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# Evaluation of Autoantibodies in patients with Systemic Sclerosis

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Systemic sclerosis is an autoimmune disease clinically characterized by vascular and immune dysfunction, leading to fibrosis that can damage multiple organs. The presence of non-overlapping SSc-associated autoantibodies best presents the autoimmune nature of systemic sclerosis. The primary purpose of this study was to investigate the autoantibody profile in Iranian patients with systemic sclerosis. Sera from 481 patients with systemic sclerosis were collected from 2013 to 2016. Levels of anti-nuclear antibodies (ANA) were quantitatively detected using the indirect immunofluorescence (IIF) method, and levels of specific autoantibodies including anti-topoisomerase I antibody (ATA), anti-centromere antibody (ACA) and anti RNA polymerase III antibody (anti-RNAP III) were determined qualitatively using the enzyme-linked immunosorbent assay (ELISA) technique. Among all patients evaluated, a predominance of females (86.7%) was found, and 434 (90.2%) patients showed positive ANA results by IIF. ANA was detected in 87.3% and 92.0% of limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc) patients, respectively, which was not significantly different. The frequency of anti-RNAP III, ACA, and ATA was 5.19%, 6.09%, and 72.3%, respectively. Furthermore, anti-RNAP III, ATA, and ANA levels were correlated with dcSSc, whereas ACA levels were correlated with lcSSc. It was confirmed that ATA expression is significantly higher in dcSSc patients. This study had a lower frequency of ACA (6.09%) than most previous cohorts. The results demonstrated that the clinical subtype of systemic sclerosis may correlate positively with the presence of specific autoantibodies.

**Keywords:** Systemic Sclerosis; autoantibody; anti-nuclear antibody; anti-topoisomerase I antibody; anti-centromere antibody; anti RNA polymerase III antibody

## Introduction

Systemic sclerosis (SSc, scleroderma) is a multi-systemic chronic autoimmune connective tissue disorder characterized by extensive fibrosis, vascular abnormalities, and immune dysfunction [1]. The exact etiopathogenesis of SSc remains unknown, although in genetically susceptible individuals, environamental triggers and dysregulated epigenetic changes contribute to the development of SSc [2]. Systemic sclerosis is more prevalent in black people; however, it is found in all racial groups and all geographic regions [3].

A serological hallmark of systemic sclerosis is the presence of serum autoantibodies against various intracellular antigens. Autoantibodies have been observed at first diagnosis in more than 95% of patients affected with systemic sclerosis [4]. Each of the autoantibodies is beneficial in the diagnosis of affected patients. Different autoantibodies have been associated with different disease subtypes and with differences in disease severity, including the extent of skin involvement, internal organ manifestations, as well as determining the prognosis [5]. All of these antibodies are directed against structures within the nucleus of the cell and, thus, are ANAs. Some ANAs, including ACA, ATA/anti-Scl-70 antibody, anti-fibrillarin/anti-U3-ribonucleoprotein (AFA), anti-PM/Scl antibody, anti-Th/To antibody, and anti-RNAP III are found in the sera of systemic sclerosis patients [4].



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The subgrouping of systemic sclerosis patients based on their serum ANA in the early stages of the disease may be practical for assessing the risk and nature of organ involvement and estimating survival in these patients [6]. Each subgroup is associated with a different type of autoantibody [7].

The presence of ACA is most often associated with a variant of SSc, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) [8], and has a better prognosis than ATA-positive SSc patients [7].

ACAs are mostly found in patients with lcSSc [9, 10] and pulmonary hypertension [7], whereas the presence of ATA is associated with dcSSc, pulmonary fibrosis, musculoskeletal and cardiac involvement, and proteinuria; thus, it represents a poorer prognosis and higher rate of mortality [11-14].

The presence of both anti-RNAP I and III and a higher prevalence of renal crisis but not pulmonary fibrosis are seen with dcSSc [15, 16]. Thus, the survival rate in patients with anti-RNAP is better than in patients with ATA [4, 17].

It has been documented that purified human ATAs impede relaxation of super helical DNA [18], anti-RNAP I /III autoantibodies inhibit RNA transcription [19], and ACAs disrupt mitosis [20, 21]. However, how these autoantibodies bind to the intracellular antigen resulting in cellular damage is not clear [5]. Henault et al. demonstrated that by binding to the surface of dermal fibroblast cell lines, topo I provides a binding site for anti-topo I and stimulates adhesion and activation of monocytes in vitro [22].

The current study assessed the prevalence of ANA, ATA, ACA, and anti-RNAP III among Iranian systemic sclerosis patients and investigated their prevalence separately in patients with dcSSc and lcSSc.

## Materials and Methods

### Patients

The study population comprised 481 systemic sclerosis patients from the rheumatology clinic of Shariati Hospital, Tehran, Iran, and the Iran Rheumatism Center (IRC) from 2013 to 2016. All of the cases fulfilled the American College of Rheumatology (ACR) 2013 criteria [23], and clinical data was available for all cases. The patients were categorized into dcSSc and lcSSc according to the 2013 classification criteria for SSc [23]. Written informed consent was obtained from all participants, and ethical approval for this study was obtained from Tehran University of Medical Sciences (TUMS).

Whole blood samples from each patient were collected in test tubes, and the sera were separated by centrifugation. Aliquots of sera were stored at –70 °C until used.

### Indirect immunofluorescence (IIF) method for detection of ANA

The ANAs were quantitatively detected using the indirect immunofluorescence method via mosaic HEp-20-10 cells and primate liver cells (EUROIMMUNE, Medizinische Labordiagnostika AG, Germany). Sera were diluted with phosphate buffered saline (PBS) at a ratio of 1/160 and 1/640. Twenty-four microliters of each serum sample was added to each well; then the chambers were capped and incubated for 30 minutes at room temperature (RT). Each slide was washed quickly two times with PBS. The slides were located again in the humidifier chamber, subjected to appropriate diluted goat anti-human IgG fluorescein isothiocyanate (FITC) conjugated antibody, and further incubated for 30 minutes. Subsequently, the slides were rinsed thrice with PBS for 5 minutes each. Completely blotted slides were then overlaid with a coverslip and positioned on the slide tray. The slides were stored at 4 °C until visualized using a Zeiss fluorescence microscope.

### Enzyme-linked immunosorbent assay (ELISA) for detection of ATA, ACA, and anti-RNAP III

The ATA and ACA of IgG class in sera were detected using an ELISA kit (Euroimmune, Medizinische Labordiagnostika AG, Germany), and the anti-RNAP III was also determined qualitatively by an ELISA kit (Cusbio, China) according to the manufacturer's protocols.

### Statistical Analysis

Statistical analyses were performed using statistical package SPSS version 22 (SPSS Inc., Chicago IL, USA), and $p\ value < 0.05$ was considered to be statistically significant.
The Benjamini and Hochberg method was used to control for false discovery rate in multiple comparisons [24].

## Results

### Demographic data and disease classification.

The demographic features of the participants are shown in Table 1. According to the data on accessible cases, 417 (86.7%) were women, and 64 (13.3%) were men. The female-to-male ratio was 6.5:1, the mean age was 43.8 ± 11.5 years, and the mean disease duration was 10.5 ± 6.5 years. Among all participants, 181 (37.6%) were classified as lcSSc, and 300 (62.4%) were classified as dcSSc. Among those with lcSSc, 165 (91.2%) were female, and 16 (8.8%) were male, and of dcSSc patients, 252 (84%) and 48 (16%) were female and male, respectively.

### The level of non-specific and specific systemic sclerosis-relevant autoantibodies

The frequency rates of autoantibodies detected in these patients are shown in Table 2. Among all patients, 434 (90.2%) were ANA positive and the remaining 47 (9.7%) were ANA negative (Table 3). Overall, ANA was detected in 158 (87.3%) and 276 (92.0%) of lcSSc and dcSSc patients, respectively, which was not significantly different ($p\ Value = 0.092$).

**Table 1.** Demographic feature of the SSc patients.

| Demographic Variable | Number (Frequency) – Mean ± SD | | |
|---|---|---|---|
| | lcSSc N (%) | dcSSc N (%) | Total N (%) |
| N (%) | 181 (37.6) | 300 (62.4) | 481 |
| Gender :Male | 16 (8.8) | 48 (16.0) | 64 (13.3) |
| Gender :Female | 165 (91.2) | 252 (84.0) | 417 (86.7) |
| Age (year) | 45.6 ± 11.4 | 42.7 ± 11.5 | 43.8 ± 11.5 |
| Disease duration (year) | 10.4 ± 6.6 | 10.5 ± 6.4 | 10.5 ± 6.5 |

**Abbreviations:** lcSSc, limited cutaneous Systemic sclerosis; dcSSc, diffuse cutaneous Systemic sclerosis; SD, Standard deviation

**Table 2.** The prevalence of the autoantibodies in SSc patients.

| | **lcSSc N (%)** | **dcSSc N (%)** | ***P value*** | ***P value*$^{adj}$** | **OR (CI 95%)** |
|---|---|---|---|---|---|
| **Anti-RNAP III** | 5 (2.8) | 20 (6.7) | 0.062 | 0.077 | 2.514 (0.927-6.821) |
| **ACA** | 16 (8.8) | 13 (4.3) | 0.044 | 0.073 | 0.467 (0.219-0.995) |
| **ATA** | 98 (54.1) | 250 (83.3) | <0.001 | <0.001 | 4.235 (2.778-6.455) |
| **ANA** | 158 (87.3) | 276 (92.0) | 0.092 | 0.092 | 1.674 (0.915-3.064 |
| **Seronegative** | 7 (3.9) | 2 (0.7) | 0.012 | 0.03 | 0.167 (0.034-0.812) |

**Abbreviations:** lcSSc, limited cutaneous Systemic sclerosis; dcSSc, diffuse cutaneous Systemic sclerosis; ANA, anti-nuclear antibody; Anti-RNAP III, anti RNA polymerase III antibody; ACA, anti-centromere antibody; ATA, anti-topoisomerase I antibody

**Table 3.** The prevalence of the autoantibodies in different populations.

| | **Iran n=481(%)** | **French n=127(%)** [30] | **U.S n=247 (%)** [30] | **Mexico n=139 (%)** [31] | **German Network n=863 (%)** [16] | **Kuwana Japanese n=275 (%)** [6] | **Midwesten region of Brazil n=46 (%)** [34] |
|---|---|---|---|---|---|---|---|
| dcSSc | 181 (37.6) | 24 (19) | 116 (47) | 60 (43.1) | 173 (20.1) | 71 (NC) | 16 (34.8) |
| lcSSc | 300 (62.4) | 69 (54) | 104 (42) | 79 (56.8) | 513 (59.4) | 112 (NC) | 22 (47.8) |
| ANA | 434 (90.2) | 125 (98.4) | 245 (99.2) | 139 (100) | 813 (94.2) | | |
| ACA | 29 (6.09) | 23 (18) | 52 (21) | 41 (29.5) | 310 (35.9) | 45 (93) | 24 (52.2) |
| ATA | 348 (72.3) | 45 (35) | 54 (22) | 39 (28.1) | 260 (30.1) | 78 (66) | 15 (32.6) |
| Anti-RNAP III | 25 (5.19) | 5 (4) | 61 (25) | 2 (1.4) | - | | 7 (15.2) |

**Abbreviations:** lcSSc, limited cutaneous Systemic sclerosis; dcSSc, diffuse cutaneous Systemic sclerosis; ACA, anticentromere antibody; ANA, antinuclear antibody; Anti-RNAP III, anti RNA polymerase III antibody; ATA, anti-topoisomerase I antibody; NC, not calculated

According to the specific autoantibody profile, 25 patients were anti-RNAP III-positive (5.19%), 29 were positive for ACA (6.02%), and 348 were positive for ATA (72.3%) (Table 3).

Among all patients, anti-RNAP III was present in 5 (2.8%) and 20 (6.7%) lcSSc and dcSSc patients, respectively, without any significant difference (*p value* = 0.077).

The presence of ACA was found in 16 (8.8%) and 13 (4.3%) individuals with lcSSc and dcSSc, respectively, which lost its significant correlation after adjustment with the Benjamini and Hochberg method (*p value* = 0.073).

The presence of ATA was detected in 98 (54.1%) lcSSc patients and 250 (83.3%) dcSSc patients. ATA was the most common autoantibody in both subtypes, and it was significantly higher in dcSSc (adjusted *p value* < 0.001).

In addition, 7 (3.9%) lcSSc patients and 2 (0.7%) dcSSc cases were seronegative, which was statistically significant with an adjusted *p value* of 0.03.

## Discussion

Systemic sclerosis is a highly heterogeneous disorder with connective tissue involvement and an autoimmune nature, clinically characterized by the triad of endothelial dysfunction, inflammation and autoimmunity, and tissue fibrosis [25]. The importance of the study of autoantibodies lies in the fact that some of them are involved in the disease pathogenesis. Therefore, laboratory examinations to detect systemic sclerosis-relevant autoantibodies (e.g., ANA, ATA, anti-RNAP III, and ACA) provide an effective tool for the diagnosis of systemic sclerosis and the classification of disease subsets [26]. It has been estimated that over 95% of patients with systemic sclerosis have a positive ANA test, and over 85% have one or more serum autoantibodies [27]. DNA topoisomerase I (topo I), centromere proteins, RNA polymerases I, II, and III are antigens defined as intracellular targets for ANAs [28].

Systemic sclerosis is more common in women than men; however, the course of the disease may be more progressive in men [29]. Similar to other populations, i.e. U.S and French [30], Mexican [31], German Network [16], EULAR[1] Scleroderma Trials And Research (EUSTAR) group [13], and the mid-western region of Brazil [32], patients in the current study were mostly female.

In this study, it was found that 434 (90.2%) patients were ANA positive and 47 (9.7%) were ANA negative. Similarly, other studies have reported a high percentage of ANA [3, 16, 30, 31, 33].

ACA is directed against centromere proteins, and it has been detected in 20–25% of SSc populations that are strongly correlated with lcSSc [27]. The frequency of ACA in different studies varies from 4-52.2% [16, 30-34], although in a study of Japanese patients by Kuwana et al., 93% were ACA positive [6]. In the current study, the frequency of ACA, which is more associated with lcSSc [9, 10], was low and 6.09% were ACA positive.

Different studies on different ethnic groups have reported 13-41.2% ATA positivity [16, 30-34]. In a study of Japanese patients by Kuwana et al., 66% were ATA positive [6]. Based on the current findings among Iranian patients, 72.3% were ATA positive, which is correlated to dcSSc and poor prognosis [11].

Anti–RNAP III has 98% to 100% specificity for SSc and occurs in 16%-20% of patients, mainly in those with the dcSSc subtype [35]. Several researchers have reported 1.4-9.9% anti-RNAP III positivity in SSc patients [30, 31, 34]. Consistent with these studies, the current results demonstrated that 5.19% of SSc patient were anti-RNAP III positive. However, two studies showed U.S and Brazil SSc patients were 25% and 15.2% anti-RNAP III positive, respectively [30, 32].

Taken together, the current data showed that anti-RNAP III and ATA were higher in dcSSc patients, whereas ACA was higher in lcSSc patients. In comparison with other studies, the frequency of ATA was significantly high in Iranian patients, but ACA frequency is low. With regards to the correlation of these autoantibodies with disease prognosis, it seems that more Iranian SSc patients have a poor prognosis. However, a study has reported that survival rates in Iranian SSc patients are 93% and 83% at 5 and 10 years from diagnosis, respectively [36], which is not different from other countries [37-41].

There were several discrepancies in frequency rates of autoantibodies among different studies. These conflicting results may be attributable in part to differences in sample size, ethnic background, and geographic factors, differences in the ACR criteria, the system assay used to detect autoantibodies, and diverse statistical tests.

## Conclusion

A systemic sclerosis diagnosis is mainly clinical; nevertheless, quantification and identification of autoantibodies can play important roles in diagnosis. The present study illustrated differences in the autoantibodies associated with SSc disease. In accordance with the literature, this study confirms that the clinical subtype of SSc may correlate positively with the presence of specific autoantibodies. Further research is required in order to investigate the exact role of autoantibodies in SSc patients and the cause of their dissimilar frequencies among different cohorts.

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## Conflict of interest

The authors report that they have no conflicts of interest to declare.

[1] The European League Against Rheumatism

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