

The role of muscarinic acetylcholine receptor type 3 polypeptide (M3RP²⁰⁵⁻²²⁰) antibody in the saliva of patients with primary Sjögren's syndrome

J. He¹, L. Qiang², Y. Ding¹, P. Wei², Y.-N. Li¹, H. Hua², Z.-G. Li¹

¹Department of Rheumatology and Immunology, Peking University People's Hospital, Beijing,

²Department of Oral Medicine, Peking University School and Hospital of Stomatology, Beijing, People's Republic of China.

Abstract

Background

Sjögren's syndrome (SS) is a chronic autoimmune disorder of unknown cause. Recent studies have shown that anti-muscarinic acetylcholine type 3 receptor (M3R) antibodies can be detected in patients with Sjögren's syndrome (SS), but little is known about the diagnostic value of this antibody.

Objective

To assess the clinical correlations of anti-M3R (muscarinic acetylcholine receptor type 3) polypeptide (M3RP²⁰⁵⁻²²⁰) antibodies in saliva from patients of primary Sjögren's syndrome (pSS).

Methods

Serum samples and unstimulated mixed saliva from 100 patients with SS were collected and examined. Their mean (SD) age was 54.2 (13.4) years, and the mean (SD) disease duration was 6.2 (3.8) years. Serum samples from 40 patients with systemic lupus erythematosus (SLE), 40 with rheumatoid arthritis (RA), and 60 healthy subjects were analysed as controls.

All the patients with SS were carefully evaluated according to European and American criteria. A circular M3RP²⁰⁵⁻²²⁰ peptide sequence was synthesized using solid-phase techniques on an applied biosystems peptide synthesizer.

The correlation between anti-M3RP²⁰⁵⁻²²⁰ antibodies and clinical manifestations of pSS was analysed.

Results

The IgG of anti-M3RP²⁰⁵⁻²²⁰ antibodies was present in 69% of patients with pSS, 27.5% with SLE, 22.5% with RA, and 23.3% of normal saliva donors. The prevalence of anti-M3RP²⁰⁵⁻²²⁰ antibodies in pSS was significantly higher than in SLE, RA, and normal controls. The specificity of anti-M3RP²⁰⁵⁻²²⁰ antibodies in pSS was 75%. The salivary flow rate in the group positive for anti-M3RP²⁰⁵⁻²²⁰ was 436 μ l/10 min, compared to a rate of 658 μ l/10 min for the negative group ($p < 0.05$).

Conclusion

The anti-M3RP²⁰⁵⁻²²⁰ antibody was detected in most patients with pSS. The presence of the antibody was closely associated with the salivary flow rate. This indicated that it may act as an autoantigen, with a role in the pathogenesis of pSS.

Key words

primary Sjögren's syndrome, saliva, type 3 muscarinic acetylcholine receptor

Jing He, MD, PhD*
 Lu Qiang, MD*
 Yan Ding, PhD
 Pan Wei, MD
 Yingni Li, MD
 Hong Hua, MD, PhD
 Zhanguo Li, MD, PhD

*These authors made an equal contribution to this work.

Please address correspondence to:
 Dr Zhanguo Li,
 Department of Rheumatology and Immunology,
 Peking University People's Hospital,
 11 Xizhimen South St.,
 Beijing 100044, P.R. China.
 E-mail: zgli@yahoo.cn

or
 Dr Hong Hua,
 Department of Oral Medicine,
 Peking University,
 Beijing 100081, P.R. China.
 E-mail: honghua1968@yahoo.com

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Introduction

Sjögren's syndrome (SS) is a chronic systemic autoimmune disease, characterised by high lymphocytic infiltration of the exocrine glands and their subsequent destruction. The syndrome is prevalent in 0.5%~5% of the population in Europe and the United States (1), and in China it affects 0.33%~0.77% of the population (2). Antibodies directed against muscarinic receptors have been identified to be of diagnostic value for SS (3). In particular, the sensitivity and specificity of the anti-muscarinic acetylcholine receptor type 3 polypeptide (M3RP²⁰⁵⁻²²⁰) antibody has been identified as a promising candidate (3, 4, 7). Autoantibodies against these receptors can impair parasympathetic neurotransmission at the postsynaptic level by blocking acetylcholine signals and mediating parasympathetic dysfunction. The inhibition of these receptors ultimately leads to the loss of secretory function in primary Sjögren's syndrome (pSS) (5, 6).

In this study, we use a conserved motif of M3R, rather than the whole protein, as an antigenic peptide to define the role of circular anti-M3RP²⁰⁵⁻²²⁰ antibodies in SS. To our knowledge, this is the first study to evaluate the prevalence of anti-M3RP²⁰⁵⁻²²⁰ antibodies in salivary samples from a small sample of Chinese patients with SS.

Materials and methods

Patients and saliva samples

Saliva samples and serum samples were collected from 100 pSS patients, 80 other rheumatic diseases patients and 60 healthy adult controls. All patients were enrolled at the department of Rheumatology and Immunology, Peking University People's Hospital and the Department of Oral Medicine and Traditional Chinese Medicine, Peking University School and Hospital of Stomatology between July 2008 and March 2009. Sixty samples were collected from healthy adults as controls. One hundred pSS patients were diagnosed according to the revised international classification criteria for Sjögren's syndrome. They had a mean age of 54.23±13.44 (range: 34–72) years and an average disease duration of 6.2±3.8

years. All of these patients fulfilled four or more of the revised US-EURO classification criteria for SS (8). Forty rheumatoid arthritis (RA) patients and 40 systemic lupus erythematosus (SLE) patients fulfilled the classification criteria (9-10). The study was approved by the Medical Ethics Committee of the People's Hospital.

Peptide synthesis

The circular peptide of M3RP²⁰⁵⁻²²⁰ (CLFWQYFVGKRTVPPGEC), which corresponds to the second extracellular loop sequence of muscarinic receptors, was synthesized using solid-phase techniques on applied biosystems peptide synthesizer (APEX396, AAPP-PEC, Kentucky, USA) at SBS Gene Technology Company (Shanghai, China). The peptide was purified by reversed-phase high-pressure liquid chromatography to a purity that was greater than 95%.

Saliva collection and salivary flow rate measurement

To reduce the possible influence of seasonal and circadian variations on the salivary flow rate, sampling collection was performed under identical conditions between 9:00 and 11:00 a.m. In addition, saliva sampling was conducted by the same dentist. Two hours prior to sample collection, all patients and healthy controls were asked not to chew gum, eat or drink. Five minutes before sampling collection, people were left to relax with their heads bent slightly forwards. Timing was begun when the saliva was swallowed. After 10 min, collection volume was measured and the unstimulated salivary flow rate was expressed in ul/10 min. Antibody detection by enzyme-linked immunosorbent assay (ELISA) of salivary supernatants was performed after a 15 min centrifugation at 3,000 r/min.

Anti-M3RP²⁰⁵⁻²²⁰ ELISA

ELISA for M3RP²⁰⁵⁻²²⁰ was performed as follows. Flat-bottom 96-well microtitre plates (Costarvinyl, Cambridge, MA, USA) were coated overnight at 4°C with 10 µg/ml circular M3RP²⁰⁵⁻²²⁰ peptide in 0.1 M PBS (pH 9.6). Between each stage, the plates were

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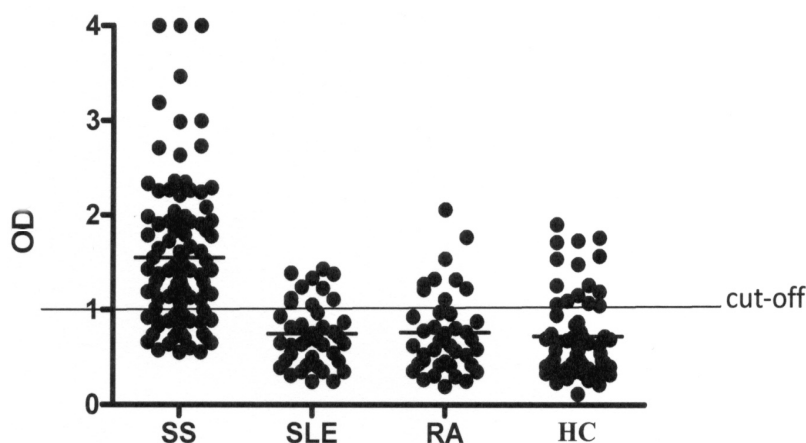


Fig. 1. OD values of anti-M3RP²⁰⁵⁻²²⁰ antibodies in the saliva of patients with pSS, other connective tissue diseases and healthy control donors. Saliva samples were collected from 100 patients with Sjögren's syndrome (SS), 40 patients with systemic lupus erythematosus (SLE), 40 patients with rheumatoid arthritis (RA), and 60 healthy controls (HC). The titer is expressed as the OD value. The cut-off level was defined by the 95th percentage of healthy sera (horizontal line).

Table I. Prevalence of anti-M3RP²⁰⁵⁻²²⁰ antibodies in patients with different rheumatic diseases and healthy controls.

	n. patients	n. anti-M3RP ²⁰⁵⁻²²⁰ antibody-positive patients (%)
pSS	100	69 (69.0*)
SLE	40	14 (27.5)
RA	40	9 (22.5)
Healthy controls	60	11 (23.3)

* $p < 0.05$ compared with other groups. pSS: primary Sjögren's syndrome; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis.

Table II. Clinical features of pSS patients with anti-M3RP antibody.

Groups	n.	Xerostomia (%)	Xerophthalmia (%)	Flow rate (SD) (μ l/10min)
Anti-M3RP (+)	69	66.7	33.3	436*
Anti-M3RP (-)	31	38.7	61.3	658
<i>p</i> -value		0.062	0.061	0.000*

* $p < 0.05$, compared with anti-M3RP negative group.

washed three times with 0.05% Tween 20 in PBS (Euroimmun, Lübeck, Germany). Each plate was blocked with 5% BSA for 2 h at room temperature. Saliva samples were diluted 1:10 in PBS and 100 μ l of the diluant was added to each ELISA well for 1 h at 37°C. Detection of IgG was done using goat anti-human IgG coupled to horseradish peroxidase (Zhongshan Technology Company, Beijing, China) diluted 1:2,000 in PBS and incubated for 0.5 h at 37°C. The reaction was developed with 100 μ l of O-phenylenediamine

(Zhongshan Technology Company, Beijing, China) for 5 min and then stopped with 100 μ l 2M H₂SO₄. Optical density (OD) was measured at 492 nm using an ELISA spectrophotometer (Bio-Rad Model 550; Microplate Reader, Hercules California, USA). Each saliva sample was assayed in triplicate. The results for each sample were expressed as the mean \pm standard deviation (SD) of triplicate values. An OD value was considered positive when it was greater than two SDs from the mean OD value of the control saliva.

Clinical and laboratory data of pSS patients

The IgG class autoantibodies against SSA and SSB were measured by blotting techniques. Antinuclear antibodies were measured by indirect immunofluorescence, according to the manufacturer's instructions (Euroimmun, Lübeck, Germany). Rheumatoid factors (RF) IgM in serum was measured by the nephelometry rate (IMMAGE; Beckman Coulter, Fullerton, CA), values of RF above 20 U/ml were considered positive.

Statistical analysis

Data analyses were performed using SPSS for Windows, version 13.0. For normally distributed data, the results were expressed as mean \pm SD, and differences between groups were analysed using a *t*-test. Data that lacked normal distribution were expressed as median (range), and differences were analysed with the Mann-Whitney U-test. For both tests, *p*-values less than 0.05 were considered statistically significant.

Results

Sensitivity and specificity of anti-M3RP²⁰⁵⁻²²⁰ antibodies in the saliva of SS patients

Anti-IgG-M3RP²⁰⁵⁻²²⁰ antibodies in saliva from patients and healthy adults were tested by indirect ELISA. A higher concentration of anti-M3RP²⁰⁵⁻²²⁰ antibodies IgG in saliva from pSS patients was detected when compared to other connective diseases and healthy controls (mean OD \pm SD, 1.55 \pm 0.07 vs. 0.750 \pm 0.054 in SLE, 0.764 \pm 0.068 in RA and 0.726 \pm 0.057 in healthy controls, $p < 0.05$) (Fig. 1). The prevalence of M3RP antibodies in pSS, SLE and RA was 69%, 27.5%, 22.5% and the specificity was 75%, 53.5%, 52.5%, respectively (Table I). Anti-M3RP²⁰⁵⁻²²⁰ antibodies in saliva with pSS were higher than that in other connective diseases ($p < 0.05$).

Assessment of the correlation between salivary flow rate and anti-M3RP²⁰⁵⁻²²⁰ antibodies

A significant difference was detected between the salivary flow rates of the positive anti-M3RP²⁰⁵⁻²²⁰ group and

the negative anti-M3RP²⁰⁵⁻²²⁰ group ($p<0.05$, Table II). In addition, no significant difference could be seen in the dry mouth ($p=0.062$) and dry eye groups ($p=0.061$).

Associations between anti-M3RP²⁰⁵⁻²²⁰ antibodies and other autoantibodies in SS

As shown in Table III, the prevalence of mixed saliva of anti-M3RP antibodies (69%) was similar to that of anti-SSA (74%) in sera, and higher than that of anti-SSB, anti-nuclear antibodies (ANA) in sera and saliva ($p<0.05$). A high prevalence of anti-M3RP²⁰⁵⁻²²⁰ antibodies was found in patients with SS who lacked anti-SSA, anti-SSB and ANA (Table IV).

Associations between anti-M3RP²⁰⁵⁻²²⁰ and clinical features in SS

A positive Schirmer test and corneal staining was significantly higher in pSS patients that had the anti-M3RP antibody ($p<0.05$) (Table V). However, there was no correlation between the presence of anti-M3RP and a positive tear film break-up test or parotid sialography examination ($p>0.05$).

Discussion

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterised by progressive lymphocytic infiltration, which results in the destruction of the lacrimal and salivary glands. Their destruction leads to a marked reduction in tear and saliva secretion. The duration of this disease, from onset to diagnosis, normally takes 9 years (11). The disease has a severe effect on the quality of life of patients, highlighting the importance of early diagnosis. The diagnostic testing of a saliva has come under recent intensive research due to its simplicity, repeatability and noninvasive nature. Testing specific immunological molecules has been suggested as a new diagnostic measure to screen early pSS. Anti-SSA/B antibodies can be detected in the saliva and parotid fluid of pSS patients, which have a parallel relationship with serum indicators (12). However, research on other relevant pSS autoantibodies in saliva has been limited.

Table III. Associations between anti-M3RP²⁰⁵⁻²²⁰ antibodies and other autoantibodies in pSS.

Antibodies	Sample	pSS (n=100)	
		n	%
anti-M3RP(+)	saliva	69	69.0
anti-M3RP(+)	sera	48	48.0
Anti-SSA(+)	saliva	28	28.0
Anti-SSA(+)	sera	74	74.0
Anti-SSB(+)	saliva	11	11.0
Anti-SSB(+)	sera	33	33.0
ANA(+)	saliva	22	22.0
ANA(+)	sera	58	58.0

Table IV. Distribution of anti-M3RP antibodies in pSS patients lacking other autoantibodies.

Group	n	Anti-M3RP positive	
		n	%
Anti-SSA (-)	26	17	65.4
Anti-SSB (-)	67	45	67.2
ANA (-)	82	54	65.9

Table V. Comparison of examinations in patients with and without anti-M3RP.

		n	Anti-M3RP (+)		Anti-M3RP (-)		p-value
			n	%	n	%	
Schirmer test	(+)	97	69	71.1	28	28.9	0.009*
	(-)	3	0	0.0	3	100.0	
Tear film break-up test	(+)	87	62	71.3	25	28.7	0.205
	(-)	13	7	53.8	6	46.2	
Corneal staining test	(+)	55	45	81.8	10	18.2	0.002*
	(-)	45	24	53.3	21	46.7	
Parotid sialography examination	(+)	54	39	72.2	15	27.8	0.563
	(-)	0	0	0.0	0	0.0	

* $p<0.05$, compared with anti-M3RP negative group.

Antibodies to the M3 subtype of muscarinic acetylcholine receptors (mAChRs) have recently been proven to be a good serum marker for the diagnosis of SS (13). The finding of muscarinic acetylcholine receptor type 3 (m3AChR)-specific autoantibodies in a majority of pSS patients is an important step towards understanding its pathogenesis. These antibodies reflect impaired glandular function and, in some patients, the display of features associated with autonomic dysfunction (7, 13, 14).

Muscarinic receptors of the M3 are present in many tissues, including smooth muscle, lacrimal and salivary glands. Recent research indicates that the anti-M3RP²⁰⁵⁻²²⁰ autoantibody reflects the onset of SS, due to the ability

of these antibodies to aggravate the hypofunction of the impaired gland. Anti-M3RP²⁰⁵⁻²²⁰ has consistently been identified as specific to SS diagnosis. This suggests that anti-M3RP²⁰⁵⁻²²⁰ should be a relatively unique autoantibody for SS, which could give it an irreplaceable role in the diagnosis of SS. However, it remains unclear whether anti-M3RP²⁰⁵⁻²²⁰ has a relationship with disease manifestation or severity. We investigated a large cohort of Chinese SS patients for the prevalence of the anti-M3RP²⁰⁵⁻²²⁰ antibody in saliva and serum by ELISA. We found that the occurrence of the anti-M3RP²⁰⁵⁻²²⁰ antibody was linked to the salivary flow rate.

To our knowledge, this is the first study to evaluate the prevalence of anti-M3RP antibodies, specific to the sec-

ond extracellular loop within M3RP, in the saliva of pSS patients. The results showed that the anti-M3RP antibodies could differentiate between the levels of M3RP in pSS patients when compared to RA and SLE patients. In addition, there is a significant difference between the salivary flow rate of the anti-M3RP²⁰⁵⁻²²⁰ antibody-positive and negative group. This result suggests that the M3RP²⁰⁵⁻²²⁰ antibody may be linked to the severity of salivary gland destruction.

In addition, we observed that patients with SS that lacked anti-SSA antibodies were anti-M3RP²⁰⁵⁻²²⁰ positive, whilst SS patients without the anti-SSB antibody had a higher prevalence of the anti-M3RP²⁰⁵⁻²²⁰ antibody. These results indicated that anti-M3RP²⁰⁵⁻²²⁰ antibody may be a helpful diagnostic tool for SS patients that lack anti-SSA or anti-SSB antibodies.

The use of synthetic peptide antigens in serum-based ELISA can give inconsistent results; a greater sensitivity and reproducibility can be achieved with the use of recombinant GST-fused peptide epitopes (7, 15). In our study, we used a circular peptide rather than the commonly used linear peptides. Circular peptides are more stable and reproducible in ELISA tests. Furthermore, the anti-M3R antibody was tested on saliva not sera. There are also some other studies shows that autoantibodies can be detected in tear fluid of pSS patients (16). This difference gave additional clinical information, especially for those which had serious symptoms and other autoantibodies in their sera.

It is known that mAChRs are, at least in part, responsible for mediating the parasympathetic stimulation of the secreted products by the exocrine glands (14), and could lead to SS-like autoimmune sialoadenitis in M3R^{-/-} mice (15). The binding of agonists to M3R initiates a sequence of events that culminates in the activation of different intracellular processes (3). The anti-M3R autoantibodies in the sera of pSS patients can not only interact with the acinar mAChRs of the lacrimal and salivary

glands but also stimulate biological effects by mimicking muscarinic cholinergic agonists (5, 6). The engagement of such receptors therefore modifies the intracellular events associated with specific cholinergic activation. Bacman *et al.* demonstrated that IgG derived from SS patients mimicked the behavior of carbachol in the stimulation of NOS activity, which occurs secondarily to an increase in the intracellular Ca²⁺ content by the activation of calcium/calmodulin-dependent NOS (3). 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 RI has used a muscarinic agonist to treat Sjögren's syndrome (13), which may imply the antigenicity and pathology of the muscarinic receptor in primary SS. In summary, testing the levels of salivary anti-M3RP²⁰⁵⁻²²⁰ antibody by ELISA is a potential non-invasive method for the diagnosis of pSS. It might also provide insights into the pathology of the disease, since anti-M3RP²⁰⁵⁻²²⁰ antibody levels are linked to salivary gland destruction. Therefore, further studies into the molecular mechanisms associated with the anti-M3RP²⁰⁵⁻²²⁰ antibody will provide further understanding of the pathogenesis of SS.

Authors' contributions

Jing He performed most of the experiments. Zhanguo Li and Hong Hua conceived the study, participated in its design and interpretation of results. Jing He participated in drafting the manuscript. All authors read and approved the final manuscript.

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