Diagnostic significance of measuring antibodies to cyclic type 3 muscarinic acetylcholine receptor peptides in primary Sjögren’s syndrome

Jing He1, Jian-ping Guo1, Yan Ding1, Ying-ni Li1, Si-si Pan1, Yanying Liu1 and Zhan-guo Li1

Abstract

Objective. SS is an autoimmune disease characterized by salivary and lacrimal gland dysfunction leading to dry mouth (xerostomia) and dry eyes (xerophthalmia). Anti-muscarinic acetylcholine type-3 receptor (anti-M3R) autoantibodies have been shown to be a good serum marker in primary SS (pSS). The aim of this study was to assess the clinical correlations of anti-M3R-derived peptide antibodies in patients with pSS.

Methods. Sequences of the first to fourth cycle-M3R (c1M3R-c4M3R)-derived peptide was synthesized by a solid-phase technique on an Applied Biosystems Peptide Synthesizer. Synthesized cM3R peptide (cM3RP) was used as substrate in an ELISA to detect IgG anti-cM3RP antibodies in serum samples of patients and controls. The clinical and biological parameters of the diseases were also evaluated. The EULAR SS disease activity index (ESSDAI) score was used to measure disease activity in patients with primary SS.

Results. (i) Anti-c2M3RP antibodies were highly prevalent in pSS patients, and the titre is much higher than anti-c1,3,4M3RP antibodies. (ii) The prevalence of anti-c2M3RP antibodies in pSS, SLE, RA and healthy controls was 62.2, 7.1, 5.3 and 1.6%, respectively. The prevalence of anti-linear-2-M3RP antibodies in pSS, SLE and RA patients and healthy controls were 56.1, 20.0, 14.7 and 9.4%. (iii) The specificity of anti-c2M3RP antibodies was 95.1%, much higher than that of linear polypeptide (84.7%) for pSS diagnosis. (iv) In pSS patients, anti-c2M3RP positivity had significantly increased frequency in patients who were RF or ANA positive, and had several haematological abnormalities, such as leucopenia, anaemia and thrombocytopenia. Furthermore, the ESSDAI score was significantly higher in anti-c2M3RP-positive pSS patients (P < 0.05).

Conclusion. Anti-c2M3RP antibody was highly specific for patients with pSS. The presence of anti-c2M3RP antibody in pSS indicates that c2M3RP may act as an autoantigen that may play a role in the pathogenesis of pSS.

Key words: Sjögren syndrome, Type 3 muscarinic acetylcholine receptor, Antibodies, ESSDAI score.
antibodies to SSA or SSB, have reportedly been detected in the case of both pSS and secondary SS (sSS) [2, 3]. Recent studies have proved that the antibody to M3 subtype muscarinic acetylcholine receptors (mACHRs) was a good serum marker in pSS [4].

The recent finding of muscarinic acetylcholine receptor subtype 3 (m3AChR)-specific autoantibodies in the majority of the patients is an important advance towards an understanding of the pathogenesis of pSS, concerning not only the impaired glandular function, but also the associated features of autonomic dysfunction in some patients [4–6].

Synthetic peptides have been increasingly used as antigens in ELISAs. However, the results are often inconsistent [6, 7]. Reliable immobilization of the peptides on a solid support poses difficulties, and peptide–plastic interactions often alter the correct exposure of the epitopes in a manner that is difficult to predict. Through the study of the structure of m3AChR by the programme HMMTOP transmembrane topology prediction server [8], M3R includes four extracellular loops. Cavill et al. [9] has shown that the peptide M3R232–237 corresponding to the COOH-terminus of the second extracellular loop of M3R displayed a strong inhibitory activity on CCh-evoked colon contraction. Koo et al. [10] had identified that the synthetic peptide that consists of amino acids corresponding to the COOH-terminus of the second extracellular loop of M3R could bind to pSS IgG. However, the full sequence of the second loop does not bind to pSS IgG [10], which may indicate that antigen expression required folding the muscarinic receptor protein. Considering that the cyclic form of nature proteins is more stable and antigenic, we decided to evaluate whether the cyclic form of different peptides, representing four extracellular loops of human m3AChR, are sufficiently antigenic to detect autoantibodies in sera from pSS patients.

The EULAR SS disease activity index (ESSDAI) is a clinical index designed to measure disease activity in patients with pSS [11, 12]. It results from a large collaboration of European and North American experts in pSS, which is a systemic disease activity index developed to allow a standardized evaluation of disease activity in pSS patients. It is the first study in which we analysed the association between the positivity of anti-c2M3RP antibodies and ESSDAI score, to evaluate the value of anti-c2M3RP antibodies for predicting the activity of pSS.

In the present study, cyclic peptides rather than the whole protein or the linear peptide in M3R were used as antigenic peptides to define the role of anti-cM3RP antibodies in pSS. To our knowledge, it is the first study to evaluate the prevalence of anti-cM3RP peptide antibodies in a cohort of Chinese patients with pSS.

**Patients and methods**

**Patients**

Between January 2004 and December 2008, we obtained serum samples from pSS patients and healthy controls. All serum samples were collected before the initiation of CS treatment and stored at −20°C. Blood samples were obtained after all the subjects provided written informed consent and the local ethics committee (Peking U Second Affiliated Hospital, number FWA00001384) approved the study.

The characteristics of the patients are summarized in Table 1. All pSS patients (n = 148) fulfilled four or more of the revised US–EURO classification criteria for SS in 2002. All primary SS patients underwent extensive serological evaluations, including anti-SSA, anti-SSB, ANA, RF, etc. For those sera that were antibody negative, some patients also underwent histological examination of the labial gland. Eighty-four SLE patients and 95 RA patients were also included. All SLE and RA patients fulfilled ACR classification criteria for the respective diseases [13, 14], and selected without sSS development. Clinical and serological features of pSS, such as swelling of the parotid gland, arthritis, RP, haematological disorders, pulmonary, purpura, ESR, CRP, IgG, IgA, IgM were also detected. Forty age- and sex-matched healthy controls were recruited from our hospital staff without a history of autoimmune disease.

**Computer analysis and peptide synthesis**

The sequence of human M3R was referred from Swiss-Prot database (Acc. No P20309) and the amino acid sequence was analysed with the programme HMMTOP transmembrane topology prediction server [8] (Table 2). All the synthetic peptides were manually synthesized by means of the standard method of solid-phase peptide synthesis on an Applied Biosystems Peptide Synthesizer from SBS Gene Technology Company (Shanghai, China). The peptide was purified by reversed

### Table 1 Baseline characteristics of the patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of patients</th>
<th>Age, mean (s.d.) (range), years</th>
<th>Duration, mean (s.d.) (range), years</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSS</td>
<td>148</td>
<td>56.3 (8.1) (30–78)</td>
<td>9.4 (6.2)</td>
</tr>
<tr>
<td>SLE</td>
<td>84</td>
<td>35.2 (9.5) (11–82)</td>
<td>11.0 (4.2)</td>
</tr>
<tr>
<td>RA</td>
<td>95</td>
<td>42.5 (12.2) (31–68)</td>
<td>9.0 (7.2)</td>
</tr>
</tbody>
</table>

### Table 2 Peptides used in the study (M3RP means M3 receptor polypeptides, synthesized according to the corresponding sequence)

<table>
<thead>
<tr>
<th>Synthetic peptides</th>
<th>Nomenclature</th>
<th>Corresponding sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMNRWALGNLACDLWLC</td>
<td>c1M3RP</td>
<td>M3RP131–144</td>
</tr>
<tr>
<td>CLFWQYFGKRTVPGECC</td>
<td>c2M3RP</td>
<td>M3RP205–220</td>
</tr>
<tr>
<td>CFIQFLSEPTCD</td>
<td>c3M3RP</td>
<td>M3RP222–230</td>
</tr>
<tr>
<td>CNTFPDSC</td>
<td>c4M3RP</td>
<td>M3RP564–570</td>
</tr>
</tbody>
</table>
phase HPLC (RP-HPLC) with a purity of >90% and prepared for ELISA assays.

Antibody detection

Detection of anti-M3RP by ELISA

A solid-phase immunoassay for M3RP was performed. M3RP were coated in each well of a 96-well ELISA (TakaRa Peptide Coating Kit; Takara Bio Inc., Otsu, Japan) at 10 μg/ml in 100 μl of 0.5 M carbonate buffer, pH 9.6, adding N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride. Coating was performed at 4°C overnight followed by blocking with 100 μl of PBS, pH 7.4, containing 5% milk and 0.1% BSA. After washing the plate with PBS-Tween-20 (PBS-T), 100 μl of diluted sera (1:100) were added into each well and incubated at room temperature for 2 h. The plates were then washed with PBS-T five times, and 100 μl of goat anti-human IgG conjugated to peroxidase (Zhongshan Technology Company, China) diluted at 1:4000 was added to each well. After incubation for 1 h at room temperature and washing with PBS-T for five times, the bound antibodies were detected with O-phenylenediamine (OPD) as the substrate. The reaction was stopped by adding 100 μl of 2.5 M sulphuric acid to each well. Plates were read at a wavelength absorbance of 492 nm [optical density (OD) 492 nm] with an ELISA reader (Bio-Rad500 microplate reader; Bio-Rad, Hercules, CA, USA). Each serum sample was assayed in duplicate. A positive serum sample was included on each plate as a positive control and reference to correct interassay variations. The titre of anti-M3RP was expressed as arbitrary units (AU) and calculated as follows:

\[
AU = \frac{[\text{OD}_{\text{M3RP}} - \text{OD}_{\text{non-specific background}}]}{[\text{OD}_{\text{M3RP}} - \text{OD}_{\text{non-specific background}}]} \times 100
\]

Cut-off level for positivity was defined as the 95th percentile of healthy sera. The threshold for positivity is based on the cut-off value.

Other autoantibodies

ANAs were detected by indirect IF; anti-SSA, anti-SSB were detected by double ID kit (EURO-IMMUN; German) according to the manufacturer’s instructions.

Statistical analyses

Statistical analysis was carried out by using SPSS software, version 13 (SPSS 13.0). Comparison between two groups was conducted using t-test for continuous variables and χ²-test for categorical variables and percentages. \( P < 0.05 \) were considered to be statistically significant.

Results

Distribution of anti-M3RP antibodies in pSS

We failed to detect a reproducible and well measurable level of autoantibodies when using a common ELISA plate. However, after coupling the peptide using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (TakaRa Peptide Coating Kit; TakaRA, Otsu, Japan), we could stably detect autoantibodies with ODs above the background.

The distribution of five anti-M3RP antibodies in pSS (n = 44) was shown in Fig. 1. The median AU values of anti-linear-2-M3RP were 96.6 for pSS and 44.1 for controls. The median AU values of anti-c2M3RP were 123.8 for pSS and 46.2 for controls. Titre of anti-c2M3RP in SS was significantly higher than for the other loops of M3RP in SS (\( P < 0.001 \)).

![Fig. 1](image1.png) The prevalence of anti-M3RP antibodies in pSS. The distribution of five anti-M3RP were detected in SS (n = 44) and healthy controls (n = 16). The median AU values of anti-linear-2-M3RP were 96.6 for SS and 44.1 for controls. The median AU values of anti-c2M3RP were 123.8 for SS and 46.2 for controls. Titre of anti-c2M3RP in SS was significantly higher than for the other loops of M3RP in SS (\( P < 0.001 \)).

![Fig. 2](image2.png) The prevalence of anti-c2M3RP or anti-linear-2-M3RP antibodies in pSS and other autoimmune diseases. The prevalence of anti-c2M3RP antibodies in SS, SLE, RA and healthy controls was 62.2, 7.1, 5.3 and 1.6, respectively. While the prevalence of anti-linear-M3RP was 56.1, 20.2, 14.7 and 9.4%, respectively. The specificity of anti-c2M3RP antibodies was 95.1%, much higher than that of linear polypeptide (84.7%; \( P < 0.05 \)).
pSS was significantly higher than that of other cyclic M3RPs in pSS ($P < 0.001$).

The cut-off value for anti-c2M3RP and anti-linear-2-M3RP positivity was defined as the 95th percentile of healthy sera (AU = 107.3 and 93.2, respectively). As shown in Fig. 2, the prevalence of anti-c2M3RP antibodies in pSS, SLE, RA patients and healthy controls was 62.2, 7.1, 5.3 and 1.6%, respectively. The prevalence of anti-linear-2-M3RP antibodies in the pSS, SLE, RA and healthy controls was 56.1, 20.0, 14.7 and 9.4%, respectively (Fig. 2). The specificity of the anti-c2M3RP antibodies in the diagnosis of pSS was 95.1%, much higher than that of linear-2-M3RP polypeptide (84.7%; $P < 0.05$).

Anti-c2M3RP antibodies were highly prevalent even in pSS patients lacking anti-SSA, anti-SSB antibodies

A subsequent analysis revealed that the prevalence of anti-c2M3RP antibodies was 62.1 and 65.8% in pSS patients lacking anti-SSA and anti-SSB, respectively. Although there is no significant difference, this finding suggests that the anti-c2M3RP antibody is a complementary diagnostic marker for pSS.

Association of anti-c2M3RP antibodies with clinical manifestations and other pSS-related laboratory parameters

The association between anti-c2M3RP antibody and clinical manifestations was also examined in this study, but no correlation was found (Table 3). As shown in Table 4, the frequency of RF positivity, ANA positivity, white blood cell decrease, platelet decrease and haemoglobin decrease were significantly higher in anti-M3RP-positive pSS patients ($P < 0.05$). The ESSDAI score is significantly higher in anti-c2M3RP positive pSS patients ($P < 0.05$; Table 4).

**Discussion**

Previous studies have confirmed the presence of anti-cM3R autoantibodies in pSS patients and have shown that these antibodies may be involved in the pathogenesis of pSS. The presence of these autoantibodies has been confirmed in functional assays in animals [4, 5, 14–16]. These findings have led to recent attempts to develop a simple test for the detection of the anti-M3RP autoantibody in patients’ sera, wherein the antibody functions as a disease marker [6, 7]. In the present study, we analysed the structural preferences and physicochemical properties of the four extracellular loops of M3RP, which contains a pair of conserved Cys residues (Cys140 and Cys220). The Cys–Cys bonds are connected in a disulphide bond, which might enfold the M3R. From a consideration of parameters such as antigenicity, accessibility and hydrophilicity, features characteristic of antibody-binding sites and secondary structure preferences, we synthesized the

<table>
<thead>
<tr>
<th>Clinical manifestation</th>
<th>Anti-c2M3RP positive ($n = 92$)</th>
<th>Anti-c2M3RP negative ($n = 56$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (range), years, n (%)</td>
<td>58.2 (30–78)</td>
<td>55.8 (31–72)</td>
<td>0.83</td>
</tr>
<tr>
<td>Duration, mean (range), years</td>
<td>9.2 (0.2–49)</td>
<td>6.9 (0.25–35)</td>
<td>0.29</td>
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<tr>
<td>Salivary gland enlargement, n (%)</td>
<td>8 (8.7)</td>
<td>9 (16.1)</td>
<td>0.175</td>
</tr>
<tr>
<td>Arthritis, n (%)</td>
<td>34 (36.9)</td>
<td>25 (44.6)</td>
<td>0.358</td>
</tr>
<tr>
<td>Liver disease, n (%)</td>
<td>13 (14.1)</td>
<td>9 (16.1)</td>
<td>0.750</td>
</tr>
<tr>
<td>Pulmonary fibrosis, n (%)</td>
<td>8 (8.7)</td>
<td>6 (10.7)</td>
<td>0.686</td>
</tr>
<tr>
<td>Cutaneous vasculitis, n (%)</td>
<td>5 (5.4)</td>
<td>3 (5.4)</td>
<td>0.984</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Laboratory parameters</th>
<th>Anti-c2M3RP positive ($n = 92$)</th>
<th>Anti-c2M3RP negative ($n = 56$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucopenia, n (%)</td>
<td>36 (37.7)</td>
<td>14 (23.0)</td>
<td>0.041*</td>
</tr>
<tr>
<td>Anaemia, n (%)</td>
<td>9 (9.8)</td>
<td>2 (4.3)</td>
<td>0.032*</td>
</tr>
<tr>
<td>Thrombocytopenia, n (%)</td>
<td>19 (19.6)</td>
<td>6 (12.9)</td>
<td>0.049*</td>
</tr>
<tr>
<td>Anti-SSB+, n (%)</td>
<td>24 (26.2)</td>
<td>14 (26.0)</td>
<td>0.884</td>
</tr>
<tr>
<td>Anti-SSA+, n (%)</td>
<td>40 (44.4)</td>
<td>27 (47.8)</td>
<td>0.657</td>
</tr>
<tr>
<td>Globulin-$\gamma$ increase, n (%)</td>
<td>36 (37.7)</td>
<td>26 (47.8)</td>
<td>0.208</td>
</tr>
<tr>
<td>RF+, n (%)</td>
<td>62 (67.2)</td>
<td>22 (39.1)</td>
<td>0.038*</td>
</tr>
<tr>
<td>ANA+, n (%)</td>
<td>66 (72.1)</td>
<td>34 (60.9)</td>
<td>0.046*</td>
</tr>
<tr>
<td>IgA, ↑ n (%)</td>
<td>36 (37.7)</td>
<td>12 (23)</td>
<td>0.887</td>
</tr>
<tr>
<td>IgG, ↑ n (%)</td>
<td>39 (42.6)</td>
<td>22 (39.1)</td>
<td>0.712</td>
</tr>
<tr>
<td>ESSDAI score</td>
<td>7.46 (2.8)</td>
<td>3.96 (2.2)</td>
<td>0.022*</td>
</tr>
</tbody>
</table>

$*P < 0.05$.
peptides of four extracellular M3Rs and test their antigenicity in pSS and healthy control groups.

To date, various methods have been used to detect anti-M3R (or M3RP) antibodies in sera of SS patients. Gao et al. [20] used a newly constructed cell line expressing human M3R, and easily detected anti-M3R autoantibodies in sera from SS patients. Bacman et al. [4] found that sera from SS patients with dry mouth reacted with membrane lacrimal gland acinar cell antigens and the synthesized 25-mer peptide, corresponding to the second extracellular loop of human M3R. But Cavill et al. [7] failed to identify the specificity of the M3R peptide in SS. Our ELISA analysis for the detection of anti-M3RP antibodies in pSS patients showed that the antibodies against synthesized 16-mer peptide corresponding to the second extracellular loop is a good serum marker for the diagnosis of pSS, and the peptide with cyclic structure was even better, which may be due to the similarity of its structure. We also showed that the presence of anti-c2M3RP antibody was associated with certain serological parameters in the given patients, i.e. RF and ANA status. In addition, the ESSDAI was significantly higher in the anti-c2M3RP-positive group than in the negative group (Table 4). Thus, anti-c2M3RP antibody may be a valuable novel serological marker for evaluating the activity of pSS.

Furthermore, to our knowledge, this is the first study to show a significant difference between anti-C2M3RP-positive and -negative groups in terms of several haematological abnormalities, such as leucopenia, anaemia and thrombocytopenia in pSS (Table 4). We are not able to understand at the moment why the haematological abnormalities were associated with the status of anti-c2M3RP. However, it has been known that lymphocytes could express functional M3Rs to release neurotransmitters, e.g. acetylcholine, and transmit signals contributing significantly to homeostasis and activation of lymphocytes [17, 18]. Anti-M3RP antibodies could impair parasympathetic neurotransmission at the post-synaptic level by blocking acetylcholine signals and mediate parasympathetic dysfunction [19]. Thus, we speculate that haematological abnormalities occurred more frequently in anti-M3RP-positive patients possibly due to reduced secretion of neurotransmitters such as acetylcholine, and impaired parasympathetic neurotransmission signals, leading to dysfunction of the haematopoietic system. Further study, therefore, is needed to unveil the underlying mechanism(s) of the association between anti-c2M3RP positivity and haematological abnormalities.

Although other authors have described a direct relationship between the presence of anti-M3R antibodies and dry mouth and dry eyes [20, 21], we were unable to determine this relationship between them which may depend on the population we selected. It is known that mAChRs are, at least in part, responsible for mediating the parasympathetic stimulation of the secreted products by the exocrine glands. The binding of agonists to M3R initiates a sequence of events that culminates in the activation of different intracellular processes [4]. The anti-M3R autoantibodies in the sera of pSS patients may not only interact with the acinar mAChRs of the lacrimal and salivary glands, but also stimulate biological effects by mimicking muscarinic cholinergic agonists. The engagement of such receptors therefore modifies the intracellular events associated with specific cholinceptor activation. Bacman et al. [22] demonstrated that IgG derived from SS patients mimicked the behaviour of carbachol in the stimulation of nitric oxide synthase (NOS) activity, which occurs secondarily to an increase in the intracellular Ca2+ content by the activation of calcium/calmodulin-dependent NOS. NO released after immunological stimulation is cytotoxic for both the invasive organism and host cells. The immunological activation of NO accumulation by IgG derived from pSS patients may also lead to secretory dysfunction of the salivary and lacrimal glands. Fox et al. [23] has used a muscarinic agonist to treat SS, which may imply the antigenicity and pathology of the muscarinic receptor in primary SS.

In conclusion, we demonstrated the high prevalence of anti-c2M3RP antibodies in primary SS patients; the second extracellular loop of the M3R, based upon its immunogenic and functional properties, can be regarded as an immunodominant functional region on the muscarinic receptor, and may play a pathogenic role in pSS. Further studies are needed to determine autoantibodies against C2M3R underlying pSS pathogenesis.

### Rheumatology key messages

- Anti-c2M3RP antibody is highly specific for patients with pSS.
- Anti-c2M3RP seems to be related to RF, ANA and haematological abnormalities.
- The ESSDAI score is significantly higher in anti-c2M3RP-positive pSS patients.

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J.H. performed most of the experiments. Z.-G.L. conceived the study and participated in the design, in the interpretation of results. J.H. participated in drafting the article. All authors read and approved the final article.

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### Disclosure statement:

The authors have declared no conflicts of interest.

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