Identification of a novel autoantibody against heat shock factor 1 in idiopathic inflammatory myopathy

Y.-M. Zhang[1,2], L. Liang[1], H.-B. Yang[1], X.-M. Shu[1], X. Lu[1], G.-C. Wang[1,2], Q.-L. Peng[1]

[1]Department of Rheumatology, Beijing Key Lab for Immune-Mediated Inflammatory Diseases, China-Japan Friendship Hospital, Beijing, China;
[2]Graduate School of Peking Union Medical College, Beijing, China.

Abstract

Objective

Myositis autoantibodies show great utility in the diagnosis and clinico-serological phenotyping of idiopathic inflammatory myopathy (IIM). We identified a novel autoantibody against heat shock factor 1 (HSF1) and further evaluated its disease specificity and clinical significance in IIM patients.

Methods

A human protein microarray was used to identify autoantibodies in myositis sera. ELISA, immunoblot and dot blot assays were applied to examine anti-HSF1 autoantibodies in IIM patients and controls. Immunofluorescence was used to detect HSF1 expression in muscle tissues.

Results

Anti-HSF1 was identified as a novel autoantibody by protein microarray and the seroreactivity was confirmed by immunoprecipitation, ELISA, immunoblot and dot blot assays. Anti-HSF1 autoantibodies were present in 64/581 (11.0%) IIM, 4/37 (10.8%) rheumatoid arthritis, 5/40 (12.5%) primary Sjögren's syndrome, 2/40 (5%) systemic lupus erythematosus, while largely negative in healthy controls. Anti-HSF1 autoantibodies were significantly associated with pruritus, hypergammaglobulinaemia, and elevated erythrocyte sedimentation rate in IIM patients. Anti-HSF1 autoantibodies were more prevalent in cancer-associated myositis (CAM) compared to non-CAM patients (17.2% vs. 7.5%, p=0.009), nevertheless were undetectable in cancer controls. Meanwhile, cross-sectional and longitudinal analyses revealed positive correlations between anti-HSF1 levels and disease activity in IIM patients without cancer. Additionally, increased expression of HSF1 was found in regenerating muscle cells of myositis muscle tissues.

Conclusion

These data reveal anti-HSF1 as a new autoantibody associated with CAM in IIM. The autoimmunity against HSF1 may be involved in the immunopathogenesis of myositis.

Key words

idiopathic inflammatory myopathy, autoantibodies, heat shock factor 1, cancer-associated myositis, disease activity

Ya-Mei Zhang, MD
Lin Liang, PhD
Han-Bo Yang, PhD
Xiao-Ming Shu, MD, PhD
Xin Lu, MD, PhD
Guo-Chun Wang, MD, PhD
Qing-Lin Peng, PhD

Please address correspondence to:
Qing-Lin Peng,
Department of Rheumatology,
Beijing Key Lab for Immune-Mediated Inflammatory Diseases,
China-Japan Friendship Hospital,
2 Yinghua East Road,
Chaoyang District,
100029 Beijing, China.
E-mail: pqinglin@163.com

Guo-Chun Wang,
(address as above)
E-mail: guochunwang@hotmail.com

Received on November 8, 2019; accepted in revised form on January 27, 2020.

Funding: this work was supported by the National Natural Science Foundation of China (81701615, 81971531, 81571603) and Beijing Municipal Science and Technology Commission (Z181100001718063, Z171100001017208).

Competing interests: none declared.

Introduction

Idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of rare systemic diseases, characterised by proximal muscle weakness, elevated serum levels of muscle enzymes, inflammation on muscle biopsy and extra muscular manifestations, and are mainly comprised by dermatomyositis (DM), polymyositis (PM), and inclusion body myositis (IBM) (1, 2). A hallmark of IIM is the presence of serum autoantibodies which have been identified in 60–80% of IIM patients, and are traditionally divided into myositis-specific (MSAs) and myositis-associated autoantibodies (MAAs) (3). Each MSA seems to correlate with a specific clinical phenotype and was found almost exclusive in IIM patients. In contrast to MSAs, MAAs are associated with IIM and other autoimmune diseases such as systemic lupus erythematosus (SLE) and systemic scleroderma (SSc) (4). In addition to their diagnostic utility in clinical practice, MSAs exhibit great value for disease phenotyping and prognosis (5). Intriguingly, several myositis autoantigens have been found to be expressed in regenerating muscle fibers of IIM patients (6, 7), providing important clues into the potential immunopathogenic mechanisms of myositis. Therefore, further discovery of myositis autoantibodies may help to identify additional disease subsets within the myositis spectrum and gain further insights into its pathogenesis.

Most of the currently known MSAs were identified by immunoprecipitation assays and subsequent target antigen confirmation analyses. Another widely utilised approach for autoantigen identification is the serological analysis of cDNA expression libraries, which is most appropriate for cancer autoantigen identification. While clearly useful, this approach is slow, technically challenging, and unable to identify autoantigens requiring posttranslational modifications because it relies on bacterial protein expression. Functional protein microarrays are new tools that empower investigators with defined protein content for profiling serum samples to identify autoantigen biomarkers. Protein microarrays containing proteins expressed in eukaryotic cells and purified under native conditions, which therefore are expected to contain appropriate posttranslational modifications and maintain their native conformations, could be a powerful tool for autoantibody screening.

Herein, we employed serum autoantibody profiling analysis using a human protein microarray composed of about 9,374 full-length unique proteins and identified autoantibodies against heat shock factor 1 (HSF1) protein in the serum of DM patients. Further validation using ELISA, immunoblot, and dot blot assay in a large cohort confirmed HSF1 as a novel autoantigen in IIM. The clinical significance of anti-HSF1 autoantibodies in IIM and HSF1 expression in myositis muscle tissues were also investigated.

Materials and methods

Study design, patients, and clinical data

A cross-sectional and longitudinal study was designed to analyse the prevalence and clinical significance of anti-HSF1 autoantibodies in IIM. Overall, 581 IIM patients fulfilled 2017 EULAR/ACR classification criteria from China-Japan Friendship Hospital were enrolled in the cross-sectional study, including, 402 patients with DM, 71 patients with amyopathic dermatomyositis (ADM), and 108 patients with PM (8). Patients with onset age less than 18 years old or with other less common types of autoimmune myositis in China, such as IBM, were excluded. Cancer-associated myositis (CAM) was defined as a cancer diagnosis within 3 years before or after the onset of myositis (9). Interstitial lung disease (ILD) was diagnosed according to radiographic signs of ILD on HRCT and/or restrictive ventilatory defects (values of total lung capacity, vital capacity, and carbon monoxide transfer factor <80% of predicted) (10). Myositis disease activity assessment visual analog scales (MYOACT) were used to evaluate 6 extra-muscular organ systems, including constitutional, cutaneous, skeletal, gastrointestinal, pulmonary, and cardiac disease activity. Meanwhile, the disease activity of global extra-muscular, muscle, and physician global assessment

(PGA) was scored separately using a continuous 10-cm visual analog scale (VAS) (11). In the longitudinal study, 10 anti-HSF1$^+$ IIM patients were followed-up for 6 to 34 months, with a median follow-up duration of 16 months. During the follow-up, patients' serum samples were collected from every hospitalisation, and the interval between two adjacent samples was 1–20 months. Clinical improvement was defined as 2 of 3 core set measures (MYOACT, PGA and muscle scores) improved by ≥20%, with no more than 1 worsened by ≥25% (12).

The control groups included 65 healthy controls (HCs), 27 patients with cancer, and 117 patients with other autoimmune diseases, including 40 patients with primary Sjögren's syndrome (pSS), 40 patients with SLE, and 37 patients with rheumatoid arthritis (RA). Cancer diagnoses in IIM patients and control groups were all confirmed pathologically and are shown in Supplementary Table S1. The sex ratio and age distribution in HCs were matched to IIM patients.

All serum samples were routinely collected from patients before admission treatments and stored at -80°C during hospitalisation or outpatient clinic. Anti-HSF1 autoantibodies and/or MSAs in serum samples were evaluated by ELISA and/or commercially available kits (EUROIMMUN, Germany), respectively. Meanwhile, patients' demographic, clinical data and prescribed medical therapy were collected. This study was approved by the Ethnic Committee of the Institutional Review Board at the China-Japan Friendship Hospital (reference number: 2013-6), and all patients and controls provided written informed consent.

Screening new autoantigens in IIM by human protein microarray analysis

Human protein microarray analysis involving 10 DM patients and 10 HCs was applied for identifying new autoantigens that could be used to statistically differentiate the two populations. Among the 10 DM patients, 8 were negative with currently known myositis autoantibodies, 2 were positive with autoantibodies against melanoma differentiation-associated gene-5 (MDA5), which were used as internal controls for the microarray analysis. ProtoArray® Human Protein Microarray v. 5.1 (Life Technologies, USA) was applied in our study (detailed in Supplementary Methods).

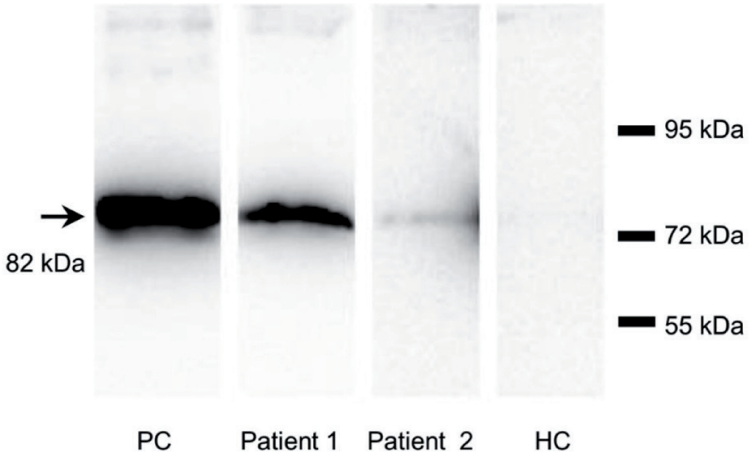

Fig. 1. Immunoprecipitation followed by western blotting analysis of anti-HSF1 autoantibodies in the serum of DM patients.
Serum from 2 DM patients who were identified as positive with anti-HSF1 autoantibodies using the protein microarray analysis, and 1 HC were used to immunoprecipitate HSF1 from buffer containing 0.6 ng/μl recombinant HSF1 protein derived from E. coli (Abcam), then immunoprecipitates were immunoblotted with a monoclonal anti-HSF1 antibody. PC: positive control; HC: healthy control; HSF1: heat shock factor 1; DM: dermatomyositis.

Anti-HSF1 ELISA

Briefly, 96-well ELISA plates (Thermo Scientific, Roskilde, Denmark), coated with 200 ng/well of recombinant full-length human HSF1 protein (Abcam, Cambridge, UK), were developed to detect anti-HSF1 autoantibodies in serum samples. In addition, another recombinant HSF1 protein (OriGene, Maryland, USA) was applied for ELISA to detect the autoantibodies. The dilution range of serum samples was examined by serial dilution ELISA in 1:2 dilution steps. All serum samples were examined in duplicate, and specific anti-HSF1$^+$ and anti-HSF1$^-$ serum measurements on every plate ensured the stability and reproductivity of the assay. A cut-off value was defined as the mean A_{450} plus 2 times the SD of the HC group to discriminate between anti-HSF1$^+$ and anti-HSF1$^-$ serum samples. Moreover, a blocking ELISA was performed to validate the specificity of the anti-HSF1 ELISA results (detailed in Supplementary Methods in the online Supplementary file).

Immunoblot and dot blot assay

HSF1 protein (Abcam, Cambridge, UK) was electrophoresed on 10% SDS-polyacrylamide gels with 1 μg/lane, and then transferred to nitrocellulose membranes. For the dot blot assay, HSF1 protein (200 ng/dot) was applied onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with serum from patients and HCs, followed by incubation with a horseradish peroxidase (HRP)-conjugated anti-human secondary antibody (Abcam, Cambridge, UK). Stained bands or dots were visualised by ECL (GE Healthcare, USA; detailed in Supplementary Methods).

Immunofluorescence staining

Frozen muscle sections from IIM patients and HCs were fixed with 95% alcohol, and non-specific protein binding was blocked with normal goat serum at room temperature (RT) for 1 hr. The sections were then incubated with antibodies against neural cell adhesion molecule (NCAM, 1:600; Abcam, Cambridge, UK) and HSF1 (1:200; Abcam, Cambridge, UK) overnight at 4°C, followed by incubation with a goat anti-mouse Alexa Fluor® 555 (Abcam, Cambridge, UK) and a goat anti-rabbit Alexa Fluor® 488 (Abcam, Cambridge, UK) secondary antibody at RT for 1 hr. Specimens were observed under a fluorescence microscope (Olympus, Japan).

Statistical analysis

Continuous data were expressed as

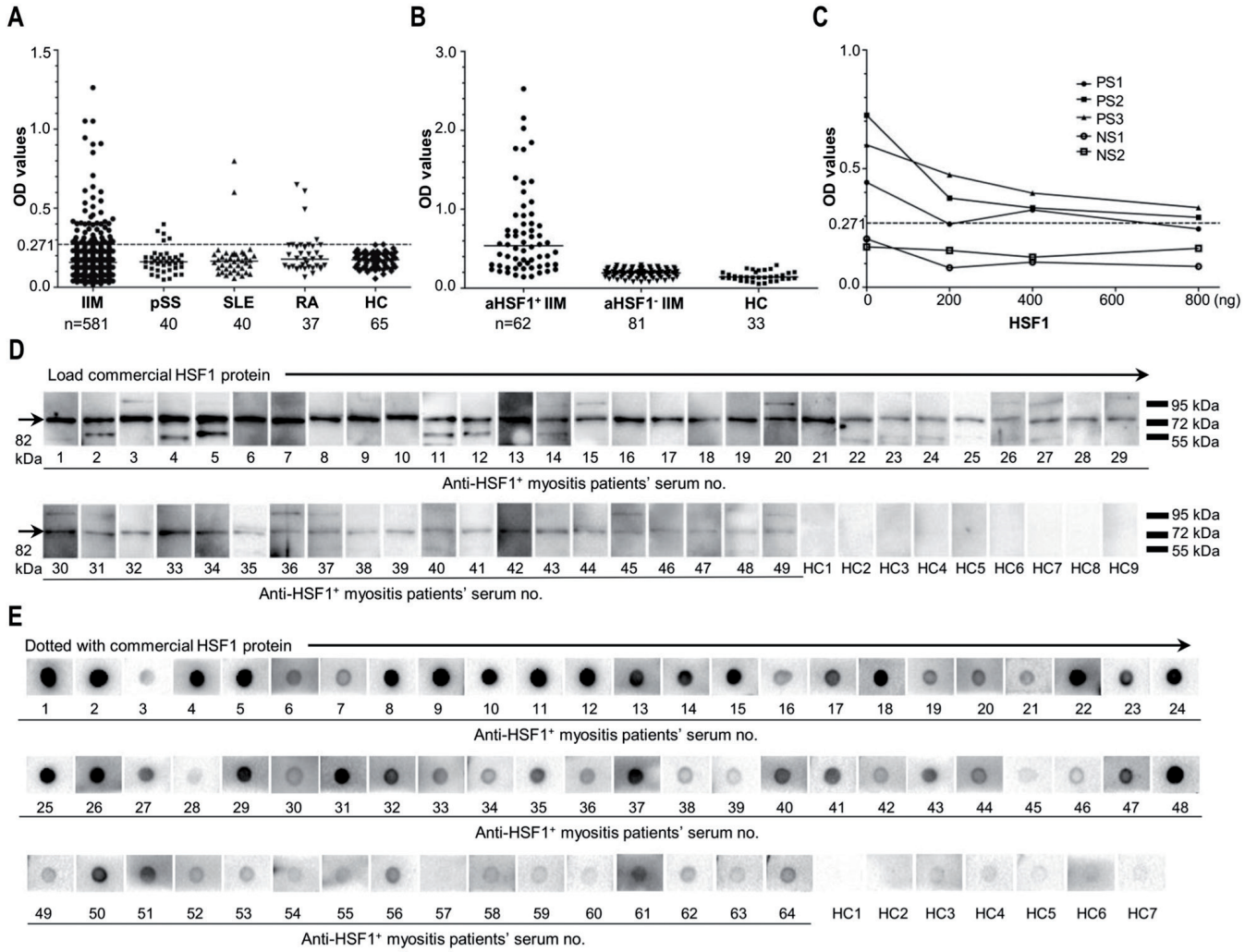

Fig. 2. The presence of anti-HSF1 autoantibodies in patients with IIM and other autoimmune diseases.
A: Serum from IIM (n=581), pSS (n=40), SLE (n=40), RA (n=37), and HCs (n=65) were examined by ELISA for anti-HSF1 autoantibodies.
B: Anti-HSF1 positivity was confirmed by another ELISA using a different commercially available recombinant HSF1 protein (OriGene). Serum from anti-HSF1$^+$ (n=62) IIM patients, randomly selected anti-HSF1$^-$ (n=81) IIM patients, and HCs (n=33) were examined.
C: Three positive samples (PS) and two negative samples (NS) for anti-HSF1 were preincubated with recombinant HSF1 protein, and then were tested by ELISA for anti-HSF1 positivity.
D: Immunoblotting results of 49 anti-HSF1$^+$ IIM patients and 9 HCs.
E: Dot blotting results of 64 anti-HSF1$^+$ IIM patients and 7 HCs. The horizontal line in A and C indicates the cut-off value (cut-off = mean [A450 HCs] + 2×SD) used for discriminating anti-HSF1$^+$ patients from anti-HSF1$^-$ patients.
HSF1: heat shock factor 1; IIM: idiopathic inflammatory myopathy; pSS: primary Sjögren's syndrome; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; HCs: healthy controls; ELISA: enzyme-linked immunosorbent assay; PS: positive samples; NS: negative samples.

median and interquartile range (IQR), and dichotomous variables were presented as percentages and absolute frequencies. Mann-Whitney U-test was used to compare continuous data, while χ^2 test or Fisher's exact test was used to compare categorical variables. Logistic regression analysis was performed to analyse the association between anti-HSF1 autoantibodies and cancer risk in IIM patients. Spearman's rank correlation test and generalised estimating equation model (GEE) were used to analyse relationships between serum anti-HSF1 levels and disease activity. SPSS (SPSS institute, USA) or GraphPad Prism 5.0 (GraphPad Software, USA) was used for data management and statistical analyses. *p*-values less than 0.05 were considered significant.

Results

HSF1 was identified as a novel autoantigen by using a protein microarray analysis

We screened 9,374 purified human proteins by a protein microarray analysis using serum from 10 DM patients and 10 HCs. In the serum from the 2 anti-MDA5$^+$ DM patients, which were referred as internal controls, we found MDA5 as one of the top ranked proteins on the list of potential autoantigens, qualifying the feasibility of the microarray analysis for broad-scale profiling of autoantibody reactivity. We identified higher serum reactivity towards HSF1 in 2 of the 10 DM patients compared to 10 HCs, which is crucial in maintaining cellular homeostasis and was demonstrated to be involved in mouse skeletal muscle plasticity

(13-16). Therefore, HSF1 was selected for further investigation.

Anti-HSF1 autoantibodies were found in IIM patients

First, we carried out immunoprecipitation analysis to confirm the seroreactivity of those two samples which were identified as anti-HSF1 positive in protein microarray analysis, and the results showed both of the samples were tested as positive (Fig. 1).

We next determined the prevalence of anti-HSF1 autoantibodies in patients with IIM and other autoimmune diseases. Recombinant HSF1 protein derived from *E. coli* (Abcam) was used to detect anti-HSF1 autoantibodies in patients' serum by ELISA. Serial dilution ELISA was used to determine the optimal dilution ratio (1:50) of serum samples for anti-HSF1 detection (Suppl. Fig. 1). We defined a cut-off value to discriminate anti-HSF1$^+$ patients from anti-HSF$^-$ patients (cut-off = mean [A450 HC] + 2 × SD = 0.271). Anti-HSF1 autoantibodies could be detected in 64/581 (11.0%) of IIM patients, 4/37 (10.8%) of RA patients, 5/40 (12.5%) of pSS patients, 2/40 (5%) of SLE patients, and 2/65 (3.1%) of HCs (Fig. 2A). Of note, the positive and negative samples identified in the protein microarray analysis were confirmed by ELISA, indicating the reliability of these different methodologies. Further, anti-HSF1 positivity in IIM was subsequently confirmed by another ELISA using a recombinant HSF1 protein derived from the eukaryotic HEK293 expression system (cut-off = mean [A450 HC]+2 × SD=0.270; OriGene, Fig. 2B). There was good agreement (κ=0.757, p<0.001) and correlation (r=0.610, p<0.001, Suppl. Fig. 2) between anti-HSF1 levels obtained from these two ELISA assays using different HSF1 proteins.

Specificity of the anti-HSF1 ELISA was confirmed by blocking anti-HSF1 autoantibodies in serum samples with recombinant HSF1 protein, and the resulting data showed a significantly decreased signal in blocked samples (Fig. 2C). Moreover, immunoblot and dot blot assay results verified anti-HSF1 positivity in patients with IIM (Fig. 2D-E) and other autoimmune diseases

Table I. Comparison of clinical characteristics of IIM patients with and without anti-HSF1 autoantibodies.

Variables	Anti-HSF1$^+$ (n=64)	Anti-HSF1$^-$ (n=517)	Total (n=581)	p-value
Sex (M/F)	27/37	153/364	180/401	0.040
Age, median (IQR), years	51.5 (40, 60)	49 (39.5, 58.5)	50 (40, 59)	0.138
Diagnosis, n (%)				
ADM	12 (18.8)	59 (11.4)	71 (12.2)	0.091
DM	45 (70.3)	357 (69.1)	402 (69.2)	
PM	7 (10.9)	101 (19.5)	108 (18.6)	
Treatment-naive, n (%)	29 (45.3)	174 (33.7)	203 (34.9)	0.065
Weight loss[a] n (%)	20 (31.3)	117 (22.6)	137 (23.6)	0.125
With CAM[b] n (%)	11 (17.2)	39 (7.5)	50 (8.6)	0.009
Muscle involvement n (%)				
Muscle weakness	38 (59.4)	343 (66.3)	381 (65.6)	0.268
Myalgia	25 (39.1)	213 (41.2)	238 (41.0)	0.743
Skin manifestations n (%)				
Gottron's papules	35 (54.7)	239 (46.2)	274 (47.2)	0.201
Heliotrope rash	27 (42.2)	215 (41.6)	242 (41.7)	0.927
Shawl sign	15 (23.4)	143 (27.7)	158 (27.2)	0.474
V-sign	22 (34.4)	197 (38.1)	219 (37.7)	0.561
Pruritus	31 (48.4)	181 (35.0)	212 (36.5)	0.035
Extramuscular involvement n (%)				
Interstitial lung disease[c]	37 (57.8)	305 (59.2)	342 (59.1)	0.829
Dysphagia	10 (15.6)	119 (23.0)	129 (22.2)	0.180
Arthritis	16 (25.0)	119 (23.0)	135 (23.2)	0.723
Autoantibodies n (%)				
ANA[d]	44 (69.8)	304 (60.6)	348 (61.6)	0.153
Myositis autoantibodies positive[e]	50 (78.1)	368 (72.9)	418 (73.5)	0.370
MDA5[e]	17 (26.6)	95 (18.8)	112 (19.7)	0.142
TIF1γ[e]	13 (20.3)	61 (12.1)	74 (13.0)	0.065
NXP2[e]	4 (6.3)	38 (7.5)	42 (7.4)	0.713
Jo-1[e]	6 (9.4)	41 (8.1)	47 (8.3)	0.731
PL-7[e]	1 (1.6)	29 (5.7)	30 (5.3)	0.266
PL-12[e]	1 (1.6)	12 (2.4)	13 (2.3)	1.000
EJ[e]	2 (3.1)	17 (3.4)	19 (3.3)	1.000
OJ[e]	0 (0.0)	0 (0.0)	0 (0.0)	-
SRP[e]	1 (1.6)	38 (7.5)	39 (6.9)	0.075
Mi-2[e]	3 (4.7)	35 (6.9)	38 (6.7)	0.498
SAE[e]	2 (3.1)	10 (2.0)	12 (2.1)	0.890
HMGCR[e]	1 (1.6)	16 (3.2)	17 (3.0)	0.748
SRP[e]	1 (1.6)	38 (7.5)	39 (6.9)	0.130
Ku[e]	2 (3.1)	9 (1.8)	11 (1.9)	0.800
PM-Scl100[e]	2 (3.1)	6 (1.2)	8 (1.4)	0.224
PM-Scl75[e]	2 (3.1)	18 (3.6)	20 (3.5)	1.000
Laboratory parameters n (%)				
CK >200IU/L[f]	26 (44.8)	214 (46.0)	240 (45.9)	0.863
LDH >250IU/L[g]	34 (55.7)	282 (55.5)	316 (55.5)	0.973
IgG >1620mg/dL[h]	23 (40.4)	88 (19.2)	111 (21.5)	<0.001
CRP ≥0.8mg/dL[i]	24 (40.7)	148 (29.7)	172 (30.9)	0.085
ESR >20mm/h[j]	31 (52.5)	172 (34.5)	203 (36.4)	0.006
SF >306.8ng/mL[k]	20 (47.6)	127 (39.4)	147 (40.4)	0.31

IIM: idiopathic inflammatory myopathy; DM: dermatomyositis; ADM: amyopathic dermatomyositis; PM: polymyositis; IQR: interquartile range; ANA: antinuclear antibodies; CK: creatine kinase; LDH: lactate dehydrogenase; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; SF: serum ferritin; [a]Weight loss: unintentional weight loss of 5% or more in body weight; [b]CAM (cancer-associated myositis): the diagnosis of cancer within 3 years before or after the onset of myositis; Myositis autoantibodies that currently known, including autoantibodies against Mi-2, TIF1γ (transcriptional intermediary factor1γ), MDA5 (melanoma differentiation-associated gene-5), NXP2 (nuclear matrix protein-2), Jo-1 (histidyl-tRNA synthetase), PL-7 (threonyl-tRNA synthetase), PL-12 (alanyl-tRNA synthetase), EJ (glycyl-tRNA synthetase), OJ (isoleucyl-tRNA synthetase), SRP (signal recognition particle), HMGCR (3-hydroxy-3-methylglutary-CoA reductase), SAE (SUMO-1-activating enzyme heterodimer), Ku, PM-Scl100, PM-Scl75. [c]Data available for 579 patients, [d]Data available for 565 patients, [e]Data available for 569 patients, [f]Data available for 523 patients, [g]Data available for 569 patients, [h]Data available for 516 patients, [i]Data available for 557 patients, [j]Data available for 558 patients, [k]Data available for 364 patients.

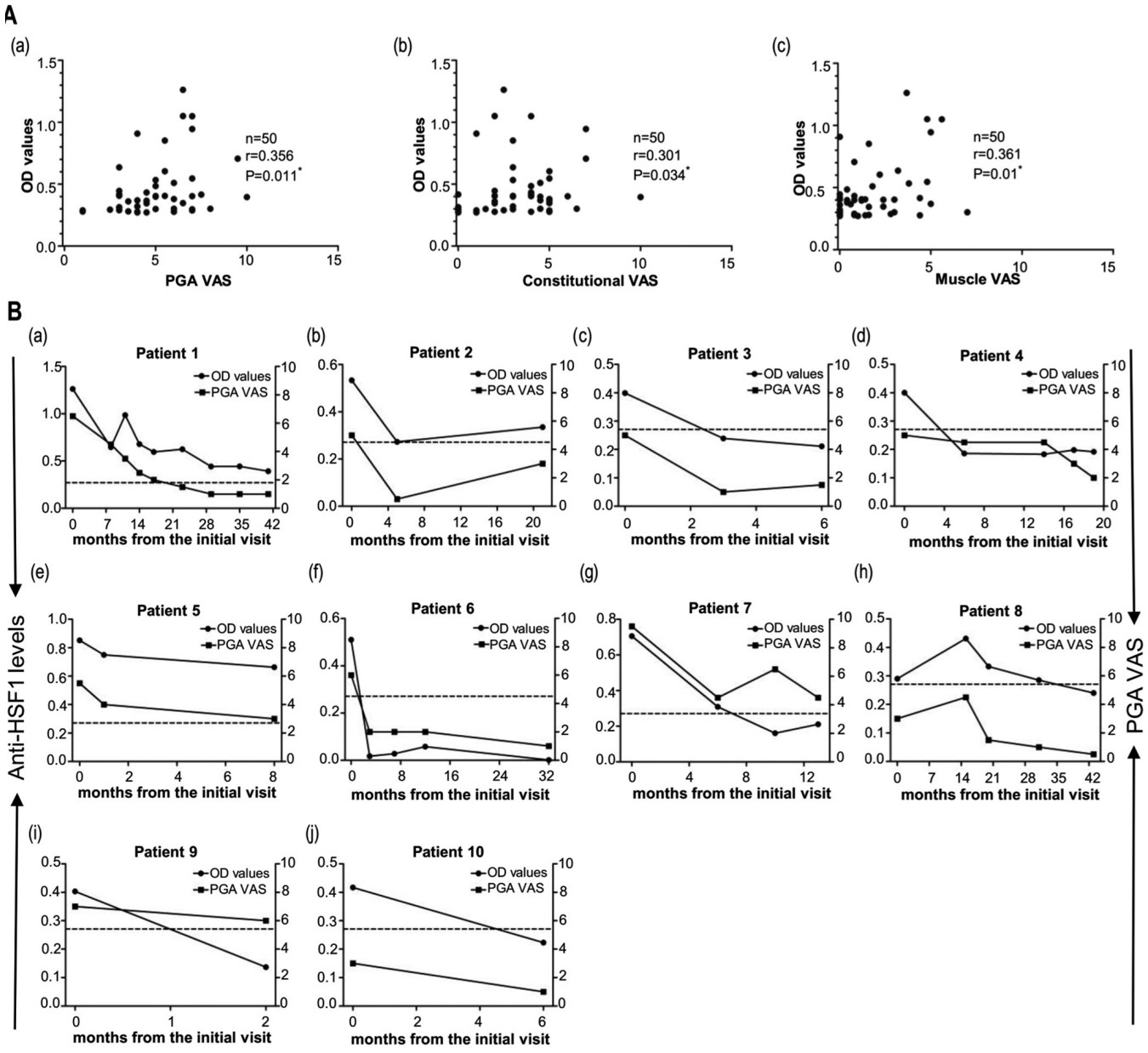

Fig. 3. Serum anti-HSF1 levels are associated with disease activity in anti-HSF1$^+$ IIM patients without cancer (cross-sectional and longitudinal analyses).
A: Spearman correlation analyses were used to detect the correlations of the serum autoantibodies levels with disease activity in 50 anti-HSF$^+$ IIM patients without cancer, resulting data showed significant positive correlations of serum anti-HSF1 with PGA VAS (r=0.356, p=0.011), constitutional VAS (r=0.301, p=0.034), muscle VAS (r=0.361, p=0.01).
B: Longitudinal changes in serum anti-HSF1 levels and PGA VAS over time in 10 anti-HSF$^+$ IIM patients without cancer. The horizonal line indicates the cut-off value (cut-off = mean [A450 HC] + 2×SD=0.271) used for discriminating anti-HSF1$^+$ patients from anti-HSF1$^-$ patients. *p<0.05.
HSF1: heat shock factor 1; IIM: idiopathic inflammatory myopathy; PGA: physician global assessment of disease activity; VAS: visual analogue scales.

(Suppl. Fig. 3A-B). We also obtained similar results in immunoblot using both commercial recombinant proteins (data not shown).

Clinical and laboratory features of IIM patients with anti-HSF1 autoantibodies

Anti-HSF1$^+$ IIM patients developed pruritus (p=0.035) more often than anti-HSF1$^-$ IIM patients. Meanwhile, patients with anti-HSF1 autoantibodies were found to have a higher frequency of CAM (17.2% *vs*. 7.5%, p=0.009), anti-transcriptional intermediary factor1γ (TIF1γ) autoantibodies (20.3% *vs*. 12.1%, p=0.065, with border statistical significance), hypergammaglobulinaemia (p<0.001), and elevated erythrocyte sedimentation rate (ESR, p=0.006) compared to anti-HSF1$^-$ patients. Interestingly, anti-HSF1$^+$ IIM patients with other myositis autoantibodies were found to have a higher prevalence of muscle weakness (66.0% *vs*. 35.7%, p=0.041) and higher serum LDH levels [291.0 (227.5, 416) IU/L *vs*. 201.5 (180.0, 295.8) IU/L, p=0.030] than anti-HSF1$^+$ IIM patients without other myositis autoantibodies. No other clinical or laboratory features of IIM were found associated with anti-HSF1 autoantibodies, such as skin manifesta-

Table II. Associations between anti-HSF1 autoantibodies and cancer risk in IIM patients.

Univariate analysis			Multivariate analysis		
Risk factors	OR (95% CI)	*p*-value	Covariates in model	OR (95% CI)	*p*-value
Sex	1.2 (0.6-2.2)	0.634	Anti-HSF1, age		
Age	1.0 (1.0-1.1)	0.001	Anti-HSF1	2.4 (1.1-4.9)	0.023
Autoantibodies			Age	1.0 (1.0-1.1)	0.002
Anti-HSF1	2.5 (1.2-5.3)	0.012	Anti-TIF1γ, age		
Anti-TIF1γ	16.1 (8.3-31.4)	<0.001	Anti-TIF1γ	14.7 (7.5-28.9)	<0.001
Anti-HSF1 or anti-TIF1γ	10.6 (5.4-20.6)	<0.001	Age	1.0 (1.0-1.1)	0.015
			Anti-HSF1 or anti-TIF1γ, age		
			Anti-HSF1 or anti-TIF1γ	9.7 (4.9-19.0)	<0.001
			Age	1.0 (1.0-1.1)	0.009

OR: odds ratio; 95% CI: 95% confidence interval; IIM: idiopathic inflammatory myopathy; HSF1: heat shock factor 1; TIF1γ: transcriptional intermediary factor1γ.

tions, ILD, dysphagia or elevated muscle enzymes, as shown in Table I. In addition, we found that the antinuclear autoantibodies (ANA) pattern of anti-HSF1$^+$ patients showed mostly speckled and cytoplasmic staining (Suppl. Fig. 4).

Associations between anti-HSF1 autoantibodies and cancer risk in IIM patients

In our cohort, cancer was present in 50/581 (8.6%) IIM patients, including patients with urinary genital (23/50, 46.0%), gastrointestinal tract (9/50, 18.0%), respiratory (7/50, 14.0%), haematological (3/50, 6.0%), and endocrine (8/50, 16.0%) cancer. Anti-HSF1 autoantibodies were found positive in 22.0% (11/50) CAM patients, however, no detectable anti-HSF1 autoantibodies were found in 27 cancer controls (Suppl. Fig. 5). All of the 11 anti-HSF1$^+$ CAM patients carried other MSAs, including 7 (63.6%) with anti-TIF1γ, 1 (9.1%) with anti-NXP2, 1 (9.1%) with anti-SAE, and 2 (18.2%) with anti-Jo1 autoantibodies. Further, we examined associations among demographic features, anti-HSF1 autoantibodies, MSAs, and cancer. On univariate analysis, we found that age (OR 1.0 [95%CI 1.0–1.1], p=0.001), autoantibodies to HSF1 (OR 2.5 [95%CI 1.2–5.3], p=0.012) and autoantibodies to either HSF1 or TIF1γ (OR 10.6 [95%CI 5.4–20.6], p<0.001) were significantly associated with cancer. By multivariate analysis, an increased cancer risk (after adjusted age) in IIM patients with autoantibodies to HSF1 (OR 2.4 [95%CI 1.1–4.9], p=0.023) and either HSF1 or TIF1γ (OR 9.7 [95%CI 4.9–19.0], p<0.001) remained, as shown in Table II.

Correlation between serum anti-HSF1 levels and disease activity in IIM patients

We first conducted a cross-sectional study among anti-HSF1$^+$ IIM patients to determine the potential associations between serum anti-HSF1 levels and disease activity. A correlation analysis was applied in 50 anti-HSF1$^+$ IIM patients without cancer, and we found that serum anti-HSF1 levels were positively correlated with PGA VAS (r=0.356, p=0.011), constitutional VAS (r=0.301, p=0.034), and muscle VAS (r=0.361, p=0.01), as shown in Figure 3A. However, no statistical correlations between anti-HSF1 levels and disease activity were found in IIM patients with cancer (Suppl. Fig. 6, all p>0.05).

We then analysed the variation of anti-HSF1 levels in longitudinal samples derived from 10 anti-HSF1$^+$ IIM patients without cancer to further explore the association between anti-HSF1 levels and disease activity. Of these 10 patients, 9 (90%) patients reached clinical improvement, and 1 (10%) patient experienced relapse according to our criteria. During the follow-up, a fluctuating reduction in serum anti-HSF1 levels was observed in all 9 patients with clinical improvement, among whom, 7 patients turned to be anti-HSF1 negative at the last visit. For the remaining 1 patient with disease relapse, serum anti-HSF1 levels increased along with worsening disease (Fig. 3B and Suppl. Fig. 7). The GEE model revealed that anti-HSF1 levels were positively correlated with PGA VAS (β=2.9, p=0.001), muscle VAS (β=4.3, p<0.001), and pulmonary VAS (β=4.7, p<0.001) (Suppl. Table S2).

HSF1 expression in myositis muscle tissues

Double staining showed co-expression of HSF1 and NCAM in muscle tissues of IIM patients, indicating that HSF1 may be highly expressed in regenerating muscle cells (Fig. 4). In addition, increased expression of HSF1 in NCAM$^+$ regenerating muscle cells was not restricted to anti-HSF1$^+$ patients, since it was also found in muscle biopsy of anti-HSF1$^-$ IIM patients.

Discussion

This study described a newly recognised autoantibody in IIM, anti-HSF1, which was identified using human protein microarray profiling analysis. Anti-HSF1 autoantibodies were detected in CAM patients but not present in cancer controls. Further, moderate correlations between anti-HSF1 levels and clinical indicators of myositis disease activity were demonstrated in anti-HSF1$^+$ IIM patients without cancer. Of interest, the autoantibody target, HSF1, was highly expressed in regenerating muscle cells, suggesting that HSF1 may play a role in the process of myogenesis. However, anti-HSF1 autoantibodies were considered to be MAAs rather than MSAs because they were also found in RA, pSS and SLE patients.

HSF1 is constitutively expressed in most human cells, appears to be in an inactive monomeric form in the nucleus and cytoplasm in a non-stressed state.

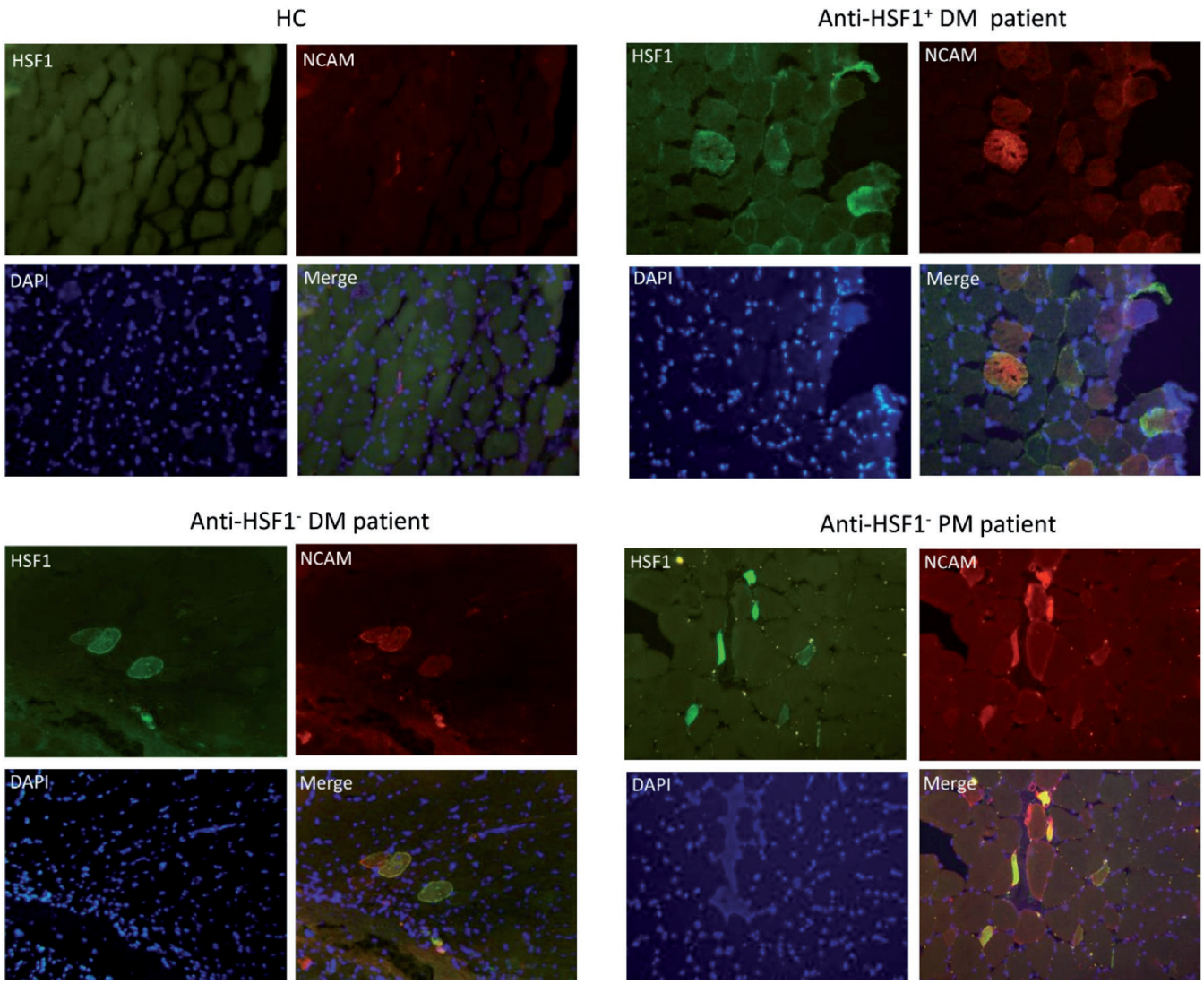

Fig. 4. HSF1 expression in myositis muscle.
Skeletal muscle frozen sections from representative DM (n=2) and PM (n=1) patients (1 was anti-HSF1 positive and 2 were anti-HSF1 negative) and 1 HC were stained with a rabbit monoclonal antibody against HSF1 (green) and a mouse monoclonal antibody against NCAM (red). DNA was visualised by staining with DAPI (blue). Merged images illustrated that HSF1 was co-expressed with NCAM.
HSF1: heat shock factor 1; DM: dermatomyositis; PM: polymyositis; HC: healthy controls; NCAM: neural cell adhesion molecule.

Once triggered, HSF1 becomes trimerised and phosphorylated, and then accumulates into the nucleus to regulate transcription of heat shock protein (HSP) and non-HSP related genes encoding proteins involved in stress-induced circumstances and normal biological processes (13, 17). In contrast to the low expression in normal tissues, HSF1 is overexpressed in various tumours such as ovarian cancer, breast cancer, endometrial carcinoma, and hepatocellular carcinoma (18-21). Increasing evidence has demonstrated that HSF1 is constitutively active in cancer cells, and plays a critical role in carcinogenesis (22). Wilson *et al*. identified IgA autoantibodies against phosphorylated HSF1 in early-stage, high-grade serious ovarian cancer (HGSOC), while other Ig types of the autoantibodies or autoantibodies towards other variants of HSF1 were not found in HGSOC (23). In our study, IgG autoantibodies against non-phosphorylated HSF1 were not found in the 5 ovarian cancer controls, but were present in 3 CAM patients with ovarian cancer. Strikingly, we also found the autoantibodies in 8 CAM patients with other cancers, including 2 with esophageal cancer, 2 with thyroid cancer, and 1 each with gastric, breast, cervical, and nasopharynx cancer. Yet, the autoantibodies could not be detected in the matched cancer controls. Thus, IgG autoantibodies against non-phosphorylated HSF1 may not be makers for cancer immunity, but may be involved in CAM.

Increased overall cancer risks, ranging from 20% to 25%, have been reported in IIM patients by numerous epidemiological studies (24). In recent years, accumulating evidence has shown that IIM patients with certain MSAs such as anti-TIF1γ, anti-NXP2, anti-HMGCR, and anti-SAE autoantibodies are at an increased cancer risk (25-28). Of particular interest, these cancer-associated autoantibodies in IIM are unlikely to improve risk stratification for cancer in

patients without rheumatic disease (29, 30). Further, it has been speculated that other currently unknown autoantigens may be involved in CAM, and MSAs$^-$ IIM patients also have an increased cancer risk (31). Thus, identifying new autoantibodies associated with CAM is of great interest. Here, we found 17.2% anti-HSF1$^+$ IIM patients had cancer, and these patients were all seropositive for MSAs such as anti-NXP2, anti-SAE, anti-Jo-1, and especially anti-TIF1γ autoantibodies. Nevertheless, anti-HSF1 autoantibodies were still associated with an increased cancer risk when multivariate analysis adjusted for age and sex was performed, suggesting that anti-HSF1 autoantibodies might be new makers for CAM.

A number of recent studies has highlighted that MSAs/MAAs can disappear with disease remission in IIM patients, which indicates the autoantibodies titers may be useful in monitoring disease activity (5). Serum levels of autoantibodies against SRP, HMGCR, MDA5, Jo-1, and NXP2 have all been demonstrated to correlate with myositis disease activity (32-36). Similarly, in the cross-sectional and longitudinal analysis of anti-HSF1$^+$ IIM patients, we found moderate correlations between autoantibodies levels and myositis disease activity, however only in IIM patients without cancer.

The implication for anti-HSF1 autoantibodies as biomarkers of clinical disease activity led to potential insights regarding the immunopathogenesis of myositis. McArdle *et al*. investigated HSF1 expression kinetics in differentiating C2C12 cells, and they found that HSF1 expression was increased and remained elevated during the 10-day differentiation induction process (37). In our study, we found high HSF1 expression in regenerating muscle cells in myositis muscle tissues, which was not detected in normal muscle cells. Accordingly, we speculated that high HSF1 expression during myogenesis may be associated with the development of anti-HSF1 autoantibodies. However, we detected high levels of HSF1 in muscle tissues from IIM patients that do not produce anti-HSF1 autoantibodies. It is, therefore, possible that HSF1 autoimmunity in myositis also requires additional contributions, including the specific human leukocyte antigen (HLA) complex. Arouche-Delaperche et al. demonstrated that anti-SRP and anti-HMGCR autoantibodies could directly induce muscle fiber atrophy and impair myoblast fusion, suggesting that myositis autoantibodies may be pathogenic (38). Previous studies identified that the cardiotoxin injection-induced and loading/heat shock-associated regrowth of soleus muscles were depressed in HSF1 null mice, suggesting that HSF1 may play a role in the plasticity of mouse skeletal muscle (14-16, 39). Therefore, anti-HSF1 autoantibodies in the serum of IIM patients may penetrate regenerating muscle cells and reach their intracellular target to affect myogenesis. Further, it has been proposed that the cross-reactivity of myositis autoantigens between cancer and regenerating muscle tissues may be a potential mechanism for the development of CAM (40). HSF1 is overexpressed in a variety of human cancers (41), and we also found increased HSF1 expression in the nucleus of HeLa cells by immunofluorescence (data not shown). Thus, there may be HSF1 cross-reactivity between cancer and immature muscle cells, which may affect muscle regeneration.

However, there were several limitations in our study. First, only a relatively small number of CAM patients were involved in our study, thus it was difficult to clearly clarify the clinical significance of anti-HSF1 autoantibodies in the CAM group. Second, further follow-up study is required to confirm the prognostic significance of anti-HSF1 autoantibodies in IIM. Third, further investigation is needed to evaluate the potential clinical associations of anti-HSF1 autoantibodies in patients with other autoimmune diseases.

Conclusions

To conclude, we identified anti-HSF1 as new cancer-associated autoantibodies. Further investigations to identify HLA alleles associated with anti-HSF1 autoantibodies and antigenic epitopes of their target may provide insights into the possible role of autoimmunity against HSF1 in IIM patients.

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