Concise Report

Anti-hnRNP and other autoantibodies in systemic sclerosis with joint involvement

Sergio Generini1,*, Günter Steiner2,3,*, Irene Miniati1, Maria L. Conforti1, Serena Guiducci1, Karl Skriner2,3, Olga Kaloudi1, Roberto Giacomelli4, Josef Smolen2,5 and Marco Matucci-Cerinic1

Objectives. To investigate joint involvement in SSc and its relationship with autoantibody to the hnRNP and to anti-cyclic citrullinated peptide (anti-CCP).

Methods. Sera from 55 SSc patients were investigated. Joint involvement was determined by clinical, radiological and ultrasonographical evaluation. Anti-hnRNP proteins A1 and A2 (anti-hnRNP-A1/A2) antibodies were determined by immunoblotting. Anti-CCP, ACA, anti-topo I (ATA), Sm, U1-RNP, ribosomal RNP, Ro(SSA), La(SSB) autoantibody and RF were determined.

Results. Six patients were positive for anti-hnRNP-A2 autoantibody and two were anti-A1 positive. Eight patients had joint erosions: seven of the eight patients positive for anti-hnRNP-A2 or A1 presented articular involvement (P < 0.04) and five of the eight erosive patients were positive for either of the two autoantibodies (P < 0.02). Of the four patients positive for anti-CCP, none had anti-hnRNP but three had erosive aspects. ATAs were found in 10 patients, six of which were also positive for anti-hnRNP (P < 0.05). RF was positive in 16 patients and in seven among those with articular involvement (P < 0.04). RF was significantly associated with anti-hnRNP in patients with erosive arthritis (P < 0.02), but not with the presence of anti-hnRNP alone. Epitope mapping of the three strongest anti-hnRNP-A2-positive sera recognized the same major epitope as patients with RA. SSc patients have higher incidence of erosions and anti-hnRNP-A2/A1 positivity. RF test and anti-hnRNP had a statistically significant diagnostic value for articular involvement.

Conclusions. These parameters might suggest that autoantibody to both hnRNP antigens might become a non-specific but useful marker for joint involvement in SSc patients and identify SSc patients prone to develop joint damage.

Key words: Systemic sclerosis, Anti-hnRNP-A2 antibodies, Articular involvement.

Introduction

SSc is an autoimmune disease affecting the microvascular system, the immune system and connective tissue [1]. The musculoskeletal system is often involved in patients with severe chronic course of SSc. In the later stages of the disease, articular alterations are very common and are mainly due to the lack of vascular supply and to the fibrosis affecting the tendons, the ligaments, the joint capsules and the synovium thus restricting movement and leading to ankylosis and bone resorption. However, a primary articular involvement and the presence of synovitis are often detectable. It has been reported that 66% of SSc patients experience joint pain and 61% have signs of joint inflammation that can configure different clinical patterns [2]. In a small number of patients, a symmetrical polyarthritis, usually seronegative, anodular and non-erosive, may be the presenting feature of SSc. In these cases, the clinical features may be similar to RA and often be confused with it [2, 3]. Erosive arthropathy is found in 20–30% of these patients, especially in the wrists [4, 5], and RF may be positive in 26–50% of the patients [6]. The co-existence of SSc and RA has been reported and is considered as an overlap syndrome [7, 8]. Although differential diagnosis between RA and SSc with articular involvement is often difficult in the early phases of disease, some different clinical and pathological features of joint involvement may help [9]. The radiographical articular manifestations of SSc are less severe than those of RA, and are usually limited to mild joint narrowing, osteoporosis and small discrete erosions at the periarticular margins (Fig. 2A and B) [10]. Pathologically, there is only a slight tendency to form pannus in SSc, and synovial lesions are characterized initially by inflammation of synovium and later by severe fibrosis of the tissue [11].

Among the autoantibodies that have been described in patients with SSc, anti-topo I autoantibodies (ATAs) are considered particularly useful for the diagnosis of diffuse SSc, whereas ACAs are mainly found in limited cutaneous SSc. At the moment, a serological marker for the early identification of SSc patients evolving towards an erosive arthritis is lacking.

In addition to RF, a number of autoantibodies have been described to occur in patients with RA [12]. Among these are autoantibodies against the RA33 antigen, i.e. the hnRNP protein A2 (hnRNP-A2), an abundant nuclear protein associated with the spliceosome [13]. These autoantibodies (anti-hnRNP-A2) occur in about one-third of patients with RA but rarely in other arthritides such as OA, PsA or reactive arthritis [14, 15]. On the other hand, anti-hnRNP-A2 may also occur in 20–40% of patients with SLE or MCTD where, contrarily to RA, they are associated with other anti-spliceosome autoantibody, such as anti-Sm or anti-U1 RNP [15]. Interestingly, in SLE patients anti-hnRNP-A2 autoantibodies were found to be significantly associated with erosive arthritis [16, 17]. Autoantibodies directed to the closely related hnRNP-A1 have also been described in RA, SLE and MCTD, but occur less frequently than anti-hnRNP-A2 and their association with erosive disease seems to be less pronounced [15, 18].

In the last years, autoantibodies directed to citrullinated antigens in the synovium have been proposed as predictive marker of RA [12]. These citrullinated autoantigens (e.g. filaggrin) are...
specifically present in inflamed synovia and the antibodies for these are locally produced. The test for anti-cyclic citrullinated peptide (anti-CCP) has a sensitivity of 51–68%, with a specificity of ~96–98% (significantly higher than that of RF) [19]. Anti-CCP can be detected in the blood of RA patients years before the first clinical signs are manifest, and high titres appear to correlate strongly with erosive disease and radiological lesions [20, 21].

The aim of our study was to evaluate the frequency of anti-hnRNP-A2/A1 and other autoantibodies in SSc patients and to investigate whether these autoantibodies may identify a subset of patients characterized by prominent articular involvement.

Patients and methods

Patients

Fifty-five consecutive Caucasian patients affected by SSc fulfilling ACR criteria (mean age 51 years; minimum 28 and maximum 77 years; 52 females and 3 males) were recruited at the Division of Rheumatology, Department of Biomedicine, University of Florence, Italy. Patients’ informed consent and local ethical committee’s approval were obtained before the study. Twelve patients were classified in the diffuse and 43 in the limited subset of the disease [22]. Patients were further sub-classified according to the disease duration in early and advanced (mean disease duration 11.3 years; 21 early and 34 advanced) [23]. Patients overlapping with SS, PsA and/or a familial history of psoriasis were excluded from the study. All patients were on alprostadil and proton pump inhibitors except one that was on iloprost and calcium channel blockers. In the previous 5 years, two patients were submitted to a course of pulse cyclophosphamide (1 g/m², for a total dosage of 7 g).

Articular evaluation

As no validated clinical and radiological criteria exist today to evaluate the articular involvement in SSc, the authors agreed to simply distinguish between the presence or absence of articular involvement on the basis of the clinical, radiological and ultrasonographical findings. Articular assessment was performed on each patient by two skilled rheumatologists that independently evaluated the presence of joint tenderness, swelling due to joint effusion or synovitis and articular deformities (due to primary joint involvement and not due to skin retraction and subcutaneous changes).

Other parameters that were considered were the onset of arthritis prior to RP, presence of elevated CRP, prolonged morning stiffness and saving of DIP joints [8]. The clinical evaluation was performed blinded with respect to autoantibody analysis.

All patients underwent X-rays of the hands and wrists. Additional articular districts were imaged if suspected to be involved on the basis of clinical symptoms. A single radiologist, unaware of patient’s clinical conditions, assessed the X-rays for the presence of juxta-articular osteoporosis, pseudo-widening of joint space, narrowing of joint space, erosions, subchondral cysts and MCP subluxation [9]. Moreover, affected joints were examined by articular ultrasonography (ESAOTE my Lab 25, linear probe 7.5–12 MHz) to detect synovial thickening, joint effusion or erosions. Scans were obtained according to guidelines for musculoskeletal ultrasound [24].

Ultrasonographical modifications were defined as follows: bone erosion: an IA discontinuity of the bone surface that is visible in two perpendicular planes; joint effusion: compressible anechoic intracapsular area; and synovitis: uncompressible hypoechoic intracapsular area. A rheumatologist certified in musculoskeletal ultrasound (O.K.) performed the ultrasound examinations [24].

The patients were considered positive for articular involvement if radiological, ultrasonographical and/or clinical signs (present or past) of arthritis were present. Only patients in which articular symptoms and signs were a clear and prominent manifestation of disease were considered. Where it was not possible to differentiate primary joint involvement from joint modifications due to skin retraction, patients were excluded from the study.

Laboratory investigations

Blood samples were drawn and centrifuged. The obtained sera were collected and stored in aliquots at −20°C until analysis.

RF and autoantibodies to nuclear antigens (ANA, ENA) were determined in all the patients. RF was analysed by nephelometry, ANA and ACA were determined by IIF on HEp-2 cells. Autoantibodies to hnRNP-A2 and A1 were assessed by immunoblotting using HeLa nuclear extracts and, additionally, a semi-purified preparation of hnRNP antigens essentially as described [15]. For epitope mapping studies, fragments of recombinant hnRNP-A2 were employed [25]. Anti-CCP were determined by an ELISA using a ‘citrullinated’ recombinant rat filaggrin [21]. Autoantibodies to other nuclear antigens including ATA, Sm, U1-RNP, ribosomal RNP, Ro and La were determined by immunoblotting using HeLa cell nuclear or cytoplasmic extracts and in house reference sera as previously described [25]. For the determination of anti-Ro autoantibody, an ELISA (Hemagen, Columbia MD, USA) was used since anti-Ro autoantibodies often escape detection by immunoblotting.

Cloning and expression of hnRNP-A2 deletion mutants

Recombinant hnRNP-A2 and overlapping fragments covering the functional domains of the antigen [the first and second RNA binding domain (RBD) and the C-terminal auxiliary domain] were expressed as fusion proteins with methyliod group 2 (MS-2) polymerase essentially as described [25]. Recombinant proteins were purified by cation exchange chromatography using carboxymethyl-Sepharose columns (Pharmacia, Uppsala, Sweden). Purity was >95% as analysed by SDS-PAGE and Coomassie protein staining. Electrophoretic separation and immunoblotting of recombinant proteins were performed as described.

Statistical analysis

Student’s t-test was used for the comparison of means. The analysis of the incidence of autoantibodies and clinical/immunological parameters was carried out with Fisher’s exact probability test.

Results

Articular involvement

Nineteen patients presented articular symptomatology but only 15 of them were considered to have an arthropathic pattern because of the presence of both clinical and X-ray/ultrasound articular alterations. Ultrasound examination showed the presence of bone erosions on longitudinal and transverse scans in the MCP and PIP in eight patients: two with bone microerosions <1 mm, three with erosions between 2 and 4 mm and three with erosions >4 mm. Synovitis was detected in three patients in which erosion was present. One patient presented synovitis (Fig. 1) and joint effusion of the II PIP. In five cases, periarticular involvement—a normally hypoechoic area around flexor tendons (tenosynovitis)—was detected in longitudinal and transverse planes.

All patients developed arthralgia and musculoskeletal manifestations after the onset of RP. In two patients, arthralgia was the initial manifestation of SSc, apart from RP. None of the patients included in the study reached the four criteria requested by ACR for the diagnosis of RA. In particular, even in the patients with positive RF and erosions, short morning stiffness, frequent
DIP involvement, no rheumatoid nodules, rare simultaneity and symmetricity of arthritis were observed. In three patients, a disease-modifying treatment was employed: Patients 1 and 6 were treated with MTX 15 mg/week and prednisone 5 mg, while Patient 2 received infliximab 5 mg/kg/every 2 months.

Two patients in whom a diagnosis of RA was formulated before the onset of SSc and fulfilling the ACR criteria were preventively excluded from the study. Only one patient, SSA-positive, was admitted in the study because patient did not develop any symptom of SS, and the parotid ultrasound, the biopsy of the minor salivary glands and the parotid scintigraphy were negative.

In two additional patients, the distinction between primary articular involvement and damages due to fibrotic skin retraction and lack of blood supply was not clear. These patients were tested for the presence of autoantibodies but were not included in the statistical analysis. HAQ data, presented in Table 1, show mainly higher values in SSc erosive patients.

All patients with joint involvement, both erosive (Fig. 2) and non-erosive, had skin involvement that included oedema and thickening in variable degree. We did not observe any association between skin involvement and erosions. Patients with longer history of disease had more severe digital thickening and tethering, and showed a higher degree of narrowing, osteoporosis and articular deformities. These were the cases in which it was more difficult to distinguish a primary joint involvement from the joint alterations due to limited vascular supply and skin and subcutaneous changes. A statistical analysis of this point was not possible because of the great variability in disease duration (from 2 to 19 years) and the low number of cases.

Autoantibodies to hnRNP-A2 and -A1
Six out of 55 patients were positive for anti-hnRNP-A2 autoantibodies and two additional patients were anti-hnRNP-A1 positive; thus, eight patients were positive for either of the two autoantibodies. Two of anti-hnRNP-A2-positive patients showed high titres (≥1:800) and appeared to react also with hnRNP-A1, while the other patients were weakly or moderately positive (from 1:50 to 1:200). Out of eight anti-hnRNP-positive patients, six patients were ATA positive and were affected by the diffuse subset of the disease.

Autoantibodies to CCP
These autoantibodies were found clearly positive in four SSc patients (three limited and one diffuse) (7.2%). Three of them (two with limited and one with diffuse subset) had articular involvement, two with erosive pattern (Fig. 2). One of these patients, initially considered as arthropathic SSc, was subsequently recognized as RA overlapping SSc and excluded from the study. None of the anti-CCP patients was positive to anti-hnRNP.

Relationships with articular involvement and disease duration
Out of the six patients positive for anti-hnRNP-A2, five were affected by articular involvement (P < 0.04) suggesting that the probability of independence between these two variables is very low. The comparison between anti-hnRNP-A2 and the presence of an erosive pattern equally showed a significant link (P < 0.05).
Both patients positive for anti-hnRNP-A1 autoantibodies also had articular involvement, one of them with erosive features. Considering the whole group of patients showing positivity for either anti-hnRNP-A2 or -A1 as a single variable, seven out of eight suffered from arthritis ($P < 0.03$). Among the seven patients showing an erosive pattern, five were positive for anti-hnRNP autoantibodies ($P < 0.02$). No association was noticed with disease duration.

Presence of other autoantibodies and relationship with anti-hnRNP antibodies

ATAs were detected in 10 patients: four of them were also positive for anti-hnRNP-A2 and two were positive for anti-hnRNP-A1. The association of ATA with anti-hnRNP was significant ($P < 0.05$). However, the association between ATA and articular involvement did not reach significance. The positivity hnRNP/ATA found in six erosive patients may not be enough to suggest a predictivity of this association for erosiveness. Twenty-five patients, all in the limited subset of disease, showed positivity to ACA and four of them had articular involvement. Anti-hnRNP-A2 was detected in only one patient without articular involvement (Table 1). One patient presented with both anti-Ro/SSA and anti-La/SSB and one patient was positive for anti-La/SSB. This last patient was also positive for anti-hnRNP-A2 autoantibodies and had an erosive articular involvement (Table 1). None of the patients had autoantibodies to Sm, U1-RNP or ribosomal RNP, respectively.

Relationship with RF

RF was positive in 16 patients and in 7 of the 12 patients with articular involvement, five of which had erosive disease ($P < 0.04$). RF was significantly associated with anti-hnRNP autoantibodies in patients with erosive arthritis ($P < 0.02$), but not with the presence of anti-hnRNP alone. The combination of these two tests showed a higher sensitivity for articular involvement than any of these two tests alone.

Epitope mapping

The hnRNP-A2 shows a modular structure and is composed of two RBDs and an auxiliary domain that is presumably involved in interactions with other proteins [20]. In previous studies, the second RBD was found to harbour a major epitope recognized by patients with RA or SLE, while patients with MCTD recognized a different epitope [26]. To investigate epitope recognition of SSc patients, the three strongest sera (Fig. 3) were tested for reactivity with a set of recombinant fragments corresponding to the major functional regions of hnRNP-A2. These experiments revealed that sera from SSc patients preferentially recognized the second RBD and reacted neither with the first RBD nor with the auxiliary domain (data not shown). Thus, epitope recognition of SSc patients appeared to be very similar or identical to that of patients with RA [27].

Discussion

Articular involvement in SSc has been neglected for a long time as it has been considered a marginal event. Today it is clear that a true arthritis, sometimes erosive, may affect the joints of SSc patients causing severe suffering and, eventually, joint destruction. In our cohort of patients, the incidence of articular erosive involvement reached 10% and was not related with the kind and severity of skin and visceral involvement. However, it should be considered that such a frequency may be due to the fact that the enrolment was done in a tertiary centre where a large number of
SSc patients, with several different clinical characteristics, are usually seen. The identification of these patients is mandatory for the clinician in order to establish an adequate therapy and avoid or at least limit severe joint derangement. Recently, two studies have addressed this problem. The first study on 120 SSc patients has identified the frequency of joint involvement characterized mainly by erosions (21%), joint space narrowing (28%) and arthritis (defined by concomitant erosion and joint space narrowing) (18%) [23]. At clinical examination, the second study on 76 SSc patients has found articular involvement, arthralgia and finger contractures more frequently than arthritis, while radiological evaluation showed mainly DIP joint space narrowing, juxta-articular osteoporosis and a significantly higher frequency of fibrotic pattern in the hands [29].

Anti-hnRNP-A2 autoantibodies have been suggested to be useful diagnostic markers for RA [14, 15, 18]. Interestingly, in SLE these autoantibodies as well as RF were found to occur particularly in patients with joint erosions thus representing a sort of marker for this (small) disease subset [16, 17]. Furthermore, anti-hnRNP-A2 autoantibodies were detected in two out of three patients with RA-limited SSc overlap syndrome and in 1 out of 20 ACA-positive patients with limited SSc [8]. In the present study, the incidence of anti-hnRNP-A2 autoantibodies was studied in a larger and homogeneous SSc population and was higher than previously reported in SSc (11%), but much lower than in RA and overlap RA–SSc [8, 14, 15]. This may be the consequence of the inclusion of diffuse cutaneous subset patients, in which the incidence of arthropathy and anti-hnRNP antibodies was somewhat higher than in the limited subset, even though not statistically significant.

Remarkably, all but one anti-hnRNP-positive patient showed articular involvement, and five of them suffered from erosive disease. Moreover, at least the three SSc patients showing the strongest reactivity against hnRNP-A2 recognized the same major epitope as patients with RA and all three had erosive arthritis. On the other hand, epitope recognition clearly differed from that of patients with MCTD [24] who may also show articular involvement and scleroderma-like features, but usually show a characteristic absence of severe erosive changes [5]. These data seem to provide further evidence that the major epitope of hnRNP-A2 is associated with erosiveness.

RF and anti-hnRNP-A2 autoantibodies had an independent and statistically significant diagnostic value for articular involvement in patients with SSc. However, the discriminative capacity of anti-hnRNP-A2 autoantibodies in the identification of an erosive articular subset in SSc appeared to be better than and complementary to that of RF.

Thus, as previously described for SLE, anti-hnRNP-A2 seems to be a marker for erosiveness in SSc. The same may be true for anti-hnRNP-A1 antibodies because both patients positive for this antibody had articular disease, one of them being erosive.

Anti-CCP autoantibodies were also present in four (7%) SSc patients. In two patients, both anti-CCP positivity and erosive arthritis were found. Previous studies [30, 31] reported that high titres of anti-CCP antibodies might help to define the diagnosis of overlap syndrome SSc/RA. Szucs et al. [30] reported anti-CCP positivity in 82% of patients with overlap syndrome suggesting that this can be considered a distinct immunological entity. The third anti-CCP positive patient with articular involvement, previously included in the study, was later classified as RA and therefore excluded from the study. Therefore, in our study anti-CCP antibody positivity did not reach a statistically significant association with articular involvement. In contrast, Ingegnoli et al. [31] found a significant association between anti-CCP pattern and marginal erosions in SSc. In the future, larger cohorts of patients will be needed to allow a clear association between anti-CCP positivity and joint involvement and erosiveness in SSc.

In conclusion, our data and the experience acquired in the other connective tissue diseases suggest that autoantibodies to both hnRNP antigens might become non-specific but useful markers for joint involvement in SSc patients, particularly during the early phases of the disease. However, a larger cohort of patients, with a multicentre and a prognostic study showing occurrence of these autoantibodies (as well as RF) before the onset of erosive arthritis, is needed to sustain these observations.

**Rheumatology key messages**

- In SSc a real arthritis, sometimes erosive, may affect the joints.
- Anti-hnRNP-A1 and -A2 may have a diagnostic value for joint involvement.
- Anti-hnRNP-A1 and -A2 may identify SSc patients at the risk of developing erosive arthritis.

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