Ocular adnexal mucosa-associated lymphoid tissue lymphoma in Northern China: high frequency of numerical chromosomal changes and no evidence of an association with *Chlamydia psittaci*

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(Received 2 July 2010; revised 28 July 2010; accepted 31 July 2010)

Abstract
Studies from different countries showed variations of genetic changes and association with *Chlamydia psittaci* in ocular adnexal mucosa-associated lymphoid tissue (MALT) lymphoma. A total of 38 ocular adnexal MALT lymphoma cases from Northern China were studied. Genetic abnormalities were investigated in 28 cases by interphase FISH. *C. psittaci* and other infectious agents that are commonly-associated with chronic eye disease were screened in 38 cases by PCR. Genetic abnormalities were detected in 60.7% of cases. Among them, only one showed a break-apart of the *IgH* gene and all others showed numerical abnormalities, including trisomy 18 in 7 cases (25%), 3 copies of *BCL6* gene in 12 cases (43%), and 3 copies of *C-MYC* gene in 2 cases (7%). *C. pneumoniae* was positive in two cases (5.3%), and *C. psittaci*, *C. trachomatis*, HSV1, HSV2, ADV8, and ADV19 were not detected in any cases. In conclusion, numerical abnormalities are frequent and the chromosomal translocations commonly associated with MALT lymphomas are rare in ocular adnexal MALT lymphoma of Northern China. *C. psittaci* and other infectious agents are not associated with ocular adnexal MALT lymphoma in these patients.

Keywords: Ocular adnexal MALT lymphoma, Chlamydia psittaci, Genetics, Interphase FISH
such as trisomies 3, 8, and 18 [2–5]. Incidences of the above mentioned genetic abnormalities are different in MALT lymphomas arising from different anatomic sites [6–8]. However, genetic studies on ocular adnexal MALT lymphoma are limited, especially for cases from China. To better understand molecular genetics underlying the development of ocular adnexal MALT lymphoma, we conducted a comprehensive interphase fluorescence in situ hybridization (FISH) study to investigate chromosomal abnormalities in ocular adnexal MALT lymphoma cases from Northern China.

MALT lymphoma arises from the lymphoid tissue acquired as a result of chronic antigenic stimulation due to inflammatory or autoimmune disorder [9]. This is exemplified by Helicobacter pylori infection in patients with gastric MALT lymphoma [10,11], Borrelia burgdorferi and Campylobacter jejuni infections in patients with cutaneous MALT lymphoma [12] and intestinal MALT lymphoma [13], respectively, and by an association of the salivary gland and thyroid MALT lymphomas with myoepithelial sialadenitis [14] and Hashimoto thyroiditis [15]. The etiology of ocular adnexal MALT lymphoma is largely unknown. Recently, Ferreri et al. [16,17] in Italy demonstrated an association of ocular adnexal MALT lymphoma with Chlamydia psittaci infection. The same authors also presented evidence to support this association by showing a complete or partial regression of ocular adnexal MALT lymphoma in some cases following anti-chlamydial antibiotic therapy [18]. However, subsequent studies from other countries or regions have failed to confirm such an association [19–24]. It seems that the association of C. psittaci with ocular adnexal MALT lymphoma may vary in different geographical regions and other etiological factors may be involved in the development of this type of lymphoma [25]. To investigate whether or not an association of infectious agents with ocular adnexal MALT lymphoma exists in Chinese patients, we screened by polymerase chain reaction (PCR) for C. psittaci and other pathogens underlying chronic eye infection, namely C. trachomatis, C. pneumoniae, (Herpes Simplex virus) HSV1, (Herpes Simplex virus) HSV2, (Adenovirus) ADV8, and (Adenovirus) ADV19 in ocular adnexal MALT lymphoma cases collected from Northern China.

Materials and methods

Tissue specimens

Sixty-eight formalin-fixed paraffin-embedded (FFPE) ocular adnexal biopsies from 38 cases of ocular adnexal MALT lymphoma, 3 cases of non-MALT lymphomas (1 follicular lymphoma and 2 diffuse large B cell lymphomas), and 27 cases of reactive lymphoid hyperplasia (RLH) were collected from the Department of Pathology of Beijing Tongrentang Hospital, Shanxi Eye Hospital and Beijing Friendship Hospital in China between April 1995 and June 2008. All cases were reviewed by two experienced hematopathologists to confirm the diagnosis according to the World Health Organization Classification of Tumours of Hematopoietic and Lymphoid Tissues [1], and appropriate immunostains were performed to assist the subclassification. Local ethical guidelines were followed for the use of archival paraffin-embedded tissues for research.

Interphase fluorescence in situ hybridization

Interphase FISH was performed on 4–5 μm thick paraffin sections of the ocular adnexal MALT lymphoma specimens as previously described [26] with some modifications. Briefly, the tissue sections were deparaffinized, rehydrated and cooked in a high pressure cooker with 1 L of 1 mM EDTA buffer for 3 min, and then digested in 0.1% of pepsin solution. This was followed by application of 2–5 μl of probe onto the appropriate areas in the tissue. The probe and target DNA were then allowed to co-denature at 80°C for 25 min and hybridize at 45°C in a humid chamber for 2 days. The slides were then rinsed by gradient SSC solution, mounted with anti-fade solution containing DAPI (Vector Labs, USA) and visualized on a fluorescence microscope (Olympus BX51, Japan) by two investigators independently.

Nine probes were used in the present study. These included IGH, MALT1, C-MYC, BCL6, BCL2, CCND1, BCL10, and FOXP1 Dual Color Break Apart Rearrangement Probes, and Chromosome 18 Enumeration Probe (CEP18). All of the probes were purchased from Vysis/Abbott Ltd, USA, except BCL10 and FOXP1 probes which were made in-house and supplied by Professor Ming-Qing Du of University of Cambridge, UK.

Around 100–200 nuclei with full FISH signals were scored for each probe. Slides with known structural or numerical abnormality for each of the above probes were used as the positive controls, and a case of reactive hyperplasia of tonsil was served as the negative control. Cut-off for each abnormality was established on five paraffin slides of the benign lymph nodes and tonsils, and was calculated to be 5% (mean plus 3 times of standard deviations).

Detection of infectious agents by touchdown enzyme time-release-polymerase chain reaction [25,27]

FFPE tissue sections were deparaffinized twice in xylene, washed in 100% ethanol and extracted for
DNA using QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer’s instructions.

The quality of DNA samples prepared from FFPE tissues was assessed by PCR amplification of variable sized human gene fragments (100, 200, 300, 400, and 600 bp) as described previously [28]. Only cases with successful amplification of a 200 bp or larger products were screened for infectious agents.

PCR amplification and electrophoresis of the PCR products were carried out in two separate rooms.

Primer sets used for detection of Chlamydiae, ADV and HSV were designed to target the sequence of 16S rRNA gene, hexon gene for major capsid protein and DNA polymerase gene, respectively, as described previously [25] and were suitable for degraded DNA in FFPE tissues. Amplification of C. psittaci, C. trachomatis, C. pneumoniae, ADV8, ADV 19, HSV1, and HSV2 was carried out using the previously described Touchdown Enzyme Time-Release (TETR) PCR [25,27], with 150 ng of template DNA and Invitrogen’s Taq DNA polymerase enzyme in each 25 μl reaction mixture.

The touchdown protocol used for C. psittaci and C. pneumoniae detection consisted of a hot start at 95°C for 75s, denaturation at 94°C for 45 s, annealing at 62°C ~ 53°C (decreased 1°C every 2 cycles), and extension at 72°C for 60 s, followed by 40 cycles with the annealing temperature at 52°C. For ADV19 and C. trachomatis detection, the touchdown annealing temperature was set from 66°C to 56°C, and other cycles conditions were the same as above.

For ADV8, HSV1, and HSV2 detection, the cycle parameters were a hot start at 95°C for 75 s, denaturation at 95°C for 30 s, annealing at 63°C ~ 59°C (decreases 1°C every 2 cycles), and extension at 72°C for 30 s, followed by 35 cycles with the annealing temperature at 58°C.

PCR products were analyzed by electrophoresis on 8% polyacrylamide gels.

To make the data confirmable, PCR amplification was performed independently three times for each sample. In each set of PCR, H2O was used as the blank control and was randomly interspersed among test samples for contamination monitoring. All primers and positive controls were provided by Professor Ming-Qing Du of University of Cambridge. Only were samples with positive PCR results in at least two of the three independent reactions regarded as true positive.

Statistical analysis

Differences in detection rate of the infectious agents among lymphoma subtypes and control subjects were analyzed using a 2-tailed Fisher’s exact test (SPSS 11.5 statistical Package), with p values <0.05 being considered to be statistically significant.

Results

Clinical – pathological features

Among the 38 patients with ocular adnexal MALT lymphoma, 24 were males and 14 were females (the male to female ratio 1.7). The ages ranged from 34 to 79 years with a mean of 57 and a median of 55 years. Among the 27 cases with RLH, 12 were males and 15 were females (the male to female ratio 0.8). The ages ranged from 16 to 72 years with a mean of 53 and a median of 52 years. The lymphoma occurred in the orbit in 27 cases, conjunctival in 6 cases, eyelid in 4 cases, and lacrimal gland in 1 case. Two cases had bilateral orbital involvement by the tumor and the rest of cases were unilateral. All 38 cases of ocular adnexal MALT lymphoma were composed of small to medium sized tumor cells that were positive for CD20 and CD79a and negative for CD3, CD5, CD10, CD23, and CCND1.

Detection of genetic abnormality by interphase fluorescence in situ hybridization

A total of 28 ocular adnexal MALT lymphoma cases had adequate materials for interphase FISH study and all these cases were successfully analyzed with the probes described above (Table I). The interphase FISH with IGH, MALT1, BCL6, and C-MYC Dual Color Break Apart Rearrangement Probes showed a break-apart of the IGH gene locus in 1 case (Figure 1). The remaining 27 cases were negative for break-apart at any of the gene loci targeted (Table I, Figure 1). To look for partner gene of the above IGH gene-involved translocation, interphase FISH with BCL10, FOXP1, BCL2, and CCND1 Dual Color Break Apart Rearrangement Probes was performed on the same specimen. None of these target loci showed a break-apart, indicating that the partner gene of the above IGH-involved translocation was unlikely the BCL10, FOXP1, BCL2, CCND1, MALT1, BCL6, or C-MYC gene in this case.

Although the translocations commonly associated with lymphoma, especially MALT lymphoma, were not found in any of the 28 ocular adnexal MALT lymphoma cases, 57% (16/28) of the cases showed three copies of the target gene locus by MALT1, BCL6, and C-MYC Dual Color Break Apart Rearrangement Probes (Figure 1). The frequency of three copies of the gene locus was 25% (7/28) for MALT1, 43% (12/28) for BCL6, and 7% (2/28) for C-MYC gene, with 4 cases (14%) carrying three copies of both the MALT1 and BCL6 gene loci and 1
Table I. Interphase FISH results in ocular adnexal MALT lymphoma.

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</table>

FISH, fluorescence in situ hybridization.
MALT, mucosa-associated lymphoid tissue.

*This case was also investigated with BCL10, FOXP1, BCL2, and CCND1 dual color break apart rearrangement probes and all the target loci were normal.

Figure 1. Representative interphase FISH images. 1 FISH with IGH dual color break-apart rearrangement probe shows nuclei with break-apart signals at the IgH locus. Signal pattern: 1 fusion, 1 red and 1 green. 2 FISH with BCL6 dual color break apart rearrangement probe shows nuclei with normal signals at the BCL6 locus. Signal pattern: 2 fusion signals. 3 FISH with MALT1 dual color break apart rearrangement probe shows three copies of MALT1 gene locus. 4 FISH with CEP18 enumeration probe shows nuclei with three copies of the centromere 18.

Detection of infectious agents by polymerase chain reaction

As shown in Table II and Figure 2, C. pneumoniae DNA was found in 2 of 38 ocular adnexal MALT lymphoma cases (5.3%) whereas C. psittaci, C. trachomatis, ADV19, ADV8, HSV1, and HSV2 were negative in all the cases analyzed. Three non-MALT lymphomas were negative for any of the infectious agents detected. The RLH were negative for C. psittaci, C. trachomatis, and C. pneumoniae DNA, but positive for HSV1, HSV2, and ADV8 in one case each. The difference in prevalence of case (4%) carrying three copies of both the BCL6 and C-MYC gene loci. In order to identify the extent of chromosomal region with a gain in copy number, CEP18 probe was applied to the cases with three copies of the MALT1 gene locus and an extra copy of the centromere 18 was shown in all the cases, suggesting probable presence of trisomy 18 in these cases (Figure 1(4)).
C. pneumoniae among ocular adnexal MALT lymphoma, non-MALT lymphomas, and RLH was not significant ($p > 0.05$).

**Discussion**

Ocular adnexal MALT lymphoma is the most common type of lymphomas of the ocular adnexa [29]. Most studies show that ocular adnexal MALT lymphoma displays a female predominance [24, 30–33]. However, our study based on Chinese cases showed a male predominance, which is in accordance with several recent reports from other Asian countries and region such as South Korea, Taiwan, and Japan [34–36]. The basis of this discrepancy is unclear, and may be associated with the geographic or racial variability. Our study also demonstrated that the median age of the ocular adnexal MALT lymphoma patients was 55 years, with the initial symptoms of exophthalmia for the lymphomas arising from the orbit, and red eyes with irritation for tumors arising from other ocular adnexal sites. The most common sites of ocular adnexal MALT lymphoma are orbit (71%). These findings are similar to those of recent reports [37,38].

Recurrent genetic abnormalities have been described in extranodal MALT lymphomas. These included $t(11;18)(q21;q21)$ involving $API2$ and $MALT1$, $t(1;14)(p22;q34)$ involving $BCL10$ and $IgH$, $t(14;18)(q32;q21)$ involving $IgH$ and $MALT1$, $t(3;14)(p14;q32)$ involving $FOXP1$ and $IgH$, and the numerical changes such as trisomies 3 and 18 [2–5], and their frequencies varied among MALT lymphomas arising at different anatomic sites [6–8]. Concerning the genetic abnormalities in ocular adnexal MALT lymphoma, most of the previous studies focused on $t(11;18)(q21;q21)$ and reported an incidence of this translocation from 0% to 13% [7,39,40]. As for other recurring structural abnormalities in ocular adnexal MALT lymphoma, Ye et al. [7] studied 73 European cases and reported an incidence of 0% and 7% for $t(1;14)(p22;q32)$ and $(14;18)(q32;q21)$, respectively. Another study [6] reported $(14;18)(q32;q21)$ in 24% of the 37 European cases. Recently, $t(3;14)(p14.1;q32)$ has been found in 20% of ocular adnexal MALT lymphoma cases that lacked $MALT1$ translocations [41]. Using interphase FISH with a substantial number of the probes, we demonstrated that $t(11;18)(q21;q21)$, $t(1;14)(p22;q32)$, $(14;18)(q32;q21)$ or $t(3;14)(p14.1;q32)$ was absent in our case series, a result similar to that found in Japan [42] and North America [8]. However, a translocation involving the $IgH$ gene was identified in one case, although the partner gene was not one of those commonly involved in translocations with the $IgH$ gene, such as $MALT1$, $BCL10$, $FOXP1$, $BCL6$, $C-MYC$, $BCL2$, and $CCND1$ genes. An unusual translocation involving the $IgH$ gene was therefore present in this ocular adnexal MALT lymphoma case, as reported by Remstein et al. [8].

Although structural abnormalities were very rare in our case series, numerical change was identified at a

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Cases with $C. pneumoniae$ positive</th>
<th>Cases with $C. psittaci$ positive</th>
<th>Cases with $C. trachomatis$ positive</th>
<th>Cases with ADV19 positive</th>
<th>Cases with ADV8 positive</th>
<th>Cases with HSV1 positive</th>
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<tr>
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</table>

MALT L, mucosa-associated lymphoid tissue lymphoma; Non-MALT L, non-mucosa-associated lymphoid tissue lymphoma; RLH, reactive lymphoid hyperplasia.

Figure 2. Detection of $C. psittaci$, $C. trachomatis$, $C. pneumoniae$, HSV1, HSV2, ADV8, and ADV19 by TETR-PCR. M: DNA size marker; “+”: positive control; H: blank control; S1–S3: the samples of ocular adnexal MALT lymphoma; $C. psittaci$ PCR product: 111 bp, all samples negative; $C. pneumoniae$ PCR product: 73 bp, positive for sample S1; $C. pneumoniae$ PCR product: 116 bp, all samples negative; $C. pneumoniae$ PCR product: 116 bp, all samples negative; $C. pneumoniae$ PCR product: 116 bp, all samples negative; $C. pneumoniae$ PCR product: 73 bp, positive for sample S1; $C. pneumoniae$ PCR product: 65 bp, all samples negative; $C. pneumoniae$ PCR product: 87 bp, all samples negative; $C. pneumoniae$ PCR product: 92 bp, all samples negative.
high frequency of 57% (16/28), shown as gain of an extra copy of BCL6 (43%), MALT1 (25%) or C-MYC (7%) genes locus. This finding is similar to that of Dierlamm et al. [2]. Trisomies 3 and 18 are the frequent genetic abnormalities in MALT lymphoma [2,3]. In order to investigate whether or not the gain of an extra copy of the MALT1 gene locus was a reflection of trisomy 18 in our series, a CEP18 probe was used and all the cases with three copies of MALT1 gene locus were shown to have three copies of centromere 18, suggesting the probable presence of a trisomy 18 in these cases. The copy number of centromeres 3 and 8 were not studied. However, it is likely that three copies of BCL6 and C-MYC gene loci may also indicate the presence of trisomies 3 and 8 in these cases.

Taken together, in this series of ocular adnexal MALT lymphoma cases from Northern China, the overall incidence of genetic abnormalities detected by the present set of FISH probes was 60.7%. The most of the genetic abnormalities (94%) were copy number gains, and the structural abnormalities at the loci targeted were very rare (4%), suggesting that the translocations common to MALT lymphomas of other sites may not contribute to the pathogenesis of ocular adnexal MALT lymphoma. Additional researches, such as high resolution array comparative genomic hybridization studies or gene expression profiling analysis may be useful to uncover other genetic changes specific to ocular adnexal MALT lymphoma [43].

MALT lymphomas arise in tissues or organs that are normally devoid of organized lymphoid tissue, but acquired organized lymphoid tissue in response to chronic antigenic stimulation due to persistent infection or autoantigenic reaction [9]. Ocular adnexal MALT lymphoma is a common subgroup of the MALT lymphoma entity and the etiological factors underlying its development are currently unknown. Using a combination of PCR and immunohistochemistry, Ferreri et al. [16,17] recently detected C. psittaci DNA in 80% of orbital lymphomas, including 87% of MALT lymphoma cases in Italy and established an association between infection of C. psittaci and the ocular adnexal MALT lymphoma. Later, the same group of authors provided further evidences that a complete or partial regression was achieved in some cases of ocular adnexal MALT lymphoma, including cases that were negative for C. psittaci, following anti-chlamydial antibiotic therapy [16–18]. However, several subsequent studies from other countries have failed to establish an association between C. psittaci infection and ocular adnexal MALT lymphoma [19–24]. A recent study [25] on ocular adnexal MALT lymphoma cases from six geographic areas including Southern China showed a 22% overall prevalence and 11% in Southern China, but marked geographic variation for C. psittaci infection. In our study, 38 cases of ocular adnexal MALT lymphoma from Northern China were all C. psittaci DNA negative, indicating that there was no association between ocular adnexal MALT lymphoma and C. psittaci infection. To examine whether or not other infectious agents are involved in the development of ocular adnexal MALT lymphoma, we examined DNA of C. pneumoniae, C. trachomatis, HSV1 and HSV 2, and ADV8 and ADV19, which are common causes of chronic eye infection, in our cases. We found that these agents were absent or present at a very low rate in both ocular adnexal MALT lymphoma and control groups. The results suggested that these infectious agents are also unlikely to be associated with the present series of ocular adnexal MALT lymphoma. As the majority cases of ocular adnexal MALT lymphoma, at least in Northern China, do not have a demonstrable association with C. psittaci, a presumptive antibiotic therapy does not seem to be warranted for these patients.

In conclusion, numerical abnormalities, such as trisomy 18 and three copies of the BCL6 and C-MYC genes are the frequent genetic aberrancy and the chromosomal translocations commonly associated with MALT lymphomas are rare in ocular adnexal MALT lymphoma in patients of Northern China. C. psittaci and other infectious agents including C. pneumoniae, C. trachomatis, HSV1 and HSV 2, and ADV8 and ADV19 are not associated with ocular adnexal MALT lymphoma in these patients.

Acknowledgements

The authors thank Professor Ming-Qing Du of Department of Pathology, University of Cambridge for donating BCL10 and FOXP1 probes and all primers. This work was supported by a grant from Beijing Municipal Commission of Education Fund, China (KM200810025017).

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