Ⅲ. 研究成果の刊行物・別刷
その3

Psychoneuroendocrinology 101 (2019) 240–245

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Hair and fingernail cortisol and the onset of acute coronary syndrome in the middle-aged and elderly men

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ARTICLE INFO

Keywords:
Cortisol
Hair
Fingernail
Acute coronary syndrome
Psychosocial stress

ABSTRACT

Cortisol levels in hair and fingernail samples could represent hormone levels that have accumulated over the past weeks and months. In this study, by using retrospective indexes, the associations between cortisol and the onset of acute coronary syndrome (ACS) were investigated among middle-aged and elderly men. We measured hair/fingernail cortisol levels in 73 ACS patients and 93 healthy controls; hair and fingernail samples for ACS patients were collected within a few weeks after the onset of ACS. The results indicated the patients exhibited significantly higher cortisol levels in their hair and fingernails compared with the healthy controls. In multivariate logistic regression analyses, adjusting for the traditional cardiovascular risk factors for ACS, high levels of hair or fingernail cortisol were associated with two- to three-fold increased risk of ACS, compared with low levels. We demonstrated that cortisol exposure over a relatively long period, assessed by hair and fingernail samples, was associated with the onset of ACS.

1. Introduction

Acute psychosocial stress triggers an activation of the hypothalamic-pituitary-adrenal axis, which causes the adrenal cortex to secrete cortisol. Traditionally, cortisol has been measured in blood and saliva samples, which revealed hormone levels for a short time period. Recently, cortisol has been reportedly measured in hair samples (Russell et al., 2012). Scalp hair grows at an average rate of 1.0 cm/month; 1.0 cm of scalp hair may be used to determine the level of the hormone secreted during a period of 1 month. Previous studies reported that psychosocial stress was associated with hair cortisol. Elevated hair cortisol levels were observed in long-term unemployed individuals (Dettenborn et al., 2010), dementia caregivers (Stalder et al., 2014), and people who had been recently exposed to major life events (Staufenbiel et al., 2014). Furthermore, hair cortisol was also associated with health outcomes such as depression (Dettenborn et al., 2012), posttraumatic stress disorder (Steudte-Schmiedgen, et al., 2015), and cognitive decline after stroke (Ben Assayag et al., 2017).

Furthermore, more recent focus has been given to cortisol measured in fingernail samples. Fingernails grow at an average rate of 1.0 mm/10 days (Gupta et al., 2005); therefore, 1.0 mm of fingernail may retrospectively reflect hormone levels over 10 days. Several months are required for nails to fully extend from the nail matrix (de Berker et al., 2007; Gupta et al., 2005). Therefore, fingernail samples may reflect cortisol levels several months prior to clipping. Previously, cortisol levels in fingernail samples were associated with those in saliva samples collected 4 or 5 months before (Izawa et al., 2015). Another study reported that psychosocial stress in the past, but not the present, was associated with elevated cortisol levels in fingernails (Izawa et al., 2017). These findings supported the notions that fingernails retrospectively represent past hormone levels. However, evidence concerning fingernail cortisol and health outcomes has been lacking. Only one study reported elevated fingernail cortisol levels in subjects experiencing a major depressive episode (Herane-Vives et al., 2018). Fingernail samples can easily be collected by the participant, and only small amounts are required. Therefore, further evidence is needed regarding fingernail cortisol.

In this study, we focused on coronary heart disease (CHD), one of the well-known stress-related diseases. Chronic psychosocial factors such as socioeconomic status (e.g., lower income and educational level), as well as episodic psychosocial distress, accelerate the development of arteriosclerosis in the coronary artery and contribute to the onset of acute coronary syndrome (Kop, 1997). One cross-sectional study using blood samples (Reynolds et al., 2010) and one prospective study using saliva samples (Schoorlemmer et al., 2009) supported the associations between cortisol and coronary heart disease; however,

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https://doi.org/10.1016/j.psyneuen.2018.11.021
Received 7 May 2018; Received in revised form 3 September 2018; Accepted 13 November 2018

other prospective studies using blood samples did not (Phillips et al., 2010; Rod et al., 2010; Smith et al., 2005). Cortisol levels in blood and saliva represent hormone levels for a short time period with large diurnal rhythms, which might produce inconsistent results. Hair and fingernail cortisol represent hormone levels accumulated over the past weeks and months, which could show some advantages for investigating this association. Previously, only a few studies have investigated the association between hair cortisol and CHD (Abell et al., 2016; Manenschijn et al., 2013; Pereg et al., 2011), and two of them (Abell et al., 2016; Manenschijn et al., 2013) only investigated the cross-sectional associations of hair cortisol and a self-reported history of CHD. To our knowledge, no studies have investigated the association between fingernail cortisol and CHD.

The purpose of this study was to investigate the associations between the onset of acute coronary syndrome (ACS) and cortisol levels in hair and fingernail samples. We measured hair/fingernail cortisol levels in ACS patients and healthy controls. Hair and fingernail samples for ACS patients were collected within a few weeks after the onset of ACS to retrospectively assess cortisol levels before the onset. We expected higher cortisol levels in hair and fingernails to be found in ACS patients, because psychosocial stress could contribute to the onset of ACS (Kop, 1997). We further investigated the associations between hair/fingernail cortisol and ACS, adjusting for ACS risk factors (e.g., dyslipidemia, hypertension) and psychosocial factors (e.g., stressful life events), since these risk factors may have confounded the associations. Furthermore, only one study previously reported the association between hair and fingernail cortisol (Izawa et al., 2015); therefore, this study investigated this association.

2. Methods

2.1. Participants

A case-control study was designed to investigate the relationships between hair/fingernail cortisol and ACS. The patient group comprised 73 men with ACS (acute myocardial infarction and unstable angina pectoris) who were admitted to a hospital located in Fuchu-city in Tokyo from September 2012 to October 2015. A medical doctor and psychologists approached the patients and asked them to participate in this study a few weeks after the onset of ACS (mean (SD) = 20.6 (8.9) days). The definite diagnosis of ACS was based on the patients' clinical histories as taken by a cardiologist, standard ECG readings, and plasma enzyme elevations.

The control group consisted of healthy 93 men living in Tokyo and its surrounding area (Kanagawa, Saitama, and Chiba). They were recruited via an internet-based survey during from October to November 2013. A portion of the data was identical to that used in a previous study (Izawa et al., 2015).

The inclusion criteria of patients and controls were as follows: (1) men; (2) no recurrent ACS (patients), or no previous history of cardiovascular diseases (controls); (3) aged 35–79 years; (4) no adrenal dysfunction (e.g., Cushing syndrome); and (5) not having taken steroid medications in the previous six months.

We were unable to measure cortisol levels in 9 hair samples and 2 fingernail samples due to technical reasons (e.g., insufficient sample volume, technical errors, measured absorbance being over the standard range of the kit). Further, some hair cortisol values (1 control and 1 patient) and fingernail cortisol values (2 controls) were over 3 SD above the mean, and we excluded these data from the analyses. Therefore, 155 hair cortisol samples (92 controls and 63 patients) and 162 fingernail cortisol samples (90 controls and 72 patients) were ultimately analyzed.

Written informed consent was obtained from each participant, and the study was approved by the institutional review board of the Sakakibara Heart Institute and the National Institute of Occupational Safety and Health, Japan.

2.2. Questionnaires

Participants were asked about their marriage status, job status, smoking status, educational background (e.g., high school, university), frequency of hair washing and manicures, and use of hair dye.

Additionally, to determine whether the participants had recently experienced a stressful life event, they were asked to complete the Japanese version of the Social Readjustment Rating Scale (Nomura, 1994), which included 28 stressful life events (e.g., death of spouse, unemployment). Participants were asked whether they had experienced any of 28 life events during the previous year using a yes/no format. If they had, they were asked when the event had occurred. In this study, we counted the total number of stressful life events in the previous six months, because the hair/fingernail samples could reflect hormone levels at any point within the previous weeks or months, up to six.

2.3. Biological risk factors

Data concerning low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, hemoglobin A1c, height, weight, and use of medications for dyslipidemia, hypertension, and diabetes mellitus were also obtained from both groups.

2.4. Procedure

Hair strands were collected by carefully cutting with fine scissors as close as possible to the scalp from a posterior vertex region. Samples of up to 6 cm long were obtained to determine the cortisol levels over the previous 6 months. However, for patients who had longer periods of time between the ACS onset and hair collection, hair strands between 0 and 1 cm from the scalp were not obtained, because these portions could reflect cortisol levels after hospitalization.

For the collection of fingernail samples, participants were asked to grow their fingernails for 2 weeks and provide samples from every digit by clipping the nail directly into a reclosable poly bag to avoid losing any part of the sample.

2.5. Hair and fingernail hormone extraction and enzyme immunoassay

Our hair and fingernail hormone extraction method was identical to that used in a previous study (Izawa et al., 2015). Hair samples were washed 3 times in 2.5 ml of isopropanol, the nail samples were washed twice in 5 ml of isopropanol, and these samples were dried overnight. Hair and nail samples were ground using a mixer mill (Retsch MM300, Germany) at 30 Hz for 15 min and 40 min, respectively. Fifteen milligrams of hair and nail powder were weighted out, and 1.5 ml of pure methanol was added for cortisol extraction over a period of 24 h under slow rotation. Following this, the samples were spun in a microcentrifuge at 10,000 rpm for 2 min, and 1.0 ml of the clear supernatant was evaporated at 60 °C until completely dry.

Cortisol level was determined by an enzyme immunoassay method using the EIA Kit (Salimetrics LLC, USA). The evaporated samples were re-suspended in 100 µl of the assay diluent included in the EIA Kit, and the levels of cortisol in the diluent were analyzed according to the manufacturer's instructions. For hair cortisol, the intra-assay and inter-assay variations were 4.2% and 4.7%, respectively. For nail cortisol, the intra-assay and inter-assay variations were 3.6% and 4.7%, respectively. The findings are presented as pg cortisol /mg hair or fingernail (pg/mg).

2.6. Statistical analyses

Independent t-tests and chi-squared tests were conducted to compare biological factors, lifestyle factors, and psychosocial factors between the controls and patients. Mann–Whitney U-tests were conducted to compare the cortisol levels between the controls and patients.

Multivariate logistic regression analyses were performed to estimate the odds ratios (ORs) of the cortisol levels for ACS, with ACS status (patient = 1, control = 0) as a dependent variable and hair or fingernail cortisol as an independent variable. Hair/fingernail cortisol levels were categorized into three groups (low, medium, or high) based on tertile values because hair and fingernail cortisol values were not normally distributed even after conducting a statistical transformation (e.g., a logarithmic transformation), and it was previously reported that the associations between cortisol and health outcomes are not always linear (e.g., cortisol awakening response and depression, as reported by Chida and Steptoe, 2009). High and medium groups were compared against the low group. We estimated crude ORs, as well as ORs adjusted for the ACS traditional risk factors (age, obesity, dyslipidemia, diabetes mellitus, hypertension, and current smoking status). Obesity was defined as body mass index $\geq$ 25 kg/m^2. Dyslipidemia was defined as LDL $\geq$ 140 mg/dl and/or HDL $<$ 40 mg/dL, and/or medical treatment for dyslipidemia. Diabetes mellitus was defined as hemoglobin A1c $\geq$ 6.5%, and/or medical treatment for diabetes mellitus. Hypertension was defined as medical treatment for hypertension. Furthermore, multivariate logistic regression analyses were performed to estimate ORs adjusting for psychosocial factors as a means to test whether psychosocial factors could be confounding the associations between hair/fingernail cortisol and ACS. In these analyses, we also estimated P values for trends from the logistic regression analyses, including an ordinal variable for cortisol levels (low = 1, medium = 2, high = 3) in the model.

In the preliminary analyses, we further investigated the logistic regression analyses while excluding the participants who reported use of hair dye (N = 39) or the use of manicure (N = 2). However, the results were not largely different; therefore, we reported the results of logistic regression analyses including those participants.

Pearson and Spearman rank-order correlations were also conducted to investigate the relationships between hair cortisol, fingernail cortisol, traditional risk factors, and psychosocial factors.

3. Results

3.1. Characteristics of the controls and patients

As shown in Table 1, the patients exhibited lower levels of HDL cholesterol, and the percentage of persons with dyslipidemia was higher among the patients. Current smokers were more prevalent among the patients than the controls. For the psychosocial factors, more highly educated persons were more prevalent among the controls, and the patients reported higher frequency of stressful life events within the previous six months.

3.2. Hair/fingernail cortisol levels in the controls and patients

Medians (ranges) of hair cortisol were 9.2 (3.1–45.9) pg/mg and 11.9 (1.3–67.2) pg/mg for controls and patients, respectively. Medians (ranges) of fingernail cortisol were 7.0 (2.2–56.7) pg/mg and 8.2 (3.9–85.6) pg/mg for controls and patients, respectively. Patients significantly exhibited higher cortisol levels in both hair (U = 2339.5, p = 0.042) and fingernails (U = 2493.5, p = 0.012, Fig. 1).

3.3. Hair/fingernail cortisol, traditional risk factors, and ACS risk

In multivariate logistic regression analyses, high levels of hair cortisol were associated with an elevated risk of ACS in the crude (OR = 2.36 [95% CI 1.07–5.19], P trend = 0.032) as well as the traditional risk factor-adjusted models (OR = 3.27 [95% CI 1.28–8.33], P trend = 0.012, Table 2). Further, we also found that high levels of fingernail cortisol were associated with an elevated risk of ACS in the crude (OR = 2.23 [95% CI 1.02–4.88], P trend = 0.046) as well as the traditional risk factor-adjusted models (OR = 2.48 [95% CI 1.02–6.06], P trend = 0.049, Table 3).

The proportion of traditional risk factors did not differ among the low, medium, and high hair cortisol groups or among the low, medium, and high fingernail cortisol groups. We also computed rank-order correlations between hair/fingernail cortisol and traditional risk factors, and we found a significant correlation between hair cortisol and the diabetes mellitus status of the patient (r_s = .18, p = 0.023).

3.4. Hair/fingernail cortisol, psychosocial factors, and ACS risk

We found significant differences in educational background and number of stressful life events between patients and controls. Therefore, in the subsequent multivariate logistic regression analyses, we estimated ORs additionally adjusting for psychosocial factors (specifically, stressful life events and educational level) to test whether or not inclusion of these psychosocial factors in the model altered the associations between hair/fingernail cortisol and ACS. High levels of hair cortisol were still associated with an elevated risk of ACS (OR = 2.90 [95% CI 1.12–7.46], P trend = 0.026, Table 2). However, the association between high fingernail cortisol and ACS was attenuated and not significant (OR = 2.14 [95% CI 0.85–5.38], P trend = 0.105, Table 3).

The proportion of marriage status, job status, and educational background as well as number of stressful life events did not differ among the hair cortisol groups or the fingernail cortisol groups. We also computed rank-order correlations between hair/fingernail cortisol and psychosocial factors, and we found significant correlations between hair and fingernail cortisol and the number of stressful life events (r_s = .17, p = 0.036 and r_s = .15, p = 0.052, respectively). Further, men with a higher educational levels (r_s = -.16, p = 0.044) and married men (r_s = -.15, p = 0.061) exhibited lower fingernail cortisol.

3.5. Correlations between hair and fingernail cortisol

We found a significant rank-order, but not a Pearson correlation, between hair and fingernail cortisol level (r_s = 0.23, p = 0.004; r = .04, p = .619). However, when excluding two data with higher fingernail cortisol levels (56.7 and 85.6 pg/mg), we found both significant rank-order and Pearson correlations (r_s = 0.24, p = 0.003; r = 0.22, p = 0.006, respectively; Fig. 2).

4. Discussion

The purpose of this study was to investigate the associations between the onset of acute coronary syndrome and cortisol levels in hair and fingernail samples. We found higher cortisol levels in hair and fingernail samples among the ACS patients compared with healthy controls. Additionally, less educated persons were more prevalent among the patients, and they reported higher frequency of stressful life events over the previous six months, implying that the patients had been under unfavorable psychosocial conditions before the onset, as we expected. The findings on the psychosocial conditions were also consistent with the previous prospective findings (e.g., Hirokawa et al., 2006; Iso et al., 2002). The persons with traditional risk factors such as dyslipidemia and smoking habits were more prevalent among the patients, but the associations between hair/fingernail cortisol and ACS remained after adjusting for the effects of the risk factors in the logistic regression analyses. Further, inclusion of psychosocial factors in the logistic regression analyses attenuated the association between fingernail cortisol, but not hair cortisol, and ACS.

We found higher hair cortisol levels among the patients, indicating higher cortisol levels for several months before ACS onset, because hair samples can retrospectively reveal accumulated hormone levels (Russell et al., 2012). Additionally, the logistic regression analyses revealed that high level of hair cortisol was associated with a three-fold increase in the risk of ACS. Previously, the reported associations between cortisol in saliva and blood and CHD were inconsistent (Reynolds

Table 1
Characteristics of the controls and patients.

	Controls (N = 93)	ACS patients (N = 73)	*t* scores or x^2 scores[a]	*p*
Biological factors				
Age, mean ± SD	59.6 ± 9.2	60.5 ± 9.4	0.57	0.567
Gender (men), n (%)	93 (100.0)	73 (100.0)		
Body mass index, mean ± SD	23.7 ± 3.2	24.4 ± 2.8	1.50	0.137
Obesity, n (%)[b]	23 (31.5)	29 (31.2)	0.00	0.964
LDL (mg/dL), mean ± SD	121.2 ± 27.8	126.1 ± 37.6	1.05	0.313
HDL (mg/dL), mean ± SD	58.6 ± 14.8	46.5 ± 12.8	5.53	< 0.001
Dyslipidemia, n (%)[c]	30 (32.3)	53 (72.6)	26.62	< 0.001
Diabetes mellitus, n (%)[d]	10 (10.8)	13 (17.8)	1.71	0.192
Hypertension, n (%)[e]	24 (25.8)	29 (39.7)	3.65	0.056
Lifestyle factors				
Smoking status (current smoker), n (%)	18 (19.4)	26 (35.6)	5.55	0.011
Hair dye use, n (%)[f]	24 (26.1)	14 (22.2)	0.30	0.583
Hair wash (frequency per week), mean ± SD[f]	5.1 ± 2.3	5.2 ± 2.2	0.24	0.810
Manicure use, n (%)[g]	1 (1.1)	1 (1.4)	0.03	0.874
Psychosocial factors				
Marriage status (married men), n (%)	76 (81.7)	59 (80.8)	0.02	0.883
Education (university graduates or those with higher education), n (%)	72 (77.4)	43 (58.9)	6.59	0.010
Job status (working men), n (%)	67 (72.0)	58 (80.0)	1.21	0.272
Number of stressful life events in the previous six months, mean ± SD	0.37 ± 0.64	0.74 ± 1.30	2.25	0.027

[a] independent t-tests or chi-squared tests were conducted.
[b] defined as body mass index ≥ 25.
[c] defined as LDL ≥ 140 mg/dl and/or HDL < 40 mg/dL, and/or medical treatment for dyslipidemia.
[d] defined as hemoglobin A1c ≥ 6.5%, and/or medical treatment for diabetes mellitus.
[e] defined as medical treatment for hypertension.
[f] n = 92 and 63 for controls and ACS patients, respectively.
[g] n = 90 and 72 for controls and ACS patients, respectively.

et al., 2010; Phillips et al., 2010; Rod et al., 2010; Schoorlemmer et al., 2009; Smith et al., 2005), perhaps because saliva and blood samples represented hormone levels for a short time period with a large diurnal rhythm. Pereg et al. (2011) reported that hair cortisol was associated with an increased risk of acute myocardial infarction when hair samples were collected a few days after hospitalization. It is worth noting that both their study (Pereg et al., 2011) and this study reported that CHD patients exhibited higher cortisol levels in hair by approximately 30% compared with controls, although reported cortisol levels differed substantially, which may be due to the fact that in the previous study (Pereg et al., 2011) hair strands were not washed with alcohol solvent before the extraction. Furthermore, two previous studies cross-sectionally investigated the associations between hair cortisol and CHD, and one supported the association in elderly participant samples (Manenschijn et al., 2013), while the other did not in a large observational cohort (Abell et al., 2016). However, these studies only investigated the associations of hair cortisol with a self-reported history of CHD, not with onset of CHD.

We also found a higher fingernail cortisol level among the patients. A previous study found that 3–4 months were required for nails to fully extend from the nail matrix (Buzalaf et al., 2006). Our previous study (Izawa et al., 2015) also indicated that cortisol levels in fingernail samples were associated with those from saliva samples collected 4 or 5 months previously. Therefore, the findings of this study could indicate

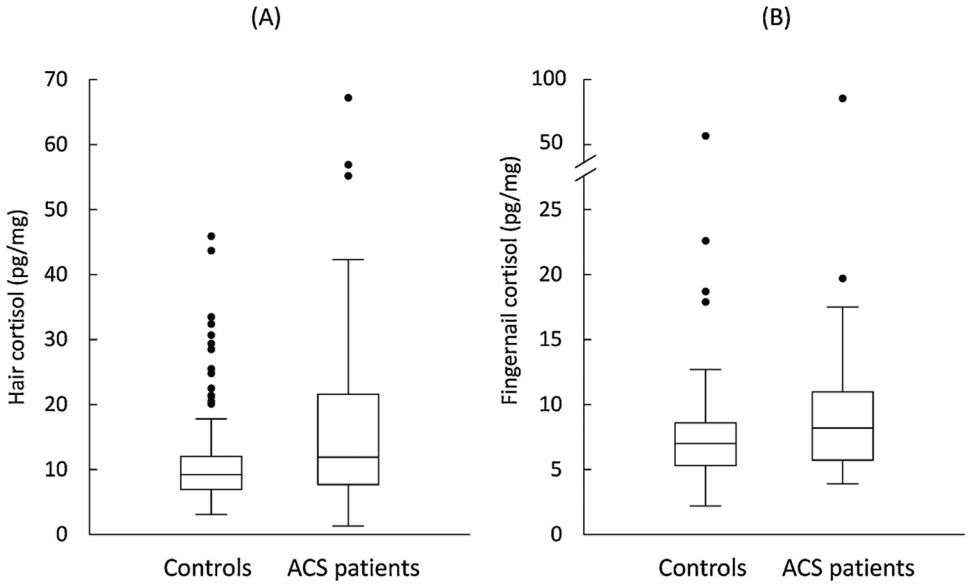

Fig. 1. Boxplots of hair (A) and fingernail (B) cortisol levels in the controls and ACS patients. Patients significantly exhibited higher cortisol levels in hair and fingernails (ps < 0.05, Mann–Whitney U-test).

Table 2
Risk of ACS and hair cortisol (N = 155)[a].

Risk factors	Crude OR (95% Cl)	OR (95% Cl) adjusting for traditional risk factors	OR (95% Cl) adjusting for psychosocial factors
Hair cortisol			
Low	1.00	1.00	1.00
Medium	0.75 (0.33-1.70)	0.91 (0.35-2.35)	0.85 (0.32-2.27)
High	**2.36 (1.07-5.19)**	**3.27 (1.28-8.40)**	**2.90 (1.12-7.46)**
Age (per yr)		1.01 (0.97-1.06)	1.01 (0.97-1.06)
Current smoking		**2.42 (1.01-5.80)**	2.28 (0.92-5.67)
Obesity		0.92 (0.40-2.11)	0.86 (0.36-2.07)
Dyslipidemia		**7.65 (3.44-17.00)**	**7.35 (3.26-16.59)**
Diabetes mellitus		0.92 (0.29-2.88)	0.91 (0.28-2.94)
Hypertension		1.52 (0.65-3.52)	1.68 (0.70-4.03)
Stressful life events (per event)			1.44 (0.84-2.46)
Higher education[b]			0.52 (0.22-1.24)

[a] Logistic regression analyses were conducted to estimate the crude and adjusted odds ratios (ORs) and 95% confidence intervals (CI) of ACS. Significant ORs ($p < .05$) are indicated in bold.
[b] University graduates or those with higher education.

Table 3
Risk of ACS and fingernail cortisol (N = 162)[a].

Risk factors	Crude OR (95% Cl)	OR (95% Cl) adjusting for traditional risk factors	OR (95% Cl) adjusting for psychosocial factors
Fingernail cortisol			
Low	1.00	1.00	1.00
Medium	1.27 (0.60-2.72)	1.06 (0.45-2.50)	0.93 (0.38-2.27)
High	**2.23 (1.02-4.88)**	**2.48 (1.02-6.07)**	2.14 (0.85-5.38)
Age (per yr)		1.01 (0.97-1.05)	1.01 (0.97-1.05)
Current smoking		2.14 (0.96-4.80)	1.98 (0.86-4.56)
Obesity		0.87 (0.39-1.90)	0.80 (0.35-1.83)
Dyslipidemia		**5.52 (2.68-11.37)**	**5.31 (2.53-11.13)**
Diabetes mellitus		1.01 (0.36-2.82)	1.02 (0.35-2.97)
Hypertension		1.81 (0.82-3.98)	1.91 (0.83-4.35)
Stressful life events (per event)			1.47 (0.90-2.40)
Higher education[b]			0.50 (0.22-1.13)

[a] Logistic regression analyses were conducted to estimate the crude and adjusted odds ratios (ORs) and 95% confidence intervals (CI) of ACS. Significant ORs ($p < .05$) are indicated in bold.
[b] University graduates or those with higher education.

higher cortisol levels among the patients several months before ACS onset. Further, the logistic regression analyses revealed that high levels of fingernail cortisol were associated with a two-fold increase in the risk of ACS. To our knowledge, this is the first study demonstrating the association between fingernail cortisol and ACS. Compared with hair samples, fingernail samples have some advantages: the samples can be self-collected, and only small amounts are required. Considering that fingernail cortisol was significantly correlated with hair cortisol in this study, future studies could investigate the value of fingernail samples for predicting health outcomes.

The pathophysiological mechanisms underlying the relationship between ACS and chronically elevated cortisol need to be considered. Psychosocial factors could contribute to the development of arteriosclerosis in the coronary artery and the onset of ACS by a number of different biological pathways (Kop, 1997). In a previous study, for example, heightened cortisol reactivity to mental stress was associated with coronary artery calcification (Hamer et al., 2012). Other studies also reported that higher cortisol levels contributed to hypercoagulable states such as increased concentration of fibrinogen (Lippi et al., 2008; von Känel et al., 2007). These pathophysiological states could link

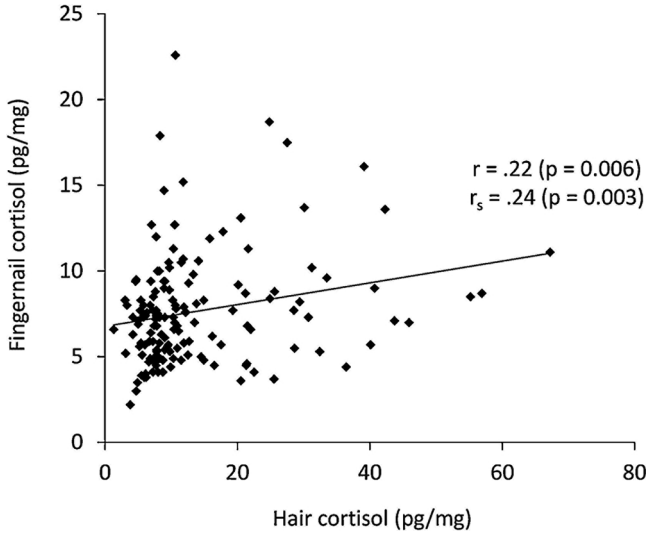

Fig. 2. Scatter plots illustrating the relationship between the cortisol levels in hair and fingernail samples (n = 149). r: Pearson product-moment correlation, r_s: Spearman rank-order correlation.

cortisol and ACS onset.

In this study, we observed associations between hair/fingernail cortisol and the onset of ACS, but these associations should be interpreted carefully. First, in the logistic regression analyses, inclusion of psychosocial factors (stressful life events and educational level) attenuated the association between fingernail cortisol and ACS, which could imply that psychosocial factors confounded the association between fingernail cortisol and ACS and that fingernail cortisol might not be directly associated with the onset of ACS. There is a possibility that, in this study, fingernail cortisol could reflect the stress level of the participants before the onset of ACS, but its physiological effect on ACS was relatively small, which could be related to the fact that fingernails reflects hormone levels for shorter periods of time compared with hair samples. Second, although cortisol measured in the hair and fingernails reflect cortisol levels prior to experiencing the ACS event, it is still possible that subclinical disease (e.g., chest pain) before the onset of ACS may have increased cortisol levels. Third, we only compared the ACS patients with healthy controls, but not with patients hospitalized for the other diseases. Therefore, it is not possible to say that the association is specific to ACS, and cortisol levels could possibly be associated with other diseases. However, a previous study (Pereg et al., 2011) reported the association between CHD and hair cortisol, in which CHD patients were compared with patients hospitalized for other diseases. Their results suggested that cortisol could be specific to ACS. Fourth, we also found a significant correlation between hair cortisol and diabetes mellitus, which was consistent with previous findings (e.g., Abell et al., 2016; Manenschijn et al., 2013; Stalder et al., 2013). Careful attention should be paid to the role of traditional cardiovascular risk factors, including diabetes mellitus, when considering the relationship between cortisol and CHD. Although we found that hair/fingernail cortisol levels were independently associated with ACS after controlling for cardiovascular risk factors, it is still possible that high cortisol levels additionally contributed to ACS through more severe cardiovascular risk factors.

We found a statistically significant association between hair and fingernail cortisol, but the association was relatively weak. In this study, the fingernail samples could show cortisol levels for anywhere from two weeks to several months prior to the ACS onset, because we collected fingernail samples that had grown for two weeks, and several months are required for nails to fully extend from the nail matrix (de Berker et al., 2007; Gupta et al., 2005). The hair samples, however,

could show cortisol levels for as long as the previous six months. The time gap between the two specimens may contribute to the weak correlation. For example, cortisol elevation by stressful experience one month before the onset of ACS could be reflected in the hair but not the fingernail samples. Although fingernail cortisol was previously investigated in several studies, the validity of the measurement was not fully established. Two studies (Frugé et al., 2017; Izawa et al., 2015) confirmed the associations between cortisol levels in the fingernail samples and other specimens. Future studies need to systematically investigate the validity of fingernail cortisol.

This study has several other limitations as well. The patients were from one hospital, and the controls were recruited via an internet-based survey. This may have caused sampling bias, although we carefully checked the backgrounds of the participants, as demonstrated in Table 1. Furthermore, the patients and controls consisted of only male participants. This was because among the Japanese, CHD was more common in males than in females. Further, considering the obvious hormonal differences between males and females, we only included male participants in this study. Future studies could investigate the associations among psychosocial stress, cortisol, and onset of ACS in a larger, less-biased sample.

In conclusion, we retrospectively investigated the associations between onset of ACS and cortisol levels using hair and fingernail samples from middle-aged and elderly men. We found higher cortisol levels in hair and fingernail samples among the ACS patients compared with the healthy controls, indicating higher cortisol levels among the patients for several weeks or months before the onset of ACS. The associations remained after the adjustments for the other ACS risk factors. High levels of hair or fingernail cortisol were associated with a two- to three-fold increased risk of ACS. The association between fingernail cortisol and ACS was, however, attenuated after the adjustments for psychosocial factors. We demonstrated that cortisol exposure over a relatively long period, as assessed by hair and fingernail samples, was associated with the onset of ACS.

Funding

This work was supported by a fundamental research grant from the National Institute of Occupational Safety and Health, Japan (N-F23-01).

Conflict of interest

All authors report having no financial or other relationships that represent actual or potential conflicts of interest relevant to the content of this paper.

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The Pharmacological Effects of Herbs on Catecholamine Signaling

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Abstract

Herbs have many biologically and pharmacologically active compounds such as flavonoids and stilbenes. They have been used in remedies for various disorders. Here we review the effects of herbs on catecholamine synthesis and secretion in cultured bovine adrenal medullary cells.

Ikarisoside A (1.0-100 μM), a flavonol glycoside, inhibited the catecholamine secretion induced by acetylcholine (0.3 mM). This inhibition was associated with the suppression of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ influx induced by acetylcholine. Ethanol extract (0.0003–0.005%) of matsufushi (extract of pine nodules) inhibited the catecholamine secretion induced by acetylcholine. SJ-2, one of the stilbene compounds isolated from matsufushi, inhibited acetylcholine-induced catecholamine secretion. Matsufushi extract and SJ-2 reversibly inhibited acetylcholine-induced Na^{+} currents in *Xenopus* oocytes expressed with α3β4nicotinic acetylcholine receptors. Sweet tea is the processed leaves of *Hydrangea macrophylla*. Extract of sweet tea (0.3–1.0 mg/ml) suppressed catecholamine secretion induced by acetylcholine (0.3 mM). Moreover, sweet tea (0.1 – 1.0 mg/ml), ikarisoside A (1.0–100 μM), and matsufushi (0.001–0.003 %) or SJ-2 (10-30 μM) inhibited acetylcholine-induced ^{14}C-catecholamine synthesis from ^{14}C-tyrosine.

These findings indicate that ikarisoside A, matsufushi (or SJ-2), and sweet tea inhibit the catecholamine secretion and synthesis induced by acetylcholine in cultured bovine adrenal medullary cells and probably in sympathetic neurons.

Keywords: Adrenal medullary cells, Catecholamine secretion, Ikarisoside A, Matsufushi, Sweet tea.

Abbreviations: ACh, acetylcholine, nAChR, nicotinic acetylcholine receptor

1. Introduction

Since herbs have many biologically and pharmacologically active compounds such as flavonoids and stilbenes, they have been used in remedies for various disorders. A high dietary intake of herbs has become a focus of research because of herbs' potential to reduce the risks of diseases such as hypertension, coronary heart disease, diabetes, and cancers [1, 2]. Flavonoids are a group of plant secondary metabolites with variable phenolic structures, and they are found in plants, fruits, vegetables, roots, stems, flowers, wine, and tea [3, 4]. Over 5,000 individual flavonoids have been reported [5], and six principal groups of flavonoids (flavones, flavonols, flavanones, flavanols, isoflavones, and anthocyanidins) are relatively common in human diets [1]. Polyphenol stilbenes have attracted scientific attention. For example, resveratrol (*trans*-3,4',5-trihydroxy-stilbene) is a natural phytoestrogen found in grapes, berries, and red wine [6, 7] that was reported to be implicated in the beneficial effect of red wine, i.e., the lower incidence of coronary artery disease in certain populations such as the French and the Greeks, despite diets rich in saturated fat and a rate of high smoking, which has been dubbed the 'French Paradox' [8].

In the human body, the most abundant catecholamines are adrenaline, noradrenaline, and dopamine, all of which are produced from phenylalanine and/or tyrosine. Catecholamines are biosynthesized mainly in the adrenal medulla, the postganglionic fibers of the sympathetic nervous system, and the central nervous system. Catecholamines play very important roles in aspects of the cardiovascular system such as heart rate and blood pressure, blood glucose levels, and the general functions of the central and peripheral sympathetic nervous system [9].

Adrenal medullary cells derived from embryonic neural crests are functionally homologous to sympathetic postganglionic neurons. Our research demonstrated that in cultured bovine adrenal medullary cells, at least three distinct types of ionic channels participate in catecholamine secretion, including nicotinic acetylcholine receptor (nAChR)-ion channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels [10]. In these cells, the Na^+ influx induced by acetylcholine (ACh) via nAChR-ion channels or by veratridine via voltage-dependent Na^+ channels, is a prerequisite for Ca^{2+} influx via the activation of voltage-dependent Ca^{2+} channels and subsequent catecholamine secretion; in contrast, high K^+ directly gates voltage-dependent Ca^{2+} channels to increase the Ca^{2+} influx without increasing the $^{22}Na^+$ influx [10] (Figure 1). ACh-induced Ca^{2+} influx is also a prerequisite for the stimulation of catecholamine synthesis associated with the activation of tyrosine hydroxylase [11-14].

The mechanisms underlying the stimulation of catecholamine synthesis and secretion mediated by these ion channels in adrenal medullary cells are thought to be similar to those of noradrenaline in the sympathetic neurons and brain noradrenergic neurons. Thus, adrenal medullary cells have provided a good model for the detailed analysis of antipsychotic [15], cardiovascular [16], and analgesic [17] drugs that act on catecholamine synthesis, secretion, and reuptake.

We have demonstrated the effects of several flavonoids and polyphenol stilbenes on catecholamine synthesis and secretion. For example, the treatment of bovine adrenal medullary cells with daidzein (an isoflavone derived from soy beans) stimulated basal catecholamine synthesis, but inhibited the catecholamine synthesis and secretion induced by ACh [18]. Genistein (another isoflavone in soy beans) but not daidzein stimulated the function of noradrenaline transporter in a human neuroblastoma cell line, SK-N-SH cells [19]. Nobiletine (a compound of polymethoxy flavone in citrus fruits) stimulated the basal synthesis and secretion of catecholamines, but it suppressed ACh-induced both the ACh-induced synthesis of catecholamines and ACh-induced secretion of catecholamines [20]. Resveratrol also inhibited the catecholamine synthesis and secretion induced by ACh [21].

The present review summarizes our recent and current studies of the pharmacological effects of herbs and their components, i.e., ikarisoside A (a flavonol glycoside), matsufushi (extract of pine nodules), one of matsufushi's stilbene components (SJ-2), and sweet tea on the catecholamine signaling induced by ACh in cultured bovine adrenal medullary cells and on ACh-induced Na^+ current in *Xenopus* oocytes expressing α3β4 nAChRs.

2. Inhibitory effects of ikarisoside A, but not its aglycon, on the catecholamine secretion and synthesis induced by ACh

Ikarisoside A is a natural flavonol glycoside derived from plants of the genus *Epimedium*, which have been used in traditional Chinese medicine as tonics, antirheumatics, and aphrodisiacs [22]. Ikarisoside A has antioxidant and anti-inflammatory effects [23] and anti-osteoporosis effects [24].

We observed that ikarisoside A (1–100 μM) concentration-dependently inhibited the secretion of catecholamines induced by ACh (0.3 m) (Figure 2A), but not the secretion of catecholamines induced by veratridine and 56 mK^+ [25]. Ikarisoside A also suppressed the $^{22}Na^+$ influx and $^{45}Ca^{2+}$ influx induced by ACh in a concentration-dependent manner similar to that of catecholamine secretion (Figure 2B, 2C). Ikarisoside A is a flavonol glycoside with one rhamnose at the 3 position in the chemical structure. The aglycon of ikarisoside A is 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4*H*-chromen-4one (Figure 3A). It is interesting to note that the aglycon of ikarisoside A had little effect on catecholamine secretion induced by ACh (0.3 m) (Figure 3B), suggesting that the rhamnose moiety at the 3 position of ikarisoside A is essential to inhibit the function of nAChR-ion channels. Ikarisoside A (1.0– or 10–100 μM) inhibited ACh (0.3 mM)-induced ^{14}C-catecholamine synthesis from ^{14}C-tyrosine and tyrosine hydroxylase activity [25].

3. Inhibitory effects of matsufushi and its stilbene component, SJ-2, on the catecholamine

synthesis and secretion induced by ACh

Pine nodules of *Pinus tabulaeformis* or *Pinus massoniana* are formed by pine bark proliferation at places on the trunk or limbs that have undergone damage, either by pests or physical injury. The effective curative components in pine nodules extract (matsufushi) have been used as an analgesic for joint pain, rheumatism, neuralgia, dysmenorrhea and other complaints in traditional Chinese medicine [26, 27].

Matsufushi ethanol extract (0.0003%–0.005%) concentration-dependently inhibited the catecholamine secretion and $^{45}Ca^{2+}$ influx induced by ACh (0.3 mM) and veratridine (0.1 mM), but not 56 mM K^+ in cultured bovine adrenal medullary cells [28]. Four compounds (SJ-2, SL-3, SJ-4, and SJ-16) were isolated from matsufushi extract (Figure 4). SJ-2, a phenol stilbene, and the mixture of four compounds (Mix4; SJ-2, SJ-3, SJ-4, and SJ-16), but not each of the other separate compounds, inhibited the catecholamine secretion (Figure 5) and $^{45}Ca^{2+}$ influx [28] induced by ACh (0.3 mM). In *Xenopus* oocytes expressed with α3β4 nAChRs, matsufushi extract and SJ-2 reversibly inhibited ACh (0.2 mM)-induced Na^+ currents (Figure 6A, 6C). Matsufushi extract (0.00003%–0.001%) (Figure 6B) and SJ-2 (1–100 μM) (Fig. 6D) significantly suppressed the Na^+ current in a concentration-dependent manner. In addition, matsufushi and SJ-2 suppressed the ACh (0.3 mM)-induced ^{14}C-catecholamine synthesis from ^{14}C-tyrosine and tyrosine hydroxylase activity [28]. These results suggest that matsufushi extract inhibits ACh-induced catecholamine synthesis and secretion mainly due to SJ-2 via the suppression of Na^+ influx mediated through nAChR-ion channels.

4. Effects of extract of sweet tea on catecholamine secretion and synthesis in adrenal medullary cells

Sweet tea is the processed leaves of *Hydrangea macrophylla var. thumbergii* Makino (Hydrangeae Dulcis Folium), which is listed in the Japanese Pharmacopoeia XV and used as a sweetening agents for diabetic patients. It also has anti-microbial and anti-allergic effects [29, 30]. There is, however, little evidence regarding sweet tea's effects on the sympathetic nervous system activity.

We investigated the effects of extract of sweet tea on adrenal medullary cell function. A dry powder of sweet tea prepared from fermented leaves of Hydrangeae Dulcis Folium was solubilized at 5.0 mg/ml and extracted at 90°C for 60 min. Extracted solution of sweet tea was used after centrifugation and filtration. Extract of sweet tea (1.0 mg/ml) slightly increased the basal secretion of catecholamines (Figure 7A), whereas it suppressed the catecholamine secretion induced by ACh (0.3 mM) in a concentration-dependent manner (300–1000 μg/ml) (Figure 7A). In addition, extract of sweet tea (300– or 100–1000 μg/ml) inhibited basal and ACh (0.3 mM)-induced ^{14}C-catecholamine synthesis from ^{14}C-tyrosine, respectively (Figure 7B). Sweet tea at concentrations of 3 mg/ml is usually used for drinking as a tea.

5. The insight of pharmacological potential of herbs in the catecholamine signaling induced by ACh in adrenal medulla

Adrenal medullary cells are derived from the embryonic neural crest and share many physiological and pharmacological properties with postganglionic sympathetic neurons. The stimulation of AChRs in these cells

increases the synthesis of catecholamines and causes the secretion of catecholamines into the systemic circulation [10, 13]. In adrenal medullary cells, the Na^+ influx induced by ACh via nAChR-ion channels is a prerequisite for Ca^{2+} influx via the activation of voltage-dependent Ca^{2+} channels and the subsequent catecholamine secretion and synthesis; in contrast, high K^+ directly gates voltage-dependent Ca^{2+} channels to increase $^{45}Ca^{2+}$ influx [10] (Figure 1).

As we noted, ikarisoside A and matsufushi (or SJ-2) inhibited the catecholamine secretion induced by ACh, but not the secretion induced by 56 mM K^+. In addition, ikarisoside A [25] and matsufushi or SJ-2 [28] suppressed the Na^+ current induced by ACh in *Xenopus* oocytes expressing $\alpha3\beta4$ nAChRs. These results suggest that the herbs and their components used as described herein inhibit the ACh-induced secretion and synthesis of catecholamines via a suppression of Na^+ influx mediated through nAChRs in adrenal medullary cells.

It is well known that catecholamines have important roles in the regulation of normal function in the central and peripheral sympathetic nervous systems as a neurotransmitter but also in the adrenal medulla as an endocrine hormone. Strong and prolonged stress causes massive amounts of catecholamine release, which can lead to cardiovascular diseases (such as hypertension, coronary heart disease, heart failure, and atherosclerosis), and such stress also suppresses the immune system to induce some cancers [2, 9]. Indeed, chronic heart failure is associated with the activation of the sympathetic nervous system as manifested by an increased circulating level of noradrenaline and increased regional activity of the sympathetic nervous system [31]. It was reported that the stress hormone adrenaline stimulates β2-adrenoceptors to activate the Gs-protein-dependent protein kinase A and the β-arrestin-1-mediated signaling pathway, which, in turn, suppresses p53 levels and triggers DNA damage [32]. On the basis of these previous and present results, it appears that the herbs and their components such as ikarisoside A, matsufushi (or SJ-2), and sweet tea suppress the induction of a hyperactive catecholamine system induced by strong stress or emotional excitation (Figure 8).

6. Future perspective

Although the *in vitro* effects of the herbs and herb components described herein have been well clarified using cultured bovine adrenal medullary cells and *Xenopus* oocytes, the *in vivo* results are not yet clear. To confirm the pharmacological effects of these herbs on the catecholamine system, further in vivo studies of the effects of the administration of herbs to animals or humans are needed. We observed a disturbance of the autonomic nervous balance in women with climacteric symptoms measured by a power spectral analysis of heart rate variability [33]. Using this assay method, we will examine the effect of herbs on the autonomic nervous activity under some stress conditions.

7. Concluding remarks

We have reviewed the evidence that herbs and their components such as ikarisoside A, matsufushi (or SJ-2), and sweet tea inhibit the catecholamine synthesis and secretion induced by ACh in cultured bovine adrenal

medullary cells. These findings may provide new insights into the pharmacological potentials of herbs on the hyperactive catecholamine system induced by stress.

8. Funding

This work was supported, in part, by Grant-in-Aid (26350170) for Scientific Research (C) from the Japan Society for the Promotion of Science and Grant-in-Aid (160701 and 170701) for Health Labor Sciences Research Grant, and by Tokujun Co. (Kobe, Japan), and Nozaki Kampo Pharmacy (Gifu, Japan).

9. Conflict of interest

The authors have no conflict of interest to declare.

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11. **Figure legends**

Figure 1. The mechanism underlying the regulation of catecholamine synthesis, secretion, and reuptake in bovine adrenal medullary cells.

Figure 2. Effects of ikarisoside A on ACh-induced catecholamine secretion (A), $^{45}Ca^{2+}$ influx (B), and $^{22}Na^{+}$ influx (C). (A) Cultured bovine adrenal medulary cells (10^6/well) were stimulated with ACh (300 μM) in the

presence or absence of ikarisoside A (0.3–100 μM) for 10 min at 37°C. Catecholamine secretion is expressed as a percentage of the total catecholamines in the cells. (B and C) Cells (4x10^6/well) are stimulated with ACh (300 μM) and 1.5 μCi of $^{45}CaCl_2$ (B) or $^{22}NaCl$ (C) in the presence or absence of ikarisoside A (0.3–100 μM) for 5 min at 37°C. $^{45}Ca^{2+}$ influx and $^{22}Na^{+}$ influx were expressed as nmol/4 × 10^6 cells. Data are means ± SEM from three separate experiments carried out in triplicate. $^{**}P<0.01$ and $^{***}P<0.001$ vs. ACh alone (by one-way ANOVA with Dunnett's multiple comparison *post hoc* test) (cited from [25]).

Figure 3. Structures of ikarisoside A and its aglycon (A) and the effect of aglycon of ikarisoside A on the catecholamine secretion induced by ACh (0.3 mM) (B). (A) Chemical structures of ikarisoside A and its aglycon (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-8- (3-methylbut-2-enyl)-4*H*-chromen-4-one). (B) Cells (10^6/well) were incubated with or without aglycon of ikarisoside A (1–100 μM) and ACh (300 μM) for 10 min at 37°C. Catecholamine secretion is expressed as a percentage of the total. Data are means ± SEM from three separate experiments carried out in triplicate (cited from [25]).

Figure 4. Chemical structures of SJ-2, SJ-3, SJ-4, and SJ-16 (cited from [28]).

Figure 5. Effects of SJ-2, SJ-3, SJ-4, SJ-16, and their mixture (Mix4) on catecholamine secretion induced by ACh in cultured bovine adrenal medullary cells. The cells (10^6/well) were incubated with or without SJ-2 (10 μM), SJ-3 (10 μM), SJ-4 (10 μM), C-16 (10 μM) and their mixture (Mix 4) (10 μM) for 10 min at 37°C. Catecholamines secretion is expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate. $^{***}P<0.001$ vs. ACh alone. Rha: rhamnose, (cited from [28]).

Figure 6. Effects of ethanol extract of matsufushi and SJ-2 on inward currents induced by ACh in *Xenopus* oocytes expressing rat α3β4 nAChRs. Representative traces from a single *Xenopus* oocyte are shown. The currents of matsufushi (Figure 6A)- and SJ-2 (Figure 6C)-treated oocytes were recorded for 10 min after recording of the control currents, and the washout currents were obtained for 10 min after matsufushi extract and SJ-2 treatment. Matsufushi extract (0.0001%) and SJ-2 (10 μM) suppressed the currents induced by the EC50 (0.2 mM) of ACh, and the inhibitory effects were reversible. Concentration-response curve for the inhibitory effects of matsufushi extract (Figure 6B) and SJ-2 (Figure 6D) on ACh-induced currents. The peak current amplitude in the presence of matsufushi extract and SJ-2 was normalized to that of the control and the effects are expressed as percentages of the control. Data are presented as means ± SEM from four separate experiments carried out in triplicate. $^{*}P<0.05$ and $^{***}P<0.001$ vs. the control (cited from [28]).

Figure 7. Effects of sweet tea on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (Figure 7A) and tyrosine hydroxylase activity (Figure 7B) in the cells. (Figure 7A) Cells (4×10^6/dish) were incubated with L-[U-^{14}C] tyrosine (20 μM, 1 μCi) in the presence or absence of sweet tea (100–1,000 μg/ml) and with or without 300

μM ACh at 37°C for 20 min. The ^{14}C-catecholamines formed were measured. (Figure 7B) Cells (10^6/well) were incubated with L-[1-^{14}C] tyrosine (18 μM, 0.2 μCi) in the presence or absence of sweet tea (100-1,000 μg/ml) and with or without 300 μM ACh at 37°C for 10 min, and tyrosine hydroxylase activity was measured. Data are means ± SEM from three separate experiments carried out in triplicate. $^{*}P<0.05$, compared with 37°C (control), and $^{**}P<0.01$ and $^{***}P<0.001$ vs. ACh alone in Figure 7A, and $^{**}P<0.01$vs. 37°C (control) and $^{***}P<0.001$ vs. ACh alone in Figure 7B.

Figure 8. Inhibitory mechanism of plant herbs (ikarisoside A, matsufushi, and sweet tea) on stress or excitation-induced excessive catecholamine secretion. Prolonged and strong stress or excitation stimulates the brain cortex, limbic system, and hypothalamus which evoke acetylcholine release from the splanchnic sympathetic nerves. Released acetylcholine induces a massive secretion of catecholamines from the adrenal medulla which may cause various deleterious symptoms or diseases such as high blood pressure (hypertension), vasculature proliferation (atherosclerosis), blood coagulation (thrombus), immune suppression, and cancers ([2] modified).

Internal Medicine
The Japanese Society of Internal Medicine
doi: 10.2169/internalmedicine.1007-18
Intern Med 58: 427-431, 2019
http://internmed.jp

【 CASE REPORT 】

Small Cell Lung Cancer Patient with Anti-transcriptional Intermediary Factor 1γ Antibody Who Developed Dermatomyositis after Successful Chemoradiotherapy

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Abstract:
We herein report a 63-year-old woman with small-cell lung cancer (SCLC) who developed dermatomyositis (DM) after initial chemoradiotherapy despite tumor reduction. Serum anti-transcriptional in termediary factor (TIF) 1γ antibody was detected before the development of DM, and its levels increased over time. She died five months after the diagnosis of SCLC. Anti-TIF1γ antibody is known to be a marker for cancer-associated DM (CAM); however, the present case indicates that the antibody can be found in cancer patients without DM. This case is also unusual, as DM developed later despite successful chemoradiotherapy.

Key words: anti-transcriptional intermediary factor 1 γ antibody, chemoradiotherapy, dermatomyositis, autoantibody, small-cell lung cancer

(Intern Med 58: 427-431, 2019)
(DOI: 10.2169/internalmedicine.1007-18)

Introduction

Dermatomyositis (DM) and polymyositis (PM) are systemic autoimmune rheumatic diseases characterized by proximal muscle weakness, myalgia, and characteristic skin rash. An increased risk of cancer in DM/PM, particularly DM, has been well described for many years. In recent years, anti-transcriptional intermediary factor 1 γ (TIF1γ) antibody was shown to be strongly associated with cancer-associated DM/PM (CAM), as confirmed by a meta-analysis (1-4).

We herein report a rare case of small-cell lung cancer (SCLC) in which anti-TIF1γ antibody was detected prior to the appearance of DM. The patient developed muscle weakness, elevated muscle enzymes, and typical skin rash of DM after initial chemoradiotherapy for SCLC despite tumor reduction.

Case Report

A 63-year-old woman with a smoking history of 20 pack-years, alcoholic liver injury, and hypertension had onset of cough and hoarseness.

A chest X-ray showed left hilar enlargement, and chest computed tomography (CT) revealed a 5-cm mass in the left upper lobe and 8-cm diameter mediastinal lymph node swelling (Fig. 1a). The levels of serum tumor markers, such as pro-gastrin-releasing peptide (pro-GRP) (4,490 pg/mL; normal range, <46 pg/mL) and nerve-specific enolase (NSE) (138 ng/mL; normal range, <10 ng/mL), were elevated. The serum levels of aspartate transferase (35 IU/L) and creatine kinase (57 U/L) were within normal limits, while those of lactate dehydrogenase (286 IU/L) and C-reactive protein (0.57 mg/dL) were slightly increased. Pathology of biopsy specimens obtained from the mediastinal lymph node and left main tumor by flexible bronchoscopy revealed SCLC,

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Received: February 13, 2018; Accepted: July 22, 2018; Advance Publication by J-STAGE: September 12, 2018
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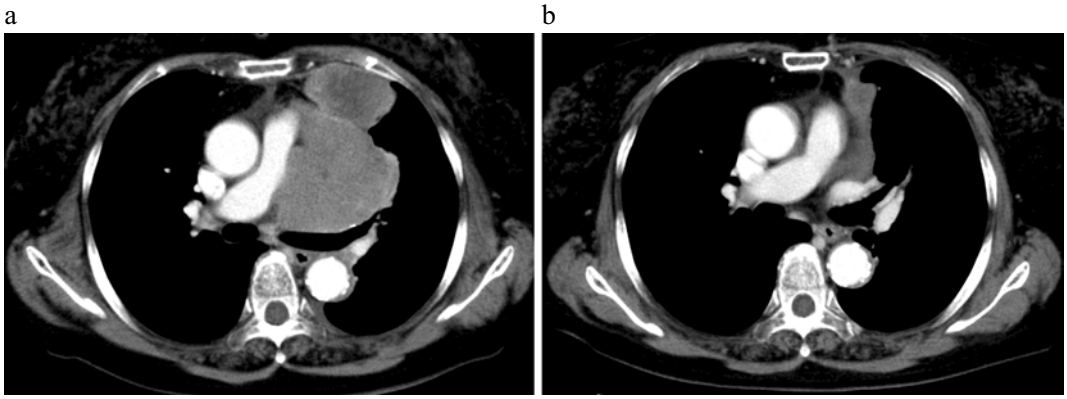

Figure 1. **Computed tomography (CT) image of the chest. CT at the initial admission showing a mass in the left upper lobe (5 cm in diameter) and a mediastinal lymph node (8 cm in diameter) (a), and CT five weeks after chemoradiotherapy showing reductions in the size of the lung and mediastinal lymph node masses (b).**

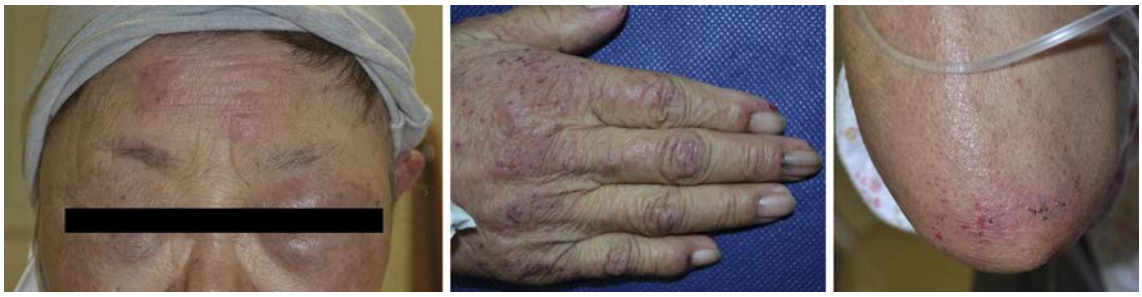

Figure 2. **Cutaneous findings upon admission. Heliotrope (a), nail-bed bleeding (b), and Gottron's sign (b) (c) were observed.**

and a systemic image analysis showed no distant metastasis; thus, she was diagnosed with limited stage SCLC. Systemic chemoradiotherapy with carboplatin plus etoposide was initiated, which led to a significant reduction in the tumor size in the lung and mediastinal lymph node on chest X-ray and a decrease in the levels of serum tumor markers. She was discharged without any clinical signs or symptoms of PM/DM at 27 days after chemotherapy. However, a week later, general malaise; facial edema; erythema on the hand, back, and body; proximal myalgia; muscle weakness; and dysphagia developed, and she was urgently readmitted to our hospital.

At the time of readmission, she was alert, and physical findings were as follows: height 146.5 cm, body weight 47.5 kg, body temperature 36.9 °C, blood pressure 134/85 mmHg, pulse rate 108 beats/min, and respiratory rate 17 cycles/min. The Eastern Cooperative Oncology Group Performance Status (ECOG-PS) was grade 4. Breathing and heart sounds were normal. Upon an examination, erythema on the face; heliotrope rash; aphthous ulcer in the oral cavity; extensive edematous erythema in the neck extending to the chest, back, and belt line; nail-bed bleeding; and Gottron papule were observed (Fig. 2). Muscle grasping pain was noted in both upper arms and thighs. Muscle weakness in the proximal muscles of the upper and lower limbs was also observed, and the results of a manual muscle test of the bilateral pectoralis major muscles, deltoid muscle, triceps brachii, iliopsoas, quadriceps muscle, and hamstrings were 4 of 5.

The serum levels of myogenic enzymes were elevated (aspartate transferase, 166 IU/L; lactate dehydrogenase, 486 IU/L; creatine kinase, 3,272 U/L; aldolase, 15.2 U/L; myoglobin, 1,854 ng/mL). The serum C-reactive protein levels (1.59 mg/dL) and blood sedimentation rate (71 mm/h) were elevated. The tumor marker levels were decreased compared with those at the time of the diagnosis (pro-GRP, 62.8 pg/mL; NSE, 30.9 ng/mL). Chest CT showed a marked reduction in the tumor size in the lung and mediastinal lymph node (Fig. 1b). Antinuclear antibodies were positive, with a titer of 1:640 in speckled and cytoplasmic patterns. Serum autoantibodies were evaluated according to immunoprecipitation and enzyme-linked immunosorbent assays, as described previously (5). Anti-TIF1γ/α, anti-Ro60, and anti-Ro52 antibodies were positive at the diagnosis of SCLC before the development of DM and chemoradiotherapy (Fig. 3). The levels of anti-TIF1γ/α antibodies increased by 2- to 6-fold after the onset of DM, whereas those of anti-Ro 52 antibodies decreased (Fig. 3b). Antibodies to aminoacyl-

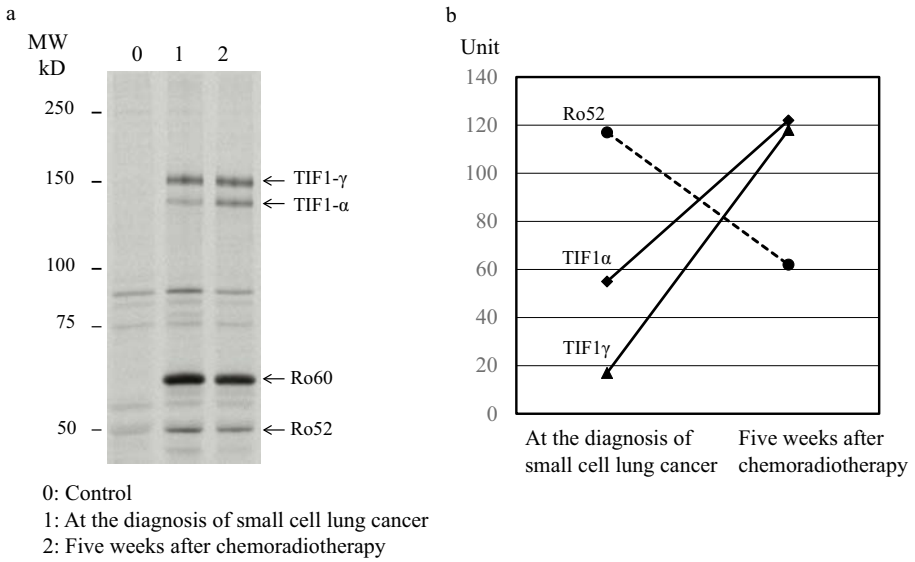

Figure 3. **An analysis of autoantibodies. (a) Immunoprecipitation. Autoantibodies in the sera were tested using ^{35}S-methionine labeled K562 cell lysate. Anti-transcriptional intermediary factor 1γ /α (anti-TIF1-γ /α) and Ro60 antibodies were positive at the diagnosis of small-cell lung cancer and five weeks after chemoradiotherapy when dermatomyositis developed. Although a protein band of approximately 50 kD was noted, Ro52 could not be clearly seen in this condition. (b) ELISA. Levels of autoantibodies to TIF1-γ /α and Ro52 by ELISA at the diagnosis of lung cancer and five weeks after chemoradiotherapy are shown. Anti-Ro60 was negative on ELISA.**

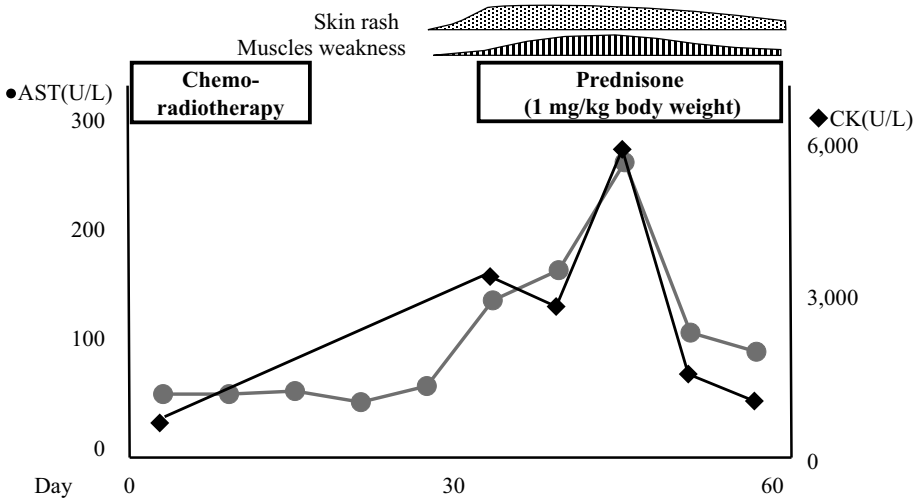

Figure 4. **Clinical course.**

tRNA synthetases and -Mi-2 were negative. She was diagnosed with classic DM based on muscle weakness, elevated levels of muscle enzymes, and typical skin rash. In addition, CAM was diagnosed because of the evidence of SCLC with positive results for anti-TIF1γ antibody (6). Although anti-Ro60 and anti-Ro52 antibodies were positive, she did not have sicca symptoms of Sjögren's syndrome.

High-dose corticosteroid therapy (prednisone 1 mg/kg body weight) was initiated for DM, resulting in only partial improvement in muscle and skin symptoms (Fig. 4). Her general condition and performance status did not improve because of complicating bacterial pneumonia, a worsened mental condition, and SCLC progression. Thus, additional systemic chemotherapy could not be administered, and she died five months after the diagnosis of SCLC at another hospital where she had been transferred for terminal care.

Discussion

We herein report a rare case of CAM that developed after initial chemoradiotherapy for SCLC despite tumor reduction. Serum anti-TIF1γ antibody was detected when the diagnosis of SCLC was made before the treatment of cancer and the development of DM, and its levels increased over time.

In patients with CAM, the occurrence of DM/PM after the diagnosis of malignant tumors is not rare (2, 7-9). Indeed, one study reported that more than 30% of malignancies preceded the diagnosis of DM/PM (7). Regarding the association between the cancer and activity of DM/PM, successful treatment of cancer, typically removal of cancer by surgery, leads to an improvement of the activity of DM/PM, and the relapse of cancers usually lead to flare-up of DM/PM (10-13). Given previous findings, the present case appears to be a very rare one because CAM developed after a marked reduction in tumor size by chemoradiotherapy and anti-TIF1γ antibodies were detected in a patient with SCLC without any signs of DM/PM.

The mechanism through which CAM developed after initial chemoradiotherapy for SCLC despite tumor reduction with increased levels of TIF1γ antibody in our patient is unclear. Since *TIF1* is a known tumor-suppressor gene, a mutation in *TIF1* may have been the primary event, leading to the development of SCLC, with mutated TIF1 antigens inducing an autoimmune response (4). Abnormal proteins expressed by tumor tissues may lead to an immune response, and the expression of mutated antigens may lead to the production of specific autoantibodies, such as anti-p53 and RNA polymerase III antibodies (14, 15). Furthermore, a large amount of tumor antigens released during chemoradiotherapy might play a role in stimulating the immune system, accelerating the autoantibody production and the subsequent development of DM in the present case. However, our speculation cannot be definitively confirmed, and the development of DM in this case might simply be a natural course.

Another interesting point of this case is the detection of anti-TIF1γ antibody at the cancer diagnosis before the onset of DM. The detection of anti-TIF1γ antibody in patients with malignancy without DM has not been well documented; only three cases of borderline positivity of the antibody in breast cancer patients without rheumatic diseases have been reported (16). The present case suggests that the detection of anti-TIF1γ antibody in cancer patients may be a useful marker for predicting DM/PM onset. Of further note, the levels of anti-Ro52 antibodies decreased while those of anti-TIF1γ and anti-TIF1α increased by 2- to 6-fold during the development of DM (Fig. 3b). Some autoantibodies may share common mechanisms of regulation, although non-parallel changes in different specific autoantibodies have also been reported. The development of anti-U1RNP or ribosomal P antibodies and a reduction in anti-RNA helicase A antibody levels in SLE has been reported (17). Our data therefore suggest different mechanisms of regulation for anti-TIF1γ and anti-TIF1α antibodies and for anti-Ro52 autoantibodies.

In the present case, anti-TIF1γ and anti-TIF1α antibodies were both positive. Fujimoto et al. showed that anti-TIF1α antibody often coexists with anti-TIF1γ antibody (18). Another report demonstrated that anti-TIF1α antibody can be found in patients with anti-Mi-2 antibody but without anti-TIF1γ antibody. In the present case, anti-Mi-2 antibody was negative, and cases with both anti-TIF1γ and TIF1α antibodies developed cancer more frequently than those with only anti-TIF1α antibodies (19). However, the clinical significance of these antibodies in lung cancer patients is not well understood, and the accumulation of more cases is desired.

The prognosis of patients with CAM generally depends on the prognosis of cancer (20-23); therefore, the treatment of cancer is essential for CAM. Treatment with corticosteroids and/or immunosuppressants is considered when necessary. In a review of 12 patients with CAM with SCLC, a very poor prognosis of 2 weeks to 9 months (median: 5 weeks) was reported (24). Compared with the median survival of 9.4 to 12.8 months in patients with extensive-disease SCLC, the prognosis of SCLC patients with CAM may be worse than that in patients with SCLC without CAM (25). An inadequate response of myopathy to corticosteroid treatment in certain patients may limit the treatment options for cancer, as seen in our case, leading to a poor prognosis.

Conclusion

We herein report a rare case of SCLC with anti-TIF1γ antibody and the subsequent development of DM despite tumor reduction by systemic chemoradiotherapy along with an increase in the levels of anti-TIF1γ antibody. Anti-TIF1γ antibody is known to be a marker for CAM; however, the present case indicates that the antibody can be found in cancer patients without DM. It should be noted that CAM might be a poor prognostic factor for SCLC.

The authors state that they have no Conflict of Interest (COI).

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RHEUMATOLOGY

Rheumatology 2019;58:120–130
doi:10.1093/rheumatology/key257
Advance Access publication 28 August 2018

Original article

Significance of nailfold videocapillaroscopy in patients with idiopathic inflammatory myopathies

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Abstract

Objective. The aim of this study was to investigate the clinical and immunological significance of nailfold videocapillaroscopy (NVC) abnormalities in patients with idiopathic inflammatory myopathies (IIMs).

Methods. Seventy consecutive Japanese patients with untreated IIMs, enrolled between April 2014 and August 2017, were prospectively studied. Clinical features, NVC findings, autoantibody profile by immunoprecipitation and ELISA, and histopathological findings of skin biopsies of DM rash were assessed at baseline and after 1-year of immunosuppressive therapy.

Results. NVC abnormalities were found in 55.7% (39/70) of IIM patients, with significantly higher prevalence in DM (65.4%) compared with PM (27.8%) ($P = 0.01$). In subsets of patients classified by autoantibody specificities, the prevalence of NVC abnormalities was significantly higher in patients with anti-MDA5 (87.5%) and anti-transcriptional intermediary factor 1γ (88.9%) *vs* anti-aminoacyl-tRNA synthetase (26.9%, $P < 0.001$). Perivascular lymphocytic infiltration in the upper dermis of skin rash biopsy of DM was more severe in patients with NVC abnormalities ($P < 0.05$). Unexpectedly, NVC abnormalities disappeared in 75% of IIM patients after 1-year of immunosuppressive therapy in contrast to stable NVC changes seen in scleroderma patients.

Conclusion. Nailfold microvascular abnormalities were common in DM patients, associated with anti-MDA5 and transcriptional intermediary factor 1γ antibodies, and perivascular inflammation in skin histology. NVC abnormalities in IIMs may become clinically useful markers for defining subsets of DM and understanding the pathogenesis of the clinical features seen in these patients.

Key words: DM, PM, nailfold videocapillaroscopy, myositis-specific autoantibodies, systemic sclerosis

CLINICAL SCIENCE

Rheumatology key messages

- Nailfold videocapillaroscopy abnormalities were common in inflammatory myopathy patients and observed in 55.7%.
- The nailfold videocapillaroscopy abnormalities were correlated with the severity of perivascular lymphocyte infiltration.
- The nailfold videocapillaroscopy abnormalities in more than half of the patients disappeared following immunosuppressive therapy.

Introduction

Idiopathic inflammatory myopathies (IIMs) are systemic autoimmune rheumatic diseases characterized by inflammatory myopathy accompanied by heterogeneous clinical features [1, 2]. IIMs in adults can be roughly divided into DM, PM, and inclusion body myositis (IBM) [3]. Two major extramuscular manifestations that influence

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Submitted 6 April 2018; revised version accepted 17 July 2018

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prognosis include interstitial lung disease (ILD) and malignancy [4, 5]. Myositis-specific autoantibodies (MSAs) are important in the diagnosis, classification, predicting clinical features and prognosis of IIMs. Clinical subsets of IIM can be defined based on MSAs such as antibodies to aminoacyl-tRNA synthetase (ARS), Mi-2, melanoma differentiation-associated protein-5 (MDA5), transcriptional intermediary factor 1γ (TIF1γ), signal recognition particle and associated clinical features [6, 7]. In particular, patients with anti-MDA5 antibodies associated with treatment-resistant, rapidly progressive ILD and anti-TIF1γ antibodies associated with malignancy are known to have a poor prognosis [6–8]. Patients with DM show not only perivascular necrosis but also perivascular lymphocyte infiltration in muscle pathology [9], suggesting that microvascular abnormalities may be a common and basic pathological process associated with muscular, skin and pulmonary inflammation.

Microvascular scleroderma spectrum disease–associated abnormalities (scleroderma patterns) are characteristic of SSc and can be detected in the nailfold of >80% of SSc patients [10]. The detection of microvascular scleroderma patterns helps understanding of the pathogenesis and enables early diagnosis of SSc [11, 12]. Microvascular scleroderma patterns are detected by nailfold videocapillaroscopy (NVC), which allows non-invasive, safe and real-time assessment of the vascular abnormalities in the nailfold [10]. NVC is used as an imaging modality in a scoring system for microvascular abnormalities in SSc [13, 14]. Thus, the significance of microvascular scleroderma patterns has been suggested for various aspects of SSc, including diagnosis [11, 12], association with organ damage [15, 16] and SSc-related autoantibodies [17, 18], treatment response, and disease progression [19].

However, only a few studies have examined nailfold microvascular abnormalities in IIMs, compared with extensive use of NVC in SSc. Several studies have reported microvascular scleroderma patterns in IIMs, with inconsistent descriptions of their clinical significance [20–23]. These inconsistent findings can be mostly explained based on differences in the methods of assessment, small numbers of subjects, as well as the lack of evaluation of untreated patients.

In this study, we report a prospective analysis in 70 previously untreated patients with IIMs to evaluate the microvascular abnormalities by NVC and analyse their association with clinical features and MSAs. Comprehensive analysis of MSAs was performed by immunoprecipitation and ELISA. In addition, the association of NVC changes with histopathological findings in skin biopsy was analysed to understand the pathogenesis of DM, focusing on the microvasculature.

Methods

Patients

Seventy consecutive patients with IIMs were enrolled to this multicentre prospective study between April 2014 and August 2017. All patients had been diagnosed previously with PM/DM using the PM/DM Diagnostic Criteria of the Ministry of Health, Labour and Welfare in Japan (Supplementary Table S1, available at *Rheumatology* online), which is commonly used in Japan [24]. Signs consistent with inflammatory myopathy were confirmed in 68.0% (34/50) of patients by electromyography, 60.0% (33/55) by MRI, and 90.9% (20/22) by muscle biopsy. Twenty-eight (11 definite and 17 probable) patients fulfilled the Bohan and Peter criteria of PM/DM [1, 2], while 19 fulfilled the Sontheimer's criteria for clinically amyopathic DM (CADM) [25]. In addition, 98.6% (69/70) of the patients fulfilled the ACR/EULAR 2017 classification criteria (63 cases fulfilled definite IIMs and 6 cases fulfilled probable IIMs) [26, 27]. According to this classification, 21 patients were classified amyopathic DM, 31 patients were classified DM, 16 patients were classified PM, and 1 patient was classified immune-mediated necrotizing myopathy. IIM patients with other systemic autoimmune rheumatic diseases were excluded.

The study also included 60 age- and gender-matched SSc patients who fulfilled the ACR/EULAR 2013 classification criteria [11, 12] and 40 healthy individuals as controls. The mean age of healthy individuals was 58.6 years old, and 62.5% were female. The human ethics review committee of our university reviewed and approved the study (SCORPION study, UMIN ID 000014293). A signed informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and its subsequent modifications.

Clinical measurements

The clinical signs, including fever, muscle weakness, Gowers' sign, dysphagia, RP, heliotrope rash, Gottron's sign/papules, palmar papules, arthritis, and presence of internal malignancy, were evaluated. The laboratory tests, including CRP, ESR, creatine kinase, IgG, ferritin, partial pressure of oxygen in arterial blood (PaO_2) and fraction of inspiratory oxygen (FiO_2), were also evaluated. For the assessment of ILD, chest CT was used in all patients. For the evaluation of ILD by CT, the thorax was divided into upper, middle and lower segments, and CT scores (0–3) in each segment were recorded. The CT score was a mean value of the independent scoring by three physicians based on the previously described method [28, 29]. Vital capacity as a percentage of predicted capacity (%VC) was evaluated by respiratory function test. Clinical data and serum samples were collected at the beginning and 1 year after treatment. Eight patients (8/58, 13.8%) were lost during follow-up and excluded from the analyses.

NVC images

All patients underwent NVC evaluation of nailfold microvascular damage, as described previously [30]. Briefly, NVC examination was performed using an optical probe videocapillaroscope equipped with a 200-fold contact lens and connected to image analysis software

(Videocap 3.0; DS Medica, Roma, Italy). The same operator, who was blinded to the patient's clinical diagnosis, disease severity and other clinical features except for skin lesions, performed the NVC examination in all patients. Nailfolds of the second, third, fourth and fifth fingers were examined in each evaluation. The following capillaroscopic parameters were recorded: presence of enlarged (defined as increase in capillary diameter $>20\,\mu m$) and giant capillaries (defined as homogeneously enlarged loops with a diameter $>50\,\mu m$), haemorrhages (recognized as dark masses due to hemosiderin deposits), loss of capillaries (reduced number of capillaries, i.e. <9 capillaries per linear millimetre at the distal row of the nailfold), disorganization of the microvascular array (seen as irregular capillary distribution and orientation, along with heterogeneity in the loop shape) and capillaries with abnormal morphologies and ramification (e.g. branching, bushy or coiled capillaries that often originated from a single normal-sized capillary) [14, 31].

The NVC scleroderma spectrum abnormalities were defined as described previously (early, active, late, and scleroderma-like pattern) [13, 18]. The early pattern is characterized by few giant capillaries, few capillary microhaemorrhages, and no evident loss of capillaries. The active pattern comprises abundant giant capillaries, several capillary microhaemorrhages, and moderate loss of capillaries. The late pattern is characterized by irregular enlargement of capillaries, almost no giant capillaries or microhaemorrhages, severe loss of capillaries, abnormal shapes, and intense disorganization of the normal capillary array [19]. The scleroderma-like pattern is defined as a capillary pattern showing mixed microvascular markers of the scleroderma NVC patterns, but not fully fitting the definition for the single early, active or late pattern. (Supplementary Fig. S1, available at *Rheumatology* online) [13, 32].

Skin biopsies and scoring

Biopsies from the skin with Gottron's sign/papules and palmar papules were obtained from 40 patients. Infiltration of inflammatory cells in the biopsy sections was assessed by a pathologist blinded to the clinical information. The number of inflammatory cells around capillaries in the upper dermis layer was counted and scored as follows: mild (<30 cells), moderate (30–99 cells) and severe ($\geqslant 100$ cells) (Fig. 1A).

Immunoprecipitation

MSAs and other autoantibodies in the sera were analysed by immunoprecipitation of K562 cell extract radiolabelled with ^{35}S-methionine as described previously. The specificity of each autoantibody was determined by using a specific reference serum sample [33]. Analysis of RNA components of the target antigen was also performed by immunoprecipitation, and silver staining when necessary.

Anti-MDA5 and Jo-1 ELISA

Anti-MDA5 and Jo-1 antibodies were tested by ELISA, using recombinant proteins (0.5 μg/ml, Diarect, Freiburg, Germany) and 1: 250 diluted sera as described previously [33, 34]. The optical density was measured and converted into units using a standard curve created by a prototype positive serum.

Statistical analysis

Data are expressed as mean ± standard deviation or number (%). Differences between groups were computed using the Mann–Whitney *U* test, Fisher's exact test, and one-way analysis of variance. The Wilcoxon signed-rank test and McNemar's test were used to detect statistically significant differences in NVC findings, clinical data, and laboratory data between baseline and 1-year measurements. All reported *P* values were two-sided and were not adjusted for multiple testing. The level of significance was set at $P < 0.05$. All analyses were conducted using JMP version 11.0 (SAS Institute Inc., Cary, NC) or SPSS software version 22.0 (SPSS Inc., Chicago, IL).

Results

Baseline characteristics

The clinical characteristics in the 70 patients with IIMs and 60 SSc patients are shown in Table 1. All were Asians, and none was on immunosuppressive drugs or glucocorticoids at baseline. For the IIM patients, the mean duration of illness was 0.7 years; 67.1% of the patients had muscle weakness, and 75.7% of the patients had ILD. The baseline characteristics of SSc patients were comparable with those of IIM patients with regard to age and gender (Table 1). When patients were classified into PM and DM, the prevalence of muscle weakness and the levels of creatine kinase were higher in PM patients (Supplementary Table S2, available at *Rheumatology* online). Anti-MDA5 and anti-TIF-1γ antibodies were found only in DM patients.

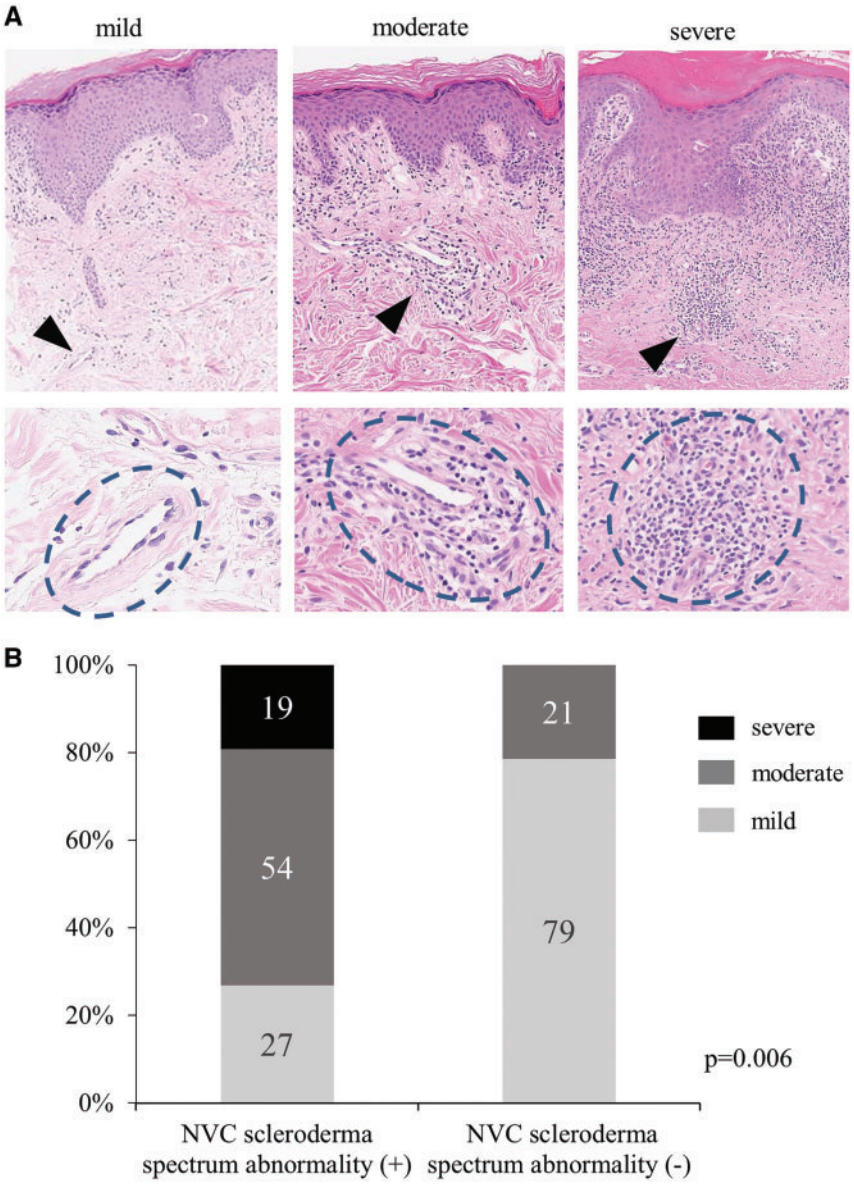
Prevalence of nailfold capillary scleroderma spectrum abnormalities in IIM patients

The prevalence of scleroderma spectrum abnormalities by NVC in patients with IIMs and SSc are summarized in Table 2. The scleroderma spectrum abnormalities by NVC were common in IIM patients and observed in 55.7%, though the prevalence was lower compared with SSc patients ($P = 0.005$) and healthy individuals. The percentage of patients with positive NVC scleroderma spectrum abnormalities was significantly higher in patients with DM than in those with PM ($P = 0.01$). Of note, the scleroderma-like pattern, which was not seen in patients with SSc, was observed in 30.9% of patients with IIMs.

Association between NVC scleroderma spectrum abnormalities and clinical findings

To analyse the clinical and laboratory features associated with NVC scleroderma spectrum abnormalities in IIMs, the

FIG. 1 NVC scleroderma spectrum abnormalities correlated with pathological findings of the skin



(**A**). Representative skin pathology images illustrating the grades (mild, moderate and severe) of perivascular inflammatory infiltrate in upper dermis. (**B**) The percentage of grades of perivascular inflammatory infiltrate in patients with *vs* without NVC abnormalities. NVC: nailfold videocapillaroscopy.

characteristics were compared between patients with *vs* without NVC scleroderma spectrum abnormalities (Table 3). Certain clinical findings (heliotrope rash, Gottron's sign/papules, and palmar papules) were significantly more prevalent in patients with NVC scleroderma spectrum abnormalities ($P = 0.003$, 0.04 and 0.001, respectively); however, levels of serum myogenic enzymes, acute phase proteins, IgG and pulmonary dysfunction were comparable between the two groups. Surprisingly, the prevalence of RP, which we predicted to have an association with scleroderma-spectrum microvascular changes detected by NVC, was also comparable. Strikingly, there were significant differences in the prevalence of several MSAs. Namely, the prevalence of anti-MDA5 antibodies ($P = 0.004$), which have strong association with CADM accompanied by rapidly progressive ILD, and anti-TIF1γ antibodies ($P = 0.04$), associated with DM with malignancy, were significantly higher in patients with NVC scleroderma spectrum abnormalities (Table 3). These trends were similar even when the analysis was limited to patients with DM, except for differences in the prevalence of Gottron's sign/papules and anti-TIF1γ antibodies (Supplementary Table S3, available at *Rheumatology* online). In sharp contrast, prevalence of anti-ARS was

Table 1 Baseline characteristics of patients with IIMs, SSc and HCs

Variables	IIMs ($n = 70$)	SSc ($n = 60$)	*P*-value IIMs *vs* SSc
Age, years	60.4 (16.1)	59.9 (16.1)	0.85
Female, *n* (%)	47 (67.0)	48 (80.0)	0.12
Disease duration, years	**0.7 (1.2)**	**6.4 (8.3)**	**<0.001**
Current smokers, *n* (%)	6 (9.0)	11 (18.6)	0.12
Symptoms, %			
Fever	45.7		
Myalgia	84.3		
Muscle weakness	67.1		
Gowers' sign	14.3		
Dysphagia	14.3		
RP	**11.4**	**93.3**	**<0.001**
Heliotrope rash	28.6		
Gottron's sign/papules	68.6		
Palmar papules	30.0		
Arthritis	**44.3**	**25.0**	**0.027**
ILD	**75.7**	**38.3**	**<0.001**
Malignancy	15.7		
Laboratory data			
CRP, mg/dl	**2.2 (4.2)**	**0.7 (1.7)**	**<0.001**
ESR, mm/h	**46.7 (29.1)**	**27.7 (24.9)**	**<0.001**
CK, U/l	**1878.2 (2747.6)**	**93.6 (72.8)**	**<0.001**
IgG, mg/dl	**1612.8 (527.5)**	**1418.1 (415.9)**	**0.033**
Ferritin, ng/ml	**488.2 (840.9)**	**107.4 (106.1)**	**<0.001**
PaO_2/FiO_2	388.5 (80.9)		
CT score	0.93 (0.78)		
VC, l	2.29 (0.95)		
%VC	75.8		
Autoantibodies, %			
Antinuclear abs	**67.1**	**96.6**	**<0.001**
Anti-MDA5 abs	22.9		
Anti-ARS abs	37.1		
Anti-TIF1γ abs	12.9		
Anti-Mi2 abs	1.4		

Data are mean (S.D.) unless otherwise stated. *P*-values are for IIMs *vs* SSc and were determined by Fisher's exact test. Values highlighted in bold indicate statistical significance ($P < 0.05$). Abs: antibodies; ARS: aminoacyl tRNA synthetase; CK: creatine kinase; HCs: healthy controls; IIMs: idiopathic inflammatory myopathies; ILD: interstitial lung disease; MDA5: melanoma differentiation-associated protein-5; PaO_2/FiO_2: ratio of partial pressure of oxygen in arterial blood to fraction of inspiratory oxygen; TIF1γ: transcription intermediary factor 1gamma; VC vital capacity.

significantly lower in patients with NVC scleroderma spectrum abnormalities ($P < 0.001$).

Based on the observed significant differences in the prevalence of different MSAs in patients with *vs* without NVC scleroderma spectrum abnormalities (Table 3), clinical and laboratory features were compared according to the specificity of autoantibodies (anti-ARS antibodies including anti-Jo-1, PL-7, PL-12, EJ, KS and OJ, anti-MDA5 antibodies, anti-TIF1γ antibodies, and other antibodies including anti-Mi-2, MJ/NXP-2, signal recognition particle, Ku, and cohesin complex) (Table 4).

The differences in the prevalence of NVC scleroderma spectrum abnormalities were more evident when patients were classified based on their autoantibody specificities; ~90% of the patients with anti-MDA5 and anti-TIF1γ antibodies had NVC scleroderma spectrum abnormalities compared with only 26.9% in anti-ARS-positive patients ($P < 0.001$) (Table 4). The clinical features associated with each autoantibody were mostly consistent with previous literature [6–8]; myopathy was less prominent in patients with anti-MDA5 antibodies, malignancy was common in patients with anti-TIF1γ antibodies, and skin rash characteristic of DM was more common in anti-MDA5- and TIF1γ-positive patients as they were exclusively found in DM or CADM (Table 4).

Relationship between NVC scleroderma spectrum abnormalities and skin pathology

The above results demonstrated a higher prevalence of NVC scleroderma spectrum abnormalities in DM patients, especially in patients with anti-MDA5 and anti-TIF1γ antibodies. However, the significance of the NVC scleroderma spectrum abnormalities in the pathogenesis and clinical features of IIMs is unclear. The NVC abnormalities represent microvascular injuries, whereas perivascular inflammation is often observed in the biopsy specimen of the DM skin rash. The pathogenesis and pathological process in these microvascular abnormalities may be common; however, their relationship has not been studied. To address this issue, the histopathological changes around the skin capillaries in skin rash of DM were evaluated and compared between patients with *vs* without NVC scleroderma microvascular changes. Perivascular lymphocytic infiltration was often observed in skin rash biopsy tissue from patients with DM (Fig. 1A), although the extent of such infiltration varied from one patient to another. When the severity (mild, moderate or severe) of perivascular lymphocytic infiltration was compared between patients with *vs* without scleroderma microvascular changes by NVC, the former had more severe perivascular lymphocytic infiltration (Fig. 1B). Furthermore, severe lymphocytic infiltration was noted only in patients with scleroderma spectrum abnormalities by NVC. These findings suggest that NVC scleroderma spectrum abnormalities in DM correlate with perivascular lymphocytic infiltration in skin rash, and local immune inflammatory perivascular reaction may contribute to the pathogenesis in these two features of the skin.

Effects of immunosuppressants on NVC changes

Finally, the changes in NVC abnormalities after 1-year treatment with CYC or calcineurin inhibitors, in addition

TABLE 2 Comparison of NVC findings (NVC pattern, %) among IIMs (PM or DM) and SSc

Variables	IIMs ($n = 70$)	PM ($n = 18$)	DM ($n = 52$)	SSc ($n = 60$)	*P*-value (IIMs *vs* SSc)
NVC scleroderma spectrum abnormalities, %	**55.7**	27.8	65.4	**80.0**	**0.005**
Early pattern	**15.7**	5.6	19.2	**43.3**	**0.001**
Active pattern	**7.1**	0.0	9.6	**26.7**	**0.004**
Late pattern	2.9	0.0	3.8	10.0	0.143
Scleroderma-like pattern	**30.0**	22.2	32.7	**0.0**	**<0.001**

P-values are for IIMs *vs* SSc and determined by Fisher's exact test. Values highlighted in bold indicate statistical significance ($P < 0.05$). IIMs: idiopathic inflammatory myopathies; NVC: nailfold videocapillaroscopy.

TABLE 3 NVC scleroderma spectrum abnormalities and clinical/laboratory findings in IIM

Variables	With NVC scleroderma spectrum abnormality ($n = 39$)	Without NVC scleroderma spectrum abnormality ($n = 31$)	*P*-value
Age, years	59.7 (15.1)	61.4 (17.4)	0.49
Female, *n* (%)	27 (69.2)	20 (64.5)	0.80
Disease duration, years	0.6 (1.1)	0.7 (1.3)	0.22
Current smokers, *n* (%)	5 (12.8)	1 (3.2)	0.22
Symptoms, %			
Fever	51.3	38.7	0.34
Myalgia	79.5	90.3	0.32
Muscle weakness	64.1	71.0	0.31
Gowers' sign	46.2	48.4	1.00
Dysphagia	17.9	9.7	0.50
RP	12.8	9.7	1.00
Heliotrope rash	**43.6**	**9.7**	**0.003**
Gottron's sign/papules	**79.5**	**54.8**	**0.04**
Palmar papules	**46.2**	**9.7**	**0.001**
Arthritis	46.2	41.9	0.81
ILD	74.4	77.4	1.00
Internal malignancy	20.5	9.7	0.32
Laboratory data			
CRP, mg/dl	1.8 (3.0)	2.7 (5.4)	0.93
ESR, mm/h	49.3 (29.3)	43.5 (29.0)	0.36
CK, U/l	1686.3 (2262.1)	2119.5 (3282.3)	0.96
IgG, mg/dl	1602.1 (515.3)	1626.4 (550.7)	0.89
Ferritin, ng/ml	570.4 (910.0)	387.5 (750.0)	0.13
PaO_2/FiO_2	387.2 (84.6)	389.8 (78.5)	0.17
CT score	0.90 (0.76)	0.97 (0.80)	0.79
VC, l	2.2 (1.2)	2.2 (0.71)	0.88
%VC	74.5	75.7	0.93
Autoantibodies, %			
Antinuclear abs	71.8	61.3	0.44
Anti-MDA5 abs	**35.9**	**6.5**	**0.004**
Anti-ARS abs	**17.9**	**61.3**	**<0.001**
Anti-TIF1γ abs	**20.5**	**3.2**	**0.04**
Anti-Mi2 abs	2.6	0	1.00

Data are mean (S.D.) unless otherwise stated. *P*-values were determined by Fisher's exact test or the Mann–Whitney test. Values highlighted in bold indicate statistical significance ($P < 0.05$). Abs: autoantibodies; ARS: aminoacyl tRNA synthetase; CK: creatine kinase; IIM: idiopathic inflammatory myopathies; ILD: interstitial lung disease; NVC: nailfold videocapillaroscopy;

to high-dose glucocorticoids, were analysed (Supplementary Table S4, available at *Rheumatology* online), excluding 10 deceased cases. The levels of myogenic enzyme, IgG and acute-phase proteins decreased and CT score improved after 1 year in all survivors (Supplementary Fig. S2, available at *Rheumatology* online, $P < 0.001$). Unexpectedly, NVC scleroderma spectrum abnormalities disappeared in more than half of the

Table 4 NVC findings and clinical and laboratory features in patients with different myositis-specific antibodies

Variables	Anti-MDA5 ($n = 16$)	Anti-ARS[a] ($n = 26$)	Anti-TIF1γ ($n = 9$)	Others[b] ($n = 19$)	*P*-value
NVC scleroderma spectrum abnormalities, %	**87.5**	**26.9**	**88.9**	**52.6**	**<0.001**
Age, years	56.3 (12.8)	63.3 (17.8)	59.7 (12.7)	60.4 (18.1)	0.36
Disease duration, years	**0.2 (0.2)**	**1.1 (1.6)**	**0.2 (0.2)**	**0.7 (1.1)**	**0.03**
Female, *n* (%)	12 (75.0)	16 (65.4)	5 (55.6)	14 (73.7)	0.63
Current smokers, *n* (%)	2 (12.5)	0 (0.0)	2 (22.2)	2 (10.5)	0.17
Symptoms, %					
Fever	62.5	50.0	44.4	26.3	0.18
Myalgia	**50.0**	**92.3**	**100**	**94.7**	**<0.001**
Muscle weakness	**37.5**	**69.2**	**100**	**73.7**	**0.01**
Gowers' sign	18.8	50.0	66.7	57.9	0.06
Dysphagia	**6.3**	**3.8**	**55.6**	**15.8**	**0.001**
RP	6.3	15.4	0	15.8	0.50
Heliotrope rash	**50.0**	**15.4**	**55.6**	**15.8**	**0.01**
Gottron's sign/papules	**100**	**46.2**	**100**	**57.9**	**<0.001**
Palmar papules	**100**	**3.8**	**22.2**	**10.5**	**<0.001**
Arthritis	**62.5**	**57.7**	**0.0**	**31.6**	**0.006**
ILD	**100**	**96.2**	**0.0**	**63.2**	**<0.001**
Internal malignancy	**12.5**	**7.7**	**55.6**	**10.5**	**0.006**
Laboratory data					
CRP, mg/dl	**0.9 (1.0)**	**3.1 (3.3)**	**2.3 (4.5)**	**1.9 (6.4)**	**0.01**
ESR, mm/h	57.6 (25.8)	49.5 (30.4)	42.7 (36.7)	35.6 (23.4)	0.09
CK, U/l	**427.3 (851.2)**	**2050.5 (2754.5)**	**2429.4 (2223.6)**	**2603.1 (3602.7)**	**0.02**
IgG, mg/dl	1593.3 (303.2)	1764.6 (637.9)	1352.4 (367.1)	1544.9 (541.0)	0.29
Ferritin, ng/ml	**847.3 (1282.5)**	**447.5 (796.3)**	**650.7 (604.5)**	**146.8 (107.1)**	**0.006**
PaO_2/FiO_2	397.4 (107.1)	374.3 (79.2)	—	406.7 (32.9)	—
CT score	**1.10 (0.52**	**1.3 (0.72)**	**0.0**	**0.71 (0.79)**	**<0.001**
Antinuclear abs, %	**68.8**	**46.2**	**100.0**	**78.9**	**0.01**

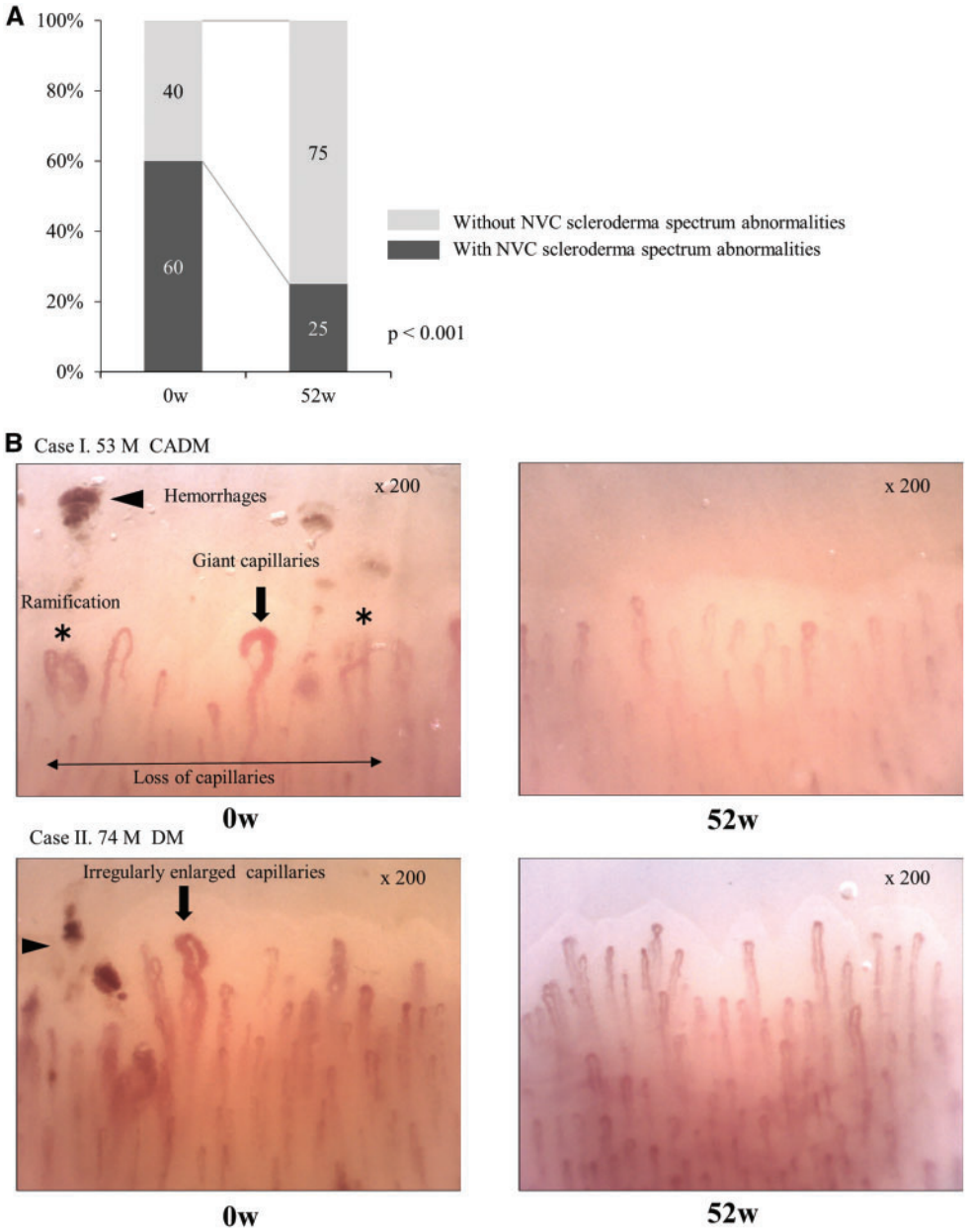
Data are mean (S.D.) unless otherwise stated. *P*-values were determined by the Kruskal–Wallis test. Values highlighted in bold indicate statistical significance ($P < 0.05$). [a]Anti-ARS ($n = 26$) including Jo-1($n = 10$), PL-7 ($n = 1$), PL-12 ($n = 2$), EJ ($n = 6$), KS ($n = 2$), OJ ($n = 5$). [b]Others ($n = 19$) include Mi-2 ($n = 1$), MJ/NXP-2 ($n = 1$), SRP ($n = 3$), Ku ($n = 2$), cohesin complex ($n = 1$), and no myositis-specific antibodies ($n = 11$). Abs: autoantibodies; ARS: aminoacyl tRNA synthetase; CK: creatine kinase; IIMs: idiopathic inflammatory myopathies; ILD: interstitial lung disease; MDA5: melanoma differentiation-associated protein-5; NVC: nailfold videocapillaroscopy; PaO_2/FiO_2: ratio of partial pressure of oxygen in arterial blood to fraction of inspiratory oxygen; SRP: signal recognition particle; TIF1γ: transcription intermediary factor 1gamma.

IIM patients following immunosuppressive therapy; NVC scleroderma spectrum abnormalities were seen in 60.0% of the 40 patients before treatment, which reduced to only 25.0% after treatment ($P < 0.001$, Fig. 2A). Such improvement was independent of the different NVC scleroderma patterns (data not shown). Also, the treatment responses to the muscle and lung involvement were independent of the different NVC scleroderma patterns. In contrast, no improvements in NVC scleroderma spectrum abnormalities were seen in SSc patients (data not shown). Fig. 2B shows the NVC images of two representative cases, and Supplementary Fig. S3 (available at *Rheumatology* online) shows NVC images before and after treatment of all cases. Severe NVC scleroderma spectrum abnormalities, seen in some cases before treatment, disappeared completely after 1 year of treatment (Fig. 2A and Supplementary Fig. S3, available at *Rheumatology* online).

Discussion

This is the first report to examine the significance of microvascular abnormalities in IIMs combined with skin pathology and detailed autoantibodies analysis. Nailfold microvascular abnormalities in SSc, which reflect disease status and are often used as a marker for early diagnosis [10], are characteristic of the disease and included in the recent classification criteria of SSc [11, 12]. However, the significance of nailfold microvascular abnormalities in other autoimmune diseases has not been extensively investigated. The present study was conducted to examine the nailfold microvascular scleroderma spectrum abnormalities and analyse their potential significance in IIMs. This is also the first study to examine the association between nailfold microvascular abnormalities, MSAs and histopathological findings of the skin rash in DM.

FIG. 2 Effects of immunosuppressant on the NVC scleroderma spectrum abnormalities



(**A**) The changes in NVC abnormalities at week 52 after treatment. (**B**) Two representative cases with improved NVC abnormalities after treatment. NVC: nailfold videocapillaroscopy.

Previous studies on the nailfold microvascular abnormalities in IIMs had only small numbers of subjects, with the reported prevalence ranging from 26.9% [35] to 57.1% [36]. The limitations of the previous studies included small number of patients, use of different assessment methods and the evaluation of a mixture of untreated and previously treated patients. To address these issues, NVC spectrum abnormalities in untreated patients with IIMs were evaluated by an established standard method used in SSc [13, 14]. In the present study, 65.4% of DM patients and 27.8% of PM patients had NVC scleroderma spectrum abnormalities. It is of interest that microvascular changes detected by NVC are much more common in DM than PM, considering the classic difference in muscle pathology between DM and PM; the key pathological change in muscle in DM is due to perivascular inflammation involving perimysial capillaries and arterioles [3]. These findings might provide a new theory in which DM and PM are considered separately. Although the prevalence of these changes in IIM patients

was lower than those in SSc, our data indicated that a significant number of IIM patients have NVC scleroderma spectrum abnormalities. However, no association was observed between NVC scleroderma spectrum abnormalities patterns and the levels of myogenic enzymes, acute-phase proteins, %VC, or CT score at baseline. Although a previous study [21] showed an association between NVC scleroderma spectrum abnormalities patterns and RP, there were no association between them in this study. From our study, the significance and pathogenesis of RP in patients with IIMs could be different from that in patients with SSc.

One of the most striking and new findings in the present study is an association of NVC scleroderma spectrum abnormalities with certain MSAs; NVC changes were seen at high prevalence in patients with anti-MDA5 and anti-TIF1γ antibodies, but not in anti-ARS antibodies (Tables 3 and 4, Supplementary Table S3, available at *Rheumatology* online). Although one study reported an association of anti-Jo-1 antibodies with microvascular abnormalities [37], no comprehensive analysis of other MSAs using immunoprecipitation and ELISA have been performed in the past to examine the association between microvascular abnormalities and specific MSAs. IIMs can be classified into subsets according to the various specific MSAs, each of them associated with a unique set of clinical features [6]. Of these, anti-ARS and anti-Mi-2 antibodies are associated with typical symptoms of myositis, while anti-MDA5 and anti-TIF1γ antibodies are associated with somewhat atypical features; anti-MDA5 antibodies are often seen in patients with CADM complicated with treatment-resistant, rapidly progressive ILD, while anti-TIF1γ antibodies are often positive in patients with cancer-associated myositis. Previous papers have also reported an association between cancer-associated myositis and NVC findings [21, 38]. The NVC scleroderma pattern was seen in the majority of patients with anti-MDA5 and anti-TIF1γ antibodies but not in those with anti-ARS antibodies, suggesting that NVC scleroderma pattern was an additional feature of IIMs associated with certain subsets defined by specific MSAs. Importantly, the trends were similar even when the analysis was limited to patients with DM. The NVC data suggest that common pathogenesis involving microvascular inflammation may play a role in the pathologic process and clinical features in anti-MDA5- and anti-TIF1γ-positive DM patients. This hypothesis was further supported by the pathological analysis of the skin rash in DM, which showed an association between the severity of perivascular lymphocyte infiltration in the skin rash and the presence of NVC scleroderma spectrum abnormalities. Nevertheless, it remains to be clarified whether the micorovascular inflammation detected by NVC and the pathology of perivascular lymphocytic infiltration often seen in the skin tissue [39, 40] directly contribute to the pathogenesis of myositis and ILD.

Another important finding in the present study was the improvement and disappearance of NVC scleroderma spectrum abnormalities in IIM patients after 1 year of treatment with immunosuppressive drugs. A previous two case reports showed similar findings [41, 42]. Although improvement of NVC abnormality to some degree by certain treatment has been reported in SSc [43–47], the effects are limited, and microvascular abnormalities in SSc usually take a chronic progressive course [48, 49]. Since the presence of NVC abnormalities is associated with perivascular lymphocyte infiltration in the skin rash, it is tempting to speculate that NVC abnormalities in IIMs result from a perivascular immune reaction and inflammation, which can be suppressed by immunosuppressive therapy, leading to unexpected disappearance/improvement of NVC abnormalities.

Our study has several limitations. First, all subjects were Japanese, and whether the findings would be similar in other ethnicities is uncertain. Collaborative work among scientists from around the world is needed to validate the results, in coordination with the EULAR Study Group on Microcirculation in Rheumatic Diseases. Second, some of the patients who participated in the study were not followed up due to death or transfer to other medical facilities. Finally, whether similar microvascular changes were present in muscular and pulmonary tissues was not studied.

Despite the above limitations, the present study highlighted the clinical and pathological significance of microvascular abnormalities in IIMs. They were associated with subsets of IIMs, defined by anti-MDA5 and TIF1γ antibodies and severe perivascular inflammation in the skin rash. Furthermore, NVC abnormalities in IIMs disappeared or improved in the majority of cases after 1 year of immunosuppressive therapy, in contrast to their chronic progressive course in SSc, suggesting a different significance of NVC abnormalities in IIMs than in SSc. Further studies with a special focus on previously under-recognized microvascular abnormalities in IIMs may help us to understand further aspects of the pathogenesis.

Acknowledgements

The authors thank all medical staff at all participating medical facilities for providing the data. We thank Ms Tomoko Hasegawa and Dr Shin Tanaka for the excellent technical assistance in autoantibody analysis.

Funding: This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the University of Occupational and Environmental Health, Japan, through a UOEH Grant for Advanced Research.

Disclosure statement: S.K. has received speaking fees from Bristol-Myers, Pfizer. T.S.N. has received speaking fees from Bristol-Myers, UCB, Astellas, Abbvie, Eisai, Pfizer and Takeda, and research grants from Mitsubishi-Tanabe, Novartis and MSD. K.N. has received speaking fees from UCB, Astellas, Mitsubishi-Tanabe, and research grants from Mitsubishi-Tanabe and Eisai. V.S. has received research grants from Actelion Pharmaceuticals, Boehringer Ingelheim, Bayer, Hoffmann-La Roche. M.C. has received research grants from Actelion Pharmaceuticals, Bristol-Myers, Celgene Corporation,

and Boehringer Ingelheim. Y.T. has received consulting fees, speaking fees, and/or honoraria from Abbvie, Daiichi-Sankyo, Chugai, Takeda, Mitsubishi-Tanabe, Bristol-Myers, Astellas, Eisai, Janssen, Pfizer, Asahi-kasei, Eli Lilly, GlaxoSmithKline, UCB, Teijin, MSD, and Santen, and research grants from Mitsubishi-Tanabe, Takeda, Chugai, Astellas, Eisai, Taisho-Toyama, Kyowa-Kirin, Abbvie, and Bristol-Myers. All other authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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Modern Rheumatology Case Reports

ISSN: (Print) 2472-5625 (Online) Journal homepage: https://www.tandfonline.com/loi/tmcr20

The two cases of acute acalculous cholecystitis associated with systemic lupus erythematosus (SLE) presented different clinical aspects

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To cite this article: Yurie Satoh, Kazuhisa Nakano, Yusuke Miyazaki, Shingo Nakayamada, Shunsuke Fukuyo, Satoshi Kubo, Ippei Miyagawa, Hiroko Yoshinari, Kazuyoshi Saito, Minoru Satoh & Yoshiya Tanaka (2019) The two cases of acute acalculous cholecystitis associated with systemic lupus erythematosus (SLE) presented different clinical aspects, Modern Rheumatology Case Reports, 3:2, 114-118, DOI: 10.1080/24725625.2018.1549928

To link to this article: https://doi.org/10.1080/24725625.2018.1549928

Published online: 09 Jan 2019.

MODERN RHEUMATOLOGY CASE REPORTS
2019, VOL. 3, NO. 2, 114–118
https://doi.org/10.1080/24725625.2018.1549928

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CASE REPORT

The two cases of acute acalculous cholecystitis associated with systemic lupus erythematosus (SLE) presented different clinical aspects

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ABSTRACT

Case 1. A 23-year-old female with polyarthralgia, facial erythema, pleuritis, carditis, nephropathy and cytopenia, the diagnosis of systemic lupus erythematosus (SLE) was established. She did not have abdominal pain, but abdominal ultrasonography and CT scan demonstrated acute acalculous cholecystitis (AAC) due to SLE. AAC was improved with glucocorticoid therapy. *Case 2*. A 26-year-old female with cytopenia, anti ds-DNA antibody, low complements, nephropathy and pleuritis, the diagnosis of SLE was established. She was hospitalized due to epigastralgia and high fever, abdominal ultrasonography and CT scan demonstrated pericholecystic edoema without gallstones. AAC was diagnosed and which was thought to be developed in accordance with active SLE. Reports of AAC in patients with SLE are rare and it sometimes difficult to diagnose because the findings of abdominal symptoms or immunological abnormalities are not specific. Because of the high risk of morbidity, surgical intervention remains as mainstay treatment of AAC. Pathophysiology of AAC is still unclear, but present reports of AAC in SLE, its pathology is thought as vasculitis. It is said that there is some possibility of avoiding surgical treatment if we could choose intensive treatment of SLE with glucocorticoid after excluding infections. Here we report 2 cases of AAC associated with SLE and which successfully treated with "non-invasive therapy". It would be illuminating in clinical practice of treating AAC with SLE.

ARTICLE HISTORY
Received 8 February 2018
Accepted 15 May 2018

KEYWORDS
Acute acalculous cholecystitis (AAC); systemic lupus erythematosus (SLE); high-dose steroids; vasculitis; serositis

Introduction

Acute acalculous cholecystitis (AAC) is likely to occur in critically ill patients with multiple trauma, high invasive abdominal surgery due to ischemia of gallbladder and biliary stasis. Because of its high mortality, surgical treatment is often recommended [1]. Variety of intraperitoneal manifestations such as peritonitis, and lupus hepatitis may occur in patients with systemic lupus erythematosus (SLE), while rarely gall bladder involvement such as AAC is reported [2–6]. All of the cases had abdominal pain or other symptoms, and because of the high risk of morbidity surgery is the main treatment of AAC even in SLE cases [1,6,7]. Recently there are some reports in the literature addressing successful treatment with high dose corticosteroid therapy as the first choice in patients with AAC, if their general condition are well and have no complications of infection [3,5]. However there is no particular consensus of treatment for AAC in SLE, surgical therapy would be chosen according to other AAC because of its rapid progression with a high incidence of gangrene (>50%) and perforation (>10%). We experienced two cases of AAC associated with SLE, which presented different clinical aspects and got improved with treatment with high dose corticosteroid therapy and could avoid surgical treatment.

Case presentation

Case 1

A 23-year-old female was admitted to our hospital with polyarthralgia, fever, chest pain, general malaise and pharyngeal pain. She also had facial erythema and Raynaud phenomenon. Her abdomen was flat and soft, without tenderness and Murphy's sign at the admission. Urinalysis showed proteinuria (Upro +2) with granular casts without any occult blood. Blood laboratory evaluations revealed elevated white blood cells (WBC;11,900/μl), decreased hemoglobin (Hb; 7.3 g/dl) with elevated hepatobiliary enzyme (AST 42 IU/L, ALT 64 IU/L, γ-GTP 75 U/L and ALP 796 IU/L). Serum creatinine level was normal (Cre; 0.82 mg/dl). CRP and ESR were elevated at

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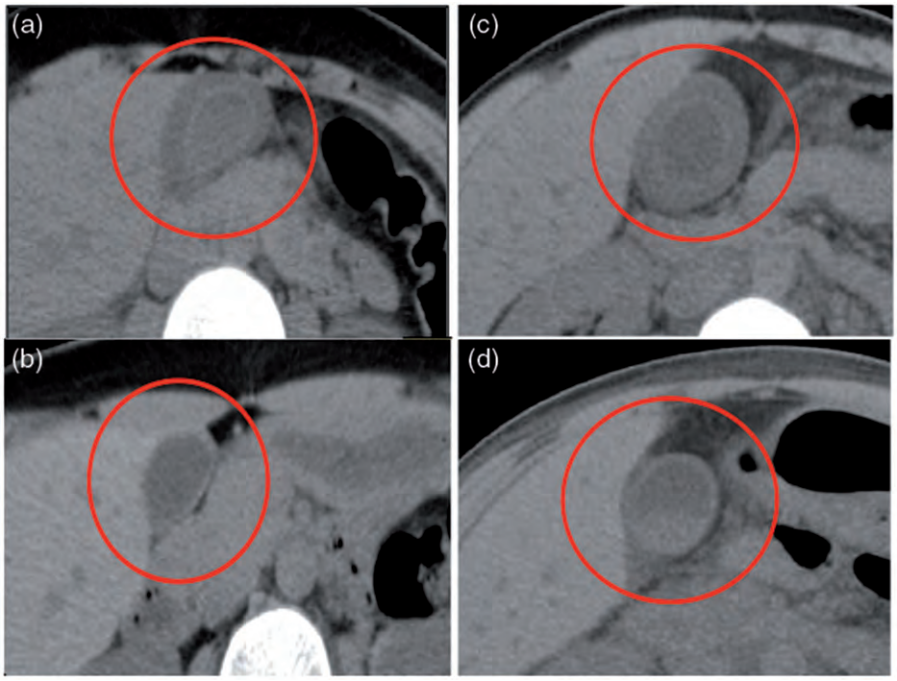

Figure 1. Abdominal computed tomography showing gallbladder. (a,c) edematous gallbladder without gallstones, (b,d) improvement of bladder wall thickness.

27.5 mg/dl and 98 mm/h, respectively. Elevation of serum IgG (1901 U/ml), low complements (C3 84 mg/dl, C4 6 mg/dl, CH50 20 mg/dl) and high titre of antinuclear antibody (x5120, Speckled pattern) with positive anti- dsDNA antibody (34.5 IU/ml), anti-Sm antibody (97 IU/l) and anti-U1-RNP antibody (>200 IU/ml) were revealed. Anti-SS-A antibody, anti-phospholipid antibody, MPO-ANCA and PR3-ANCA were negative. Transthoracic echocardiography and chest CT showed pericardial effusion and a small amount of pleural effusion, indicating complication of pericarditis and pleuritis. Also, abdominal CT and ultrasonography showed edematous gall bladder without gallstones or sludge, indicating acalculous cholecystitis (Figure 1(a)). Sonographic Murphy's sign was negative and no other abnormal findings in the intestinal tract were presented at that time. Clinical criteria as malar rash, arthritis, serositis, kidney failure and immunologic criteria as low complements, antinuclear antibody positive, anti ds-DNA antibody positive, anti Sm antibody positive, she was diagnosed as SLE. The day after administration, she slightly started feeling right upper quadrant abdominal pain. Generally the gall bladder drainage adaptation will be concerned if the severity of cholecystitis is moderate or higher, or if it is impossible to undergo cholecystectomy and does not respond to the initial treatment. But we determined this case did not have urgent drainage because of her condition was not poor. We also considered of bacterial acute cholecystitis but we determined bacterial infection is not a main pathology because there was no significant evident rise of procalcitonin or positive blood culture. Since present reports of acalculous cholecystitis in SLE were developed with high disease activity, AAC of this case (SLEDAI 23 points and BILAG 22 points) was thought to be one of the manifestations of SLE. So we administered glucocorticoid (GC) pulse therapy for 3 days followed by high-dose GC therapy (1mg/kg) and cyclophosphamide pulse therapy (IVCY) for the treatment of AAC. After administering treatments, fever, inflammation, pleuritis, cardial effusion, hepatobiliary enzymes and also bladder wall thickness were improved with decrease activity of SLE (Figure 1(b), Figure 2).

Case 2

A 23-year-old female, she was diagnosed as SLE with kidney dysfunction, pancytopenia, low complements, positive antinuclear antibody and anti-ds-DNA antibody. High-dose GC therapy and IVCY were started and had been keeping its remission with low-dose of GC and cyclosporine (CsA). On March in 20xx, CsA therapy was discontinued because of her desire for pregnancy. Two weeks after CsA was discontinued, she felt fever and abdominal pain. Three weeks after, the symptom got worse and she was admitted to the hospital. Physical examination showed fever, facial erythema, discoid rash and tenderness in the right upper quadrant of the abdomen with a positive Murphy's sign. Urinalysis showed proteinuria without any occult blood and granular casts. Blood laboratory evaluations revealed elevated WBC (8000/μl), decreased hemoglobin (Hb; 10.5 g/dl) with elevated hepatobiliary enzymes (AST 39 IU/L, ALT 55 IU/L, LDH 593 IU/l, γ-GTP 133 U/L, and ALP 867 IU/L). BUN and creatinine were normal. CRP and ESR were elevated

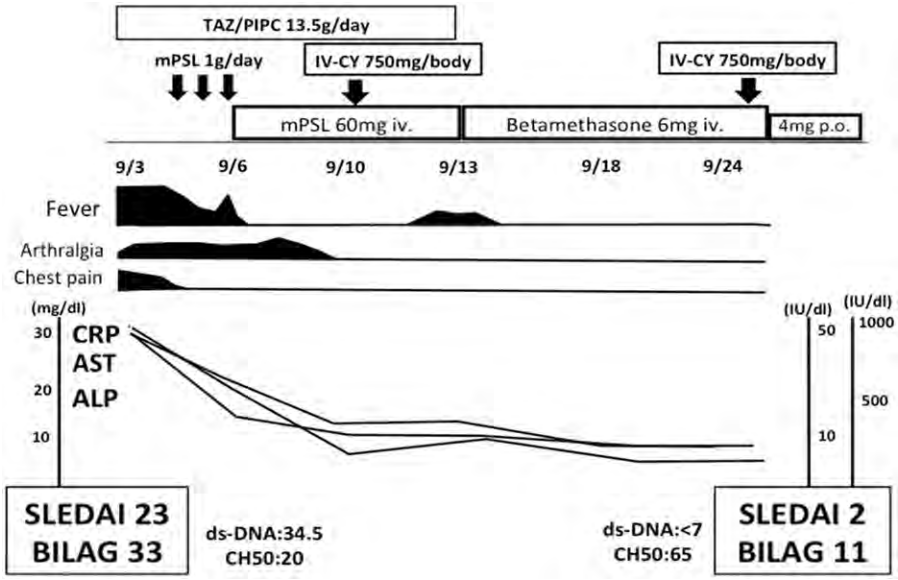

Figure 2. Clinical course of case 1.

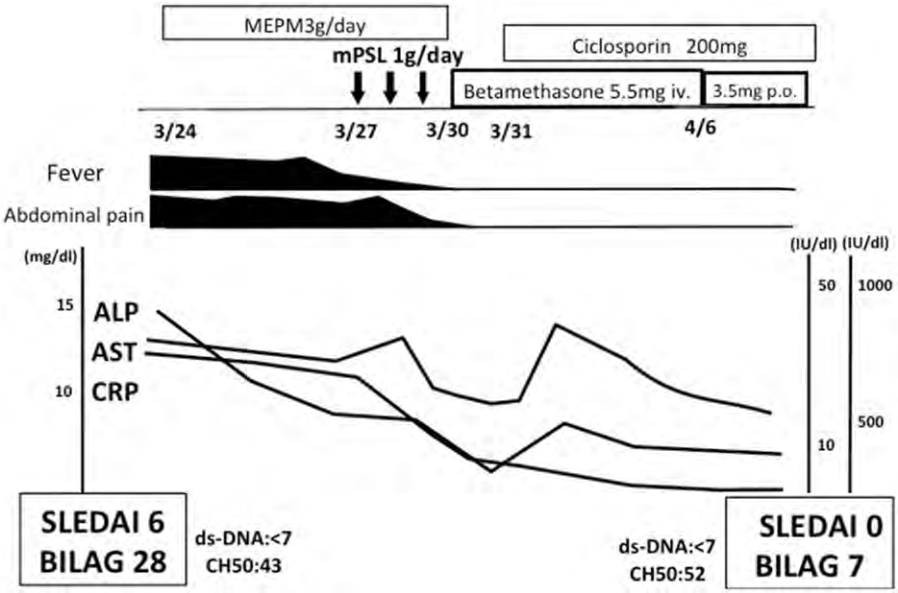

Figure 3. Clinical course of case 2.

at 14.1mg/dl and 58 mm/h, respectively. Serum immunoglobulin and complements were normal. Antinuclear antibody was x1280 (Speckled pattern). Anti-dsDNA antibody, anti-Sm antibody, anti-U1-RNP antibody, anti-SS-A antibody, anti-phospholipid antibody, MPO-ANCA and PR3-ANCA were all negative, but anti RNA helicase antibody were positive. Abdominal CT and ultrasonography showed gallbladder wall thickening and edematous gallbladder without gallstones therefore acalculous cholecystitis was suspected (Figure 1(c)). As a cause of AAC, vasculitis by AAV or infections were also suspected but ANCA and blood cultures were both negative and antibiotics were ineffective. During the examination, the symptoms of AAC such as vomiting, abdominal pain and fever got worse. Since AAC was developed with high disease activity of SLE (SLEDAI 6 points and BILAG 23 points), we selected GC pulse therapy followed by high-dose GC therapy and restarting CsA. After the administration, fever, abdominal pain, pleuritis, inflammation, abnormal hepatobiliary enzyme got improved (Figure 3). With decrease of disease activity of SLE, improvement of gall bladder thickness and edema were confirmed by image (Figure 1(d)).

Discussion

AAC usually occurs in special circumstances, such as severe trauma, sepsis and postoperative period of major non-billiary surgery and/or prolonged parenteral nutrition. It may also be observed in the course of medical disorders such as diverse infections, diabetes mellitus and vasculitis. AAC is rare, occurring in only 2% to 15% of patients with acute

Table 1. Reports of AAC developed with SLE.

	Age/sex	Symptom	Autoantibody	Treatment
M. Basiratnia et al. [16]	10/Male	Fever, abdominal pain, vomitting	ANA, ds-DNA antiphospholipid	High dose GC → Operation
D. Martinez et al. [12]	39/female	Fever, abdominal pain	–	Operation
C.R. Swanepoel et al. [6]	22/female	Abdominal pain, vomitting	ANA	High dose GC, CYC → Operation
A.J. Rhoton et al. [13]	22/female	Abdominal pain	–	Operation
S. Uetsuji et al. [11]	53/female	Abdominal pain	–	Operation
J.A. Mendonca et al. [4]	12/female	Fever, abdominal pain, vomiting, anorexia	ANA	High dose GC, CYC
T. Kamimura et al. [14]	27/female	Fever, abdominal pain	ANA, ds-DNA Sm, RNP	High dose GC
K.M. Newbold et al. [7]	28/female	Abdominal pain, vomiting, diarrhea	ANA, ds-DNA	GC, CYC → Operation
K.M. Newbold et al. [7]	38/female	Fever, abdominal pain, vomitting	ANA, ds-DNA	Operation
S.J. Shin et al. [15]	39/female	Abdominal pain, vomitting	ANA	High dose GC
Case 1	23/female	Fever, fatigue	ds-DNA, RNP Sm	High dose GC, CYC
Case 2	25/female	Fever, abdominal pain, vomitting	ANA	High dose GC

AAC: Acute acalculous cholecystitis, GC; Glucocorticoid, CYC; Cyclophosphamide.

cholecystitis, and its diagnosis remains less well defined [1,8,9]. The diagnosis is usually made by image inspection, most often by sonographic examination of the gall bladder. The most reliable diagnostic clues to cholecystitis are thickness of the gall bladder wall and accumulated fluid around the gall bladder without gallstones. The pathophysiology of AAC is multifactorial and likely results from bile stasis or ischemia. Bile inspissation that is directly toxic to gall bladder epithelium and ischemia causes leukocyte margination. Inflammatory cells infiltrated in gall bladder wall due to biliary stasis, it increase endothelium disorder of the gall bladder [1,8]. The mortality rate remains about 15-30% because the affected patients are critically ill, and the disease itself can progress rapidly and cause high incidence of perforation and secondary infection [8]. The perforation rate in AAC with SLE does not have a report, but there are some reports that surgical treatment had been chosen at the time of a diagnosis when the patient's general condition is poor [6,7]. Because of this, the mainstay of therapy for AAC has been cholesystectomy [1]. It was no exception in patients with SLE, cholesystectomy and drainage was main therapy until 1990s and cooperation with surgeons was thought to be necessary. Since the report of Swanepoel et al.[6], 14 reports of AAC developed with SLE can confirm until now including our cases (Table 1) [3–7,10–16]. The average age of onset is 28.6 years old and 34 cases in 35 were women, which consistent to susceptible age for general SLE. Acalculous cholecystitis occurs in patients with SLE when its disease activity is high. In general, fever, gastrointestinal symptom such as abdominal pain or vomiting originating and Murphy's sign can be seen, but in some cases lack gastrointestinal symptoms at the time of diagnosis like our case 1 [9]. The majority of cases shows liver dysfunction, but some cases do not present. About the association between complications and the autoantibody of SLE, it is reported that the positive percentage of the Sm/RNP antibody is significantly higher in cases complicating polyserositis than those of Ro/La antibody positive cases [17]. In AAC cases, ANA was positive in all cases, but the positive rate of the specific antibody was erratic. Our case (case2) presented RNA helicase antibody which is detected around 10% of Japanese SLE and is thought to be a specific autoantibody in SLE. However, the association with clinical manifestation is not clear [18]. About the image views, there are not the views that are specific for AAC with SLE. Although there is one case report presented the gall bladder atrophy as image views unlike most of the cases presented characteristic views of AAC such as all lap-related thickenings of the gall bladder wall and edema. AAC in SLE, vasculitis in the neighborhood capillary of gall bladder is confirmed histologically in five of eight cases, the main pathophysiology is thought as cholestasis by ischemia of gall bladder wall and vascular obstructions due to the chorionic vasculitis [1]. It is suggesting that the main pathophysiology of AAC in patients with SLE might be serositis with vasculitis. Therefore, AAC predominantly occurs during exacerbations of disease activity, mostly presenting pleuritis, carditis and high level of CRP. Considering the pathophysiology of AAC from this perspective, there is no doubt about complicating other abdominal vasculitis such as mesenteric vasculitis. But as far as we searched, there are no reports that developed other abdominal vasculitis with the onset of AAC. There is no particular consensus of treatment for AAC in SLE. However, from the pathophysiological point of view, controlling the disease activity of SLE with high-dose GC therapy would be considered as the first line of treatment for AAC in patients with SLE. In most cases in previous reports, abdominal symptoms, inflammatory reactions, liver dysfunction and the gall bladder wall thickening are significantly improved after the administration of high-dose GC therapy.

From the above, the immediate treatment with GC is important when AAC is suspected in patients with SLE with high disease activity and no other risk factor for AAC or its serious complications. We here report it is thought with enlightening cases in clinical practice of treating SLE with AAC.

Patient consent

For this case report, informed consent was obtained in writing from the patient in all of the cases.

Conflict of interest

None

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RHEUMATOLOGY

Rheumatology 2019;58:1784–1793
doi:10.1093/rheumatology/kez123
Advance Access publication 22 April 2019

Original article

Autoantibodies to a novel Rpp38 (Th/To) derived B-cell epitope are specific for systemic sclerosis and associate with a distinct clinical phenotype

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Abstract

Objective. Detection of antinuclear antibodies and specific autoantibodies is important in the diagnosis and classification of SSc. Several proteins of the Th/To complex, including Rpp25, Rpp38 and hPop1 are the target of autoantibodies in SSc patients. However, very little is known about the epitope distribution of this autoantigen. Consequently, we screened Rpp25, Rpp38 and hPop1 for B cell epitopes and evaluated their clinical relevance.

Methods. Serum pools with ($n = 2$) and without ($n = 1$) anti-Th/To autoantibodies were generated and used for epitope discovery. Identified biomarker candidate sequences were then utilized to synthesize synthetic, biotinylated, soluble peptides. The peptides were tested to determine reactivity with sera from SSc cohorts ($n = 202$) and controls ($n = 159$) using a chemiluminescence immunoassay. Additionally, samples were also tested for antibodies to full-length recombinant Rpp25 antibodies by chemiluminescence immunoassay.

Results. Several immunodominant regions were found on the three proteins. The strongest reactivity was observed with an Rpp38 peptide (aa 229–243). Autoantibodies to the Rpp38 peptide were detected in 8/149 (5.4%) limited cutaneous SSc patients, but not in any of 159 controls ($P = 0.003$ by two-sided Fisher's exact probability test). Although reactivity to the novel antigenic peptide was correlated with the binding to Rpp25 (rho = 0.44; $P < 0.0001$), subsets of patient sera either reacted strongly with Rpp25 or with the novel Rpp38-derived peptide.

Conclusion. A novel Rpp38 epitope holds promise to increase the sensitivity in the detection of anti-Th/To autoantibodies, thus enhancing the serological diagnosis of SSc.

Key words: systemic sclerosis, autoantibodies, interstitial lung disease, diagnosis, epitope, peptide

CLINICAL SCIENCE

Rheumatology key messages

- Epitope mapping identified several linear epitopes on Th/To antigens.
- The novel Rpp38 (Th/To) derived peptide helps to increase sensitivity.
- Antibodies to the novel Rpp38 (Th/To) B-cell epitope are specific for limited cutaneous SSc.

Introduction

SSc is characterized by the presence of circulating autoantibodies that bind a variety of intracellular antigens, generally referred to as antinuclear antibodies (ANA) [1, 2]. The most prevalent autoantibodies in SSc target topoisomerase I (topo-I, Scl-70), centromere proteins (CENP) and RNA polymerase III, which are also key components of the revised SSc classification criteria [1, 3]. Several other autoantibodies have been reported in SSc sera including autoantibodies targeting the PM/Scl complex (also known as the exosome) [4], U3RNP/fibrillarin [5, 6], U11/12 snRNA [7, 8] and the Th/To autoantigens [9–12].

Anti-Th/To antibodies are typically associated with a homogeneous nucleolar staining by conventional IIF on HEp-2 cells [9, 13], a pattern now designated as AC-08 by the International Consensus on ANA patterns (www.anapatterns.org). In addition to the contribution to accurate diagnosis of SSc, ANA can also be used to stratify SSc patients according to clinically relevant

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Submitted 9 November 2018; accepted 8 March 2019

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phenotypes [14–16]. For example, anti-Th/To antibodies have been associated with the limited cutaneous SSc (lcSSc) subset where the reported prevalence of these autoantibodies ranged from 1% to 13% [9, 17, 18]. In addition to SSc, a few reports have described anti-Th/To antibodies in localized scleroderma, RA and interstitial lung disease (ILD) [19–21].

The Th/To autoantigen is a macromolecular protein-RNA complex (human RNase MRP complex) consisting of at least 10 proteins and catalytic RNA [2, 10]. RNase MRP is a ubiquitously expressed eukaryotic endoribonuclease that cleaves various RNAs, including ribosomal, messenger and mitochondrial RNAs [10]. Almost all protein components of the RNase MRP and the evolutionarily related RNase P complex have been reported as autoantibody targets in patients with ANA-associated rheumatic diseases [10, 11, 20], although Rpp25 [22], Rpp38 [10] and hPop1 [10] have been described as the major target autoantigens. While some studies tested serological cohorts, other investigations analysed selected samples initially identified on the basis of a nucleolar IIF staining pattern [9]. Historically, anti-Th/To antibodies have been mostly detected by immunoprecipitation (IP) [9]. Several years ago, a commercial line immunoassay (LIA) for the detection of anti-Th/To antibodies based on the hPop1 target became available and was evaluated in two independent studies [23, 24]. Just recently, a fully automated chemiluminescent immunoassay (CIA) for the detection of anti-Rpp25 antibodies (research use only) has been developed and evaluated [22, 25]. Lastly, an IP real-time PCR assay has been evaluated [26].

Except for their association with lcSSc, the reported clinical association of anti-Th/To antibodies over the past two decades has been inconsistent. Furthermore, due to technical challenges and limited availability of the IP assay or alternative reliable immunoassays, anti-Th/To autoantibody tests are rarely performed in routine diagnostic algorithms. In addition, very little is known about the B-cell epitope distribution of the major Th/To autoantigens. Consequently, the primary goal of this study was to analyse the epitope distribution on Rpp25, Rpp38 and hPop1 as an approach to adapting the identified epitopes to a high performance immunoassay that may be easily adopted by research and diagnostic laboratories.

Methods

Patient sera

Three pools of sera were generated, each containing equal volumes of three individual samples. The first two pools (Th/To Pool 1 and Th/To Pool 2) were based on samples monospecific for anti-Th/To autoantibodies (anti-Th/To by CIA; reactivity to other SSc-related antigens were excluded using commercial methods) derived from SSc patient sera and a third pool combining three SLE sera (SLE Pool 3) served as negative control pool. Reactivity to Th/To and other autoantigens was measured using CIA (Inova Diagnostics, San Diego, CA). For the evaluation of the assay based on the Rpp38-derived biotinylated peptide, patients fulfilling the classification criteria for SSc [3] were enrolled including 149 with limited cutaneous (lcSSc), 49 with diffuse cutaneous SSc (dcSSc) and four with sine SSc (absence of external skin involvement, but the presence of RP and fibrosis in one or more internal organs) [27].

As controls, samples from patients with SLE ($n = 15$), SS ($n = 5$), idiopathic inflammatory myopathies ($n = 27$), patients with HBV ($n = 20$), HCV ($n = 21$), HIV ($n = 18$), syphilis infection ($n = 20$) and various other conditions ($n = 33$) were included.

Longitudinal samples were available for all anti-Rpp38 antibody positive samples. In addition, 13 patients with primary RP without signs of SSc as well as samples from various disease controls were included ($n = 159$).

Under the terms of this study, all patient information was anonymized prior to analysis, precluding the requirement of written informed consent. All clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki. Biobank and clinical data collection procedures were approved by the CHUM Ethical Review Board.

Recombinant Rpp25 antigen and anti-Rpp25/Rpp38 immunoassay

Recombinant full-length, his-tagged human Rpp25 was generated and purified as previously described and used for CIA [22]. The Rpp25 assay (Inova Diagnostics) is a novel CIA that is currently used for research purposes only and utilizes the BIO-FLASH® instrument (Biokit, Barcelona, Spain), fitted with a luminometer, as well as the hardware and liquid handling accessories necessary to fully automate the assay [28]. For the Rpp25 and Rpp38 screening assays, the two antigens were immobilized on separate beads. Bead mixtures with different ratios of the two beads were generated and a 50 : 50 ratio showed the best discrimination between positive and negative samples.

Epitope mapping using solid phase peptides

The amino acid sequences of the Rpp25 (#NP_060263), Rpp38 (#NP_006405) and hPop1 (#NP_001139333) autoantigens were translated into 15-mer peptides with peptide-to-peptide overlaps of 14 amino acids (aa, Rpp25 and Rpp38) and 13 aa (hPop1) resulting in 1041 different peptides printed in duplicate (2082 peptide spots in all) using solid phase peptide arrays (PEPperPrint, Heidelberg, Germany) [18, 29, 30]. The three serum pools, Th/To Pool 1, Th/To Pool 2 and SLE Pool 3 were used. Epitope mapping was carried out as previously described [29–31].

Synthesis of candidate peptides and detection

Soluble biotinylated peptides were synthesized by FMOC define chemistry as previously reported [32] and purity verified by HPLC. After reconstitution, the peptides were coupled to streptavidin beads and tested by CIA.

IIF on HEp-2 cells

ANA of SSc sera were detected by IIF performed on HEp-2 cells (Antibodies Incorporated, Davis, CA, USA) [21]. Patterns were reported in concordance with the International Consensus on ANA Patterns (ICAP) classification [33].

Statistical evaluation

The data were statistically evaluated using Analyse-it software (Version 3.90; Analyse-it Software, Ltd, Leeds, UK). χ^2, Spearman's correlation and Cohen's kappa agreement test were carried out to analyse agreements between portions and P values <0.05 were considered significant. Descriptive statistics were used to summarize the baseline characteristics of the patients. χ^2, Fisher's exact and Mann–Whitney U tests were used as appropriate and P values <0.05 were considered statistically significant.

Results

Discovery of linear epitopes using high density solid phase peptide array

When the two serum pools containing anti-Th/To autoantibodies and the control SLE pool were used to probe the Rpp25, Rpp38 and hPop1 peptide arrays, the highest intensity signals were found in peptides representing amino acids 226–246 of the Rpp38 antigen (Fig. 1). The identical peptides were identified by both Th/To serum pools, but not by the SLE negative control serum pool. Moreover, the assays identified some additional but weaker epitopes of Rpp38 and hPop1 (Fig. 1).

Next, all peptides that yielded at least 200 units of reactivity (at 1 : 250 serum dilution) with at least one of the Th/To serum pools, but not with the SLE pool, were selected for further analyses. For Rpp25, one peptide was positive for both pools, 10 peptides for Th/To pool 1 and 7 peptides for Th/To pool 2. Rpp38 displayed a greater number of reactive peptides. Seven peptides were positive for both pools, six peptides for Th/To pool 1 and 24 peptides for Th/To pool 2. Last, two hPop1 peptides were positive for both Th/To pools, 16 peptides for Th/To pool 1 and three peptides for Th/To pool 2. The number and percent of positive peptides (>200 units) and highest signals are summarized in Fig. 2.

Verification of identified epitopes using synthetic Th/to derived soluble peptides

A total of eight soluble biotinylated peptides were designed based on the identified sequences and used for further studies (Table 1). Two peptides were based on the hPop1 sequence, three on Rpp25, two on Rpp38 and one was a Rpp38 and hPop1 hybrid (see Table 1). In further analysis, peptide Rpp38- ^{229} RELLDTSFEDLSKPK ^{243}, Rpp38- ^{233} DTSFEDLSKPKRKLA ^{247} and Rpp38- ^{233}DTSFEDLSKPKRKLA ^{247}/hPop1- ^{418} TGIIISDLTMEMNRF ^{432} were most reactive. Further comparative descriptive analysis showed significantly higher reactivity against peptide Rpp38- $^{229–243}$ and Rpp38- $^{233–247}$ with the SSc sera. The best discrimination between SSc and controls was observed with Rpp38- ^{229} RELLDTSFEDLSKPK ^{243}, which was then used for further analysis in a clinical study.

Prevalence and titres of anti-Rpp25 and Rpp38 antibodies in different diseases

When the prevalence and the titres of anti-Rpp25 and anti-Rpp38 peptide antibodies were studied in SSc and various other diseases, the highest prevalence and titres were found with SSc sera (Fig. 3). Anti-Rpp25 antibodies showed a higher sensitivity, but lower specificity compared with anti-Rpp38 antibodies. Strikingly, the reactivity to the Rpp38 derived peptide was very specific for SSc and restricted to the limited cutaneous form.

Correlation between anti-Rpp25 and anti-Rpp38 peptide antibodies and development of anti-Th/to screening assay

Antibodies to the novel Rpp38 peptide showed significant correlation with antibodies to full length human recombinant Rpp25 as measured by CIA. Using 299 serum samples (202 SSc, 97 controls), the quantitative agreement according to the Spearman equation was (rho = 0.44, 95% CI 0.35, 0.53; $P < 0.0001$). However, there were subsets of SSc sera that either reacted strongly with Rpp25 or with the novel Rpp38 derived peptide (see Fig. 4). When beads coupled with Rpp25 were mixed with Rpp38 coupled beads in a 50 : 50 ratio and tested with four samples positive for Rpp25 but negative for Rpp38 and two samples positive for Rpp38 but negative for Rpp25, it was found that the bead mixture detected both autoantibody specificities. Negative samples remained negative and double positive samples remained positive.

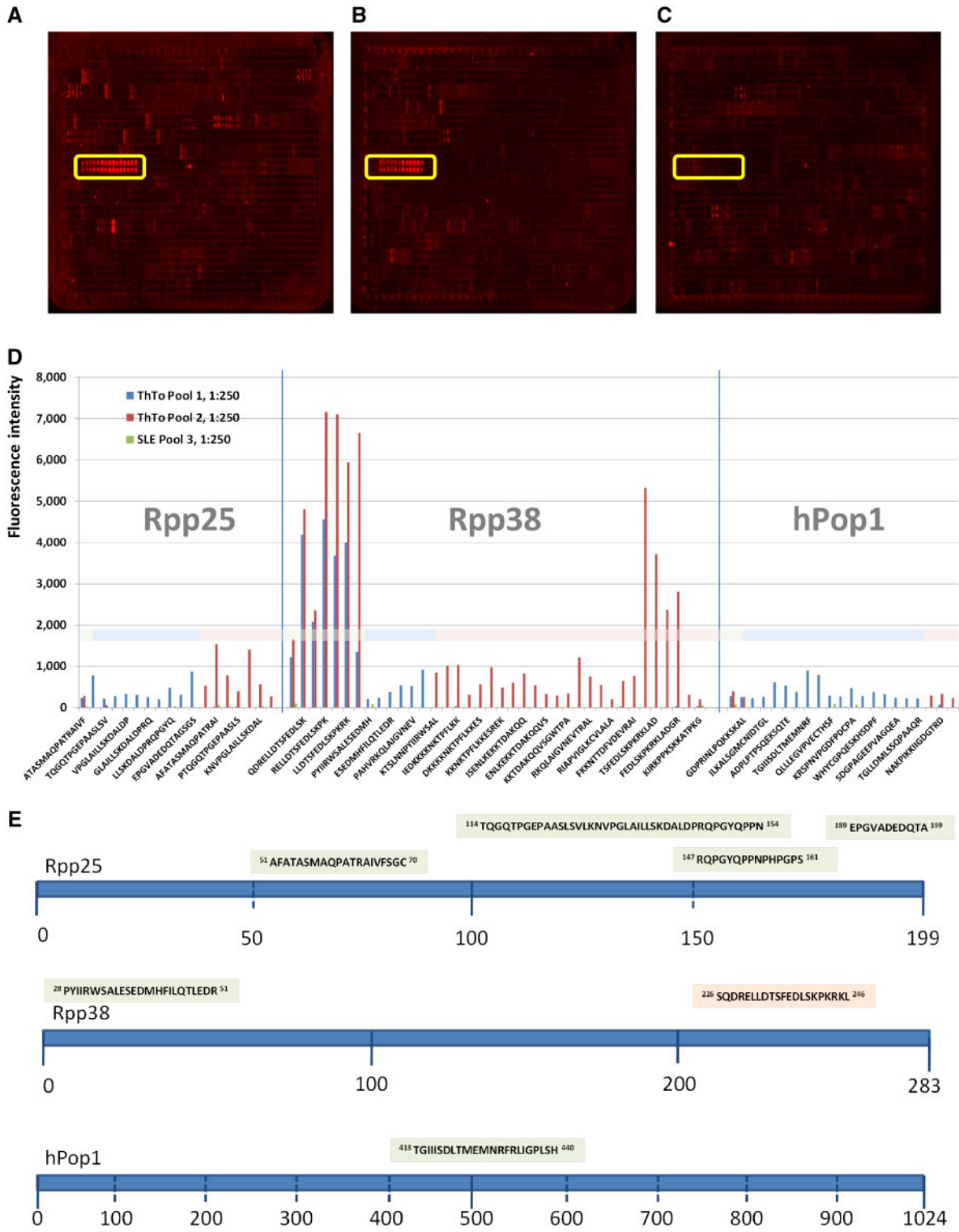
Sequence comparisons between Rpp25 and Rpp38

Due to the observed correlation between the reactivity of autoantibodies to Rpp25 and the Rpp38 derived peptide, a sequence alignment was carried out that showed limited similarity/identity between the two molecules (Supplementary Fig. S1, available at *Rheumatology* online). A total of four regions were identified on both proteins that showed >30% sequence identity, none of them overlap with the identified major Rpp38 epitope.

Clinical features of anti-Rpp38 peptide antibody positive patients

We identified eight (seven females and one male) patients that tested positive for anti-Rpp38 peptide antibodies in at least one serum sample and for which clinical data could be retrieved from medical records. Seven had lcSSc and one had early lcSSc. The median age at disease onset was 34.6 years (7.8–48.7 years) and the median age at blood sampling was 51.0 years (32.2–73.9 years). Of the eight patients, two had co-morbid pulmonary fibrosis, two had SS and two had primary biliary cholangitis. In six of the eight patients, anti-Rpp38 antibodies were detectable in the first longitudinal serum sample. The remaining two patients developed anti-Th/To antibodies over the course of their disease. Although anti-Rpp38

FIG. 1 Epitope mapping of Rpp25, Rpp38 and hPop1 using high density peptide arrays



The peptide arrays stained with pools of Th/To positive SSc serum samples (**A**, **B**) show several immunoreactive regions in all proteins. Reactivity with the pool of samples derived from SLE sera showed minor reactivity (**C**). Peptides that showed reactivity (>200 units) with at least one of the two Th/To serum pools, but not with the SLE pool are shown in **D**. In **E**, epitope map highlights main reactive peptides.

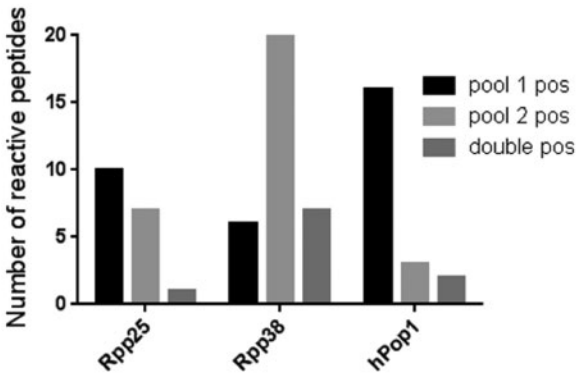
antibodies were exclusively found in patients with lcSSc (8/149 lcSSc *vs* 0/49 dcSSc; OR 6.0, 95% CI 0.33, 104.94), the difference between the two clinical subsets did not reach statistical significance ($P = 0.2235$).

Nevertheless, anti-Rpp38 antibodies were significantly more common in patients with lcSSc than in all other controls including the dcSSc (8/149 *vs* 0/225, $P = 0.0006$ by two-sided Fisher's exact probability test, OR 27.1, 95% CI 1.6, 473.04). Of the eight patients with anti-Th/To antibodies, five had other antibodies in addition to anti-Th/To, with anti-Ro52 being the most common one followed by anti-centromere and anti-PM/Scl (see Table 2).

Association of anti-Rpp38 antibodies with IIF pattern

Of the eight anti-Rpp38 peptide positive samples, three showed a homogeneous nucleolar pattern (ICAP AC-08) as the main pattern. In the remaining five samples, AC-08 was the secondary IIF pattern. Within the group of dcSSc patients, 12/49 (24.5%) showed a nucleolar pattern, but all were negative for anti-Rpp38 antibodies confirming the association with lcSSc.

Fig. 2 Number of linear peptides on Rpp25, Rpp38 and hPop1 identified by Th/To pools



The number of peptides that showed a reactivity of >200 units with at least one of the Th/To pools, but not with the SLE control pool, are displayed. For both Rpp25 and hPop1, more peptides reacted with pool 1, but not with pool 2. Rpp38 showed the highest number of peptides that reacted with both Th/To sample pools.

Discussion

This is the first study to describe linear epitopes on three Th/To antigens. In addition, we developed a particle-based assay using the newly identified Rpp38 epitope that showed high specificity for lcSSc and identified patients with a distinct clinical phenotype.

ANA are detected in >90% of SSc patients' sera and are valuable tools in establishing the diagnosis, predicting the onset, internal organ involvement and the prognosis of the disease [1, 34]. Besides anti-centromere, anti-topo I/Scl-70 and anti-RNA polymerase III antibodies, which are part of the classification criteria of SSc, autoantibodies to the Th/To complex have also been described in up to 10% of SSc sera [9–12, 18]. Almost all protein components of the Th/To complex have been reported to be targets of the anti-Th/To autoantibody repertoire [10, 20]. Moreover, recent studies using ELISA and CIA confirmed Rpp25 as the major Th/To autoantigen, being detected in ~60–100% of anti-Th/To positive patients (as identified by IP) [10, 22].

The observation that they are primarily detectable in SSc makes this specificity an important serological adjunct in the diagnosis and clinical stratification of SSc. In addition, a high prevalence (~25%) of anti-Th/To antibodies was found in ANA-positive/ENA-negative SSc patients [25]. In our study, all samples with anti-Rpp38 antibodies showed the AC-08 (nucleolar) pattern, which is consistent with the presence of anti-Th/To antibodies [35]. The anti-Th/To antibody test may also have applications for non-SSc patients such as those with ILD, as anti-Th/To antibodies were reported in ~50% of anti-nucleolar antibody-positive idiopathic pulmonary fibrosis patients [13]. However, this requires further studies on large idiopathic pulmonary fibrosis cohorts as only a portion of these patients expressed nucleolar pattern.

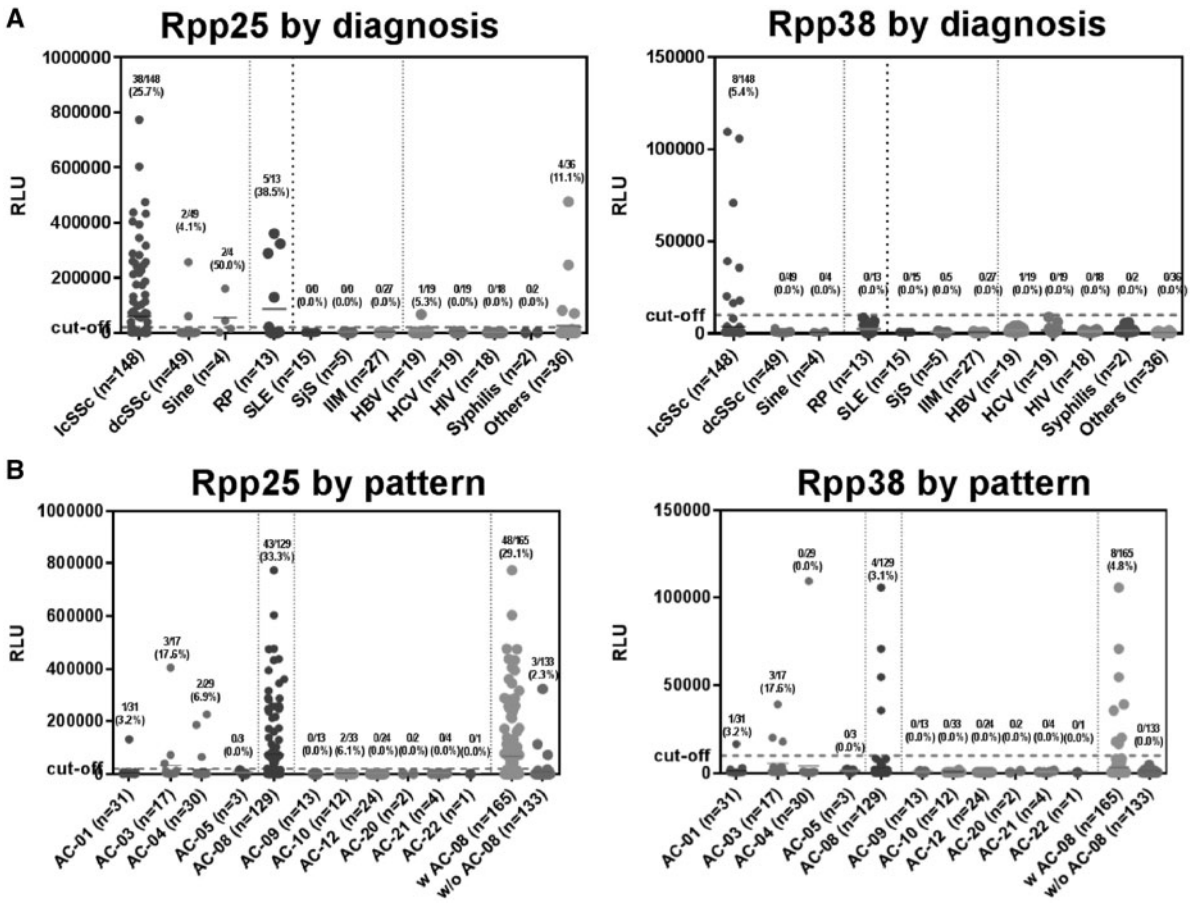
When comparing the prevalence of anti-hPop1 or anti-Rpp25 antibodies in SSc patients based on published studies, similar prevalences were found (2.1–3.3%) [22–24]. However, statistically significant differences in the clinical specificities were reported 98.7% [23] and

Table 1 Designed Rpp25, Rpp38 and hPop1 peptides

No.	Antigen	aa	Short ID	ID
1	hPop	15	^{400} hPop1 ^{414}	hPop- ^{400} IGDGTRDPCLPYSWI ^{414}
2	Rpp25	20	^{51} Rpp25 ^{68}	Rpp25- ^{51} AFATASMAQPATRAIVFSGC ^{68}
3	Rpp38	15	^{229} Rpp38 ^{243}	Rpp38- ^{229} RELLDTSFEDLSKPK ^{243}
4	Rpp25	30	^{54} Rpp25 ^{68} / ^{123} Rpp25 ^{137}	Rpp25- ^{54} TASMAQPATRAIVFS^{68} / Rpp25- ^{123} AASLSVLKNVPGLAI ^{137}
5	Rpp25	26	^{189} Rpp25 ^{199} / ^{123} Rpp25 ^{137}	Rpp25- ^{189} EPGVADEDQTA^{199} / Rpp25- ^{123} AASLSVLKNVPGLAI ^{137}
6	Rpp38	15	^{223} Rpp38 ^{247}	Rpp38- ^{233} DTSFEDLSKPKRKLA ^{247}
7	hPop1	23	^{418} hPop1 ^{440}	hPop1- ^{418} TGIIISDLTMEMNRFRLIGPLSH ^{440}
8	Rpp38/hPop1	30	^{233} Rpp38 ^{247} / - ^{418} hPop1 ^{432}	Rpp38- ^{233} DTSFEDLSKPKRKLA ^{247} / hPop1- ^{418}TGIIISDLTMEMNRF ^{432}

aa: amino acid; ID: identification code.

FIG. 3 Autoantibody reactivity to Rpp25 and to the major Rpp38 peptide epitope

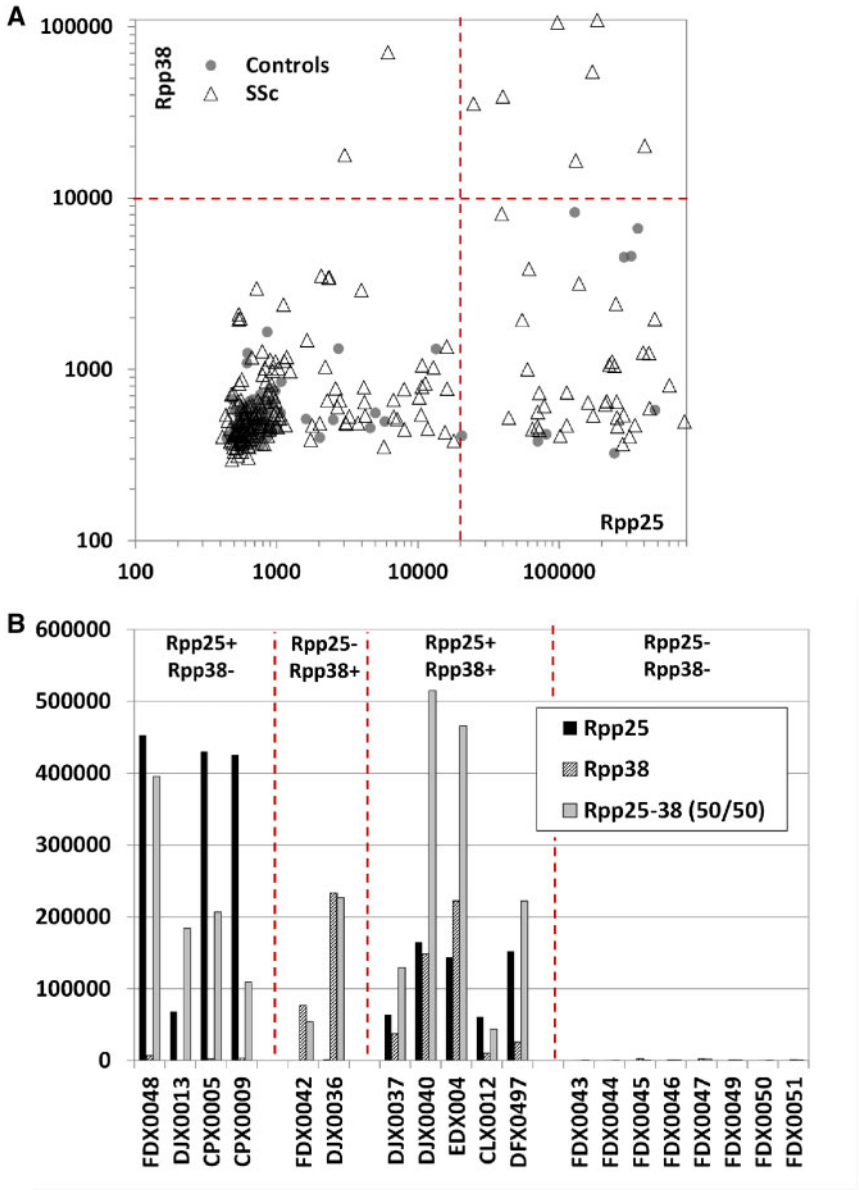


The levels of anti-Rpp25 (recombinant full-length protein) and anti-Rpp38 (the major Rpp38 peptide) antibodies are shown as measured by chemiluminescence immunoassay (CIA) in **A**. In **B**, reactivity is displayed according to the IIF pattern. The individual ICAP pattern as well as two groups [with (w) and without (w/o) any AC-08 pattern] are presented. lcSSc: limited cutaneous SSc; dcSSc: diffuse cutaneous); IIM: idiopathic inflammatory myopathies; HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; AC-01: homogeneous; AC-03: centromere; AC-04: fine speckled; AC-05: large coarse speckled; AC-08: homogeneous nucleolar; AC-09: clumpy nucleolar; AC-10: punctate nucleolar; AC-12: punctate nuclear envelop; AC-20: cytoplasmic fine speckled; AC-21: cytoplasmic anti-mitochondrial; AC-22: cytoplasmic Golgi-like.

97.8% [24] for hPop1 by LIA *vs* 99.5% for Rpp25 by CIA [22]. Whether the differences are attributable to different control groups is unknown and should be analysed in future studies.

In a recent study on a large cohort of Canadian SSc patients, the LIA used to detect anti-hPop1 missed a significant number ($n = 18$) of anti-Th/To antibody positive samples that were identified by IP [25]. This may be due to low prevalence of anti-hPop1 autoantibodies among anti-Th/To positive patients in this cohort or low sensitivity of the hPop1 antigen used in LIA. However, 20% of the anti-Th/To antibodies positive samples were also missed using the Rpp25 CIA when compared with IP. An analysis of the lack of concordance and the potential complementarity of anti-Rpp25 and anti-hPop1 antibodies is currently ongoing. In a study by Kuwana *et al.* [20], anti-hPop1 autoantibodies were significantly more prevalent in anti-Th/To positive SSc patients as detected by IP, compared with anti-Th/To positive patients with other AARDs. In contrast, Rpp30 and Rpp38 were equally targeted by autoantibodies from SSc and non-SSc ANA-associated rheumatic disease patients. This is inconsistent with our findings, which showed that reactivity of an SLE serum pool to linear peptides of hPop1 was higher compared to Rpp25 and Rpp38. However, discontinuous conformational epitopes were not analysed in our study. During the course of this study, we also aimed to compare the reactivity to the individual Th/To components using a recombinant Rpp38 and hPop1. However, we were unable to develop an immunoassay or functional assays that might be related to the recombinant constructs. Nevertheless, when indirectly comparing the reactivity to

FIG. 4 Autoantibodies to Rpp25 and Rpp38



A: Correlation between anti-Rpp25 and anti-Rpp38 $^{229-243}$ peptide antibodies. The reactivity between anti-Rpp25 and anti-Rpp38 peptide antibodies measured in 299 samples (203 SSc, 81 controls and 13 with unknown diagnosis) using a chemiluminescence assay showed significant correlation (rho = 0.44, 95% CI 0.35, 0.53; $P < 0.0001$). However, subsets of some patients either reacted with Rpp25 or with the novel Rpp38 derived peptide. **B**: Using a mixture of Rpp25 and Rpp38 coupled beads, samples that were positive for either of the two antigens were detected. All values on the ordinate axis are expressed as relative light units (RLU). QF: QUANTA Flash.

Rpp38 described in the original study (6/303, 2%), the identified Rpp38 epitope showed comparable reactivity.

Although autoantibodies to Th/To have been known for over two decades, the clinical associations of anti-Th/To antibodies are not yet fully established. Previous studies are mostly consistent in showing an association with lcSSc; however, association with more specific clinical features is somewhat inconsistent. Small numbers of anti-Th/To positive patients, differences in ethnicity and environment, differences in the detection methods, recruitment bias and other factors could explain the inconsistencies [9, 11, 36–38]. Anti-Th/To antibodies have also been associated with pericarditis, ILD and a high frequency of isolated pulmonary hypertension [9, 17]. Compared with anti-CENP IIF positive patients, anti-Th/To lcSSc patients have milder cutaneous, vascular and

TABLE 2 Clinical features of SSc patients with anti-Rpp38 antibodies

#	Sex	Diagnosis	Other	Age at onset	Age at sampling	ILD at blood sampling	ILD during disease	Pulmonary hypertension	Capillaroscopy	Other aab
1	F	lcSSc	PBC	7.8	53.1	0	0	0	1-Late pattern	Ro52, CENP
2	F	lcSSc	PBC	34.6	48.8	0	0	0	1-Late pattern	Ro52, PM/Scl
3	M	lcSSc		23.3	36.6	1	1	0	1-Late pattern	none
4	F	lcSSc	C, DU	43.1	73.9	0	0	0	1-Late pattern	Ro52
5	F	lcSSc	DU	NA	36.1	0	1	0	NA	Ro52, CENP, PM/Scl
6	F	early SSc	SS	30.5	32.2	0	1	0	1-Early pattern	CENP
7	F	lcSSc	SS	45.6	73.9	0	1	0	1-Late pattern	none
8	F	lcSSc	none	48.7	57.7	0	0	0	1-Late pattern	none

lcSSc: limited cutaneous SSc; C: calcinosis; CENP: centromere protein; DU: digital ulcers; NA: not available; PBC: primary biliary cholangitis; f: female; m: male; 0: absent; 1: present.

gastrointestinal involvement, but more often have pulmonary fibrosis, renal crisis and reduced survival [36]. Like other SSc-related autoantibodies, the presence of anti-Th/To antibodies in patients with isolated RP is predictive of early SSc [39]. Also, anti-Th/To positive SSc patients demonstrated earlier development of nailfold capillary abnormalities than anti-CENP B positive patients [39]. In addition, anti-Th/To positive patients were younger and more frequently male compared with anti-CENP IIF-positive patients [9]. The prevalence of anti-Th/To antibodies might be higher in Caucasian Americans compared with African and Latin American patients [37]. Lastly, in the Canadian SSc cohort preselected for autoantibody reactivity, the association of anti-Th/To and anti-Rpp25 antibodies with ILD and nailfold capillary abnormalities was confirmed [25]. In light of the importance of stratifying SSc patients in more clinically meaningful subsets of patients [14, 38], it is interesting to note that all eight anti-Rpp38 antibody positive subjects had lcSSc and four patients developed ILD during disease cause. This potential association is of interest as anti-Th/To antibodies fluctuate over time and might predict future development of ILD [40]. However, because the prevalence of ILD in the anti-Rpp38 positive individuals is not significantly different from general SSc cohorts [41], future research might also consider severity of ILD and prognostic aspects. Although not statistically significant, it is intriguing that two of the eight (25%) patients had primary biliary cholangitis (PBC), an uncommon autoimmune liver condition that may coexist with lcSSc [42]. This observation deserves further investigation.

Several studies have analysed the epitope distribution on SSc-related autoantigens, including CENP, PM/Scl [43], RNA Pol III and topo I [1]. For CENP-A and PM/Scl, linear epitopes could be identified using the SPOT technology and these epitopes were then used to develop sensitive and specific ELISA [44, 45]. In contrast, very little has been published about the epitope specificity and distribution on Th/To autoantigens. This is the first study to describe linear epitopes on the Rpp25, Rpp38 and hPop Th/To autoantigens. The identified major epitope on Rpp38 was then used to develop an immunoassay showing significant correlation to the results obtained with the anti-Rpp25 immunoassay, which raised questions about potential cross-reactivity. A sequence comparison between Rpp25 and Rpp38 revealed low sequence similarity. In addition, the major Rpp38 epitope does not show sequence similarity to any region on Rpp25. Consequently, the correlation might be explained by a polyclonal autoantibody response to several members of the protein complex rather than by cross-reactivity. In the future, studies using, for example, the entire Canadian Scleroderma Research Group (CSRG) SSc cohort, or patients registered at the EULAR Scleroderma Trials and Research (EUSTAR) [18, 46] or the German Network for systemic scleroderma [18] and/or the Australian SSc cohort [47] would be of great interest to thoroughly analyse the clinical associations of autoantibodies to the new Th/To epitopes. This could also provide further insights about the future use of Rpp25 and Rpp38 peptide, either as single analytes, or as a screening approach to assess anti-Th/To antibodies as demonstrated herein.

Despite the relatively low prevalence of anti-Th/To antibodies in SSc, testing for these antibodies and the sub-specificities (anti-Rpp25, anti-Rpp38 and anti-hPop1 antibodies) may have significant value for patient stratification [5, 14]. The association with ILD is of particular importance because lung disease accounts for 33% of SSc-related deaths [48].

In summary, this is the first study to describe linear epitopes on the Th/To antigen. In addition, the assay based on the identified epitope was specific for lcSSc and identified patients with a distinct clinical phenotype.

Acknowledgements

We thank Meifeng Zhang, James Wick and Haiyan Hou at the University of Calgary for their technical assistance.

This research was supported in part by grants to J.-L.S. and M.K. from Sclérodermie Québec, Scleroderma Society of Ontario, Scleroderma Society of Canada, the Scleroderma Association of Saskatchewan and the Scleroderma Association of British Columbia.

Funding: No specific funding was received from any funding bodies in the public, commercial or not-for-profit sectors to carry out the work described in this manuscript.

Disclosure statement: M.M. and C.B. are employed at Inova Diagnostics, a company that manufactures and markets autoantibody assays. M.J.F. is the Director of Mitogen Advanced Diagnostics Laboratory (Calgary, Alberta, Canada), which performs autoantibody testing and is also a consultant to Inova Diagnostics Inc. and has received gifts in kind from ImmunoConcepts (Sacramento, CA) and Euroimmun GmbH (Luebeck, Germany). The other authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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Autoimmunity Reviews 18 (2019) 658–664

Contents lists available at ScienceDirect

Autoimmunity Reviews

journal homepage: www.elsevier.com/locate/autrev

Review

Anti-OJ autoantibodies: Rare or underdetected?

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ARTICLE INFO

Keywords:
Idiopathic inflammatory myopathy
Myositis
Autoantibody
Anti-synthetase syndrome
OJ
Isoleucyl-tRNA synthetase

ABSTRACT

Anti-OJ autoantibodies are rare myositis-specific autoantibodies that have been described to target isoleucyl-tRNA synthetase. Routinely used multiplex assays perform poorly in detection of anti-OJ antibodies. In this manuscript, we review the existing literature on critical issues in detection of anti-OJ and the clinical features associated with anti-OJ. The challenging detection with line/blot immunoassays and ELISAs is most likely related to the characteristics of the autoantigen involved, which is part of a multi-enzyme synthetase complex. Anti-OJ autoantibodies might therefore be more aptly termed anti-OJ complex autoantibodies. Anti-OJ autoantibodies are associated with the anti-synthetase syndrome, with interstitial lung disease (ILD) frequently being the sole manifestation. Myositis, present in the majority of patients with anti-OJ antibodies, is more severe than in patients with other anti-aminoacyl-tRNA synthetases. Most patients respond to glucocorticoid therapy. As detection of anti-OJ is relevant for treatment, reliable and practical detection is needed. Meanwhile, clinicians need to be aware of the possibility of anti-OJ in patients with ILD, isolated or in combination with myositis.

1. Introduction

Anti-OJ autoantibodies are myositis-specific autoantibodies (MSAs) that can be found in < 5% of patients with idiopathic inflammatory myopathies (IIMs) [1,2]. They presumably target isoleucyl-tRNA synthetase and, as such, are part of the group of the anti-aminoacyl-tRNA synthetases (anti-ARS, Table 1), of which anti-Jo-1 is the most prevalent [1,3]. Collectively, anti-ARSs can be found in 11–40% of patients with IIM, depending on the detection method and the examined cohort [1,4].

The anti-ARSs are associated with the anti-synthetase syndrome (ASS), a subtype of IIM. ASS consists of the triad of myositis, interstitial lung disease (ILD) and arthritis, with mechanic's hands, Raynaud phenomenon and unexplained fever as frequently accompanying manifestations [3,5–7]. In addition to ASS, which is sometimes seen as a form of overlap myositis (OM), four more IIM subtypes are recognized: dermatomyositis (DM), immune-mediated necrotizing myopathy (IMNM), inclusion-body myositis (IBM) and polymyositis (PM) [8]. Within these subtypes, other autoantibody-defined syndromes are being considered, highlighting the relevance of reliable autoantibody detection [9,10].

Although the detection methods for autoantibodies in IIM are evolving rapidly, detection of anti-OJ autoantibodies with newer immunoassays proves to be especially difficult. Therefore, knowledge of the clinical features associated with anti-OJ is relevant for the physician in order to avoid misdiagnosis or misclassification. To this end, we provide an overview on the detection, with emphasis on the target of anti-OJ, and associated clinical features of anti-OJ autoantibodies.

2. Methods

In PubMed and Embase the following search was performed: “Anti-OJ” OR ‘OJ autoantibodies” OR “anti-isoleucyl-tRNA synthetase”. After removal of duplicates and exclusion of articles based on article type (reviews and conference abstracts were excluded) and content (as based on abstract) a full-text review was performed for articles available in English. Additional articles were hand searched. Patients described in multiple articles, if explicitly stated, were only included once for review of associated clinical features.

Antigens recognized by sera were analyzed by immunoprecipitation (IP) of radiolabeled K562 (human erythroleukemia) cell extract and

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https://doi.org/10.1016/j.autrev.2019.05.002
Received 5 January 2019; Accepted 11 January 2019
Available online 03 May 2019
1568-9972/

Table 1
Autoantibodies targeted at aminoacyl-tRNA synthetases or proteins associated with translation.

Autoantibody	Autoantigen	ARS class	Molecular weight in kDa (IP)	Clinical phenotype	Prevalence in IIM (%)
Aminoacyl-tRNA synthetases					
Anti-Jo-1 [55]	Histidyl-tRNA synthetase (HARS)	II	50	ASS/ILD	25–30%
Anti-PL-12 [56]	Alanyl-tRNA synthetase (AARS)	II	110	ASS/ILD [57]	2–5%
Anti-PL-7 [58]	Threonyl-tRNA synthetase (TARS)	II	80	ASS/ILD [59]	2–5%
Anti-EJ [11]	Glycyl-tRNA synthetase (GARS)	II	75	ASS/ILD	< 2%
Anti-KS [60]	Asparaginyl-tRNA synthetase (NARS)	II	65[60]	ILD, arthritis, sicca syndrome [60,61]	< 2% [60]
Anti-OJ [11]	Components of the MSC	I (IRS)	150 + 170/130/75	ASS/ILD	< 5% [1,2]
Anti-YRS/Anti-Ha [62]	Tyrosyl-tRNA synthetase (YARS)	I	59	ASS/ILD	Rare
Anti-Zo [63]	Pheynlalanyl-tRNA synthetase (FARS)	II	60/70	ASS/ILD	Rare
Anti-WRS [64]	Tryptophanyl-tRNA synthetase (WARS)	I	120	SLE, RA, malignancy [65,66]	NA
Proteins associated with translation					
Anti-Mas [67]	Selenocysteine-seryl-tRNA-protein complex	NA	48	AIH, IIM	2% [68]
Anti-KJ [69]	Translocation factor	NA	30/43	ASS-like syndrome	Rare
Anti-Wa [70]	NEFA/nucleobindin-2 [71]	NA	48	ASS-like syndrome	Rare
Anti-Fer [72]	Eukaryotic elongation factor Ia [73]	NA	Unknown	ASS-like syndrome	Rare

ASS antisynthetase syndrome, IIM idiopathic inflammatory myopathy, IP immunoprecipitation, IARS isoleucyl-tRNA synthetase, MSC multienzyme synthetase complex, NA not applicable, NEFA DNA binding/EF-hand/acidic amino acid rich region.

SDS-PAGE. In brief, cells were labeled for 14 h with 35S-L-methionine and 35S-L-cysteine (NEG772, PerkinElmer, Waltham, MA, USA) and lysed in NET/IGEPAL CA-630 buffer (500 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.3% IGEPAL CA-630) containing 0.5 mM PMSF and 0.3 TIU/ml aprotinin. Cell extract was cleared by centrifugation and immunoprecipitated on Protein A Sepharose beads (17-0780-01, GE Healthcare, Marlborough, MA, USA) coated with antibodies from 8 µl of human serum. Beads were then washed with 0.5 M NaCl NET/ IGEPAL CA-630 buffer (500 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.3% IGEPAL CA-630). Immunoprecipitated proteins were subjected to 8% and 12.5% SDS-PAGE followed by autoradiography.

3. The anti-OJ target

Anti-OJ autoantibodies were, concurrently with anti-EJ autoantibodies, discovered by IP and aminoacylation inhibition experiments by Targoff in 1990 (Fig. 1) [11]. The immunoprecipitate of sera of two patients, who had the initials OJ and EJ, revealed identical patterns of tRNA, which co-immunoprecipitated with the ARS, and a distinct protein pattern, consistent with the multi-enzyme synthetase complex (MSC). These sera inhibited the enzymatic activity of isoleucyl-tRNA synthetase (IARS), a component of the MSC, most strongly in an aminoacylation assay, though reactivity with other components of the MSC were noted in the initial and subsequent study by Targoff [11,12]. Interestingly, immunoblotting with anti-OJ positive sera did not show reaction with the presumed main target IARS in most cases. These findings challenge the notion of IARS as a singular isolated target and points to considering IARS as a part of the MSC if we want to further elucidate the target.

The MSC consists of 9 synthetases, including IARS, and 3 non-catalytic components (Fig. 2a) [13,14]. It has a key role in protein synthesis but is a central hub for many signaling pathways as well [14]. In addition to IARS, anti-OJ autoantibodies have shown reactivity with lysyl-tRNA synthetase (KARS), a 160 kD synthetase protein that most likely corresponds to the bifunctional glutamyl-prolyl-tRNA synthetase (EPRS) or glutaminyl-tRNA synthetase (QARS), and possibly leucyl- (LARS) and arginyl-tRNA synthetase (RARS) [12,15]. No reactivity with methionyl-, arginyl- and asparaginyl-tRNA synthetases, the remaining synthetases of the MSC, has been described. The 3 non-catalytic components, p43, p38 and p18, are instrumental for stabilization of the interactions between the components [14,16]. Next to these established components, additional components have been suggested, such as threonyl-tRNA synthetase like-2 (TARSL2), an enzyme with similar aminoacylation activity to threonyl-tRNA synthetase (TARS) [17], which is a known autoantigen for anti-PL-7 autoantibodies [18].

These components are assembled into multiple subcomplexes and ultimately into the MSC, which has an estimated weight of 1.5 MDa [14]. Cryogenic electron microscopy [19] and small angle X-ray scattering [20] has allowed the construction of a low resolution model of the entire native MSC. These structural studies reveal a large and elongated, but probably flexible, structure. This organization befits the dual role of the MSC in protein synthesis and signaling.

Considering the confounding results in defining the primary target (s) of anti-OJ and the expanded knowledge of the components and structure of the MSC, the epitope might be based on quaternary interactions between MSC components. If the binding of autoantibody and the OJ epitope would indeed be dependent on quaternary interactions, anti-OJ autoantibodies may rather be seen as anti-OJ complex autoantibodies. Viewing anti-OJ autoantibodies as targeted at a complex has direct implications for the development of an assay for detecting anti-OJ autoantibodies.

4. Detection of anti-OJ autoantibodies

In current clinical practice detection of anti-OJ autoantibodies is problematic. IP is the preferred method as sera with anti-OJ autoantibodies have a specific pattern on RNA and protein IP (Fig. 2b). However, differences between direct antigen-antibody interactions that can be detected by solid phase assays vs. direct, indirect and quaternary interactions captured by IP could negatively impact the agreement between these methods. Furthermore, IP is laborious and technically demanding, leading to increasing use of other assays in routine practice.

Line immunoassays (LIA) and dot blot assays (DBA) are the current detection method for MSAs in many hospitals. These multiplex assays, which are based on immunoblotting, agree only for some MSAs with IP [21]. For anti-OJ, they perform poorly. In a comparison of a LIA (Euroimmun Myositis profile, Lübeck, Germany) with IP in three studies, none of the 25 anti-OJ-positive sera (all three studies combined), as confirmed by RNA and protein IP [1,22] or protein IP alone [21], were positive on the LIA. Moreover, in large cohorts tested with LIA or DBA, detection of anti-OJ was exceedingly rare, even more so than in cohorts tested with IP with a similar number of patients [4,23–25].

Few other methods besides LIA and DBA have been investigated. In a study of an ELISA for a mixture of 6 anti-ARSs, anti-OJ was the only anti-ARS that could not be detected in RNA IP-confirmed anti-OJ positive sera [26]. The recombinant antigen was expressed in insect cell expression system (Hi-5 cells). A novel detection technique called

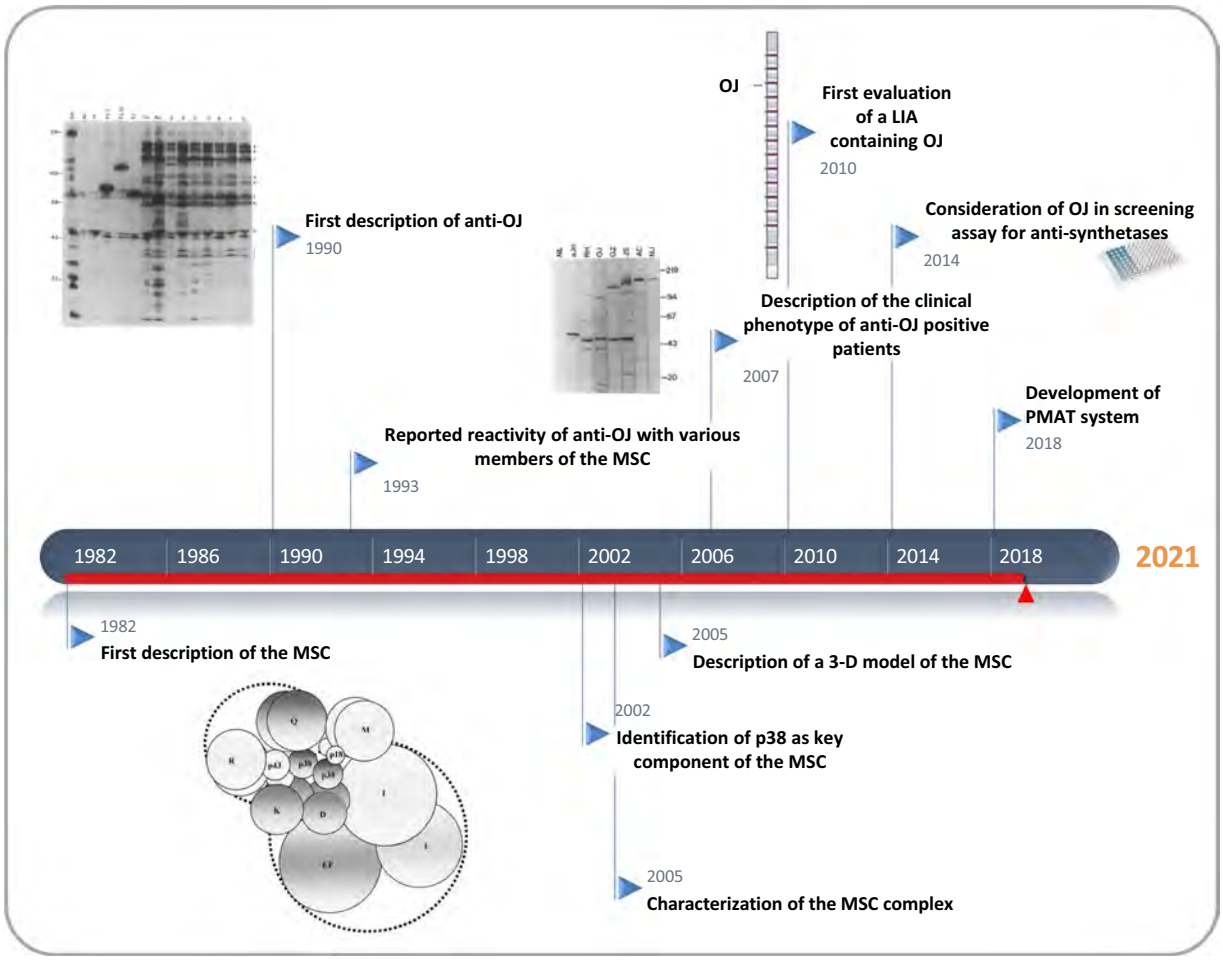

Fig. 1. The history of anti-OJ autoantibodies from biochemical and immunological perspective. MSC = multi-synthetase complex; LIA = Line immunoassay; PMAT = particle-based multi-analyte technology.

particle-based multi-analyte technology (PMAT) has an excellent correlation with IP for the most frequently encountered MSAs, but has not been evaluated with anti-OJ positive sera as detected by IP [27].

The role of indirect immunofluorescence (IIF) in IIM is limited [28]. A cytoplasmic pattern can be seen in a proportion of patients with anti-OJ, as is the case for other anti-ARS [29–31]. Notably, ARSs can also be found in nonconventional localizations such as the nucleus and mitochondria [32]. Lack of sensitivity of IIF on HEp-2 cells for various cytoplasmic antigens, including the ARSs, could be a consequence of low relative antigen concentration, cell preparation or fixation protocols [33]. As such, a negative result on IIF, even with separate reporting of cytoplasmic staining [34], does not exclude MSAs such as anti-OJ. Therefore, IIF alone is not adequate for screening patients with possible anti-OJ or other anti-ARSs. Furthermore, identification of the specific autoantibody is relevant for management of these diseases, even within the ASS, given the heterogeneity in clinical features among anti-ARSs [28,35].

Considering the detection issues with other techniques, IP remains the preferred method for detection of anti-OJ autoantibodies, despite its own limitations. The disappointing results with the other assays reflect the complexity associated with OJ testing and the need to establish the primary target/targets that represent anti-OJ reactivity. This in turn may lead to development of a more practical immunoassay that captures the direct and indirect (quaternary) interactions involving anti-OJ antibodies.

5. Coexistence with other autoantibodies

Of the patients described in literature, only one patient with anti-OJ had coexisting anti-Jo-1 autoantibodies, both detected by RNA IP [15]. This is the only documented case of reactivity with a synthetase that is not a part of the MSC. Furthermore, coexistence of anti-SS-A/Ro60 and anti-SS-B/La, two autoantibodies frequently present in patients with systemic autoimmune rheumatic diseases, have been reported in more than one case [12,36,37]. In patients positive for anti-Jo-1 and other anti-ARS, the anti-Ro52 status delineates a subgroup with more severe muscular, respiratory and articular involvement [38]. The paucity of patients with anti-OJ does not allow a similar analysis. Finally, several patients were positive for rheumatoid factor, though this has no clear relevance in IIMs [39].

6. Associated clinical features and treatment experience

Anti-OJ autoantibodies are reportedly associated with the ASS. Within the ASS, there is heterogeneity in clinical features depending on the specific anti-ARS [35,40]. In general, patients with non-Jo-1 anti-ARS have a poorer prognosis than patients with anti-Jo-1 [41,42]. For anti-OJ, we identified 52 published cases with sufficient description of clinical features (summarized in Table 2) [1,12,15,29–31,35–37,43–48]. These patients

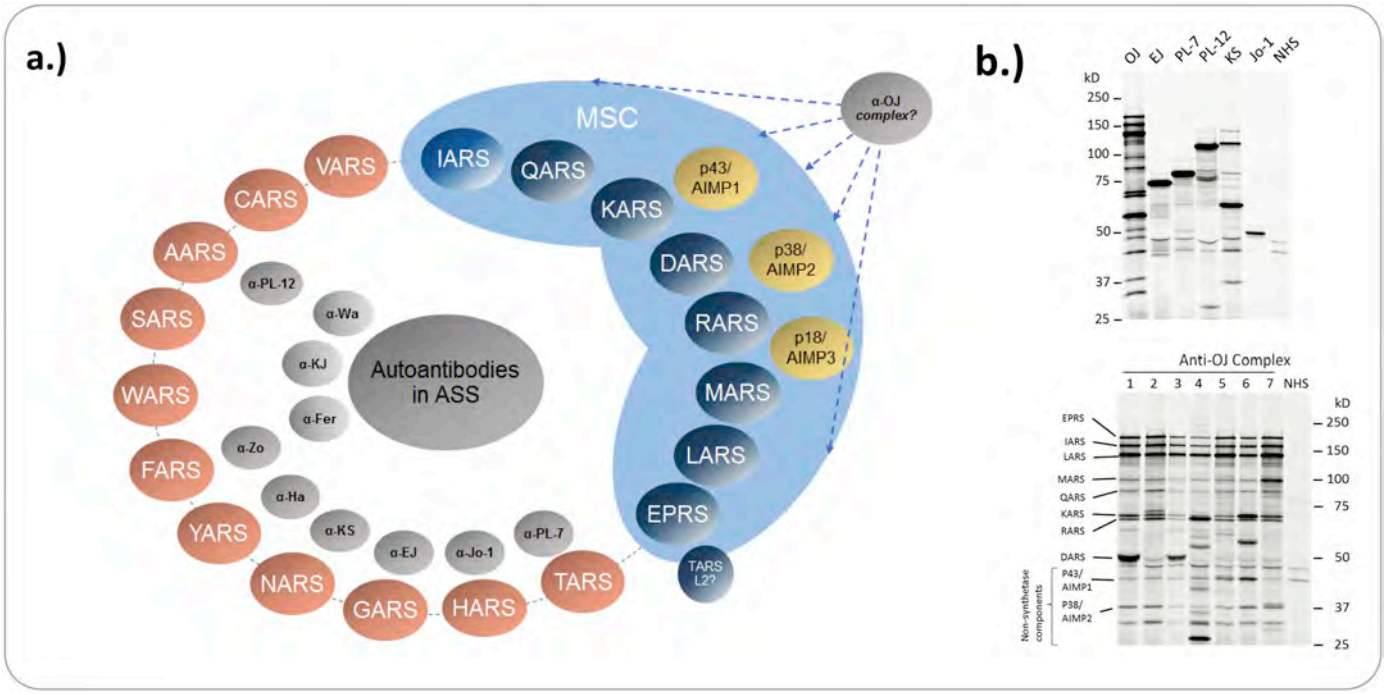

Fig. 2. Autoantibodies associated with the antisynthetase syndrome (ASS) or ASS-like syndrome. In a.) the composition of the multi-synthetase complex (MSC) is illustrated. The panel b.) shows the reactivity profile of anti-synthetase autoantibodies including anti-OJ autoantibodies. α- = anti-, ASS = antisynthetase syndrome, AARS = alanyl-tRNA synthetase, CARS = cysteine-tRNA synthetase, DARS = aspartyl-tRNA synthetase, EPRS = glutamyl-prolyl-tRNA synthetase, FARS = phenylalanyl-tRNA synthetase, GARS = glycyl-tRNA synthetase, HARS = histidyl-tRNA synthetase, IARS = isoleucyl-tRNA synthetase, KARS = lysyl-tRNA synthetase, LARS = leucyl-tRNA synthetase, MARS = methionyl-tRNA synthetase, MSC = multienzyme synthetase complex, NARS = asparagyl-tRNA synthetase, QARS = glutaminyl-tRNA synthetase, RARS = arginyl-tRNA synthetase, SARS = seryl-tRNA synthetase, TARSL2 = threonyl-tRNA synthetase like 2, TARS = threonyl-tRNA synthetase, VARS = valyl-tRNA synthetase, WARS = tryptophanyl-tRNA synthetase.

were between 13 and 79 years old at disease onset and had a female-to-male ratio of 1.7:1. All but one patient, who was diagnosed with systemic sclerosis (SSc), had a primary diagnosis of PM, DM, ILD or ASS. Given the detection issues described in the previous section it is logical that anti-OJ was detected by IP in all but two patients, of which one had confirmation by IP afterwards [36,48].

ILD is present in 90% of patients and is in most cases the first and presenting clinical feature [35]. Based on the results of high-resolution computed tomography (CT) of chest or open lung biopsy, usual interstitial pneumonia (UIP), organizing pneumonia (OP) and non-specific pneumonia (NSIP) are the most encountered ILD patterns [36,46,49]. Data on respiratory function tests are scarce. In one study there was a statistically significant difference in forced vital capacity between anti-OJ and anti-ARS-negative patients while no difference in CT chest score or diffusion capacity for carbon monoxide was noted. However, only 2 anti-OJ positive patients were included [46]. Death due to respiratory failure was not reported in the studies listed in Table 2. As ILD is the main and frequently sole feature, the possibility of anti-OJ needs to be considered in patients with 'idiopathic' ILD, as is the case for all anti-ARSs [50]. Moreover, follow-up should include regular respiratory function tests and high-resolution CTs of the chest.

The majority of anti-OJ positive patients have muscle involvement. Seventy-five percent of patients described in Table 2 had myositis, but, importantly, muscle weakness was an inclusion criterion in the study by Noguchi et al., which described the largest group of patients with anti-OJ [1]. Interestingly, the prevalence of anti-OJ was higher in this study (14/461) than in other studies, even rivalling the frequency of anti-Jo-1 (15/461 patients), though the frequency of anti-Jo-1 was notably lower than reported in other studies. In a reply to this study, Castañeda et al. stated that in a currently unpublished series of their patients with anti-OJ, 40% had hypomyopathic or amyopathic forms of ASS [6]. If muscle involvement is present, it might be more severe as compared to myositis patients with other anti-ARSs. In the study by Noguchi et al., an increased incidence of severe limb and neck muscle weakness, dysphagia and muscle atrophy on muscle biopsy was noted [1]. Illustrative of this potentially severe muscle involvement is a case report in which the presenting symptom was rhabdomyolysis [48]. Accordingly, clinicians should consider the possibility of anti-OJ autoantibodies in patients with severe muscle involvement, besides anti-SRP and anti-HMGCR autoantibodies or non-immune types of myopathy.

The remaining features of ASS vary in prevalence. Arthritis was present in nearly half of patients, mostly as a polyarthritis. In a limited number of patients arthritis was the presenting symptom, which contrasts with patients with anti-Jo-1 antibodies in whom arthritis is found in a quarter of patients [29]. Several of these patients had a concomitant alleged diagnosis of rheumatoid arthritis [43,44], a finding which has also been noted in patients with other anti-ARSs [51–53]. Fever is regularly present while Raynaud phenomenon occurs in a minority of patients (5/49). Mechanic's hands have not been reported consistently, but they have been described in at least 5 patients [30,35,45]. A complete ASS is hence the exception, rather than the rule.

Skin involvement can occur in anti-OJ positive patients. Approximately 30% of patients had a DM-associated skin lesion. More specifically, heliotrope rash, Gottron's sign or papules, V sign, shawl sign and holster sign have been reported [45]. Other skin lesions include ulceration and sclerodactyly, both described in 3 patients. Two of these patients, who had both ulceration and sclerodactyly, could be classified as both SSc and ASS [5,54].

Few cases of malignancies have been reported in patients with anti-OJ. In one study, there was a history of a malignancy in 2 patients: one with a gastric neuro-endocrine tumor and one with colon cancer [35]. In another study 2 patients with anti-OJ (of 5 in total) died due to cancer without further specification of the type of cancer [41]. Due to the small number of patients with anti-OJ, it is difficult to assess a possible association with cancer.

Most cases of patients with anti-OJ had a good response to

Table 2
Clinical features of patients with anti-OJ autoantibodies described in literature.

Study (authors) (year, reference)	Primary diagnosis	Patients (n)	Myositis (n/n)	DM skin lesions (n/n)	MH (n/n)	ILD (n/n)	Arthritis (n/n)	RP (n/n)	Fever (n/n)
Detected or confirmed by RNA and/or protein IP									
Targoff et al. (US, 1993, [12])	IIM	9	8/9	3/9	NA	8/9	6/9	1/9	NA
Friedman et al. (US, 1996, [31])	CTD/ILD	2	1/2	0/2	NA	2/2	1/2	0/2	0/2
Gelpi et al.[a] (US, 1996, [15])	ASS	1	1/1	0/1	NA	1/1	1/1	0/1	1/1
Ohosone et al. (Japan, 1998, [43])	PM-RA	1	1/1	0/1	NA	1/1	1/1	1/1	0/1
Sato et al.[b] (Japan, 2007, [44])	IIM/ILD	7	4/7	0/7	NA	7/7	4/7	0/7	NA
Koreeda et al. (Japan, 2010, [29])	ILD	1	0/1	1/1	NA	1/1	1/1	1/1	0/1
Noda et al. (Japan, 2011, [45])	DM	1	1/1	1/1	1/1	1/1	0/1	0/1	0/1
Kunimasa et al. (Japan, 2012, [30])	IIM-ILD	2	2/2	0/2	1/2	2/2	0/2	0/2	1/2
Hamaguchi et al.[b] (Japan, 2013, [35])	ASS	8	2/8	1/8	3/8	8/8	1/8	1/8	NA
Johnson et al. (US, 2014, [46])	ILD	2	1/2	0/2	0/2	2/2	NA	NA	0/2
Hamada et al. (Japan, 2017, [37])	JPM	1	1/1	0/1	0/1	0/1	1/1	1/1	1/1
Noguchi et al.[b] (Japan, 2017, [1])	IIM[c]	14	14/14[c]	8/14	NA	12/14	4/14	0/14	7/14
Pauling et al. (GB, 2017, [47])	SSc	1	1/1	0/1	NA	NA	1/1	1/1	NA
Kapoor et al. (US, 2018, [48])	ASS	1	1/1	1/1	NA	0/1	1/1	0/1	0/1
Total (n/n, %)		51	38/51 (75%)	15/51 (29%)	5/14 (36%)	45/50 (90%)	15/49 (31%)	6/49 (12%)	10/26 (38%)
Detected by IB									
Hervier et al. (France, 2011, [36])	ASS	1	1/1	0/1	0/1	1/1	0/1	1/1	NA

Incomplete data.
ASS = antisynthetase syndrome, CTD = connective tissue disease, DM = dermatomyositis, GB = Great Britain, IB = immunoblot (in both studies Euroimmun Myositis Profile, Lübeck, Germany), IIM = idiopathic inflammatory myopathy, ILD = interstitial lung disease, IP = immunoprecipitation, JPM = juvenile polymyositis, MH = mechanic's hands, NA = data not available or absence not explicitly stated, OM = overlap myositis, PM = polymyositis, RA = rheumatoid arthritis, SSc = systemic sclerosis. USA = United States of America. Myositis was defined as one or more of the following: clinically apparent muscle weakness, elevation of CK levels or findings compatible with myositis on muscle biopsy. ILD was defined by one or more of the following: clinical diagnosis of ILD, ILD pattern on CT chest, restrictive pattern on lung function testing or findings compatible with ILD on open lung or transbronchial biopsy.

[a] Coexistence of anti-Jo-1 autoantibodies.

[b] Overlap of patients could not be excluded.

[c] Presence of muscle involvement was an inclusion criterion for this study.

glucocorticoid therapy with improvement of ILD and/or myositis. The dose of oral prednisolone used in literature varied between 20 and 60 mg or 0.5–1 mg/kg daily. More intensive regimens described include pulse doses of glucocorticoids (1 g methylprednisolone) and cyclophosphamide [30,36,44,48]. Intravenous immunoglobulins have been used for cases refractory to glucocorticoid therapy [1]. As corticosteroid-sparing agents, azathioprine and cyclosporine were used [30,44,48]. The response to immunosuppressive drugs in the majority of patients underlines the importance of detection of anti-OJ in ILD patients.

7. Conclusion

Anti-OJ is a rare but frequently missed myositis-specific autoantibody. The precise target of anti-OJ remains elusive, but the epitope might be dependent on quaternary interactions in the MSC. IP remains the preferred detection method but is difficult to implement in daily practice for many hospitals. The need for practical and reliable immunoassays for detection of anti-OJ is high as the detection of this antibody is relevant for diagnosis and treatment. The performance of available immunoassays, such as LIA or DBA, should improve and the role of novel detection methods, such as PMAT, further explored. Anti-OJ is associated with ASS, with ILD often being the sole manifestation. Myositis, if present, seems to be more severe than in patients with other anti-ARSs. Overlap with or classification as other connective tissue-diseases are possible. While awaiting a widely available and reliable assay, clinicians need to consider anti-OJ in their workup of patients with ILD, be it isolated or combined with severe muscle involvement.

Take home messages

- Detection of anti-OJ autoantibodies lacks standardization due to the miss-understanding of the antigenic target.
- The reactivity of anti-OJ autoantibodies is heterogeneous and targets several components of the MSC.
- Clinically, anti-OJ autoantibody-positive patients have features of ASS, but further studies are required to further define the precise clinical phenotype.

Declaration of interest

XB has been a consultant for Inova Diagnostics. MM and KM are employees of Inova Diagnostics, a company commercializing autoimmune assays.

Acknowledgements

JBV is supported by a Doctoral Grant Strategic Basic Research from the Fonds Wetenschappelijk Onderzoek (Vlaanderen) and a bursary from the Fonds Joël Hurlet. The authors acknowledge the technical assistance of Tomoko Hasegawa and Shin Tanaka (University of Occupational and Environmental Health, Kitakyushu, Japan).This work was supported by JSPS KAKENHI (Grants-in-Aid for Scientific Research, grant number 15K08790) for Dr. Minoru Satoh.

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Establishment of an international autoantibody reference standard for human anti-DFS70 antibodies: proof-of-concept study for a novel Megapool strategy by pooling individual specific sera

https://doi.org/10.1515/cclm-2019-0087
Received January 25, 2019; accepted March 14, 2019

Abstract

Background: International autoantibody standards, traditionally based on material obtained from plasmapheresis of single subjects, represent individual immune response and may not comprehend the heterogeneity of the general population. The anti-DFS70 autoantibody yields a characteristic dense fine speckled (DFS) nuclear pattern on indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA) and speaks against autoimmunity. We propose a novel strategy for developing autoantibody reference standards, based on stepwise pooling of serum samples from hundreds of individuals with anti-DFS70 antibodies.

Methods: Within a 2-year period, serum samples were selected from routine HEp-2 IFA according to the following criteria: DFS HEp-2 IFA pattern at titer ≥1:640; anti-DFS70 reactivity in three analyte-specific tests (Western blot [WB], enzyme-linked immunosorbent assay [ELISA] and chemiluminescent immunoassay [CLIA]). Aliquots of individual samples were combined into progressively larger pools with stepwise validation of intermediary pools as for individual samples. Validated intermediary pools were merged into a final pool for lyophilization.

Results: A total of 741 validated samples yielded a 750 mL final pool that was lyophilized into thousands of 200 µL-aliquots. Reconstituted aliquots yielded the expected anti-DFS70 reactivity in ELISA, CLIA and WB, as well as high-titer DFS HEp-2 IFA pattern. The appropriate anti-DFS70 reactivity of the lyophilized pool was confirmed by seven international expert centers, using HEp-2 IFA, ELISA, WB and immunoprecipitation.

Conclusions: This proof-of-concept study provides an innovative and efficient strategy to build serum reference standards for autoantibody testing. The anti-DFS70 standard will integrate the panel of standards of Autoantibody Standardization Committee (ASC, www.autoab.org), contributing to education for proper assay validation and interpretation of the DFS pattern and other HEp-2 IFA patterns.

Keywords: antinuclear antibodies; autoantibodies; autoimmunity testing; DFS70; HEp-2 cells; reference standards.

List of abbreviations: AC, anti-cell; AC-1, homogeneous nuclear pattern; AC-2, dense fine speckled; AC-3, centromere nuclear pattern; AC-5, coarse speckled nuclear pattern; AC-29, anti-topo I-like pattern; AMA-M2, anti-mitochondrial antibody type 2; ANA, antinuclear antibodies test; Sm, Smith antigen; U1-RNP, U1 small nuclear ribonucleoprotein; ASC, Autoantibody Standardization Committee; AU, arbitrary units; CENP-A, anti-centromere protein A; CENP-B, anti-centromere protein B; CLIA,

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chemiluminescence immunoassay; CONEP, Brazilian Research Ethics Committee; DFS, dense fine speckled; EASI, European Auto antibody Standardization Initiative; ECFSG, European Consensus Finding Study Group; EJ, anti-glycyl-tRNA synthetase; ELISA, enzyme-linked immunosorbent assay; ERM-DA470k/IFCC, certified reference material 470; HEp-2 IFA, indirect immunofluorescence assay on HEp-2 cells; HI, healthy individuals; ICAP, International Consensus on ANA Patterns; IgG, immunoglobulin G; IP-MS, immunoprecipitation and mass spectrometry; IUIS, International Union of Immunology Societies; Jo-1, Histidyl-tRNA synthetase; KS, asparaginyl-tRNA synthetase; Ku, DNA-binding protein (p70/p80); LEDGF, lens epithelium derived growth factor; LKM-1, liver kidney microsome type 1; MDA5, melanoma differentiation-associated gene 5; min, minute; MJ/NXP-2, nuclear matrix protein; MOLT-4, T cell lines (human acute lymphoblastic leukemia); MT-PBS, skim milk and Tween 20 in PBS; OJ, Isoleucyl-tRNA synthetase; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PL-12, alanyl-tRNA synthetase; PL-7, threonyl-tRNA synthetase; PM-Scl, polymyositis-scleroderma; PSG, Plasma Services Group; Rib-P, ribosomal P protein; RNA, ribonucleic acid; SARD, systemic autoimmune rheumatic diseases; Scl-70, 70 kDa scleroderma antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus; SRP, signal recognition particle; SS-A/Ro, Sjögren's syndrome-related antigen A; SS-A/Ro52, SS-A/Ro 52 kDa; SS-A/Ro60, SS-A/Ro 60 kDa; SS-B/La, Sjögren's syndrome-related antigen B; Th/To, RNase P nucleolar protein complex, Rpp 25 subunit; U3-RNP, U3-ribonucleoprotein (fibrillarin); WB, Western blot.

Introduction

Autoantibodies are helpful elements in the diagnosis of systemic autoimmune rheumatic diseases (SARD), establishment of prognosis and monitoring disease activity. The indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA), historically known as the antinuclear antibody (ANA) test, is a standard method for screening antibody against antigens in the nucleus, cytoplasm and mitotic apparatus. HEp-2 IFA allows the identification of numerous morphological patterns that reflect the autoantibody binding to cellular targets in several subcellular domains, thus providing preliminary indication of the autoantibody specificities in the sample being tested. This property of HEp-2 IFA prompted a team of specialists to set up the International Consensus on ANA Patterns initiative (ICAP) [1–4]. ICAP has established a classification algorithm (www.anapatterns.org) comprising 30 relevant HEp-2 IFA patterns, each one receiving an alphanumeric code from AC-0 to AC-29 (AC for anti-cell). AC patterns bear distinctive immunologic and clinical associations [5]. For example, AC-1 (homogeneous nuclear pattern) and AC-5 (coarse speckled nuclear pattern) are correlated with anti-native DNA/nucleosome and with anti-Sm/U1-RNP, respectively, which are valuable biomarkers for systemic lupus erythematosus (SLE) [4, 6]; AC-29 is highly suggestive of anti-topoisomerase 1 [7], which is associated with systemic sclerosis [8, 9].

A rather frequent HEp-2 IFA pattern in the clinical laboratory is AC-2, the dense fine speckled (DFS) nuclear pattern (Figure 1), which is highly correlated with autoantibodies to the 70/75 kDa DFS70 antigen [10, 11], also referred to as lens epithelium derived growth factor (LEDGF) [12, 13]. Anti-DFS70 antibodies and the AC-2 pattern have been observed in healthy individuals (HI) and in patients with miscellaneous chronic inflammatory conditions [10–18]. Intriguingly, mono-specific anti-DFS70 reactivity is frequently observed in HI but rarely in SARD patients [14]. As the AC-2 pattern is characteristically observed when anti-DFS70 is the only antibody in the sample, this HEp-2 IFA pattern is seldomly observed in SARD patients and relatively common in HEp-2 IFA-positive subjects in the general population. Therefore, the isolated presence of anti-DFS70 antibodies (and the resultant AC-2 pattern) has been proposed to represent a strong evidence against

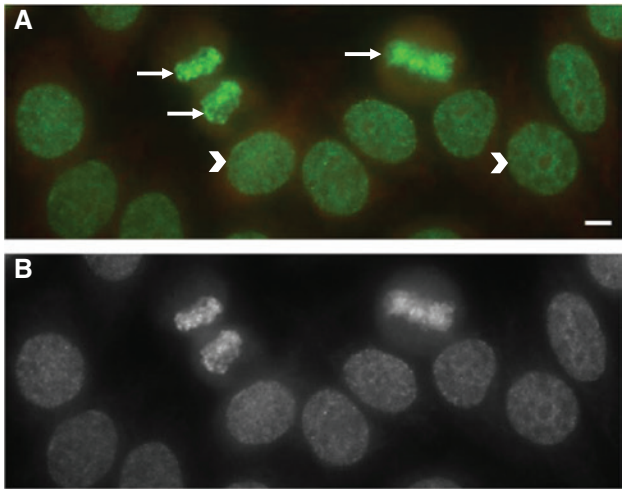

Figure 1: Nuclear dense fine speckled pattern on HEp-2 IFA. HEp-2 slides stained with human serum diluted 1:80 and anti-human IgG goat immunoglobulin labeled with fluorescein isothiocyanate (MBL Bion Enterprise Ltd, Des Plaines, EUA). Full arrows: Mitotic cells; arrowheads: Interphase cells. (A) Color image; (B) black and white image. Scale bars 5 μm.

SARD [10, 11, 19–21]. This contrasts with the consistent association of the Homogeneous (AC-1) [22, 23], Large/Coarse Speckled (AC-5) [22, 23] and Centromere (AC-3) [24] nuclear patterns with SARD, emphasizing that the precise identification of the HEp-2 IFA pattern is relevant to the appropriate clinical interpretation of a positive HEp-2 IFA test as well as to guide further testing for autoantibodies possibly present in the sample.

However, the proper interpretation of HEp-2 IFA patterns may be challenging due to inter-observer variability in skill and heterogenity of HEp-2 slide manufacturer and microscope characteristics. In particular, the topographic features of the AC-2 pattern may eventually lead to misinterpretation [25]. An inexperienced observer may erroneously interpret AC-2 as a homogeneous pattern (AC-1), a regular nuclear fine speckled pattern with stained mitotic chromatin (not defined by ICAP), or a mixed pattern composed of AC-1 and AC-5 patterns. In fact, the distinctive features among these patterns can be subtle, based on the texture and distribution of the staining throughout the nucleus.

Reference standards are crucial to the generation of reliable and interpretable laboratory results, which in turn are vital for accurate patient diagnosis and management [26]. For several HEp-2 IFA patterns with meaningful clinical associations, reference standards have been developed by the Autoantibody Standardization Committee (ASC, www.autoab.org), a subcommittee of the International Union of Immunology Societies (IUIS, http://www.iuisonline.org) Quality Assessment and Standardization Committee [27], and distributed by Plasma Services Group (https://www.plasmaservicesgroup.com/). Most of these international reference standards originated from large plasmapheresis batches from single donors. Standards obtained by the plasmapheresis strategy represent the immune response of single individuals to a particular autoantigen, comprising a collection of polyclonal autoantibodies peculiar to that individual. It can be argued that these plasmapheresis-derived standards do not necessarily reflect the heterogeneity of autoantibodies to that particular antigen present in other patients with the same general autoantibody specificity. In fact, polyclonal antibodies to any particular antigen differ from one individual to another with respect to targeted epitopes, avidity, isotype, etc. This is particularly relevant because the available methods for autoantibody detection may show significantly different inter-patient results depending on the availability and display of epitopes. Therefore, the use of standards based on large batches from a single donor ensures the optimization of kits according to that specific donor's immune response, but that may not be ideal for other individuals [26, 28]. Theoretically, a reference material comprised of well-characterized samples from hundreds of individuals would better represent the variability in the humoral response the relevant antigen.

This study investigates the immunologic characteristics of a reference standard for anti-DFS70 antibodies established by pooling samples from hundreds of individuals with high-titer mono-specific reactivity to DFS70.

Materials and methods

Samples

From March/2013 to July/2015, serum samples from individuals with high-titer characteristic AC-2 pattern were selected among routine samples from a large clinical laboratory in São Paulo, Brazil. Sample selection criteria included: HEp-2 IFA titer ≥1:640; sample volume ≥0.8 mL; bona fide AC-2 pattern (Figure 1) confirmed by two experienced and independent technologists; and confirmation of anti-DFS70 antibodies in the three specific tests: Western blot (WB), enzyme-linked immunosorbent assay (ELISA) and chemiluminescence immunoassay (CLIA) [29–31]. Samples were stored at −80 °C.

HEp-2 indirect immunofluorescence assay

Samples were screened by HEp-2 IFA using slides from MBL-Bion Enterprise Ltd. (Des Plaines, IA, USA), according to manufacturer's instructions. Incubation steps were performed at room temperature in a moist chamber. Samples diluted 1:80 in sample buffer were incubated with HEp-2 cell substrate for 30 min. After washing twice in PBS for 10 min, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-human IgG for another 30 min in the dark. After washing twice as before, slides were fitted with buffered glycerol pH 8.5 and cover slips. HEp-2 IFA titer was determined by testing successive 2-fold dilutions of the serum up to endpoint. All slides were analyzed on an Olympus BX50 fluorescence microscope at 400× magnification by two expert observers.

Immunoassays for detection of anti-DFS70 antibodies

Detection and semi-quantitation of anti-DFS70 antibodies were performed using CLIA (QUANTA Flash®, Inova Diagnostics, San Diego, CA, USA) with a cutoff of 20 arbitrary units (AU) and ELISA (Anti-DFS EA-159z-9601-G, Euroimmun Medizinische Labordiagnostika, Luebeck, Germany) with a cutoff of 1.0 AU. We also determined reactivity to DFS70 antigen in WB as reported previously [10]. Briefly, HEp-2 cell whole extracts were separated in 3%–8% NuPAGE® Novex Bis-Tris Gel SDS-PAGE and transferred onto nitrocellulose membrane using the iBlot® 2 Dry Blotting System (Thermo Fisher Scientific Inc., Waltham, MA, USA). All steps were performed at room temperature. Test samples and an anti-DFS70 standard (kindly donated by Professor Carlos Casiano, Loma Linda University, USA) were incubated with nitrocellulose strips at 1:100 dilution in 5% skim milk and 0.05% Tween-20 in

PBS (MT-PBS) for 1 h. After washing in 0.05% Tween PBS, strips were incubated with horseradish peroxidase-labeled goat anti-human IgG (Bio-Rad Laboratories, Hercules, USA) diluted 1:1500 in MT-PBS for 1 h. After washing as before, the strips were incubated with chromogenic solution (6 mg 4-chloro-1-naftol in 2 mL methanol, 10 mL PBS and 20 µL 30% H_2O_2). The reaction was stopped with distilled water once bands became visible.

Preparation and validation of ranked pools

A total of 778 anti-DFS70 sera were validated according to the predefined inclusion criteria: AC-2 pattern ≥1/640; anti-DFS70 reactivity on CLIA and ELISA; clear-cut 75 kDa-band co-migrating with the band elicited by the DFS70 standard. If these samples were pooled together as one standard, it would not be possible to troubleshoot any potential interference from putatively problematic sera. Mixing each serum one to one to observe potential interference would also be technically challenging to analyze due to the sheer number of possible serum combinations. A novel pooling process, provisionally named mega-pooling, was designed with a small aliquot of each sample, obeying a hierarchical strategy based on consecutive pooling of penta-pools (five individual samples), icosa-pools (four penta-pools), centum-pools (three to five icosa-pools) and final pool (Figure 2). Individual samples were ranked according to the intensity of CLIA anti-DFS70 reactivity and the penta-pools were formed with neighboring groups of five samples, as considered by the CLIA intensity ranking. The generated penta-pools were also ranked according to increasing intensity of CLIA anti-DFS70 reactivity. Icosa-pools were formed with four neighboring penta-pools. The same process applied to the ranking of the icosa-pools and the assembly of centum-pools. Each penta-pool, icosa-pool and centum-pool underwent the same validation process applied to the individual samples, and only the validated ones were further used.

Screening for infectious agents and other autoantibodies in the samples

Antibodies against *Treponema pallidum*, hepatitis B virus, hepatitis C virus and HIV, as well as autoantibodies to native DNA, SS-A/Ro, SS-B/La, Sm, U1RNP, Jo-1, Scl-70, smooth muscle, mitochondria, LKM-1, parietal cell, IgG Fc (rheumatoid factor) and cyclic citrullinated peptide were screened accordingly. Samples presenting any reactivity in these tests were discarded prior to inclusion to pooling.

Preparation and validation of the final pool

All validated samples were thawed and carefully mixed. The final pool was centrifuged at 689 *g* for 30 min and filtered consecutively through sterile gauze and through a non-pyrogenic sterile 0.22 µm polystyrene filter system (Corning Inc., NY, USA) in a sterile laminar flow hood. The final filtered pool had the DFS70 reactivity further tested in ELISA and CLIA, as well as by absorption with recombinant DFS70 antigen using the NOVA Lite HEp-2 Select product (Inova Diagnostics).

A 30-mL aliquot of the final filtered and sterile pool was lyophilized at Butantan Institute (São Paulo, Brazil) and 0.5 mL-individual vials were used for preliminary evaluation by four HEp-2 IFA specialists in Sao Paulo, Brazil, Gainesville, USA, Calgary, Canada and Kitakyushu, Japan, respectively. After this preliminary validation step, the whole pool was shipped under dry ice to PSG (Huntington Valley, PA, USA) for lyophilization and preparation of vials

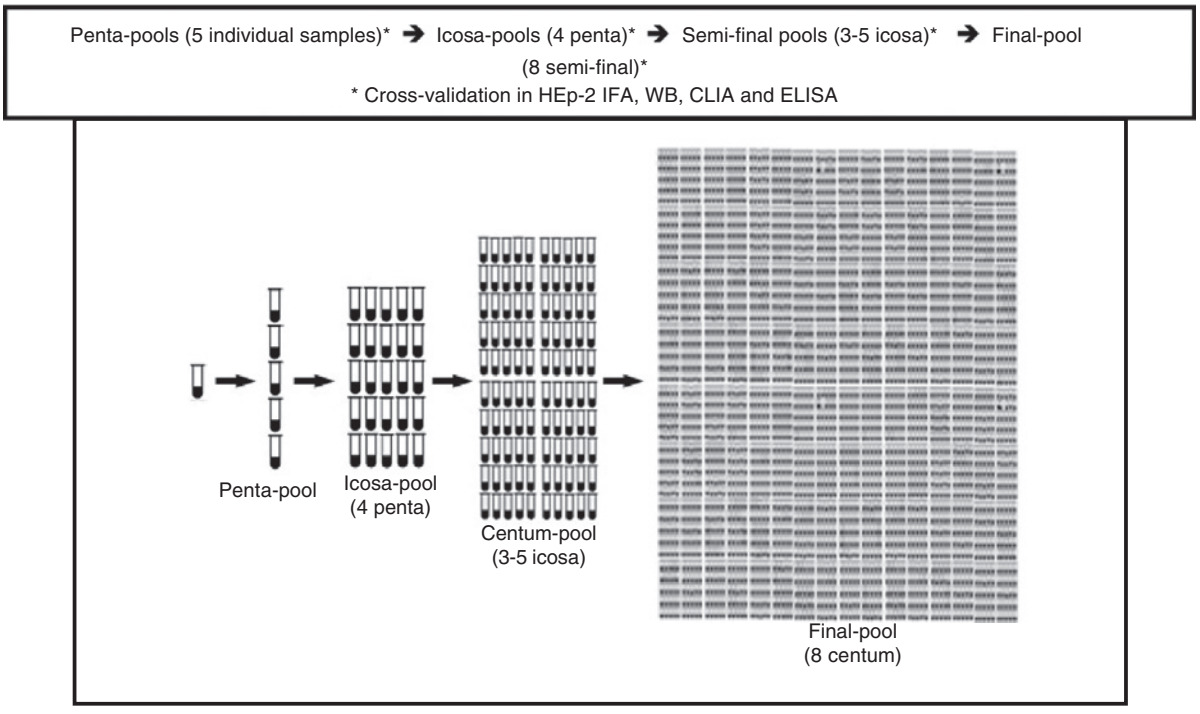

Figure 2: Stepwise production strategy to develop the pooled standard with anti-DFS70-positive samples from hundreds of individuals.

for worldwide distribution. Vials of the lyophilized material were submitted to reference centers across the world (LA, São Paulo; EC, Gainesville; MF, Calgary; FH, Berlin, Germany; MS, Kitakyushu; IGT, Guadalajara, Mexico; MW, Seattle, USA as well as evaluated as an unknown sample within the European Consensus Finding Study Group on autoantibodies [ECFSG]).

All steps of sample selection, immune serological assays and construction of intermediate pools and the final pool were performed at the Research and Development Department of Fleury Medicine and Healthy Laboratory (São Paulo, Brazil) with the approval of the Brazilian Research Ethics Committee (CONEP-http://conselho.saude.gov.br/web_comissoes/conep/index.html) under protocol 69959517.3.0000.5474.

Reactivity of the Megapool standard on HEp-2/DFS70 Knock-out cells

The reconstituted Megapool standard was tested by IFA on HEp-2 ELITE slides containing a 9:1 ratio of DFS70 knock-out and regular HEp-2 cells, respectively, according to the manufacturer's instructions (Immco Diagnostics, Buffalo, NY, USA). In brief, HEp-2 IFA-positive, HEp-2 IFA-negative, and anti-DFS70 positive controls from the manufacturer were stained as recommended, and the Megapool was analyzed at dilutions 1:40, 1:80, and 1:160.

Immunoprecipitation and mass spectrometry (IP-MS) analysis

A 10 µL-aliquot of the Megapool standard was mixed with Protein A Dynabeads (Dynal Biotech Inc., Lake Success, USA) for 10 min at room temperature. These IgG-bound beads were cross-linked by dimethyl pimelimidate dihydrochloride (Sigma) for 30 min at room temperature and then incubated with MOLT-4 whole cell lysate at 4 °C for 1 h. Negative controls included IP reactions without the addition of cell lysate and controls with other unrelated HEp-2 IFA positive standards. IP products without further purification were submitted to direct analysis using Nano-liquid chromatography tandem mass spectrometry (nano-LC/MS/MS) at the University of Florida Mass Spectrometry Research and Education Center.

Results

Samples

Altogether 778 serum samples were initially identified as high-titer bona fide AC-2 pattern and 741 of these samples (95.2%) were approved according the selection criteria resulting in a total volume of 800 mL. The 37 non-approved samples were rejected because of low volume (25 samples), hemolysed or icteric samples (four samples), presence of other autoantibodies (one anti-LKM-one positive and one anti-gastric mucosa-positive sample) and evidence of infectious agents (one HBsAg-positive and five anti-HCV-positive samples). Along centrifugation, filtering and pilot analytic steps, 50 mL of the final pool was used, yielding a 750 mL pool, which was shipped for final lyophilization at PSG.

Anti-DFS70 reactivity of individual samples, hierarchical pools and final pool

All 741 individual samples presented the expected anti-DFS70 reactivity in CLIA (Table 1). Likewise, the 152 generated penta-pools, 38 icosa-pools, eight centum-pools and the final pool also yielded consistent reactivity in anti-DFS70 CLIA and ELISA (Table 1). The final pool also yielded a clear-cut 75 kDa band in WB (Figure 3B) and the typical AC-2 pattern on HEp-2 IFA at 1:1280 dilution according to the blinded analysis by six experienced and independent readers. In addition, the final pool exhibited the expected fading in AC-2 pattern with no additional HEp-2 IFA reactivity after absorption with the recombinant DFS70 antigen using the NOVA Lite HEp-2 Select kit (Inova Diagnostics).

Table 1: ELISA and CLIA anti-DFS70 reactivity along the sequential stages of assembly of the final pool.

	n	Composed by	CLIA (AU) Median (Max and min value)	ELISA (AU) Median (Max and min value)
Individual samples	741	N/A	337.7 (26.1–3547)	N/D
Penta pools	152	5 validated individual samples	420.6 (60.4–3518)	5.5 (2.8–6.8)
Icosa pools	38	4 validated penta pools	407.2 (77.5–1823)	5.5 (3.7–6.7)
Semi-final pools	8	3–5 validated icosa pools	N/D	5.2 (3.8–6.2)
Final pool before lyophilization	1	8 validated semi-final pools	241.2 (221.7–260.7)	7.4 (7.25–7.55)
Final pool reconstituted after lyophilization	1	Total 741 samples	339.2 (329.3–349.0)	4.77 (4.58–4.96)

N, number of samples or pools; N/A, not applicable; N/D, not done.

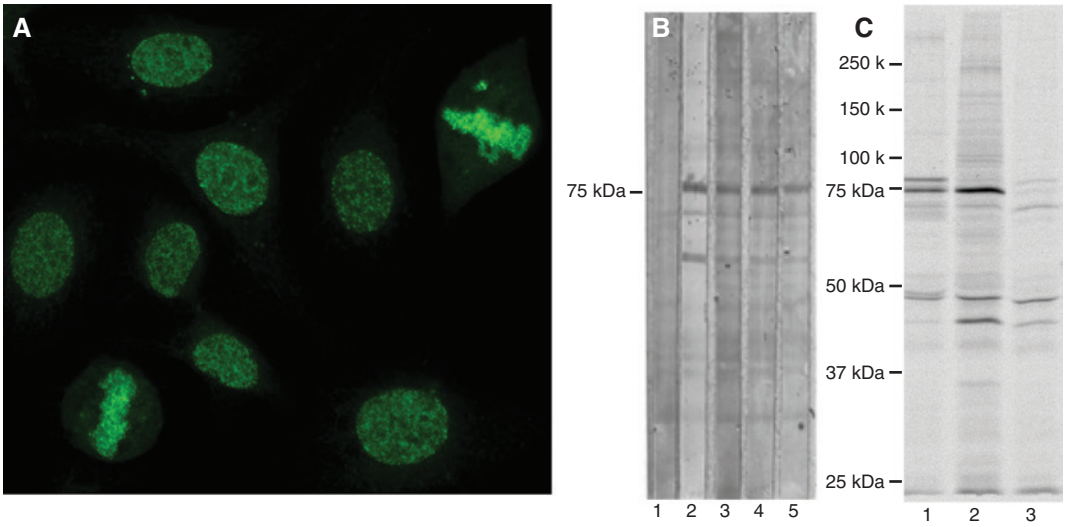

Figure 3: Reactivity of Megapool reference material in three methodological platforms.
(A) Dense fine speckled pattern on HEp-2 IFA with reconstituted lyophilized final pool diluted 1:80 (provided by MS). (B) Western blot with whole HEp-2 cell extract separated in 3%–8% SDS-PAGE and probed with sera diluted in the ratio 1:100. [1] negative sample; [2] positive control; [3] final pool before lyophilization process; [4] final pool lyophilized and reconstituted in distilled water; [5] final pool lyophilized and reconstituted in PBS + 10% FBS; (provided by AD). (C) Autoradiograph of 8.5%–11% gradient SDS-PAGE of 35S-labeled proteins from total HeLa cell extract immunoprecipitated by human serum reactive with [1] anti-DFS70 serum confirmed by ELISA, [2] reconstituted Megapool, [3] normal human serum; (provided by MS).

Validation of lyophilization of the DFS70 Megapool

After reconstitution with distilled water, vials of the DFS70 Megapool aliquot lyophilized at the Butantan Institute yielded the expected anti-DFS70 reactivity when tested by ELISA and CLIA (Table 1), as well as recognized a clear-cut 75 kDa band in WB and yielded the expected AC-2 pattern on HEp-2 IFA at 1:1280 dilution. Aliquots of this preparation were kindly evaluated by the ECFSG in 38 clinical laboratories across Europe, with 32 (84%) reporting the expected AC-2 pattern. Vials with the DFS70 Megapool lyophilized at PSG were evaluated in seven expert laboratories worldwide (one each in Brazil, Canada, Germany, Japan, Mexico, and two in the USA) and all reported the expected AC-2 reactivity in HEp-2 IFA (Figure 3A), WB (Figure 3B), immunoprecipitation (Figure 3C) and in anti-DFS70 CLIA and ELISA.

The Megapool lyophilized at PSG was further validated using commercially available DFS70 knock-out HEp-2 cells (Figure 4). On this substrate, the Megapool had practically identical results, as compared to the anti-DFS70 positive control (Figure 4C), by producing the expected AC-2 pattern on non-knock-out cells and no staining on knock-out cells (Figure 4D–F).

IP-MS analysis identified DFS70, gene name PSIP1, as the 75 kDa antigen recognized by the Megapool sample. Common autoantigens such as SS-A/Ro60, SS-B/La, Sm, U1-RNP, CENP-B, Jo-1, PCNA, PM-Scl, Scl-70, Rib-P, RNA polymerase I/II/III, Th/To, U3-RNP, PL-7, PL-12, EJ, OJ, KS, SRP, Ku, Ki, MJ/NXP-2, RNA helicase A, Replication protein A, mitochondria complex, and SMN complex were not detected in this IP-MS analysis as well as in standard IP. Consistently, the Megapool sample was negative in ELISA for antibodies against U1-RNP, Sm, Scl-70, SS-B/La, SS-A/Ro60, Ro52, CENP-A, CENP-B, MDA5, and Jo-1. PSIP1 was not identified with other unrelated normal human sera (n = 2) and 10 autoantibody positive sera in the IP-MS analysis.

Discussion

Autoantibodies are useful biomarkers for the diagnosis and clinical management of SARD. Therefore, accurate and standardized laboratory tests are needed to provide reliable and useful clinical information. However, uncoordinated actions from academic researchers and industry innovators have generated a plethora of novel immunoassays with differing performance characteristics. This prompted the need for standardization and harmonization of current and novel methods for detection and measurement of autoantibodies. International initiatives, such as the Autoantibody Standardization Committee (ASC) of the IUIS (www.autoab.org), ICAP (www.anapatterns.org),

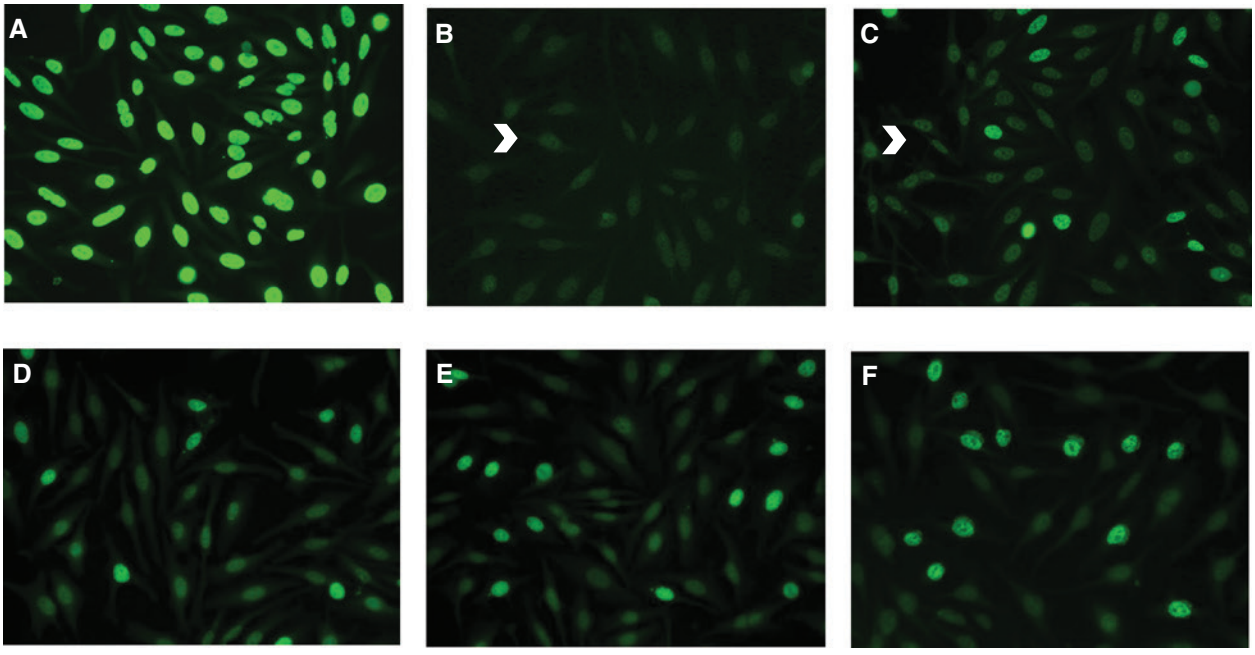

Figure 4: Indirect immunofluorescence assay on DFS70 knock-out HEp-2 cells.
Standard IFA was performed on a mosaic culture containing 10% normal HEp-2 cells and 90% DFS70 knockout HEp-2 cells (Elite DFS70-KO HEp-2 Immco Diagnostics/Trinity Biotech). Serum samples were diluted 1:40 according to manufacturer's instructions, except otherwise stated. (A) positive control (anti-native DNA antibodies), (B) negative control, (C) positive anti-DFS70 serum, (D) DFS70 Megapool 1:40, (E) DFS70 Megapool 1:80, and (F) DFS70 Megapool 1:160.

the European Autoantibody Standardization Initiative (EASI) and the ECFSG, focus on developing guidelines and recommendations for the use and interpretation of immunodiagnostic assays [27, 32–34]. In particular, the ASC has established several reference sera for calibration and harmonization of immunoassays with the goal of improving inter-laboratory harmonization of results [27].

Antibodies have a greater degree of molecular heterogeneity than any other serum protein due to the inherent variability of the antigen-binding site, existence of multiple chain isotypes, extensive variation in avidity and heterogeneous degree of glycosylation [35]. In fact, the humoral immune response comprises a polyclonal population of antibodies directed against different epitopes of the relevant antigens. Different individuals develop different repertoires of antibodies against any given antigen and this applies to the autoantibody response as well. Therefore, it is entirely plausible that the set of autoantibodies against a hypothetical autoantigen X can differ substantially between any two patients with the same autoantibody "specificity". There may be a variable degree of overlap in the panel of anti-X autoantibodies across patients in the general population, but there are likely patients that differ considerably in epitopes targeted by their autoantibodies against the same antigen. This may be even more relevant in diverse ethnic populations.

The time-honored convenient strategy of obtaining large volumes of serum or plasma from a single donor has been used for the establishment of reference materials for several autoantibody specificities related to SARD [27]. Such reference materials have been extensively used to standardize and calibrate immunoassays worldwide. However, considering the above-mentioned heterogeneity in polyclonal antibodies against any given antigen, one concern is that the plasmapheresis-based autoantibody standards derived from single individuals may not be always representative of the general population. An apparently appropriate alternative would be the establishment of standards by building a serum pool from hundreds of different individuals [26, 28]. An autoantibody reference material based on pooled samples with the same antigenic specificity but derived from different individuals should preserve individual immunologic differences, thus representing the diversity across patients worldwide. The use of pooled serum for protein standardization is well established for some certified reference preparations, such as the ERM-DA470k/IFCC human plasma protein standard [36]. However, this material is based on samples from normal donors, whereas samples with autoantibodies usually derive from patients with SARD, who often show high concentrations of rheumatoid factor or immune complexes, which may potentially interfere to

varying degrees in immunoassays. Therefore, any attempt at mixing samples from different patients to build a reference material should be carefully evaluated for the possibility of mutual interference and spurious findings.

Autoantibodies against the DFS70 autoantigen were originally identified in the 1990s in patients with interstitial cystitis [12] and subsequently were shown to be present in a host of clinical conditions [10, 13–18]. Eventually, it was demonstrated that anti-DFS70 antibodies and the DFS pattern are relatively prevalent in the general population with as high as 4%–5% frequency reported in some studies [11, 16, 19, 20]. It has been generally agreed that high-titer HEp-2 IFA results caused by anti-DFS70 should not be considered as a factor in favor of a SARD diagnosis [11, 21]. This scenario indicates that the prompt recognition of the DFS70-associated HEp-2 IFA pattern (AC-2) is very important. However, the proper recognition of AC-2 pattern is not trivial and requires considerable expertise [25]. The ICAP team provides information and representative images on the www.anapatterns.org website. In addition, a mono-specific anti-DFS70 reference material for onsite processing of HEp-2 slides would be helpful in improving the expertise regarding recognition of the AC-2 pattern. As with any other HEp-2 IFA pattern, the anti-DFS70 reactivity in samples yielding the AC-2 pattern needs to be confirmed in follow-up specific immunoassays. To fulfill this demand, some kit manufacturers have developed solid phase immunoassays that are now commercially available. However, the concordance rate among the several specific immunoassays for anti-DFS70 antibodies is not optimal [37]. The availability of an international reference standard for anti-DFS70 antibodies would be highly useful for the calibration and harmonization of such assays.

Taking into consideration that the bona fide AC-2 pattern is usually elicited by mono-specific anti-DFS70 samples from apparently normal individuals [10, 11], we reasoned that this would be an appropriate opportunity to develop and test a multiple-sample pooling strategy as an approach to developing more reliable and representative autoantibody reference materials. Accordingly, we established a careful stepwise strategy in which small pools were successfully tested and merged into progressively larger pools that were themselves tested. In fact, with a few minor exceptions the vast majority of intermediate pools behaved as expected.

The anti-DFS70 antibody system has several peculiarities that may have contributed to the favorable results obtained using this mega-pooling strategy. First, high-titer mono-specific anti-DFS70 samples produce a characteristic and distinctive DFS nuclear HEp-2 IFA pattern. This property provided a simple and effective method for screening thousands of samples of interest using the routine HEp-2 IFA. Any serum that had other antinuclear or anti-cytoplasmic antibodies was excluded after the initial screening step. Second, as isolated anti-DFS70 antibodies occur predominantly in HI, the selected samples had low probability of containing elements that might interfere with anti-DFS70 antibodies present in the pool, such as rheumatoid factor, immune complexes and high immunoglobulin concentration.

One outstanding finding in the present study was the extremely strong association between AC-2 pattern and anti-DFS70 antibodies, as all samples selected at the HEp-2 IFA screening stage by presenting the characteristic AC-2 pattern yielded the expected anti-DFS70 reactivity in specific immunoassays. However, as pointed out before, HEp-2 IFA pattern interpretation is challenging and subjective. Therefore, similar to other HEp-2 IFA patterns, the definition of anti-DFS70 antibodies cannot rely solely on the identification of the AC-2 pattern, but must be confirmed by specific anti-DFS70 immunoassays.

The reference material produced by pooling serum samples from hundreds of individuals with high-titer mono-specific anti-DFS70 reactivity resulted in the production of a large volume of human serum reference material with strong and apparently 'mono-specific' anti-DFS70 reactivity in different immunoassays, including WB, immunoprecipitation, ELISA and CLIA, as well as the expected AC-2 pattern in the HEp-2 IFA test using kits from several manufacturers. This material has been lyophilized and stored for global distribution as 0.2 mL aliquots. The reconstituted material reproduced the anti-DFS70 reactivity profile of the original material in seven expert laboratories across the world. In addition, the HEp-2 IFA pattern elicited by this reagent was correctly assigned as AC-2 by 84% of 38 laboratories participating in a European external quality assessment program, which is very acceptable as ICAP nomenclature had been introduced the year before. One limitation of the study is that all samples were retrieved from a single country – Brazil. However, given the highly heterogeneous ethnical composition of the Brazilian population [38, 39] and the high number of individuals sampled, this first proof-of-concept study offers a reasonable proxy to future approaches based on samples evenly retrieved from several regions in the world.

This novel reference material should contribute to continuing education for proper interpretation of the AC-2 pattern, as well as to the harmonization and calibration of specific anti-DFS70 immunoassays, such as

ELISA, CLIA and WB. This novel autoantibody standard will add to the panel of autoantibody standards of the IUIS Autoantibody Standardization Committee. Aliquots of the anti-DFS70 reference material will be made available for free distribution (subjected to shipping charges) to qualified academic or commercial clinical laboratories through PSG (https://www.plasmaservicesgroup.com) as catalogue number IS2726.

Acknowledgments: The authors are deeply indebted to Vitor Anselmo Sakihara and Paulo Ho, from Butantan Institute, São Paulo, Brazil for lyophilizing the preliminary serum pools; Inova Diagnostics, Inc, San Diego, CA, and Euroimmun Medical Laboratory Diagnostics AG, Lübeck, Germany, for providing the anti-DFS70 ELISA and CLIA kits, respectively; Trinity Biotech, Jamestown, NY, for providing the HEp-2 ELITE slides; Kathryn Kohl, at the Plasma Service Group, for preparing the lyophilized aliquots of the Megapool; Kari B. Basso at the University of Florida Mass Spectrometry Research and Education Center supported by NIH S10 OD021758-01A1; Tomoko Hasegawa and Shin Tanaka from the Department of Clinical Nursing, University of Occupational and Environmental Health, Kitakyushu, Japan.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: Fleury Medicine and Health Laboratory provided the material support for the selection and testing of all samples (NP-084). Luis Eduardo C. Andrade receives a research grant (#232711/2014-3) from the Brazilian Research Agency CNPq.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication. MJF is a consultant or received honoraria form Inova Diagnostics, Euroimmun GmbH and Alexion Canada.

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Internal Medicine
The Japanese Society of Internal Medicine
doi: 10.2169/internalmedicine.1516-18
Intern Med 58: 837-841, 2019
http://internmed.jp

【 CASE REPORT 】

Clinical Features of Anti-MDA5 Antibody-positive Rapidly Progressive Interstitial Lung Disease without Signs of Dermatomyositis

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Abstract:

Anti-melanoma differentiation-associated gene 5 (anti-MDA5) antibody is associated with rapidly progressive interstitial lung disease (RP-ILD) in patients with clinically amyopathic dermatomyositis (CADM) or dermatomyositis (DM). We herein report three Japanese cases of anti-MDA5 antibody-positive RP-ILD without signs of CADM or DM. High-resolution computed tomography revealed patchy or subpleural distribution of consolidations and/or ground-glass opacities accompanied by traction bronchiectasis. All patients succumbed to respiratory failure within two months. Anti-MDA5 antibody-positive RP-ILD without signs of CADM or DM should be included in the differential diagnosis of acute/subacute ILD. Measurement of anti-MDA5 antibody and an intensive immunosuppressive regimen might rescue these patients from RP-ILD.

Key words: acute interstitial pneumonia, anti-melanoma differentiation-associated gene 5, clinically amyopathic dermatomyositis

(Intern Med 58: 837-841, 2019)
(DOI: 10.2169/internalmedicine.1516-18)

Introduction

Clinically amyopathic dermatomyositis (CADM), defined as the presence of cutaneous features of dermatomyositis (DM) without clinical muscle weakness, may be complicated by life-threatening rapidly progressive interstitial lung disease (RP-ILD) (1). The anti-melanoma differentiation-associated gene 5 (anti-MDA5) antibody, also known as anti-CADM140 antibody, is associated with RP-ILD in patients with CADM or DM (2). We herein report three Japanese cases of anti-MDA5 antibody-positive RP-ILD without signs of CADM or DM.

Case Reports

Case 1

A 72-year-old woman visited our hospital complaining of general fatigue. She had undergone surgery for left-sided breast cancer two years earlier and subsequent hormonal treatment with letrozole before this admission. Lung auscultation on admission revealed normal vesicular sounds in both lungs and no signs of DM or CADM in the skin or muscle. Laboratory investigations revealed an increased Krebs von den Lungen-6 level and a normal creatine kinase level (Table). High-resolution computed tomography (HRCT) of the chest on admission showed a patchy distribu-

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Received: May 11, 2018; Accepted: September 9, 2018; Advance Publication by J-STAGE: November 19, 2018
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Table. Characteristics of Patients with Interstitial Lung Disease with Anti-MDA5 Antibody.

Patient number	1	2	3
Gender	Female	Female	Male
Age (years)	72	68	70
Smoking	Ne	Ne	Ex
Dust exposure	-	-	+
Complications	HT	HT, Comlete AV block	HT, Dyslipidemia
Month of onset	October	July	May
Malignancy	Breast cancer	-	Prostate cancer
Laboratory data			
CK (IU/L)	183	140	105
Aldolase (U/L)	NA	5.3	NA
Ferritin (ng/dL)	1,486	235	1,428
ANA	-	×80 (S)	×40 (H, S)
SP-D (ng/mL)	40.9	320.0	55.7
KL-6 (U/mL)	858	2,330	526
Pulmonary function test	NA	NA	NA
Bronchoalveolar lavage fluid findings			
Total cell counts ($\times 10^5$/mL)	5.7	NA	0.6
Macrophages (%)	83.9	NA	81.3
Lymphocytes (%)	15.2	NA	15.1
Neutrophils (%)	0.9	NA	0.8
Eosinophils (%)	0.0	NA	2.3
CD4/CD8 ratio	1.00	NA	1.69
Treatment	mPSL, PSL, IVCY, CyA	mPSL, PSL, IVCY, TAC	mPSL, PSL, IVCY
Pneumomediastinum	-	+	+
Outcome	death	death	death
	42 days	27 days	44 days
Anti-MDA5 antibody index	>150	>150	>150

M: male, F: female, Ne: never-smoker, Ex: ex-smoker, HT: hypertension, AV: atrioventricular block, CK: creatine kinase, NA: not assessed, ANA: anti-nuclear antibody, SP: surfactant protein, KL: Krebs von den Lungen, S: speckled, H: homogeneous, mPSL: methyl prednisolone pulse therapy, PSL: prednisolone, CyA: cyclosporine, TAC: taclorimus, IVCY: intravenous cyclophosphamide

tion of consolidations accompanied by traction bronchiectasis (Figure). An analysis of the bronchoalveolar lavage fluid revealed increased total cell counts with a slightly increased proportion of lymphocytes (Table). Transbronchial lung biopsy specimens revealed organizing inflammation accompanied by fibrin deposition, suggesting acute lung injury. Methylprednisolone pulse therapy followed by oral prednisolone and subsequent treatment with oral cyclosporine, intravenous cyclophosphamide, and invasive positive pressure ventilation did not improve the patient's status. She died of respiratory failure 42 days after admission. After her death, anti-MDA5 antibody in serum obtained at 35 days after admission was found to be positive.

Case 2

A 68-year-old woman was referred to our hospital because of deterioration of dyspnea and abnormal shadows on a chest radiograph. She had been treated previously for third-degree atrioventricular block and had undergone surgery for aortic dissection. Lung auscultation on admission revealed fine crackles in both lungs but no signs suggestive of DM or CADM. Laboratory investigations revealed slightly increased Krebs von den Lungen-6 and ferritin levels and a normal creatine kinase level (Table). HRCT of the chest on admission showed peripleural ground-glass opacity (GGO) and consolidations accompanied by traction bronchiectasis (Figure). Anti-MDA5 antibody in serum obtained on admission was positive. Methylprednisolone pulse therapy followed by treatment with oral prednisolone, oral tacrolimus, and intravenous cyclophosphamide supported by high-flow nasal oxygen did not improve the patient's status, and she died of respiratory failure 27 days after admission.

Case 3

A 70-year-old man visited our hospital complaining of deterioration of dyspnea. He worked as an automobile mechanic and had been receiving treatment with enzalutamide for prostate cancer immediately before this admission. Lung auscultation on admission revealed fine crackles in both lungs but no signs suggestive of DM or CADM. Laboratory investigations revealed increased Krebs von den Lungen-6 and ferritin levels and a normal creatine kinase level (Table). HRCT of the chest on admission showed peripleural GGO and consolidations that were accompanied by traction bronchiectasis (Figure). An analysis of the bronchoalveolar lavage fluid revealed slightly increased proportions of lymphocytes and neutrophils (Table). Transbronchial lung biopsy specimens did not suggest a specific disease. The patient did

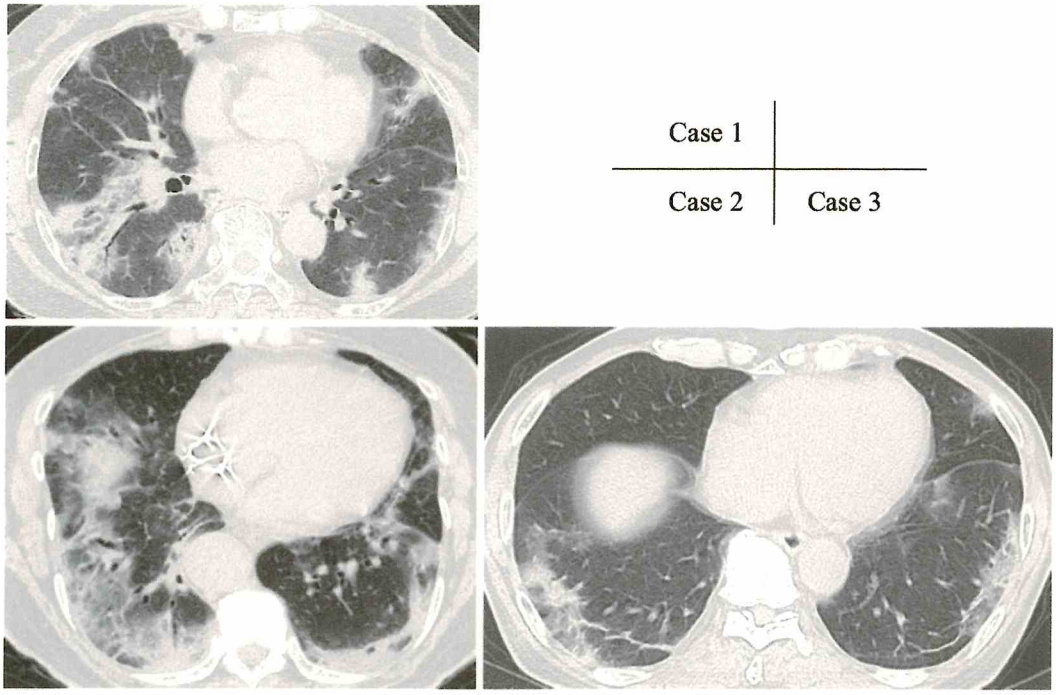

Figure. Findings on high-resolution computed tomography of the chest at the time of admission. Patchy distribution of areas of consolidation accompanied by traction bronchiectasis (case 1). Peripleural ground-glass opacity and areas of consolidation accompanied by traction bronchiectasis (case 2). Peripleural ground-glass opacity and areas of consolidation (case 3).

not improve on methylprednisolone pulse therapy followed by treatment with oral prednisolone and intravenous cyclophosphamide, and he died of respiratory failure 44 days after admission. After the patient's death, anti-MDA5 antibody in serum obtained 35 days after admission was found to be positive.

Discussion

We have reported three cases of anti-MDA5 antibody-positive RP-ILD without signs of DM or CADM. Anti-MDA5 antibody is a myositis-specific autoantibody that is specific for CADM and DM and is associated with RP-ILD in patients with CADM or DM but not in those with idiopathic interstitial pneumonias (IIPs) (2, 3). However, it was reported that ILD preceded skin and muscle symptoms in 2 of 43 patients with anti-CADM-140 antibody-positive CADM and DM (4). There have also been case reports of RP-ILD with anti-CADM-140/MDA5 antibody-positive CADM preceding cutaneous symptoms (5, 6). Consistent with those reports, the patients described in the present report might have had RP-ILD that preceded CADM. There has been a recent report of RP-ILD without skin involvement (7). That case and our experience in the present three cases indicate that patients who are anti-MDA5 antibody-positive might be considered to have an IIP, such as acute interstitial pneumonia (AIP). AIP is a major IIP and is characterized by rapidly progressive hypoxemia with a mortality rate exceeding 50% (8). There is still no proven treatment for AIP. Biopsy specimens in patients with AIP show an acute and/or organizing form of diffuse alveolar damage that is typically seen in patients with RP-ILD of the anti-MDA5 antibody-positive CADM type (9).

It has been reported that GGO/consolidation in a subpleural, lower, or random distribution is a common HRCT finding in patients with anti-MDA5 antibody-positive dermatomyositis, whereas a peribronchovascular distribution and intralobular reticular opacities are significant in those with anti-MDA5 antibody-negative dermatomyositis (10, 11). However, no marked difference in the loss of lung volume or presence of traction bronchiectasis was found between these two groups of patients (11). Pneumomediastinum has also been reported to be more common in patients with anti-MDA5 antibody-positive DM than in anti-MDA5 antibody-negative DM (12). Our three cases had features of anti-MDA5 antibody-positive dermatomyositis on HRCT, suggesting an association between the anti-MDA5 antibody and HRCT findings, regardless of the skin involvement in these cases.

All three patients in the present series died within two months of admission. In previous reports, all deaths in patients with anti-MDA5 antibody-positive DM occurred within the first six months (12, 13). These reports are consistent with our own experience. The anti-MDA5 antibody titers in patients with RP-ILD and DM have been reported to be lower before treatment and to decline significantly more in survivors than in non-survivors after treatment (13, 14). Anti-MDA5 antibodies were measured about 1 month after treatment in 2 of our 3 patients, both of whom had a titer of ≥150. The serum ferritin levels are re-

ported to be higher in anti-MDA5-positive patients with ILD and DM than in anti-MDA5 antibody-negative ILD with DM as well as higher in non-survivors than in survivors of anti-MDA5-positive ILD with DM (12, 13). In addition, pneumomediastinum has been reported to be more common in anti-MDA5-positive patients with ILD and DM than in anti-MDA5 antibody-negative patients with ILD and DM (12, 15). These reports indicate that our patients who all had a high anti-MDA5 antibody titer, a high ferritin level, and the complication of pneumomediastinum had either RP-ILD preceding CADM or a type of RP-ILD without CADM.

An intensive immunosuppressive regimen of high-dose glucocorticoids, oral cyclosporine, and intravenous cyclophosphamide pulse therapy is reported to be effective (16, 17), and additional immunosuppressive therapy, such as tofacitinib and rituximab, was recently identified as being potentially useful for treating refractory anti-MDA5 antibody-positive ILD accompanied by DM (18, 19). Two of our patients (cases 1 and 3) were treated initially with high-dose glucocorticoids and subsequently with oral calcineurin inhibitors (cyclosporine/tacrolimus) and/or intravenous cyclophosphamide pulse therapy two weeks after the first treatment. The early measurement of the anti-MDA5 antibody should be considered in patients who are found to have GGO/consolidation in a subpleural, lower, or random distribution on HRCT. Although the efficacy of these therapies should be confirmed in prospective trials, the early detection of the anti-MDA5 antibody in patients with an AIP-like presentation might guide the use of these intensive immunosuppressive regimens and improve the prognosis.

Signs of skin involvement from dermatomyositis were sought by experienced pulmonologists in the present cases (except in case 2). However, slight skin involvement from dermatomyositis might be missed when the skin examination is performed by pulmonologists who are not experienced in checking for dermatomyositis.

We herein report three Japanese patients with anti-MDA5 antibody-positive RP-ILD without signs of CADM or DM. Anti-MDA5 antibody-positive RP-ILD without CADM or DM should be included in the differential diagnosis of acute/subacute ILD. The measurement of the anti-MDA5 antibody level and an intensive immunosuppressive regimen might rescue these patients from RP-ILD.

The authors state that they have no Conflict of Interest (COI).

Financial Support

This work was supported by JSPS KAKENHI JP17K09617 (to HM) and JP15K08790 (to MS) and the Non-Profit Organization Aimed to Support Community Medicine Research in Nagasaki (to NS).

Acknowledgement

We thank Dr. Tomohiro Koga (a rheumatologist) and Dr. Yuta Koike (a dermatologist) for the evaluation of the skin involvement in case 2.

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Clinical relevance of HEp-2 indirect immunofluorescent patterns: the International Consensus on ANA patterns (ICAP) perspective

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Handling editor Josef S Smolen

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2018-214436).

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Received 13 September 2018
Accepted 23 January 2019
Published Online First
12 March 2019

To cite: Damoiseaux J, Andrade LEC, Carballo OG, *et al*. *Ann Rheum Dis* 2019;**78**:879–889.

ABSTRACT

The indirect immunofluorescence assay (IIFA) on HEp-2 cells is widely used for detection of antinuclear antibodies (ANA). The dichotomous outcome, negative or positive, is integrated in diagnostic and classification criteria for several systemic autoimmune diseases. However, the HEp-2 IIFA test has much more to offer: besides the titre or fluorescence intensity, it also provides fluorescence pattern(s). The latter include the nucleus and the cytoplasm of interphase cells as well as patterns associated with mitotic cells. The International Consensus on ANA Patterns (ICAP) initiative has previously reached consensus on the nomenclature and definitions of HEp-2 IIFA patterns. In the current paper, the ICAP consensus is presented on the clinical relevance of the 29 distinct HEp-2 IIFA patterns. This clinical relevance is primarily defined within the context of the suspected disease and includes recommendations for follow-up testing. The discussion includes how this information may benefit the clinicians in daily practice and how the knowledge can be used to further improve diagnostic and classification criteria.

INTRODUCTION

Autoantibodies, as detected by the indirect immunofluorescence assay (IIFA) on HEp-2 cells (IIFA HEp-2), are recognised as important diagnostic markers in a plethora of autoimmune diseases, in particular the systemic autoimmune rheumatic diseases (SARD).[1] Although somewhat dated by today's standards, members of the American College of Rheumatology (ACR) prepared an evidence-based guideline for the usefulness of the HEp-2 IIFA results for diagnostic and prognostic purposes and also for meeting diagnostic criteria.[2] That guideline was based on reactivity with nuclear antigens as detected by IIFA on rodent tissue or HEp-2 cells. More recently, the IIFA on HEp-2 cells was reinforced as the gold standard for autoantibody screening in SARD.[3]

Interestingly, the HEp-2 IIFA test reveals much more information than the mere absence or presence of autoantibodies, that is, the level of antibody as well as the HEp-2 IIFA pattern. Based on titration or appropriate evaluation of the fluorescence intensity, the antibody level can be determined and this information has general concordance with the clinical relevance of the test result. Indeed, higher antibody levels are better associated with SARD and have an increased likelihood to identify the autoantigen in follow-up testing.[4–6] The importance of the level of autoantibodies is also recognised in the ACR guideline as well as by the recommendations issued by the European Autoimmunity Standardization Initiative (EASI) and the International Union of Immunologic Societies (IUIS) Autoantibody Standardization Subcommittee.[2 7]

The HEp-2 IIFA pattern may also reveal clinically relevant information. This information is not restricted to giving direction to follow-up testing for antigen-specificity, but, for instance, the centromere pattern is included in the classification criteria for systemic sclerosis,[8] while the nuclear dense fine speckled pattern is reported to be more prevalent in apparently healthy individuals as compared with patients with SARD.[9] To harmonise the names and descriptions of the distinct HEp-2 IIFA patterns, an ordered classification taxonomy was proposed.[10] This proposal was subsequently elaborated on by the International Consensus on ANA Patterns (ICAP), initiated in parallel to the 12th International Workshop on Autoantibodies and Autoimmunity (2014) held in Sao Paulo, Brazil. During this workshop, a consensus was reached on the nomenclature and definitions of 28 HEp-2 IIFA patterns. Each HEp-2 IIFA pattern was ascribed an alphanumeric code from AC-1 to AC-28.[11] The consensus nomenclature for each pattern and representative images were also made available online at the ICAP website (http://www.ANApatterns.org).

In addition to the nuclear patterns, important cytoplasmic and mitotic patterns may also be observed in HEp-2 IIFA analysis. Although reporting non-nuclear patterns is considered clinically relevant,[7] for various jurisdictional reasons there is no clear-cut consensus viewpoint on reporting non-nuclear patterns as a negative or positive test.[12] With the understanding that the term 'Antinuclear antibody (ANA) test' may be inappropriate to designate a test that also addresses autoantibodies to antigens in the cytoplasm and mitotic apparatus, an alternative name, anticellular antibodies, was suggested in the EASI/IUIS recommendations.[7] Recent publications from ICAP have preferred the term HEp-2 IIFA as it covers the

whole spectrum of patterns that can be observed when using the HEp-2 cells as substrate.[13 14]

Originally, the HEp-2 IIFA patterns were associated with diseases, but it was anticipated that many of these associations are only valid if the antigen-specificity was confirmed by follow-up testing. In subsequent ICAP workshops, it was agreed that the disease associations should be replaced by clinical relevance. In this current paper, we present the consensus on the clinical relevance of the distinct HEp-2 IIFA patterns as achieved by consecutive workshops and discussions among the executive ICAP members.

MATERIALS AND METHODS

For discussion about the structure of clinical relevance templates were prepared for AC-2 (LECA), AC-3 (JD) and AC-5 (MS). This formed the basis of a guideline for description of each AC pattern (EC). Of highest importance, it was agreed that the information should be objective and helpful for the clinician, the pattern–antigen associations should be put in the right clinical context and information should be evidence-based.

In preparation for the third ICAP workshop in Kyoto (2016), composition of the clinical relevance documents was started for the nuclear patterns (JD, LECA, MS), cytoplasmic patterns (CAvM, EKLC) and mitotic patterns (MH, TM). As far as already available, the documents were commented on by the ICAP executive board and, after appropriate adjustment, discussed with the workshop participants. The feedback from participants mainly focused on the structure of the information provided, on the required level of detail and the format of recommended follow-up testing.

In anticipation of the fourth ICAP workshop in Dresden (2017), the set of clinical relevance documents was completed for all patterns. Further comments from the ICAP executive board were included. The resulting documents were individually discussed with the workshop participants for nuclear (JD), cytoplasmic (CAvM) and mitotic (MH) patterns. Besides several substantive comments, there was general agreement that the information should be provided in tabular format at two distinct levels. The first level should contain information on relevant follow-up testing in the respective clinical context, the recommended follow-up tests should be commercially available and detailed test characteristics should not be given because of potential geographic and jurisdictional differences. Information based on case reports or small patient cohorts, as well as information on possible follow-up testing that is only available in specialised research laboratories, should only be provided in the second level information.

Tables for nuclear, cytoplasmic and mitotic patterns were prepared for first and second level information (JD). These tables were commented by the ICAP executive board and finalised by JD. Of note, since the starting point of the tables on clinical relevance is the HEp-2 IIFA pattern and not the clinically suspected disease, the tables do not list all autoantibodies related to the respective disease.

RESULTS

Nuclear HEp-2 IIFA patterns

To date, a total of 15 nuclear HEp-2 IIFA patterns have been described, that is, AC-1–AC-14 and AC-29. Table 1 summarises the clinical relevance of these patterns.[8 9 14–79] Since AC-29 was only recently described,[14] the advice for follow-up testing for autoantibodies to topoisomerase I (Scl-70) in case of clinical suspicion of systemic sclerosis is also added as a note to the clinical relevance of AC-1. In particular, disease-specific immunoassays, like autoimmune liver disease profile, inflammatory myopathy profile, systemic sclerosis profile, are often only available in specialty clinical laboratories.

For six nuclear HEp-2 IIFA patterns (AC-3, 5, 7, 8, 12 and 13), additional information about clinical relevance is summarised in online supplementary table S1. Although some assays for anti-CENP-A antibodies are commercially available, these antibodies are included in online supplementary table S1 because the majority of sera revealing the AC-3 pattern are also reactive with CENP-B. In contrast to CENP-A, CENP-B is included in many routine extractable nuclear antigens profiles.

Cytoplasmic HEp-2 IIFA patterns

Table 2 summarises the clinical relevance of the nine cytoplasmic HEp-2 IIFA patterns, that is, AC-15–AC-23.[26 33 80–101] It is recognised that the distinction between AC-19 (dense fine speckled) and AC-20 (fine speckled) can be challenging. Moreover, within the spectrum of anti-tRNA synthetase antibodies, not all produce an HEp-2 IIFA pattern and only some anti-Jo-1 antibodies are considered to give the AC-20 pattern, while the other anti-tRNA synthetase antibodies (EJ, KS, OJ, PL-7 and PL-12) are more likely to reveal the AC-19 pattern. Solid information on the pattern of two additional anti-tRNA synthetase antibodies (Ha and Zo) is lacking. Overall, the relation between these two cytoplasmic HEp-2 IIFA patterns and the distinct anti-tRNA synthetase antibodies is subject to further discussion. In clinical practice, the complete spectrum of the anti-tRNA synthetase antibodies should be determined irrespective of the subtype of cytoplasmic speckled pattern, that is, AC-19 or AC-20.

For seven cytoplasmic HEp-2 IIFA patterns (AC-15–AC-19, AC-22 and AC-23), more detailed information is provided in online supplementary table S2. In particular, for AC-16–AC-18, the clinical associations are quite diverse, depending on the antigen recognised. Overall, the clinical associations provided are primarily based on antigen-specific immunoassays and not on the HEp-2 IIFA pattern as such.

Mitotic HEp-2 IIFA patterns

The clinical relevance of the five mitotic patterns is summarised in table 3,[102–122] with more detailed information in online supplementary table S3. As for the cytoplasmic patterns, clinical associations for the mitotic patterns are primarily based on antigen-specific immunoassays and not on the HEp-2 IIFA pattern as such.

DISCUSSION

In the current paper, we present the ICAP consensus on the clinical relevance of 29 HEp-2 IIFA patterns defined by ICAP.[11 14] The consensus on clinical relevance is defined in the clinical context of the patient, that is, suspected disease, and includes recommended follow-up testing within the spectrum of antigen-specificities that are commercially available. Obviously, if follow-up testing identifies the antigen, the clinical relevance can be further refined.[123]

Defining the clinical relevance of HEp-2 IIFA patterns in the context of disease manifestations is meant to be an important tool for the clinician in the diagnostic work-up of patients suspected of SARD. Unfortunately, good data on the association between HEp-2 IIFA patterns and the distinct diseases are lacking, probably due to reasons summarised below. There are several reasons for not finding a perfect association between HEp-2 IIFA patterns and diseases. First, pattern assignment in

Table 1 Nuclear HEp-2 IIFA patterns

Code	AC pattern—clinical relevance	Refs
AC-1	HOMOGENEOUS	
	► Found in patients with SLE, chronic autoimmune hepatitis or juvenile idiopathic arthritis	
	► If SLE is clinically suspected, it is recommended to perform a follow-up test for anti-dsDNA antibodies, alone or in combination with dsDNA/histone complexes (nucleosomes/chromatin); anti-dsDNA antibodies are included in the classification criteria for SLE	15, 16
	► If chronic autoimmune hepatitis or juvenile idiopathic arthritis is suspected, follow-up testing is not recommended because the respective autoantigens revealing the AC-1 pattern are not completely defined	17
	Notes: Although autoantibodies to Topoisomerase I (formerly Scl-70) may be reported as nuclear homogeneous, they typically reveal a composite AC-29 HEp-2 IIFA pattern; as such, clinical suspicion of SSc may warrant follow-up testing for reactivity to this antigen.	14, 18
	Although AC-1 is the most prevalent pattern in chronic autoimmune hepatitis, other HEp-2 IIFA patterns may occur, but also for these patterns the autoantigens are not completely defined.	19
AC-2	DENSE FINE SPECKLED	
	► Commonly found as high titer HEp-2 IIFA-positive in apparently healthy individuals or in patients who do not have a systemic autoimmune rheumatic disease (SARD)	9
	► The negative association with SARD is only valid if the autoreactivity is confirmed as being directed to DFS70 (also known as LEDGF/p75) and if no other common ENA is recognized	20, 21
	► Both in apparently healthy individuals as well as patients who do not have a SARD the AC-2 pattern may be caused by autoantibodies to other antigens than DFS70	22
	Note: Confirmatory assays for anti-DFS70 antibodies may be available only in specialty clinical laboratories.	
AC-3	CENTROMERE (see online supplementary table S1 for further details	
	► Commonly found in patients with limited cutaneous SSc, and as such included in the classification criteria for SSc	8, 15, 23
	► In combination with Raynaud phenomenon, the AC-3 pattern is prognostic for onset of limited cutaneous SSc	15, 23
	► Strongly associated with antibodies to CENP-B; especially in case of low titers, confirmation by an antigen-specific immunoassay is recommended to support the association with limited cutaneous SSc; the CENP-B antigen is included in many routine ENA profiles	15
	► The AC-3 pattern is also apparent in a subset of patients with PBC; these patients often have both SSc as well as PBC	15
AC-4	FINE SPECKLED	
	► Present to a varying degree in distinct SARD, in particular SjS, SLE, subacute cutaneous lupus erythematosus, neonatal lupus erythematosus, congenital heart block, DM, SSc, and SSc-AIM overlap syndrome	15
	► If SjS, SLE, subacute cutaneous lupus erythematosus, neonatal lupus erythymatosus, or congenital heart block is clinically suspected, it is recommended to perform follow-up tests for anti-SS-A/Ro (Ro60) and anti-SS-B/La antibodies; in most laboratories these antigens are included in the routine ENA profile	15
	► Autoantibodies to SS-A/Ro are part of the classification criteria for SjS (the criteria do not distinguish between Ro60 and Ro52/TRIM21)	25
	► If SSc, AIM, or to a lesser extend SLE, is clinically suspected, it is recommended to perform follow-up tests for detecting autoantibodies to Mi-2, TIF1γ, and Ku; these antigens are typically included in disease specific immunoassays (i.e., inflammatory myopathy profile*)	26
	► Autoantibodies to Mi-2 and TIF1γ are associated with DM; autoantibodies to TIF1γ in patients with DM, although rare in the overall AC-4 pattern, is strongly associated with malignancy in old patients	26, 27
	► Autoantibodies to Ku are associated with SSc-AIM and SLE-SSc-AIM overlap syndromes	26
	Notes: Anti-SS-A/Ro (Ro60) and AIM-specific autoantibodies may be undetected in HEp-2 IIFA-screening.	28
AC-5	LARGE/COARSE SPECKLED (see online supplementary table S1 for further details)	
	► Present to a varying degree in distinct SARD, in particular SLE, SSc, MCTD, SSc-AIM overlap syndrome, and UCTD (i.e, patients with rheumatic symptoms without a definite SARD diagnosis)	29
	► If SLE is clinically suspected, it is recommended to perform follow-up tests for anti-Sm and anti-U1RNP antibodies; these antigens are commonly included in the routine ENA profile; anti-Sm antibodies are included in the classification criteria for SLE	16, 30, 31
	► If SSc is clinically suspected, it is recommended to perform a follow-up test for anti-RNApol III antibodies (e.g., SSc profile*); the anti-RNApol III antibodies are included in the classification criteria for SSc	8
	► If MCTD is clinically suspected, it is recommended to perform a follow-up test for anti-U1RNP antibodies; the antigen is commonly included in the routine ENA profile; anti-U1RNP antibodies are included in the diagnostic criteria for MCTD	32
	► If the SSc-AIM overlap syndrome is clinically suspected, it is recommended to perform follow-up tests for anti-U1RNP and anti-Ku antibodies; these antigens are included in the routine ENA profile (U1RNP), or in disease specific immunoassays (Ku, i.e., inflammatory myopathy profile* and SSc profile*)	26, 33
	► In non-SARD individuals in the general population, the presence of the AC-5 pattern is not associated with the autoantigens mentioned above and most often concerns low antibody titers	

Continued

Table 1 Continued

Code	AC pattern—clinical relevance	Refs
AC-6	MULTIPLE NUCLEAR DOTS	
	► Found in a broad spectrum of autoimmune diseases, including PBC, AIM (DM), as well as other inflammatory conditions	34
	► If PBC is clinically suspected, it is recommended to perform follow-up tests for anti-Sp100 (and PML/Sp140) antibodies; in particular anti-Sp100 antibodies have the best clinical association with PBC and have added value, especially when associated with AMA; the Sp100 (and PML-Sp140) antigen is included in disease specific immunoassays (ie, liver profile*)	17, 35, 36
	► If DM is clinically suspected, it is recommended to perform a follow-up test for anti-MJ/NXP-2 antibodies; these anti-MJ/NXP-2 antibodies are highly specific for AIM, are found in up to one third of patients with juvenile DM, and have been reported to be associated with malignancies in adult AIM patients; the antigen is included in disease specific immunoassays (i.e., inflammatory myopathy profile*)	37–39
AC-7	FEW NUCLEAR DOTS (see online supplementary table S1 for further details)	
	► The AC-7 pattern has low positive predictive value for any disease	40, 41
	► Antigens primarily localized in the dots include p80-coilin and SMN complex; specific immunoassays for these autoantibodies are currently not commercially available	42, 43
AC-8	HOMOGENEOUS NUCLEOLAR (see online supplementary table S1 for further details)	
	► Found in patients with SSc, SSc-AIM overlap syndrome, and patients with clinical manifestations of other SARD	44–46
	► If limited cutaneous SSc is clinically suspected, it is recommended to perform a follow-up test for anti-Th/To antibodies; the antigen is included in disease specific immunoassays (ie, SSc profile*)	44, 45
	► If SSc-AIM overlap syndrome is clinically suspected, it is recommended to perform a follow-up test for anti-PM/Scl antibody reactivity; the antigen may be included in the routine ENA profile and is included in disease specific immunoassays (i.e., inflammatory myopathy profile* and the SSc profile*); in general, anti-PM/Scl antibodies yield a diffuse nuclear fine speckled staining in addition to the AC-8 pattern	46
	► Other antigens recognized include B23/nucleophosmin, No55/SC65, and C23/nucleolin, but the clinical significance of these autoantibodies is not well established; specific immunoassays for these autoantibodies are currently not commercially available	
	Notes: Although some anti-Th/To antibody immunoassays are commercially available, technical issues relating to the limited sensitivity of these immunoassays should be taken in to consideration.	44, 47
AC-9	CLUMPY NUCLEOLAR	
	► Found in patients with SSc	48
	► If SSc is clinically suspected, it is recommended to perform a follow-up test for anti-U3RNP/fibrillarin antibodies; the antigen is included in disease specific immunoassays (i.e, SSc profile*)	48
	► If confirmed as anti-U3RNP/fibrillarin reactivity by immunoassay, the clinical association is with diffuse SSc, increased incidence of pulmonary arterial hypertension, skeletal muscle disease, severe cardiac involvement, and gastrointestinal dysmotility	23, 48–50
	► Among SSc patients, anti-U3RNP/fibrillarin antibodies are most commonly found in African American and Latin American patients	48, 49, 51
	Notes: Although some anti-U3RNP/fibrillarin immunoassays are commercially available, technical issues relating to the limited sensitivity of these immunoassays should be taken into consideration.	24
AC-10	PUNCTATE NUCLEOLAR	
	► The AC-10 pattern can be seen in various conditions, including SSc, Raynaud's phenomenon, SjS, and cancer	52–56
	► If the AC-10 pattern is observed in the serum of patients with conditions mentioned above, follow-up testing for anti-NOR90(hUBF) antibodies is to be considered; the antigen is included in disease specific immunoassays (i.e. SSc profile*)	54, 55
	► While AC-10 is associated with anti-RNApol I antibodies, these antibodies almost always coexist with anti-RNApol III antibodies which reveal the AC-5 pattern; therefore, if SSc is clinically suspected, it is recommended to perform a follow-up test for anti-RNApol III antibodies (See also AC-5); specific immunoassays for anti-RNApol I antibodies are currently not commercially available	52, 53, 57
AC-11	SMOOTH NUCLEAR ENVELOPE	
	► The AC-11 pattern is infrequently found in routine autoantibody testing and has been described in autoimmune-cytopenias, autoimmune liver diseases, linear scleroderma, APS, and SARD; current information on clinical associations is based mainly on case reports and small cohorts	58–60
	► Antigens recognized include lamins (A, B, C) and LAP-2; specific immunoassays for these autoantibodies are currently not commercially available	58–60
AC-12	PUNCTATE NUCLEAR ENVELOPE (see online supplementary table S1 for further details)	
	► Found in patients with PBC, as well as patients with other autoimmune liver diseases and SARD	61
	► If PBC is clinically suspected, it is recommended to perform a follow-up test for anti-gp210 antibodies; the antigen is included in disease specific immunoassays (ie, extended liver profile*)	62–64
	► Other antigens recognized include p62 nucleoporin, LBR, and Tpr; specific immunoassays for these autoantibodies are currently not commercially available	65–68

Continued

Table 1 Continued

Code	AC pattern—clinical relevance	Refs
AC-13	PCNA-like (see online supplementary table S1 for further details)	
	► The AC-13 pattern has formerly been considered highly specific for SLE, but this specificity is debated	69, 70
	► If SLE is clinically suspected, it is recommended to perform a follow-up test for anti-PCNA antibodies; the antigen is included in several routine ENA profiles	69
	► Recent studies with antigen-specific immunoassays show clinical associations also with SSc, AIM, RA, HCV, and other conditions	70–73
AC-14	CENP-F-like	
	► The majority of sera exhibiting the AC-14 pattern are from patients with a diversity of neoplastic conditions (breast, lung, colon, lymphoma, ovary, brain); paradoxically, the frequency of the AC-14 pattern in patient cohorts with these malignancies is low	
	► The AC-14 pattern is also seen in inflammatory conditions (Crohn's disease, autoimmune liver disease, SjS, graft-versus-host disease); current information on clinical associations is based mainly on case reports and series of cases	
	► Possible associations only hold if the reactivity to CENP-F is confirmed in an antigen-specific immunoassay; current information on clinical associations is based mainly on case reports and series of cases; specific immunoassays for this autoantibody are currently not commercially available	74–78
AC-29	*TOPOI-like*	
	► The AC-29 pattern is highly specific for SSc, in particular with diffuse cutaneous SSc and more aggressive forms of SSc	14, 18, 23
	► If SSc is clinically suspected, it is recommended to perform a follow-up test for anti-Topoisomerase I (formerly Scl-70) antibodies; the anti-Topoisomerase I antibodies are included in the classification criteria for SSc and the antigen is included in routine ENA profiles	8, 23, 79

*Availability of the inflammatory myopathy profile, the SSc profile and the (extended) liver profile may be limited to specialty clinical laboratories.
AIM, autoimmune myopathy; AMA, antimitochondrial antibodies; APS, antiphospholipid syndrome; CENP, centromere-associated protein; DFS, dense fine speckled; DM, dermatomyositis; ENA, extractable nuclear antigens; HCV, hepatitis C virus; IIFA, indirect immunofluorescence assay; LAP, lamin-associated polypeptide; LBR, lamin B receptor; LEDGF, lens epithelial derived growth factor; NOR, nucleolus organiser region; NXP, nuclear matrix protein; PBC, primary biliary cholangitis; PCNA, proliferating cell nuclear antigen; PML, promyelocytic leukaemia; PM/Scl, polymyositis-scleroderma; RA, rheumatoid arthritis; RNApol, RNA polymerase; RNP, ribonucleoprotein; SARD, systemic autoimmune rheumatic diseases; SLE, systemic lupus erythematosus; SMN, survival of motor neuron; SSc, systemic sclerosis; SjS, Sjögren's syndrome; TIF, transcription intermediary factor; TRIM, tripartite motif; Tpr, translocated promoter region; UCTD, undifferentiated connective tissue disease; dsDNA, double stranded DNA; hUBF, human upstream binding factor.

clinical laboratories is rather inconsistent as shown by external quality assessments.[14 124 125] This is exactly the reason why ICAP was initiated: the consensus on nomenclature and definitions of HEp-2 IIFA patterns allows to align pattern description across laboratories. Also, the integration of computer-aided immunofluorescence microscopy (CAIFM) may further improve the consistency in pattern assignments.[126–131] As such, it is promising that several companies involved in CAIFM have declared their intention to accommodate to the ICAP classification. Second, even apparently healthy individuals may have autoantibodies as detected by the HEp-2 IIFA. Such autoantibodies, being either innocent bystander antibodies or predictive antibodies, may still be present on development of SARD and interfere with the SARD-related pattern. Interestingly, the pattern best associated with apparently healthy individuals is the nuclear dense fine speckled pattern (AC-2), but this association only holds if the specificity is confirmed as monospecific for DFS70.[20 21 132] Third, the HEp-2 IIFA patterns may slightly differ depending on the cellular substrate used. For this reason, the ICAP website contains for each pattern multiple pictures taken from different brands of HEp-2 slides. Fourth, diseases like systemic lupus erythematosus and autoimmune inflammatory myopathies may be associated with distinct autoantibodies, each associated with a distinct HEp-2 IIFA pattern. If the autoantigens are ill defined, as is the case, for instance, in autoimmune hepatitis, only the most prevalent patterns are included. Altogether, it is evident that, with the exception of the centromere pattern (AC-3), all patterns are to be confirmed by antigen-specific immunoassay for a solid association with the respective autoimmune diseases.

While consensus statements have been generated for all 29 HEp-2 IIFA patterns, and it is highly recommended to report patterns,[7 11] it is anticipated that laboratories may restrict their reports to the so-called 'competent level' patterns (http://www.ANApatterns.org).[133] Although, for instance, the nucleolar patterns may not be reported as distinct entities (AC-8, AC-9 and AC-10), all three subtypes represent autoantibodies reactive with antigens associated with systemic sclerosis, either alone or in combination with autoimmune inflammatory myopathies. Follow-up testing, therefore, anyhow involves the systemic sclerosis multiparameter assay including all the relevant autoantibodies. Traditionally, only nuclear HEp-2 IIFA patterns have been considered as a true positive HEp-2 IIFA test, and this is most likely related to the time-honoured terminology 'Antinuclear Antibody Test',[12] but it is evident from this report that even for nuclear HEp-2 IIFA patterns, the clinical associations are quite diverse. In particular, the nuclear dense fine speckled pattern (AC-2) seems to have an inverse association with SARD.[9 134] On the other hand, the cytoplasmic HEp-2 IIFA patterns, and to a lesser extent the mitotic patterns, are also clinically relevant and may demand dedicated follow-up testing in daily clinical practice. Therefore, the ICAP executive board advocates that information on HEp-2 IIFA patterns should be reported to the clinician and should also be incorporated in diagnostic and classification criteria instead of the simple assignment '*ANA-positive*'.[135]

Although the HEp-2 IIFA has been considered the gold standard for autoantibody detection in SARD,[3] the limitations of this assay are understood.[136–138] Indeed, up to 35% of healthy controls may be positive if a screening dilution of 1/40 is used.[139] Therefore, in the EASI/IUIS recommendations, it is advocated that each laboratory verifies that the screening dilution is defined by a cut-off set at the 95th percentile.[7] However, by

Table 2 Cytoplasmic HEp-2 IIFA patterns

Code	AC pattern——clinical relevance	Refs
AC-15	FIBRILLAR LINEAR (see online supplementary table S2 for further details)	
	► Found in patients with AIH type 1, chronic HCV infection, and celiac disease (IgA isotype); rare in SARD	17
	► If AIH type 1 is clinically suspected, it is recommended to confirm reactivity with smooth muscle antibodies (IgG isotype), typically detected by IIFA on rodent tissue (liver, stomach, kidney); anti-smooth muscle antibodies are included in the international criteria for AIH type 1	17,80
	► F-actin is the main target antigen of anti-smooth muscle antibodies in AIH type 1; autoantibodies to F-actin are of more clinical importance than antibodies to G-actin	81–83
	Notes: Although anti-F-actin immunoassays are commercially available, technical issues relating to the sensitivity of these immunoassays should be taken into consideration.	
AC-16	FIBRILLAR FILAMENTOUS (see online supplementary table S2 for further details)	
	► Found in various diseases, but AC-16 is not typically found in SARD	
	► Antigens recognized include cytokeratins 8, 18, & 19, tubulin, and vimentin; specific immunoassays for these autoantibodies are currently not commercially available	
AC-17	FIBRILLAR SEGMENTAL (see online supplementary table S2 for further details)	
	► Found very infrequently in a routine serology diagnostic setting	
	► Antigens recognized include α-Actinin and Vinculin; specific immunoassays for these autoantibodies are currently not commercially available	
AC-18	DISCRETE DOTS (see online supplementary table S2 for further details)	
	► Autoantibodies revealing the AC-18 pattern have been reported in distinct SARD and in a variety of other diseases; their prevalence in unselected or specified disease cohorts has not been thoroughly studied	84
	► Antigens recognized include GW-body (Processing or P body) antigens (Ge-1/Hedls, GW182, and Su/Ago2) and endosomal antigens (EEA1, CLIP-170, GRASP-1, and LBPA); specific immunoassays for these autoantibodies are currently not commercially available	
	Notes: Autoantibodies to GW-bodies and endosomes may yield slightly different HEp-2 IIFA patterns.	84, 85
AC-19	DENSE FINE SPECKLED (see online supplementary table S2 for further details)	
	► Found in patients with SLE and the anti-synthetase syndrome (a subset of AIM), interstitial lung disease, polyarthritis, Raynaud's phenomenon, and mechanic's hands; these features may occur in various combinations or as an isolated manifestation, especially interstitial lung disease	33, 86, 87
	► If SLE is clinically suspected, follow-up tests for antibodies to ribosomal P phosphoproteins (P0, P1, P2, C22 RibP peptide) are recommended; these antigens may be included in the routine ENA profile	
	► Anti-RibP antibodies have been associated in some studies with neuropsychiatric lupus, and in childhood-onset SLE with autoimmune hemolytic anemia	86, 88, 89
	► If AIM, in particular the anti-synthetase syndrome, is suspected, it is recommended to perform follow-up tests for antibodies to tRNA synthetases; antigens are included in disease specific immunoassays (ie, inflammatory myopathy profile*)	26, 33
	► If AIM, in particular necrotizing myopathy, is suspected, it is recommended to perform follow-up tests for anti-SRP antibodies; the antigen is included in disease specific immunoassays (ie, inflammatory myopathy profile*)	26
	Notes: The fine distinction between AC-19 and -20 may depend on HEp-2 substrates and/or antibody concentration; antibodies to both RibP as well as tRNA synthetases may be undetected in HEp-2 IIFA-screening.	
AC-20	FINE SPECKLED	
	► Found in patients with the anti-synthetase syndrome (a subset of AIM), interstitial lung disease, polyarthritis, Raynaud's phenomenon, and mechanic's hands; these features may occur in various combinations or as an isolated manifestation, especially interstitial lung disease	33, 90
	► Autoantibodies associated with the AC-20 pattern are primarily reported for the anti-Jo-1 antibody, which recognizes histidyl-tRNA synthetase; since AC-20 is not specific for Jo-1, it is recommended to perform a follow-up test for anti-Jo-1 antibodies; the antigen is included in the routine ENA profile, as well as in disease specific immunoassays (i.e., inflammatory myopathy profile*); the anti-Jo-1 antibodies are included in the classification criteria for AIM	91, 92
	Notes: The fine distinction between AC-19 and -20 may depend on HEp-2 substrates and/or antibody concentration; antibodies to Jo-1 may be undetected in HEp-2 IIFA-screening.	

Continued

Table 2 Continued

Code	AC pattern——clinical relevance	Refs
AC-21	RETICULAR/AMA	
	► Commonly found in PBC, but also detected in SSc, including PBC-SSc overlap syndrome and PBC-SjS overlap syndrome	93–97
	► If PBC is clinically suspected it is recommended to perform a follow-up test for AMA, historically detected by IIFA on rodent tissue (liver, stomach, kidney); these autoantibodies are primarily directed to the PDH complex, and in particular the E2-subunit (PDH-E2); the antigen is included in disease specific immunoassays (i.e., liver profile*) as well as in some routine ENA profiles	93, 94
	► Additional antigens recognized include the E1α and E1β subunits of PDH, the E3-binding protein of PDH, and the 2-OGDC; these antigens are only included in extended disease specific immunoassays (i.e., extended liver profile*)	93, 94
AC-22	POLAR/GOLGI-like (see online supplementary table S2 for further details)	
	► Found in small numbers of patients with a variety of conditions	
	► Antigens recognized include giantin/macrogolgin and distinct golgin molecules; specific immunoassays to detect autoantibodies directed to specific Golgi antigens are currently not commercially available	85
AC-23	RODS and RINGS (see (online supplementary table S2 for further details)	
	► Most commonly found in HCV patients who have been treated with pegylated interferon-α/ribavirin combination therapy, but autoantibodies revealing the AC-23 patterns were undetected prior to treatment; as the use of interferon-α/ribavirin in HCV treatment is decreasing, the frequency and clinical associations of the AC-23 pattern may change	98–101
	► Specific immunoassays to detect autoantibodies directed to specific Rods and Rings antigens, for instance IMPDH2, are not commercially available	
	Note: Presence of the AC-23 pattern depends on the HEp-2 cell substrate.	

*Availability of the inflammatory myopathy profile, the SSc profile and the (extended) liver profile may be limited to specialty clinical laboratories.
AIH, autoimmune hepatitis; AIM, autoimmune myopathy; AMA, anti-mitochondrial antibodies; APS, antiphospholipid syndrome; Ago, argonaute protein; CENP, centromere-associated protein; CLIP, class II-associated invariant chain peptide; DFS, dense fine speckled; DM, dermatomyositis; EEA, early endosome antigen; ENA, extractable nuclear antigens; HCV, hepatitis C virus; IFA, indirect immunofluorescence assay; LAP, lamin-associated polypeptide; LBR, lamin B receptor; LEDGF, lens epithelial derived growth factor; NOR, nucleolus organizer region; NXP, nuclear matrix protein; PBC, primary biliary cholangitis; PCNA, proliferating cell nuclear antigen; PML, promyelocytic leukaemia; PM/Scl, polymyositis-scleroderma; RA, rheumatoid arthritis; RNApol, ribonucleic acid polymerase; RNP, ribonucleoprotein; SARD, systemic autoimmune rheumatic diseases; SLE, systemic lupus erythematosus; SMN, survival of motor neuron; SRP, signal recognition protein; SSc, systemic sclerosis; SjS, Sjögren's syndrome; TIF, transcription intermediary factor; TRIM, tripartite motif; Tpr, translocated promoter region; dsDNA, double stranded deoxyribonucleic acid; hUBF, human upstream binding factor; tRNA, transfer ribonucleic acid.

taking into account that the HEp-2 IIFA nowadays is ordered by a wide spectrum of clinical disciplines,[1] the number of clinically unexpected positive results, that is, positive test results with no clinical evidence of an associated autoimmune disease, is ever increasing and may even equal the likelihood of a clinically true-positive result.[140 141] A study performed in a community setting concluded that many patients with a positive ANA test are incorrectly given a diagnosis of systemic lupus erythematosus and sometimes even treated with toxic medications.[142] These arguments are used to introduce a gating strategy in order to restrict test-ordering to those cases that have a sufficiently high pretest probability for having a SARD. However, it can also be argued that patients with a low pretest probability should be tested using the HEp-2 IIFA in order to prevent true cases, especially those with very early disease manifestations, from being missed. This is a paradigm shift to disease prediction and prevention.[143] In this strategy, the HEp-2 IIFA could be integrated in multianalyte 'omic' profiles for case finding and establishing an early diagnosis and preventing severe complications.[143 144] Obviously, it is anticipated that the added value of the HEp-2 IIFA in this approach can be increased by incorporating information on both patterns as well as titres in combination with well-directed advices on follow-up testing.

Although the current consensus on the clinical relevance of HEp-2 IIFA patterns has come across after extensive discussion and debate within the ICAP executive board as well as with the workshop participants, the information provided is not based on a systematic review or meta-analysis of the existing literature. Because of the short history of ICAP, being founded in 2014, inclusion of older literature might have been hampered by potential differences in pattern nomenclature and definitions. For instance, the nuclear dense fine speckled (AC-2) and topo I-like (AC-29) patterns were previously often considered homogeneous, speckled or even mixed patterns. The centromere pattern (AC-3) or the cytoplasmic reticular/AMA (AC-21) patterns, on the other hand, are examples that probably have been less prone to change in pattern definition over time. The universal use of the ICAP nomenclature and pattern definitions, both in daily clinical practice as well as in the scientific literature, may enable systematic reviews in the future, and may well fine-tune current consensus based on expert opinions only.

In conclusion, the consensus statements on clinical relevance should be readily available to clinicians and this will enable further harmonisation of test-result interpretation with respect to HEp-2 IIFA patterns. Obviously, clinicians should be aware of the clinical suspicion for the respective patient, and therefore should order specific tests accordingly, also taking into account the anticipation of prevalence of HEp-2 IIFA negative (AC-0)[13] results in SARD. The information on clinical relevance of HEp-2 IIFA patterns is intended to support the decision strategy of the clinician. Information presented in the online supplementary tables 1–3 is primarily intended to be used for complex cases in the consultation of the laboratory specialist by the clinician. Depending on various jurisdictional regulations, follow-up testing can be automated in predefined algorithms which eventually will shorten the diagnostic delay. Eventually, appropriate integration of HEp-2 IIFA pattern information may help to better define disease criteria and even enable a paradigm shift in the pretest probability paradox.

Table 3 Mitotic HEp-2 IIFA patterns

Code	AC pattern—clinical relevance	Refs
AC-24	CENTROSOME (see online supplementary table 3 for further details)	
	► The AC-24 pattern has low positive predictive value for any disease	
	► Within the spectrum of the SARD, the AC-24 pattern is found in patients with Raynaud's phenomenon, localized scleroderma, SSc, SLE and RA, either alone or in combination with other SSc-associated antibodies;	102–105
	► Antigens recognized include α-enolase, γ-enolase, ninein, Cep-250, Mob1, PCM-1/2, pericentrin; specific immunoassays for these autoantibodies are currently not commercially available	104, 106–108
AC-25	SPINDLE FIBERS (see online supplementary table 3 for further details)	
	► The AC-25 pattern has low positive predictive value for any disease	109
	► Found very infrequently in a routine serology diagnostic setting	109
	► Antigen recognized includes HsEg5; specific immunoassays for this autoantibody, or other spindle fiber targets, are currently not commercially available	110, 111
AC-26	NuMA-like	
	► Approximately one-half of the patients with the AC-26 pattern have clinical features of a SARD (SjS, SLE, UCTD, limited SSc, or RA); the AC-26 pattern is also observed in patients with organ-specific autoimmune diseases and less frequently in non-autoimmune conditions, especially when in low titer	109, 111–114
	► Found very infrequently in a routine serology diagnostic setting	109
	► Antigens recognized include NuMA, centrophilin, SP-H antigen and NMP-22; specific immunoassays for these autoantibodies are currently not commercially available	115
AC-27	INTERCELLULAR BRIDGE (see online supplementary table 3 for further details)	
	► The AC-27 pattern has low positive predictive value for any disease	116
	► Found very infrequently in a routine serology diagnostic setting	117
	► Antigens recognized include, among other, CENP-E, CENP-F, TD60, MSA36, KIF-14, MKLP-1, MPP1/ KIF20B, and INCENP; specific immunoassays for these autoantibodies are currently not commercially available	116, 118, 119
AC-28	MITOTIC CHROMOSOMAL (see online supplementary table 3 for further details)	
	► The AC-28 pattern has low positive predictive value for any disease	
	► Found very infrequently in a routine serology diagnostic setting	120
	► Antigens recognized include DCA, MCA1, and MCA5; specific immunoassays for these autoantibodies are currently not commercially available	120–122

CENP, centromere-associated protein; Cep, centrosomal protein; DCA, dividing cell antigen; IIFA, indirect immunofluorescence assay; INCENP, inner centromere protein; KIF, kinesin family; MCA, mitotic chromosomal antigen; MKLP, mitotic kinesin-like protein; MPP, M-phase phosphoprotein; MSA, mitotic spindle apparatus; NMP, nuclear matrix protein; NuMA, nuclear mitotic apparatus; PCM, pericentriolar material; RA, rheumatoid arthritis; SARD, systemic autoimmune rheumatic diseases; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; SjS, Sjögren's syndrome; UCTD, undifferentiated connective tissue disease.

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Acknowledgements We thank all the workshop participants for their constructive comments and fruitful discussions.

Contributors All authors actively participated in the respective workshops in Kyoto and Dresden. They also participated in the discussions of the executive ICAP committee. The draft of the manuscript was made by JD and was commented on by all authors. Final discussions have taken place at the international autoimmunity meeting in Lisbon. Required amendments were made by JD and approved by all authors.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests The ICAP committee is funded by unrestricted educational grants by several in vitro diagnostics companies (for details see www.anapatterns.org/sponsors.php). JD has received lecture fees from Euroimmun and Thermo Fisher. MJF is a consultant to Inova Diagnostics and Werfen International; none of the other authors declare any competing interest.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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Medication Adherence among Colorectal Cancer Patients Receiving Postoperative Adjuvant Chemotherapy: A longitudinal Study

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出版情報：福岡醫學雜誌. 110 (1), pp.24-38, 2019-03-25. 福岡医学会
バージョン：
権利関係：

Original Article

Medication Adherence among Colorectal Cancer Patients Receiving Postoperative Adjuvant Chemotherapy : A longitudinal Study

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Abstract

Administration of oral agents is a standard postoperative adjuvant chemotherapeutic regimen in colorectal cancer patients. However, little is known about medication adherence of oral chemotherapy in Japanese patients. This study was aimed to elucidate the current status of medication adherence and identify the factors associated with low adherence to postoperative adjuvant chemotherapy in colorectal cancer patients. Among 81 enrolled patients receiving postoperative adjuvant chemotherapy, 61 patients (oral anticancer agents alone : 33, combination of oral anticancer agents and IV administration : 28) were analyzed. Medication adherence (MMAS-8), and anxiety and depression (HADS) were evaluated longitudinally at 3 time-points (1-2, 3-4, 5-6 months) using questionnaires. Factors associated with low adherence were analyzed by multivariate logistic regression. The median medication adherence score and anxiety and depression score did not change significantly over the 6-months of chemotherapy. At 1-2 months after initiation of treatment, low medication adherence was associated with treatment using oral anticancer agents alone (OR : 9.49) and depression (OR : 1.30). At 5-6 months, treatment with oral anticancer agents alone was also associated with low adherence (OR : 6.39).

To maintain adherence, health care professionals should focus on patients who have higher risk for low adherence by monitoring those receiving oral chemotherapeutic agents alone and patients with depression. Thus, continuous educational and emotional support tailored each patient should be considered from the initiation of chemotherapy.

Key words : colorectal cancer, medication adherence, MMAS-8, postoperative adjuvant chemotherapy, longitudinal study

Introduction

Colorectal cancer is one of the most common malignancies worldwide. In Japan, approximately 370,000 cancer-related deaths were recorded in 2016, with colorectal cancer being the second most common cause[1)]. Adjuvant chemotherapy is administered to patients with high-risk stage II and stage III colorectal cancer to prevent recurrence and improve postoperative prognosis. In general, adjuvant chemotherapy is recommended to start by 4 to 8 weeks after surgery and to continue for 6 months[2)]. This treatment schedule puts additional burden on patients as

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they receive the chemotherapy as a new treatment at outpatient clinics while also returning to the society after surgery.

There are three types of postoperative adjuvant chemotherapy : (1) oral administration only, (2) a combination of oral and intravenous (IV) administration, and (3) IV administration only[2]. This study focused on adherence to oral postoperative adjuvant chemotherapy in patients treated with oral administration only and a combination of oral and IV administration. Oral chemotherapy requires fewer hospital visits than intravenous therapy, entailing lesser lifestyle changes than the combination of oral and IV administration. To maintain high levels of medication adherence, patients need to not only comply with treatment requirements, but also proactively participate in the treatment. It has been reported that most patients undergoing anticancer treatment prefer oral to intravenous therapy[3]. Hence, as the oral chemotherapy is increasing, improvement of adherence in this group of patients will be a key issue in cancer care. Poor medication adherence and failure to reach the desired treatment goal could result in poor prognosis and increased medical costs[4].

Previous studies on medication adherence using a patient's self-reported data or a medication event monitoring system reported that the non-adherence rates for capecitabine treatment regimens ranged 9%-25%[5~7]. In Japan, a recent longitudinal study on 338 colorectal cancer patients treated by a combination therapy of oral and IV administration (CapeOX) reported that a median adherence rate was high (94% in the first cycle, and 98% in the final cycle)[8]. Another cross-sectional study reported compliance rate of 77% in 104 patients[9]. Poor adherence was reported to be associated with many factors such as the complexity of the treatment, side effects, inconvenience of visiting the hospital, cost, dissatisfaction with the treatment, forgetfulness, and depression[10~12]. Conversely, factors associated with high adherence included good relationship with healthcare providers, social support, milder adverse events, and adequate knowledge on medication[10]. Some studies suggested that adverse events did not necessarily lead to reduced medication adherence[11], however, results have been inconsistent[12].

Furthermore, regimens for postoperative adjuvant chemotherapy differ in Japan with those in other countries due to the differences in overall survival[2], health insurance system, and economic backgrounds. For example, in Japan, all citizens are covered by universal healthcare systems having unlimited access to standard medical cares. Thus, to develop a support program to improve adherence to postoperative adjuvant chemotherapy in patients with colorectal cancer, we first need to understand the current status of their adherence and identify the factors associated with poor medication adherence, particularly psychological conditions, social backgrounds, knowledge on the treatment, and complexity of the treatment.

The objectives of this study were to elucidate the status of adherence to postoperative adjuvant chemotherapies and to analyze factors associated with low medication adherence in patients with colorectal cancer.

Materials and Methods

1. Study design

This is a prospective longitudinal study based on self-report questionnaires administered during outpatient visits at two cancer centers and one general hospital in Japan. Patients were followed, and the questionnaires were handed out in the hospital or sent through mail. The surveys were conducted from October 2014 to March 2017 at 3 time points ; 1-2 months, 3-4 months, and 5-6 months after starting postoperative adjuvant chemotherapies. This study was approved by the Institutional Review Board of University of Occupational and Environmental Health, Japan (Approval number : H26-116) and that of all the collaborating institutions.

2. Participants

The inclusion criteria for our study was patients who (1) had clinical stage II or III disease who had started adjuvant chemotherapy with oral anticancer agents with/without intravenous administrations after curative surgery for colorectal cancer, (2) were aged 20 years and above, and (3) had the intellectual ability to fill out the self-administered questionnaire. After providing verbal and written information about the study, a written informed consent was obtained from all participants prior to enrollment. The standard treatment regimens for participants were either 1) oral treatment with uracil/tegafur with leucovorin (UFT + LV) that was administered only orally or 2) oral capecitabine plus intravenous oxaliplatin (CapeOX). In the UFT + LV regimen, one cycle consisted of 3 times a day oral administration for 28 days, followed by a week of off-drug period. In the CapeOX regimen, the anticancer agents were administered both orally (capecitabine) and intravenously (oxaliplatin). One cycle consisted of intravenous oxaliplatin on day 1 at an outpatient clinic and oral administration of capecitabine twice a day for 14 days followed by a week of off-drug period. It is recommended that these regimens continue for 6 months.

3. Questionnaires

1) Medication adherence

In this study, medication adherence was evaluated through a self-report questionnaire using Morisky Medication Adherence Scale-8 (MMAS-8)[13]. The MMAS-8 is a scale designed to evaluate the psychosocial characteristics of adherence, social support, and satisfaction with care related to medication adherence. A systematic review and meta-analysis showed that MMAS-8 had acceptable internal consistency and reproducibility[14] and it has been used in more than 200 studies since its development in 2009. The MMAS-8 score ranges from 0 to 8, where higher points indicate better adherence. Patients were classified into three groups for analysis based on their total adherence scores : the low adherence group comprised patients who scored less than 6 points ; the moderate adherence group, patients who scored from 6 points to less than 8 points ; and the high adherence group, 8 points. A Japanese translation of the MMAS-8 was developed by Dr. Morisky in collaboration with the Mapi Institute. A license agreement for the use of the copyrighted MMAS-8 is available from Donald E. Morisky.

2) Anxiety and depression

To evaluate the state of anxiety and depression of the patients, a Japanese version of the Hospital Anxiety and Depression Scale (HADS) for general outpatients (registration #13105116) was used[15]. HADS consists of 14 items : 7 items for anxiety (HADS-A) and 7 items for depression (HADS-D). Higher total scores indicate more severe depression and anxiety. Each item is scored from 0 to 3, and the total score ranges from 0 to 21 points. The patients were stratified into those with normal (≦ 7 points), mild (8 to ≦ 10 points), moderate (11 to ≦ 14 points), and severe (15 to ≦ 21 points) using the HADS. The validity and reliability of the Japanese version of HADS had been assessed previously[16][17] with calculated Cronbach's α of 0.73 to 0.81.

3) Adverse events

The occurrence of adverse events was surveyed using a 10-item questionnaire of the Japanese version of Common Terminology Criteria for Adverse Events v4.0 from the Japan Clinical Oncology Group[18]. The adverse events surveyed were fatigue, anorexia, altered taste, constipation, nausea, diarrhea, rash, fever, stomatitis, and hand-foot syndrome. Basic information on the patient's cancer (e.g., diagnosis, location, stage, duration, date when treatment was started, and regimen) was collected from electronic medical records.

4. Statistical analysis

Descriptive statistics were calculated in accordance with the MMAS-8 and HADS scoring

manuals. The Mann-Whitney U-test, chi-square test, and Fisher's exact test were used to compare the 2 groups. Repeated measures ANOVA were used to analyze the longitudinal changes in medication adherence during the treatment. In the analysis of factors associated with low levels of medication adherence, individuals with low and moderate MMAS-8 scores were grouped together and categorized as the low adherence group, following the method in a previous study[19]. The low adherence group and the high adherence group were used as dependent variables. Factors associated with low adherence at each time point were analyzed via logistic regression analysis. A univariate regression analysis was used to determine significant differences between the high and low adherence group for the following independent variables : age (≤ 65 years vs. > 65 years), sex, marital status, employment status, educational background (≤ high school vs. > high school), cancer location (colon vs. rectal), stage (stage II vs. stage III), stoma, administration route of adjuvant chemotherapy (oral vs. oral + IV), dose schedule (complex vs. simple), difficulty in each of the 4 items on the original questionnaire on the treatment experience and concerns on medication, depression (as assessed via HADS-D), and anxiety (as assessed via HADS-A). SPSS (Japanese version 22.0 for Windows, IBM Japan, Inc., Tokyo) was used for statistical analysis, and all tests used a 5% significance threshold.

Results

1. Demographic and clinical characteristics of the patients

A total of 81 patients were enrolled, of which data from 61 patients who completed the survey at 3 time points were analyzed. The demographic and clinical characteristics of the 61 patients are summarized in Table 1. The average age was 69.0 ± 8.1 (range, 44-83) years, and 67% of the participants were men. Thirty-three patients (54%) received oral chemotherapeutic agents only (the oral treatment group), whereas 28 patients (46%) received a combination of oral and IV chemotherapeutics (the oral + IV treatment group). In the oral treatment group, UFT/LV therapy was administered in 27 patients (44%), capecitabine in 4 patients (7%), and S-1 therapy in 2 patients (3%), while all 28 patients (46%) in the combination group were on CapeOX therapy. There were no significant differences in demographic and clinical characteristics between the oral treatment group and the oral + IV treatment group. However, the oral + IV treatment group tended to include more stage III patients (P = .06).

2. Medication adherence and anxiety and depression

The medication adherence as measured by MMAS-8 during the treatment period is summarized in Table 2. The mean MMAS-8 scores at 1-2 months, 3-4 months, and 5-6 months from the start of treatment were 7.0 ± 1.2, 6.8 ± 1.3, and 6.9 ± 1.3, respectively. A total of 16.4% -27.9% of patients had low medication adherence (MMAS < 6) during the study ; however, no significant differences were observed in MMAS-8 scores at different time points (F (2, 120) = 1.21, P = .30).

The anxiety and depression as measured by HADS during the treatment period is summarized in Table 2. Regarding anxiety, mild and moderate levels were observed in 1.6%-11.5% of the patients throughout the study period. Regarding depression, a total of 13.1%-16.4% of patients were mild or moderate level during the study period. No significant differences were observed in HADS-A (F (2, 180) = 0.29, P = .75) and HADS-D (F (2, 180) = 0.19, P = .83) at different time points.

The comparison of MMAS-8 scores according to different attributes is shown in Table 3. MMAS-8 scores were not significantly associated with age, sex, marital status, occupational status, educational level, location, or stage of cancer, or having stoma. However, the MMAS-8 score was

Table 1 Clinical Characteristics of the Patients

	Overall	Oral treatment group	Oral + IV treatment group	P-value
Number	61	33	28	-
Age, years	69.0 ± 8.1	70.0 ± 8.6	67.9 ± 7.5	.32
Men	41 (67.2)	23 (69.7)	18 (64.3)	.65
Married	47 (77.0)	26 (78.8)	21 (75.0)	.73
Employed	22 (36.1)	12 (36.4)	10 (35.7)	.96
Educational level				
Primary	8 (13.1)	2 (6.1)	6 (21.4)	
Secondary	39 (63.9)	23 (69.7)	16 (57.1)	.21
College	14 (23.0)	8 (24.2)	6 (21.4)	
Location of cancer				
Colon	41 (67.2)	23 (69.7)	18 (64.2)	.65
Rectum	20 (32.8)	10 (30.3)	10 (35.7)	
Clinical stage of cancer				
II	23 (37.7)	16 (48.5)	7 (25.0)	.06
III	38 (62.3)	17 (51.5)	21 (75.0)	
Patients with stoma	9 (14.8)	5 (15.2)	4 (14.3)	.92
Chemotherapy regimen				
UFT/LV	-	27 (44.3)	-	
Capecitabine	-	4 (6.6)	-	-
S-1	-	2 (3.3)	-	
CapeOX	-	-	28 (46.0)	

Abbreviations : Oral, patients with oral medications only ; Oral + IV, patients with oral and intravenous medications ; UFT, Uracil-tegafur ; LV, Leucovorin ; S-1, tegafur-gimeracil-oteracil ; CapeOX, capecitabine and oxaliplatin.
The UFT/LV therapy group included 2 patients on monotherapy, and the UFT/LV/protein-bound polysaccharide K group included 3 patients.

Data are displayed as mean ± SD or number (%).
We compared differences between patients with oral medications and patients with both oral and intravenous medications.

significantly lower ($P < .001$-$.01$) in the oral treatment group than in the oral + IV treatment group at all three time points. Compared with patients taking chemotherapeutics at similar doses ("Simple" in Table 3), those taking different doses of oral anticancer agents during the day (e.g., 2 tablets in the morning, 3 tablets at noon, and 2 tablets in the evening, "Complex" in Table 3) had significantly lower MMAS-8 scores at 5-6 months ($P = .02$) or tended to have lower MMAS-8 scores at 1-2 months and 3-4 months ($P = .051$-$.08$) after treatment.

Because there were significant differences in MMAS-8 scores between the oral group and oral + IV treatment group, the answers to each item of the MMAS-8 questions were compared to analyze whether there were certain items of MMAS-8 that were responsible for the differences (Table 4). Throughout the treatment period, the non-adherent answer to the question "Do you sometimes forget to take your pills?" was significantly higher in the oral treatment group than in the oral + IV treatment group ($P < .001$). Similarly, in the first 2 months of the treatment,

Table 2 MMAS-8 Score and HADS score at Each Phase

	1-2 Months (T1)	3-4 Months (T2)	5-6 Months (T3)	P-value
MMAS-8 score	7.0 ± 1.2	6.8 ± 1.3	6.9 ± 1.3	.30
MMAS-8 adherence level				
Low	10 (16.4)	17 (27.9)	12 (19.7)	
Medium	22 (36.1)	24 (39.3)	26 (42.6)	.40
High	29 (47.5)	20 (32.8)	23 (37.7)	
HADS-A score	2.9 ± 2.9	2.7 ± 2.4	3.1 ± 2.9	.75
HADS-A score ranges				
Normal	57 (93.4)	60 (98.4)	54 (88.5)	
Mild	3 (4.9)	1 (1.6)	7 (11.5)	.12
Moderate	1 (1.6)	0 (0.0)	0 (0.0)	
Severe	0 (0.0)	0 (0.0)	0 (0.0)	
HADS-D score	3.9 ± 3.3	4.1 ± 3.2	3.7 ± 3.3	.83
HADS-D score ranges				
Normal	53 (86.9)	51 (83.6)	53 (86.9)	
Mild	5 (8.2)	8 (13.1)	5 (8.2)	.86
Moderate	3 (4.9)	2 (3.3)	3 (4.9)	
Severe	0 (0.0)	0 (0.0)	0 (0.0)	

Abbreviations : MMAS-8, Morisky Medication Adherence Scale-8.

Data are displayed as mean ± SD or number (%).

The patients were stratified into those with low adherence (< 6 points), medium adherence (6 to < 8 points), and high adherence (8 points) using the MMAS-8.

Abbreviations: HADS, Hospital Anxiety and Depression Scale

HADS-A; Anxiety scores from HADS, HADS-D; Depression scores from HADS

Data are displayed as mean ± SD or number (%).

The patients were stratified into those with normal (≦ 7 points), mild (8 to ≦ 10 points), moderate (11 to ≦ 14 points), and severe (15 to ≦ 21 points) using the HADS.

the non-adherent answer to the question "Thinking over the past two weeks, were there any days when you did not take your medicine?" and to the question "When you travel or leave home, do you sometimes forget to bring along your medication?" were also significantly higher in the oral treatment group than that in the oral + IV treatment group (P = .03 and .01, respectively). For the question "Do you ever feel hassled about sticking to your treatment plan?" the non-adherent answers were significantly higher in the oral treatment group than those in the oral + IV treatment group at 5-6 months of treatment (P = .049).

3. Adverse events

The most common adverse event throughout the treatment period was fatigue. A total of 39%, 56%, and 51% of the patients reported fatigue at 1-2, 3-4, and 5-6 months after starting treatment, respectively. The second most frequent adverse event was decrease in taste sensation that was reported by 34%, 43%, and 39% of the patients at 1-2, 3-4 months, and 5-6 months after starting treatment, respectively. There were no significant differences in MMAS-8 scores between patients with vs without adverse events.

Table 3 Differences in MMAS-8 Scores at Each Phase

	1-2 Months (T1)		3-4 Months (T2)		5-6 Months (T3)	
	MMAS-8 score	P-value	MMAS-8 score	P-value	MMAS-8 score	P-value
Age						
< 65 years old	7.3 ± 0.9	.71	6.5 ± 1.4	.42	6.7 ± 1.0	.28
≥65 years old	6.9 ± 1.3		6.9 ± 1.2		6.9 ± 1.4	
Sex						
Male	7.1 ± 1.0	.88	6.8 ± 1.3	.83	7.0 ± 1.1	.38
Female	6.9 ± 1.6		6.7 ± 1.3		6.5 ± 1.7	
Marital status						
Married	7.0 ± 1.2	.87	6.7 ± 1.3	.92	6.8 ± 1.4	.81
Single/divorced	7.0 ± 1.3		6.9 ± 1.1		7.0 ± 1.0	
Occupational status						
Employed	7.0 ± 1.3	.98	6.8 ± 1.5	.57	6.6 ± 1.5	.39
Unemployed	7.0 ± 1.2		6.8 ± 1.2		7.0 ± 1.2	
Educational level						
Primary	6.8 ± 1.1		6.8 ± 1.6		7.0 ± 0.9	
Secondary	7.0 ± 1.4	.64	6.8 ± 1.3	.92	6.8 ± 1.5	.99
College	7.3 ± 0.8		6.8 ± 0.9		7.0 ± 1.1	
Location of cancer						
Colon	7.0 ± 1.3	.92	6.8 ± 1.3	.86	6.6 ± 1.4	.11
Rectum	7.1 ± 1.2		6.8 ± 1.3		7.3 ± 1.0	
Clinical stage of cancer						
II	6.9 ± 1.4	.88	6.8 ± 1.4	.73	6.9 ± 1.6	.46
III	7.1 ± 1.2		6.8 ± 1.2		6.8 ± 1.2	
Stoma						
Yes	7.3 ± 0.9	.46	6.8 ± 1.0	.55	7.4 ± 0.9	.24
No	7.0 ± 1.3		6.8 ± 1.3		6.8 ± 1.4	
Route of administration						
Oral	6.5 ± 1.4	< .001	6.4 ± 1.3	.01	6.4 ± 1.5	< .001
Oral + IV	7.7 ± 0.6		7.2 ± 1.1		7.4 ± 0.9	
Dosage						
Simple	7.2 ± 1.1	.051	7.0 ± 1.1	.08	7.1 ± 1.0	.02
Complex	6.4 ± 1.6		6.2 ± 1.6		6.0 ± 1.8	

Abbreviations : MAAS-8, Morisky Medication Adherence Scale-8 ; Stoma, patients with stoma ; Oral, patients with oral medications ; Oral + IV, patients with both oral and intravenous medications.

Data are displayed as mean ± SD.

Table 4 Differences in Adherent Behavior Between the Oral Treatment Group and the Oral + IV Treatment Group

	1-2 Months (T1)				3-4 Months (T2)				5-6 Months (T3)			
	All	Oral	Oral + IV	P-value	All	Oral	Oral + IV	P-value	All	Oral	Oral + IV	P-value
1. Do you sometimes forget to take your pills? (Yes)	10 (16.4)	10 (30.3)	0 (0.0)	< .001	16 (26.2)	14 (42.4)	2 (7.1)	.003	14 (23.0)	13 (39.4)	1 (3.6)	.001
2. Over the past 2 weeks, were there any days when you did not take your medicine? (Yes)	9 (14.8)	8 (24.2)	1 (3.6)	.03	10 (16.4)	7 (21.2)	3 (10.7)	.32	11 (18.0)	9 (27.3)	2 (7.1)	.051
3. Have you ever cut back or stopped your medication without telling your doctor because you felt worse when you took it? (Yes)	3 (4.9)	3 (9.1)	0 (0.0)	.24	4 (6.6)	2 (6.1)	2 (7.1)	1.00	6 (9.8)	4 (12.1)	2 (7.1)	.68
4. When you travel or leave home, do you sometimes forget to bring along your medication? (Yes)	8 (13.1)	8 (24.2)	0 (0.0)	.01	6 (9.8)	5 (15.2)	1 (3.6)	.21	4 (6.6)	4 (12.1)	0 (0.0)	.12
5. Did you take your medication yesterday? (No)	4 (6.6)	4 (12.1)	0 (0.0)	.12	6 (9.8)	3 (9.1)	3 (10.7)	1.00	5 (8.2)	3 (9.1)	2 (7.1)	1.00
6. When you feel like health concern is under control, do you sometimes stop taking your medicine? (Yes)	1 (1.6)	1 (3.0)	0 (0.0)	1.00	1 (1.6)	1 (3.0)	0 (0.0)	1.00	0 (0.0)	0 (0.0)	0 (0.0)	-
7. Do you ever feel hassled about sticking to your treatment plan for colon disease? (Yes)	19 (31.1)	12 (36.4)	7 (25.0)	.34	25 (41.0)	16 (48.5)	9 (32.1)	.20	21 (34.4)	15 (45.5)	6 (21.4)	.049
8. How often do you have difficulty remembering to take all your medicine?												
All the time	0 (0.0)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)		1 (1.6)	1 (3.0)	0 (0.0)	
Usually	1 (1.6)	0 (0.0)	1 (3.6)		1 (1.6)	1 (3.0)	0 (0.0)		5 (8.2)	2 (6.1)	3 (10.7)	
Sometimes	4 (6.6)	4 (12.1)	0 (0.0)	.06	3 (4.9)	2 (6.1)	1 (3.6)	.37	3 (4.9)	2 (6.1)	1 (3.6)	.27
Once in a while	9 (14.8)	7 (21.2)	2 (7.1)		18 (29.5)	12 (36.4)	6 (21.4)		13 (21.3)	10 (30.3)	3 (10.7)	
Never/Rarely	47 (77.0)	22 (66.7)	25 (89.3)		39 (63.9)	18 (54.5)	21 (75.0)		39 (63.9)	18 (54.5)	21 (75.0)	

Abbreviations : Oral, patients with oral medications ; Oral + IV, patients with both oral and intravenous medications.

Data are displayed as number (%).

4. Factors associated with medication adherence

The results of logistic regression analyses are presented in Table 5. On univariate analysis, factors significantly associated with low adherence at 1–2 months of treatment were oral treatment only ($P < .001$) and depression ($P = .02$), while "knowing the names of medicines" was associated with high adherence ($P = .04$). Meanwhile, multivariate analysis showed that oral treatment (odds ratio (OR) : 9.49 ; 95% confidence interval (CI) : 2.63–34.23) and depression (OR : 1.30 ; 95% CI : 1.04–1.61) were significantly associated with low adherence at 1–2 months after starting treatment, whereas there were no factors significantly associated with low adherence at 3–4 months. At 5–6 months, oral therapy was significantly associated with low adherence on multivariate analysis ($P < .001$) with an OR of 6.39 (95% CI : 1.80–22.69).

Discussion

The present study aimed to analyze longitudinal changes in medication adherence and to identify the factors associated with low adherence in Japanese patients with colorectal cancer receiving oral adjuvant chemotherapy. In the present study, the MMAS-8 scores during the 6 months of adjuvant chemotherapy ranged from 6.8 to 7.0, which were similar to previous studies on adjuvant chemotherapy[19] and on patients with breast cancer patients under hormone therapy in Western countries[20]. Studies on medication adherence using the MMAS-8 in Japanese patients are limited in the studies of non-cancer patients such as type 2 diabetes (MMAS-8 scores of 5.9–6.2) and psoriasis (6.3)[21,22].

In general, patients with cancers are considered to be highly motivated to take their medication and have high adherence to chemotherapy because they understand the risks of not taking the medication and fear for potentially life-threatening disease[23]. The MMAS-8 scores in the patients with colorectal cancer in the present study are higher than those reported in Japanese patients with chronic diseases. Nevertheless, further improvement of medication adherence in patients with cancer is critical to achieve the treatment goal of postoperative adjuvant chemotherapy, i.e., to prevent recurrence and improve prognosis.

In the present study, depression as assessed by HADS-D was associated with low medication adherence at 1–2 months after starting the treatment. Regarding the types of therapy, treatment with oral anticancer agent alone was associated with low medication adherence at 1–2 and 5–6 months after starting treatment. The lower adherence in the oral treatment alone compared with the combination of oral + IV treatment was consistent with the results of previous studies[24,25] and suggested to be a common characteristic among patients receiving oral chemotherapeutics only.

One of the main reasons for these results could be the complexity of the oral chemotherapy regimen. It has been reported that the complexity of regimen such as the combination of different times and/or doses per day has a negative impact on medication adherence[10] and the schedule of medication is an important factor in adherence status[26]. Previous studies reported that the timing and times of oral administration affected adherence. One study reported that taking oral chemotherapy medication every 8 hours was significantly associated with non-adherence among patients with gastroenterological cancers[16]. UFT, the most commonly used drug in the present study, is taken every 8 hours, with recommendation to avoid taking the drug one hour before and after meals. Thus, compared with patients receiving capecitabine that is taken twice a day, patients on UFT have more concerns such as timing of taking the drug and eating meals and carrying the medication. This is consistent with our data that the patients on oral chemotherapy alone more frequently answered "Yes" to the questions "When you travel or leave home, do you

Table 5 Predictors for Low/moderate Medication Adherence

	1-2 Months (T1)				3-4 Months (T2)				5-6 Months (T3)			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
< 65 years old	1.05 (0.32-3.37)	.94	-	-	1.47 (0.40-5.36)	.56	-	-	1.29 (0.38-4.38)	.69	-	-
Men	0.86 (0.29-2.51)	.78	-	-	0.83 (0.26-2.62)	.75	-	-	0.84 (0.28-2.56)	.76	-	-
Married	0.88 (0.27-2.90)	.83	-	-	1.29 (0.35-4.77)	.70	-	-	0.76 (0.22-2.55)	.65	-	-
Employed	1.14 (0.40-3.25)	.81	-	-	0.57 (0.19-1.70)	.31	-	-	1.49 (0.50-4.48)	.48	-	-
Primary school	0.97 (0.42-2.27)	.95	-	-	1.53 (0.61-3.82)	.37	-	-	1.05 (0.44-2.53)	.91	-	-
Colon cancer	0.86 (0.29-2.51)	.78	-	-	0.58 (0.18-1.91)	.37	-	-	2.95 (0.98-8.95)	.06	-	-
Stage III of cancer	1.35 (0.45-3.81)	.57	-	-	1.58 (0.53-4.70)	.43	-	-	1.99 (0.68-5.77)	.21	-	-
Stoma	0.69 (0.17-2.85)	.60	-	-	-	-	-	-	0.42 (0.10-1.78)	.24	-	-
Oral medication	6.67 (2.17-20.48)	< .001	9.49 (2.63-34.23)	< .001	2.34 (0.79-6.99)	.13	-	-	6.96 (2.17-22.30)	< .001	6.39 (1.80-22.69)	< .001
Simple medication	0.40 (0.12-1.33)	.14	-	-	0.91 (0.27-3.10)	.88	-	-	0.46 (0.13-1.64)	.23	-	-
Depression	1.20 (1.00-1.42)	.045	1.30 (1.04-1.61)	.02	1.16 (0.96-1.40)	.11	-	-	1.05 (0.89-1.23)	.58	-	-
Anxiety	1.07 (0.89-1.28)	.47	-	-	1.15 (0.91-1.45)	.25	-	-	1.05 (0.87-1.26)	.63	-	-

Abbreviations: OR, Odds ratio; 95% CI, 95% confidence interval.

sometimes forget to bring along your medication?" and "How often do you have difficulty remembering to take all your medicine?" due to simple forgetting. Therefore, it was suggested that early educational intervention focusing on the complexity of treatments was necessary for patients receiving treatment with oral anticancer agents alone.

Another reason for the low adherence in the oral treatment group may arise from shorter time spent with health professionals aside from visiting a doctor than that of combination therapy. Jacob Arriola et al reported a significant correlation between the frequency of patients' communication with medical professionals and medication adherence[27]. Collectively, these results suggested that limited communication with medical professionals may be one of the reasons causing low medication adherence in patients receiving oral chemotherapy alone. Patients on oral chemotherapy typically receive a prescription from the hospital, bring it to a local pharmacy, and consume the drugs as prescribed. Meanwhile, oral + IV chemotherapy patients usually spend more time with health professionals as they need to stay in the infusion room and interact with health professionals when the drug is administered intravenously. Patients on oral chemotherapy alone have less opportunity for communication to get information and for consulting with nurses, pharmacists, and other health professionals compared with those on oral + IV chemotherapy. In a survey of 397 cancer institutions in Japan, the patients with oral anticancer agent alone received less medication information from pharmacists compared to the patients treated with intravenous chemotherapeutics[28].

In the present study, depression at 1–2 months after starting chemotherapy was associated with low medication adherence during the same period. Depression in patients with cancer is not only caused by psychological problems, but also by physical symptoms and the adverse effects of chemotherapy, among others. Anxiety and depression in addition to physical symptoms such as fatigue and abdominal symptoms during the postoperative period were also reported among patients with colorectal cancer[17].

On the other hand, 1–2 months after starting postoperative adjuvant chemotherapy, patients try to return to their social life while adjusting to their altered gastrointestinal function and handling adverse events of chemotherapy. Depression along with physical and psychological instability may be a reason for the low medication adherence during this period.

As an increased use of oral chemotherapy is predicted, intervention for patients at high risk for poor adherence, such as those receiving oral chemotherapy or those with depression is necessary. For this purpose, methods to evaluate medication adherence at outpatient clinics, where time and manpower are limited, are necessary. Medication adherence is defined by the World Health Organization as the extent to which a person's behavior – taking medications, following a diet, and/or executing lifestyle changes – corresponds with agreed recommendations from a health care provider[29]. Seal et al reported the usefulness of MMAS-8 as a method to briefly and effectively evaluate medication adherence[25]. Evaluating adherence from the viewpoint of patients is also important, although measuring adherence via self-report has a risk for overestimation of the actual adherence status[23].

Our data indicate that "simple forgetting" is one of the main reasons for low adherence, which suggests that factors like remembering the time to take medications and carrying the drugs are obstacles to maintaining adherence. Patients on postoperative adjuvant chemotherapy struggle to add medication management as a new daily routine, and this issue may lead to low adherence. Thus, nurses will need to identify problems in taking medication in individual patients and provide success stories from other patients to solve problems, such as suggesting methods for simple packing of medication to carry, using the

reminder function in mobile phones, and obtaining support from family members.

The limitations of the present study include the small number of patients recruited in a few medical facilities. As such, comparison with previously published data on the association of demographic factors with adherence was incomplete. Additionally, the statistical power was low due to the small number of subjects, which might lead to failure in achieving statistical significance. Another limitation is the relatively short surveillance period, which is set to 6 months based on the length of the standard postoperative adjuvant chemotherapy. However, the treatment period can be extended in certain patients, and clinical trials with longer treatment period are ongoing. Thus, the surveillance period has to be extended corresponding to each treatment period and follow-up studies will be necessary to address these issues. Furthermore, establishing a support program to help medication adherence in patients receiving oral chemotherapy, according to the setting of medical practice would be possible by accumulating data on medication adherence in Japan.

Conclusions

Medication adherence of the patients with colorectal cancer who received postoperative adjuvant chemotherapy was longitudinally evaluated by questionnaire survey. The results suggested that treatment with oral chemotherapeutic agent alone and depressive state were significantly associated with low medication adherence. Thus, additional attention might be given to these patients who are at a high risk of low medication adherence, and nurses in collaboration with other medical professionals would need to develop individualized patient education programs and provide emotional intervention to improve medication adherence in this patient population. However, the generalization of the results needs to be evaluated by future studies because they were based on the small number-survey at only three facilities.

Acknowledgements

We thank the staffs at First Department of Surgery, University of Occupational and Environmental Health, Japan, and Japan Community Health Care Organization (JCHO) Kyusyu Hospital, for their help in recruitment of participants and arrangement for the study.

This work was supported by the Japan Society for the Promotion of Science (KAKENHI (C) Grant Number JP 26463364 and 18K10336 to YN).

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(Received for publication December 3, 2018)

（和文抄録）

術後補助化学療法中の大腸がん患者の服薬アドヒアランスに関する縦断調査

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大腸がんの術後補助化学療法として経口抗がん剤を使用した療法は標準治療の一つとなっているが，日本において経口抗がん剤を用いた術後補助化学療法中の服薬アドヒアランスに関する報告は僅かである．

本研究の目的は，経口抗がん剤を使用する術後補助化学療法を受ける大腸がん患者の服薬アドヒアランスの実態と服薬アドヒアランスの低下に関連する要因を明らかにすることである．61 名の患者（経口抗がん剤単独群 33 名，経口抗がん剤に静注薬併用群 28 名）を対象として，6ヶ月間の術後補助化学療法の治療開始から 3 時点（1〜2，3〜4，5〜6ヶ月後）において服薬アドヒアランス（MMAS-8 scores），不安と抑うつ（HADS）に関する質問紙調査を実施した．アドヒアランス低下に関連する要因の解析はロジスティック回帰分析を使用した．

MMAS-8 scores によるアドヒアランスや HADS による不安と抑うつの術後 6ヶ月間の経時的有意な変化は無かった．服薬アドヒアランスの低下に関連する要因は，治療開始 1〜2ヶ月は経口抗がん剤単独による治療（OR：9.49）と抑うつ（OR：1.30）であった．治療開始 5〜6ヶ月は経口抗がん剤単独治療（OR：6.39）が関与していた．

本調査結果から，アドヒアランスを維持するためには，医療者はアドヒアランスの低下のリスクが高い経口抗がん剤単独治療を受ける患者や抑うつに焦点を当て，治療開始初期から継続的に教育的，精神的な支援を検討する必要があることが示唆された．

キーワード：大腸がん，服薬アドヒアランス，MMAS-8，術後補助化学療法，縦断調査

RHEUMATOLOGY

Original article

doi:10.1093/rheumatology/kez244

Distinctive association of peripheral immune cell phenotypes with capillaroscopic microvascular patterns in systemic sclerosis

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Abstract

Objective. The pathological changes in SSc include immune system dysregulation and microvascular damage. However, the association of immune cell phenotype heterogeneity and microvascular abnormalities is unclear. The aim of this study is to elucidate this association in SSc.

Methods. Peripheral blood mononuclear cells obtained from 150 SSc patients were used for comprehensive flow cytometric analysis based on the Human Immunology Project. Hierarchical cluster analysis was used to classify SSc patients into subgroups and their association with microvascular abnormalities, as assessed by nailfold videocapillaroscopy (i.e. 'early', 'active' and 'late' patterns), was analysed.

Results. The proportions of activated $CD4^+$ T cells, T cells re-expressing CD45RA, activated Th1 and Th17 cells and IgD^-CD27^- B cells were higher in SSc patients than in healthy individuals. Hierarchical cluster analysis stratified SSc patients into three groups: patients with few immune abnormalities (fewer abnormalities group), patients with high proportions of activated T and Treg cells (Treg-dominant group) and patients with high proportions of Tfh and plasmablasts (Tfh-dominant group). Age and disease duration were comparable among the groups. On the other hand, microvascular abnormalities, especially the 'late' nailfold videocapillaroscopy pattern, correlated with internal organ involvement. Among the groups stratified according to immune cell phenotype, the progression to the 'late' nailfold videocapillaroscopy pattern was more frequent in the Tfh-dominant group.

Conclusion. Our study confirmed the presence of immunophenotypic abnormalities in SSc. Immunological abnormalities were not uniform but rather limited to subpopulations, particularly the Tfh-dominant group, where they were highly associated with microvascular abnormalities and organ involvement.

Key words: Systemic sclerosis, nailfold videocapillaroscopy, microvascular damage, cluster analysis, the human immunology project

Rheumatology key messages

- This study confirmed the immunophenotypic abnormalities by comprehensive flow cytometric analysis in systemic sclerosis patients.
- Immunological abnormalities in systemic sclerosis patients were not uniform but rather limited to subpopulations.
- Tfh-dominant immune cell phenotype in systemic sclerosis patients was associated with the progression of microvasculopathy.

Introduction

SSc is an autoimmune disease characterized by fibrosis in multiple organs [1]. The pathological changes in SSc include immune system activation, microvascular damage and fibrosis [2]. However, the exact pathogenic mechanisms have not been fully elucidated and there is no disease-specific treatment [3].

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Submitted 10 February 2019; accepted 9 May 2019

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CLINICAL SCIENCE

One of the early pathological changes observed in patients with SSc is microvasculopathy. The pathological role of this process in organ involvement has been examined extensively [4–7]. In addition, the efficacy of various vascular-targeting therapies has been demonstrated in certain organs involved in patients with SSc, such as for skin ulceration and pulmonary arterial hypertension [2].

On the other hand, SSc is also known as an immune-mediated disease [2] with distinctive specificity of autoantibodies associated with unique clinical subsets. Several studies stressed the roles of pathogenic lymphocytes in SSc, including T helper (Th) 17 cells [8], regulatory T (Treg) cells [9], as well as abnormal activation and function of B cells [10, 11]. However, the pathogenic role of autoimmunity in SSc is so far less convincing compared with that in other autoimmune diseases, such as SLE. In fact, the above studies focused on specific subsets of lymphocytes, rather than comprehensive analysis of the immunological abnormalities in SSc. In addition, there is little information on the effects of molecular targeted therapy on these immune cells [3]. Although B cell depletion therapy [12] and anti-IL-6 receptor antibody [13] are considered beneficial, there are no double-blind placebo controlled studies of any compound with clear effects on skin sclerosis. One of the most important reasons for the lack of specific treatment is the heterogeneity of SSc. In other words, therapeutic management of individual cases based on one kinetic molecular theory is likely to be associated with large variability in treatment outcome, which makes interpretation of the data of any clinical study difficult. Moreover, there is so far no convincing evidence for a link between microvasculopathy, which is directly associated with organ involvement in SSc [4] and immune abnormalities. This fact is reflected in EULAR treatment recommendations for SSc [14]. The recommendations advise separate treatment regimens for various affected organs, and the use of a combination of immunosuppressants and vasoactive drugs.

The reasons for these limited clinical efficacies of immunosuppressive therapy include the unclear role of immune dysregulation in microvascular damage and fibrosis and the diversity of SSc patients. Accordingly, the lack of appropriate molecular targets is one of the major clinical challenges. Based on the above background, it is important both clinically and pathologically to elucidate the relationship between microvasculopathy and the immunological heterogeneity of SSc.

The aim of this study was to determine the immunophenotypes and microvascular abnormalities, as detected by nailfold videocapillaroscopy (NVC) [15, 16], in patients with SSc. For this purpose, we conducted comprehensive analysis of immune cell phenotypes in peripheral blood of SSc patients using multicolour flow cytometric analysis, as recommended by the Human Immunology Project Consortium [17]. We also divided SSc patients into immunological subgroups, using hierarchical cluster analysis methods, to investigate the relationship between immune cell phenotypes and morphological markers of microvasculopathy.

Methods

Subjects

One hundred and fifty SSc patients seen at our clinics between March 2014 and September 2017 and diagnosed according to the classification criteria for SSc [18–20] were enrolled in this study. The study also included a control group of age- and sex-matched healthy individuals. The Ethics Committee of Medical Research, University of Occupational and Environmental Health, Japan, reviewed and approved this study, which was named the SCORPION study (UMIN ID 000014293). A signed informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and its subsequent modifications.

Clinical measurements

Skin sclerosis was assessed using the modified Rodnan skin score (mRSS) [21, 22]. The assessment of interstitial lung disease (ILD) was by high-resolution chest computed tomography in all patients. Pulmonary arterial hypertension (PAH) was screened by Doppler echocardiography, and once identified it was confirmed with right heart catheterization. The diagnosis of PAH was made when the mean pulmonary arterial pressure exceeded 25 mmHg. Oesophageal motility disorders were evaluated by barium oesophagography, and oesophageal manifestations were diagnosed based on findings indicative of a dilated oesophagus, barium retention in the oesophagus and abnormal oesophageal motility. Patients who required antibiotics for inhibition of intestinal bacteria overgrowth or who had history of pseudo ileus obstruction were judged to have lower gastrointestinal lesions. In addition, the presence of joint contractures and digital ulcers was noted. SSc-related antibodies were evaluated by enzyme-linked immunosorbent assay.

Flow cytometric analysis

Immunophenotyping analysis of peripheral blood mononuclear cells was conducted by multi-colour flow cytometry. Flow cytometric analysis was conducted as described previously [23]. Briefly, the cells were incubated in blocking buffer and then suspended in FACS solution with fluorochrome-conjugted monoclonal antibodies. After staining with the indicated antibodies (Supplementary Fig. S1, available at *Rheumatology* online), the cells were analysed with a FACSVerse (BD Biosciences, San Jose, CA, USA) and analysed with Flow Jo software (Tree Star, Ashland, OR, USA). Isotype-matched mouse IgG controls (BD Biosciences) were used to evaluate the background. The phenotype of immune cell subsets was defined based on the Human Immunology Project Consortium protocol of comprehensive eight-colour flow cytometric analysis proposed by NIH/FOCIS with some modification for detecting Tfh cells [17]. Details of the gating strategy are described in Supplementary Figs S1 and S2, available at *Rheumatology* online.

Nailfold videocapillaroscopy images

All patients underwent nailfold videocapillaroscopy (NVC) evaluation of nailfold microvascular damage, as described previously [24, 25]. Briefly, NVC examination was performed using an optical probe videocapillaroscope equipped with a 200-fold contact lens and connected to image analysis software (Videocap; DS MediGroup, Milano, Italy). The same operator, who was blinded to the patient's clinical diagnosis and/or disease severity, performed the NVC examination. The following capillaroscopic parameters were recorded: presence of enlarged and giant capillaries, haemorrhages, loss of capillaries, disorganization of the microvascular array, and capillaries with abnormal morphologies and ramification [15, 26].

The NVC scleroderma spectrum abnormality pattern was defined as described by Cutolo and colleagues ('early', 'active' and 'late' patterns) [27, 28]. The 'early' pattern is characterized by a few giant capillaries, few capillary microhaemorrhages and no evident loss of capillaries. The 'active' pattern comprises abundant giant capillaries, several capillary microhaemorrhages and moderate loss of capillaries. The 'late' pattern is characterized by severe loss of capillaries, abnormal shapes and intense disorganization of the normal capillary array [29].

Hierarchical cluster analysis

Hierarchical cluster analysis was performed by the Ward method [30], using the immunophenotypes of SSc patients. The number of clusters was determined based on a scree plot (Supplementary Fig. S3, available at *Rheumatology* online), which showed the sum of within-cluster dissimilarities. Consequently, the appropriate number of clusters was judged as three and a tree diagram was created accordingly.

Other statistical analysis

Differences between groups were compared using Student's *t*-test or Tukey's test. Correlation analysis was performed using Pearson's correlation coefficient. Changes in values were represented with colours (blue for negative changes and red for positive changes), as shown in Fig. 1. All reported *P*-values were two-sided and were not adjusted for multiple testing. The level of significance was set at $P < 0.05$. All analyses were conducted using JMP version 11.0 (SAS Institute Inc., Cary, NC, USA).

Results

Patients' disposition and baseline characteristics

A total of 150 SSc patients and 30 healthy individuals were enrolled in this study. The mean age of the patients was 64.8 years. The mean duration of illness was 7.2 years and the mean mRSS was 9.6 (Table 1). ILD was identified in 41.6% of the patients, while PAH was in 12.8%. Other involvements included joint contractures and digital ulcerations in 9.4 and 16.4% of the study patients, respectively. Anti-nuclear antibodies (dilution $\geqslant$160) were positive in 89.7% of the patients. Among them, 61.8, 13.8 and 10.7% were also positive for anti-centromere antibodies, anti-Scl-70 antibodies and anti-RNA polymerase III antibodies, respectively (Table 1).

Peripheral immune cell phenotypes of SSc

The immune cell phenotypes of SSc patients based on peripheral blood analysis are shown in Fig. 1. For helper T cells, significantly higher proportions of T cells re-expressed CD45RA (termed TEMRA) ($P = 0.02$) and activated T helper cells ($P < 0.001$) were significantly more common in SSc than in healthy controls. Similarly, the proportion of central memory $CD8^+$ T cells was significantly higher in SSc than in healthy controls.

We also assessed the properties of functional subsets of T helper cells, such as Th1, Th17, Treg and Tfh cells. Among them, the proportions of activated Th1 ($P < 0.001$) and activated Th17 ($P < 0.001$) were higher in SSc, but no differences in Th1, Th17, Treg and Tfh cells were observed between the two groups (Fig. 1).

For B cells, the proportions of IgM memory ($P < 0.001$) and class-switched B cells ($P = 0.02$) were lower, while that of IgD^-CD27^- (double negative) B cells ($P = 0.03$) was higher in SSc than in healthy controls. The proportion of plasmablasts was not different between the two groups.

The proportions of dendritic cells subsets and monocyte subsets were comparable.

In addition, we investigated the change of immunophenotypes in patients who were not treated with immunosuppressive therapy. At baseline, the immune phenotypes except the proportion of activated Th17 were similar to those of patients with immunosuppressive therapy (data not shown). No changes in immunophenotypes were observed after 1 years' observation in 18 patients who were not on immunosuppressive therapy (Supplementary Table S1, available at *Rheumatology* online).

Cluster analysis based on immune phenotype in SSc

Activation and differentiation abnormalities of T and B cells have been described in patients with SSc. However, the clinical efficacy of immunosuppressive therapy in patients with SSc generally seems limited, although a clinically meaningful response has been described in at least some patients [14]. In order to clarify such heterogeneous pathology, we classified SSc patients into subgroups. In this regard, hierarchical cluster analysis of 150 SSc patients suggested that they can be divided into three subgroups. These subgroups are coloured red, green and blue in Fig. 2, and Fig. 3 shows details of their immunophenotypes. In the group shown in blue (which comprised about half of the patients), the proportions of naïve T helper cells and naïve B cells were higher, whereas that of activated helper T cells was not, unlike the high proportion found in the entire group of SSc patients. In other words, about half of the SSc patients exhibited poor immunological abnormalities in the peripheral blood (it can therefore be named the fewer abnormalities group).

On the other hand, the proportion of naïve T helper cells was lower and that of activated T cells was higher in the groups shown in green and red, and abnormal immunophenotypes were noted. In particular, in addition to the

Table 1 Characteristics of participating patients

Variable	SSc patients ($n = 150$)	Healthy control ($n = 30$)	*P*-value
Age, mean (S.D.), years	64.8 (14.4)	59.3 (12.3)	0.07
Gender, % female	91.3	80.0	0.22
Disease duration, mean (S.D.), years	7.2 (8.0)		
mRSS, mean (S.D.)	9.6 (8.5)		
Organ involvement, % of patients			
Interstitial lung disease	41.6		
Pulmonary arterial hypertension	12.8		
Oesophageal manifestations	59.2		
Lower gastrointestinal lesions	10.1		
Joint contracture	9.4		
Digital ulceration	16.4		
Positivity, % of patients			
Anti-nuclear antibodies	89.7		
Anticentromere antibodies	61.8		
Anti-Scl70 antibodies	13.8		
Anti-RNA polymerase III antibodies	10.7		
Anti-U1RNP antibodies	6.2		
Laboratory tests			
CRP, mean (S.D.), mg/dl	1.1 (2.8)		
ESR, mean (S.D.), mm/h	21.9 (25.0)		
IgG, mean (S.D.), mg/dl	1500 (581)		

mRSS: modified Rodnan skin score.

higher proportion of activated T helper cells, a higher proportion of Treg cell was found in the group shown in green (it can therefore be named the Treg-dominant group) compared with the other two groups.

Moreover, the group shown in red had markedly high percentages of TEMRA and activated helper T cells, and the highest degree of immune cell phenotypic abnormality. Another distinct feature of the group shown in red was the high proportion of Tfh cells and plasmablasts, which was not seen in the other two groups (it can therefore be named the Tfh-dominant group).

The clinical features of patients of the three groups according to the immunophenotype are summarized in Supplementary Table S2, available at *Rheumatology* online. While the age and disease duration were comparable among the three groups, progression of skin sclerosis, prevalence of lower gastrointestinal lesions and prevalence of digital ulceration were more common in the Tfh-dominant group, while the prevalence of these involvements was lower in the Treg-dominant group. With regard to the autoantibodies, although anti-RNA polymerase III antibody was more commonly found in the Treg-dominant group, the proportions of anti-centromere antibody, anti-Scl-70 antibody and anti-U1RNP antibody were comparable among the groups. Also comparable were the serological inflammatory findings and serum immunoglobulin.

Microvascular abnormalities according to peripheral blood immune phenotypes

The above results showed that SSc patients could be divided immunologically into three subgroups. Therefore, we next investigated the correlation between these immune cell phenotypes and the morphology of microvasculopathy. The NVC-based scleroderma spectrum abnormalities were observed in 70% of SSc patients.

Of note, comparison of the different NVC patterns ('early', 'active' and 'late' patterns) among the three immune cell phenotype subgroups demonstrated more common progression of microvasculopathy to the 'late' pattern in the Tfh-dominant group ($P = 0.055$), and progression to the 'active'/'late' pattern in the Tfh-dominant group (Fig. 4A, B). The results suggested earlier progression of microvasculopathy in the Tfh-dominant group since the mean disease duration was comparable in the three groups. This tendency was clearer in patients with disease duration of <10 years (Fig. 4C, D). We also compared the mean disease duration according to the NVC pattern. The results showed a shorter mean disease duration in patients of the Tfh-dominant group with the 'late' pattern (Fig. 4E). Furthermore, a linear progression of NVC pattern was noted in the Tfh-dominant group, whereas a slow progression from the 'active' pattern to the 'late' pattern was seen in the other two groups. These results suggest rapid progression of the NVC pattern in the Tfh-dominant group.

Association of microvascular abnormalities and organ involvement

Finally, we investigated the association between NVC patterns and organ involvement using the data of 200 SSc patients, including those evaluated for immune cell phenotype. The clinical background of these patients is

Fig. 1 Differences in immune cell phenotypes between patients with systemic sclerosis and age-sex-matched healthy control subjects

		SSc patients (n = 150)	Healthy control (n = 30)	P-value
CD4+ T cells	Naive	44.7 (16.3)	45.7 (15.8)	0.75
	Central memory	29.9 (14.2)	35.2 (12.7)	0.06
	Effector memory	18.1 (12.0)	15.3 (9.6)	0.23
	TEMRA	**7.1 (11.0)**	**3.8 (6.0)**	**0.02**
	Activated	**8.1 (4.7)**	**6.4 (2.2)**	**<0.001**
CD8+ T cells	Naive	32.0 (17.7)	35.3 (18.6)	0.36
	Central memory	**21.3 (16.6)**	**14.7 (13.7)**	**0.03**
	Effector memory	23.0 (14.3)	20.2 (15.1)	0.33
	TEMRA	23.7 (18.1)	29.8 (16.9)	0.09
	Activated	13.2 (8.1)	12.4 (6.6)	0.61
CD4+ T cells subsets	Th1	22.0 (7.5)	21.6 (6.5)	0.81
	Activated Th1	**2.0 (1.7)**	**1.4 (0.6)**	**<0.001**
	Th17	12.0 (4.9)	11.9 (5.9)	0.94
	Activated Th17	**1.3 (0.9)**	**0.9 (0.4)**	**<0.001**
	Treg	4.6 (1.6)	4.3 (1.4)	0.29
	Activated Treg	1.7 (0.9)	1.6 (0.9)	0.52
	Naive Treg	1.0 (0.6)	0.9 (0.5)	0.70
	Memory Treg	3.7 (1.6)	3.4 (1.2)	0.34
	Tfh	1.0 (0.6)	0.9 (0.4)	0.66
	Activated Tfh	0.3 (0.2)	0.3 (0.2)	0.83
B cells	Naive	**71.5 (13.1)**	**61.2 (16.7)**	**<0.001**
	IgM memory	**13.0 (6.9)**	**21.2 (8.8)**	**<0.001**
	Class-switched	**8.6 (7.6)**	**12.2 (8.2)**	**0.02**
	Double negative	**6.9 (3.6)**	**5.4 (2.4)**	**0.03**
	Plasmablast	4.7 (6.8)	3.2 (4.5)	0.29
Monocytes	Classical	87.5 (6.1)	88.4 (4.4)	0.45
	Non-classical	11.2 (5.8)	10.2 (4.3)	0.38
Dendritic cells	Myeloid	1.3 (1.1)	1.4 (0.6)	0.67
	Plasmacytoid	0.1 (0.1)	0.1 (0.0)	0.16
NK cells	CD16+	**84.5 (15.3)**	**90.8 (10.2)**	**0.03**
	CD16−	**14.8 (15.3)**	**8.3 (10.1)**	**0.03**

The extent of changes, compared with healthy control, is shown in colour

Data are mean (S.D.) of percentages of patients, with levels that were significantly different in the patient group compared with the healthy control group highlighted in colour. The colour shows the ratio of ×0.5 to ×2.0 (blue for decreased; red for increased). *P*-values in the univariate analysis were determined by Student's *t*-test. TEMRA: terminally differentiated effector memory cells; Tfh: follicular helper T cells.

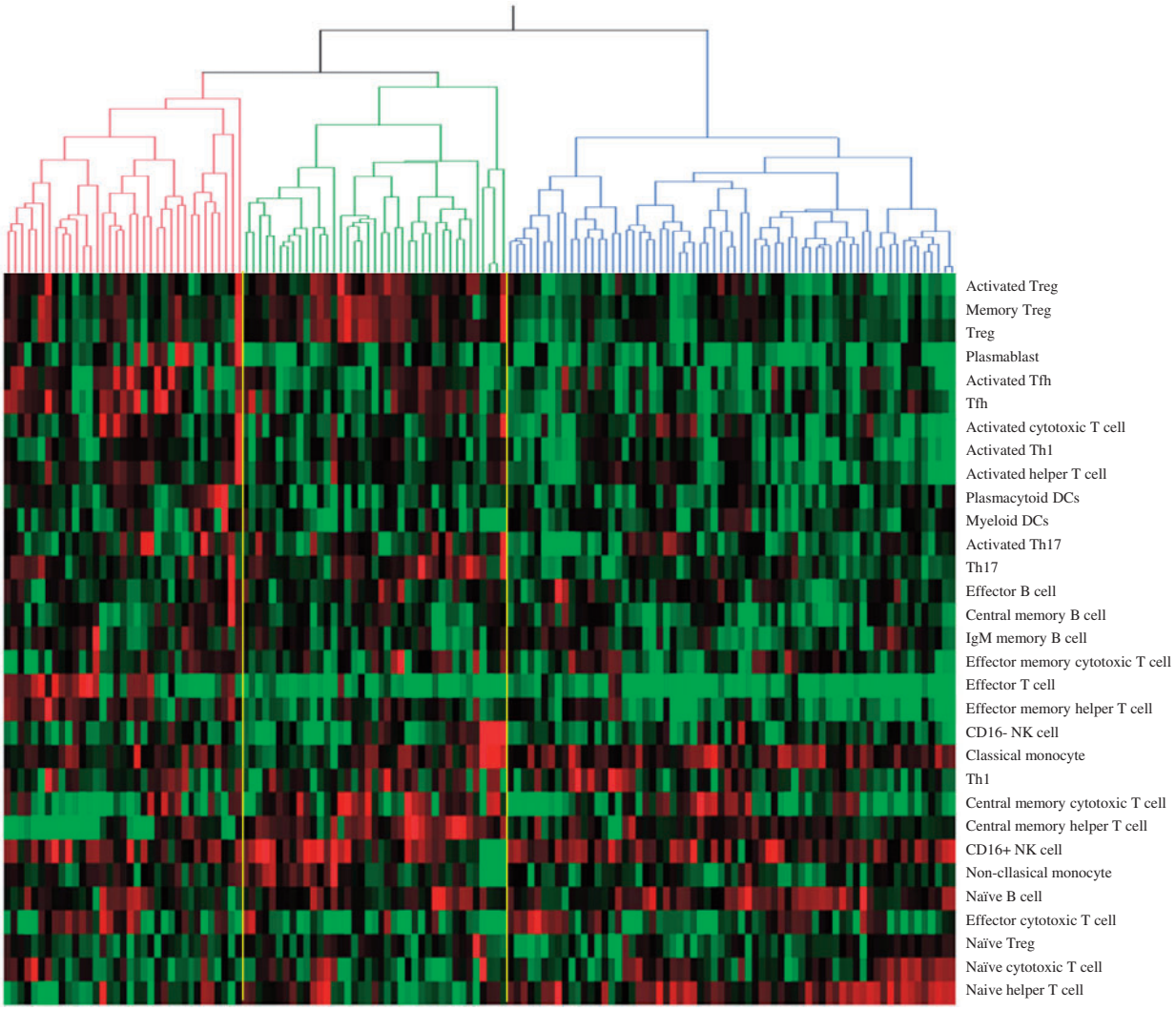
shown in Supplementary Table S3, available at *Rheumatology* online.

Division of the 200 patients into those with and without NVC patterns ('normal' *vs* 'early', 'active' and 'late' patterns) showed differences in mRSS, oesophageal motility disorders ($P = 0.07$) and digital ulcers ($P = 0.06$) (Supplementary Table S4, available at *Rheumatology* online). On the other hand, division of the patients into those with and without 'late' pattern ('normal', 'early' and 'active' *vs* 'late' pattern) showed differences in all organ involvement related to SSc (Supplementary Table S5, available at *Rheumatology* online). In other words, the skin score, presence of oesophageal dismotility and digital ulcers increased linearly with the progression of NVC scleroderma spectrum abnormality, while the likelihood of severe organ involvement, such as ILD and PAH, increased suddenly between the 'active' pattern and the 'late' pattern (Fig. 5B). These results indicate that progression to the late pattern is important for organ involvement in patients with SSc.

Discussion

This is the first study to examine how both microvasculopathy, as assessed by capillaroscopy, and the autoimmune response, evaluated by comprehensive immune

Fig. 2 Results of statistical cluster analysis based on immune cell phenotypes in patients with systemic sclerosis



Hierarchical statistical clustering of scleroderma patients. Subgroups are coloured red, green and blue.

cell phenotypic analysis, are strictly linked in patients with SSc. In particular, the study showed higher proportions of activated Th1 and Th17 cells in SSc patients compared with healthy individuals and the presence of abnormal B cell differentiation in SSc patients.

One of the most important goals of this study was to elucidate immunological and pathophysiological heterogeneity in SSc patients. Our results showed that SSc patients could be subclassified into three groups and each group had unique vasculopathic and clinical characteristics.

The characteristic of the Treg-dominant group was the high proportion of Treg cells. Conflicting results have been reported on the status of Treg cells in peripheral blood and skin of SSc patients, with one study showing high proportion [31] while other studies showed a low proportion [32] or no changes in the proportion of Treg cells [33]. One of the reasons for the inconsistent results on Treg may be the small number of patients in these studies. The present study with 150 cases showed no differences in the proportion of Treg cells in the entire SSc patients though some SSc patients had a higher proportion of Treg cells after classification of the patients into subgroups.

Patients of the Tfh dominant group had markedly high proportions of Tfh cells and plasmablasts, as well as plasmacytoid dendritic cells. These results further suggest the potential involvement of the Type I IFN-B cell axis in the SSc pathology [10, 11]. Furthermore, these patients also had a significantly high proportion of activated T cells compared with other groups. However, contrary to our expectations, there was no substantial difference in the prevalence of autoantibodies between these three groups.

Fig. 3 Statistical cluster analysis based on immune cell phenotypes in patients with systemic sclerosis

		Tfh dominant (red group) (n = 35)	Treg dominant (green group) (n = 39)	Fewer abnormalities (blue group) (n = 66)	P-value
CD4+ T cells	Naive	33.2 (15.4)	38.0 (14.7)	53.9 (11.6)	<0.001
	Central memory	17.2 (14.1)	41.6 (12.1)	29.7 (8.9)	<0.001
	Effector memory	29.0 (15.0)	17.3 (9.0)	13.3 (8.0)	<0.001
	TEMRA	19.7 (16.3)	3.2 (4.1)	3.1 (3.4)	<0.001
	Activated	11.1 (6.2)	8.3 (4.0)	6.2 (3.1)	<0.001
CD8+ T cells	Naive	29.4 (17.0)	29.6 (18.9)	35.1 (17.4)	0.18
	Central memory	17.5 (15.3)	28.1 (17.8)	18.6 (15.0)	<0.001
	Effector memory	24.6 (14.1)	24.9 (17.3)	22.0 (12.9)	0.54
	TEMRA	28.1 (19.0)	17.4 (15.6)	24.3 (17.1)	<0.03
	Activated	16.4 (10.3)	12.9 (7.4)	11.3 (6.8)	<0.01
CD4+ T cells subsets	Th1	21.8 (7.4)	22.6 (7.6)	21.5 (7.4)	0.75
	Activated Th1	2.6 (2.5)	2.1 (1.3)	1.4 (0.8)	<0.001
	Th17	13.1 (4.9)	15.2 (5.6)	9.7 (2.8)	<0.001
	Activated Th17	1.5 (1.0)	1.5 (0.9)	1.1 (0.6)	0.01
	Treg	4.7 (1.8)	5.8 (1.6)	3.8 (0.9)	<0.001
	Activated Treg	1.7 (0.8)	2.2 (0.8)	1.2 (0.5)	<0.001
	Naive Treg	0.9 (0.4)	1.1 (0.8)	0.9 (0.5)	0.03
	Memory Treg	3.9 (1.6)	4.6 (1.6)	2.9 (0.8)	<0.001
	Tfh	1.3 (0.7)	1.0 (0.5)	0.7 (0.3)	<0.001
	Activated Tfh	0.4 (0.2)	0.2 (0.1)	0.2 (0.1)	<0.001
B cells	Naive	65.8 (17.1)	71.7 (10.2)	74.2 (11.6)	0.01
	IgM memory	15.5 (8.6)	12.7 (5.6)	12.1 (6.0)	0.05
	Class-switched	11.9 (11.7)	8.4 (5.2)	7.0 (5.7)	0.05
	Double negative	6.8 (3.6)	7.1 (3.5)	6.7 (3.6)	0.86
	Plasmablast	9.2 (10.1)	3.2 (2.5)	2.2 (1.8)	<0.001
Monocytes	Classical	86.6 (4.7)	84.9 (8.7)	89.7 (3.3)	<0.001
	Non-classical	11.8 (4.3)	13.7 (8.0)	9.1 (3.4)	<0.001
Dendritic cells	Myeloid	1.7 (1.7)	1.1 (0.7)	1.2 (0.8)	0.15
	Plasmacytoid	0.2 (0.2)	0.1 (0.0)	0.1 (0.1)	<0.001
NK cells	CD16+	85.9 (9.2)	79.2 (22.9)	87.2 (8.6)	0.11
	CD16−	13.3 (9.0)	20.1 (22.9)	12.2 (8.6)	0.13

The extent of changes, compared with healthy control, is shown in colour

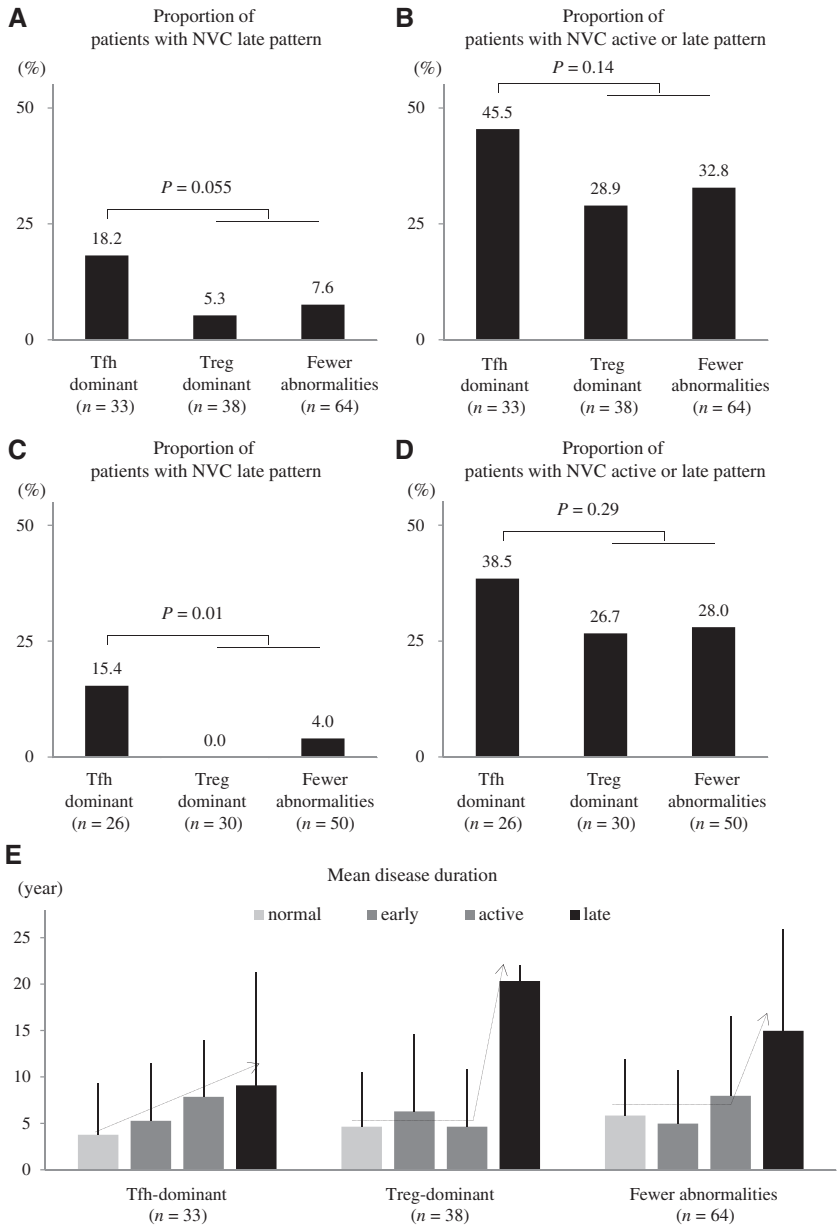
× 0.5 × 2.0

Data are mean (S.D.) of percentage of baseline immunophenotypes of each group. Levels that were significantly different in the patient group compared with the healthy control group are highlighted in colour. The colour shows the ratio between ×0.5 to ×2.0 (blue for decreased; red for increased). P-value in the univariate analysis were determined by one-way analysis of variance. See Fig.1 for definitions.

On the other hand, although there were no differences among subgroups, there were significant differences between SSc patients and healthy controls in terms of the peripheral CD16− NK cells. In addition, the proportion of CD16− NK cells was increased in patients who had ILD, PAH and oesophageal manifestations (data not shown). CD16− NK cells are considered to have high ability to produce cytokines such as IFNγ and TNFα [34]. Thus, CD16− NK cells might contribute to these organ involvements.

Several studies have reported the association of microvascular abnormality with the pathological abnormalities and organ involvement in SSc patients [35]. In the present study, we applied the most commonly used method [26, 28, 36] to assess semi-quantitatively the nailfold capillary abnormality. Our results showed that microvascular abnormality in SSc is associated with organ involvement in SSc patients, which is consistent with the previous studies [4]. Moreover, our study also demonstrated two

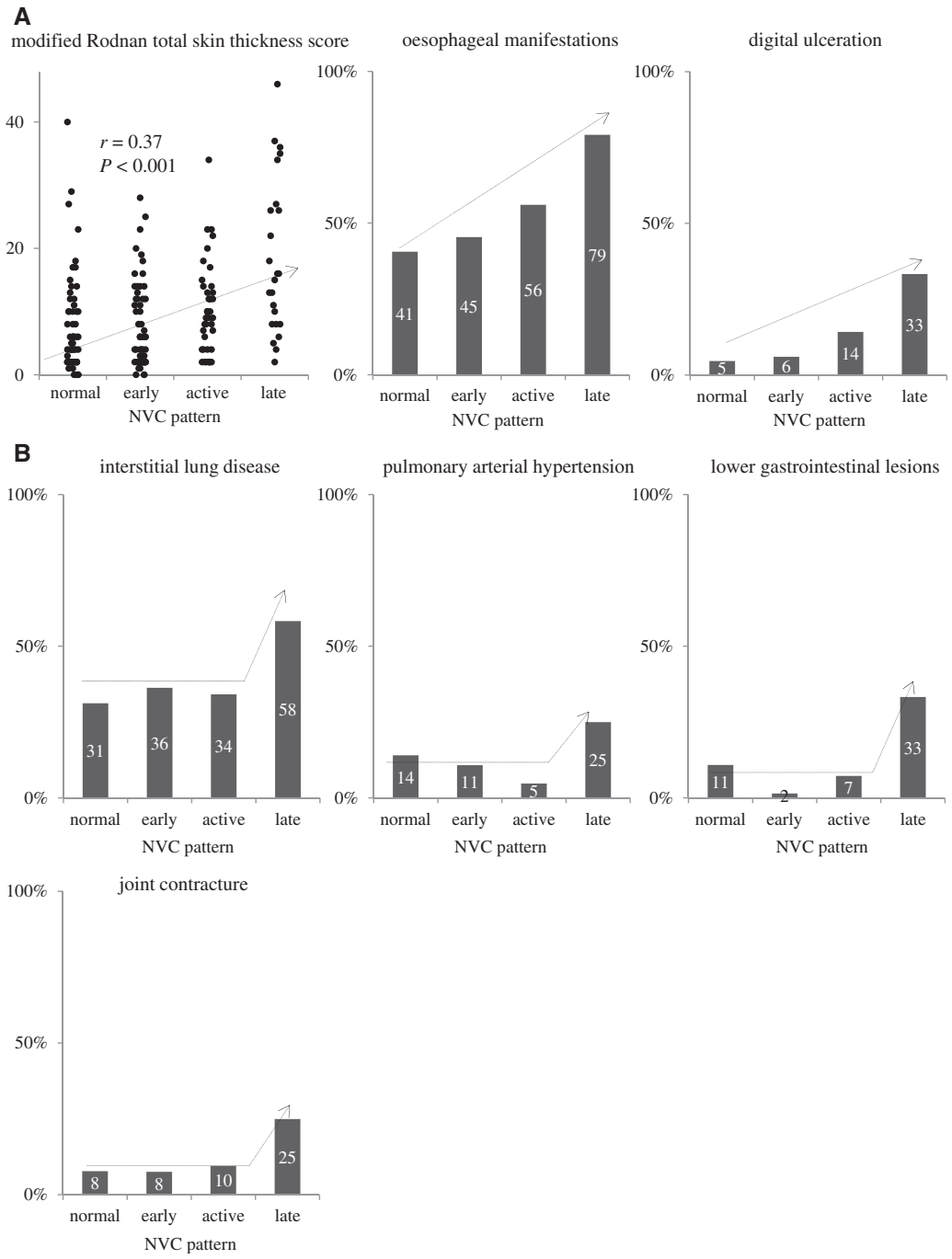
Fig. 4 Nailfold videocapillaroscopy scleroderma spectrum abnormalities in the three subgroups



(**A**, **B**) Proportion of patients with NVC late pattern (**A**) and NVC active or late pattern (**B**). (**C**, **D**) Proportion of patients (with disease duration of <10 years) with NVC late pattern (**C**) and NVC active or late pattern (**D**). (**E**) Mean disease duration in each pattern of nailfold videocapillaroscopy scleroderma spectrum abnormalities.

patterns of organ involvement in SSc patients. One pattern was characterized by a linear increase in organ involvement with advancement of NVC abnormalities. The other pattern showed a rapid increase in organ involvement after NVC abnormality exceeds a certain threshold ('late' pattern). Namely, mild vasculopathy in organs with large vascular beds [37], such as the lungs and lower oesophagus, does not immediately result in organ involvement, whereas mild vasculopathy may result in organ involvement in fingers and the oesophagus. In this respect, attention needs to be paid to the progression of nailfold capillary abnormality to 'late' pattern, since such progression may ultimately progress to lethal organ involvement. It is noteworthy that organ involvement was observed in some patients without NVC changes. This finding suggests that the mechanism(s) of organ involvement may include pathological processes other than vasculopathy.

Fig. 5 Correlations between organ involvement and nailfold videocapillaroscopy scleroderma spectrum abnormalities



(**A**) Correlations between nailfold videocapillaroscopy scleroderma spectrum abnormalities and modified Rodnan total skin thickness score, oesophageal manifestations and digital ulceration. (**B**) Correlations between nailfold videocapillaroscopy scleroderma spectrum abnormalities and interstitial lung disease, pulmonary arterial hypertension, lower gastrointestinal lesions and joint contractures.

We also examined the relationship between microvascular abnormality and immune cell phenotypes. Progression to the 'late' pattern was observed in many patients of the Tfh-dominant group. The disease duration was also shorter in patients of the Tfh-dominant group who progressed to 'late pattern', compared with those of the other groups. Thus, it seems that progression of vasculopathy tends to occur in patients with the most

severe form of immunophenotypic abnormality, suggesting that we need to focus on the development of organ involvement.

From these results, several speculations were raised involving the pathogenesis and therapeutic application. First, the results demonstrated the heterogeneity of immune cell phenotypes in patients with SSc is more than was originally recognized. Of note, nearly half of SSc patients did not show severe immune dysregulation. The molecular targeted therapy for chronic fibrosis may be better than aggressive immunosuppressive therapy for these patients, since they had a slow progression in microvascular damage. On the other hand, the results identified a subgroup with Tfh-plasmablast-dominant phenotype, which was highly associated with the progression of microvasculopathy and concurrent organ involvement. The early intervention using molecular target therapy for T cells or B cells, such as rituximab, might be expected in the patients included in this group.

Our study has the following three limitations. First, we did not evaluate the therapeutic response in each group. In addition, it was a single-centre Asian study with inherent selection bias. For instance, this study included high frequency of the patients who had anti-centromere antibody. Thus, further studies are needed to validate our results in a multicentre prospective study. Second, the results did not verify the causal relationship between vasculopathy and immunophenotypic abnormality. We were unable to demonstrate statistically that advanced vasculopathy results in a high degree of immune cell phenotypic abnormality or that advanced immunophenotypic abnormality is directly linked to significant vasculopathy. It is always difficult to demonstrate such a causal relationship in humans. This finding suggests that the immune cell phenotypic abnormality contributes to microvasculopathy. Third, we focused on immunophenotypic abnormalities by analysing peripheral blood only, but not in the skin and organs. It is important to ascertain that the observed immunophenotypic abnormalities in peripheral blood are also present in different organs.

Despite these limitations, we were successful in detecting abnormal immune cell phenotypes in SSc patients and subclassifying SSc patients. Accumulation of more data should help in the elucidation of pathological mechanisms of SSc as well as in the design of new therapies for SSc [38].

Acknowledgements

The authors thank all medical staff at all institutions for providing the data. The authors thank Narumi Sakaguchi for helping in flow cytometric analysis.

Funding: This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan (JSPS KAKENHI 15K08790, 17K16218), and the University of Occupational and Environmental Health, Japan, through UOEH Grant for Advanced Research.

Disclosure statement: S.K. has received speaking fees from Bristol-Myers, Pfizer, Takeda and Eli Lilly. S.N. has received speaking fees from Bristol-Myers, Sanofi, Abbvie, Eisai, Eli Lilly, Chugai, Pfizer, Takeda and also research grants from Mitsubishi-Tanabe, Novartis and MSD. K.N. has received speaking fees from UCB, Astellas, Mitsubishi-Tanabe and research grants from Mitsubishi-Tanabe and Eisai. V.S. has received research grants from Actelion Pharmaceuticals, Boehringer Ingelheim, Bayer and Hoffmann-La Roche. M.C. has received research grants from Actelion Pharmaceuticals, Bristol-Myers, Celgene Corporation and Boehringer Ingelheim. Y.T. has received speaking fees and/or honoraria from Daiichi-Sankyo, Astellas, Eli Lilly, Chugai, Sanofi, Abbvie, YL Biologics, Bristol-Myers, Glaxo-Smithkline, UCB, Mitsubishi-Tanabe, Novartis, Eisai, Takeda, Janssen, Asahi-kasei and has received research grants from Mitsubishi-Tanabe, Bristol-Myers, Eisai, Chugai, Takeda, Abbvie, Astellas, Daiichi-Sankyo, Ono, MSD, Taisho-Toyama. The other authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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産業ストレス研究(Job Stress Res.), 26(3), 305-306(2019)

【特集　産業ストレス2040】

産業医学における睡眠研究の未来

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2019年４月から政府による「働き方改革関連法」が施行され，いま改めて日本人の働き方に対する考え方が問われている。この法律の成立には，多くの過労死・過労自殺を教訓としたことは言うまでもないが，これよりも前から施行されていた「労働時間等の設定の改善に関する特別措置法」「過労死等防止対策推進法」「自殺対策基本法」などの法律は，数字上は期待するほど過労死・過労自殺を抑止する力はなかった。

何故，期待ほどの成果が上がらなかったのであろうか。我が国の多くの職場においては業績重視の体質を変化させることができず，また働きやすく労働意欲を高めるように環境を整備する余裕がなかったことが大きな要因であることは言うまでもないが，労働に伴う休息や睡眠のあり方に目をあまり向けてこなかったことも看過できない一因である。そこで本稿では労働と表裏一体にある睡眠，特に睡眠覚醒リズムに目を向けて，これまでの成果と今後の産業領域が目指すべき産業睡眠医学研究の方向性について私見を述べる。

日本を含む先進諸国は24時間社会に突入して久しいが，我が国では多くの労働者が常日勤以外の時間帯で仕事をしている。例えば，夜勤・交代勤務に従事している労働者は約３割存在し，この割合は年々増加してきた。その内，深夜業従事者は労働者全体の21.8%にも上る[1]。こうした交替勤務者では睡眠覚醒リズムが乱れ，睡眠障害を発生することが多くなる（概日リズム睡眠障害）。この場合，睡眠障害だけでなく，慢性疲労や胃腸障害などを併発することが多い。長い期間交替勤務に従事すると，がんに罹患しやすいことも知られている。

概日リズムが乱れる他の仕事上の要因としては海外出張も挙げられるであろう。日系企業の海外進出に伴い，海外出張も増加していると推測されるが，海外出張を頻繁に行う者では時差ぼけによって睡眠覚醒リズムが乱れる。海外出張に伴う環境の変化や衛生状態も異なることから身体的負荷は多くなる[2]。

このように時間帯が異なる環境で働いた結果，睡眠覚醒リズムの乱れが誘発され健康を害することは，長年産業医学領域で研究されてきた。しかし，近年は常日勤者であっても睡眠負債や社会的時差ぼけ（Social jetlag）により健康障害が生じることが判明し，注目を集めている。社会的時差ぼけとは，手短に言えば平日の睡眠時間帯と休日の睡眠時間帯の中央の時間に大きなずれがあることを指す（図）。多忙な労働者では残業などによってこの社会的時差ぼけが発生しやすい状況にあるが，問題はこのような習慣に慣れ親しんでしまい，定着化させてしまうことである。これまでの研究では，社会的時差ぼけが２時間以上になると抑うつ，不安，肥満，メタボリック・シンドローム，糖尿病などの疾患が急増することが報告されており，パフォーマンスや学業成績の低下，攻撃性の増加などに

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も影響することも示唆されている[3]。では，社会的時差ぼけ状態の労働者はどの程度存在するのであろうか。我が国の日勤労働者を対象とした研究では，約３割が１時間以上の社会的時差ぼけを有することが報告され[4]，我々独自の調査でも１時間以上の社会的時差ぼけを有する者は４割程度存在した[5]。社会的時差ぼけは今や看過できない産業衛生上の問題である。

以上挙げたような労働者だけを各々単純に合計しただけでも，我が国の約６割近くの労働者に睡眠覚醒リズム障害が生じている可能性があることが伺える。こうした背景から，産業睡眠医学領域では，より一層時間生物学的要素を取り入れた研究が推進される必要があると言える。幸いにして，最近ではフィットビット等のウエラブルデバイスが開発され，個人の詳細な客観的な睡眠データが追跡可能になり，このようなデバイスを用いた睡眠研究が少しずつ報告されるようになった。これらのデータは比較的人工知能（AI）やIoT（物のインターネット）と親和性が高いことから，今後20年は，IoTから吸い上げた個人の精密な生体ビッグデータと健診データや医療情報とを紐付けてAIで解析する研究が進むであろう。恐らく，こうしたデータの解析結果から個人のテーラーメイドの睡眠コーチングなども可能になると思われる。

産業領域における睡眠研究の未来は明るいかもしれない。

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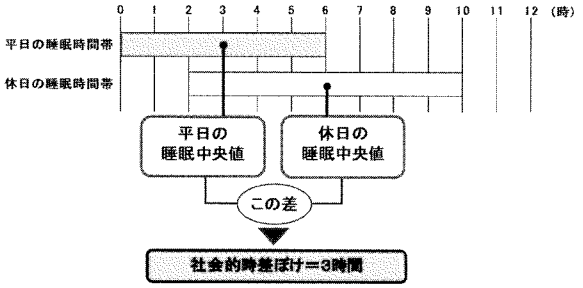

図　社会的時差ぼけの具体例．この場合，平日は午前０時に入眠し，午前６時に起床．休日は午前２時に入眠し，午前10時に起床．実際の計算は睡眠負債も考慮する．

（受付　2019年５月28日，受理　2019年５月29日）

睡眠が労働に果たす役割

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はじめに

日本人は世界的に見ても睡眠時間が短い国民である．経済協力開発機構(Organisation for Economic Co-operation and Development: OECD)が発表した2018年のデータによれば，日本人の1日の平均睡眠時間は7時間22分と，参加30カ国中，最も短い．また，NHKが1960年から5年ごとに行っている「国民生活時間調査」では，日本人の睡眠時間は調査開始以来，一貫して短縮しており，時間にすれば平日は平均して毎年約40秒短くなっていることが示されている．睡眠時間を削って活動(労働)時間を長くすれば，生産性は向上し，競争社会の勝者となれるのだろうか．近年の睡眠科学による発見は，これまで信じられてきた「根性論」とは異なる結果を示している．睡眠をおろそかにすれば，睡眠不足や睡眠の質の低下が引き起こされ，日中の眠気の増加や疲労の蓄積が起こり，メンタルヘルス不調や生活習慣病の発症が加速化される．産業の現場では，集中力の低下による生産性の低下，労災，病欠やプレゼンティーズムの増加，企業の医療費の負担が増える．

本稿では，働く人々の睡眠の実態を主に睡眠時間の観点から概観するとともに，睡眠と表裏一体にある労働との関係について解説する．そして，労働者の短時間睡眠や睡眠不足を解消するヒントを提示する．

日本人労働者の睡眠時間の実態と，世界における位置付け

上述したNHKの国民生活時間調査には，対象者の就労の有無も調査項目に含まれており，有職者のみの睡眠時間も抽出可能である．1995年以降の計5回の調査結果を**図1**[1]に示す．本図は男女合算のデータであるが，平均睡眠時間は平日，土曜，日曜ともに経年的に減少している．平日は過去20年で19分，土曜は12分，日曜は16分減少している．

一方，総務省統計局が5年おきに実施している「社会生活基本調査」では，就労男女の平日，土曜と日曜の睡眠時間のデータを収集している．1976年から2016年までのわが国の就労男女の平日と休日の平均睡眠時間のデータを**図2**[2]に示す．本図が示すとおり，男女ともに平均睡眠時間は平日，日曜ともに経年的に減少している．1986年までは土曜が勤務日であったため，睡眠時間は平日とほぼ同じように推移しているが，それ以降はほぼ横ばいである．男性は過去42年間で平日の平均睡眠時間が約49分，土曜が約15分，日曜が約36分短縮し，女性では平日の睡眠時間が約36分，土曜が約8分，日曜が約22分短縮している．これらの2つの調査を並行して眺めると，調査方法や対象が異なっても睡眠時間の減少の推移がほぼ同じであることは注目に値する．

その他，社会生活基本調査から読み取れるのは，女性の方が男性よりも一貫して平日，土曜，日曜ともに睡眠時間が短いことである．特

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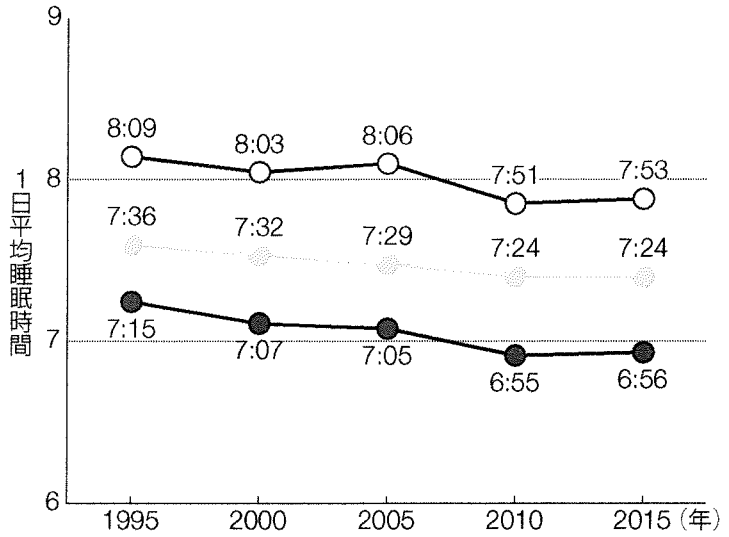

図 1　就労者の曜日別の睡眠時間の推移

●平日，◌土曜日，○日曜日．文献 1)〔2015 年国民生活時間調査報告書 p 47「4. 食事と睡眠(1)睡眠」〕より作成．

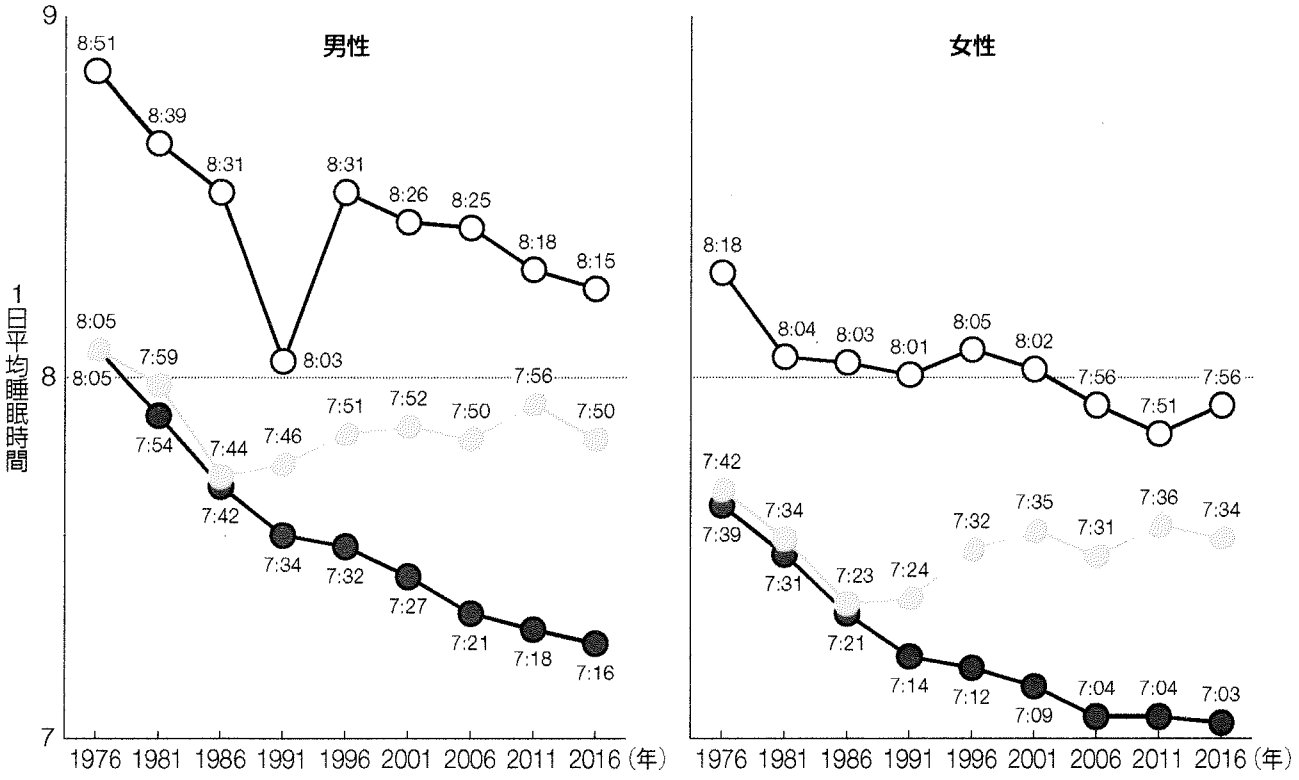

図 2　就労男女別・曜日別の睡眠時間の推移

●平日，◌土曜日，○日曜日．　　(文献 2 より作成)

に，女性は 45 歳を超えると平均睡眠時間が 7 時間未満となり，50 歳代後半までその傾向が持続する(**図 3**)[2]．就労女性は仕事の他，家事，育児，介護などの負担が多く，このため睡眠時間が短くなることが理由の一つと考えられる．一方，女性は睡眠障害や更年期障害が増加し始

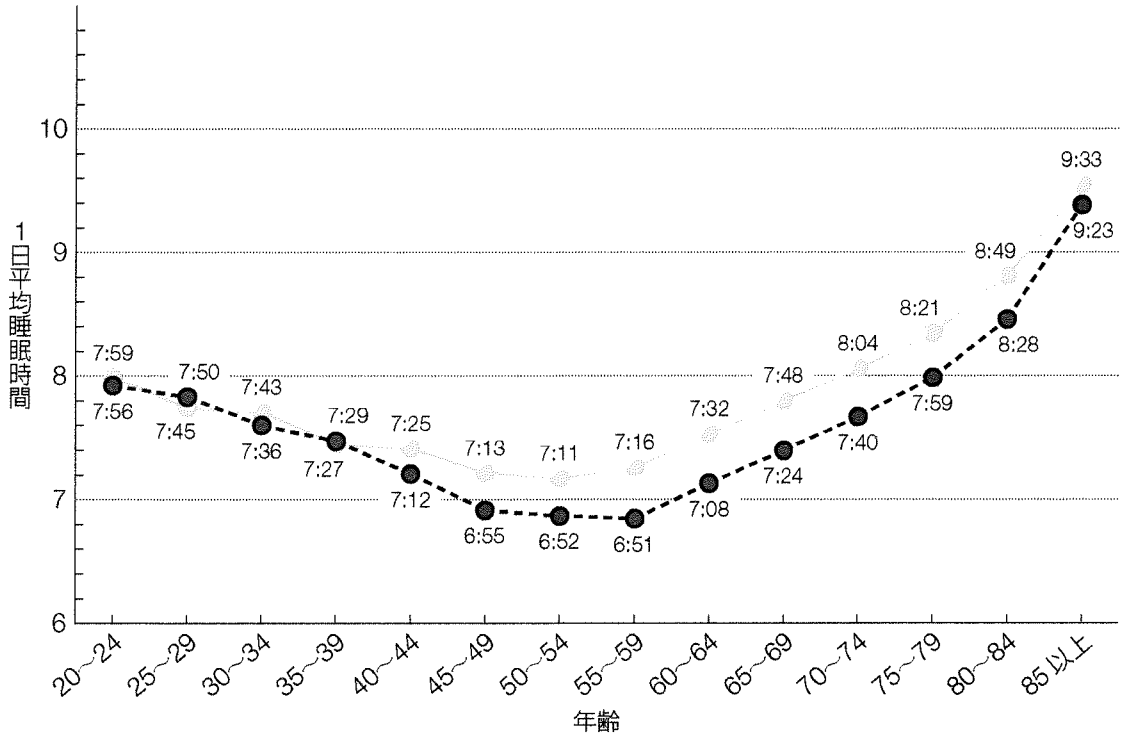

図3　就労男女別・年齢別の睡眠時間(週全体から算出)

男性，-●-女性．　　(文献2より作成)

める年齢であるため，この年齢層においては労働衛生上の配慮が必要と思われる．

女性の睡眠時間が男性よりも短いことは，他の国々と比較しても日本の特徴であるといえる．2018年のOECDのデータを基に作成した16カ国の就労男女別の睡眠時間を**図4**[3]に示す．参加30カ国中，女性の方が睡眠時間が短い国はわずか5カ国(日本，インド，スペイン，エストニア，イタリア)で，最も差があるのは日本とインド(共に14分)，ついでスペイン(9分)，エストニア(4分)，イタリア(2分)である．しかし，睡眠時間が男女ともに短い国は日本のみである．なお，女性の方が睡眠時間が長い国は長い順にスウェーデン(40分)，オランダ，アイルランド(共に18分)である．

以上から，日本の就労者は世界的に見ても睡眠時間が短く，年々短縮し，特に男女ともに40歳から50歳代にかけて短時間睡眠であるが，女性ではその傾向が顕著であるといえる．

労働時間と睡眠時間のバランスと健康・安全

1．勤務日における労働者の1日の時間の使い方

日本人労働者の睡眠時間はなぜ短くなるのであろうか．その最大の原因の一つとして疑われるのが長時間労働や過度の残業である．OECDのデータでは，日本人労働者の労働時間は年々減少していることが報告されているが，日本のデータにはサービス残業や社外での労働時間が含まれないことや，非正規雇用やパートタイムなどが増加したことによる実労働時間数の減少などが大きく影響しているものと考えられる．

労働者の勤務日の1日の典型的な時間の使い方の例を**図5**に示す．1日は24時間であることから，労働時間を8時間とすると，昼休みが1時間，通勤が1時間，夕食・お風呂・休憩・運動・その他が4時間と仮定され，残りは10時間となる．睡眠がこの10時間内に含まれる

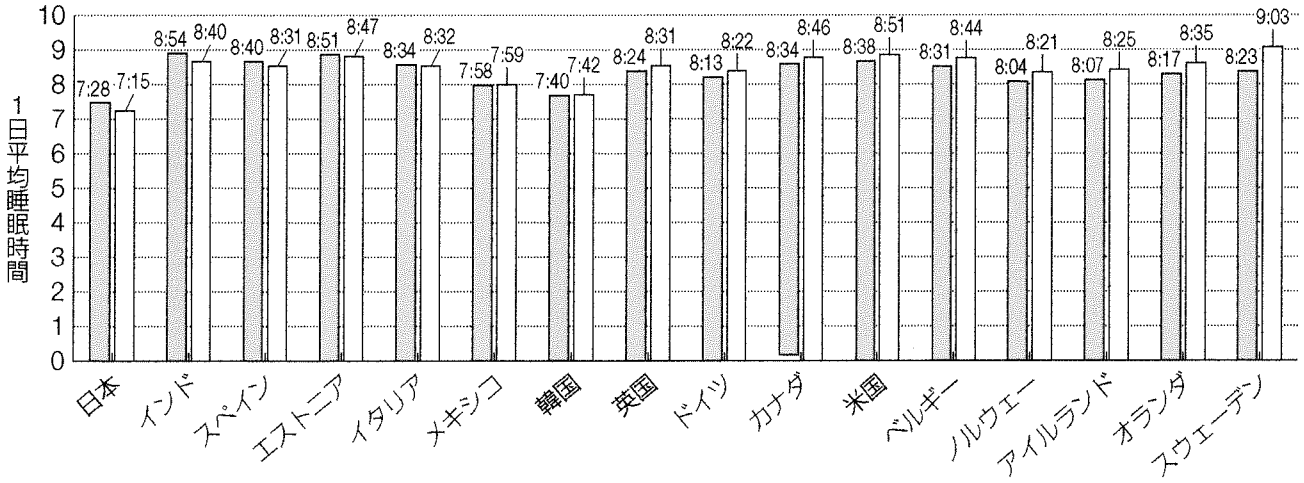

図 4　OECD の就労男女の 1 日平均睡眠時間

男性，女性．　（文献 3 より作成）

とすると，8 時間の睡眠を確保するためには 1 日残業 2 時間まで(**図 5** の右下)，7 時間の睡眠を確保するためには 1 日残業 3 時間まで，6 時間の睡眠を確保するためには 1 日残業 4 時間までとなる．残業時間が 1 日 6 時間を超えると睡眠は 4 時間しか確保できない計算となる．月の残業が 80～100 時間以上，すなわち，1 日 12 時間以上の労働が続くと過労死の頻度が高まることを考えると，労働者が健康を維持するためには最低 6～7 時間の睡眠が確保される必要がある．

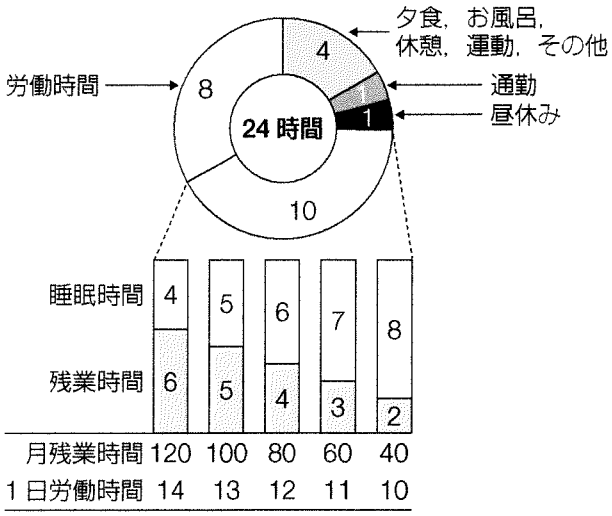

図 5　働く人の勤務日における典型的な 1 日の例

2. 労働時間と睡眠時間のバランスとメンタルヘルス

筆者らは，前項で述べた 1 日の時間の使い方を踏まえて，中小企業労働者約 2,600 人を対象にして，労働時間と睡眠時間のバランスの観点から健康と安全との関連を検討した[4]．まず，労働時間と睡眠時間そのものと抑うつの関連では，労働時間が 6 時間以上 8 時間未満の労働者(参照群)と比べると，労働時間が 8 時間より多く 10 時間以下の者で抑うつ自己評価尺度(CES-D)の得点が 16 点以上(抑うつあり)の調整オッズ比は 1.03 (95% 信頼区間 (CI) 0.85～1.26)，10 時間より多い者は 1.37 (95%CI：1.02～1.83) であり，長時間労働の抑うつに対する直接効果は有意ではあるが，それほど大きいものとは認められなかった．一方，睡眠時間と抑うつの関連は，睡眠 6 時間以上 8 時間未満の労働者(参照群)に比べて，睡眠 6 時間未満の者で CES-D 得点が 16 点以上の者の調整オッズ比は 1.43 (95%CI：1.19～1.71)，睡眠 8 時間以上の者で 0.96 (95%CI：0.62～1.47) であり，短時間睡眠の者で抑うつのオッズが有意に上昇した．

次に，労働時間と睡眠時間の組み合わせが抑

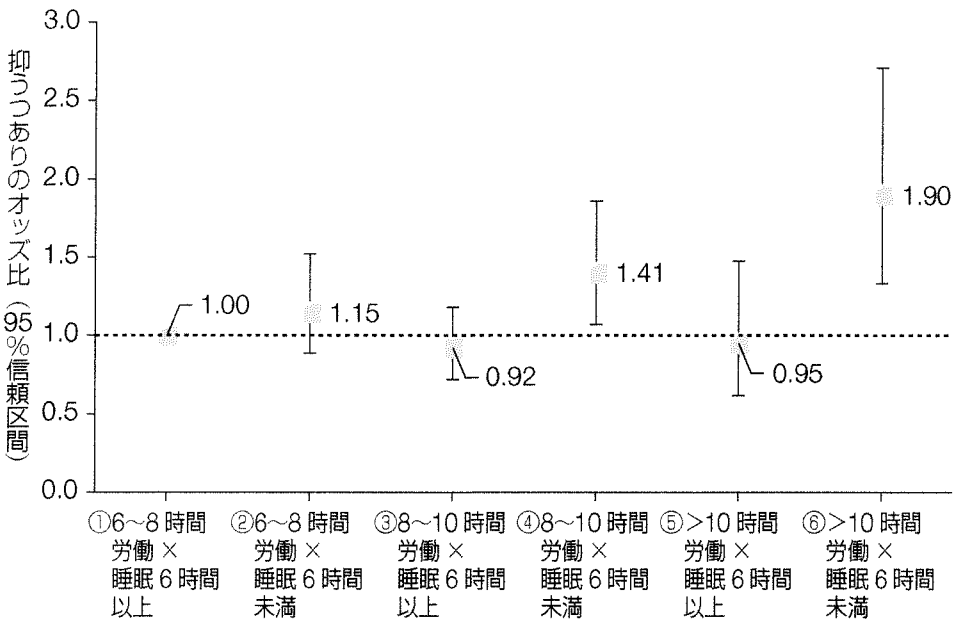

図6　労働時間と睡眠時間の組み合わせと抑うつの関連

（文献4より作成）

うつに対してどのように関連するかを検討した（**図6**）[4]．組み合わせとしては，上記の3とおりの労働時間と，2とおりの睡眠時間をかけ合わせ，計6つの群となった．具体的には，**図6**[4]の左側から順に，①労働6時間以上8時間未満かつ睡眠6時間以上（この群を「参照群」とする），②労働時間が6時間以上8時間未満かつ睡眠6時間未満，③労働時間が8時間より多く10時間以下かつ睡眠6時間以上，④労働時間が8時間より多く10時間以下かつ睡眠6時間未満，⑤労働時間が10時間より多くかつ睡眠6時間以上，⑥労働時間が10時間より多くかつ睡眠6時間未満である．

結果，労働時間が長くなればなるほど抑うつ労働者は増加するが，増加するのは不十分な睡眠時間（6時間未満）の者に限られ（上記④と⑥），睡眠6時間以上確保している者は抑うつが増加しない（上記③と⑤）ことが判明した．

近年，労働時間とうつ病の発症について7つのコホート研究をまとめたメタ分析が報告[5]されたが，残業時間自体はうつ病のリスクを高めないという結果であった〔残業なしに対して，残業ありでうつ病のリスク比が1.075（$p>0.05$）〕．これには上記のように睡眠時間が十分に取れている者も含まれているため，残業によって睡眠不足に陥って，その結果，うつ病を発症した者が過小評価されている可能性が考えられる．極端な長時間労働も危険因子であることには間違いないが，長時間労働による睡眠不足への直接影響も同等に重要である．

3．労働時間と睡眠時間のバランスと労働災害

上記の抑うつの関連と同じように，筆者らは労働時間と睡眠時間の組み合わせと，労災の有無の関連について検討した（**図7**）[6]．その結果，労災については労働時間の影響も強いが，⑥の労働時間が10時間より多くかつ睡眠6時間未満の者で顕著に労災が増加することが示された．

以上から，労働時間か睡眠時間のどちらかに注目するのではなく，労働者が働く一日においてどのような時間の使い方をしているのかを精査しない限り，真の意味で健康の維持・増進は達成できないと考えられる．

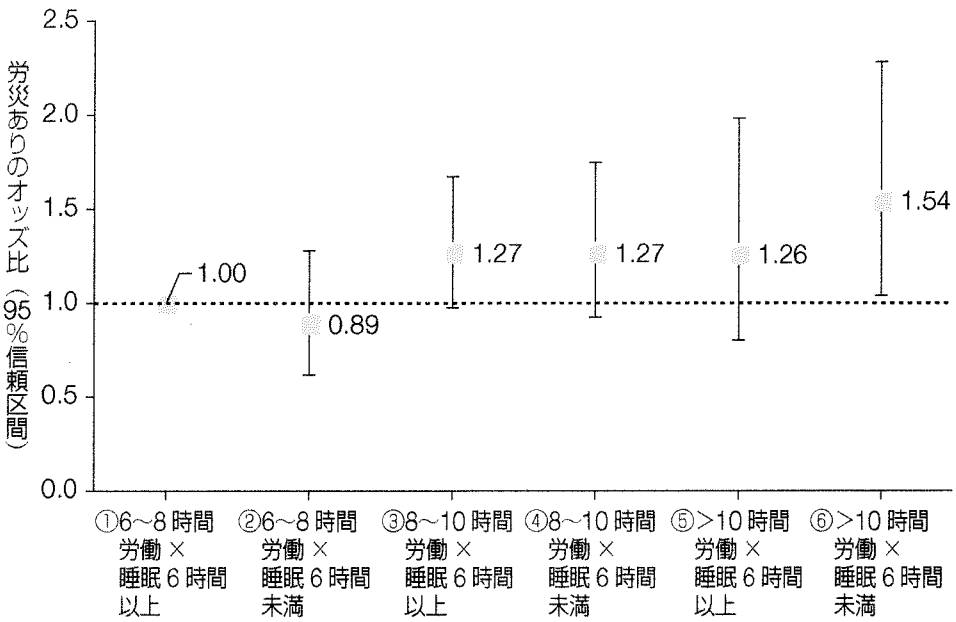

図7　労働時間と睡眠時間の組み合わせと労災の関連

（文献6より作成）

週末の寝だめ（社会的時差ぼけ）の健康影響

働く人々は平日働いている時間が忙しいため，どうしても睡眠時間が不足がちになる．週単位での仕事の場合，平日5日働いて週末2日休みとなるが，この場合，週末に朝寝坊をして平日の睡眠不足を解消しようとする．このような睡眠行動を当たり前のように行っている労働者も多いと思われるが，近年，この週末朝寝坊が思いのほか健康に悪影響がある可能性が示されている．専門的には，平日と休日の睡眠時間帯がずれることによって生じる不調を「社会的時差ぼけ」と呼び，この社会的時差ぼけが睡眠の質の低下ばかりでなく，メタボリック・シンドローム，肥満，抑うつなどの危険因子として注目されている．参考までに，社会的時差ぼけと時差による時差ぼけ（通常の時差ぼけ）の健康影響の差異を**表1**に示す．

次に，社会的時差ぼけの具体例を**図8**に示す．例えば，平日の就寝時刻を午前0時とし，起床時間を午前6時とする．一方，休日の就寝時刻を午前2時とし，起床時間を午前10時とすると，それぞれの中央の時刻（睡眠中央時刻）は午前3時と午前6時となる．社会的時差ぼけの大きさを計算するには，この睡眠中央時刻の差を求める．この例の場合は，3時間である．ただし，社会的時差ぼけの計算は，厳密には，就寝時刻は布団やベッドに入った時間ではなく眠りに実際に入った時刻，起床時刻は目が覚めた時刻から計算される．

表1　社会的時差ぼけと通常の時差ぼけの差異

	社会的時差ぼけ	時差による時差ぼけ
	平日と休日の睡眠時間帯のずれによって生じる不調	数時間以上の時差がある地域間を飛行機などで短時間で移動した際に起こる心身の不調
体内時計の乱れ	あり	あり
時差	なし	あり
影響期間	長期的・永続的	短期的・一過性
健康影響	中等度～大	現地時間に順応後は最小
関連疾患など	うつ病，心疾患，糖尿病，メタボ，肥満，パフォーマンス低下など	一過性の睡眠障害，疲労，食欲不振，日中の眠気，胃腸症状，集中力低下など

図8　社会的時差ぼけ（平日と休日の睡眠時間帯のずれ）の例

日本人労働者を対象とした研究では，社会的時差ぼけが1時間未満の者と比べると，2時間以上の者でメタボリック・シンドロームの調整オッズ比（aOR）が1.92（95%CI：1.01～3.67）であることが報告されている[7]．一方，筆者らが国内の227の日勤の企業従業員約7万人を対象に希死念慮について検討したところ，社会的時差ぼけが1時間未満の者に比べると，1時間以上2時間未満の者で希死念慮のaORが0.94（95%CI：0.85～1.04），2時間以上3時間未満のaORが1.20（95%CI：1.04～1.37），3時間以上のaORが1.97（95%CI：1.57～2.47）であった．2つの研究結果から，2時間以上の社会的時差ぼけには睡眠週間の改善が必要であるといえよう．

おわりに

本稿では働く人々の睡眠時間に着目し，日本人労働者の睡眠時間の推移ならびに睡眠時間とメンタルヘルス・労災の関連，近年注目されている社会的時差ぼけの健康影響について解説した．労働者が健康で生き生きと働くためには，十分な睡眠時間の確保と睡眠の規則性が保てるよう家庭環境や労働環境を整備する必要がある．わが国が「睡眠危機社会」を脱出するためには，今後，個人だけでなく企業や組織，行政機関も睡眠を改善する施策に積極的に取り組んでいくことが期待される．

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産業ストレス研究(Job Stress Res.), **26**(3), 335-339(2019)

【小特集　古典を知る】

仕事の要求度—コントロールモデル

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キーワード

Occupational stress model（職業性ストレスモデル）, Job strain model / job demands-control model（仕事の要求度—コントロールモデル）, Demand-control-support model（要求度—コントロール—社会的支援モデル）, Job strain（仕事のストレイン）, Iso-strain（孤立ストレイン）, Stress management（ストレスマネジメント）

ポジションペーパー

Karasek, R. A.: Job demands, job decision latitude, and mental strain: implications for job redesign. Admin. Sci. Quart., **24**, 285-307 (1979)

Johnson, J. V. and Hall, E. M.: Job strain, work place social support, and cardiovascular disease: a cross-sectional study of a random sample of the Swedish working population. Am. J. Public Health, **78**, 1336-1342 (1988)

ポジションペーパーの要約（歴史的意義を含む）

- Karasek（1979）は，仕事の要求度—コントロールモデルを初めて提唱した。
- これまでの疫学研究では別個に扱われてきた「仕事の要求度」と「仕事の裁量度」を融合し，その交互作用から労働者の精神的緊張を予測しようとした。
- 仕事の要求度を「作業負荷の遂行，予期しない仕事，対人葛藤などの心理的なストレス要因となるもの」，仕事の裁量度を「その人が自身の職務を潜在的にコントロールできる度合いで，意思決定権と技能の活用度によって構成されるもの」と定義した。
- 仕事の要求度が高く，仕事の裁量度が低い状況を「仕事のストレイン」と呼んだ。
- 仕事の要求度と仕事の裁量度の高低の組み合わせから，「高ストレイン・ジョブ（高要求度—低裁量度：仕事のストレインに相当）」，「能動的ジョブ（高要求度—高裁量度）」，「受動的ジョブ（低要求度—低裁量度）」，「低ストレイン・ジョブ（低要求度—高裁量度）」の４つの象限を構成し，高ストレイン・ジョブに該当する労働者は，精神的緊張のリスクが最も高いことを報告した。
- JohnsonとHall（1988）は，仕事の要求度—コントロールモデルに「職場の社会的支援」という新たな軸を追加し，３次元に拡張した，要求度—コントロール—社会的支援モデルを初めて提唱した。
- 職場の社会的支援の高い状況を「集団的」，低い状況を「孤立的」と呼び，高ストレイン・ジョブで孤立的な状況に置かれている労働者は，その対極にある労働者に比べて，冠動脈疾患の有病オッズ比がおよそ２倍であることを報告した。

解説

仕事の要求度—コントロールモデル（Job strain model / Job demands-control model）は，アメリカの産業・労働社会学者Karasekによって，ストレスマネジメントの理論モデルとして提唱された。本モデルが提唱される以前の1940年代から，職場を対象とした事例報告では，仕事の要求度（job demands）と仕事の裁量度（job decision latitude）の交互作用（組み合わせ）が，労働者の精神的緊張や職場組織のひずみを把握するのに重要であるということが言われていたが，1979年にAdministrative Science Quarterly誌に掲載された本論文[1]は，このような交互作用の考え方を疫学研究に導入した初めての論文と位置づけられている。

実際，本モデルが提唱される以前の疫学研究では，仕事の要求度に着目する流派と仕事の裁量度に着目する流派が存在し，いずれの流派も労働者の精神的緊張や疾病との関連について，一貫した知見を見出すことができていなかった。Karasekは，その理由を「それぞれの流派が，互いにもう一方の流派の考え方を排除してきた（すなわち，仕事の要求度に着目していた流派は，仕事の裁量度を含めた議論を行わず，仕事の裁量度に着目していた流派も，仕事の要求度を含めた議論を行わなかった）ためである」と考え，職場環境を「仕事の要求度」だけ，あるいは「仕事の裁量度」だけ，といった1つの軸で捉えるのではなく，「仕事の要求度」と「仕事の裁量度」という2つの軸に明確に区別し，その交互作用を見ることが重要だと主張した（このような主張をする契機となったのが，「管理職と製造ライン作業者は，ともに要求度の高い業務に就いているにもかかわらず，両者の間で仕事満足度に有意な差が認められ（管理職は高く，製造ライン作業者は低い），仕事の要求度は，この差を説明することができなかった」とする先行研究の知見であり[2]，本研究では測定されなかった仕事の裁量度によって，この差を説明できるのではないかと考えたためである）。

この主張に基づき，Karasekは，仕事の要求度と仕事の裁量度の交互作用から労働者の精神的緊張を予測する，仕事の要求度—コントロールモデルを提唱した。本モデルは，職業性ストレスの理論モデルとして紹介されることが多いが，厳密には，仕事の要求度を「個人に活動を起こす潜在エネルギーを与えるもの」，仕事の裁量度を「潜在エネルギーを活動エネルギーに変換し，放出するのを調整するもの」と捉えた，言わば，職場環境に基づいたストレスマネジメントの理論モデルであり，何も活動を起こすことができなかったり，仕事の裁量度が低く，エネルギーを放出することができなかった場合に，精神的緊張という形で兆候が現れると考えている（そのため，本稿の冒頭でも，厳密に「ストレスマネジメントの理論モデル」として紹介した）。本モデルでは，仕事の要求度を「作業負荷の遂行，予期しない仕事，対人葛藤などの心理的なストレス要因となるもの」，仕事の裁量度を「その人が自身の職務を潜在的にコントロールできる度合いで，意思決定権（decision authority）と技能の活用度（intellectual discretion / skill discretion）によって構成されるもの」と定義し，これらを合成したもの（とくに，仕事の要求度が高く，仕事の裁量度が低い状況）を「仕事のストレイン（job strain）」と呼んだ。更に，仕事の要求度と仕事の裁量度の高低の組み合わせから4つの象限を構成し，（1）仕事の要求度が高く，仕事の裁量度が低い（仕事のストレインに相当し，労働者の精神的緊張を予測する）職務を「高ストレイン・ジョブ（high strain job）」，（2）仕事の要求度と仕事の裁量度がともに高い（業務上外における労働者の新たな行動パターンを生み出すと予測される）職務を「能動的ジョブ（active job）」，（3）仕事の要求度と仕事の裁量度がともに低い（全般的な活動や問題解決の活動を低下させると予測される）職務を「受動的ジョブ（passive job）」，（4）仕事の要求度が低く，仕事の裁量度が高い職務を「低ストレイン・ジョブ（low strain job）」と呼んだ（図1）。また，本モデルには，構成した4象限の中に2種類の交互作用を想定した対角線が描かれている。このうち，仕事の要求度と仕事の裁量度の高低が互いに異なっている領域（すなわち，高ストレイン・ジョブと低ストレイン・ジョブが含まれる領域）に描かれた対角線は「解消されない緊張（unresolved strain）」の度合いを表すものとし，仕事の要求度と仕事の裁量度の高低が互いに一致している領域（すなわち，能動的ジョブと受動的ジョブが含まれる領域）に描かれた対角線は「活動レベル（activity level）」の度合いを表すものとされた。

尚，本論文では，アメリカとスウェーデンの労働者データ（前者は横断データ，後者は縦断データ）を用い，上記の4カテゴリーと精神的緊張（疲労，抑うつ）

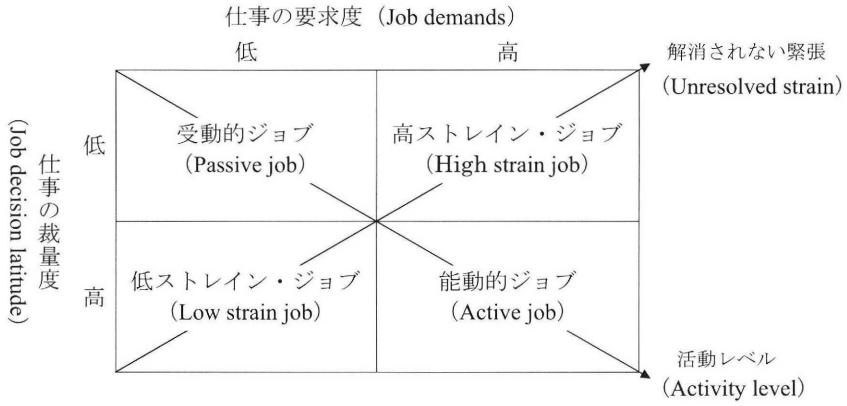

図１．仕事の要求度—コントロールモデル

およびその代替指標（年５日以上の疾病休業，精神安定剤や睡眠薬の服用，仕事に対する不満）との関連を検討し，いずれの指標においても，高ストレイン・ジョブに該当する労働者は，そのリスクが最も高かったことが報告されている。

その後の変遷

１．理論モデルの拡張（要求度—コントロール—社会的支援モデルの提唱）

仕事の要求度—コントロールモデルが提唱された後，高ストレイン・ジョブに該当する労働者は，冠動脈疾患の発症リスクが高いという知見が数多く報告されたが，同時に，本モデルは「仕事の要求度」と「仕事の裁量度」以外の重要な職場特性に着目していないという批判が起きていた。とくに，先行研究では，職場における孤立が労働者の健康に影響を及ぼすことや，職場の社会的支援が仕事のストレインによる労働者への健康影響を緩和する（すなわち，緩衝効果（buffering effect）がある）という知見が認められていたことから，アメリカの社会・行動科学者JohnsonとHallは，仕事の要求度—コントロールモデルに「職場の社会的支援」の軸を追加し，３次元に拡張した，要求度—コントロール—社会的支援モデル（Demand-control-support model）を提唱し，1988年にAmerican Journal of Public Health誌に掲載された（図２）[3]。本モデルでは，社会的支援が多い状況を「集団的（collective）」，少ない状況を「孤立的（isolated）」と呼び，高ストレイン・ジョブに孤立的な状況が重なった場合に，最も健康リスクが高くなると考えている（本モデルを紹介する際，職場の社会的

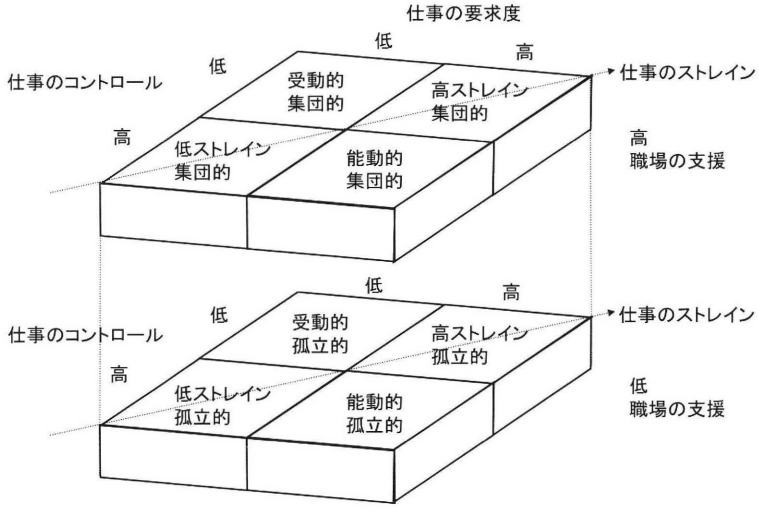

図２．要求度—コントロール—社会的支援モデル

支援は「上司の支援（supervisor support)」と「同僚の支援（co-worker support)」によって構成されるものとして紹介されることが多いが，本論文では，同僚の支援しか測定していない。また，高ストレイン・ジョブに孤立的な状況が重なった状態を「孤立ストレイン（iso-strain)」と呼ぶ場合もあるが，本論文では「iso-strain」という用語は出てこない)。

尚，本論文では，スウェーデンの労働者データ（横断データ）を用いて冠動脈疾患との関連を検討し，高ストレイン・ジョブで孤立的な状況に置かれている労働者は，低ストレイン・ジョブで集団的な状況に置かれている労働者に比べて，冠動脈疾患の有病オッズ比がおよそ２倍であることが報告されている。

２．高ストレイン・ジョブの定義方法の拡張

仕事の要求度―コントロールモデルに基づいて労働者への健康影響を検討する際，仕事の要求度と仕事の裁量度を自記式評価尺度によって測定し（代表的な評価尺度は，Karasekが開発したJob Content Questionnaire[4]であり，仕事の要求度を５項目，仕事の裁量度を９項目（意思決定権３項目＋技能の活用度６項目）で測定可能である)，高ストレイン・ジョブの者を定義する必要がある。最も古典的な定義方法は，前述の４象限に分類する方法（図１）に基づき，仕事の要求度得点が中央値（あるいは全国平均値）よりも高く，仕事の裁量度得点が中央値（あるいは全国平均値）よりも低い者を高ストレイン・ジョブとする方法である。この定義方法は，象限に基づいていることから「quadrant term」と呼ばれているが，Landsbergisらは，仕事の要求度と仕事の裁量度の交互作用を連続量として捉えたうえで，高ストレイン・ジョブの者を定義する方法を紹介した[5]。

１つ目の方法は，仕事の要求度得点を仕事の裁量度得点で除した値(要求度／裁量度比)を算出し，カットオフ値を設けて対象者を２群に分け，値が大きい方の群を高ストレイン・ジョブと定義する方法である。Landsbergisらは，カットオフ値の設定方法は任意であるとしているが，実際の疫学研究では，要求度／裁量度比の四分位点で対象者を４群に分け，第４四分位群（上位４分の１）に該当する者を高ストレイン・ジョブとする場合が多い。この方法は，割り算（比率）に基づいていることから「quotient term」と呼ばれている。

もう１つの方法は，仕事の要求度得点と仕事の裁量度得点の取り得る値の範囲が同一になるように重み付けした後，仕事の要求度得点から仕事の裁量度得点を引いた値を算出し，カットオフ値を設けて対象者を２群に分け，値が大きい方の群を高ストレイン・ジョブと定義する方法である（この方法も，quotient termと同様，カットオフ値の設定方法は任意であるとしている)。この方法は，仕事の要求度と仕事の裁量度の交互作用を線形の関数と捉えていることから「linear term」と呼ばれている。

尚，Landsbergisらは，アメリカの労働者を対象に仕事のストレインと24時間血圧との関連を検討し，収縮期血圧については，上記のいずれの定義方法を用いても，概ね一致した結果が認められたことを報告している。

まとめ

本稿では，仕事の要求度―コントロールモデル，要求度―コントロール―社会的支援モデルについて，歴史的背景も含めてその概要を説明したが，この中で最も混乱を招きやすいのは「仕事のストレイン（job strain)」という用語であろう。というのも，ストレス理論の中では，「ストレイン」という用語は，外部からの刺激によって生じる生体の心理的・生理的変化（すなわち，ストレス反応）を意味する用語であるにもかかわらず，本モデルでは，「仕事の要求度が高く，仕事の裁量度が低い状況」という，ストレス要因に近い意味合いで用いられているからである。実際，「ストレイン」という用語は，必ずしもストレス反応だけを意味するものではなく，例えば循環器学など，他の学問領域では「負荷」という意味で「ストレイン」という用語が用いられている。また，人に不安や緊張を与えるような状況に対して「ストレイン」という用語が用いられる場合もある。Karasekの論文[1]では，様々な場面でstrainという用語が用いられているが，job strain やhigh strain jobなど，仕事を表す用語と一緒に用いられている場合は「負荷」を，mental strainやpsychological strainなど，精神や心理を表す用語と一緒に用いられている場合は「緊張」や「歪み」などのストレス反応を意味していると捉えれば理解しやすいであろう。尚，「仕事のストレイン」という用

語は固有名詞化しており，論文中でjob strainと記述されている場合は，仕事の要求度—コントロールモデルに基づく研究であると考えて差し支えない（逆に言うと，本モデル以外の理論モデルに基づく研究では，この用語は用いられない）。「仕事のストレイン」という用語が，前述のような混乱を招く恐れがあるにもかかわらず，大きな批判を受けることもなく用いられ続けたのは，本モデルが提唱された1970年代当時は，ストレスに関連する用語が研究者によって様々な意味合いで用いられていた時期であり，用語の整理が精緻化されていなかったことに起因するものと推察される。

最後に，本稿で紹介した通り，高ストレイン・ジョブの定義方法が複数存在するため，実際に研究を行う際は，使用した評価尺度を明確にするとともに，どの方法を用いて高ストレイン・ジョブの者を定義したかについて，論文中に明記する必要がある。

謝辞

本稿は，平成31年度労災疾病臨床研究事業費補助金「医学的知見に基づく裁量労働を含む長時間労働者に対する適切な面接指導実施のためのマニュアルの作成に関する研究（180701-01）」（研究代表者：堤明純）の成果物である。

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（受付　2018年12月25日　受理　2019年４月11日）