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Lack of association of conjunctival MALT lymphoma with *Chlamydiae* or *Helicobacter pylori* in a cohort of Chinese patients

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Ji-Ping Cai* [ABCDEF], **Jin-Wei Cheng** [ABCDEF], **Xiao-Ye Ma** [BCD], **Yu-Zhen Li** [BCD], **You Li** [BCD], **Xiao Huang** [BCD], **Rui-Li Wei** [AEFG]

* Ji-Ping Cai and Jin-Wei Cheng contributed equally to this work

Department of Ophthalmology, Shanghai Changzheng Hospital, 2nd Military Medical University, Shanghai, China

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Summary

Background: This study was conducted to detect microbial pathogens in conjunctival mucosa-associated lymphoid tissue (MALT) lymphoma specimens in an attempt to determine possible associations between conjunctival MALT lymphoma and microbial infections.

Material/Methods: Using PCR technique, freshly obtained tumor specimens from 16 cases of conjunctival MALT lymphoma, as confirmed by postoperative pathology, were analyzed for DNA of *Chlamydia psittaci* (*C. psittaci*), *Chlamydia trachomatis* (*C. trachomatis*), *Chlamydia pneumoniae* (*C. pneumoniae*) and *Helicobacter pylori* (*H. pylori*). Synthetic *C. psittaci*, *C. trachomatis*, *C. pneumoniae* and *H. pylori* DNA were used as positive control, and blank plasmid DNA as negative control.

Results: Electrophoresis showed that no bands corresponding to the positive control were observed in the specimens, indicating that no DNA of the 4 microorganisms was detected in the specimens of the 16 cases of conjunctival MALT lymphoma.

Conclusions: The PCR technique was able to detect the positive control quickly and accurately, but the results of PCR in analyzing the 16 specimens were negative, indicating that there is no association between conjunctival MALT lymphoma and the 4 microorganisms in Chinese patients.

key words: **ocular adnexal MALT lymphoma • *Chlamydia* • *Helicobacter pylori***

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Author's address: Rui-Li Wei, Department of Ophthalmology, Shanghai Changzheng Hospital, #415 Fengyang Road, Shanghai 200003, China, e-mail: ruiliwei@gmail.com

Background

Chlamydiae are prokaryotic organisms that differ from both bacteria and viruses [1]. There are 3 known types of *chlamydiae* associated with human diseases: *Chlamydia psittaci* (*C. psittaci*), *Chlamydia trachomatis* (*C. trachomatis*) and *Chlamydia pneumoniae* (*C. pneumoniae*). They may cause multiple diseases, including pneumonia, trachoma, cervicitis, pelvic inflammatory disease, urethritis and epididymitis [2]. Recent studies in other countries revealed a close association between *C. psittaci* and ocular adnexal MALT lymphoma [3–6], but no such association has been reported in China.

MALT lymphoma is an extra-nodal marginal zone B cell lymphoma, and is the second most common inert tumor originating from mucosa-associated lymphoid tissue. There is definite evidence that occurrence of some B-cell lymphomas is associated with long-term chronic stimulation of microbials or autologous pathogens, which is most clearly exemplified by *H. pylori*-associated gastric MALT lymphoma [7–9].

Similar to the association of gastric MALT lymphoma with chronic stimulation of *H. pylori*, many pathogenic microbials, especially *Chlamydiae*, are also reported to be associated with the formation of MALT lymphoma. Italian researchers were the first to study ocular MALT lymphoma, and detected *C. psittaci* DNA in 80% of their ocular adnexal lymphoma specimens. They found that the tumor subsided after *C. psittaci* was eliminated with medical treatment, thus confirming a close correlation [3,4]. However, the results of subsequent similar studies in different parts of the world were controversial – there were great differences in the detection rate of *C. psittaci* DNA from ocular adnexal lymphoma specimens, seemingly suggesting a regional correlation [10–14].

The present study used the PCR method to detect *C. psittaci*, *C. trachomatis*, *C. pneumoniae* and *H. pylori* DNA in specimens of conjunctival MALT lymphoma freshly obtained from Chinese patients in our hospital to determine if these microorganisms were present Chinese patients with conjunctival MALT lymphoma.

Material and Methods

Material

Genomic DNA Mini Preparation Kit was purchased from MN NucleoSpin®, Germany. Tissue 740952 and Hotstart Taq were the products of TaKaRa DR028 (Dalian, China). All materials used, including tubes and pipette tips, were imported from AXYGEN. *C. psittaci*, *C. trachomatis*, *C. pneumoniae* and *H. pylori* DNA positive controls were synthesized in our laboratory.

Methods

Patient and sample collection: The study was approved by the local Ethics Review Board. Conjunctival MALT lymphoma specimens in the present study were from 14 patients (16 eyes) who were admitted at Shanghai Changzheng Hospital of the Second Military Medical University (Shanghai, China) between January 2008 and December 2009, all of whom were Han ethnicity and resided mainly in East China, without histories of keeping birds or having close contacts with birds. The patients included 9 males and 5 females who ranged in aged from 14 to 83 years, with a mean age of 55±16.76 years. Of the 2 patients with both eyes infected, both were female. The common presentation of the condition included the presence of subconjunctival (especially subfornical) pink tumors (salmon patch), whose course ranged from 3 months to 2 years. No history of lymphoma was elicited, nor was any tumor detected in another part of the body on physical examination. All patients received surgical treatment, and postoperative pathology and immunohistochemistry confirmed the diagnosis of MALT lymphoma in all patients. The tumor tissues, about 5mm in diameter, were obtained surgically, immediately washed with normal saline, stored in tubes and plunged directly in liquid nitrogen. The whole procedure was completed within 5 min. After 2–4 h preservation in liquid nitrogen, the 16 specimens were transferred to a –80°C freezer for preservation until later use.

Genome extraction

Genome extraction of the specimens was performed according to the manufacturer's instructions. In brief, about 25 mg of freshly obtained MALT lymphoma tissue was cut off, digested in 180 µl T1 and 25 µl proteinase K at 56°C for 3 h, boiled in 200 µl B3 at 70°C for 10 min, and added to 210 µl ethanol. The mixture was put into the column, centrifuged at 11000g for 1 min, washed with 500 µl BW once, and again with 600 µl B5. The column was spun without loading for 1 min to dry the membrane, and was put into BE which had been preheated up to 70°C, and then centrifuged at 11,000g for 1 min. Purity of the extracted DNA was measured by spectrophotometry, and integrity was measured by electrophoresis.

Primer design and synthesis

The 16S rRNA sequences of *C. psittaci*, *C. trachomatis*, *C. pneumoniae* and *H. pylori* were from NCBI DataBank and the previous literature [16]. Primer design was according to microbial 16S rRNA gene sequences.

Expected length of *C. trachomatis* product: 315 bp
C. trachomatis p5 5' GGCGATATTTGGGCATCCGAGTAACG 3'
C. trachomatis p3 5' TCAAATCCAGCGGGTATTAACCGCCT 3'

Expected length of *C. pneumoniae* product: 197 bp
C. pneumoniae p5 5' GGTCTCAACCCCATCCGTGTCGG 3'
C. pneumoniae p3 5' TGCGGAAAGCTGTATTTCTACAGTT 3'

Expected length of *C. psittaci*: 111 bp
C. psittaci p5 5' CCCAAGGTGAGGCTGATGAC 3'
C. psittaci p3 5' CAAACCGTCCTAAGACAGTTA 3'

Expected length of *H. pylori*: 305bp
H. pylori p5 5' TGGCGTGTCTATTGACAGCGA-3'
H. pylori p3 5' CCTGCTGGGCATACTTCACCA-3'

The above primers were synthesized by Shanghai Bioengineering Co., Shanghai, China.

Detection of the specimens

We used 100 ng of tissue from each specimen as the template, and *C. psittaci* as touchdown of each 0.25°C cycle from 62°C to 50°C, pre-degeneration at 95°C for 5 min, and 45 cycles.

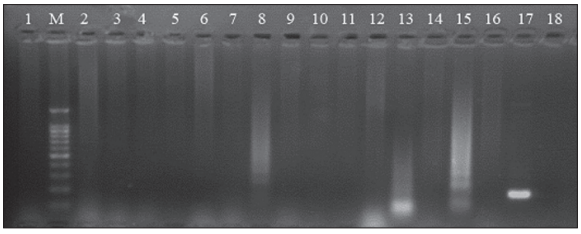

Figure 1. 16S rDNA specific segment of *C. pneumoniae*, where M refers to Marker; the bands are 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, and 1000bp in size sequence, of which 500 bp is the brightest. 1–16 refer to the serial number of the 16 specimens; 17 is the positive control (plasmid DNA); and 18 is the negative control. The target band is 197 bp. The results of electrophoresis after PCR indicate that no *C. pneumoniae* DNA was detected in the 16 tumor tissue specimens.

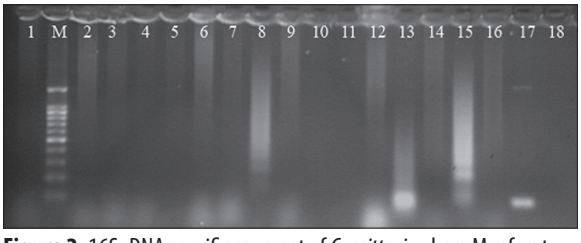

Figure 2. 16S rDNA specific segment of *C. psittaci*, where M refers to Marker; the bands are 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, and 1000 bp in size sequence, of which 500 bp is the brightest. The target band is 111 bp. The specimen numbers are the same as before. Electrophoresis shows that no *C. psittaci* DNA is detected in all 16 specimens except No. 13, which is suspected and needs to be confirmed.

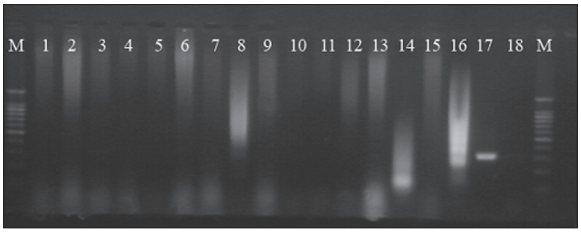

Figure 3. 16S rDNA specific segment of *C. trachomatis*, where M refers to Marker; the bands are 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, and 1000 bp in size sequence, of which 500bp is the brightest. The target band is 315 bp. The specimen numbers are the same as before. Electrophoresis shows that no *C. trachomatis* DNA is detected in all 16 specimens except No. 16, which is suspected and needs to be confirmed.

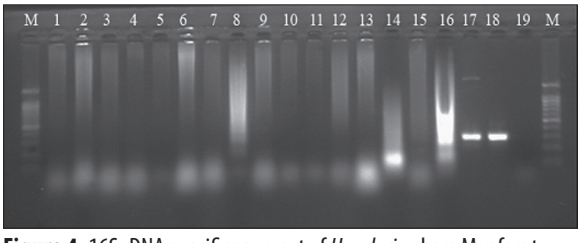

Figure 4. 16S rDNA specific segment of *H. pylori*, where M refers to Marker; the bands are 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, and 1000 bp in size sequence, of which 500 bp is the brightest; the target band is 305 bp. 1–17 are the same as before, 18 refers to the gel recovery product of the positive control, and 19 is the negative control. Electrophoresis shows that no *H. pylori* DNA is detected in all 16 specimens except No. 8 and 16, which need to be confirmed.

PCR cycling of *C. pneumoniae*, *C. trachomatis* and *H. pylori*: annealing at 55°C for 20 s, degeneration for 20 s, extension for 30 s, and 45 cycles. Electrophoresis was performed after PCR.

Results

No *C. psittaci*, *C. trachomatis* and *C. pneumoniae* DNA of the 16 MALT lymphoma specimens from our patients were detected by PCR assay. The results are shown in Figures 1–4.

We observed that the electrophoretic bands of PCR amplification of specimens No. 8 and 16 *C. trachomatis* were similar to those of *H. pylori*, and there was a suspected band at the place corresponding to the positive control. There was also a suspected band in specimen No. 13 *C. psittaci*. Sequencing after gel cutting and recovery revealed that they were not significantly correlated with the 16S rRNA sequences of *C. trachomatis*, *H. pylori* and *C. psittaci* reported. This meant that there was no target product detected in these suspected bands and indicated that the results of these specimens were also negative. Appearance of such bands might be related to the environment of the templates themselves.

Discussion

Research on the transformation of lymphocytes to cancer cells due to chronic microbial stimulation is of intense scientific interest world-wide. To confirm their correlation, it is necessary to detect the microorganisms in tumor tissues. In previous similar studies, DNA extraction was mostly from paraffin-embedded tumor pathologic specimens, and some of these tissues had been preserved for years before research; therefore, a portion of the DNA may have been degraded. The present study was a prospective study, different from previous reports in that the specimens used in the present study were freshly obtained from surgically resected and immediately frozen tumor tissues. Specimen collection and preservation were completed by the same person, thus minimizing DNA degradation and inter-specimen differences arising from personal influences, thereby maximizing the detection rate of the target DNA.

There is ample clinical and experimental evidence that *H. pylori* is the causative agent of gastric MALT lymphoma [7,8]. But there have been controversies over the conclusions in the research on ocular adnexal lymphoma. In 2004, Chan et al. [16] detected positive *H. pylori* in 4 (80%) of their 5 specimens of ocular adnexal lymphoma. Lee et al. [17] reported a 100% detection rate of *H. pylori* (15/15). Both studies pointed out that there might be a correlation between this agent and *H. pylori*. However, Sjo et al. [18] reported a conflicting result, finding that no *H. pylori* DNA was detected in their 13 specimens of ocular adnexal lymphoma. The present study searched for the presence of *H. pylori* in

our MALT lymphoma specimens, and the results were negative in all specimens. Based on other related studies and reports [19–21], our conclusion seems to support that *H. pylori* infection is unlikely to be associated with the occurrence of ocular adnexal MALT lymphoma.

MALT lymphoma is the most common pathology in ocular adnexal lymphoma, accounting for 50–78% in developed countries [22–26], 80–90% in South Korea and Japan [27,28], and more than 80% in China [29–31]. Ocular adnexa include the eyelid, conjunctiva, orbit and lacrimal apparatus, of which the conjunctiva is most frequently exposed to the external environment directly, and therefore most susceptible to infection by microbial pathogens. If microbial infection is truly associated with ocular adnexal MALT lymphoma, the occurrence of the tumor is most likely to be related to chronic stimulation by exogenous microbials. Knowing that the incidence of conjunctival MALT lymphoma has been rising annually in recent years, we selected it as the subject of research in the present study, hoping to discover pathologic factors related to the etiology of the tumor in a limited number of cases. To our knowledge, this is the first study in China using fresh specimens of conjunctival MALT lymphoma from a cohort of Chinese patients to explore possible correlations between MALT lymphoma and microbial infections.

Italian researchers reported the presence of *C. psittaci* in 32 (80%) of their 40 specimens of ocular adnexal lymphoma [3], which aroused much attention at the time of the discovery. Later, South Korean [5] and Austrian [6] researchers confirmed this correlation. However, American [32], Dutch [10] and Japanese [11] research groups did not find any evidence to confirm the correlation between ocular adnexal lymphoma and *C. psittaci*. Husain et al. [33] published an overview of related studies and concluded that there were serious controversies over *C. psittaci* DNA detection in ocular adnexal lymphoma specimens. Out of 458 cases of ocular adnexal lymphoma that they reviewed, positive *C. psittaci* was detected in 104 cases (23%), but 90% of the 104 cases were from 3 of the 12 reports reviewed. A recent study [14] indicates that the *C. psittaci* detection rate in ocular adnexal lymphoma specimens varied from place to place: 17% in Italy and 0% in Kenya. The positive rate is geographically biased: it is relatively high in Italy and South Korea, relatively low in the United States and Japan, and 0% in our study. Interestingly, the prevalent rate of *C. psittaci* infection in MALT lymphoma was 11% in specimens from Southern China in a study whose results showed that *C. psittaci* was associated with ocular adnexal MALT lymphoma and that this association was variable in 6 different geographical areas [13]. The glaring difference between the only 2 studies in Chinese patients is that the cases in this present study resided mainly in East China, while most of the samples in the other study were collected from Southern China. A similar situation occurred in the studies of Italian patients. The *C. psittaci* detection rate in ocular adnexal lymphoma specimens varied from study to study (from 13% to 80%) [3,13,14].

Although we cannot deny possible errors and differences arising from specimen selection and detection methodology, the main problem is that we can neither rule out nor confirm the cause-effect relationship between them. In addition, *C. psittaci* infection is most likely to occur in luminal mucosa that has the contact with the external environment, such as the respiratory, reproductive and urinary tracts. It is therefore difficult to explain how MALT lymphoma in the deep orbit is caused by *C. psittaci* infection. The correlation between gastric MALT lymphoma and *H. pylori* infection has been confirmed [7,8]. This is understandable, because *H. pylori* exists extensively in normal human gastric mucosa, while *C. psittaci* is not commonly parasitic in human ocular adnexa. However, positive detection of *C. psittaci* is an objective finding, suggesting that *C. psittaci* infection may be one of the pathogenetic factors contributing to ocular adnexal MALT lymphoma. Whether it is an initiating factor or a predisposing factor needs further study.

Conclusions

No *C. psittaci*, *C. trachomatis*, *C. pneumoniae* or *H. pylori* DNA was detectable in the 16 freshly obtained specimens of conjunctival MALT lymphoma in the present study. There is no evidence to confirm the correlation between the above 4 microorganisms and conjunctival MALT lymphoma in patients from the East China area.

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