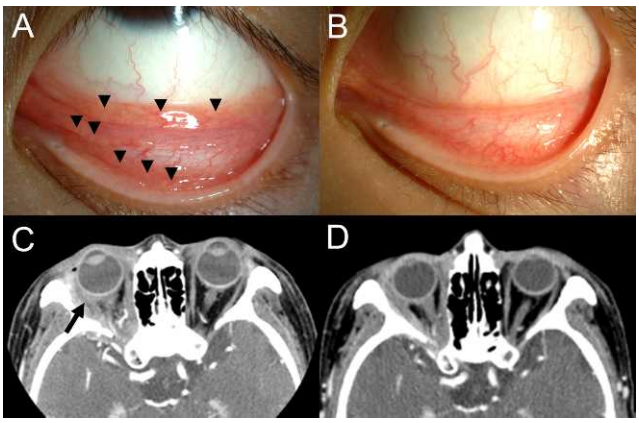

Figure 3. Representative cases with ocular adnexal MALT lymphoma showing response to doxycycline treatment. (**A**) and (**B**) Case 1. Left conjunctival MALT lymphoma case. (**A**) Pinkish masses on forniceal and tarsal conjunctiva (arrow heads) revealing ocular adnexal mucosa-associated lymphoid tissue (MALT) lymphoma. (**B**) Five months after double course of doxycycline treatment, masses were completely regressed. (**C**) and (**D**) Case 2. Right orbital MALT lymphoma case. (**C**) Contrast-enhanced CT scans showing well-enhancing, diffuse orbital mass (arrow). (**D**) The mass was much decreased 7 months after double-course doxycycline treatment.

DAPK, the most frequently methylated (94.3%) gene in this study, is a proapoptotic gene, and its inactivation by promoter hypermethylation has previously been frequently observed in various human cancers. Moreover, *DAPK* hypermethylation was recently found to be associated with chronic inflammation-associated carcinogenesis in ulcerative colitis. The association of *C. psittaci* and ocular adnexal MALT lymphoma is also thought to be a result of chronic inflammation caused by chronic antigenic stimulation by *C. psittaci*, and *DAPK* hypermethylation may be thought of as one of the underlying mechanisms of this association. However, positivity of *C. psittaci* DNA and *DAPK* hypermethylation was not found to be significantly correlated, and the association of methylation of this gene and *C. psittaci* DNA remains unclear. Furthermore, RASSF1A, a putative tumor suppressor with proapoptotic activity, is frequently observed in solid tumors and Hodgkin's lymphoma, and *p14* is known to have frequent methylation in cutaneous marginal zone B-cell lymphoma[3] and *p14*'s hypermethylation is known to be a poor prognostic factor in adult leukemia.[47] However, in this study, these two genes failed to show hypermethylation in any cases.

Interestingly, the ocular adnexal tissues are devoid of native lymphoid tissue, indicating that lymphoma at these sites arises from MALT acquired as a result of chronic antigenic stimulation by microbial pathogens or autoimmune disorder.[48,49] In the past few years, several studies have reported the possible role of *C. psittaci* in the development of ocular adnexal lymphoma (OAL). The first evidence of such an association came from a study performed on an Italian cohort of patients, where *C. psittaci* DNA was detected in 87% of the cases of OAL.[9] Since then, many groups worldwide have investigated the possible association between *C. psittaci* and OAL occurrence but with discordant results, and the causative role of *C. psittaci* is not well established.[9,49] It must be considered, in fact, that the genetic background of different populations, as well as epidemiological risk factors, may vary among different

Table 4. Clinical Characteristics of the Ocular Adnexal MALT Lymphoma Patients According to the *C. psittaci* Infection Status ($n = 33$)

	C. psittaci Positive	*C. psittaci* Negative	*P* Value
No. of patients	25	8	
Mean age (y)	53.5 ± 15.1	48.9 ± 15.7	0.550*
Gender (M/F)	10/15	6/2	0.118†
Laterality			
Unilateral vs. bilateral	18:7	7:1	0.643†
Clinical stage			
T1N0M0 vs. higher than T1N0M0	18:7	2:6	0.035†

* Mann-Whitney test.
† Fisher's exact test.

geographic areas and may affect the incidence of lymphomas.[50–52]

It has been demonstrated that specific cytogenetic abnormalities represent alternative pathogenic events to *H. pylori*-driven lymphomagenesis in gastric lymphomas. In gastric lymphoma, the average number of methylated genes significantly increased with *H. pylori* infection and, furthermore, aberrant CpG methylation of specific genes, such as *p16, MGMT,* and *MINT31,* was consistently associated with *H. pylori* infection.[21] As to the ocular adnexal MALT lymphoma, recently there was a first attempt to report a correlation of *C. psittaci* infection with genetic lesions and epigenetic change, showing that *p16* hypermethylation was observed only in *C. psittaci*-negative cases. However the frequencies of *p16* hypermethylation and *C. psittaci* positivity were quite different between Italian and African patients.[23]

In the present study, there was no specific correlation between *p16* hypermethylation and *C. psittaci* positivity. However, *ECAD* hypermethylation was significantly associated with *C. psittaci* positivity ($P = 0.041$, Fisher's exact test). *ECAD*, as one of the cadherin molecules, is important in maintaining cell-to-cell contact, and the inactivation of this gene can increase tumor invasion or metastasis. Silencing of *ECAD* by promoter CpG island methylation was shown to be associated with tumor invasion and metastasis in gastric cancer.[53] Also, *ECAD* methylation was frequently observed in the precancerous lesions of *H. pylori*-infected chronic gastritis and could be reversed after *H. pylori* eradication therapy.[54,55] In another report, *H. pylori*-related inflammation was found to induce this methylation in the stomach.[55] Therefore, *H. pylori*-related inflammation may cause epigenetic silencing of this gene by methylation, which may further lead to gastric carcinogenesis. The fact that *ECAD* was significantly highly methylated in *C. psittaci*-positive cases in this study implies that epigenetic silencing of this gene may also be involved in the similar inflammation-associated carcinogenesis of *C. psittaci*-related ocular adnexal MALT lymphoma. Although not statistically significant, *MT1G* methylation was more frequently detected in *C. psittaci*-positive (60%) than *C. psittaci*-negative (25%) cases ($P = 0.11$). Further larger-scale studies could clarify this point.

This is the second study confirming a high prevalence of *C. psittaci* in Korean patients with ocular adnexal MALT lymphomas. Yoo et al.[56] previously reported that *C. psittaci* DNA was detected in 26 (78%) of 33 patients, comparable with our results of a 75.8% *C. psittaci* detection rate. *C. psittaci* positivity was significantly more frequent in T1 (18 of 25 conjunctival MALT lymphomas) than above-T1 (2 of 8 orbital or eyelid MALT lymphoma) cases ($P = 0.035$). In 18 patients who received doxycycline monotherapy, 12 patients (66.7%) showed a response to the treatment. The response to doxycycline treatment was known to have a geographical difference, and our results were comparable to those of an Italian study that showed a 48% response rate.[49] We used a single-course treatment in 5 patients and double-course treatment in 13 patients. Kim et al.[57] reported that patients receiving the double-course treatment had a tendency to a greater and more rapid response than those receiving the single course treatment, Similarly, the double-course treatment showed a better response rate (76.9%) than single-course treatment (40.0%), although there was no statistically significant difference in this study. Analysis of the chemotherapy response according to the *C. psittaci* status was unavailable due to the small numbers of patients (*C. psittaci* positive in six cases and *C. psittaci* negative in two cases).

In this study, *p16* hypermethylation was observed in 28.0% of *C. psittaci*-positive cases and in none of the *C. psittaci*-negative cases, while the previous report showed that *p16*

Table 5. Comparison of the Frequencies of Methylation with Methylation in Other Lymphomas in Previous Studies*

Gene	Our Cases	Gastric Lymphoma[6]	DLBCL[44]	DLBCL[46]	NHL[45]	OAL[23]
DAPK	94.2	55.1	59	76.1		
ECAD	77.1	32.7				
MT1G	48.6			76.1		
THBS1	37.1			69.6		
RAR β	31.4				26	
p 16	20.0	26.5	52	52.2		43.6
MGMT	5.71	44.9		30.4		
RASSF1A	0		15			
p14	0		41			
Methylation detection method	MSP	MSP sequencing	MSP	MSP	MSP RT-PCR	MSP

RT-PCR, reverse transcriptase-polymerase chain reaction.
* Data are the frequencies of methylation (%) in each type of lymphoma. Reference numbers for the previous studies are shown in the column heads.

hypermethylation was observed only in *C. psittaci*-negative cases.[23] The methylation of *ECAD* was shown in 88% of the *C. psittaci*-positive cases but only in half of the *C. psittaci*-negative cases. In the case of *MT1G*, the methylation was detected in 60% of *C. psittaci*-positive cases and in 25% of *C. psittaci*-negative cases. Thus, these findings suggest that *C. psittaci* infection may cause the aberrant DNA hypermethylation of specific genes. Further studies with larger sample sizes will certainly be warranted in the future to verify these results.

These results indicated that epigenetic inactivation of certain genes might play a critical role in the pathogenesis of ocular adnexal MALT lymphoma. However, validation of these MSP data using another technique and possibly another cohort of OAL patients is essential to confirm these findings. It could be suggested from the results of our study that epigenetic inactivation of *DAPK* and *ECAD* may be strongly correlated with ocular adnexal MALT lymphoma in South Korea and that hypermethylation of *ECAD* may be closely associated with *C. psittaci* infection.

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