Published in final edited form as:

J Immunol Methods. 2016 October ; 437: 28–36. doi:10.1016/j.jim.2016.07.003.

Muscarinic type 3 receptor autoantibodies are associated with anti-SSA/Ro autoantibodies in Sjögren's syndrome

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Abstract

Anti-muscarinic type 3 receptor autoantibodies (anti-M3R) are reported as potential inhibitors of saliva secretion in Sjögren's syndrome (SjS). However, despite extensive efforts to establish an anti-M3R detection method, there is no clinical test available for these autoantibodies. The purpose of this study was to propose inclusion of anti-M3R testing for SjS diagnosis through investigation of their prevalence using a modified In-Cell Western (ICW) assay. A stable cell line expressing human M3R tagged with GFP (M3R-GFP) was established to screen unadsorbed and adsorbed plasma from primary SjS (n = 24), rheumatoid arthritis (RA, n = 18), systemic lupus erythematosus (SLE, n = 18), and healthy controls (HC, n = 23). Anti-M3R abundance was determined by screening for the intensity of human IgG interacting with M3R-GFP cells by ICW assay, as detected by an anti-human IgG IRDye800-conjugated secondary antibody and normalized to GFP. Method comparisons and receiver-operating-characteristic (ROC)-curve analyses were performed to evaluate the diagnostic value of our current approaches. Furthermore, clinical parameters of SjS were also analyzed in association with anti-M3R. Anti-M3R was significantly elevated in SjS plasma in comparison with HC, SLE, or RA ($P < 0.01$). SjS anti-M3R intensities were greater than two-standard deviations above the HC mean for both unadsorbed (16/24, 66.67%) and adsorbed (18/24, 75%) plasma samples. Furthermore, anti-M3R was associated with anti-SjS-related-antigen A/Ro positivity ($P = 0.0353$). Linear associations for anti-M3R intensity indicated positive associations with focus score ($R^2 = 0.7186$, $P < 0.01$) and negative associations with saliva flow rate ($R^2 = 0.3052$, $P < 0.05$). Our study strongly supports

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our rationale to propose inclusion of anti-M3R for further testing as a non-invasive serological marker for SjS diagnosis.

Keywords

Sjögren's syndrome; Autoantibodies; Muscarinic type 3 receptor; Sjögren's syndrome antigen A

1. Introduction

Sjögren's syndrome (SjS) is a systemic autoimmune disorder characterized by lymphocytic infiltration in the salivary and lacrimal glands, resulting in severe dry mouth or eyes (Fox, 2005). Extraglandular manifestations, such as fatigue, arthritis, renal and nervous system dysfunction, or Raynaud's phenomenon are common in SjS (Jonsson et al., 2011). SjS predominantly affects perimenopausal women with a female to male ratio of 9:1 (Mavragani and Moutsopoulos, 2010). SjS is the third most common rheumatic autoimmune disorder after rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), affecting approximately 4 million Americans (Fox et al., 2000). However, due to lack of universally accepted diagnostic criteria, previously estimated prevalence varies depending on criteria used (Patel and Shahane, 2014).

The 2002 modified European-American diagnostic criteria considered signs and symptoms of dryness as key elements of diagnosis and was most frequently used in clinical settings (Vitali et al., 2002b). In 2012, criteria proposed by Sjögren's International Collaborative Clinical Alliance (SICCA) focused mainly on objective tests (signs) (Shiboski et al., 2012). The SICCA proposed less emphasis on subjective feelings of dryness and distinction in classification between primary and secondary SjS (Shiboski et al., 2012). Main components of both diagnostic criteria include histological examinations of minor salivary glands for lymphocytic infiltration (focus score) and serology. However, presence or extensiveness of lymphocytic infiltration in salivary glands does not always indicate disease severity or degree of secretory dysfunction (Humphreys-Beher et al., 1999). Therefore, other potential factors contributing to SjS dryness are actively investigated. Interestingly, antibodies to muscarinic type 3 receptor (anti-M3R) have been proposed to contribute to secretory dysfunction in SjS (Cavill et al., 2004; Dawson et al., 2006a).

M3R is the primary receptor subtype to promote fluid secretion in salivary acinar cells (Matsui et al., 2000; Gautam et al., 2004). Previously, anti-M3R were identified in SjS mouse models and patients and indicated to have critical roles in secretory dysfunction and neurological disturbances in a subset of SjS patients (Gao et al., 2004; Li et al., 2004; Wang et al., 2004; Kovacs et al., 2005; Marczinovits et al., 2005; Cha et al., 2006; Dawson et al., 2006a; Scarselli et al., 2007; Koo et al., 2008; Tsuboi et al., 2010; He et al., 2011; Sumida et al., 2014). Furthermore, our recent study confirmed SjS anti-M3R inhibits water channel aquaporin-5 trafficking *in vitro*, thus potentially disturbing secretion in SjS (Lee et al., 2013). Serological markers used for SjS diagnosis include rheumatoid factor (RF), anti-nuclear antibody (ANA), anti-SjS-related antigen A(SSA)/Ro, and anti-SjS-related antigen B (SSB)/La autoantibodies (Vitali et al., 2002b; Delaleu et al., 2005; Fox, 2005; Shiboski et

al., 2012; Tincani et al., 2013). Of note, anti-SSA/Ro are positive in 60 to 80% and anti-SSB/La in 40 to 60% of SjS patients, also showing association with extraglandular manifestations in SjS (Garcia-Carrasco et al., 2002; ter Borg et al., 2011; Scofield et al., 2012). Current SjS diagnosis relies on anti-SSA/Ro and anti-SSB/La for their high prevalence in SjS although they are not exclusive to SjS.

Various methods have previously indicated the sensitivity and specificity of anti-M3R detection in SjS, such as radioisotope-labeled ligand binding (Bacman et al., 1996), Ca^{2+} fluorimetry (Koo et al., 2008), immunofluorescence staining (Lee et al., 2013), enzyme-linked immunosorbent assay (ELISA) (Cavill et al., 2002; Kovacs et al., 2005; Marczinovits et al., 2005; Nakamura et al., 2008; Tsuboi et al., 2010; He et al., 2011; Roescher et al., 2011), flow cytometry (Gao et al., 2004; Schegg et al., 2008), and surface plasmon resonance (Scarselli et al., 2007). However, many assays utilized synthetic linear peptides as antigens for detection, resulting in inconsistent data among studies (Cavill et al., 2002; Kovacs et al., 2005). The reported frequency of anti-M3R for ELISA ranged from 42% to 97% with increased frequency when peptides were affixed to maintain native M3R structures (Kovacs et al., 2005; Marczinovits et al., 2005; Nakamura et al., 2008; Tsuboi et al., 2010; He et al., 2011). However, some studies reported complete absence of anti-M3R in SjS or no difference between controls and SjS using ELISA (Cavill et al., 2002; Dawson et al., 2004; Zigon et al., 2005; Schegg et al., 2008; Roescher et al., 2011), suggesting linear peptides are unlikely to substitute for the native conformation of M3R in the detection of anti-M3R.

In our present study, to evaluate anti-M3R as a serological marker for SjS diagnosis, we determined prevalence and clinical significance of anti-M3R by developing a simple and high throughput In-Cell Western (ICW) assay. ICW is a cell-based immunofluorescence assay utilized to detect proteins in fixed culture cells, providing a sensitive and quantitative method for evaluating variations in protein expression in their native cellular environment. We utilized a cell line to stably express human M3R as a green fluorescent protein (GFP)-tagged recombinant protein. Adsorbed and unadsorbed blood plasma from primary SjS, SLE, RA, and healthy controls (HC) were screened for anti-M3R. Our approach provides qualitative and quantitative evaluation of anti-M3R from plasma samples. Moreover, anti-M3R was evaluated for associations with clinical parameters to determine if anti-M3R could substitute for any parameters of minor significance in current SjS diagnosis.

2. Materials and methods

2.1. Recombinant human M3R (rhM3R) expression

The 1.77 kb full-length recombinant hM3R DNA fragment was generated by PCR using human M3R cDNA with 3 $\times$ -hemagglutinin-tagged pcDNA3.1+ as the template (UMR cDNA Resource Center). Sequences of the forward and reverse primers were 5'-ATGACCTTGAC AATAACAGTAC-3' and 5'-CTACAAGGCCTGCTCGGGTG-3', respectively. PCR was performed for 30 cycles at 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 2 min and 30 s. The PCR product included the initiating ATG-codon and no intervening in-frame stop codons. The amplicon was confirmed by sequencing and cloned into *EcoRI* and *Apal* sites in the multiple cloning region at the N-terminus of the *Aequorea coerulescens*

AcGFP1 gene of the pAcGFP1-N1 GFP expression vector (Clontech Laboratories Inc.), generating the pAcGFP1-N1-rhM3R construct (referred to as M3R-GFP herein). Sequencing confirmed no mutations (reference sequence GenBank accession number NM000740).

2.2. Cell culture and stable M3R-GFP cell line generation

The human embryonic kidney (HEK) 293 cell line from the American Type Culture Collection was grown in Dulbecco's modified Eagle medium (DMEM) and L-glutamine with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and penicillin-streptomycin (Life Technologies, Inc.) in a 5% CO₂ incubator at 37 °C. Briefly, HEK293 cells stably expressing M3R-GFP were generated through transient transfection of M3R-GFP expression vector (2.5 µg) with Lipofectamine™ 2000 (Invitrogen), following the manufacturer's instructions. After 24 h incubation, cells were passed into selection medium (growth medium containing 1 mg/mL of geneticin) and incubated for 2 weeks. Monoclonal populations were created by limiting serial dilution and picked based on expression level of M3R-GFP observed under a microscope. HEK293 cells expressing M3R-GFP vector were maintained in growth media containing 0.5 mg/mL geneticin. Following expansion, one clone was selected based on highest level of M3R-GFP expression and gene stability was verified for at least 15 passages.

2.3. Plasma samples derived from patients and controls

Veinous blood samples (20 mL/participant) were collected in glass Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) from 23 HC, 24 SjS, 18 SLE, and 18 RA patients and processed within 30 min of collection. Peripheral blood treated with EDTA anticoagulant was utilized to allow for subsequent isolation of peripheral blood leukocytes. To isolate the plasma fraction, whole blood samples were centrifuged at 4000 ×g for 10 min at 4 °C and plasma supernatants collected and stored at -80 °C. Stored plasma samples were thawed once on ice and aliquoted for later evaluation. SjS diagnosis was based on 2002 modified European-American criteria (Vitali et al., 2002a). SLE or RA diagnosis was based on the American College of Rheumatology criteria (Hochberg, 1997; Aletaha et al., 2010). HC ages 18–65 years were pre-screened to exclude individuals with known autoimmune conditions or viral/bacterial conditions. Patients' and HC demographic, clinical and laboratory characteristics are summarized in Table 1. This study was approved by the University of Florida Institutional Review Board and a written permission was obtained from all who participated in the study by the involved collaborators at the University of Florida Rheumatology & Clinical Immunology clinic. De-identified samples were tested.

2.4. Western blotting

To confirm for M3R expression, cultured cells were lysed at three days post-transfection on ice for 15 min using RIPA (radio-immunoprecipitation assay) buffer containing 1% NP40, 1 mM EDTA, 10 mM Tris-hydrogen chloride (pH 7.5), 0.2% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablet (Roche Applied Science). Cell lysates were centrifuged at 15,000 rpm for 15 min at 4 °C. Aliquots of 20 µg

protein were mixed with loading buffer and separated on 10% SDS-polyacrylamide gels under reducing conditions, then transferred to polyvinylidene fluoride membranes. Matched blots were blocked for 1 h with 5% non-fat dry milk and incubated overnight at 4–8 °C with polyclonal rabbit anti-human M3R antibody (Santa Cruz Biotechnology, 1:500) or polyclonal anti-GFP antibody (Life Technologies, 1:500). Blots were washed and incubated for 1 h with horseradish-peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, 1:1000), followed by visualization with ECL detection system (Thermo Scientific).

2.5. Immunocytochemistry

M3R-GFP positive cells (4×10^4 cells/well) grown on 8-well chamber slides were fixed with 4% paraformaldehyde for 15 min at room temperature. Following three washes with phosphate-buffered saline (PBS) and blocking with 5% goat serum in PBS for 1.5 h, the cells were incubated overnight at 4–8 °C with polyclonal rabbit anti-M3R antibodies (Santa Cruz Biotechnology, 1:300 dilution in blocking solution) and 1 h at room temperature with goat anti-rabbit AlexaFluor568-conjugated antibody (Molecular Probes, 1:400 dilution) in darkness, respectively. After a final wash, images were obtained using a Zeiss Axiovert 200 M microscope equipped with a Zeiss AxioCam MRm camera and AxioVs40 software (Ver. 4.7.1.0, Zeiss). Frozen plasma aliquots from HCs or SjS were thawed on ice and centrifuged at $1250 \times g$ and were used as sources of primary antibody (1:400 dilution with 1% bovine serum albumin (BSA) in PBS) and detected with a goat anti-human IgG (H + L) AlexaFluor568-conjugated secondary antibody (Molecular Probes Life Technologies, 1:400). SjS and HC samples were blind scored by three researchers from duplicate experiments. M3R-GFP localization with anti-IgG was used as a positive indication of anti-M3R. Semi-quantification of staining was divided into four categories: strong positive, 3; positive, 2; slight positive, 1; and negative, 0. Evaluation of M3R staining pattern was based on the comparison of staining intensity to SjS-positive control SjS-16 as previously identified (Lee et al., 2013).

2.6. In-Cell Western assay

In-Cell Western (ICW) was performed with several modifications to the manufacturer's protocol (LI-COR Bioscience). Specifically, permeabilization steps were not included and GFP was used for M3R protein normalization instead of conventional DRAQ5 DNA staining or Sapphire700 cell labeling strategies for normalization in the ICW assay system. Briefly, M3R-GFP positive cells were seeded into 96-well plates (1.5×10^4 cells/well) and cultured until 85–90% confluent. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Following three washes with PBS, M3R-GFP cells were blocked with 5% goat serum in PBS for 1.5 h and incubated overnight at 4 °C with unadsorbed plasma samples (1:400 dilution with 1% BSA in PBS) from HC, SjS, SLE, or RA patients. Frozen plasma aliquots were thawed on ice and centrifuged at $1250 \times g$ prior to dilution. Adsorbed samples were generated following 2 h incubation of 1:400 diluted samples with 1% BSA in PBS at 37 °C with untransfected HEK293 cells prior to transfer to fixed M3R-GFP cells for overnight incubation. The M3R-GFP cells incubated with unadsorbed or adsorbed plasma were washed three times for 5 min each with 0.1% Tween-20 in PBS and incubated for 1 h at room temperature in the dark with goat anti-human IgG (H + L) IRDye800-labeled

secondary antibody (Rockland Immunochemicals, Inc.) diluted 1:1000 in 1% BSA PBS. Wells were washed three times with 0.1% Tween-20 in PBS for 5 min each prior to scanning. Plates were scanned on an Odyssey Reader (LI-COR Bioscience) at 800 nm to detect the integrated intensity (equivalent to total pixel intensity minus background) of bound human IgG and analyzed using Odyssey Software version 2.0. Expression levels of GFP were next determined by a fluorescence microplate reader (BioTeck, using a 485 nm/20 nm excitation filter and a 528 nm/20 nm emission filter). Relative fluorescence units of GFP were used to normalize the 800 channel of IRDye800 to correct for level of M3R-GFP in each well, instead of the company's standards of DRAQ5 or Sapphire700 cell density stains.

2.7. Statistical analyses

Statistical analyses were performed using the GraphPad Prism Software package version 5.0 (Graph Pad Software). *P*-values <0.05 were considered significant. Data presented as dot plots represent patient and control values obtained from three-independent experiments. One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-tests were used to compare between HC, SjS, SLE, and RA anti-M3R signal intensities. Unadsorbed and adsorbed ICW experiments were performed using separate passages of M3R-GFP cell line for each independent experiment. Comparison of unadsorbed and adsorbed plasma ICW anti-M3R signal intensities were performed using Deming regression analyses and Bland-Altman plot was utilized to determine bias. Diagnostic efficacies were examined by receiver-operating characteristic (ROC) curves from unadsorbed and adsorbed plasma samples. Optimum test cut-off values for anti-M3R intensity were selected based on maximum positive likelihood ratios (+LR) obtained from HC and SjS ROC curve analyses. The anti-M3R detection from adsorbed SjS patients' plasma were compared to laboratory detection of anti-SSA/ Ro, anti-SSB/La, ANA, focus score, and salivary flow rate using two-sided Fisher's exact test, linear regression, or one-phase decay analyses where appropriate.

3. Results

3.1. Establishment of M3R-GFP stable cell line

Human M3R cDNA was successfully amplified and inserted into the pAcGFP1-N1 vector. To verify stable expression of M3R-GFP, we performed western blotting for M3R and GFP protein expression in our stable cell line (M3R-GFP) and transiently-transfected vector control (pAcGFP1-N1). Endogenous M3R was detected as a monomer at ~65 kDa (Dawson et al., 2004; Dawson et al., 2005) in M3R-GFP transfected cells (left panel of Fig. 1A). GFP expression from the vector was detected at ~27 kDa, consistent with size information for pAcGFP1-N1 vector (Fig. 1A, right). The M3R-GFP protein migrated at ~92 kDa as expected. Concurrent detection of diffuse bands (~190–300 kDa) may correspond to dimerized or oligomerized M3R-GFP proteins, based on predicted size calculations (Fig. 1A, right), likely formed during the protein sample preparation.

Furthermore, GFP localization of M3R-GFP was compared to untransfected or pAcGFP1-N1 cells (Fig. 1B). Untransfected cells showed no GFP expression (Fig. 1Ba) and cells transfected with pAcGFP1-N1 showed dispersed GFP (Fig. 1Bb), whereas M3R-GFP cells showed cell surface GFP localization (Fig. 1Bc). In addition, M3R-GFP expressing cells

were examined for co-localization of GFP and M3R (Fig. 1C). Cells incubated with secondary antibody only were used as a negative control (Fig. 1Ca). M3R-GFP expressing cells showed strong M3R expression and GFP co-localization at the cell surface (Fig. 1Cb, arrowheads).

3.2. Anti-M3R detection with semi-quantitative immunostaining

M3R-GFP positive cells were incubated with either plasma from HC (n = 23) or SjS (n = 24), followed by goat anti-human IgG AlexaFluor568-conjugated secondary antibody. Co-localization of M3R-GFP and anti-human IgG was detected, as indicated by white arrowheads (Fig. 2A). Staining pattern was consistent with polyclonal rabbit anti-M3R primary antibodies as in Fig. 1Cb. Samples were scored semi-quantitatively and the grading score distributions for HC and SjS are shown in Fig. 2B. SjS patients showed 0% negative (n = 0), 8% slight positive (n = 2), 54% positive (n = 13), and 38% strong positive (n = 9), whereas HCs showed 17% negative (n = 4), 57% slight positive (n = 13), 26% positive (n = 6), and 0% strong positive (n = 0) (Fig. 2B).

3.3. Detection of anti-M3R by quantitative ICW assay

To verify anti-M3R levels in SjS samples quantitatively, plasma samples were tested by a modified ICW assay (Fig. 3). Of note, SjS patients visibly showed higher levels of anti-M3R intensity as compared to HCs for unadsorbed and adsorbed plasma experiments (Fig. 3A). Deming regression analyses for unadsorbed and adsorbed SjS samples (open squares, slope = 0.9448 ± 0.09467 , 95% CI = 0.7484 to 1.141) and HC samples (closed circles, slope = 0.4114 ± 0.08582 , 95% CI = 0.2329 to 0.5898) indicate positive association between unadsorbed and adsorbed anti-M3R signal intensities ($P < 0.0001$) (Fig. 3B, left). However, concurrent line comparisons indicated that the slope of HC anti-M3R signal intensity is significantly different from the SjS slope ($P = 0.01643$). The Bland-Altman difference plot for SjS unadsorbed and adsorbed plasma samples did not indicate significant systematic bias (Fig. 3B, right).

ICW anti-M3R signal intensities were evaluated by one-way ANOVA with Bonferroni post-tests to compare results from HC, SjS, SLE, and RA patient unadsorbed or adsorbed plasma. Our analyses indicate anti-M3R is elevated ($P < 0.01$) in SjS plasma samples in comparison to HC, SLE, or RA samples (Fig. 3C). Most SjS samples showed anti-M3R signal intensities greater than two standard deviations (SD) above the HC mean for both unadsorbed (dashed line, mean + 2SD = 6.24, 16/24 (66.67%)) and adsorbed (dashed line, mean + 2SD = 3.31, 18/24 (75%)) samples (Fig. 3C).

Furthermore, ROC curve analyses for anti-M3R were applied for unadsorbed and adsorbed blood plasma tests (Fig. 3D). P -values of comparisons were all considered statistically significant ($P < 0.05$, Fig. 3D). Area under the curve (AUC) was used to determine the efficiency of the ICW tests for detection of anti-M3R in SjS. The AUC was 0.8243 (unadsorbed) and 0.8913 (adsorbed) comparing SjS to HC (Fig. 3D). Additionally, AUC for comparing SLE to SjS was 0.7199 (unadsorbed) and 0.7384 (adsorbed) and AUC for comparing RA to SjS was 0.8032 (unadsorbed) and 0.8530 (adsorbed) (Fig. 3D). Optimum test cut-off values for anti-M3R were selected based on maximum positive likelihood ratios

(+LR) obtained from HC and SjS ROC curve analyses. Unadsorbed anti-M3R ICW test cutoff was 6.13 (+LR 15.33, sensitivity 66.67% and specificity 95.65%) whereas adsorbed cutoff was 3.21 (+LR 17.25, sensitivity 75% and specificity 95.65%). As expected, immunocytochemistry scoring of SjS plasma was positively associated ($P = 0.0005$, $R^2 = 0.4261$) with anti-M3R intensity observed from the ICW assay (data not shown). Comparisons were made with anti-M3R detection and patient clinical parameters to identify any associations.

3.4. Anti-M3R and associations with SjS clinical features

Frequency of anti-M3R was analyzed in comparison with clinical parameters used for SjS diagnosis (Table 2). Anti-M3R positivity from adsorbed samples was distributed in anti-SSA/Ro-positive (17/20, 85%), anti-SSB/La-positive (10/11, 90.9%), ANA >1:320 titer (11/12, 91.7%), and SjS patients with salivary flow less than or equal to 0.1 mL/min (6/6, 100%). Two-sided Fisher's exact tests were applied to evaluate between frequencies of biological factors listed in Table 2. Only anti-SSA/Ro was significantly associated with anti-M3R frequency ($P = 0.0353$). Of note, anti-M3R was detected in SjS patients with anti-SSA/Ro-negative (1/4, 25%), anti-SSB/La-negative (8/13, 61.5%), ANA <1:320 titer (5/8, 62.5%) and salivary flow rates >0.1 mL/min (6/11, 54.4%) (Table 2).

Furthermore, linear regression analyses were performed to evaluate associations of currently detected anti-M3R to previously determined focus score (Fig. 4A) and salivary flow rates (Fig. 4B). Anti-M3R from adsorbed plasma from SjS were significantly associated with focus score ($n = 10$, $R^2 = 0.5392$, slope = 0.7101 ± 0.2321 , intercept = 1.422 ± 1.348 , $P = 0.0156$) (Fig. 4A). With respect to saliva flow, anti-M3R was significantly associated with salivary flow rate by linear regression ($n = 17$, solid line, $R^2 = 0.3052$, slope = -0.01042 ± 0.00406 , intercept = 0.2299 ± 0.035 , $P < 0.0215$). Further model analysis indicated one phase exponential decay may better represent data distribution (dashed line, $R^2 = 0.6025$, $Y = 5.996$, Plateau = 0.1231, $K = 2.935$, $1/2$ life = 0.2362, Tau = 0.3408, Span = 5.873) (Fig. 4B).

In Fig. 4C and D, SjS patients were characterized based on the distribution of anti-SSA/Ro, -SSB/La, and anti-M3R positivity. Of the ten focus score positive SjS patients 70% (7/10) showed anti-SSA/Ro-positivity and of those patients 71.4% (5/7) were also anti-M3R positive (Fig. 4C). Notably, of the three focus score positive, but anti-SSA/Ro- and anti-SSB/La-negative SjS patients, one patient was anti-M3R positive (Fig. 4C). In addition, in fourteen SjS patients who did not undergo lip biopsy for diagnosis, 92.9% (13/14) were anti-SSA/Ro-positive and 85.7% (12/14) were anti-M3R positive (Fig. 4D).

4. Discussion

We established a stable cell line expressing M3R-GFP to be utilized in a modified ICW assay for detection of anti-M3R directly from patient plasma samples. Expression of native M3R in a cell line allowed detection of autoantibodies with conformation-dependent epitope(s). We hope our study provides a foundation for inclusion of anti-M3R as a serological marker, which can ultimately replace serologic parameters with minor significance or reduce requirement for an invasive lip biopsy for SjS diagnosis. Our results

indicate high prevalence of anti-M3R in SjS plasma, which distinguished SjS from HC, RA, or SLE samples. In addition, our study provides fresh insight into the utilization of anti-M3R detection in monitoring SjS disease with respect to focus score and salivary flow rate.

As previously mentioned, M3R controls fluid secretion in salivary acinar cells and presence of anti-M3R has critical roles in secretory dysfunction in subsets of SjS patients (Cavill et al., 2004; Kovacs et al., 2005; Marczinovits et al., 2005; Dawson et al., 2006b; Manoussakis et al., 2007; Daniels et al., 2011; Jin et al., 2012). Despite significant roles of anti-M3R, no test is currently accepted for clinical detection of anti-M3R. In our present study, we stably overexpress M3R-GFP in HEK293 cell line. Of note, HEK293 cells have low levels of endogenous M3R expression (Luo et al., 2008), which ensures proper protein post-translational modifications, folding, and localization of M3R-GFP in our ICW system. We confirmed expression of our recombinant M3R-GFP by western blotting and immunofluorescence (Fig. 1). As expected, only cells stably expressing M3R-GFP showed membrane localization of GFP. Furthermore, polyclonal antibodies for M3R showed extensive co-localization with GFP at the outer membranes, indicating intact M3R-GFP expression (Fig. 1).

Our M3R-GFP cell line was first tested using HC and primary SjS plasma by a conventional immunofluorescence assay for anti-M3R. Co-localization of GFP and anti-human IgG at the cell membrane was consistent with rabbit polyclonal anti-M3R antibodies staining pattern as in Fig. 1C (Fig. 2A). Semi-quantitative scoring of anti-M3R positivity indicated SjS patient plasma gave stronger staining for anti-M3R than HC samples (Fig. 2B). As the grading of positive signals could be subjective with this type of approach, it was imperative to develop a quantitative system for detection of anti-M3R. Therefore, in this study we modified a commercially developed ICW assay to utilize GFP for more accurate quantitation and standardization of anti-M3R detection.

To eliminate possible background reactivity of our plasma to cellular antigens other than M3R in ICW assays, we also adsorbed plasma by incubation with untransfected HEK293 cells prior to ICW. Anti-M3R was readily detected and visualized in the majority of SjS samples, but were mostly absent from HC samples for both unadsorbed and adsorbed samples (Fig. 3A). To evaluate the level of background reactivity in our assay, unadsorbed and adsorbed plasma preparation methods were directly compared by Deming regression analysis. Ideally, if unadsorbed and adsorbed plasma methods are equivalent then the slope of the regression line would be equal to 1. Our analysis shows SjS anti-M3R in unadsorbed plasma is significantly associated with adsorbed plasma anti-M3R intensity (Fig. 3B, left). Furthermore, SjS samples do not show significant bias between unadsorbed and adsorbed samples based on the Bland-Altman difference plot (Fig. 3B, right). However, HC plasma developed lower overall signal intensities and a comparison of the HC with SjS regression line indicated slopes were significantly different ($P = 0.01643$) (Fig. 3B). Taken together, our results indicate HC samples have some background in unadsorbed plasma whereas in SjS, unadsorbed and adsorbed plasma samples were highly comparable for anti-M3R detection. Therefore, adsorption of SjS plasma for anti-M3R detection may be unnecessary for clinical application.

Interestingly, SjS patient plasma showed significantly higher anti-M3R intensities in comparison to HC, SLE, or RA plasma from both unadsorbed and adsorbed plasma samples (Fig. 3C). Of note, unadsorbed (66.67%, 16/24) and adsorbed (75%, 18/24) SjS samples frequently had anti-M3R signal intensities greater than two standard deviations above the HC mean (Fig. 3C). Therefore, we concluded anti-M3R detection to be highly prevalent in SjS, but not in HCs or other related autoimmune diseases.

In addition, we utilized ROC curve analyses to evaluate the potential effectiveness of our modified ICW test in diagnosing SjS (Fig. 3D). Comparing SjS patients to controls, ICW was able to distinguish SjS ($P < 0.05$) (Fig. 3D). For our study, we selected test cut-off values for anti-M3R intensity based on the highest +LR from the HC and SjS sample comparisons, which corresponded closely to the HC mean + 2SD, and utilized these values for further analyses. Our ROC curve analyses also indicated lower AUC values in SLE vs SjS (Fig. 3D), which was not surprising since SLE frequently coincides with SjS, and can share many common serological features (Tincani et al., 2013). For instance, in our anti-M3R positive SLE patients by ICW, 66.7% (2/3, unadsorbed) and 83.3% (5/6, adsorbed) were either anti-SSA/Ro or anti-SSA/Ro/anti-SSB/La positive (data not shown). Therefore, further studies to follow-up SLE or RA patients with anti-M3R positivity would be useful to determine if they are more likely to develop SjS or have undiagnosed SjS.

SjS diagnosis relies heavily on the presence of anti-SSA/Ro or anti-SSB/La. We tested association of anti-M3R detection from adsorbed SjS patient plasma to other SjS diagnostic markers (Table 2). RF and focus score were not tested by Fisher's exact test due to limited data available for SjS patients with negative test results. Our analyses showed only anti-SSA/Ro was significantly associated with anti-M3R. Therefore, our results support a previous study showing anti-SSA was significantly associated with anti-M3R, but not anti-SSB or saliva flow rate (Tsuboi et al., 2010). Notably, a portion of SjS patients with negative anti-SSA/Ro, anti-SSB/La, or salivary flow rate tests were positive for anti-M3R (Table 2); suggesting some individuals may benefit from inclusion of anti-M3R testing for SjS diagnosis.

Focus score and salivary flow rate are also important indicators of immune activation and damage that may occur during SjS. Unstimulated whole salivary flow rates have been associated with focus score (Daniels et al., 2011), although presence or extensiveness of lymphocytic infiltration in salivary glands is not always indicative of disease severity or degree of secretory dysfunction (Daniels et al., 2011). We show focus scores are positively associated with anti-M3R signal intensities (Fig. 4A). Over-interpreting these data needs to be avoided, though, since this analysis was performed on a small number of samples, one of which appeared to largely determine the slope. Nonetheless, our results are supported by previous studies observing M3R-reactive T-cells in SjS patients and in mice following immunization with M3R (Naito et al., 2006; Iizuka et al., 2010), indicating local immune activation against autoantigens such as M3R in SjS. Furthermore, although SjS patient salivary flow rates were not associated with anti-M3R detection by Fisher's exact test (Table 2), regression analysis indicated a negative association with anti-M3R intensities (Fig. 4B, solid line). With respect to optimum line fit, one-phase exponential decay better describes the association of anti-M3R with salivary flow rate (Fig. 4B, dashed line). Therefore, our

results pose an interesting question of at what stage anti-M3R exerts a pathogenic effect on salivary secretion. Based on our findings, we speculate patients with relatively high salivary flow rate and relatively low anti-M3R intensity may reflect a stage in SjS progression where anti-M3R could be associated with antibody-mediated suppression. As SjS progresses, patients may develop increasing concentrations or affinity of anti-M3R, which may correspond with increased foci number, suppressed secretory function, and inflammatory damage to salivary glands.

To describe distributions of anti-M3R, anti-SSA/Ro, and anti-SSB/La in SjS, we subdivided our SjS cohort into patients who had undergone lip biopsy and those who had not required a lip biopsy. In the clinical setting, methods other than lip biopsy are preferred for reaching diagnosis or monitoring SjS progression due to fewer potential complications and feasibility. Of the ten SjS patients in our study who underwent lip biopsy, we detected anti-M3R in 55.5% (5/9), of whom 80% (4/5) were also anti-SSA/Ro positive (Fig. 4C). In addition, of the fourteen SjS patients who were not required to undergo lip biopsy 85.71% (12/14) were anti-M3R positive and anti-SSA/Ro positive (Fig. 4D). Therefore, we conclude both anti-SSA/Ro and anti-M3R reflect a critical distribution and are most likely to enhance noninvasive identification of individuals with SjS. In future multi-center cohort studies, it would be important to determine if inclusion of anti-M3R testing can help differentiate SjS from other autoimmune disorders, especially SLE. Furthermore, anti-M3R associated with secretory dysfunction or cognitive impairment in SjS patients would be important to investigate further. Currently, an independent cohort study is in progress to help determine if anti-M3R may replace any parameters with minor significance in current SjS diagnosis.

5. Conclusions

Based on our results, anti-M3R is detected by our modified ICW and may enhance the likelihood of diagnosing SjS patients when evaluated alongside anti-SSA/Ro. Although at this time we are unable to define population distributions of anti-M3R and anti-SSA/Ro among SjS, with inclusion of anti-M3R it is conceivable in the future to eliminate anti-SSB/La testing and drastically reduce requirements for lip biopsies. Our simple assay system enables inclusion of anti-M3R detection for SjS diagnosis, thus improving autoantibody-based diagnostics and expediting discovery of clinical implications of anti-M3R in SjS.

Acknowledgments

This work was supported by the grants National Institutes of Health/ NIDCR DE019644 (SC) & T90DE21990 (AEGW) and NIH/NCATS CTSA UL1 TR000064/TL1 TR000066 (AEGW). This work was also supported by a National Research Foundation of Korea Grant, through the Oral-Maxillofacial Dysfunction Research Center for the Elderly (No. 2014050477) at Seoul National University in Korea (KP). We acknowledge JinTeak Kwon for optimizing the experimental condition to detect the recombinant protein expression.

Abbreviations

M3R	muscarinic type 3 receptor
SjS	Sjögren's syndrome
ICW	In-Cell Western

GFP	green fluorescent protein
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
HC	healthy controls
SICCA	Sjögren's international collaborative clinical alliance
SSA	Sjögren's syndrome-related antigen A
SSB	Sjögren's syndrome-related antigen B
ROC	receiver-operating-characteristic
ANA	anti-nuclear antibody
RF	rheumatoid factor
CCP	cyclic citrullinated peptide
ELISA	enzyme-linked immunosorbent assay
HEK	human embryonic kidney
FBS	fetal bovine serum
DMEM	Dulbecco's modified Eagle medium
EDTA	ethylenediaminetetraacetic acid
RIPA	radio-immunoprecipitation assay
LR	likelihood ratio
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
BSA	bovine serum albumin
ANOVA	analysis of variance
IgG	immunoglobulin G
AUC	area under the curve
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
NA	not available

SD standard deviation

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Fig. 1.

Stable over-expression of muscarinic type 3 receptor-GFP in HEK293 cell line. (A) Western blots of protein lysates from the M3R-GFP stable HEK293 cell line (lane 1), transiently transfected pAcGFP1 vector (lane 2) and untransfected HEK293 (lane 3) probed for M3R, GFP, and GAPDH. Original molecular weight of M3R is ~65 kDa and GFP is ~27 kDa. The M3R-GFP fusion protein is represented by ~92 kDa band and diffuse band ~190–300 kDa. Theoretical sizes of homo- or hetero-oligomerized M3R-GFP are indicated by black bars. (B) HEK293 cells were untransfected (a), transiently transfected with the control pAcGFP1-N1-vector (b) or stably transfected with M3R-GFP1 (c). Representative images of GFP (green) fluorescence and phase light microscopy at 100× magnification. (C) HEK293 cell line expressing M3R-GFP were labeled with either anti-rabbit-Alexa647 secondary antibody alone (a) or polyclonal anti-M3R primary and anti-rabbit-Alexa647 secondary antibodies (b).

(b). Representative images (200× magnification) show distribution of M3R (red) immunoreactivity in HEK293 cells stably expressing M3R-GFP (green). White arrows indicate co-localized M3R-GFP and anti-hM3R at the cell membranes.

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Fig. 2.

Detection of anti-muscarinic type 3 receptor autoantibodies in primary Sjögren's syndrome patient (n = 24) and healthy control (n = 23) blood plasma. (A) Representative immunofluorescence images showing localization of anti-human IgG (red) from blood plasma on HEK293 cells expressing M3R-GFP (green) for grading anti-M3R detected from SjS (a–c) and HC (d–f) blood plasma samples (200× magnification). White arrows indicate colocalized M3R-GFP with anti-human IgG (yellow). Grading score of immunofluorescence of anti-human IgG staining was subjectively classified as strong positive (a), positive (b, d), slight positive (c, e), and negative (f). (B) Bar graphs represent percent distribution of number of participants with grading score (strong positive, black; positive, gray; slight positive, light gray; and negative, white) from duplicate experiments.

Fig. 3. Unadsorbed and adsorbed plasma anti-muscarinic type 3 receptor detection is specific for primary Sjögren's syndrome patients in comparison to healthy controls, systemic lupus erythematosus, and rheumatoid arthritis patients. (A) Representative ICW images indicate anti-M3R autoantibodies (white shading) from unadsorbed (Unad.) and adsorbed (Ads.) HC (n = 23) and primary SjS (n = 24) blood plasma. Wells 1–5 are individual HC samples and well 6 is the secondary antibody only negative control (2°Ab). Wells 7–11 are individual SjS samples and well 12 the positive SjS control sample (+Ctrl). (B) Deming regression analyses for HC and SjS (left) and Bland-Altman difference plot for unadsorbed and adsorbed blood SjS plasma sample anti-M3R intensities (right). (C) Dot plots showing anti-M3R intensities for unadsorbed and adsorbed HC, SjS, SLE, and RA samples. Horizontal line and error bars represent mean $\pm$ SEM. Horizontal dashed lines indicate HC mean + 2 standard deviations (SD). (D) Receiver-operating characteristic (ROC) curve analyses of unadsorbed and adsorbed blood plasma samples comparing HC, SLE, or RA patients to SjS patients. Area under the curve (AUC) and P -value of each analysis are reported.

Fig. 4.

Sjögren's syndrome anti-muscarinic type 3 receptor detection is associated with clinical parameters. Adsorbed primary SjS sample anti-M3R signal intensity plotted against focus score ($n = 10$), (A) or salivary flow ($n = 17$), (B) with linear regression (solid) and one phase decay (dotted) lines. Squares represent individual SjS patients. (C) Distribution of focus score positive (FS+) SjS patients ($n = 10$) or (D) patients without lip biopsies ($n = 14$). Anti-SSA/Ro+ (solid line, gray fill), anti-M3R+ (dashed line, light gray fill), anti-SSB/La+ (dotted line) positive and negative test results are indicated.

Table 1

Demographic and clinical characteristics of healthy controls, primary Sjögren's syndrome, systemic lupus erythematosus, and rheumatoid arthritis patients.

	Healthy controls (n = 23)	Patients with SjS (n = 24)	Patients with SLE (n = 18)	Patients with RA (n = 18)
Age, mean $\pm$ SD years	34.7 $\pm$ 10.4	53.5 $\pm$ 15.4	52.7 $\pm$ 15.3	53.9 $\pm$ 14.4
Sex, no. (%) female	20 (87.0)	23 (95.8)	17 (94.4)	14 (77.8)
Anti-SSA+, no. (%)	NA	20/24 (83.3)	7/18 (38.9)	0/5 (0.0)
Anti-SSB+, no. (%)	NA	11/24 (45.8)	4/18 (22.2)	0/5 (0.0)
RF+, no. (%)	NA	8/11 (72.7)	2/13 (15.4)	14/18 (77.8)
ANA+, no. (%)	NA	16/20 (80.0)	13/18 (72.2)	2/3 (66.7)
Anti-CCP+, no. (%)	NA	NA	NA	15/17 (88.2)
Rheumatoid nodules, no. (%)	NA	NA	NA	3/18 (16.7)
Erosive changes, no. (%)	NA	NA	NA	9/17 (52.9)
Focus score, no. (mean $\pm$ SD)	NA	10/10 (4.7 $\pm$ 3.7)	NA	NA
Salivary flow 0.1 mL/min, no. (%), mean $\pm$ SD mL/min)	NA	6/17 (35.3, 0.16 $\pm$ 0.10)	NA	NA
Renal disorder, no. (%)	NA	1/24 (4.2)	6/18 (33.3)	NA

NA: Data is not available in the UF Clinical Rheumatology database, SD: standard deviation, SSA: Sjögren's syndrome-related antigen A, SSB: Sjögren's syndrome-related antigen B, RF: rheumatoid factor, ANA: anti-nuclear antibody; CCP, cyclic citrullinated peptide.

Table 2

Comparison of laboratory features in adsorbed Sjögren's syndrome patients' plasma between those positive for anti-muscarinic type 3 receptor and those negative for anti-muscarinic type 3 receptor autoantibodies.

Laboratory parameters, no. (%)	Anti-M3R positive	Anti-M3R negative	P-value
Anti-M3R positive, 18/24 (75.0)			
Anti-M3R negative 6/24 (25.0)			
Anti-SSA/Ro+, 20/24 (83.3)	17/20 (85.0)	3/20 (15.0)	0.0353 *
Anti-SSA/Ro-, 4/24 (16.7)	1/4 (25.0)	3/4 (75.0)	
Anti-SSB/La+, 11/24 (45.8)	10/11 (90.9)	1/11 (9.1)	0.1660
Anti-SSB/La-, 13/24 (54.2)	8/13 (61.5)	5/13 (38.5)	
ANA >1:320, 12/20 (60.0)	11/12 (91.7)	1/12 (8.3)	0.2553
ANA <1:320, 8/20 (40.0)	5/8 (62.5)	3/8 (37.5)	
Salivary flow 0.1 mL/min, 6/17 (35.3)	6/6 (100)	0/6 (0)	0.1023
Salivary flow > 0.1 mL/min, 11/17 (64.7)	6/11 (54.5)	5/11 (45.5)	

M3R: muscarinic acetylcholine type 3 receptor, SSA: Sjögren's syndrome-related antigen A, SSB: Sjögren's syndrome-related antigen B.

* $P < 0.05$ Fisher's exact test.