treated with prednisolone alone underwent tracheotomy. CPA, CYA and AZP treatment in conjunction with steroid administration also reduced the prevalence of airway involvement in patients with RP (54.5%, 50.0% and 57.0%, respectively, Fig.2).

Discovery of the central role of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in autoimmune/inflammatory diseases and subsequent development of anti-cytokine agents have quickly led to the clinical application of them in treatment of refractory RP.

In our survey, infliximab, an anti-TNF-α agent, treatment resulted in a response in 6 cases of 10 RP patients with airway involvement that had not responded to conventional immunosuppressants. Etanercept (anti-TNF-α) and tocilizumab (anti-IL-6) treatment also showed a sustained response in 1 case of 3 patients with refractory RP.

Prognosis

We summarized the prognostic outcome of patients of RP in our cohort in Fig.3. Medication was discontinued without any manifestation in 11 patients (4.6%). All these patients exhibited auricular chondritis without respiratory involvement and 2 of the patients had scleritis.

One hundred and fifty-nine patients (66.5%) were well controlled and, in total, 71.1% of patients in our cohort responded to the treatments. 32 patients (13.4%) showed limited response and 9 patients (3.8%) suffered from progressive disease or relapse. 22 patients (9.0%) died and the causes of death were as follows; respiratory failure (8 patients), pulmonary infection (4), cardiovascular disease (2), cerebrovascular disease (2), suicide (1), myelodysplasia (1), leukemia (1) and unknown reason (2).

Discussion

We conducted a large-scale survey of patients with RP in Japan. Surveyed physicians dispersed widely on geographic location and a large part of survey responses were limited in patient number even in the main surveys. Considering the survey response rate and the number of collected patient data, the RP prevalence in Japan was estimated to be similar to that in the United States[5].

The disease severity and prognosis of RP largely depends on airway and cardiovascular involvement[10]. It has been reported that airway involvement were seen in approximately half of all RP patients during follow-up, while the manifestation were observed in only 20% of the patients at the onset of disease[12, 13]. 10-30% of patients with airway involvement were treated with tracheotomy and the leading cause of death was airway collapse and/or pulmonary infection[12-14]. These study results were similar to those in our study (Table 2, Fig.3). Several studies reported a female predominance in RP patients with airway involvement (male-female ratio, 1:2.3-2.8)[12, 13] but the ratio in our study was approximately 1:1.

It was suggested that the detection of tracheal wall thickness in CT scan was remarkably effective to the diagnosis of airway involvement in patients with RP and dynamic expiratory CT scan was more useful to indicate the lesions than conventional CT scan[12, 15-18]. Despite of the advances in CT scanning techniques, bronchoscopy is essential for the diagnosis because it identify additional findings in approximately 25% of RP patients who received the CT scan[12]. Miyazawa et al. described the endobronchial ultrasonography was useful to indicate fragmentation and edema of cartilage in patients with RP[11].

It has been reported that cardiac involvement were seen in 15-46% RP patients and the second cause of RP death[10, 19]. The male-to-female ratio was high (1:0.4) in RP patients with cardiac complications[20]. A retrospective chart review of 33 RP patients with cardiac surgery recommended that ultrafast chest computed tomography, magnetic resonance imaging or trans-esophageal echocardiography should be repeated every 6 months because subclinical development of cardiovascular involvement was occasionally observed in RP patients[20].

Certainly, several reports have described the latent phase of cardiovascular complications for a few years after the onset of RP in relatively young patients[21-24]. Several RP patients developed febrile vasculitis after RP onset with or without anti-neutrophil cytoplasmic antibody (ANCA)[25-30]. The activity of the vasculitis correlated well with severity of scleritis in patients with RP[2, 31, 32].

In our study, we found that cardiovascular involvement was less frequent in Japan (7.1 %) as compared with other reports[10, 19]. The reason for the low prevalence of cardiovascular disease remains unclear. Low prevalence of cardiac complications was reported in Japanese patients with rheumatoid arthritis as well[33]. We speculate that this is a public health issue of Japanese people regardless of the presence or absence of diseases.

No specific laboratory diagnostic test exists for RP and the diagnosis is made by clinical features and pathological

finings of chondritis[28]. Typical pathological changes began with the loss of proteoglycans' basophilic staining of cartilage. Then lymphocytes, plasma cells and neutrophils infiltrated into perichondrial area, degenerated and decreased the number of chondrocytes. Finally, the cartilage was replaced by fibrous tissue[34].

In this study, tissue biopsies were conducted in 95.4% of patients with RP and a definitive diagnosis was obtained in 60.5% of patients who underwent biopsy. To reach accurate diagnosis of RP, it was essential that physicians perform a deep biopsy to obtain the chondral tissue in the site with acute inflammation[28].

In laboratory experiments of biopsy specimen, immunoglobulin and C3 component of complement deposited to margin of cartilage and perichondrial vessel wall[34]. Antibody to type II collagen was detected in patients with RP from the disease onset and the titers were correlated with disease activity[35]. Hyper-activation of macrophage/monocytes in peripheral blood of RP patients was reported using cytokine profile analysis[36].

We found that serum level of soluble triggering receptor expressed on myeloid cells 1 (TREM1), an inflammatory receptor on macrophage/monocytes, was correlated with disease activity[37]. These data suggest that over-activation of immune system in the whole organism of RP patients converge on the chondritis of RP in a polyphyletic manner.

Several studies reported the possibility that combination therapy with prednisolone and immunosuppressants was effective for patients with RP[21, 38, 39], especially that with methotrexate[1, 2, 40, 41]. In agreement with the studies, our survey revealed high prevalence of airway involvement in patients with prednisolone monotherapy and relatively low prevalence of the involvement in patients with the combination therapy. We recommend use of the combination therapy using prednisolone and immunosuppressants in RP patients with airway involvement.

We found several case reports showed the effectiveness of anti-cytokine antibodies,such as infliximab[42], adalimumab[43], anakinra[44, 45] and abatacept[46]. We presume that the biological agents are applicable for patients with refractory RP based on the results of this survey. However, it is important to control infections of respiratory tracts before administrating such biological agents.

Endoscopic and surgical interventions are sometimes unavoidable for respiratory distress and such interventions with experienced clinicians were effective for the treatment of airway involvement in patients with RP[12, 47]. The progression of airway involvement occurs even under intensive medication and intervention in some patients with RP and a new modality is awaited for treating such patients. We are currently planning to conduct a prospective study using a patient conducted patient registry system which allows us to collect detailed status data of patients.

Conclusions

We described here patient profiles and major clinical features in patients with RP in Japan. Airway involvement of RP was fundamentally progressive and required frequent clinical checks and appropriate medications. Combination therapy with prednisolone and immunosuppressants may be beneficial for controlling airway involvement of RP than prednisolone monotherapy.

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Disclosure

All authors have no conflict of interest.

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Rapid Article

Neurological involvement of relapsing polychondritis in Japan: An epidemiological study

Noboru Suzuki*, Jun Shimizu, Hiroshi Oka, Yoshihisa Yamano and Kazuo Yudoh

Institute of Medical Science and Department of Immunology and Medicine, St. Marianna University School of Medicine, Kawasaki, Japan

We conducted a large scale epidemiological study in Japan and revealed a high mortality rate in RP patients with neurological involvement. Japanese RP patients developed encephalitis/meningitis (12 out of 239 cases, 5.0%), cerebral infarct/bleeding (5 cases, 2.1%) and cerebral vasculitis (4 cases, 1.7%). The mortality rate was 18%, in contrast to 8.1% of RP patients without neurological involvement. We suggested that neurological involvement appeared to be a major determinant of disease severity in patients with RP.

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*Correspondence should be addressed to:
Noboru Suzuki, Department of Immunology and Medicine, St. Marianna University School of Medicine, Sugao 2-16-1, Miyamae-ku, Kawasaki 216-8511, Japan. Phone: 81-44-977-8111(ext.3547), Fax: 81-44-975-3315, E-mail: n3suzuki@marianna-u.ac.jp

Key words relapsing polychondritis, epidemiology, encephalitis, meningitis, cerebral stroke, auricular cartilage

Relapsing polychondritis (RP) is a relatively rare disease, exhibiting swelling of the ear, destruction of the nose, fever, and arthritis. Tracheobroncheal involvement was potentially lethal through the occlusion[1]. Neurologic complications of RP have begun to attract increasing attention. There are some reports presenting neurological symptoms of RP[2]. In a multi-center study which enrolled 62 patients, CNS involvement was reported to be 10%[3].

We conducted a large scale epidemiological study in Japan[4] and revealed a high mortality rate in RP patients with neurological involvement. We reanalyzed the data in view of neurological involvement in patients with RP. A Multi-institutional surveillance study of Japanese major medical facilities was conducted from July to December, 2009. All subjects to whom the questionnaire was sent were informed of the purpose of the study and the responses would be kept confidential. All authors reviewed the questionnaire.

We obtained responses from 121 facilities with clinical information of 239 RP patients. The average age of onset was to be 52.7 years (range, 3-97) and the male-to-female ratio was 1.1:1 (127 males to 112 females)[4]. Biopsies were performed in 228 patients (95.4%) and histological confirmation of RP was obtained in 138 patients (57.7%).

Among 239 RP patients, 28 cases (12%) developed neurological involvement, excluding cochlear-vestibular symptoms (Table 1). The mean age of onset of RP with neurological involvement was 60 years. The ratio of men

Table 1 Characteristics of RP patients with neurological involvement in Japan

	Patients without neurological involvement (n=211)		Patients with neurological involvement (n=28)	
Profile				
Male-female ratio	106:105		21:7	
Mean age	57 (range 6-104)		64 (range 45-80)	
Mean age of disease onset	53 (range 3-97)		60 (range 38-78)	
Disease duration (yr)	5.3 (range 1-33)		4.2 (range 1-26)	
Mortality rate (%)	8.1		17.9	
Clinical features,	Number of patients (% in each group)			
	Onset	Follow-up	Onset	Follow-up
Neurological	0 (0)	0 (0)	6 (21)	28 (100)
External ear	124 (59)	159 (75)	12 (44)	27 (96)
Internal ear	7 (3.3)	53 (25)	2 (7.4)	12 (43)
Nasal cartilage	5 (2.4)	88 (42)	0 (0)	5 (18)
Airway	40 (19)	114 (54)	1 (3.6)	6 (21)
Eye	15 (7.1)	92 (44)	7 (25)	18 (64)
Arthritis	15 (7.0)	84 (40)	0 (0)	9 (32)
Cardiovascular	0 (0)	12 (5.7)	0 (0)	5 (18)

Table 2 Frequencies of central nervous system manifestations in relapsing polychondritis in Japan

Central nervous system manifestations	No. (%) of patients	No. of death
Encephalitis/meningitis	12 (43%)	2[a]
Cerebral vascular disease	5 (18%)	3[b]
Cerebral vasculitis	4 (14%)	
Brain abscess	2 (7.1%)	
Cerebral aneurysm	1 (3.6%)	
Hypertrophic pachymeningitis	1 (3.6%)	
The depression	5 (18%)	
Schizophrenia	3 (11%)	
Dementia	1 (3.6%)	
Insomnia	1 (3.6%)	
Parkinsonism	1 (3.6%)	
Tonic spasm and loss of consciousness	1 (3.6%)	

[a] Two deaths caused by encephalitis (76 year-old female) and myocardial infarction (54 year-old male)

[b] Three deaths caused by cerebral bleeding (77 year-old male), cerebral embolism (60 year-old male) and cerebral infarction (67 year-old female)

to women was 2.7 to 1 and thus men predominantly developed neurological symptoms.

RP patients with neurological involvement were diagnosed with the diagnostic criterion[4]. In addition, histological confirmation of RP was obtained 17 patients (64% of the 28 patients).

Based on the results of our study, we described incidence of the neurological symptoms and their outcome observed in patients with RP in Japan. Differential diagnosis of cerebrovascular disease and/or cerebral vasculitis, from encephalopathy, encephalitis, and meningitis, was not completely clear from this type of epidemiological studies.

Percentages of the RP patients who developed encephalitis/meningitis (12 out of 239 cases), cerebral infarct/bleeding and cerebral vasculitis were 5.0, 2.1 and 1.7%, respectively (Table 2). Our survey revealed that the RP death rate in Japan was 9%[4]. When we focused on RP with neurological involvement, 5 cases have died out of 28

cases; accordingly the death rate was 18%. Four deaths were caused by encephalitis, cerebral bleeding, cerebral embolism, and cerebral infarction. The remaining 54-year-old male patient had ten years of history of RP, during which he developed meningoencephalitis and died of acute myocardial infarction (Table 2). With regard to the atherosclerotic cardiovascular disease, two RP patients with neurological involvement (aseptic meningitis and cerebral infarction) had old myocardial infarction.

In our survey, 96% RP patients with neurological involvement accompanied inflammation in the head, such as auricular chondritis (Table 1)[1, 5]. Four RP patients (14% of 28 patients with neurological involvement) suffered from cerebral vasculitis and one of them had noninfectious aortitis. Systemic lupus erythematosus, Behcet's disease, Wegener's granulomatosis and infectious diseases were included in the differential diagnosis of the inflammatory disorders in the head and neck[6]. Further studies are needed to disclose the entire clinical pictures of RP patients with neurological involvements.

In conclusion, 12% of Japanese patients with RP developed relatively severe neurological involvement. Conventional treatment, such as administration of steroids and immunosuppressants, was not fully satisfactory and establishment of a new therapeutic strategy for neurological symptoms in patients with RP is awaited.

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Conflict of interests

None

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ORIGINAL ARTICLE

Serum level of soluble triggering receptor expressed on myeloid cells-1 as a biomarker of disease activity in relapsing polychondritis

Tomoo Sato · Yoshihisa Yamano · Utano Tomaru · Yukiko Shimizu · Hitoshi Ando · Takahiro Okazaki · Hiroko Nagafuchi · Jun Shimizu · Shoichi Ozaki · Teruomi Miyazawa · Kazuo Yudoh · Hiroshi Oka · Noboru Suzuki

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Abstract

Objectives We aimed to identify a serum biomarker for evaluating the disease activity of relapsing polychondritis (RP).

Methods We measured and compared serum levels of 28 biomarkers potentially associated with this disease, including soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), high-sensitivity C-reactive protein (hs-CRP), and cartilage oligomeric matrix protein (COMP), in 15 RP patients and 16 healthy donors (HDs). We divided the 15 RP patients into active RP ($n = 8$) and inactive RP ($n = 7$) groups, depending on the extent of the disease, and compared candidate markers between groups. The localization of membrane-bound TREM-1 in the affected tissue was examined by immunohistochemistry.

Results Serum levels of sTREM-1, interferon-γ, chemokine (C–C motif) ligand 4, vascular endothelial growth factor, and matrix metalloproteinases-3 were significantly higher in RP patients than HDs. Among these markers, sTREM-1 had the highest sensitivity and specificity (86.7 and 86.7 %, respectively). Furthermore, the serum level of sTREM-1 was significantly higher in active RP patients than inactive RP patients ($p = 0.0403$), but this was not true for hs-CRP or COMP. TREM-1 was expressed on endothelial cells in RP lesions.

Conclusions The serum level of sTREM-1 may be a useful marker of disease activity in RP.

Keywords Relapsing polychondritis · Serum marker · Soluble triggering receptor expressed on myeloid cells-1

T. Sato · Y. Yamano · Y. Shimizu · H. Ando · K. Yudoh
Institute of Medical Science, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8512, Japan

U. Tomaru
Department of Pathology, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan

T. Okazaki · H. Nagafuchi · S. Ozaki
Division of Rheumatology and Allergy, Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8512, Japan

J. Shimizu · N. Suzuki (✉)
Departments of Immunology and Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8512, Japan
e-mail: n3suzuki@marianna-u.ac.jp

T. Miyazawa
Division of Respiratory and Infectious Diseases, Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8512, Japan

H. Oka
Rheumatic Disease Center, Tokyo Medical University Hachioji Medical Center, 1163 Tate-machi, Hachioji 193-0998, Japan

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Introduction

Relapsing polychondritis (RP) is a rare inflammatory disorder of unknown etiology; it is characterized by recurrent, widespread chondritis of systemic cartilages, specifically those in the ear, eye, nose, large airways, and joints [1–3]. RP is occasionally life-threatening, as its progression leads to fatal dyspnea due to cartilage destruction in large airways. To detect such disease progression, the accurate assessment of disease activity is important. Today, this assessment is performed by analyzing a combination of clinical manifestations, laboratory findings, and imaging results.

However, it is still difficult to conduct proper evaluations. This is partly because there are no established biomarkers for evaluating the disease activity of RP, although several potential biomarkers—such as CRP, antibody to type II collagen, and cartilage oligomeric matrix protein (COMP)—have been reported previously [3–7]. For example, CRP is the most commonly used marker of inflammation, and its serum level is frequently used to assess RP disease activity [3, 4]. However, RP patients with normal CRP levels are often observed to experience advanced fibrosis of the airways, suggesting insidious chronic inflammation in those tissues, which is difficult to detect by CRP [8]. It has also been reported that antibodies to type II collagen reflect RP disease activity [6]. However, these antibodies were only detected in 30–50 % of RP patients [6, 9]. Furthermore, it has been reported that this measure lacks sensitivity and specificity [10]. Therefore, in the current study, we aimed to identify more sensitive biomarkers that would be able to detect those small differences that cannot be detected by antibodies to type II collagen or CRP.

To do so, this study excluded highly active RP patients. We measured 28 candidate markers that had been previously shown to be involved in RP, inflammation, or cartilage destruction. The levels of these markers were compared not only between RP patients and healthy donors (HDs) but also between active RP and inactive RP patients. Our results showed that the serum level of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) is most suitable as a disease-activity marker in RP.

TREM-1 is a type I transmembrane receptor of the immunoglobulin superfamily. The soluble form of TREM-1 (sTREM-1) is thought to be released from TREM-1-expressing cells by proteolytic cleavage of membrane-bound TREM-1 [11]. The serum level of sTREM-1 has been found to be elevated in patients with sepsis and has therefore been considered as a marker of microbial infection [12].

Materials and methods

Patients and samples

Fifteen patients (8 women and 7 men) diagnosed with RP according to Damiani's criteria [13, 14] and 16 healthy donors (HD) serving as age-matched and sex-matched controls (Table 1) were recruited from St. Marianna University Hospital, Kanagawa, Japan. They were enrolled between November and December 2009. In this study, we used the patient information (disease condition, disease duration, medication, etc.) obtained at the time of enrollment (Table 1). None of the patients had any other inflammatory disorders, such as overt infections or collagen diseases. To detect small differences that cannot be detected by CRP, this study enrolled RP patients in the chronic phase—not the acute phase—and further excluded patients who had highly active RP, such as those with acute respiratory failure. From among them, we divided the 15 RP patients into two groups (active RP and inactive RP) according to the definition by Lekpa et al. [7]. Briefly,

Table 1 Demographics, clinical characteristics, and medication of subjects

	HD	RP		
	(n = 16)	Total (n = 15)	Active (n = 8)	Inactive (n = 7)
Demographics				
Age (years)[a]	40.5 [27–67]	47 [10–81]	50.5 [10–74]	44 [27–81]
Female sex	50.0 %	53.3 %	50.5 %	57.1 %
Clinical characteristics				
Disease duration (years)[a]		5 [1–19]	12 [4–19]	4 [1–8]
Auricular chondritis		46.7 %	62.5 %	28.6 %
Nasal chondritis		40.0 %	62.5 %	14.3 %
Laryngotracheal chondritis		66.7 %	87.5 %	42.9 %
Ear symptoms		53.3 %	87.5 %	14.3 %
Arthritis		46.7 %	75.0 %	14.3 %
Ocular inflammation		33.3 %	50.0 %	14.3 %
Medication				
Prednisolone		86.7 %	87.5 %	85.7 %
Methotrexate		33.3 %	50.0 %	28.6 %
Azathioprine		20.0 %	25.0 %	14.3 %

HD healthy donor, *RP* relapsing polychondritis

[a] Data are expressed as median [range]

patients were defined as having active RP if they were affected with chondritis involving at least two of three sites (auricular, nasal, or laryngotracheal cartilage) at the time of blood collection or if they were affected in one of these sites and also had two other manifestations, which could include ocular inflammation, audiovestibular symptoms, or seronegative inflammatory arthritis. Fourteen patients with HTLV-1-associated myelopathy (HAM), 10 with progressive systemic sclerosis (PSS), 19 with systemic lupus erythematosus (SLE), and 20 with rheumatoid arthritis (RA) also participated in this study.

All blood and cartilage samples were obtained with written informed consent and full ethical approval. The study protocol was approved by the Ethics Committee of St. Marianna University School of Medicine.

Measurement of serum levels of marker candidates

High-sensitivity CRP (hs-CRP) was determined by nephelometry using N-latex CRP II (Siemens Healthcare Diagnostics, Tokyo, Japan). Serum concentrations of sTREM-1; matrix metalloproteinases (MMP)-1, MMP-2, MMP-3, MMP-13; cartilage oligomeric matrix protein (COMP); interleukin (IL)-17A; and anti-type II collagen antibody (α-COLII Ab) were measured using commercially available ELISA kits (sTREM-1, MMP-1, and MMP-2: R&D Systems, Minneapolis, MN, USA; MMP-3: Daiichi Fine Chemical, Toyama, Japan: MMP-13: GE Healthcare, Chalfont St Giles, UK; COMP: Abnova, Taipei, Taiwan; IL-17A: Gen-Probe, San Diego, CA, USA; α-COLII Ab: Chondrex, Redmond, WA, USA). Serum concentrations of

Table 2 Serum concentrations of biomarker candidates in healthy donors and patients with RP

Biomarker candidates[a]	Units	Methods of measurement	HD (n = 16) Mean ± SD	RP (n = 15) Mean ± SD	p*
sTREM-1	pg/ml	ELISA	92.48 ± 56.45	281.87 ± 150.42	**0.0002**
IFN-γ	pg/ml	CBA	N.D.[c]	5.65 ± 6.25	**0.0035**
CCL4	pg/ml	CBA	64.38 ± 66.03	133.76 ± 68.13	**0.0075**
VEGF	pg/ml	CBA	131.03 ± 104.66	267.46 ± 187.03	**0.0212**
MMP-3	ng/ml	ELISA	35.96 ± 29.23	243.12 ± 313.50	**0.0229**
CXCL10	pg/ml	CBA	154.72 ± 91.72	229.50 ± 114.03	0.0552
CCL5	ng/ml	CBA	2.70 ± 1.43	37.66 ± 15.66	0.0582
hs-CRP	ng/ml	Nephelometry	0.04 ± 0.05	0.30 ± 0.50	0.0643
IL-17A	pg/ml	ELISA	1.17±1.52	0.33 ± 0.79	0.0673
TNF	pg/ml	CBA	N.D.[c]	0.76 ± 2.01	0.1646
IL-4	pg/ml	CBA	N.D.[c]	0.80 ± 2.13	0.1671
IL-6	pg/ml	CBA	N.D.[c]	1.27 ± 3.38	0.1686
COMP	ng/ml	ELISA	14.38 ± 4.28	24.33 ± 26.72	0.1750
MMP-13	ng/ml	ELISA	0.31 ± 0.04	0.28 ± 0.09	0.2367
MMP-2	ng/ml	ELISA	125.01 ± 10.45	133.01 ± 28.45	0.3191
IL-1α	pg/ml	CBA	N.D.[c]	0.54 ± 2.09	0.3343
IL-1β	pg/ml	CBA	N.D.[c]	0.58 ± 2.24	0.3343
IL-10	pg/ml	CBA	N.D.[c]	0.69 ± 2.69	0.3343
IL-12p70	pg/ml	CBA	N.D.[c]	0.35 ± 1.36	0.3343
CX3CL1	pg/ml	CBA	N.D.[c]	6.55 ± 25.38	0.3343
CXCL8	pg/ml	CBA	12.93 ± 11.52	16.24 ± 7.05	0.3413
MMP-1	ng/ml	ELISA	5.19 ± 3.15	4.30 ± 3.67	0.5129
CCL2	pg/ml	CBA	67.08 ± 43.78	72.29 ± 59.36	0.7842
αCOLII Ab[b]	U/ml	ELISA	51.75 ± 37.95	263.93 ± 577.87	0.2109

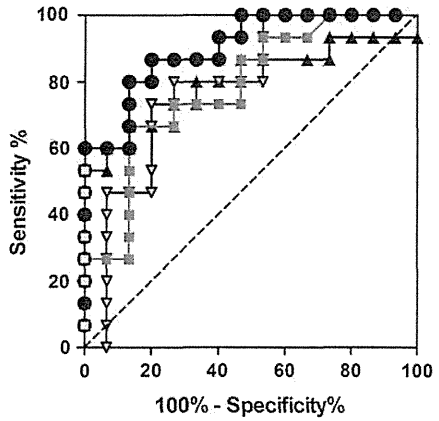
HD healthy donor, *RP* relapsing polychondritis, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *ELISA* enzyme-linked immunosorbent assay, *IFN* interferon, *CBA* cytometric bead array, *ND* not detected, *CCL* chemokine (C–C motif) ligand, *VEGF* vascular endothelial growth factor *MMP* matrix metalloproteinase, *CXCL* chemokine (C–X–C motif) ligand, *hs-CRP* high-sensitivity C-reactive protein, *IL* interleukin, *TNF* tumor necrosis factor, *COMP* cartilage oligomeric matrix protein, *CX3CL* chemokine (C–X3–C motif) ligand, *αCOLII Ab* anti-type II collagen antibody

* By Welch's t test. p values of less than 0.05 are indicated in boldface

[a] The serum levels of IL-2, IL-5, GM-CSF, and CCL3 were below the detection limits in all cases

[b] The sample size of this item is different from that of the others due to the lack of some serum samples (HD: n = 13, RP: n = 13)

[c] For the statistical analyses, values of zero were substituted for the "N.D. (not detected)" entries



symbol	candidate markers	AUC	95%CI
●—	sTREM-1	0.90	0.80 to 1.01
□—	IFN-γ	0.77	0.59 to 0.94
▽—	CCL4	0.79	0.62 to 0.96
■—	VEGF	0.78	0.62 to 0.95
▲—	MMP-3	0.80	0.63 to 0.97

--- reference line

Fig. 1 Receiver operating characteristic (ROC) analysis of marker candidates of relapsing polychondritis (RP). We compared the sensitivity and specificity of soluble triggering receptors expressed on myeloid cells-1 (sTREM-1), interferon (IFN)-γ, chemokine (C–C motif) ligand 4 (CCL4), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-3 (MMP-3) for discriminating RP patients from healthy donors (HDs) using ROC analysis. Closer proximity of the ROC curve to the upper left corner indicates higher sensitivity and specificity of the marker

IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70; interferon (IFN)-γ; tumor necrosis factor (TNF); chemokine (C–C motif) ligand (CCL) 2, CCL3, CCL4, CCL5; chemokine (C–X–C motif) ligand 8 (CXCL8), CXCL10; chemokine (C–X3–C motif) ligand 1 (CX3CL1); granulocyte–macrophage colony-stimulating factor (GM-CSF); and vascular endothelial growth factor (VEGF) were measured using a cytometric bead array (CBA; BD Biosciences, San Jose, CA, USA). All assays were conducted according to the respective manufacturers' instructions.

Immunohistochemistry

Biopsy specimens from three patients with RP chondritis were subjected to immunohistochemical analysis. Formalin-fixed tissue sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Slides were processed for antigen retrieval by a standard microwave-heating technique and incubated with anti-TREM-1 antibody (Sigma), followed by detection with streptavidin–biotin-horseradish peroxidase (Dako Cytomation Japan, Tokyo, Japan). All sections were visualized using 3,3′-diaminobenzidine (DAB).

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot graphs and perform statistical analyses. Mean serum concentrations of biomarker candidates were compared between RP patients and HDs using Welch's *t* test (Table 2). Receiver operating characteristic (ROC) analysis was used to examine the sensitivity and specificity of the selected markers (Fig. 1). Serum concentrations of biomarker candidates in patients with active RP and patients with inactive RP were analyzed by Welch's *t* test (Table 3). To compare serum sTREM-1 levels between healthy donors and patients with some inflammatory diseases (Fig. 3), we employed the Kruskal–Wallis test followed by Dunn's post hoc test. In all analyses, statistical significance was set at $p < 0.05$.

Results

Serum biomarker candidates in RP patients

First, we measured the serum levels of 12 cytokines, 7 chemokines, 4 MMPs, VEGF, hs-CRP, sTREM-1, COMP, and anti-type II collagen antibody in RP patients and age- and sex-matched HDs (Table 1), and compared the results from these two groups (Table 2). Serum samples from RP patients showed significantly higher concentrations of five molecules (sTREM-1, IFN-γ, CCL4, VEGF, and MMP-3) than the samples from HDs (Table 2). The serum levels of several other molecules (including hs-CRP, COMP, and anti-type II collagen antibody) tended to be higher in RP patients than in HDs, though the differences were not statistically significant.

Then, using ROC analysis, we compared the performances of the above five molecules in distinguishing RP patients from HDs. As shown in Fig. 1, the ROC analysis demonstrated that sTREM-1 had the highest sensitivity and specificity of the five molecules (area under the ROC curve [AUC] = 0.90; 95 % confidence interval [CI] 0.80–1.01; $p = 0.0002$). A sTREM-1 cut-off value of 158 pg/ml had a sensitivity of 86.7 % with a specificity of 86.7 %.

Table 3 Serum concentrations of biomarker candidates in patients with active RP and patients with inactive RP

Biomarker candidates[a]	Units	Active RP ($n = 8$) Mean ± SD	Inactive RP ($n = 7$) Mean ± SD	p*
sTREM-1	pg/ml	353.39 ± 158.03	200.14 ± 95.11	**0.0403**
VEGF	pg/ml	339.19 ± 218.10	185.48 ± 106.88	0.1066
hs-CRP	ng/ml	0.48 ± 0.64	0.10 ± 0.08	0.1342
TNF	pg/ml	1.43 ± 2.65	N.D.[c]	0.1708
IL-6	pg/ml	2.38 ± 4.45	N.D.[c]	0.1752
IL-17A	pg/ml	0.05 ± 0.14	0.71 ± 1.14	0.2129
MMP-3	ng/ml	334.71 ± 400.33	138.44 ± 135.59	0.2254
MMP-1	ng/ml	5.35 ± 4.35	3.07 ± 2.51	0.2658
MMP-13	ng/ml	0.30 ± 0.11	0.26 ± 0.05	0.3469
IL-1α	pg/ml	1.01 ± 2.86	N.D.[c]	0.3506
IL-1β	pg/ml	1.09 ± 3.07	N.D.[c]	0.3506
IL-10	pg/ml	1.30 ± 3.68	N.D.[c]	0.3506
IL-12p70	pg/ml	0.66 ± 1.87	N.D.[c]	0.3506
CX3CL1	pg/ml	12.29 ± 34.75	N.D.[c]	0.3506
MMP-2	ng/ml	139.68 ± 25.79	125.38 ± 31.39	0.3589
COMP	ng/ml	30.26 ± 35.31	17.56 ± 10.53	0.3598
CXCL10	pg/ml	251.14 ± 110.78	204.78 ± 121.20	0.4563
IFN-γ	pg/ml	4.54 ± 7.29	6.93 ± 5.06	0.4703
CXCL8	pg/ml	17.31 ± 6.34	15.01 ± 8.11	0.5571
CCL2	pg/ml	80.59 ± 78.04	62.80 ± 30.33	0.5660
CCL4	pg/ml	141.68 ± 90.46	124.71 ± 33.26	0.6332
IL-4	pg/ml	0.83 ± 2.36	0.76 ± 2.02	0.9509
CCL5	ng/ml	37.87 ± 17.21	37.42 ± 15.05	0.9585
αCOLII Ab[b]	U/ml	382.34 ± 808.48	162.44 ± 311.65	0.5525

RP relapsing polychondritis, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *VEGF* vascular endothelial growth factor *hs-CRP* high-sensitivity C-reactive protein, *TNF* tumor necrosis factor, *N.D.* not detected, *IL* interleukin, *MMP* matrix metalloproteinase, *CX3CL* chemokine (C–X3–C motif) ligand, *COMP* cartilage oligomeric matrix protein, *CXCL* chemokine (C–X–C motif) ligand, *IFN* interferon, *CCL* chemokine (C–C motif) ligand, *αCOLII Ab* anti-type II collagen antibody

* By Welch's t test. p values of less than 0.05 are indicated by boldface

[a] The serum levels of IL-2, IL-5, GM-CSF, and CCL3 were below the detection limits in all cases

[b] The sample size of this item is different from that of the others due to the lack of some serum samples (active RP: $n = 6$, inactive RP: $n = 7$)

[c] For the statistical analyses, values of zero were substituted for the "N.D. (not detected)" entries

Identification of serum markers of disease activity in RP

Next, to identify a serum marker that correlates with RP disease activity, we divided the 15 RP patients into two groups based on the extent of inflammation (see "Methods" for details) (Table 1): active RP ($n = 8$) and inactive RP ($n = 7$). We then compared serum levels of all tested molecules in the two RP groups. The results showed that only serum sTREM-1 level was significantly higher in active RP patients than in the inactive RP patients ($p = 0.0403$) (Table 3). Moreover, to investigate the association of serum sTREM-1 level with disease activity in RP, we examined the clinical course of one patient with active RP. As shown in Fig. 2, treatment with methotrexate (MTX) provided symptomatic improvement in this case; simultaneously, the patient's abnormally high sTREM-1 level was reduced to almost the same level as healthy donor (720.5 pg/ml in Nov 2009 → 106.6 pg/ml in June 2011). Importantly, before the MTX treatment, the patient's CRP level was almost normal, even when the sTREM-1 level was abnormally high (CRP 0.41 mg/dl, sTREM-1 720.5 pg/ml).

Serum levels of sTREM-1 in patients with other immunological disorders

To investigate the disease specificity of sTREM-1, we measured the serum levels of this molecule in patients with other immunological disorders, including HTLV-1-associated

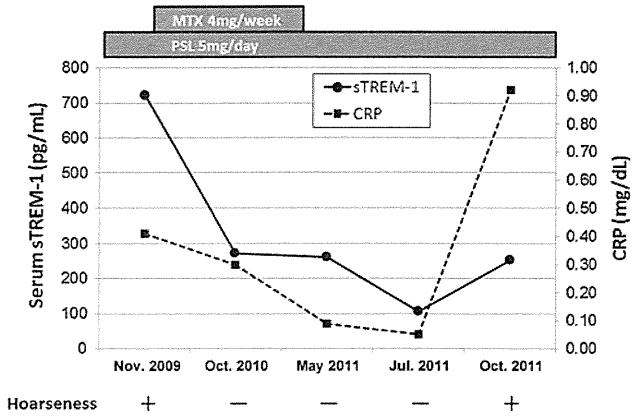


Fig. 2 Clinical course of a patient who was classified as having active RP at the time of enrollment, in 2009. The *line chart* shows the time courses of the serum sTREM-1 level (*closed circles*, *solid line*) and the CRP level (*closed squares*, *dashed line*) in an RP patient treated with prednisolone (PSL) and methotrexate (MTX). A plus sign (+) indicates the presence of hoarseness as a respiratory tract symptom, while a minus sign (−) indicates the absence of that symptom

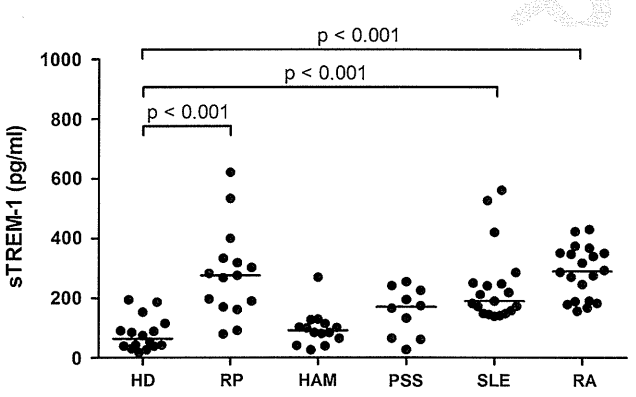


Fig. 3 Comparison of serum sTREM-1 levels between HDs and patients with other immunological disorders, including RP. Individual values are plotted, and the *bars* represent medians of the values. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post hoc tests. *HAM* HTLV-1-associated myelopathy, *PSS* progressive systemic sclerosis, *SLE* systemic lupus erythematosus, and *RA* rheumatoid arthritis

myelopathy (HAM), progressive systemic sclerosis (PSS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). Serum sTREM-1 levels were higher by a statistically significant amount in patients with RP and in patients with SLE or RA when compared to the levels in HDs (Fig. 3). This result indicates that elevation of the serum sTREM-1 level is not specific to RP.

TREM-1 expression in chondritis-affected areas of RP patients

Finally, we examined the expression of membrane-bound TREM-1 in chondritis-affected areas of RP patients. Immunohistochemistry demonstrated that TREM-1 was expressed on vascular endothelial cells in perichondral granulation foci but not on chondrocytes (Fig. 4). No positive cells were observed in a control sample (nonspecific inflammatory granulation tissue derived from a ruptured epidermal cyst) (Fig. 4).

Discussion

In this study, we identified serum sTREM-1 level as a novel biomarker for RP. We produced several results indicating the strength of this candidate marker: first, our results indicated that serum sTREM-1 level could discriminate RP patients from HDs more successfully than could other candidate biomarkers (Table 2; Fig. 1). Second, serum sTREM-1 level gave better discrimination between active RP patients and inactive RP patients than 27 other tested molecules, including hs-CRP, COMP, and anti-type II collagen antibody (Table 3). Third, the time course of serum sTREM-1 level was associated with the clinical course in an RP patient who was treated with prednisolone and MTX (Fig. 2). However, sTREM-1 showed some limitations in disease specificity, as its serum level was also elevated in patients with SLE or RA (Fig. 3). These results suggest that serum sTREM-1 level is suitable for use as a disease-activity marker for RP, but not as a diagnostic marker for the disease.

TREM-1, as the name suggests, has been shown to express on myeloid cells such as neutrophils and monocytes/macrophages [15]. Recently, it has been reported that TREM-1 is also expressed on endothelial cells (a type of non-myeloid cell) in liver tissue from lipopolysaccharide-treated mice [16]. In this study, our immunohistochemical analyses demonstrated that TREM-1 is expressed on human endothelial cells in chondritis-affected areas of RP patients (Fig. 4). The increase in sTREM-1 in the blood of RP patients might be due to its presence on the surfaces of endothelial cells in those inflammatory lesion sites. This hypothesis is supported by the finding that there was no difference in the expression level of TREM-1 on peripheral blood mononuclear cells between healthy donors and RP patients (data not shown). However, further investigations are needed to clarify the source of the increased sTREM-1.

It was previously reported that the expression of TREM-1 is induced by bacterial infection and that levels of circulating sTREM-1 are important as a diagnostic and prognostic marker of sepsis [17–19]. More recently, however, it has been reported that the serum sTREM-1 level is elevated in non-infectious chronic inflammatory diseases such as RA and inflammatory bowel diseases [20, 21]. Therefore, our finding that serum samples from patients with chronic inflammatory diseases (including RP, RA, and

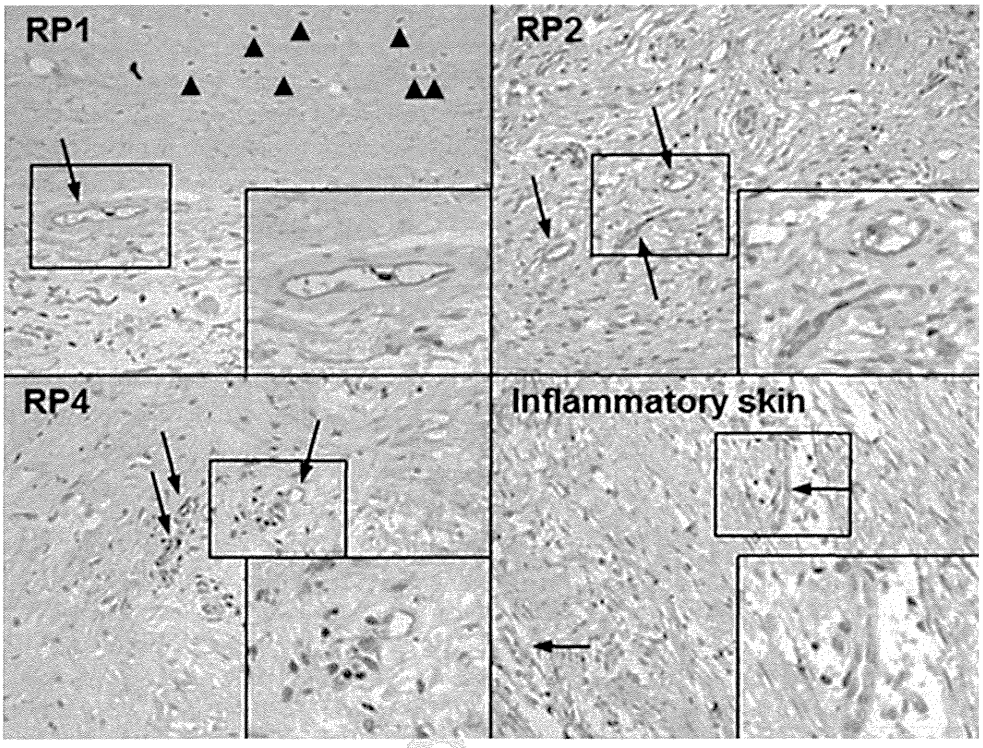


Fig. 4 Immunohistological staining showing the expression of TREM-1 in chondritis-affected areas. Inflammatory granulation tissue from a patient with a ruptured epidermal cyst was used as a negative control (*lower right panel*: inflammatory skin). TREM-1-positive cells were stained brown using 3,3′-diaminobenzidine (DAB) and are displayed at a higher magnification in the *lower right inset*. *Arrows* and *arrowheads* indicate vascular endothelial cells and chondrocytes, respectively

SLE) had significantly higher concentrations of sTREM-1 is consistent with previous reports. On the other hand, serum level of sTREM-1 in patients with HAM—a chronic inflammatory neurologic disease caused by human T cell leukemia virus-1—was not significantly higher than the level in HDs. This indicates that the serum level of sTREM-1 differs among patients with different chronic inflammatory diseases. Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a chronic inflammatory disease. Patients with AAV show elevated levels of serum sTREM-1 [22]. Intriguingly, as in RP, sTREM-1 levels in active AAV have been shown to be significantly higher than those for inactive AAV [22]. Thus, elevated levels of serum sTREM-1 have been observed in several chronic inflammatory diseases.

Such disorders with elevated sTREM-1 levels often overlap in the same patient. For example, 14 % of patients with RP have clinically evident vasculitis [23] and 35.5 % of patients have other collagen diseases, such as RA or SLE [24]. These examples imply the existence of common mechanisms in the pathogenesis of these disorders. In this regard, because TREM-1 works as an amplifier of inflammatory responses through the production of multiple pro-inflammatory cytokines and chemokines, TREM-1 may play an important role in the common pathomechanisms of these disorders [15, 21, 25, 26]. A previous study provided in vivo evidence that the blockade of TREM-1 can ameliorate collagen-induced arthritis in mice [27].

One of the molecules that has been reported as a disease-activity marker for RP is COMP [7]. This is a non-collagenous protein found in the matrix of cartilage. Lekpa et al. reported that serum COMP levels during the active phase were significantly higher than those seen during the inactive phase in the same patients. However, our results showed no significant differences in the serum levels of this molecule in active RP patients compared to inactive RP patients (Table 3). This discrepancy could be attributed to the different study designs employed, including differing disease conditions of the RP patients, sample sizes, and measurement methods.

To further characterize this molecule, we checked for correlations between serum levels of COMP and the other tested molecules. Interestingly, serum COMP levels in RP patients had a strong positive correlation only with serum MMP-3 levels ($rs = 0.7357$, $p = 0.0018$, by Spearman rank correlation test, data not shown). This suggests that serum levels of MMP-3 and COMP might reflect the degree of cartilage destruction in RP patients, since serum

MMP-3 level is considered a predictor of the degree of cartilage destruction in patients with early RA [28].

In conclusion, this study suggests that serum sTREM-1 level can serve as a more sensitive marker for disease activity in RP patients than other candidate molecules, such as CRP, COMP, and anti-type II collagen antibody.

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Conflict of interest None.

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Ⅴ．平成 26 年度班員名簿

平成 26 年度　難治性疾患等政策研究事業(難治性疾患政策研究事業)

研究分野	ライフサイエンス		
研究課題名	難治性疾患等政策研究事業(難治性疾患政策研究事業) 再発性多発軟骨炎の診断と治療体系の確立		
課題番号	H26-難治等(難)－一般－080		
区分	氏名	所属	職名
研究代表者	鈴木　　登	聖マリアンナ医科大学 難病治療研究センター・医学部免疫学	教　授
研究分担者	岡　　寛	東京医科大学八王子医療センター リウマチ性疾患治療センター	教　授
研究分担者	遊道　和雄	聖マリアンナ医科大学 難病治療研究センター	教　授
研究分担者	山野　嘉久	聖マリアンナ医科大学 難病治療研究センター	准教授
研究分担者	清水　潤	聖マリアンナ医科大学 免疫学・病害動物学	准教授