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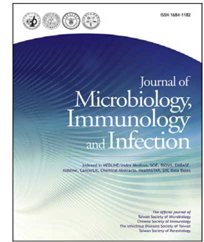

ORIGINAL ARTICLE

Detection of anti-p155/140, anti-p140, and antiendothelial cells autoantibodies in patients with juvenile dermatomyositis

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Juvenile dermatomyositis (JDM)

Background/Purpose: The pathogenesis of juvenile dermatomyositis (JDM), the most common idiopathic inflammatory myopathy in children, is unclear. The identification of novel autoantibodies in JDM may have clinical implications. The aim of this study was to assess the presence of anti-p155/140, anti-p140 antibodies, and antiendothelial cells antibodies (AECA) in patients with JDM and to correlate autoantibodies with clinical manifestations.
Methods: Serum AECA against human umbilical vein endothelial cells were detected by enzyme-linked immunosorbent assay in 25 patients with JDM and 17 normal controls. Immunoblotting was performed to detect serum anti-p155/140 and anti-p140 antibodies.
Results: Patients with JDM had significantly higher serum levels of AECA than healthy controls ($p = 0.002$). Nineteen patients (76%) and five control patients (29.4%) had positive AECAs ($p = 0.003$). The cutoff point of serum levels of AECA was determined by the receiver operating characteristic (ROC) curve analysis. Anti-p155/140 and anti-p140 antibodies were detected in 9 patients and 7 patients with JDM (36% and 28%, respectively). Anti-p155/140 antibodies were significantly associated with higher proportion of ESR elevation (100% vs. 0%, $p = 0.006$), higher erythrocyte sedimentation rate levels at diagnosis (40.3 ± 15.5 vs. 13.4 ± 5.3, $p = 0.019$), and a younger age at diagnosis (5.2 ± 3.2 years vs. 8.0 ± 3.0 years, $p = 0.03$).

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Conclusion: anti-p155/140, anti-p140, and AECA antibodies are significantly associated with JDM. The roles of autoantibodies in the pathogenesis await further investigation.
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Introduction

Juvenile dermatomyositis (JDM) is a rare autoimmune disease and is the most common subset, representing up to 85% of idiopathic inflammatory myopathy (IIM) in children. It is a systemic vasculopathy characterized by symmetrical proximal muscle weakness, raised serum concentrations of muscle enzymes, and pathognomonic skin rashes that include the heliotrope rash over the eyelids and Gottron papules over the extensor joint surfaces. In addition to the skin and skeletal muscle, vasculopathy of JDM also can affect the gastrointestinal tract, lungs, kidneys, eyes, and heart.[1] In contrast to adults with dermatomyositis (DM), cancer-associated myositis is rare in JDM.[2] The etiology of JDM remains unclear. The pathogenesis involves environmental triggers (such as viral infection), immune dysfunction with upregulation of type I interferon response, and perivascular infiltration of B and $CD4^+$ T cells and dendritic cells in the muscle, skin and small vessel endothelium, in genetically susceptible individuals.[1]

Because of the heterogeneity of the nature of the disease, the clinical outcome is difficult to predict. Autoantibodies as serological markers are associated with specific clinical features, immunogenetics, treatment response, and prognosis. These associations are less well characterized in JDM as compared to adult DM. Previous reports have described a low frequency (<10%) of myositis-specific autoantibodies (MSA) including antiaminoacyl-transfer RNA (tRNA) synthetase, antisignal recognition particle (SRP), and anti-Mi-2 antibodies in JDM.[1,3] Antibodies noted not only in IMM but also in other conditions are called myositis-associated autoantibodies (MAA).

Targoff et al[4] have described novel autoantibodies that immunoprecipitated a 155-kd protein along with a weaker 140-kd protein in serum of patients with DM and JDM. Anti-p155/140 antibodies have been proved to target transcriptional intermediary factor-1 (TIF-1) family protein recently, present in 22–29% of patients with JDM, 13–21% of adult DM, and in most patients with cancer-associated DM.[4,5,6,7] Anti-p155/140 autoantibodies appear to define a distinct clinical phenotype with severe skin manifestations in JDM.[8] Another anti-p140 antibody targeting a 140-kd protein has been identified in 18–27% of patients with JDM, but is rare in adult DM or cancer-associated DM.[7,9,10,11] The 140kD autoantigen was originally termed MJ antigen, or MORC3, which was further identified as nuclear matrix protein NXP2.[7]

However, antiendothelial cell antibodies (AECA), a heterogeneous group of antibodies directed against a variety of antigen determinants on endothelial cells, have been detected in healthy individuals, in patients with autoimmune diseases and systemic vasculitis such as Kawasaki disease, Wegener granulomatosus, and Takayasu arteritis.[12,13,14,15] In addition to immunoglobulin (Ig)M or IgG AECAs, our previous studies have found that IgA AECA from patients with Henoch-Schonlein purpura could directly activate endothelial cells to produce interleukin-8 to enhance inflammation.[16] AECA play a pathogenic role by mediating endothelium activation and vascular damage.[17,18] The detection of AECA in JDM has not been previously studied. In this study, we detected anti-p155/140, anti-p140 antibodies, and AECA in the serum of JDM patients. We also correlated the clinical manifestations with the presence of these myositis autoantibodies in our patients with JDM.

Methods

Patients and controls

Children in whom definite JDM was diagnosed and who were younger than age 18 years ($n = 25$) were recruited on visits to the pediatric rheumatology department at the National Taiwan University Hospital, Taipei, Taiwan. The diagnosis of JDM was based on the Bohan and Peter criteria developed in 1975, which included characteristic skin rash and two or more of the following symptoms and signs: (1) symmetric proximal muscle weakness, (2) raised muscle enzymes, (3) myopathic changes on electromyogram, and (4) biopsy-proven myositis.[19] Serial clinical data, including the degree of skin involvement, muscle weakness, internal organ involvement, serum muscle enzymes and inflammatory markers, magnetic resonance imaging, muscle biopsies, duration of follow-up, and current clinical status were collected. Demographic data, clinical features at diagnosis and at follow-up, medications and current status of mortality, disability, or remission are listed in Table 1. Complete clinical remission was defined as no evidence of active myositis or dermatitis for more than 6 months without receiving any medications. Seventeen healthy controls (12 female, 5 male, mean age 16.4 ± 6.1 years) were also recruited at our hospital by advertisement. Informed consent and institutional approval were obtained. Blood samples of patients were all drawn at the time at the active stage. Serum samples from patients with JDM and controls were collected and stored at −20°C prior to testing.

Human umbilical vein endothelial cells culture

Endothelial cells were obtained from human umbilical vein by collagenase (GIBCO BRL Life Technologies, Rockville, MD, USA) digestion as described previously.[20] The separated cells were seeded in 75-mL flasks precoated with 1% gelatin solution and grown in medium 199 (GIBCO BRL Life

Table 1 Clinical characteristics and medications of patients with juvenile dermatomyositis

Patients with JDM	$N = 25$
Female: male	1.5:1
At diagnosis	
Diagnosis age (y)	6.9 ± 3.3
Age of onset (y)	6.3 ± 3.2
Gottron papules	21/25 (84)
Heliotrope sign	18/25 (72)
Malar/facial rash	15/24 (62.5)
Muscle weakness	23/25 (92)
Elevation of muscle enzymes[a]	21/25 (84)
At follow-up	
Calcinosis	7/25 (28)
Lung involvement	2/25 (8)
Gastrointestinal involvement	4/25 (16)
Disability	3/25 (12)
Malignancy	0
Mortality	0
Remission[b]	3 (12)
Disease duration (y)	5.6 ± 4.6
Medication	
NSAIDs	19 (76)
Corticosteroids	22 (88)
Hydroxychloroquine	21 (84)
Cyclosporine	19 (76)
Azathioprine	22 (88)
Methotrexate	6 (24)
Cyclophosphamide	1 (4)
Intravenous immunoglobulins	11 (44)

[a] Elevation of serum levels of creatine phosphokinase, lactate dehydrogenase, or aspartate aminotransferase.
[b] Complete clinical remission was defined as no evidence of active myositis or dermatitis for >6 months without receiving any medications.
Data are presented as n/N (%), n (%), or mean ± SD.
JDM = juvenile dermatomyositis; NSAIDs = nonsteroidal anti-inflammatory drugs.

Technologies) supplemented with 15% heat inactivated fetal calf serum, heparin sulfate, L-glutamine, endothelial cell growth factor (final concentration, 20 μg/mL) and 100 μg/mL penicillin/streptomycin. All cultures were incubated at 37°C in 5% CO_2 and the cells were used between the second and the sixth passage.

AECA IgG detection by cell-based enzyme-linked immunosorbent assay

Human umbilical vein endothelial cells were seeded on gelatin-coated 96-well microtiter plates at a concentration of 1×10^5 cells/well. When the cellular growth became confluent 3–4 days later, cells were fixed with 0.2% glutaraldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature and incubated with blocking buffer for 60 minutes at 37°C to prevent nonspecific binding. After washing with PBS/Tween 20, the serum samples of patients and controls diluted at 1:100 for IgG detection were incubated for 2 hours at 37°C. The sera were then removed and the plates were washed, 100 μL of peroxidase-conjugated rabbit antihuman IgG immunoglobulins were added to each well for 2 hours at 37°C. After washing, tetramethyl benzidine (TMB) solution was added for 15 minutes, and stop solution (1M hydrochloric acid) for 5 minutes. The optical density of each well was read at 450 nm by an enzyme-linked immunosorbent assay (ELISA) reader. The results were expressed using EU (ELISA unit):

$$EU = (A_{sample} - A_{blank})/(A_{positive} - A_{blank})$$

Immunoblotting

Total cell lysates of K562 cells were extracted by Gold lysis buffer. K562 cell lysates (50 μg/well) were loaded into the wells of 10% polyacrylamide gel. Electrophoresis was performed at a fixed current for 90–120 minutes, and the separated gel was transferred to a polyvinylidene difluoride membrane, washed with 0.05% TBST, and incubated with blocking buffer (5% milk in TBST) at 25°C for 1 hour. The membrane was further incubated with patients' serum (1:1000 dilution), a commercial anti-TIF1γ antibody (1:500; Bethyl, Montgomery, TX, USA) or anti-NXP2 antibody (1:500; MBL, Nagoya, Japan) as positive control at 4°C overnight. Horseradish peroxidase-conjugated antirabbit IgG antibody (1:10000) was then added for another hour at 37°C. Finally ECL Plus™ substrate was added onto the membrane and exposed the signal to the hyperfilm.

Statistical analysis

The values of AECA titers (EU) and clinical continuous parameters were expressed as mean ± standard deviation (SD). The receiver operating characteristic (ROC) curve analysis of the serum levels of AECA of patients and controls was performed to determine the optimal cutoff point (at which the sum of the sensitivity and specificity is highest) for distinguishing between positive and negative results. The area under the ROC curve (AUC), which can be used as a measure of accuracy of the test, was calculated. The continuous data were compared by Mann–Whitney *U* test. The frequencies of autoantibodies or clinical features between JDM patient groups were compared by the Chi-square test or Fishers exact test. Statistical analyses were done through SPSS software (IBM SPSS Statistics, version 20). A two-tailed $p < 0.05$ was considered significant. Power calculations were performed at a type 1 error rate (αvalue) of 0.05, current sample size, and proportions between patients and controls by z tests using the G*Power 3.1 (Heinrich Heine University Düsseldorf, Düsseldorf, Germany).[21]

Results

Detection of AECA, anti-p155/140, and anti-p140 antibodies

The levels of AECA in 25 patients with JDM were significantly higher than that in 17 healthy controls (6.7 ± 5.2 vs. 3.1 ± 1.7 EU, $p = 0.002$; Fig. 1A). The ROC curve analysis of the serum levels of AECA of patients and controls yielded an AUC of 0.78 and recommended at cutoff point of 3.35 EU.

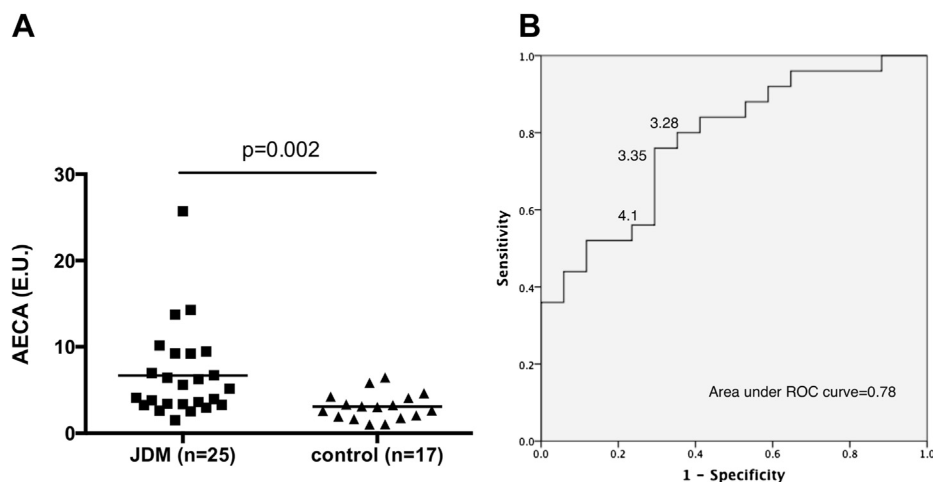

Figure 1. (**A**) Serum levels of antiendothelial cells IgG antibodies (AECAs) in 25 patients with JDM and 17 controls, presented as ELISA units (EU). The receiver operating characteristic (ROC) curve obtained from analysis of the serum levels of AECA of patients and controls. The true-positive rate (sensitivity) is plotted against the false-positive rate (1−specificity) for each cutoff point applied. (B) An optimal cutoff point of 3.35 EU is indicated with sensitivity 0.76 and specificity 0.71. ELISA = enzyme-linked immunosorbent assay; Ig = immunoglobulin.

At this cutoff point, the sensitivity estimate was 0.76, and the specificity estimate was 0.71 (Fig. 1B). Nineteen patients and five controls (76% and 29.4%, respectively) had positive AECAs (≥3.35 EU; $p = 0.003$; Fig. 1 and Table 2). Using immunoblotting with K562 cell lysates as antigen source, anti-p155/140 antibodies and anti-p140 antibodies were detected in nine patients and seven patients (36% and 28%), respectively (Fig. 2 and Table 2). Anti-p155/140 and anti-p140 antibodies were not detected in the serum of healthy controls (data not shown).

Clinical features in patients with autoantibodies

Anti-p155/140 antibodies were significantly associated with higher proportion of ESR elevation (100% vs. 0%, $p = 0.006$), higher ESR levels at diagnosis (40.3 ± 15.5 vs. 13.4 ± 5.3 mm/hr, $p = 0.019$), and a younger diagnostic age (5.2 ± 3.2 years vs. 8.0 ± 3.0 years, $p = 0.03$; Table 3). Gottron papules were seen in 100% of patients with anti-p155/140 and 71.4% of patients with anti-p140. Heliotrope rash was seen in 66.7% of patients with anti-p155/140 and 71.4% of patients with anti-p140. Calcinosis was seen in 11.1% of patients with anti-p155/140 and in 14.3% of patients with anti-p140 antibodies. The frequencies of clinical signs including heliotrope signs, calcinosis, muscle weakness or muscle enzymes elevation, internal organs involvement, disability, or mortality were not significantly different among children with or without anti-p155/140 antibodies, anti-p140 antibodies, or AECA (Table 3).

Table 2 Laboratory results and antibodies profiles in patients with juvenile dermatomyositis

Patients with JDM	$N = 25$
Positive antinuclear antibodies	6/22 (27.3)
C3/C4 elevation	0
ESR elevation	3/11 (27.3)
CRP elevation	4/14 (28.6)
Anti-p155/140 antibody	9/25 (36)
Anti-p140 antibody	7/25 (28)
Antiendothelial cells antibodies	19/25 (76)

Data are presented as n/N (%), n (%), or mean ± SD.
CRP = C-reactive protein; ESR = erythrocyte sedimentation rate in 1 h; JDM = juvenile dermatomyositis.

Discussion

Our study describes the presence of anti-p155/140 antibodies (36%), anti-p140 antibodies (28%), and AECA (76%) in our patients with JDM. Anti-p155/140 and anti-p140 present in 23−38% and 18−27% of patients with JDM, respectively.[4,6,8,9,10,22] The frequencies of anti-p155/140 and anti-p140 antibodies in our study were compatible with other previous reports. Based on the multiple bands detected between 130 kD and 170 kD area in our immunoblotting, our study could be better demonstrated by using recombinant protein TIF-1γ or NXP2 as antigen in immunoblotting.

Studies investigating anti-p155/140, anti-p140 antibodies, and other MSA have shown that these autoantibodies are mutually exclusive.[7] It is apparent that

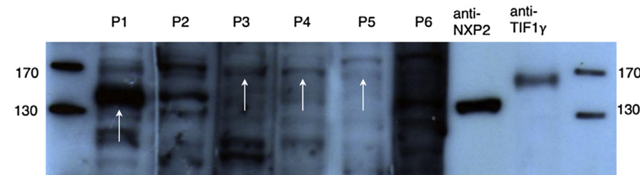

Figure 2. Detection of serum anti-p155/140 and anti-p140 antibodies using immunoblotting. K562 cell lysates were hybridized with patients' serum (P1−P6), or commercial anti-NXP2 and anti-TIF1γ antibody as positive control. P1: anti-p140 positive; P3, P4, P5: anti-p155 positive.

Table 3 Demographic, clinical, and laboratory features in patients with juvenile dermatomyositis with and without anti-p155/140, anti-p140 autoantibodies, and antiendothelial cells antibodies

	Anti-p155/140 positive (n = 9)	Anti-p155/140 negative (n = 16)	Anti-p140 positive (n = 7)	Anti-p140 negative (n = 18)	AECA positive (n = 19)	AECA negative (n = 6)
Dx age (y)	5.2 ± 3.2*	8.0 ± 3.0*	6.4 ± 3.3	7.1 ± 3.3	7.3 ± 3.3	5.7 ± 3.2
Onset age (y)	4.7 ± 2.8	7.2 ± 3.2	5.8 ± 3.6	6.5 ± 3.2	6.9 ± 3.3	4.0 ± 2.0
Male	33.3	43.8	57.1	33.3	31.6	66.7
Heliotrope rash	66.7	75	71.4	72.2	73.7	66.7
Facial rash	66.7	60	50	66.7	63.2	60
Gottron papules	100	75	71.4	88.9	84.2	83.3
Calcinosis	11.1	12.5	14.3	11.1	10.5	16.7
Muscle weakness	100	87.5	100	88.9	94.7	83.3
GI	11.1	18.8	14.3	16.7	15.8	16.7
Arthritis	0	18.75	28.6	5.6	10.5	16.7
Disability	22.2	6.25	0	16.7	15.8	0
Elevated muscle enzymes	77.8	87.5	100	77.8	84.2	83.3
ESR (mm/h)	40.3 ± 15.5**	13.4 ± 5.3**	15.7 ± 4.5	22.6 ± 17.4	25.6 ± 16.7	12.7 ± 8.5
CRP (mg/dL)	0.2 ± 0.2	0.43 ± 0.63	0.6 ± 0.8	0.2 ± 0.3	0.43 ± 0.55	0.05 ± 0.02
ESR elevation	100***	0***	0	37.5	42.8	0
CRP elevation	16.7	37.5	50	20	30	25
ANA positivity	37.5	21.4	0	40	29.4	20

Data are expressed as % or mean ± SD.
*p = 0.03 by Mann-Whitney U test.
**p = 0.019 by Mann-Whitney U test.
***p = 0.006 by Fisher exact test.
ANA = antinuclear antibodies; CRP = C-reactive protein (normal < 0.8 mg/dL); Dx age = diagnostic age (y), GI = gastrointestinal involvement.

autoantibodies have a role in distinguishing between subtypes of the patients. The association between serotype and clinical phenotype suggests that autoantibodies may play a role in the pathogenesis of JDM. Our study described the patients with anti-p155/140 autoantibodies appear to define a subset of JDM with significantly younger age of disease diagnosis and a higher inflammatory marker ESR at diagnosis. Our result is compatible with a previous report that showed significantly more skin involvement [including ulceration (51.9%), edema (63%), heliotrope rash (92.6%), and Gottron papules (100%)] in patients with JDM with anti-p155/140 autoantibodies.[8,10]

The 155kD autoantigen target was first identified as TIF-1γ by immunoaffinity purification and mass spectrometry in a preliminary report.[7] Fujimoto et al[6] further confirmed that anti-p155/140 antibodies target TIF-1 family of proteins, including TIF-1γ (the 155kD band), TIF-1α (a second weaker 140kD band), and TIF-1β (a 100kD band in a small portion of the patients). The TIF-1 protein family is a nuclear protein that mediates gene transcription. It also plays an intriguing role in carcinogenesis.[23] TIF-1γ has been shown to inactivate smad-4, which regulates transforming growth factor-β (TGF-β), thus promoting cell growth and differentiation including malignancy.[24] A recent meta-analysis showed that anti-p155/140 is a valuable tool for diagnosis of cancer-associated myositis in adult patients with DM with a high sensitivity (78%), specificity (89%), and negative predictive value (95%).[23] Cancer was not associated with anti-p155/140-positive patients with JDM, like our cases. However, further follow-up of our cases is needed to clarify these patients with anti-p155/140 are at risk for malignancy.

Anti-p140 antibody was shown to be associated with calcinosis in Caucasian patients with JDM.[10] The association of anti-p140 antibody with rapid progressive interstitial lung disease and malignancy has been reported in adult DM.[9,25] Such association was not seen in our study, probably because of the small sample size. The target antigen NXP2 may have a role in diverse nuclear functions including regulation of transcriptional and RNA metabolism.[26] NXP2 has also shown to repress transcription by post-transcriptional modification of transcription factors.[27] The role of NXP2 in the disease mechanism is still unclear.

Identification of IgG AECA in patients with JDM has not been previously reported. Based on the typical changes on JDM biopsy of endothelial dysfunction, damage, depletion of capillaries, and neovascularization, JDM is considered to be a small-vessel vasculopathy.[28,29] A study using gene expression profiling demonstrated that genes in angiogenesis, leukocyte trafficking, and complement cascade were highly upregulated in juvenile and adult DM.[29] Binding of AECA may induce endothelial damage, complement activation, upregulation of adhesion molecules, cytokines and chemokines production, and induction of apoptosis and thrombosis in both muscle and involved skin.[17,28,30] Although further studies are necessary to explore the candidate antigens for AECA and the pathogenic roles of AECA in JDM, their presence in patients with JDM shows that AECA might be involved in vascular injury.

There are several limitations of our study. First, the sample size is small. The current study showed that AECA, anti-p155/140, and anti-p140 antibodies were associated with JDM with statistical power of 0.87, 0.9, and 0.7, respectively. In addition, a recent study showed that the serum levels of these autoantibodies decreased after treatment for 6 months in most patients with JDM.[6] We only detected these autoantibodies during the active disease status, not in remission status. The roles of these autoantibodies need to be clarified in further large-scale studies with longitudinal follow-up. Second, there were no disease control groups including patients with other rheumatic diseases. Although AECA is not specific for JDM, anti-p155/140 and anti-p140 antibodies are considered highly specific for JDM or DM.[12,13,14,15] Anti-p155/140 antibody has been detected in 1 patient (2%) with systemic lupus erythematosus and 1 patient (5%) with polymyositis in previous studies.[4,31] Anti-p140 antibodies have not been found in patients with other rheumatic diseases or inflammatory myopathies.

Although the exact role of autoantibodies in the pathogenesis of JDM remains unknown, evidence suggests that the production of autoantibodies reflects upregulated autoantigen expression within the tissue during inflammation. A major drawback for routine detection of anti-p155/140 and anti-p140 antibodies is the costly and time-consuming technique using immunoprecipitation or immunoblotting. It has been shown that an excellent concordance between anti-p155 antibodies detected by immunoprecipitation and anti-TIF1γ antibodies determined by ELISA.[32] The application of autoantibodies detection by commercial ELISA kits in clinical practice is promising. Increasing our understanding of myositis autoantibodies and their corresponding targets may provide insight into the pathogenesis of JDM.

Conflicts of interest

All authors declare that they have no conflicts of interest relevant to this article.

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