

Distinct dermatomyositis populations are detected with different autoantibody assay platforms

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ABSTRACT

Objective. To compare autoantibody-defined dermatomyositis sub-populations using immunoprecipitation-based assays, a commercially available line immunoblot assay and alternate commercial ELISA assays.

Methods. Banked plasma from 261 carefully phenotyped dermatomyositis patients was studied. Immunoprecipitation-based assays were used to detect antibodies against Mi2, TIF1- γ , MDA5, NXP2, SAE1 and PM-Scl, while anti-Jo1 antibodies were assayed using ELISA. These data were compared with that obtained using a commercial line immunoblot, and, additionally, for Mi2, TIF1- γ , MDA5, commercially available ELISA kits. Test agreement was measured using Cohen's kappa statistic, and phenotypic differences between differentially identified groups are described.

Results. Line immunoblot, immunoprecipitation, and ELISA detected increasingly larger nested pools of anti-TIF1- γ samples, with increasing frequency of concurrent anti-Mi2 reactivity and decreasing incidence of malignancy. Line immunoblot and immunoprecipitation showed fair concordance for identifying anti-NXP2 antibodies (Cohen's kappa=0.71) but very good agreement for identifying antibodies against Mi2, MDA5, and SAE1 (Cohen's κ =0.9, 0.94, 0.88, respectively). Anti-PM-Scl results showed moderate agreement (Cohen's κ =0.48) between immunoblot and immunoprecipitation.

Conclusion. Our results demonstrate that for some specificities, especially anti-TIF1- γ , antibody results obtained using different assay platforms vary, and identify significantly different patient populations. These findings highlight the need for standard adoption of carefully validated platforms to detect dermatomyositis autoantibodies.

Introduction

In recent years a number of novel autoantibody targets have been identified in patients with dermatomyositis (DM) which can be associated with distinct phenotypes (1-2). Data regarding antibody prevalence and associated phenotypes can vary between studies, and choice of antibody assay platform may

be an important variable. In general, immunoprecipitation is performed using native conditions and radiolabelled cell extracts, and is considered the gold standard for autoantibody detection (3). Limitations of this methodology include lack of quantitation and standardisation, as well as being labour-intensive. Other assays can be substituted provided they are rigorously validated in multiple patient cohorts.

In this study we used banked plasma from a large, meticulously phenotyped cohort of U.S. patients with DM. Detection of antibodies against TIF1- γ , Mi2, MDA5, NXP2, SAE1 and PM-Scl was compared using immunoprecipitation, immunoblot and ELISA-based assays, and clinical characteristics of the patients identified were evaluated.

Methods

Patients

An ongoing research protocol was developed to prospectively collect clinical data and serum from dermatomyositis patients presenting at the Stanford University Medical Center for clinical care – this protocol was approved by the Stanford Panel on Human Subjects initially in 2004 and is renewed annually. All patients provided verbal and written informed consent to participate in the study. The current analysis pertains to patients seen between July 2004 and August 2017. All patients were >18 years old with a diagnosis of probable or definite DM based on the Bohan and Peter criteria (4), or, for patients with clinically amyopathic disease, based on characteristic skin findings (5). Patients were considered to have cancer-associated DM if they had a malignancy diagnosis within 5 years of DM onset. Rapidly progressive interstitial lung disease (RP-ILD) was defined as acute and progressive worsening of dyspnea secondary to ILD requiring hospitalisation, supplementary oxygen, or respiratory failure requiring intubation within 3 months of the diagnosis of ILD.

Antibody assays

• *Immunoprecipitation using ³⁵S-methionine-labelled in vitro transcription/translation (IVTT) proteins to detect antibodies* cDNAs encoding full-length human

MDA5, NXP2, SAE1 and PM-Scl were purchased (OriGene, Rockville, MD). For PM-Scl, DNA encoding the 100- and 75 kDa subunits was used, since these constitute the multi-component exosome complex subunits targeted by the auto-immune response (6). Mi2- β cDNA was a gift (Dr Seelig, Institute of Molecular Genetics, Germany). DNAs were used to generate ^{35}S -methionine-labelled proteins by IVTT (Promega, Madison, WI). Immunoprecipitations were performed using these products as input. Immunoprecipitates were electrophoresed on 10% SDS-PAGE gels and visualised by fluorography (7). A positive reference sample was included in each set.

• *Immunoprecipitation/blot (IP/blot) to detect anti-TIF1- γ antibodies*

Since IVTT product generation with TIF1- γ was inconsistent, we optimised an alternate assay for this specificity (detailed in (8)). A positive reference sample was included in each set.

Line immunoblot

A commercial line immunoblot assay (EUROLine, EUROIMMUN, Germany) was used to assay a panel of myositis autoantibodies (Autoimmune Inflammatory Myopathies 16 Ag panel). Samples were considered positive for anti-Mi2 if they had reactivity against either Mi2- α , Mi2- β or both, and were considered positive for anti-PM-Scl if they had reactivity towards either PM-Scl-75, PM-Scl-100 or both. In accordance with the manufacturer's recommendation, for all specificities, ≥ 15 units (U) was considered positive.

ELISA assays

ELISA kits (MBL, Japan) were used to test for antibodies against Mi2- β , MDA5 and TIF1- γ (9-10). Cut-offs for assigning antibody positivity were defined by assaying 44 healthy control sera banked on our site. The mean of the controls +3SD was used for Mi2 and MDA5 as the positive cut-off: Mi2 cut-off: 22U (range for positive samples 23–206U); MDA5 cut-off: 20U (range for positive samples 34–169U). For TIF1- γ a cut-off of 3SD above the control mean was very low (4U), so 7U was used (positive range 7–135U).

Jo-1 antibody detection

Jo-1 antibodies were assayed using an ELISA kit (Inova Diagnostics).

Statistical methods

All data were analysed using non-parametric tests. Median values were calculated and compared using Mann-Whitney testing. Agreement between assays was measured by calculating Cohen's kappa (GraphPad Software, San Diego, CA).

Results

Discordance of assays for detecting anti-TIF1- γ antibodies

The patient characteristics are shown in Table I. Patient plasma were initially tested comparing immunoprecipitation assay (IP) (including either IVTT IP or IP/blot) with line immunoblot assay for multiple specificities (Table II). All samples were also tested using ELISA assays for selected specificities (Mi2- β , MDA5 and TIF1- γ).

There was a striking difference between assays for anti-TIF1- γ antibody detection: the IP identified 107 positives compared to 41 by line immunoblot (Cohen's $\kappa=0.39$, fair agreement). The antibody-positive samples detected by line immunoblot were almost completely (39/41) contained within the larger group of samples detected by IP. A commercial ELISA detected another 26 novel positive samples in addition to capturing nearly all (105/107) samples testing positive on IP. Thus, the line immunoblot had poor concordance with both the IP and ELISA assays (Table II). We examined the characteristics of the three anti-TIF1- γ positive groups defined by the various assays. For the 39 samples that tested positive on all 3 assays, 14 (36%) had an associated malignancy, as would be expected for this antibody group (1); for the 66 samples positive by IP and ELISA, 12 (18%) had an associated malignancy; for the 26 samples positive only on ELISA, 2 (8%) had an associated malignancy. This difference was statistically significant ($p=0.022$, chi squared). Since age is a risk factor for cancer, we tested this and found no significant difference in median age between the 3 groups (not shown). The median anti-TIF1- γ titres

Table I. Patient characteristics.

Variable	n=261
Gender, n (%)	
Male	68 (26)
Female	193 (74)
Race, n (%)	
Caucasian	153 (59)
Latino	53 (20)
Pacific Islander	8 (3)
Asian	33 (13)
African American	8 (3)
Age at diagnosis, yr ¹	47 (17)
Duration of disease at blood draw, yr ²	1.6 (0.71-4.9)
Length of follow-up, yr ²	2.2 (0.59-5.0)
Cancer-associated, n (%)	41 (16)
Interstitial lung disease, n (%)	55 (21)
Rapidly progressive ILD, n (%)	14 (5.5)
Clinically amyopathic, n (%)	52 (20)
Raynaud's, n (%)	66 (25)
Mechanic's hands, n (%)	53 (20)
Erythematous palmar papules, n (%)	28 (11)
Arthralgia/arthritis, n (%)	126 (48)
CK, maximum ²	274 (113-1200)
Aldolase, maximum ²	9.2 (6.2-15.5)

CK: creatine kinase.

¹Data expressed as mean (standard deviation).

²Data expressed as median (first quartile-third quartile).

(by ELISA) for these groups were significantly different at 84, 39, and 30, respectively (Fig. 1).

For the 39 samples positive for anti-TIF1- γ on all assays, only two were clearly positive (by at least two assays) for another specificity. For the 66 anti-TIF1- γ positive samples by IP and ELISA, 14 tested positive (by IP) for anti-Mi2 but for no other specificities (Fig. 1). Of the 26 anti-TIF1- γ positive samples identified only by ELISA, 12 were reactive (by at least two assays) against another DM-specific antigen (8 Mi2, 2 MDA5, 1 SAE1, 1 NXP2) (Fig. 1). Thus, even using the IP as a gold standard for both specificities, Mi2/ TIF1- γ is a common combination, although, interestingly, the line immunoblot assay appears to detect a population of anti-TIF1- γ patients that do not also target Mi2.

It has recently been reported that, using ELISA assays as the readout, many anti-TIF1- γ /Mi2 double positive sera have antibodies primarily directed against the Mi2 antigen which are also weakly cross-reactive with TIF1- γ (9). Of the 36 samples with anti-Mi2 antibodies (by

Table II. Comparison of assay data.

Antigen	IP+IB+* (#ELISA+) [§]	IP+IB-* (#ELISA+) [§]	IP-IB+* (#ELISA+) [§]	IP-IB-* (#ELISA+) [§]	Cohen's k IP vs. IB (95% CI)	Cohen's k IP vs. ELISA (95% CI)	Cohen's k IB vs. ELISA (95% CI)
TIF1-γ	39 (39)	68 (66)	2 (2)	152 (26)	0.39 (0.29-0.49)	0.79 (0.71-0.86)	0.31 (0.23-0.40)
Mi2	26 (26)	2 (2)	3 (1)	230 (7)	0.90 (0.82-0.99)	0.86 (0.76-0.95)	0.81 (0.7-0.92)
MDA5	36 (36)	3 (3)	1 (0)	221 (1)	0.94 (0.88-1.0)	0.99 (0.95-1.0)	0.92 (0.86-0.99)
NXP2	18	8	5	230	0.71 (0.56-0.86)	--	--
SAE1	20	0	5	236	0.88 (0.77-0.98)	--	--
Jo-1**	11	1	1	248	0.91 (0.79-1.0)	--	--
PM-Scl	10	1	18	232	0.48 (0.29-0.68)	--	--

*number of samples that scored positive or negative (+ or -) in IP and IB assays

§number of samples that scored positive on ELISA (manufactured by MBL) in each group

**For this antigen, "IP" represents the commercial INOVA ELISA assay (see *Methods*).

IP: immunoprecipitation; IB: line immunoblot; ELISA: enzyme-linked immunosorbent assay;

TIF1-γ: Transcriptional Intermediary Factor 1-gamma; MDA5: melanoma differentiation-associated protein 5; NXP2: nuclear matrix protein 2; SAE1/2: small ubiquitin-like modifier activating enzyme 1.

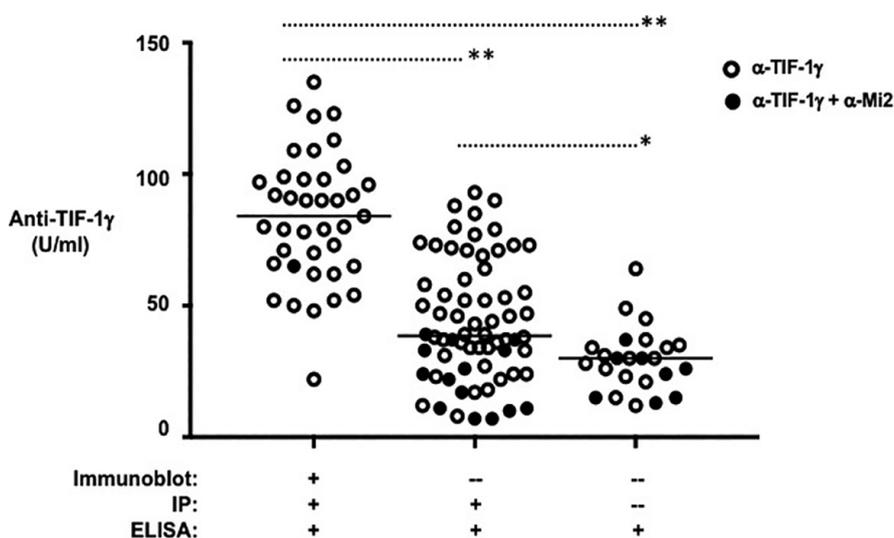


Fig. 1. Anti-TIF-1γ titres and co-existing anti-Mi2 reactivity vary depending on how anti-TIF1-γ reactivity is defined. 3 groups of plasma were defined on basis of anti-TIF1-γ positivity on immunoblot, IP, and/or ELISA. Each circle represents the anti-TIF1-γ titre (by ELISA) of a plasma sample from an individual patient. Open circles represent plasma with only anti-TIF1-γ reactivity, filled circles represent plasma with both anti-TIF1-γ and anti-Mi2 reactivity (the latter by IP). Horizontal solid lines represent median values in each assay group. Dotted horizontal lines represent statistical significance of difference between median values of indicated groups (* $p=0.0032$, ** $p<0.0001$, Mann-Whitney test).

ELISA), 29 (81%) also had antibodies (by ELISA) against TIF1-γ. The anti-TIF1-γ/anti-Mi2 samples had lower median anti-TIF1-γ titres compared to those targeting only TIF1-γ, (24U vs. 54U, $p<0.0001$). However, we found no difference in anti-Mi2 titres between samples that did and did not also target anti-TIF1-γ (not shown).

Discordance of assays for detecting anti-NXP2 antibodies

There were significant discrepancies between the IP and line immunoblot assays in identifying anti-NXP2 antibod-

ies (Table II, Cohen's $\kappa=0.71$). For the 8 patients uniquely identified as anti-NXP2 positive by IP, 3/8 had calcinosis and 3/8 had cancer-associated DM (both features previously associated with anti-NXP2)(1-2), and none had other DM-specific antibodies. However, for the 5 samples uniquely identified as anti-NXP2 positive by line immunoblot, none had cancer-associated DM or calcinosis, all were identified as anti-TIF1-γ positive by both IP and ELISA, and all had a significantly lower anti-NXP2 titres (35 vs. 146, respectively) ($p=0.011$).

Assay concordance for antibodies against Mi2, MDA5, SAE1 and Jo1

All three assays showed very good agreement for detecting anti-Mi2 and anti-MDA5 antibodies (Table II). Several samples ($n=7$) were positive for anti-Mi2 uniquely by ELISA; these were lower titre than those detected by all three assays (median 37 vs. 133, $p<0.0001$). Unlike the other anti-Mi2-positive samples, 2/7 of these also had antibodies against SAE1 and NXP2. For anti-SAE1, the 5 discordant samples (positive only on line immunoblot) were all low titre (median 17U) compared to the concordant samples (range: 34–142U, median 101U) ($p<0.0001$). Additionally, all 5 discordant samples also possessed another DM-specific antibody (3 anti-TIF1-γ and 2 anti-MDA5). Assays detecting anti-PM-Scl antibodies showed only moderate agreement (Table II, $\kappa=0.48$); those for anti-MDA5 and anti-Jo1 antibodies showed excellent agreement ($\kappa=0.91$).

Discussion

Maximising the utility of autoantibody testing in a rare disease like DM depends on the universal adoption of validated assays. We found significant discordance between selected assays for detecting antibodies against TIF1-γ and NXP2. Without a true "gold standard" it is difficult to interpret these differences.

The most striking finding was the widely discordant results for detecting antibodies against TIF1-γ--both IP and

ELISA produced many discordantly positive samples compared to line immunoblot. For both groups, many of these patients had anti-Mi2 antibodies, the significance of which is unclear and may represent cross-reactivity (9).

Given these data, we suggest that the ELISA assay is the preferred option for testing for both MDA5 and Mi2, although for the Mi2 the cut-off could be raised to increase specificity without impacting sensitivity. For TIF1- γ , the ELISA seems to be an appropriate screening test with virtually all samples with ELISA titres >50 U also scoring positive on IP assay; unfortunately, lower titre samples (between 5 and 50 U) will have a significant chance having a negative IP, and thus the clinician needs to interpret these modest titre samples with caution. For SAE1 and NXP2, we were unable to test an ELISA assay, and we conclude that there are significant differences between the IP and line blot assays – using expected clinical characteristics and absence of another DM-specific antibody as criteria for true positives, we conclude that the IP assay is more specific and, for NXP2, also more sensitive than line

blot for SAE1 and NXP2 testing. Both line blot and ELISA performed well for testing for Jo1 antibodies, while the significance of the discordance of line blot and IP for PM-Scl requires further study.

The findings in this study highlight how autoantibody assay platform can influence results. Having high performance, universally adopted antibody assays is a critical priority for studies in which patients are sub-grouped by antibody profile. Ultimately, these subsets will be important in aiding clinicians to both understand disease pathogenesis and to make wise, informed diagnostic and treatment decisions.

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