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Multiple Serum Cytokine Profiling to Identify Combinational Diagnostic Biomarkers in Attacks of Familial Mediterranean Fever

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Abstract: The precise cytokine networks in the serum of individuals with familial Mediterranean fever (FMF) that are associated with its pathogenesis have been unknown. Here, we attempted to identify specific biomarkers to diagnose or assess disease activity in FMF patients.

We measured serum levels of 45 cytokines in 75 FMF patients and 40 age-matched controls by multisuspension cytokine array. FMF in ''attack'' or ''remission'' was classified by Japan College of Rheumatology-certified rheumatologists according to the Tel Hashomer criteria. Cytokines were ranked by their importance by a multivariate classification algorithm. We performed a logistic regression analysis to determine specific biomarkers for discriminating FMF patients in attack. To identify specific molecular networks, we performed a cluster analysis of each cytokine.

Twenty-nine of the 45 cytokines were available for further analyses. Eight cytokines' serum levels were significantly elevated in the FMF attack versus healthy control group. Nine cytokines were increased in FMF attack compared to FMF remission. Multivariate classification algorithms followed by a logistic regression analysis revealed that the combined measurement of IL-6, IL-18, and IL-17 distinguished FMF patients in attack from the controls with the highest accuracy (sensitivity 89.2%, specificity 100%, and accuracy 95.5%). Among the FMF patients, the combined measurement of IL-6, G-CSF, IL-10, and IL-12p40 discriminated febrile attack periods from remission periods with the highest accuracy (sensitivity 75.0%, specificity 87.9%, and accuracy 84.0%).

Our data identified combinational diagnostic biomarkers in FMF patients based on the measurement of multiple cytokines. These findings help to improve the diagnostic performance of FMF in daily practice and extend our understanding of the activation of the inflammasome leading to enhanced cytokine networks.

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Abbreviations: FMF = familial Mediterranean fever, G-CSF = granulocyte colony stimulating factor, IFN = interferon, IL = interleukin, MEFV = Mediterranean fever, RFA = random forest analysis, SAA = serum amyloid A, sCD54 = soluble CD54, Th = T-helper, TNF-α = tumor necrosis factor-alpha.

INTRODUCTION

Familial Mediterranean fever (FMF) is an inherited autoinflammatory disorder characterized by unpredictable attacks of fever with arthritis, abdominal pain, and/or serositis.[1,2] The usual clinical manifestations of FMF are acute episodes of inflammation and there is no residual symptom between attacks. However, chronic subclinical inflammation may persist despite achieving remission.[3] FMF is associated with a number of mutations of the *Mediterranean fever (MEFV)* gene, which codes for a protein named pyrin. Pyrin acts as a major regulatory component of the NACHT, LRR, and PYD domains-containing protein 3 inflammasome complex.[4] Dysfunction of pyrin causes autoinflammatory disease, resulting in the aberrant production of interleukin (IL)-1β and IL-18.[5,6] These cytokines activate nuclear factor κB signaling pathways that lead to increased amounts of tumor necrosis factor-alpha (TNF-α) and IL-6.[7,8]

In line with these observations, it is widely known that elevated acute-phase proteins such as serum amyloid A and C-reactive protein and inflammatory cytokines including IL-6 and IL-18 are implicated in the disease activity of FMF in clinical practice.[3,9,10] However, a specific biomarker for FMF is not yet available, and the cytokine profile in serum from FMF patients in attacks associated with its pathogenesis has not been established. There has been no extensive study examining multiple cytokines and investigating their importance and pathogenic networks.

In the present study, in order to identify the utility of the measurement of multiple cytokines including a specific

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combination of biomarkers for clinical application, we analyzed the serum from FMF patients in attack or in remission by using a multisuspension cytokine array. Our findings demonstrated that the serum from FMF patients in attack had higher levels of several inflammatory cytokines compared to the serum from FMF patients in remission and a normal population. We also identified a specific combination of cytokines that distinguished FMF patients in attack from those in remission and the normal population.

METHODS

Patients and Controls

This study was registered with the University Hospital Medical Information Network Clinical Trials Registry (http://www.umin.ac.jp/ctr/) as UMIN000015881. The study population consisted of 75 Japanese patients with FMF who were recruited consecutively and prospectively between May 2010 and October 2015 from Nagasaki University, Shinshu University, Kanazawa University and Nagasaki Medical Center. Each of the FMF patients fulfilled the Tel Hashomer criteria.[11,12] All participants undergo a clinical assessment and provide a blood sample for the assay at the same time. The control group was 40 age- and sex-matched healthy Japanese individuals recruited from staff at Nagasaki University. Sixty-four of the 75 patients (84%) expressed a typical FMF attack, and the remaining 11 patients (16%) expressed an incomplete attack as defined by the Tel Hashomer criteria.[11]

The distribution of the *MEFV* genotype was as follows: M694I/M694I, 5 patients; M694I/–, 7; M694V/–, 3; M694I/E148Q, 23; M694I/P715L, 1; M694I/E148Q/L110P, 7; M680I/V726A, 1; E148Q/–, 6; L110P/E148Q/E148Q, 1; L110P/E148Q, 4; L110P/E148Q/R202Q, 2; L110P/E148Q/P369S, 1; E148Q/P369S/R408Q, 2; E84K/–, 2, E84K/G304R, 1; E84K/R410H, 1; G304R/–, 1; S503C/–, 1; no mutation, 6 patients.

Serum samples were centrifuged at 3000 *g* for 5 minutes, and the supernatants were collected and stored at −80 °C before the assay was performed. We obtained 82 serum samples from FMF patients in total (21 patients for attack only, 47 patients for remission only, and 7 patients for both attack and remission; thus, 28 samples in attack and 54 samples in remission). All patients gave their signed informed consent to be subjected to the protocol, which was approved by the Institutional Review Board of Nagasaki University and related centers (Approval No. 14092946).

Multiplex Cytokine Assay

We performed a multiplex cytokine bead assay blindly and in parallel using the Bio-plex Pro Human Cytokine assay (Bio-Rad, Hercules, CA) and Milliplex MAP Human Cytokine/Chemokine panel 1 (Millipore, Billerica, MA) according to the manufacturers' instructions. We were able to further analyze 29 of the 45 cytokines: IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-17, IL-18, TNF-α, interferon-α (IFN-α), IFN-γ, granulocyte macrophage colony stimulating factor, basic granulocyte colony stimulating factor (G-CSF), vascular endothelial growth factor, soluble CD54 (sCD54), sCD106, fibroblast growth factor 2, CCL2 (monocyte chemoattractant protein-1/MCAF), CCL3 (macrophage inflammatory protein-1a), CCL4 (macrophage inflammatory protein-1b), CCL22 (human macrophage-derived chemokine), CXCL1 (growth-regulated protein alpha precursor), CXCL10 (IFN-γ inducible protein 10), and CX3CL1 (fractalkine).

Statistical Analysis

The subjects' baseline demographic characteristics were compared using Fisher exact tests for discrete variables and Wilcoxon test for continuous variables. The Kruskal–Wallis test followed by a Dunn multiple comparisons test was used to compare cytokine levels between groups. To rank the cytokine levels, we performed a multivariate classification algorithm termed random forest analysis (RFA),[13] using the R package RandomForest (http://cran.r-project.org/web/packages/randomForest/) ver. 4.6–12 software, as described.[14] We subsequently selected a classifier consisting of a combination of cytokine markers yielding the best classification performance to predict FMF attacks by a multiple logistic regression analysis. We then calculated the sensitivity, specificity, accuracy, a receiver operator characteristic curve, the area under the curve, and Akaike information criterion. Correlations between pairs of serum markers were calculated using Spearman rank correlation test. We used Wilcoxon signed-rank tests to evaluate the continuous change of cytokines between FMF in attack and FMF in remission. The cytokine profiles of the FMF patients with or without mutations in exon 10 were compared with Wilcoxon test. Statistical analyses were performed in R (ver. 3.2.3) and JMP pro 11.2 software (SAS Institute, Cary, NC). All reported *P*-values are 2-sided. A P-value <0.05 was considered significant.

RESULTS

Cytokine Profiles of the FMF Patients and the Healthy Controls

Table 1 shows the characteristics of the FMF patients. Out of the 75 patients, 48 patients (64%) were female. The patients' median age at onset was 22 years, and their median age at diagnosis was 38 years. We found that the serum levels of 8 cytokines (IL-4, IL-6, IL-7, IL-17, IL-18, G-CSF, sCD54, and CXCL10) were significantly elevated in the FMF attack group compared to the healthy control group. In addition, 9 cytokines (IL-6, IL-8, IL-10, IL-12p40, IFN-α, IFN-γ, G-CSF, sCD106, and CXCL10) were significantly increased in the FMF attack group compared to the FMF patients in remission (Table 2).

Identification of Combinational Biomarkers Specific for FMF in Attack by the Random Forest Analysis and Logistic Regression Analysis

To determine the most important predictor that can discriminate FMF in attack from FMF in remission or healthy controls, we ranked the cytokines by their importance, using the RFA (Figure 1A, healthy controls vs FMF in attack; Figure 1B, FMF in remission vs FMF in attack) (see Supplementary Digital Content, http://links.lww.com/MD/A953). We subsequently performed a multiple logistic regression analysis and constructed receiver operator characteristic curves to calculate the sensitivity, specificity, accuracy, area under the curve, and Akaike information criterion. Table 3 shows these variables in each combination. The data revealed that IL-6, IL-18, and IL-17 were the best combination to distinguish FMF patients in attack from the normal population with high accuracy (sensitivity 89.2%, specificity 100%, and accuracy 95.5%, Figure 2A). The prediction of attack used the following formula:

Probability (attack) $= \exp(A)/\{+\exp(A)\}$ [$A = 4.208 - 0.318^{*}$IL-6 $- 0.010^{*}$IL-18 $- 0.037^{*}$IL-17].

As shown in Figure 2B, we also observed that the combined measurement of IL-6, G-CSF, IL-12p40, and IL-10 discriminated the attack periods from the remission periods

TABLE 1. Patients Demographic Profile*

Characteristic	Total (n = 115)	Healthy Control (n = 40)	FMF Patients (n = 75)	*P*-Value (Healthy Control Vs FMF)
Age at onset, year			22 (10–44)	
Age at diagnosis, year	39 (29–48)	40 (33–46)	38 (26–51)	0.64
Female	71 (62)	23 (58)	48 (64)	0.55
Typical FMF			64 (84)	
MEFV mutation in exon 10				
M694I/M694I			5	
M694I/–			6	
M694V/–			3	
M694I/E148Q			23	
M694I/P715L			1	
M694I/E148Q/L110P			7	
M680I/V726A			1	
Total			47 (63)	

FMF = familial Mediterranean fever, MEFV = Mediterranean fever.
*Median (interquartile range) or number (percentages) is shown.

TABLE 2. Cytokine Profile of FMF Patients and Healthy Control*

Cytokine	Healthy (n = 40)	FMF Attack (n = 28)	*P*-Value (Healthy Vs Attack)	FMF Remission (n = 54)	*P*-Value (Attack Vs Remission)
IL-1β	0.9 (0.3–1.5)	0.92 (0.1–3.1)	0.52	0.4 (0.1–3.4)	0.98
IL-1RA	38.7 (27.5–68.7)	38.7 (27.5–68.7)	0.63	29.6 (19.8–56.0)	0.086
IL-2	1.1 (0.5–2.1)	2.3 (0.5–5.7)	0.2	1.2 (0.3–2.5)	0.12
IL-4	1.0 (0.11–4.2)	6.6 (0.9–27.1)	**0.036**	6.9 (0.1–13.4)	0.39
IL-5	1.1 (0.9–1.5)	0.9 (0.4–3.0)	0.44	0.9 (0.4–1.6)	0.66
IL-6	0.6 (0.1–1.9)	22.5 (5.1–47.9)	**<0.001**	2.5 (0.1–6.8)	**<0.001**
IL-7	6.0 (2.4–9.6)	11.3 (7.8–18.1)	**0.021**	9.2 (1.7–15.8)	0.17
IL-8	36.0 (14.0–112.4)	34.3 (19.2–44.2)	0.63	15.8 (1.0–35.1)	**0.028**
IL-10	1.4 (0.1–2.5)	2.2 (0.5–9.9)	0.11	0.8 (0.2–2.4)	**0.046**
IL-12p40	8.9 (0.9–16.7)	12.3 (8.0–21.8)	0.3	4.3 (0.1–12.9)	**0.021**
IL-12p70	4.5 (3.0–9.5)	5.6 (3.9–23)	0.91	2.5 (1.5–6.3)	0.099
IL-17	4.7 (2.5–7.8)	17.4 (3.2–34.4)	**0.019**	7.0 (3.5–16.5)	0.17
IL-18	59.6 (44.8–93.2)	345.5 (53–864.2)	**0.004**	190.1 (69.6–845.0)	0.93
TNF-α	7.2 (5.3–8.9)	8.6 (5.7–14.0)	0.29	7.9 (5.4–15.8)	0.82
IFN-α	30.2 (3.3–48.5)	37.4 (21.5–70.6)	0.26	25.8 (13.1–63.5)	0.26
IFN-γ	13.08 (6.9–22.4)	17.7 (8.6–75.0)	0.21	8.8 (3.9–18.3)	**0.011**
GM-CSF	17.7 (13.3–31.5)	27.7 (13.9–52.2)	0.34	15.2 (7.0–30.6)	**0.093**
G-CSF	24.3 (21.5–36.5)	37.2 (25.3–74.2)	0.053	20.1 (11.4–44.2)	**0.005**
VEGF	172.72 (111.4–270.5)	216.6 (154.8–495.5)	0.39	223.45 (111.0–398.5)	0.49
sCD54†	90.9 (79.0–101.3)	147.1 (116.9–198.3)	**<0.001**	124.5 (87.0–175.6)	0.13
sCD106†	128.4 (115.2–140.1)	144.1 (119.7- 213.4)	0.12	123.8 (113.3–157.0)	**0.044**
FGF2	70.5 (55.5–88.8)	84.4 (55.1–112.0)	0.67	52.6 (36.1–95.0)	0.14
CCL2	596.31 (482.0–789.5)	440.5 (324.69–752.16)	0.45	415.2 (309.21–559.26)	0.67
CCL3	10.6 (5.7–14.7)	11.8 (0.15–17.4)	0.82	7.3 (1.4–12.1)	0.45
CCL4	65.3 (38.9–87.0)	60.9 (47.4–75.9)	0.59	47.0 (30.3–68.0)	0.17
CCL22	860.6 (633.0–968.9)	899.7 (434.8–1348)	0.93	879.0 (607.2–1154)	0.65
CXCL1	772.3 (669.5–986.5)	1142 (635.1–1553)	**0.017**	1117 (711.2–1674)	0.88
CXCL10	218.4 (178.6–302.2)	351.6 (205.1–994.0)	**0.041**	181.4 (108.7–317.9)	**0.004**
CX3CL1	92.0 (67.3–145.8)	120 (89.1–161.6)	0.38	87.4 (51.7–178.0)	0.29

P-values were established using Kruskal–Wallis test followed by a Dunn multiple comparisons test. Bold indicates significant values. FGF = fibroblast growth factor, GM-CSF = granulocyte macrophage colony stimulating factor, G-CSF = granulocyte colony stimulating factor, IFN = interferon, IL = interleukin, RA = receptor antagonist, sCD54 = soluble CD54, TNF-α = tumor necrosis factor-alpha, VEGF = vascular endothelial growth factor.
*Values are the median (interquartile range) pg/mL.
†Values are the median (interquartile range) ng/mL.

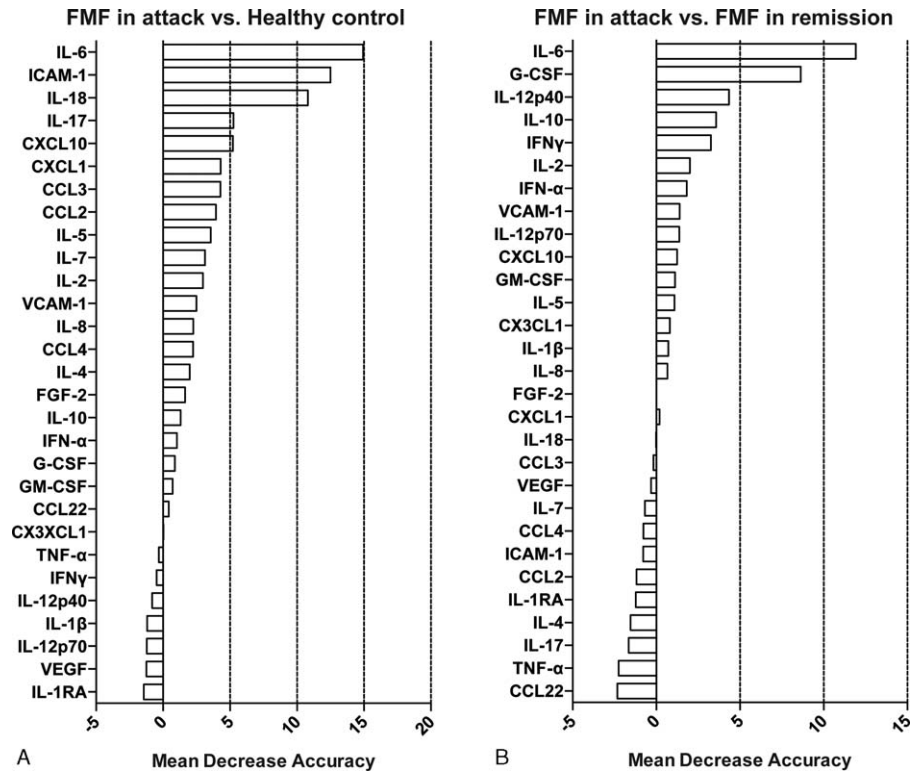

FIGURE 1. RFA, cytokines are ranked by their relative importance for discriminating FMF in attack from healthy subjects (A) or FMF in remission (B). The horizontal axis represents the average decrease in classification accuracy. FMF = familial Mediterranean fever, RFA = random forest analysis.

TABLE 3. ROC Curve in Each Subset Determined by Multiple Logistic Regression Analysis*

Variables (Healthy Vs Attack)	Sensitivity	Specificity	Accuracy	AUC	AIC
IL-6	88.9	90.9	90	0.932	44.5
IL-6 + sCD54	92.6	93.9	93.3	0.964	31.9
IL-6 + IL-18	85.2	100	93.3	0.980	30.0
IL-6 + IL-17	89.3	87.9	88.5	0.918	48.5
IL-6 + sCD54 + IL-18	92.6	93.9	93.3	0.975	30.6
IL-6 + sCD54 + IL-17	92.6	93.9	93.3	0.975	30.6
IL-6 + IL18 + IL-17	**89.2**	**100**	**95.5**	**0.986**	**29.1**
IL-6 + sCD54 + IL18 + IL-17	88.5	100	94.9	0.984	31.5
IL-6 + sCD54 + IL18 + IL-17 + CXCL10	96.1	93.9	94.4	0.986	30.5
Variables (attack vs remission)					
IL-6	66.7	90.3	82.2	0.833	76.4
IL-6 + G-CSF	66.7	92.3	83.5	0.819	77.7
IL-6 + IL12p40	64.3	90.4	81.2	0.803	81.9
IL-6 + IL-10	78.6	78.9	78.8	0.875	74.2
IL-6 + G-CSF + IL-12p40	66.7	92.3	83.5	0.819	79.5
IL-6 + G-CSF + IL-10	78.6	80.8	80.0	0.873	76.2
IL-6 + IL-12p40 + IL-10	75.9	84.6	81.5	0.873	76.3
IL-6 + G-CSF + IL-12p40 + IL-10	**75.0**	**87.8**	**84.0**	**0.889**	**73.9**
IL-6 + G-CSF + IL-12p40 + IL-10 + IFN-γ	81.5	78.8	79.7	0.873	76.2

AIC = Akaike information criterion, AUC = area under the curve, G-CSF = granulocyte colony stimulating factor, IFN = interferon, IL = interleukin, sCD54 = soluble CD54.

*Bold indicates the minimum number of cytokines among the subsets.

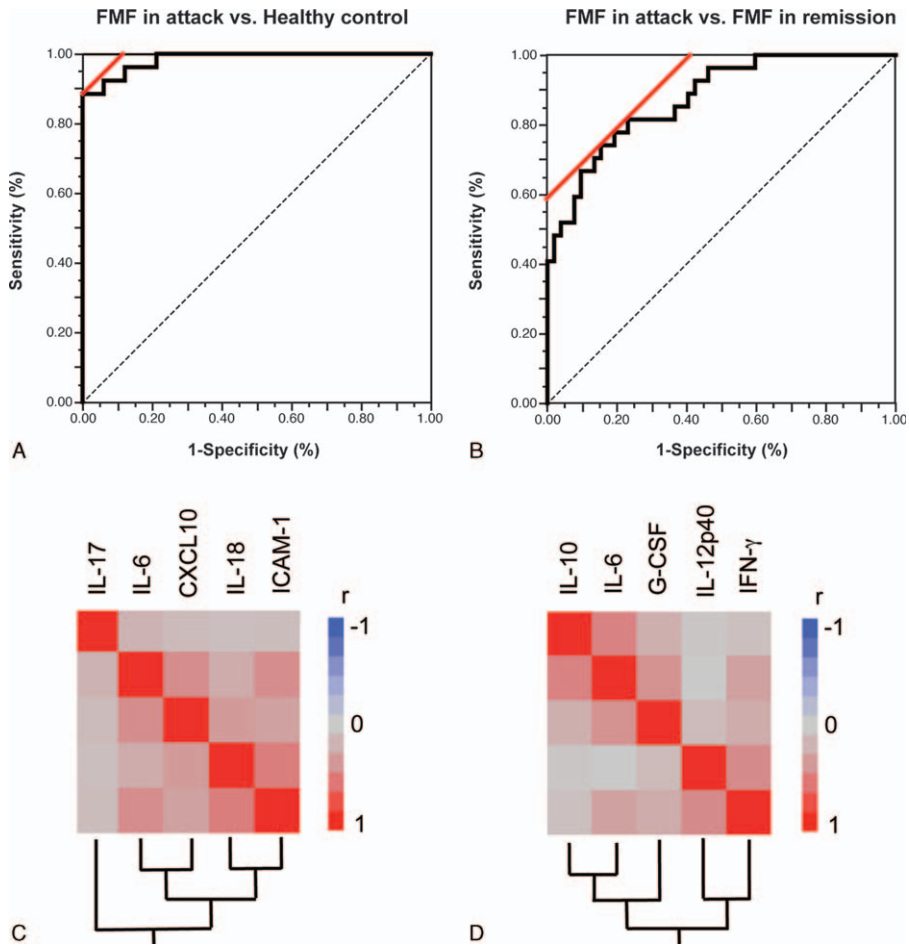

FIGURE 2. ROC curve analysis for the prediction of FMF in attack by a specific set of cytokines. (A) Healthy control versus FMF in attack; the combined measurement of IL-6, IL-18, and IL-17. (B) FMF in remission versus FMF in attack; the combined measurement of IL-6, G-CSF, IL-12p40, and IL-10. Hierarchical clustering with a Spearman correlation heat map of serum cytokine levels among (C) the FMF in attack and healthy control groups and (D) the FMF in attack and FMF in remission groups. FMF = familial Mediterranean fever, G-CSF = granulocyte colony stimulating factor, IL = interleukin, ROC receiver operator characteristic.

with the highest accuracy (sensitivity 75.0%, specificity 87.9%, and accuracy 84.0%) The prediction of attack used the following formula:

Probability (attack) = exp(A)/{1 + exp(A)} [A = 2.550 − 0.085*IL-6 − 0.002*G-CSF − 0.001*IL-12p40 − 0.231*IL-10].

Activated Cytokine Networks Among the FMF in Attack and Healthy Control Groups and Among the FMF in Attack and FMF in Remission Groups

To identify activated cytokine networks of FMF in attack, we further examined the correlations between the serum concentrations of the top 5 individual cytokines ranked by the RFA. In the FMF in attack and healthy control groups, there were significant correlations between sCD54 and IL-18 ($r = 0.547$, $P = 0.010$), IL-6 and sCD54 ($r = 0.487$, $P < 0.001$), CXCL10 and sCD54 ($r = 0.343$, $P = 0.006$), CXCL10 and IL-6 ($r = 0.474$, $P = 0.001$), and CXCL10 and IL-18 ($r = 0.559$, $P = 0.005$).

In the FMF in attack and FMF in remission groups, there were significant correlations between G-CSF and IL-6 ($r = 0.463$, $P < 0.001$), IL-6 and IL-12p40 ($r = 0.282$, $P = 0.040$), IL-10 and IL-6 ($r = 0.531$, $P < 0.001$), IL-10 and IL-12p40 ($r = 0.731$, $P < 0.001$), IL-10 and G-CSF ($r = 0.494$, $P = 0.047$), IFN-γ and IL-12p40 ($r = 0.457$, $P < 0.001$), IFN-γ and G-CSF ($r = 0.276$, $P = 0.012$), and IFN-γ and IL-6 ($r = 0.356$, $P = 0.0010$). Hierarchical clustering with heatmaps based on the Spearman correlation coefficients are shown in Figure 2C (healthy control and attack) and D (attack and remission).

Validation Analysis of the Combinational Biomarkers in the Serial Serum Samples From the FMF Patients

To determine whether the identified combinational biomarkers in this study are also relevant in serial serum samples, we analyzed serial cytokine changes in attack and in remission in 7 patients with FMF. As shown in Figure 3, the serum levels of IL-6, G-CSF, IL-12p40, and IL-10 were significantly decreased in FMF remission compared to FMF attack.

Comparison of *MEFV* Genotypes With Cytokine Profiling

Since it was proposed that mutations at position 680 to 694 in exon 10 present a risk of severe FMF,[15] we investigated

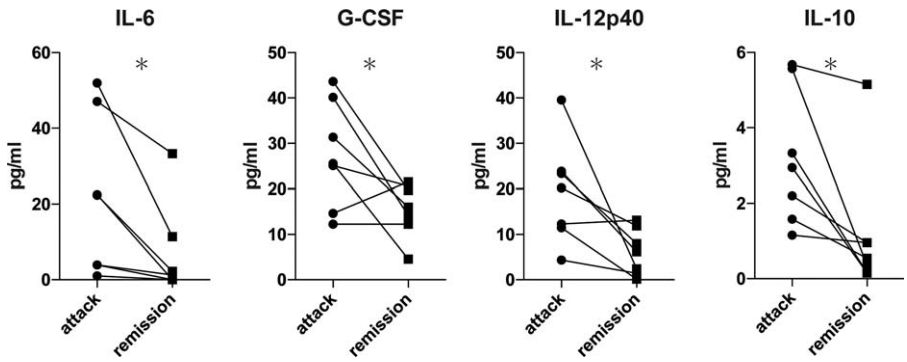

FIGURE 3. Serial cytokine changes in attack and in remission. The lines link the same patients. The changes from baseline were compared using Wilcoxon signed rank test ($^*P < 0.05$).

whether these mutations can affect the levels of cytokines. To this end, we compared 7 important cytokines of FMF in attack and in remission determined by the RFA in the absence or presence of *MEFV* mutation in exon 10. As shown in Table 4, no significant difference was observed among these cytokines in attack. In contrast, the serum level of IL-18 in remission was significantly higher in the patients with *MEFV* mutation in exon 10 (Table 4).

DISCUSSION

FMF is a hereditary autoinflammatory disease caused by the mutation of pyrin, which is involved in inflammasome complex formation.[16] Accordingly, activated cytokine networks are implicated in the pathogenesis of FMF.[3,10,17] However, these prior studies focused on a few individual cytokines in FMF. Our present work suggested cytokine networks in FMF patients by using a multisuspension cytokine array system, and we were able to identify a set of possible diagnostic and disease activity markers for FMF with high accuracy.

IL-6 is an inflammatory cytokine that plays a pivotal role in autoimmune and chronic inflammatory diseases. Increased levels of IL-6 in the serum during FMF attacks were reported.[10,17,18] Our present findings demonstrated that IL-6 had the best performance for distinguishing FMF in attack from healthy controls or FMF in remission. Other recent case reports have shown the efficacy of an IL-6 inhibitor in clinical practice for colchicine-resistant FMF or secondary amyloidosis in FMF patients.[19–22] Taken together, our data support the notion of IL-6 as a main inflammatory cytokine in FMF and, thus, as a promising target in this disease.

Inflammasomes can activate caspase-1, which processes pro-IL-1β and pro-IL-18 from their inactive to mature and biologically active forms[23] and these cytokines are closely associated with T-helper (Th) cell differentiation.[24] The abnormal regulation of the innate immune response and Th17 cell polarization via IL-1 signaling plays pathogenic roles in the development of autoinflammatory diseases including FMF.[25] On the other hand, IL-18 has the ability to induce Th1 cell differentiation by enhancing INF-γ production.[26,27] As also observed in an earlier study,[28] our findings demonstrated that the serum IL-17 and IL-18 levels of FMF patients both in attack and in remission were significantly higher than those of healthy controls, and that the levels of serum IL-17 and IL-18 were comparable in FMF patients in attack and in remission.

In addition, CXCL10, IL-12p40, and IFN-γ, which are known as Th1-related chemokines and cytokines,[29–31] were significantly elevated in FMF patients in attack. The data obtained in the present study indicate that aberrant Th1/Th17 differentiation or activation

TABLE 4. Comparison of Cytokine Profile in FMF With or Without Mutations in Exon 10*

	In Attack			In Remission		
	Exon 10 Mutations			Exon 10 Mutations		
Cytokine	(+) (n = 13)	(–) (n = 15)	*P*-Value	(+) (n = 39)	(–) (n = 15)	*P*-Value
IL-6	21.0 (3.9–50.2)	26.4 (16.6–49.2)	0.17	2.9 (0.1–8.4)	1.61 (0.8–3.5)	0.85
IL-10	2.2 (1.4–8.2)	3.0 (1.2–14.9)	0.53	0.4 (0.2–2.4)	1.4 (0.2–2.2)	0.19
IL-12p40	12.3 (5.7–20.1)	11.0 (7.1–23.9)	0.98	1.5 (0.1–12.3)	6.2 (0.4–75.5)	0.17
IL-17	25.5 (5.4–59.7)	7.0 (2.8–22.9)	0.062	7.1 (2.6–16.5)	6.5 (2.2–16.8)	0.79
IL-18	673.2 (162.5–1245)	83.5 (50.6–691.6)	0.084	378.8 (107.2–944.1)	66.5 (28.7–475.7)	**0.007**
G-CSF	26.0 (13.5–56.6)	43.6 (31.4–97.6)	0.16	18.8 (5.3–45.6)	20.6 (14.1–27.8)	0.72
sCD54*	144.8 (99.4–168.4)	150.3 (116.9–225.0)	0.37	106 (92.2–163.3)	146.5 (105.1–255.5)	0.061

P-values were established using Wilcoxon rank-sum test. Bold indicates significant values. G-CSF = granulocyte colony stimulating factor, IL = interleukin, sCD54 = soluble CD54.

*Values are the median (interquartile range) pg/mL.

is involved in acute and subclinical chronic inflammation in FMF. A plausible explanation is thus that the combination of IL-17 and IL-18 with IL-6 is very characteristic in activated cytokine patterns for FMF patients in attack.

We observed that the serum levels of sCD54 were significantly higher in the FMF attack group compared to the healthy controls but not significantly higher than the levels of the FMF patients in remission. This finding suggests that CD54 is involved in the development of FMF and that it could be useful as a diagnostic marker. CD54 is an intercellular adhesion molecule expressed mainly by the vascular endothelium, macrophages, and lymphocytes. This protein can be induced by pathogenic cytokines related to autoinflammatory diseases, such as IL-1β, IL-18, and TNF-α.[32–34] Indeed, it has been reported that the level of sCD54 was increased in the serum from patients with FMF and patients with Adult Still Disease.[35,36] These finding are consistent with our present observation that sCD54 clearly correlates with IL-18 in the serum from FMF patients in attack.

In our comparison of the FMF patients in attack and those in remission, we found highly elevated serum levels of IL-6, G-CSF, IL-12p40, and IL-10. Although elevated G-CSF in the serum from FMF patients had not been reported previously, Stojanov et al observed that the serum level of G-CSF was significantly higher in patients with periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) attacks.[37] We also detected the elevated expression of CD64 on neutrophils in patients with FMF.[38,39] These observations support the importance of G-CSF in FMF attack.

IL-10, considered a regulator of inflammation, was also elevated in FMF attack. Elevated serum levels of IL-10 along with proinflammatory cytokines were reported in FMF patients in attack[10] and other inflammatory diseases or macrophage activation syndrome.[40–43] Accordingly, this increase in IL-10 could be partly explained by a counter response that functions to regulate the aberrant production of inflammatory cytokines.

IL-12, a heterodimeric protein of two subunits (p35, p40; p70 is a heterodimer of p35 and p40) secreted by phagocytic cells in response to pathogens, is crucial in Th1 cell polarization.[44] Increased levels of IL-12 in the serum of FMF patients in both attack and remission were reported,[3,45] but the distinction of each subunit was not clear in the previous reports, and our present data showed the difference in the IL-12p40 subunit only. The increase in serum IL-12 is considerable among FMF patients in attack. In addition, although only 7 patients were available for our examination of the cytokine profiles in both attack and remission, all 4 of the above-mentioned cytokines (IL-6, G-CSF, IL-12p40, and IL-10) in remission are decreased in total or individually. Accordingly, we speculate that the serum levels of these combinational biomarkers would be appreciable in cases of serial measurements in FMF patients.

Although the efficacy of an IL-1 inhibitor and a TNF inhibitor in severe FMF patients has been shown,[46] we could not find any significant differences in these cytokines among our patients with FMF. Possible reasons for this discrepancy are that: the amount of the detectable soluble form of IL-1β is limited due to the intracellular localization of pro-IL-1β or binding to target proteins such as IL-1RA in the serum; IL-1β and TNF-α are produced mainly in inflamed local tissues; and the concentration of these cytokines in the serum is not great enough for the assay that we used to detect significant differences. Thus, we cannot exclude the possibility that the contributions of IL-1β and TNF-α to the pathogenesis of FMF are underestimated in the present study.

We also attempted to determine whether cytokine levels are affected by *MEFV* exon 10 mutation, as an association between *MEFV* exon 10 mutation and subclinical inflammation in FMF patients was described.[3] In the present study, we found that there was no significant difference in 7 important cytokines in FMF attack. These observations indicate that the mutation in exon 10 does not affect cytokine levels in attack among Japanese FMF patients and that the utility of combinational biomarkers is assured regardless of *MEFV* gene mutations.

Importantly, our findings also showed that serum IL-18 in remission was significantly higher in the FMF patients with *MEFV* exon 10 mutation than in those without mutation, whereas other cytokines were not significantly different in either attack or remission. A 2014 study of Japanese FMF patients also demonstrated that the serum IL-18 level in FMF patients in remission was significantly higher in typical FMF with M694I mutation than in atypical FMF with exon2 variants including E148Q.[47] Based on our present data and previous findings, we speculate that in FMF, a pathogenic mutation in exon 10 facilitates IL-18-dependent subclinical inflammation via an aberrant regulation of the pyrin inflammasome cascade.

Our study has several limitations. Serial serum sampling was possible for only some of the patients. Therefore, large validation studies are required to characterize the performance of this assay. In addition, a considerable number of our patients in remission were treated with colchicine, the mode of action of which is to inhibit the NALP3 inflammasome.[48] Thus, inflammatory cytokines in remission could be pharmacologically suppressed by colchicine, and this may not reflect the nature of cytokine networks of FMF patients in remission. However, no large-scale, comprehensive analysis has been conducted to test the findings of past studies and clarify the association between each pathogenic cytokine in FMF. Our present data show, for the 1st time, specific cytokine networks in FMF. These findings help to improve the diagnostic performance of FMF in daily practice and facilitate our understanding of the inflammatory mechanisms of FMF patients.

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