Increased immunoreactivity against human cytomegalovirus UL83 in systemic sclerosis

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Received on September 7, 2016; accepted in revised form on January 7, 2017.

Clin Exp Rheumatol 2017; 35 (Suppl. 106): S31-S34.

Key words: autoantibodies, biomarkers, immunology, rheumatic diseases, scleroderma/systemic sclerosis

Funding: ELKE, University of Thessaly, Greece.

Competing interests: T. Scheper and W. Meyer are employees of Euroimmun; the other authors have declared no competing interests.

Part of this work was presented at the 12th Dresden Symposium on Autoantibodies, Dresden, Germany, 23-26 September, 2015 (10).

ABSTRACT

Objective. *To study immunoreactivity against human cytomegalovirus (HCMV) in systemic sclerosis (SSc), since HCMV has been put forward as a candidate infectious cause.*

Methods. *Eighty four patients with SSc (67 females; median age 60 years, range 25-81), 30 patients with multiple sclerosis (MS) (23 females; median age 44, range 20-69 years) and 28 healthy controls (NCs), all pre-tested positive for IgG anti-HCMV antibodies, were studied. IgG anti-UL83 HCMV antibodies were tested by western immunoblotting and expressed in arbitrary units (AUs). Reactivity to UL83 HCMV was assessed in relation to clinical manifestations and SSc-related autoantibodies (autoAbs), tested by an IgG SSc autoantibody profile line immunoassay (Euroimmun) that detects autoAbs against Scl-70, CENPA, CENPB, RNA polymerase III subunit 11 (RP11), RP155, fibrillarin, NOR90, Th/To, PM-Scl100, PM-Scl75, Ku, PDGFR and Ro-52.*

Results. *Fifty patients (59.5%) were anti-UL83 clear positive (UL83+), including 21/40 (52.5%) lcSSc and 29/44 (65.6%) dcSSc, compared to 15/30 (50%) patients with MS (SSc vs MS, p=ns and 11/28 (39.29%) of NCs (SSc vs NC, p=ns MS vs NC, p=ns). Anti-UL83 antibody AU levels (mean±SD) were higher in SSc (64.3 ± 26) compared to MS (49.1±21.6, p=0.05) or NCs (40.4±13.7, p<0.001; MS vs NCs, p=ns) and were associated with pulmonary fibrosis.*

Conclusion. *Immunoreactivity to UL83 HCMV is frequent and strong in patients with SSc, implying a possible pathogenic role for this disease.*

Introduction

Systemic sclerosis (SSc) is a systemic autoimmune rheumatic disease characterised by microangiopathy, extensive collagen deposition, and activation of the immune system with many autoantibodies (1). The aetiology of the disease is not known (2). Environmental factors, mainly human cytomegalovirus (HCMV), have been considered as potential triggers for the development of SSc (3-6). Elevated levels of anti-HCMV antibodies (abs) were detected in SSc patients (3-6). Moreover, elevated levels of anti-HCMV UL94 abs were found in patients with SSc (6) and these abs cross-recognise NAG-2, a cell surface antigen expressed on endothelial cells (7). Such cross-reactive anti-viral responses can induce SSc-associated endothelial cell apoptosis and fibroblast proliferation (7-9). However, NAG-2 and UL94 are not the most immunodominant antigens during HCMV infection in SSc (6). We recently investigated ab responses against HCMV UL44 and UL57 but we failed to find any differences between SSc, and healthy controls (10). Other Investigators have focused their research on other HCMV proteins, namely UL83 (pp66) (11), which is a nuclear protein and a major antigen targeted by immune responses during HCMV infection (12). In this paper we study the magnitude of anti-UL83 HCMV immunoreactivity in a large cohort of patients with SSc and determine if it is associated with any of 13 SSc-associated autoAbs and clinical parameters.

Material and methods

Patients and controls

Eighty four SSc patients (67 females; median age 55.1 years, range 25-81), 40 with limited cutaneous SSc (lcSSc), 44 with diffuse cutaneous SSc (dcSSc), all positive for IgG anti-HCMV abs were studied. The main demographic, clinical and immunological characteristics of the patients are shown in

Table I. Main demographic and clinical parameters of the 84 patients with systemic sclerosis (SSc) according to anti-UL83 antibody status.

	Total SSc (n=84)	Anti-UL83 pos* (n=50)	Anti-UL83 neg (n=34)
Epidemiological			
Mean age, years (±SD)	53.9 (± 13,7)	58.2 (± 13.3)	52.6 (± 13.8)
Females, n (%)/Males, n (%)	67 (79.8%)/17 (20.2%)	40(80%)/10 (20%)	27 (79.4%)/7 (20.6%)
Type of SSc			
Limited/limited cutaneous, n (%)	40 (47.6%)	21 (42%)	19 (55.9 %)
Females, n (%) / Mean age, years (±SD)	29 (72.5 %) /54.3 (± 12.9)	15 (71.4%)/ 52.1 (±13.2)	14 (73.7%)/ 50.8 (±12.9)
Diffuse cutaneous, n (%)	44 (52.4%)	29 (58%)	15 (44.1%)
Females, n (%) / Mean age, years (±SD)	38 (86.4%)/ 60.1 (±13.3)	25(86%) / 62.3 (±11.8)	13 (38.2%)/56 (±15.1)
Clinical features			
Ulcers, n (%)	44 (52.4%)	25 (50%)	19 (55.6%)
Pulmonary fibrosis, n (%)	32 (38.1%)	23 (46%)**	9 (26.4%)**
Ulcers and/or pulmonary fibrosis, n (%)	53 (63.1%)	32 (64%)	19 (55.6%)
Ulcers and pulmonary fibrosis, n (%)	20 (23.8%)	13 (26%)	7 (20.6%)
Autoantibody reactivities			
Anti-Scl-70	44 (52.4%)	25 (50%)	19 (55.6%)
Anti-CENPA	21 (25%)	16 (32%)	5 (14.7%)
Anti-CENPB	20 (23.8%)	15 (30%)	5 (14.7%)
Anti-CENPA or Anti-CENPB	21 (25%)	16 (32%)	5 (14.7%)
Anti-RNA pol III RP11	12 (14.3%)	6 (12%)	6 (17.6%)
Anti-RNA pol III RP155	15 (17.9%)	9 (18%)	6 (17.6%)
Anti-RP11 or RP155	15 (17.9%)	9 (18%)	6 (17.6%)
Anti-fibrillarin	1 (1.2%)	1 (2%)	0 (0 %)
Anti-NOR90	5 (6%)	4 (8%)	1 (2.9%)
Anti-Th/To	0 (0 %)	0 (0 %)	0 (0%)
Anti-PM/Scl100	3 (3.6%)	1 (2%)	2 (5.9%)
Anti-PM/Scl75	3 (3.6%)	2 (4%)	1 (2.9%)
Anti-PM/Scl100 or PM/Scl75	3 (5%)	2 (4%)	1 (2.9 %)
Anti-Ku	2 (2.4%)	2 (4%)	0 (0 %)
Anti-PDGFR	0 (0 %)	0 (0 %)	0 (0 %)
Anti-Ro-52	18 (21.4%)	9 (18%)	9 (26.5%)

*(anti-UL83 positive >11AU (including anti-UL83 antibody borderline); no statistically significant differences were found between anti-UL83+ and anti-UL83- SSc patients; **, p=0.067.

Table I. Conventional treatment regimens included low-dose steroids (<7.5 mg/day) plus azathioprine or methotrexate. All patients, attending the out-patient clinic of our department, were positive for antinuclear antibodies, and fulfilled the American College of Rheumatology criteria for SSc. As pathological controls, we included 30 multiple sclerosis (MS) patients positive for IgG anti-HCMV abs, (23 female, median age 44, range 20-69 years; 15 patients with relapsing-remitting MS, RRMS; 15 patients with secondary progressive MS, SPMS). Patients attended the out-patient clinic of the Department of Neurology, in our hospital. Twenty eight demographically-matched healthy subjects (NCs) (21 female, median age 45, range 19–72 years), positive for IgG anti-HCMV abs were also included.

All investigations conformed to the revised principles of the Declaration of Helsinki. A written informed consent was obtained by all patients and NCs. The protocol was approved by the Local Ethical Committee of the University General Hospital of Larissa, Thessaly, in central Greece.

Anti-UL83 antibody testing by immunoblotting

Antibody responses against UL83 HCMV were tested by western immunoblotting, as we previously described (13). Blot strips with electrophoretically separated HCMV extract (HCMV strain A169) (Euroimmun, Germany) were used as a source of UL83 antigen. Membranes were incubated with serum samples at a dilution of 1/51. Ready-made NBT/BCIP (Euroimmun) was used as substrate for ALP-conjugated abs. Strips were scanned and analysed using the EUROLineScan program (13). Pretests authenticated that the amplitude compared with the integral of the curve was a valid variable; arbitrary units (AU) of the amplitude were applied. The cut off value set by the manufacturer at 11-22 for borderline area and >23 AU for clear positivity.

Autoantibody testing by line immunoassay

AutoAb testing of SSc patients was performed using the EUROIMMUN "Systemic Sclerosis Profile (Nucleoli)" an IgG autoAb line assay profile kit which tests autoAbs against 13 autoantigens: Scl-70, CENPA, CENPB, RNA polymerase III subunit 11 (RP11), subunit 155 (RP155), fibrillarin, NOR90, Th/To, PM-Scl100, PM-Scl75, Ku, PDGFR and Ro-52. Native Scl-70 is purified from bovine and rabbit thymus, and all other antigens are recombinant. Serum samples were tested at a dilution of 1/101, as described. Tests were considered negative when <5 AU, equivocal when 6-10 AU, weakly posi-

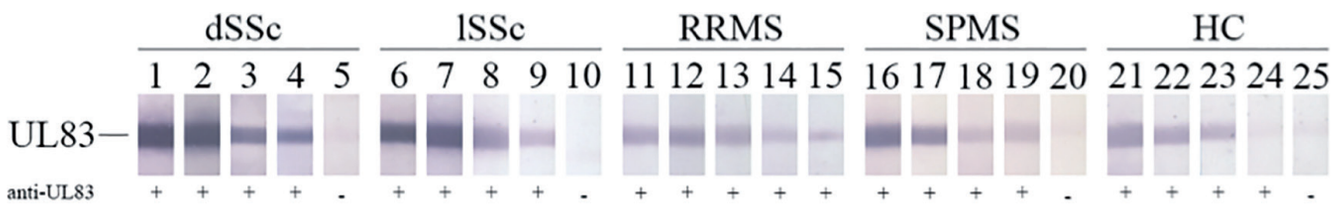

Fig. 1. Anti-UL83 human cytomegalovirus (HCMV) antibody testing by western blotting of representative patients with diffuse cutaneous systemic sclerosis (dcSSc), limited cutaneous (lcSSc), patients with multiple sclerosis and normal controls (NCs).
Lines 1-4 (dcSSc), 6-9 (lcSSc), 11-15 RRMS, 16-19 SPMS, 21-24 (NCs) show anti-UL83 reactive cases while lines, 5, 10, and 20 and 25 show anti-UL83 negative cases.

tive when 11-20 AU and positive when >20 AU.

Statistical analysis

All results are expressed as percentages (%). Differences between SSc patients, MS and NCs and between SSc groups were tested by two-tailed *t*-test. *P*-values smaller than or equal to 0.05 were considered significant. The statistical calculations were performed with Graph Pad Prism Software 5.

Results

Figure 1 illustrates representative anti-UL83 antibody testing by western immunoblotting. Anti-UL83 positivity (clear positivity and borderline) was detected in 69 (82.1%) anti-HCMV(+) SSc patients (in 35/40 [87.5%] lcSSc and in 34/44 [77.2% dcSSc patients, *p*=ns), compared to 22/30 (73.3%) of anti-HCMV(+) MS patients (SSc or SSc types *vs*. MS, *p*=ns) and in 17/28 (60.71%) anti-HCMV(+) NCs (SSc or lcSSc *vs* NC, *p*=0.02; dcSSc *vs*. NC, *p*=ns; total MS *vs*. NC, *p*=ns). Clear anti-UL83 positivity was detected in 50 (59.5%) anti-HCMV(+) SSc patients (in 21/40 lcSSc, and 29/44 dcSSc; lcSSc *vs* dcSSc, *p*=ns), compared to 15/30 (50%) anti-HCMV(+) MS patients (SSc or SSc types *vs*. MS, *p*=ns) and 11/28 (39.3%) of anti-HCMV(+) NCs (SSc *vs*. NC, *p=ns*; lcSSc *vs* NC; *p=ns*; dcSSc *vs*. NC, *p*=0.027; MS *vs*. NC, *p*=ns).

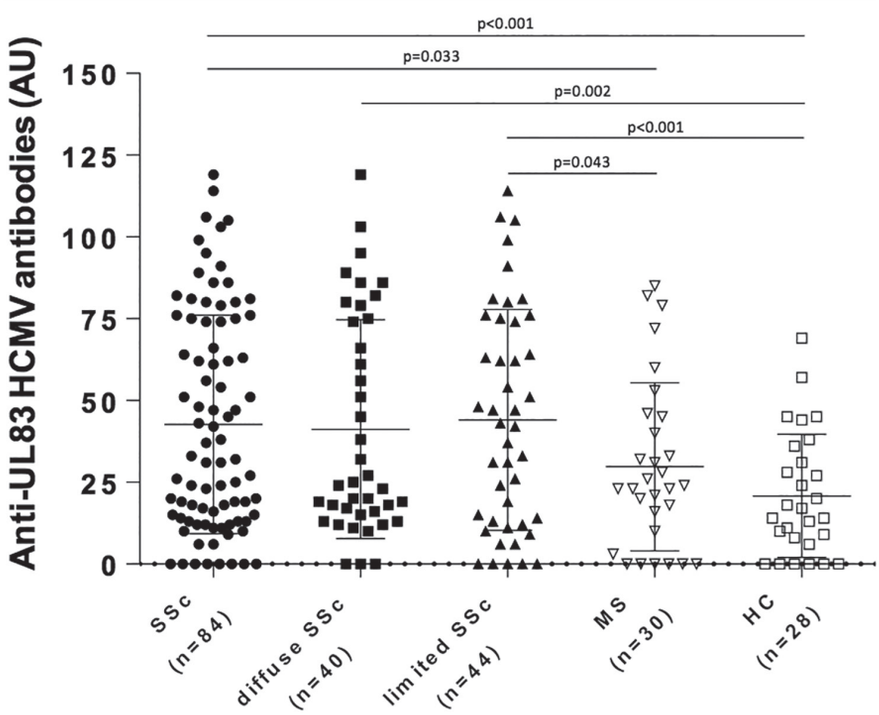

Fig. 2. Anti-UL83 human cytomegalovirus (HCMV) antibody levels in patients with systemic sclerosis (SSc), diffuse cutaneous systemic sclerosis (dcSSc) and limited cutaneous (lcSSc), patients with multiple sclerosis(MS) and normal controls (HCs). Antibody results expressed as arbitrary units (AU). The cut off value set by the manufacturer is 11 AU.

Anti-UL83 ab AU levels (mean ± SD) in patients and controls were as follows: 64.3 ± 26.0 in total SSc (62.9±25.5 in lcSSc *vs* 66.3±27.2 in dcSSc), 49.1±21.6 in total MS (44.2±18.4 in RRMS and 56.3±25.6 AU in SPMS; total SSc *vs* total MS, *p*=0.030; lcSSc *vs* total MS, *p*=ns; dcSSc *vs*. total MS, *p*=0.041; lcSSc *vs*. RRMS, *p*=0.027; lcSSc *vs*. SPMS, *p*=ns; dcSSc *vs* RRMS; *p*=0.017, dcSSc *vs* SPMS, *p*=ns) and 40.4±13.7 in NCs (total SSc or SSc types *vs*. NCs, *p*<0.001; total MS or MS types *vs*. NCs, ns) (Fig. 2).

Strong/very strong anti-UL83 immunoreactivity (>50 AUs) had 34 SSc patients (16 with lcSSc and 18 with dcSSc) 6 MS patients (2 RRMS and 4 SPMS) and 2/28 (7.1%) NCs (SSc or SSc types *vs*. NC, *p*<0.001; SSc *vs*. MS, *p*=0,043; dcSSc *vs*.. MS, *p*=0.049; lcSSc *vs*.. MS, *p*=0.074; MS *vs*. NC, *p*=ns). The strength of anti-UL83 immunoreactivity was further analysed by dilution experiments (1/200, 1/500, 1/5,000). Amongst 10 SSc patients with strongest anti-UL83 immunoreactivity, anti-UL83 positivity was detected in 10 patients at 1/200 dilution, in 8 at 1/500 dilution, and in 3 at 1/5,000 dilution.

No association was found between anti-UL83 abs and SSc-associated autoAbs (Table I). The most frequent co-existence of anti-UL83 plus autoAb reactivity was that with anti-Scl-70 (25/84, 29.8 %), followed by anti-CENP (16/84, 19%) and anti-RNA pol III (9/84, 10.7%). In patients with SSc, there was no difference between anti-UL83(+) and anti-UL83(-) in terms of demographics, type of SSc or clinical features (digital ulcers, pulmonary fibrosis or pulmonary arterial hypertension). However, pulmonary fibrosis was associated with high levels of anti-UL83 abs (clear positive) (*p*=0.039).

This association did not reach statistical significance in SSc patients with borderline and clear positive anti-UL83 abs (cut off value, 11 AUs), (46% *vs*. 26.4%, p=0.067) (Table I).

Discussion

Our study corroborates previous attempts to link HCMV, and in particular ab responses against UL83, with the development of SSc. We found that amongst anti-HCMV+ SSc patients and controls, anti-UL83 immunoreactivity was more frequent and stronger in SSc patients than in controls. In 2006, Namboodiri *et al*. (11) studied anti-UL83 ab levels in SSc patients and pathological controls (with osteoarthritis, fibromyalgia, gout, localised musculoskeletal pain syndromes), irrespective of anti-HCMV status and found increased anti-UL83 ab levels in SSc. Our study focusing on anti-HCMV+ patients and controls, showed that anti-UL83 immunoreactivity was frequent and strong in SSc. Furthermore, strong anti-UL83 rectivity was associated with SSc-associated lung fibrosis. UL83 is a likely candidate of HCMV-triggered antigen-specific autoimmune responses, since it is one of the most immunodominant antigens of virus-specific CTL and CD4 responses during natural HCMV infection (14). UL83 HCMV induces autoAb production in mice (15-16). We cannot exclude the possibility that the enhanced reactivity to UL83 may be related to immunogenetic or other factors related to SSc, since the adaptive immune system is activated in this disease.

In conclusion, our study in anti-HCMV(+) SSc patients clearly demonstrates high anti-UL83 immunoreativity that is associated with lung fibrosis. Large prospective studies investigating anti-UL83 antibody responses in early SSc patients may shed additional light as to whether or not there is a causal relation to HCMV in SSc.

Acknowledgments

We thank Dr C. Katsiari and Dr T. Simopoulou for patient recruitment. We thank Euroimmun AG, Lübeck, Germany for providing antigens, kits and reagents.

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