Insights from the ganglionic acetylcholine receptor autoantibodies in patients with Sjögren’s syndrome

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Abstract

Objective: It is not known whether autonomic neuropathy is a feature of Sjögren’s syndrome (SS) or whether it is related to circulating antiganglionic acetylcholine receptor (gAChR) antibodies. The goal of the present study was to investigate the autonomic dysfunction in patients with SS and the associations between autonomic dysfunction, anti-gAChR antibodies, and clinical features of SS.

Methods: (1) The first observational study tested for the presence of gAChR antibodies in the serum samples from 39 patients with SS (absent information regarding autonomic symptoms) and healthy volunteers. (2) In the second study, serological and clinical data from 10 Japanese patients diagnosed with SS were reviewed. These patients showed autonomic dysfunction, and luciferase immunoprecipitation systems (LIPS) test was conducted to detect anti-α3 and anti-β4 gAChR antibodies. (3) In the final analysis, we combined the data of seropositive SS patients with autonomic symptom from the first study with all of the patients from the second study, and analyzed the clinical features.

Results: (1) The LIPS assay revealed that anti-gAChRα3 and anti-gAChRβ4 antibodies were detected in the sera from patients with SS (23.1%, 9/39). Five of nine SS patients had autonomic symptoms. (2) Anti-α3 and anti-β4 gAChR antibodies were also detected in 80.0% (8/10) of patients with SS with autonomic symptoms. Six of the ten patients were diagnosed as having SS after neurological symptoms developed. These seropositive patients had predominant and severe autonomic symptoms and were diagnosed with autonomic neuropathy. (3) Thirteen of fifteen SS patients with autonomic symptoms (86.7%) were seropositive for anti-gAChR antibodies, and we confirmed sicca complex, orthostatic hypotension, upper and lower gastrointestinal (GI) symptoms, and bladder dysfunction at high rates.

Conclusion: The present results suggest the possibility of anti-gAChR antibodies aiding the diagnostics of SS with autonomic dysfunction.

Keywords

Antiganglionic acetylcholine receptor antibodies, Autonomic dysfunction, Luciferase immunoprecipitation systems, Sjögren’s syndrome

Introduction

Sjögren’s syndrome (SS) is an autoimmune disease affecting both the exocrine glands and various nonexocrine organs, including the nervous system [1,2]. Numerous studies have demonstrated peripheral neuropathic symptoms that influence the autonomic nervous system in SS [3–8]. We previously reported that SS-associated neuropathy manifests as chronic autonomic neuropathy with anhidrosis [9]. The autonomic dysfunction observed in SS has been ascribed to various immunological factors, including antimuscarinic acetylcholine receptor M3 (M3R) antibodies, autoimmune ganglionitis, and cytokines interfering with neurotransmission [10,11].

To date, five muscarinic acetylcholine receptor (M1R–M5R) subtypes have been identified, of which M3R is expressed in the exocrine glands, playing a crucial role in exocrine secretion. However, it is not clear whether a specific pathogenic factor causes autonomic dysfunction in SS. Thus, further exploration of new causative agents (e.g., other autoantibodies) related to...
autonomic involvement in SS is needed. Kondo et al. [12] previously reported on two Japanese patients with SS, who also developed chronically progressive dysautonomia. They showed an elevated titer of ganglionic acetylcholine receptor (gAChR) antibodies, which improved with the oral intake of prednisolone. This case indicated that anti-gAChR antibodies are relevant to SS.

Thus, the detection of autoantibodies associated with the gAChR, which mediates fast synaptic transmission in all peripheral autonomic ganglia (sympathetic, parasympathetic, and enteric ganglia), shows promise in the search for SS biomarkers. The gAChRs in autonomic neurons are typically composed of two α3 subunits and three additional AChR subunits [13,14]. Interestingly, gAChR antibodies are found in the serum of 50% of patients with autoimmune autonomic ganglionopathy (AAG) and have been shown to be pathogenic and correlated to disease severity [15]. AAG is an acquired immune-mediated disorder presented with various autonomic symptoms, such as orthostatic hypotension and gastrointestinal (GI) symptom. Therefore, in the current study, we used a simple in vitro technique—luciferase-reporter immunoprecipitation systems (LIPS)—to detect antibodies that specifically bind to the α3 or β4 gAChR subunits with high sensitivity. We recently reported that gAChR autoantibodies could be detected in about 48% of patients with AAG [16]. Thus, to accomplish the present detection procedure, 10 Japanese patients who had been diagnosed with primary SS (pSS) and secondary SS (sSS) with dysautonomia were reviewed. Assessments included patient histories and current clinical and laboratory evaluations. The LIPS assay was then used to identify gAChR antibodies to demonstrate the relationships between the SS clinical features, specifically between autonomic involvement and the presence of autoantibodies.

Methods

Ethics approval

All subjects gave written, informed consent prior to participating in the present study. The Ethics Committee at the Nagasaki Kawatana Medical Center and Nagasaki University Graduate School of Biomedical Sciences (Nagasaki, Japan) approved the present protocol.

Study design and participants

The first observational study (Figure 1) tested for the presence of gAChR antibodies in serum samples from 39 patients with SS at the Department of Immunology and Rheumatology, Nagasaki University Graduate School of Biomedical Sciences, between 2005 and 2010 (mean age ± standard error = 58.4 ± 11.8 years old; 2 men and 37 women). We selected patients based on the diagnostic criteria proposed by the American European Consensus Group (AECG) and/or the Japanese Ministry of Health criteria for SS diagnostics (JPN) [17,18]. AECG criteria excluded patients with the following medical histories and concomitant diseases: past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency syndrome, pre-existing lymphoma, sarcoidosis, graft-versus-host disease, and current use of anticholinergic drugs. Patients with SS were classified into pSS (n = 31) and sSS (n = 8). No information regarding the existence or nonexistence of autonomic dysfunction was available for these samples. We also tested sera from 39 healthy volunteers (mean ages = 46.0 ± 6.8 years old; 2 men and 37 women).

The second study (Figure 1) included the collection of serum samples from 10 patients with SS who presented with autonomic dysfunction (mean age = 52.6 ± 21.7 years old; 2 men and 8 women). These patients were referred from various general and teaching hospitals throughout Japan between March 2012 and March 2014. These participants were also selected based on the AECG criteria and/or JPN criteria. Eight cases were pSS, and two cases were sSS. Clinical diagnosis and neurological findings (including autonomic symptoms) were defined at each hospital. The control groups included 73 healthy adults (healthy controls, HCs: mean age = 38.3 ± 11.1 years old; 31 men and 42 women) and 34 adults with other neurological diseases (ONDs) that included any autonomic symptoms (mean age = 56.3 ± 20.4 years old; 19 men and 15 women).

Figure 1. Study design and participant information. Details regarding study design and participant recruitment for each subject group. SS: Sjögren’s syndrome.
And finally, we combined the data of seropositive SS patients with autonomic symptom from the first observational study with all of the patients from the second study, and analyzed the clinical features (Figure 1).

**LIPS assay for autoantibodies to gAChR in the first and second experiment**

Serum gAChR antibodies were detected using the LIPS assay, as described elsewhere [16]. To generate luciferase reporters for the α3 and β4 subunits (termed gAChRα3-GL and gAChRβ4-GL, respectively) of the human gAChR, full-length human AChR α3 (P32297, Promega Corporation, Madison, WI) or β4 (P30296, Promega Corporation, Madison, WI) was fused to a Guassia luciferase (GL) mutant (GL<sup>990</sup>). Human embryonic kidney 293F cells (Life Technologies Corporation, Grand Island, NY) were then transfected with the expression plasmid encoding either gAChRα3-GL or gAChRβ4-GL with FuGENE6 (Promega Corporation, Madison, WI). Two days later, the transfected cells were solubilized with a Tris-based saline containing 1% Triton<sup>TM</sup> X-100. To detect α3 or β4 gAChR antibodies, 100 µL of the soluble fraction, containing gAChR α3-GL or gAChR β4-GL, was incubated with 15 µL of human serum for 1 h at 4°C. Subsequently, the fraction was mixed with 15 µL of protein G-sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, UK), 600 µL phosphate-buffered saline (PBS) with 3% bovine serum albumin, and 0.05% Tween<sup>®</sup> 20, and incubated for several hours at 4°C. Following centrifugation and two washes with PBS containing 0.05% Tween<sup>®</sup> 20, bioluminescence activities of the luciferase reporters in protein G-sepharose were measured with a BioLux<sup>TM</sup> GL assay kit (New England Biolabs, Ipswich, MA) and a Lumat LB 9507 luminometer (BERTHOLD TECHNOLOGIES GmbH & Co. KG, Bad Wildbad, Germany); luminometer output was measured in relative luminescence units (RLUs). To confirm LIPS assay accuracy for the gAChR antibodies, we used commercially available α3 and β4 antibodies (H-100 and S-15; Santa Cruz Biotecology, Inc., Dallas, TX) as positive controls. Based on anti-gAChRα3 and β4 antibody data from the 73 HCs, cut-off values were calculated as the mean plus 3 standard deviations (SDs) from the mean. In this study, antibody levels were expressed as an antibody index (AI) that was calculated as follows:

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AI = \frac{\text{measurement value of the sample serum (RLU)}}{[\text{the cut-off value (RLU)}]}
\]

The normal value that was established in this study from healthy individuals was <1.0 AI.

To evaluate the assay’s diagnostic accuracy, we verified the cut-off points for all data collected in the previous study. Cut-off points calculated sensitivity and specificity, and ROC curves were obtained. According to the ROC curves, we confirmed the most discriminative cut-off points. At these points, the sensitivity, specificity, and positive and negative predictive values (PPV and NPV) were calculated. The AUC was 0.849 (95% confidence interval [CI]: 0.786–0.911) for the LIPS assay of the anti-gAChRα3 antibody. With an anti-gAChRα3 antibody cut-off point of 1.0, the sensitivity and specificity were 46.9% (95% CI: 33.7–60.6%) and 99.2% (95% CI: 94.8–100.0%), respectively, and the PPV and NPV were 95.8% and 81.8%, respectively. The AUC was 0.720 (95% CI: 0.632–0.807) for the LIPS assay of the anti-gAChRβ4 antibody. With the anti-gAChRβ4 antibody cut-off point of 1.0, the sensitivity and specificity were 14.3% (95% CI: 6.9–27.1%) and 100.0% (95% CI: 96.2–100.0%), respectively. The PPV and NPV were 100.0% and 74.4%, respectively.

**Assessment of clinical SS features**

Demographic, clinical, and laboratory characteristics were collected for all participants in the first study. Two of the authors (A.M. and H.N.) retrospectively obtained medical histories, examination findings, and laboratory data (results from Schirmer test, lip biopsy, Saxon test, sialography, salivary gland scintigraphy, and serological testing) via medical record reviews of identified seropositive patients. SS information was obtained from 10 patients in the second study. However, the ability to collect these data was limited since the attending or assessing physicians for these patients were not rheumatologists; thus, evaluations of the SS features were incomplete.

**Assessment of autonomic function**

Comprehensive clinical, neurological, hematologic, biochemcial, serologic, cerebrospinal fluid (CSF), and physiological assessments of all patients at baseline were reviewed at each hospital in the second study. For the assessment of autonomic nerve dysfunction, we evaluated the sicca complex; syncope or orthostatic hypotension for orthostatic intolerance; upper and lower GI symptoms, including diarrhea and constipation; dysuria or urinary retention needing catheterization for bladder dysfunction; dryness of the skin or hypohidrosis and anhidrosis for heat intolerance; pupil abnormalities; sexual dysfunction; intercurrent illness; and complications. Autonomic symptoms were generally assessed by examining or interviewing patients or their family members or reviewing clinical records. Each patient underwent autonomic testing, which involved the Schellong test, head-up tilt test, measurement of the coefficient of variation in R–R intervals (CV R–R), pupillary response to local instillation, and [123I] and metaiodobenzylguanidine ([123I-MIBG) myocardial scintigraphy. The physiologic noradrenaline analog, [123I-MIBG, traces the uptake and transport of noradrenaline in the noradrenaline presynaptic sympathetic nerve terminals and its subsequent vesicular storage. The postganglionic presynaptic cardiac sympathetic nerve endings can be noninvasively assessed using MIBG scintigraphy because a reduction in the cardiac MIBG uptake (H/M ratio) indicates postganglionic sympathetic dysfunction. Cardiac MIBG uptake is reduced in patients with Lewy body diseases, such as Parkinson’s disease, as well as dementia with Lewy bodies. In the standard procedure, the H/M ratio is calculated at 30 min (early scan) and 4 h (delayed scan) to assess the anterior chest planar images by drawing a region of interest that includes the heart (H) and the upper mediastinum (M) [19]. However, we were unable to unify the items between the autonomic testing facilities among the different hospitals.

**Statistical analyses**

Commercially available statistics software was used for data analyses (SigmaPlot<sup>®</sup>). A normally distributed AI was analyzed via a one-way analysis of variance (ANOVA) for controls and patients with SS. For autoantibody frequencies and data that were not normally distributed, a one-way ANOVA assessing ranks was employed. Data were considered statistically significant at \( p<0.05 \).

**Results**

**Frequency of gAChR antibodies in patients with SS**

In the first observational study, the LIPS assay revealed that anti-gAChRα3 and anti-gAChRβ4 antibodies were absent in all 39 HCs. In contrast, 23.1% (9 of 39) of the serum samples from patients with SS (with a lack of information regarding autonomic symptoms) were positive for autoantibodies. More specifically,
the anti-gAChR\(\alpha3\) antibodies were detected only in eight samples, while the anti-gAChR\(\beta4\) antibodies were detected in only one sample. Further, no serum samples were positive for both antibodies in the healthy volunteers. We subsequently checked the clinical records of these nine SS patients of seropositive for the anti-gAChR antibodies and confirmed the salient autonomic symptoms (except for sicca and sensory disturbance) in five of nine patients.

In the second study, the LIPS assay demonstrated that the anti-gAChR\(\alpha3\) and anti-gAChR\(\beta4\) antibodies were absent in all 73 HCs. The anti-gAChR\(\alpha3\) antibodies were detected in the serum of one patient with suspected amyloid neuropathy within the OND group. Surprisingly, 80.0% (8 of 10) of the serum samples from patients with autonomic-symptom SS were positive for auto-antibodies \((p < 0.001, \text{Figure 2a})\). The anti-gAChR\(\alpha3\) antibodies were detected in eight samples, while the anti-gAChR\(\beta4\) antibodies were not detected in any sample. Mean anti-gAChR\(\alpha3\) antibodies in HCs and ONDs were 0.305 AI and 0.336 AI, respectively. These levels were significantly lower than the anti-gAChR\(\alpha3\) antibody levels in the SS samples, with a mean level of 1.030 AI \((p < 0.001, \text{Figure 2a})\). The mean anti-gAChR\(\beta4\) levels in HCs and ONDs were 0.367 AI and 0.302 AI, respectively, which were significantly lower than those in the SS samples \((0.526 \text{ AI, } p = 0.001, \text{Figure 2b})\).

**Clinical profiles among patients with SS that were positive for the anti-gAChR antibody**

Clinical characteristics of nine patients with SS that were positive for anti-gAChR antibody from the first study are presented in Table 1 and Supplementary Table 1. Eight of nine patients were women over the age of 50. This group included four pSS and five sSS cases. All participants fulfilled the AECG classification criteria, except for patient 7, who lacked sicca symptoms. However, patient 7 satisfied the Japanese Ministry of Health criteria for an SS diagnosis. Patients 1, 2, and 3 showed only sicca symptoms, and the other six patients were recorded as having other autonomic and neurological symptoms, including orthostatic hypotension and intolerance (patient 8), upper and lower GI symptoms (patients 4, 6, 7, and 9), bladder dysfunction (patients 6, 8, and 9), coughing episodes (patient 8), sweating disorder (patient 4), and sensory disturbances (patients 4, 5, and 6). Patients 4, 6, 8, and 9 had several other autonomic symptoms, and patients 6, 8, and 9 had underlying rheumatic disease. Although the frequencies of anti-SS-A and/or SS-B antibodies were relatively low \((4/9, 44.4\%)\), the presence of anti-centromere antibody (ACA) was confirmed in patients 3 and 5. Patient 4 had pSS and was the only patient who had only anti-gAChR\(\beta4\) antibodies. Except for patient 4, all patients had the anti-gAChR\(\alpha3\) antibodies, but no correlation was found with histopathological grading (determined by Chisholm and Mason [20,21]) or between the number of complicating autonomic symptoms and antibody level.

All 10 patients fulfilled the diagnostic criteria for SS in the second study (Table 2 and Supplementary Table 2). Patients 1–8 were seropositive for anti-gAChR\(\alpha3\) antibodies, and patients 9–10 were seronegative. A high percentage of female patients was a distinct trend in this SS sample. Half of the patients (patients 3, 5, 6, 8, and 9) were diagnosed with SS after neurological symptoms developed, while the remaining half (patients 1, 2, 4, 7, and 10) were diagnosed with SS before neurological symptoms appeared. Four cases demonstrated acute onset. Two cases with acute onset and one case with chronic onset were accompanied by antecedent illness (30.0%). All patients had sicca symptoms, except for patient 10. These patients had predominant and severe autonomic symptoms and were diagnosed with autonomic neuropathy. In 9 of 10 patients \((90.0\%)\), orthostatic hypotension and upper and lower GI symptoms were confirmed. Bladder dysfunction, hypohidrosis or anhidrosis, and pupil abnormalities were also present \((60.0\%, 50.0\%, 40.0\%, \text{respectively})\). Syndrome of inappropriate antidiuretic hormone secretion (SIADH) was observed in two patients (patients 1 and 7), and, interestingly, sensory disturbances were recorded in all eight seropositive patients. From the physiological and laboratory data (Table 3), CV R–R was decreased in six of seven patients examined \((85.7\%)\). Cardiac \(^{123}\text{I-MIBG}\) uptake was reduced in five patients \((100.0\%)\). Albuminocytologic dissociation in the CSF was confirmed in four of six patients \((66.7\%)\). We
Table 1. Anti-gAChR-antibody-positive patients in 39 cases of Sjögren’s syndrome (SS).

| Patient | Age (y) | Sex | Type of SS | Diagnostic criteria | Sicca | OH | OI | LGI | Duration of AS | Antecedent infection* | Bladder dysfunction | Coughing episodes | Sweating disorder | Sensory disturbance | Underlying rheumatic disease | Complication | Anti-gAChR Abs (AI) | Anti-gAChR Abs (AI) |
|---------|---------|-----|------------|--------------------|------|----|----|-----|----------------|---------------------|---------------------|-----------------|----------------|---------------------|----------------|-----------------|-----------------|
| 1       | 55      | F   | S          | AECG JPN           | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 2       | 65      | F   | P          | AECG JPN           | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 3       | 51      | F   | P          | AECG JPN           | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 4       | 37      | F   | P          | AECG JPN           | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 5       | 73      | F   | S          | AECG               | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 6       | 74      | F   | S          | AECG JPN           | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 7       | 50      | F   | P          | JPN                | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 8       | 63      | M   | S          | AECG JPN           | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |
| 9       | 65      | F   | S          | AECG JPN           | +    |    |    |     |                |                     |                     |                 |                 |                     |                |                 |                 |

*Patients 3, 8* 1, 2, 4, 5, 6, 7 = xerophthalmia and xerostomia

†Patients 4, 6 = constipation; 7 = anorexia, nausea and vomiting; 9 = anorexia, constipation

There were no cases with pupil abnormality and sexual dysfunction.

anti-gAChR, Abs = antiganglionic acetylcholine receptor antibodies, AI = antibody index, F = female, IgG4RD = IgG4-related disease, LGI = lower gastrointestinal symptom, M = male, MCTD = mixed connective tissue disease, MS = multiple sclerosis, OH = orthostatic hypotension, OI = orthostatic intolerance, P = primary, RA = rheumatoid arthritis, S = secondary, SS = Sjögren’s syndrome, SSc = systemic sclerosis, UGI = upper gastrointestinal symptom.

Table 2. Clinical and autonomic features of Sjögren’s syndrome (SS) patients with autonomic symptoms.

| Patient | Age (y) | Sex | Onset Age | Type of SS | Onset | Diagnostic criteria | Duration of SS | Antecedent infection* | Sicca† | OH | OI | LGI | Bladder dysfunction | Coughing episodes | Sweating disorder | Pupil abnormality* | Sexual dysfunction | Endocrine disorder‡ | Sensory disturbance|| Complication |
|---------|---------|-----|-----------|------------|-------|--------------------|---------------|----------------------|--------|----|----|-----|-----------------|-----------------|-----------------|-----------------|----------------|-----------------|-----------------|----------------|
| 1       | 45      | F   | 45        | P          | AECG   | Acute             | 0             | +                    | +      | +  | +  |     | +               | +               | +               | +               | +              | +               |                 | SLE            |
| 2       | 37      | F   | 37        | P          | AECD   | Acute             | 0             | +                    | +      | +  | +  |     | +               | +               | +               | +               | +              | +               |                 | RA             |
| 3       | 37      | F   | 37        | S          | AECG   | Acute             | 0             | +                    | +      | +  | +  |     | +               | +               | +               | +               | +              | +               |                 |                 |
| 4       | 68      | F   | 66        | S          | AECG   | Chronic           | 2             | –                    | +      | +  | +  |     | +               | +               | +               | –               | –              | –               |                 |                 |
| 5       | 75      | F   | 72        | P          | JPN    | Chronic           | 3             | –                    | +      | –  | –  |     | –               | –               | –               | +               | +              | +               |                 |                 |
| 6       | 8       | F   | 7         | P          | AECG   | Acute             | 1             | +                    | +      | +  | +  |     | –               | –               | –               | –               | –              | –               |                 |                 |
| 7       | 75      | M   | 68        | P          | JPN    | Chronic           | 7             | –                    | +      | –  | –  |     | –               | +               | –               | –               | +              | +               |                 |                 |
| 8       | 71      | F   | 71        | P          | AECG   | Acute             | 0             | –                    | +      | +  | +  |     | +               | +               | +               | –               | +              | +               |                 |                 |
| 9       | 63      | F   | 41        | P          | AECG   | Chronic           | 22            | –                    | +      | +  | +  |     | +               | +               | +               | –               | –              | –               |                 | KG, CIDP       |
| 10      | 47      | M   | 43        | P          | JPN    | Chronic           | 4             | –                    | +      | –  | –  |     | –               | +               | –               | –               | +              | +               |                 |                 |

*Patient 1 = fever, arthralgia, headache, epigastralgia, erythema; 2 = fever; 6 = infectious colitis
†Patients 6, 7 = xerophthalmia; 5 = xerostomia; 1, 2, 3, 4, 8, 9 = xerophthalmia and xerostomia
‡Patients 1 = diarrrhea and abdominal pain; 2 = nausea and vomiting; 3 = nausea, vomiting, constipation, abdominal pain; 4 = anorexia and constipation; 5, 10 = constipation; 6 = abdominal pain; 9 = anorexia, early satiety, constipation; 10 = anorexia, nausea, vomiting, abdominal pain, constipation, diarrhea
*Patient 1 = light reflex was absent; 3, 5, 6 = light reflex was sluggish; 9 = photophobia
†Patients 1, 7 = SIADH was suspected; 2 = hyperthyroidism
‡Patients 1, 2 = numbness, pain, nerve conduction abnormalities; 3, 6 = numbness, pain; 5 = numbness
AECG = the diagnostic criteria proposed by American European Consensus Group, AS = autonomic symptoms, CIDP = chronic inflammatory demyelinating polyneuropathy, JPN = the Japanese Ministry of Health criteria for the diagnosis of SS, LGI = lower gastrointestinal tract symptoms, MG = myasthenia gravis, OH = orthostatic hypotension, OI = orthostatic intolerance, RA = rheumatoid arthritis, SIADH = syndrome of inappropriate antidiuretic hormone secretion, SLE = systemic lupus erythematosus, TPO = thyroid peroxidase, UGI = upper gastrointestinal tract symptoms
concluded that there was no correlation between the severity of autonomic dysfunction in SS or findings from the laboratory data and anti-gAChR antibody levels (Figure 3 and Table 3).

Table 3. Autonomic function tests at baseline of Sjögren’s syndrome (SS) with autonomic symptoms.

| Patient | Age (years) | Sex (female/male) | Anti-gAChR α3 Abs (AI) | Decreased CV R–R | Decreased H/M ratio | Abnormality of pupillary response | Albuminocytologic dissociation in CSF |
|---------|-------------|-------------------|------------------------|-------------------|---------------------|----------------------------------|----------------------------------------|
| 1       | 45          | F                 | 1.033 (+)              | +                 | +                   | +                                | +                                       |
| 2       | 37          | F                 | 1.307 (+)              | +                 | +                   | +                                | +                                       |
| 3       | 37          | F                 | 1.054 (+)              | +                 | +                   | +                                | +                                       |
| 4       | 68          | F                 | 1.476 (+)              | +                 | +                   | +                                | +                                       |
| 5       | 75          | F                 | 1.313 (+)              | +                 | +                   | +                                | +                                       |
| 6       | 8           | F                 | 1.354 (+)              | –                 | –                   | –                                | –                                       |
| 7       | 75          | M                 | 1.053 (+)              | +                 | –                   | –                                | –                                       |
| 8       | 71          | F                 | 1.023 (+)              | +                 | –                   | –                                | –                                       |
| 9       | 63          | F                 | 0.352 (–)              | –                 | –                   | –                                | –                                       |
| 10      | 47          | M                 | 0.331 (–)              | –                 | –                   | –                                | –                                       |

AI = antibody index; anti-gAChRα3 Ab = ganglionic acetylcholine receptor α3 antibody; anti-gAChRβ4 Ab = ganglionic acetylcholine receptor β4 antibody; CSF = cerebrospinal fluid; CV R–R = coefficient of variation R–R interval; H/M ratio = heart-to-mediastinum ratio in 123I-MIBG myocardial scintigraphy

Figure 3. LIPS test for anti-gAChRα3 and β4 antibodies in the sera of patients with Sjögren’s syndrome (second study). SS: Sjögren’s syndrome; OND: other neurological diseases; HC: healthy control. Sera from patients with SS, patients with OND, and HCs were tested. Using the LIPS test, we determined that 80.0% (8 of 10) of the serum samples from patients with SS were positive for anti-gAChR. Further, the anti-gAChRα3 antibodies were detected in eight samples from patients with SS (a) (p < 0.001). The mean anti-gAChRα3 level in HCs had an antibody index (AI) of 0.305, which was significantly different from SS samples with a mean titer of 0.975 AI (p < 0.001). Anti-gAChRβ4 was not detected in patients with SS (b) (p = 1.000). The mean anti-gAChRβ4 level in HCs was 0.367 AI, which was significantly lower than the SS samples that had a mean titer of 0.499 AI (p = 0.001).

Table 4. Clinical features of Sjögren’s syndrome (SS) patients with autonomic disturbance in two studies.

|                  | 15 SS patients |
|------------------|----------------|
| Age (years)      | 51.7 ± 18.8    |
| Sex (female/male)| 12/3           |
| Types of SS      | 10/5           |
| Sicca (%)        | 13 (86.7)      |
| Orthostatic hypotension/intolerance (%) | 10 (66.7) |
| Upper gastrointestinal tract symptoms (%) | 9 (60.0) |
| Lower gastrointestinal tract symptoms (%) | 10 (66.7) |
| Bladder dysfunction (%) | 9 (60.0) |
| Sweating disorders (%) | 6 (40.0) |
| Pupil abnormalities (%) | 4 (26.7) |
| Sexual dysfunction (%)* | 2 (13.3) |
| Sensory disturbance (%) | 8 (53.3) |

*We reviewed three males only.

This table summarized the data from five patients from the first observational study (patients 4, 5, 7, 8, and 9) and all of the patients from the second study.

Ultimately, we reviewed the clinical features of 15 SS patients with autonomic disturbance in the final analysis. Table 4 contained the data of five patients from the first observational study (patients 4, 6, 7, 8, and 9) and all 10 patients from the second study. Thirteen of fifteen patients (86.7%) were seropositive for anti-gAChR antibodies, and we confirmed orthostatic hypotension, upper and lower GI symptoms, and bladder dysfunction at high rates, except for the sicca complex.

Discussion

The first study in the present manuscript found a high (23.1%) prevalence of anti-gAChR antibodies in the serum from patients with SS, but information regarding the presence or absence of autonomic symptoms was lacking. Therefore, in the second study, we collected serum samples from 10 SS patients with autonomic symptoms from throughout Japan and discovered a surprisingly high frequency (80.0%) of seropositivity for anti-gAChR antibodies. All eight seropositive patients in the second study demonstrated pandysautonomia, but the autoantibody levels were not clearly high. Thus, this phenomenon may be due to a bystander effect, reflecting an abnormal immune system in patients with SS. Since SS is a systemic autoimmune disease, we hypothesized that the neuropathy may have an autoimmune
pathogenesis. In addition, as far as we can see the results for the final analysis and second studies, further studies are necessary to determine whether the anti-gAChR antibody levels correlate with autonomic dysfunction severity in SS.

With regard to autonomic symptoms in SS, several studies have demonstrated an association between autonomic dysfunction and SS, but previous results are conflicting [3–8]. Newton et al. [7] reported that the autonomic symptoms are common among patients with pSS, may contribute to the overall symptom burden, and are linked with systemic disease activity. They used the Composite Autonomic Symptom Scale to determine the prevalence of dysautonomia and assessed the pSS clinical features with the European league against rheumatism (EULAR) Sjögren’s Syndrome Disease Activity Index and Patient Reported Index. Further studies should incorporate these assessment tools and analyze the relationship between autoantibody levels and scores from well-validated and comprehensive assessments as described earlier.

Six dysautonomia patients with anti-gAChR antibodies also had sensory disturbances, suggesting that autonomic ganglionopathy has a similar etiology to sensory neuropathy. Two patients who were seropositive for anti-gAChR antibodies presented with SIADH. Japanese pediatric neuroradiologists have already reported cases of acute pandysautonomia with SIADH [22]. They have suggested that patients with autonomic neuropathy might have both peripheral and central nervous system manifestations. Nicotinic AChRs are associated with cholinergic neurotransmission, modulation of dopamine function, and activity in the hypothalamic–pituitary–adrenal axis [23,24]. From a rheumatological viewpoint, we should mention that we grouped the patients with pSS together with the patients with sSS in the first and second studies. Particularly in the first study, sSS accounted for approximately half of the patients with SS that were seropositive for anti-gAChR antibodies. It is difficult to treat pSS as equivalent to sSS when evaluating our results because of the potential differences in the immunological backgrounds between pSS and sSS. Five of the nine patients with SS had other underlying rheumatic diseases (mixed connective tissue disease, rheumatoid arthritis, systemic sclerosis, and IgG4-related disorder), and, potentially, the immunological pathogenesis in patients with SS could be more complex than in patients with pSS (e.g., cellular immunity) [25–30]. Furthermore, several studies reported that pSS could be classified into subgroups based on ACA profiles. We reported the clinical and pathological characteristics of ACA-positive pSS and differentiated the ACA-positive pSS subgroup from the conventional pSS [31]. Several aspects of immune pathogenesis in SS need to be further clarified. We identified 17 seropositive patients in the present study, and nearly all patients were positive for only anti-gAChRα3 antibodies (16/17, 94.1%), except for patient 4 in the first study. Interestingly, all of the seropositive patients were positive for only anti-gAChRα3 or only β4 antibodies with no double-seropositive patients. This tendency toward seropositivity in patients with SS was different from that in patients with AAG [16]. In AAG patients, anti-gAChRα3 and β4 antibody positivity were 46% and 14%, respectively [16]. This difference of antibody positivity between SS with autonomic disturbance and AAG might be the key to understanding immune system dysfunction in SS. Additional experiments and investigations are necessary to clarify the role of AChRβ4 antibodies in the pathogenesis of autonomic involvement in SS [32].

Mori et al. [33] classified the SS-associated neuropathy into seven forms: sensory ataxic neuropathy, painful sensory neuropathy without sensory ataxia, multiple mononeuropathy, multiple cranial neuropathy, trigeminal neuropathy, autonomic neuropathy, and radiculoneuropathy. They reported that the autonomic features are widely present in SS-associated neuropathy, particularly in the sensory ataxic, painful sensory, and autonomic neuropathic forms. Several studies have demonstrated that the autonomic symptoms may be attributed to different pathologic causes such as autonomic gangliononeuromitis and peripheral autonomic nerve involvement due to direct T-cell attacks on the nerves or ischemia due to vasculitis [34–36]. However, gAChR antibodies have the potential for impairing autonomic ganglionic synaptic transmission; since both sympathetic and parasympathetic ganglia utilize nicotinic cholinergic synapses, antibodies interfering with ganglionic transmission could cause AAG. Indeed, our clinical and serological observations suggest that pandysautonomia in SS may be mediated by autoantibodies that interfere with autonomic ganglionic transmission [37]. Furthermore, patients identified in the present study demonstrated both sympathetic and parasympathetic dysfunctions. For instance, the segmental distribution of anhidrosis corresponded to segmental variation in the autonomic ganglion cell involvement, also supporting the hypothesis that primary lesions in the autonomic ganglion cells are responsible for autonomic symptoms. The presence of pupil abnormalities, which is often associated with SS-associated neuropathy, may also be attributable to ciliary ganglion cell involvement. Orthostatic hypotension, anhidrosis, constipation, and loss of cardiac [121]-MBIF uptake were severe when the postganglionic autonomic system was predominantly involved. In the future, we will have to verify the role of gAChR antibodies in SS autonomic dysfunction. Because ganglionic acetylcholine receptor autoantibodies have been recently found in a variety of peripheral neuropathies, we screened patients for these autoantibodies as candidate biomarkers. The timing of peripheral neuropathy manifestation, including the autonomic symptoms during the course of SS, has also been debated [33,38–40]. In approximately half the patients from the present study, the sicca complex was a late event. Thus, half the patients had been diagnosed with autonomic neuropathy of unknown etiology before being diagnosed with SS. Thus, we recommend gAChR antibody measurement as a novel tool for early SS diagnosis. However, it must be noted that a sural nerve biopsy was not performed for any of the patients, and nerve conduction studies were performed on only two patients (patients 7 and 10) in the second study. Therefore, the present study did not evaluate peripheral neurological involvement in SS. It is likely that tissue examination and electrophysiological studies could be more informative in confirming or excluding the possibility of autoantibody-mediated pathogenesis. Such work is under way in an ongoing prospective study. In addition, autonomic dysfunctions have been reported in association with the other autoimmune rheumatic diseases (systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and mixed connective tissue disease [25–30]). A current project is targeting at SS and these autoimmune rheumatic diseases.

An additional limitation of this study is that we utilized a retrospective design and included only a small number of subjects. Thus, a prospective, multicenter, clinical trial with a larger sample size is necessary to confirm the relationships between gAChR autoantibodies, symptoms, and autonomic function in SS.

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**Conflict of interest**

All of the authors report no disclosures. Akihiro Mukaino had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Supplementary material available online
Supplementary Tables S1 and S2