Table 3. Clinical presentations of 80 patients with FMF.

Age at Onset, yrs, n (%)						Sex, Male,	Clinical Manifestations, n (%)					
< 10	10–19	20–29	30–39	40–49	> 50	n (%)	Fever	Pleuritis	Peritonitis	Arthritis	Erysipelas-like Erythema	Amyloidosis
19 (23.8)	32 (40.0)	20 (25.0)	6 (7.5)	2 (2.5)	1 (1.3)	33 (41.3)	79 (98.8)	49 (61.2)	44 (55.0)	22 (27.5)	8 (10.0)	1 (1.3)

Table 4. Dose of colchicine in 28 patients. Number of patients in whom colchicine was effective is given in parentheses.

Dose mg/day	No. Patients
≤ 0.5	13 (11)*
1	13 (10)**†
1.5	1 (1)
2	1 (1)
> 2.0	0

* The efficacy was unclear in 2 patients. ** Efficacy was unclear in one patient in our survey. † No efficacy was observed in 2 patients.

Table 5. Genotypes of *MEFV* gene of the 80 cases.

MEFV mutation	No. Patients (%)
E148Q/M6941	20 (25.0)
M6941/normal	14 (17.5)
L110P/E148Q/M6941	14 (17.5)
L110P/E148Q	9 (11.3)
M6941/M6941	5 (6.3)
L110P-E148Q/E148Q	4 (5.0)
L110P/M6941	2 (2.5)
E148Q/P369S/R408Q/S503C	2 (2.5)
L110P-E148Q/L110P-E148Q	2 (2.5)
R202Q/M6941	1 (1.3)
E148Q/E148Q-R761H	1 (1.3)
L110P/E148Q/P369S/R408Q	1 (1.3)
E148Q/P369S/R408Q	1 (1.3)
P369S/R408Q	1 (1.3)
E148Q/R202Q	1 (1.3)
E148Q/E148Q	1 (1.3)
E84K/normal	1 (1.3)

M694I alone (14 patients, 17.5%), and L110P/E148Q/M694I (14 patients, 17.5%). Nine patients (11.3%) had L110P/E148Q and 5 (6.3%) were homozygous for the M694I mutation. The majority of patients carried E148Q (56 patients) or M694I (56 patients) at least on an allele, but L110P was also identified in 32 patients. As minor mutations, E84K[19], R202Q, P369S, R408Q, S503C[18], and R761H were found in some patients (Table 5), but most of those mutations were detected with L110P, E148Q, or M694I. Only 2 patients, who had P369S/R408Q or E84K alone[19], did not carry L110P, E148Q, or M694I. The other mutations, including M694V, M680I, and V726A in exon 10, which were common in Mediterranean patients with FMF[28], were not found in these 80 patients.

Allele frequencies of L110P, E148Q, and M694I in 51 healthy individuals (102 alleles) were 0.039, 0.26, and 0, respectively. On the other hand, allele frequencies of these 3 mutations were examined in 39 FMF patients and the results were 0.31 (L110P), 0.44 (E148Q), and 0.35 (M694I). The differences in allele frequencies between healthy populations and those with FMF were statistically significant ($p < 0.001$ for L110P and M694I; $p < 0.02$ for E148Q).

DISCUSSION

Clinical features of Japanese patients with FMF. Our study shows that the clinical pictures of Japanese patients with FMF seem to be different from those of Mediterranean patients. The frequencies of cardinal clinical symptoms during attacks in Japanese and Mediterranean FMF patients[2] are shown in Table 6. Mediterranean patients almost always have abdominal symptoms due to peritonitis[2]. However, the frequency of abdominal symptoms in Japanese patients was relatively low (55.0%). Because the frequencies of chest symptoms due to pleuritis, arthropathy, and erysipelas-like erythema are quite variable even among Mediterranean FMF patients, no clear differences were seen in such symptoms between Mediterranean and Japanese patients. In the literature, the relation between severity of the disease and the diet low in animal fat is discussed, and in particular, it was reported that butter ingestion appeared to provoke peritonitis attacks[29]. Although the mechanism of the low frequency of abdominal symptoms in Japanese patients remains unclear, the difference in diet between Japanese and Mediterranean FMF patients may have effects on the difference of phenotype.

Because of atypical symptoms like high fever or abdominal pain, 5 patients with Behçet's disease underwent the *MEFV* gene analysis. All of them clinically met the Tel-Hashomer criteria and were therefore diagnosed as having both FMF and Behçet's disease. However, there was no significant difference between the patients with con-

Table 6. Frequency of symptoms during attack (%) in different races.

	Japanese	Mediterranean populations[2]			
	(80 cases)	Turks	Jews	Arabs	Armenians
Fever	98.8	93	100	100	100
Peritonitis	55.0	94	95	82	96
Pleuritis	61.3	31	40	43	87
Arthritis	27.5	47	77	37	37
Erysipelas-like erythema	10.0	21	46	3	8

comitant Behçet's disease and the patients with FMF alone in terms of the clinical severity of FMF symptoms.

With regard to age at disease onset, 90% of FMF patients experience their first attacks before the age of 20 years and the percentage of patients with onset at age over 30 is less than 5% in the Mediterranean area[2,3]. In Japanese patients, 63.8% of patients experienced their first attack before age 20 and 11.3% of patients after age 30, indicating that FMF onset in Japanese patients was much later than in Mediterranean patients. The Turkish FMF Study Group reported that the mean period from disease onset to diagnosis of FMF in Turkey was 6.9 ± 7.65 years[28], and there may also be a delay in the diagnosis of FMF in Japanese patients, probably due to the low recognition of this disorder in Japan.

Administration of colchicine is known to be the most effective therapy for FMF to reduce the frequency, duration, and severity of attacks in most patients, and it has commonly been used in doses of 1.0–2.0 mg/day[2]. Moreover, Pras, *et al* noted that 30% of North African Jewish patients needed 2 mg or more of colchicine to control their symptoms[30]. In our study a small dose of colchicine, not over 1.0 mg/day, showed a favorable therapeutic effect in the majority of Japanese patients, so a relatively lower dose of colchicine may control the attacks of FMF symptoms in Japanese as described[21].

Prevalence of reactive systemic AA amyloidosis in Japanese patients with FMF. Although the incidence of reactive systemic AA amyloidosis in Mediterranean FMF patients varies in different ethnic groups, AA amyloidosis occurs very frequently in North African Jews (12.4%–26%), Iraqi Jews (9.5%–15%), Ashkenazi Jews (11%), Arabs (12%), Armenians (24%), and Turks (12.9%)[28,30-32]. On the other hand, the prevalence of AA amyloidosis in our study was quite low. Of 80 patients, only one male patient[10] had AA amyloidosis, which had been detected 3 years before the *MEFV* gene mutation (M694I) was identified. At the time he was diagnosed as having amyloidosis, he did not receive treatment with colchicine. However, in 21 out of 80 FMF patients who had not been treated with colchicine, to date no patient has had AA amyloidosis. Thus, the prevalence of AA amyloidosis associated with FMF in Japanese would appear to be lower than in Mediterranean patients, regardless of treatment with colchicine.

Genotype of MEFV gene in Japanese patients with FMF. The characteristics of the genotype of the *MEFV* mutations in Japanese patients were that almost all patients were homozygous, heterozygous, compound heterozygous, and/or complex allele for L110P, E148Q, and/or M694I. The correlation between the *MEFV* genotype and phenotype (severity of the disease) in FMF has been well discussed. The C-terminal B30.2 domain of pyrin encoded by exon 10 is known to play an important role in its function, interacting directly with caspase-1 to modulate interleukin 1ß production[33]. In addition, the methionine residue in codon 694 makes a crucial contribution to the function of pyrin[34]. Thus, the mutations in codon 694 are considered to produce severe symptoms with early onset and high frequency of attacks and the necessity of a high dose of colchicine to prevent attacks[2]. In particular, the M694V mutation is regarded as a significant risk factor for secondarily developing amyloidosis[3,7,35]. However, in our study none of the 80 patients carried this mutation. While the M694I mutation was the one most frequently found in Japanese patients, the majority of the patients were compound heterozygous or complex allele for M694I and other mutations producing a relatively milder phenotype such as E148Q and/or L110P, or heterozygous for M694I alone. In addition, numbers of Japanese patients were compound heterozygous or complex allele for E148Q and L110P, so the characteristics of Japanese patients such as late onset and low prevalence of AA amyloidosis would be associated with differences of *MEFV* genotype compared with Mediterranean patients.

It remains controversial whether the E148Q mutation is a disease-causing mutation or a simple polymorphism because of high allele frequency in healthy controls[36-39]. However, it has been reported that most homozygote or compound heterozygote patients associated with other *MEFV* mutations are symptomatic[40,41], and it has also been noted that the allele frequency of E148Q is significantly higher among patients with AA amyloidosis and chronic fever of unknown origin[41]. Moreover, the E148Q mutation was described as producing a milder FMF phenotype with low penetrance[2,6]. While in our study 5 healthy controls were proved to be homozygous for E148Q, the allele frequency of E148Q in patients with FMF was significantly higher than in healthy individuals. Hence we also consider that this mutation can cause FMF, especially when patients are compound heterozygous for E148Q and other *MEFV* mutations or homozygous for E148Q[14].

The L110P mutation was first reported in 2000[42], and to date, several patients have been reported to be compound heterozygote with other mutations even in Japan[19,21]. In our study, 30 out of the 80 patients carried L110P as heterozygote with other mutations, and among these, 28 were compound heterozygous or complex allele for L110P and E148Q. Moreover, there was a significant difference in the frequency between FMF and healthy populations. Therefore, it is considered that L110P can also be associated with the onset of FMF.

Although it is true that *MEFV* gene analysis is needed to establish a definite diagnosis in suspected cases of FMF, *MEFV* mutations are not always found on both alleles even in typical FMF patients[7]. Therefore, diagnosis based on the clinical diagnostic criteria, family history, and the patient's response to colchicine treatment is of great importance in this disorder.

Our study indicates that the clinical presentations and the

MEFV genotype of Japanese patients with FMF seem to be different from those of Mediterranean patients, and our survey suggests that there will be a large number of FMF patients even in Japan.

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Protracted synovitis without systemic manifestations in familial Mediterranean fever (FMF)

Sirs,

It is now being recognised that familial Mediterranean fever (FMF) also occurs in non-Mediterranean populations, including Japanese (1). The arthritis of FMF consists of acute attacks of pain and swelling of one articulation at a time, most frequently affecting the large joints of the lower extremities within 2-3 days duration (2). In some cases, however, protracted arthritis develops, mostly in the hips and knees (3). Furthermore, disabling joint damage, including joint replacement therapies, has been reported (4). We describe a patient with FMF with protracted arthritis without elevations of acute phase proteins.

A 17-year-old Japanese girl was hospitalised because of pain and swelling of her bilateral knee joints. She suffered from repeated massive bilateral knee joint effusions, for 2 years. Synovial fluid aspirated from the knee joint was clear and cultures for microorganisms were negative. The patient was referred to our hospital because of sustained knee joint pain and effusion. Review of the patient's medical history indicated that the knee joint pain or effusion were not associated with fever, chest or abdominal pain. Physical examination showed a temperature of 36.4°C; pulse 82 beats/min; blood pressure 110/72mmHg. The knees were swollen with marked effusions. Radiographic evaluation demonstrated no positive findings except soft tissue swelling. Laboratory data indicated no abnormal findings, including a lack of anti-nuclear antibody, rheumatoid factor, or anti-cyclic citrullinated peptide (CCP) Ab. There was no elevation of CRP (<0.30mg/dl), ESR (6mm/hr) or serum amyloid A protein (SAA, 3.8μg/ml). MRI imaging of the knee demonstrated the massive fluids and synovial hypertrophy. Arthroscopy showed synovial hypertrophy, which was characterised by heavy and villously proliferative synovial tissues (Fig. 1). The histopathological findings of the biopsied synovial tissues showed severe synovial inflammation with infiltrations of plasma cells, lymphocytes and neutrophils (Fig. 2). We performed the sequencing of all 10 exons of the *MEFV* gene and detected a heterozygous mutation (GAG to AAG) in codon 84 of the exon 1 of the *MEFV* gene that resulted in a substitution of lysine for glutamic acid (E84K). We initiated daily colchicine treatment (1.0mg/day), the loss of joint effusions was confirmed and the bilateral knee joint pains disappeared.

The frequency of arthritis in FMF has been reported to be more than 70% (5). The arthritic episodes in FMF are usually associated with fever and affect the large joints of the lower extremities (6). The arthritis typically resolves spontaneously, over the course of a few days (6). However, about 5% of patients have recurrent attacks and develop chronic arthritis (7). Previous reports have estimated that only 10% patients with FMF experience arthritis as their sole manifestation (8).

We described a patient with FMF who developed long-standing, symmetrical knee joint arthritis with synovial hyperplasia. We speculate that this case is a distinct and rare clinical subtype of FMF arthritis without fever or elevations of acute phase proteins, which has not been described previously.

More recently, Tomiyama *et al.* reported a new *MEFV* mutation, E84K, in a Japanese FMF patient (9). We could not comment on linkage between the E84K genotype and the arthritis-dominant phenotype observed in our FMF case. The recent study indicated that colchicine was effective in non-typical cases of MEFV-associated diseases apart from FMF (10). Alternatively, we should reconsider the possibility of the additional *MEFV* gene mutation-associated syndromes. Future studies aimed to clarify the role of *MEFV* variants in the clinical manifestations of FMF are needed.

In conclusion, arthritis could present as the sole manifestation in FMF patients without systemic manifestations. The present case highlights the importance of FMF in the differential diagnosis of chronic symmetrical arthritis in young adults.

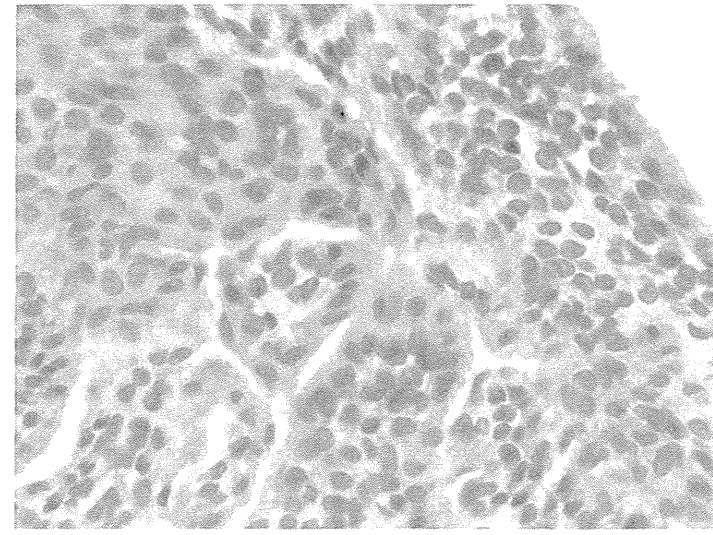

Fig. 1. High-power of photography of the biopsied synovilal tissues. Note the infiltrations of inflammatory cells, including plasma cells lymphocytes and neutrophils, into the synovial tissues (Hematoxylin-Eosin; original magnification x200)

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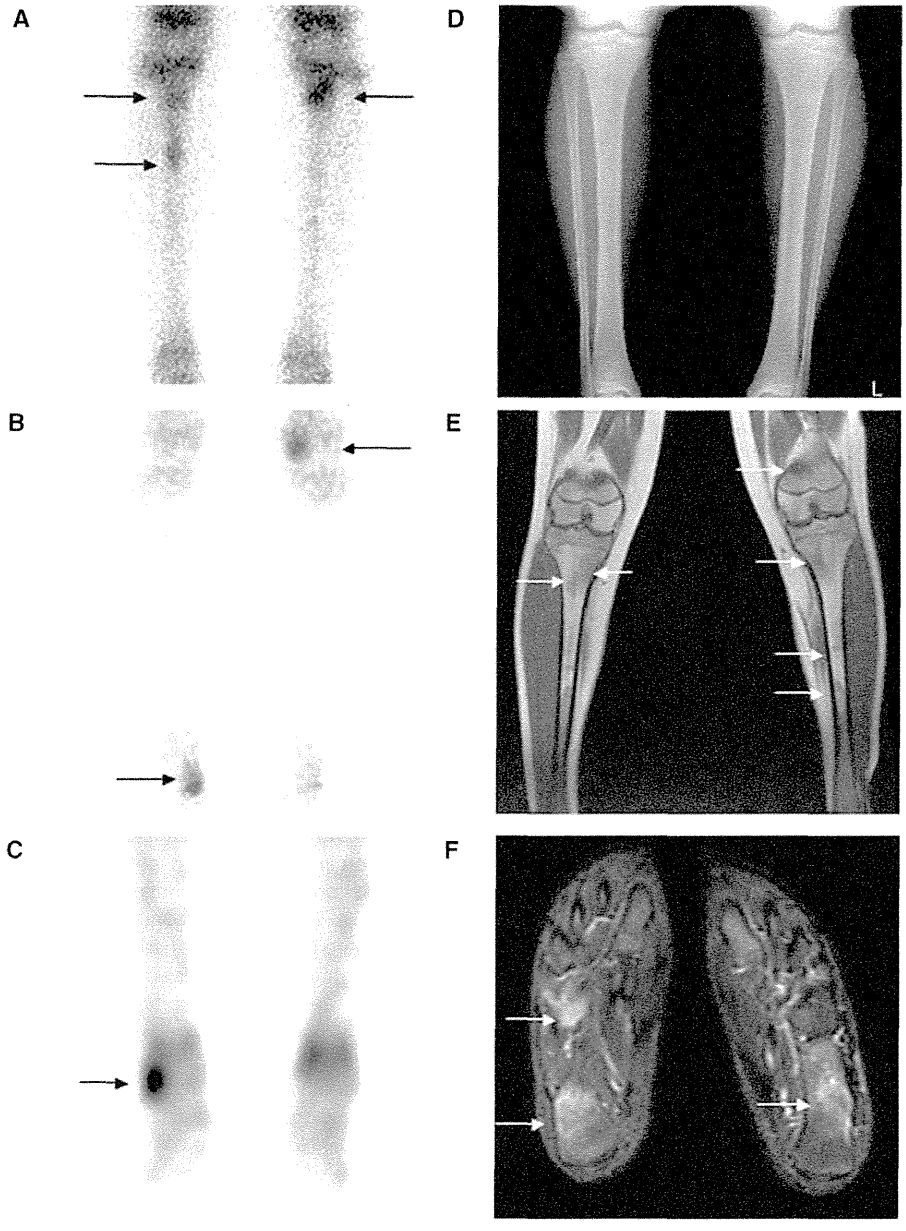
Colchicine-responsive chronic recurrent multifocal osteomyelitis with *MEFV* mutations: a variant of familial Mediterranean fever?

Sir, FMF is an autosomal recessive disease characterized by self-limited recurrent attacks of fever with serositis such as peritonitis, pleuritis and arthritis [1]. FMF is caused by mutations in the *MEFV* gene [2]. This gene had been considered to be responsible only for FMF in the past; however, recent reports show that the *MEFV* gene is associated with more than typical FMF and is linked to additional clinical presentations within the family of the autoinflammatory diseases [2–4]. Here, we describe a case of colchicine-responsive chronic recurrent multifocal osteomyelitis (CRMO) with *MEFV* gene mutations.

A 14-year-old female was referred with fever of unknown origin persisting for 15 days. Physical examination was unremarkable. Laboratory findings showed normal white blood cell count (3.9×10^9/l), high levels of CRP (3.1 mg/dl), accelerated ESR (72 mm/h), normal levels of immunoglobulins and negative autoantibodies. Blood culture was negative. Unexpectedly, gallium (Ga) scintigraphy on Day 3 after admission demonstrated significant uptake in the bilateral proximal region of the tibia (Fig. 1A). Plain radiography showed no significant findings (Fig. 1D), but MRI demonstrated multifocal lesions whose intensity was low in T_1-weighted condition and high in T_2-weighted condition in bilateral tibia (Fig. 1E). Biopsy of the left tibia showed non-specific inflammatory changes and no malignant cells. The culture of bone marrow was negative. She had severe pain of the left heel on Day 21. MRI on Day 23 demonstrated multifocal lesions whose intensity was low in the T_1-weighted condition and high in the T_2-weighted condition in bilateral tarsal bones (Fig. 1F). Ga scintigraphy on Day 38 demonstrated significant uptake in left calcaneus and bilateral femur (Fig. 1B). From these findings, the diagnosis of multifocal recurrent osteomyelitis was made. No evidence of bone destruction or hyperostosis was observed at the time of diagnosis. High fever continued despite treatment with appropriate antibiotics and naproxen for 8 weeks. However, she was relieved dramatically from high fever soon after colchicine (2 mg/day) was started. Mutation analysis demonstrated the heterozygous mutation E148Q-P369S-R408Q *in cis* on one allele of the *MEFV* gene. But no mutation was found in the *LPIN2* gene. Colchicine dose was gradually decreased to 0.5 mg/day and daily colchicine therapy (0.5 mg/day) relieved her from febrile attacks for 1 year, although she had one episode of osteomyelitis in the left fibula (Fig. 1C) when she ceased to take colchicine.

FIG. 1 (**A–C**) Ga scintigraphy: the image on Day 3 (A) showing significant uptake in the bilateral proximal region of tibia. Day 38 (B) showing significant uptake in the left calcaneus and bilateral femur. Nine months later (C) showing significant uptake in the left fibula. (**D**) A plain X-ray, frontal view, showed no significant findings including scrolesis in the bilateral tibia. (**E** and **F**) MRI: the T_1-weighted image on Day 3 (E) showing multiple lesions whose intensity was low in the bilateral tibia. The T_2-weighted image on Day 23 (F) showing multiple lesions whose intensity was high in the bilateral tarsal bones.



FMF is an autosomal recessive, inherited periodic inflammatory syndrome, characterized by self-limited recurrent attacks of fever with serositis such as peritonitis, pleuritis and arthritis [1]. The disease is common among people of eastern Mediterranean ancestry. The mainstay of treatment is cochicine, which is effective for both relieving symptoms and preventing secondary amyloidosis. The *MEFV* gene encodes a protein named pyrin, which is expressed in neutrophils and monocytes. The function of pyrin is still unknown and remains to be determined.

The *MEFV* gene had been considered to be responsible only for FMF in the past. However, about one-third of patients with FMF have a single mutation on one allele. This finding suggests that FMF might be transferred as an autosomal dominant trait with partial penetration. Another possibility is that an additional, unidentified gene might be

associated with the disease in these patients with single allele mutation.

Recently, a case with heterozygous *MEFV* mutations and distinct clinical presentations not typical of FMF has been reported, with colchicine-responsive recurrent episodes of muscle pains [3]. These reports including our case show that the *MEFV* gene is associated with more than a single disease (FMF) and is linked to additional clinical presentations within the family of the autoinflammatory diseases [4, 5] and some rheumatic diseases such as systemic-onset juvenile idiopathic arthritis [6, 7].

Mutation analysis in our patient demonstrated the heterozygous mutation E148Q-P369S-R408Q. In the whole list of 187 sequence alterations reported in Infevers—an online database for autoinflammatory mutations available at http://fmf.igh.cnrs.fr/ISSAID/infevers [8], this mutation is reported to be associated with atypical clinical presentations of FMF.

CRMO is an ill-defined inflammatory disease. In typical cases, multiple bone lesions with apparent bone destruction, hyperostosis and pustulosis of the skin are seen [9]. But there are variable clinical manifestations, which make differential diagnosis of CRMO often difficult. *LPIN2* mutation is detectable in a syndrome form of CRMO known as Majeed syndrome [10], but for most cases the responsible gene is unknown.

CRMO is unusual, or an unexpected manifestation of FMF and this is the first case of CRMO with *MEFV* mutations to our knowledge. To start the treatment with colchicines promptly, thereby relieving symptoms and preventing secondary amyloidosis, mutation analysis of *MEFV* gene should be performed in cases of CRMO.

Rheumatology key message

- The *MEFV* gene might be associated with more than typical FMF.

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Adenosine 5′-triphosphate infusions reduced disease activity and inflammation in a patient with active rheumatoid arthritis

Sir, Inhibition of inflammation by MTX is partly mediated by adenosine, accumulating through extracellular breakdown of adenosine 5′-triphosphate (ATP) [1]. Extracellular ATP is also able to down-modulate inflammation [2–5]. Previously, anti-inflammatory effects of ATP by i.v. infusion on CRP levels in lung cancer patients were observed *in vivo* [6, 7]. Since CRP in RA strongly correlates with disease activity [8], we hypothesized that ATP treatment could inhibit inflammation and disease activity in RA, and initiated a small placebo-controlled randomized clinical trial to test this hypothesis. Herein, we describe remarkable effects observed in an RA patient following ATP treatment.

The 50-year-old female patient, who had been diagnosed with seropositive, non-erosive RA 16 months prior to enrolment in the trial, received anti-rheumatic treatment with MTX at a limited maximum dose of 15 mg once weekly due to gastrointestinal side effects at higher doses, despite adequate folic acid supplementation and not resolving with parenteral MTX administration. Similar

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CASE REPORT

A case of familial Mediterranean fever associated with compound heterozygosity for the pyrin variant L110P-E148Q/M680I in Japan

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Abstract Familial Mediterranean fever (FMF) is an autosomal recessive disorder characterized by recurrent and self-limited fever attacks and serositis/arthritis. The M694V, M694I, M680I, V726A, and E148Q mutations in *MEFV*, the gene responsible for FMF, account for most FMF cases in Mediterranean populations. In Japan, M694I and E148Q are most frequently detected; M694V, M680I, and V726A have not been identified so far. We report the first case of FMF associated with M680I in Japan.

Keywords Familial Mediterranean fever · M680I · MEFV

Introduction

Familial Mediterranean fever (FMF) is an autosomal recessive disorder that is particularly common in Mediterranean populations [1]. It is characterized by recurrent and self-limited attacks of fever, serositis or arthritis and subsequent secondary amyloidosis [1–3]. The gene responsible for FMF-the Mediterranean fever gene (*MEFV*)-has been mapped to chromosome 16p13.3 [4–6]. It consists of 10 exons and encodes a protein comprising 781 amino acids called pyrin or marenostrin, which is expressed mainly in granulocytes and monocytes [4, 5]. So far, 184 mutations and polymorphisms of this gene have been identified [7]. Five common mutations (M694V, M694I, M680I, V726A, and E148Q) account for the vast majority of FMF mutations [8–10]. The clinical symptoms of FMF vary according to the mutations of *MEFV*. The M694V homozygote and compound heterozygote for M694V are associated with greater disease severity [11, 12]. Notably, E148Q is found in 16–25% of normal individuals in Japan [13], and it is known that compound heterozygous mutations of E148Q with M694V, M694I, M680I or V726A cause FMF.

In Japanese individuals, FMF is an extremely rare disease because of the low allele frequencies of the disease-causing mutations [13]. E148Q/M694I seem to be the most frequent alleles in Japanese FMF patients [13–15]. The M694V, M680I, and V726A mutations have not been found in Japan so far; herein, we report the first case in Japan of FMF in a patient who is compound heterozygote for L110P–E148Q/M680I.

Case report

A 7-year-old Japanese boy was brought to our hospital in 2007 for periodic fever accompanied by chest pain. He had experienced fever and chest pain once a year since the age of 3 years. The fever and chest pain continued for about 3 days after onset, and then disappeared spontaneously.

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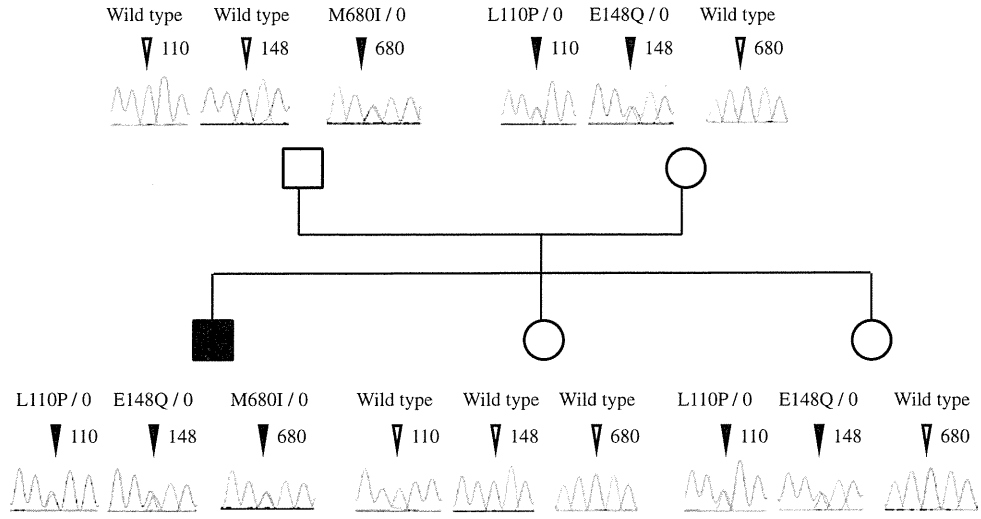

Fig. 1 Pedigree and chromatograms of the *MEFV* gene at amino acid positions 110, 148, and 680. *Black arrowheads* show heterozygous mutations for L110P, E148Q or M680I

None of his family members had these symptoms. From the age of 6 years, he experienced these symptoms about once every 2 months. At the time of the fever attack, he could not breathe deeply due to the chest pain. Subsequently rapid shallow breathing was recognized. Laboratory examinations showed mild leucocytosis (13,200 WBCs/μl) and elevated levels of C-reactive protein (CRP) (5.9 mg/dl) during an episode. Chest X-ray and electrocardiography revealed no abnormalities, and the patient did not have arthritis or rashes. Because he met the criteria for FMF based upon the Tel Hashomer criteria [2], we made a clinical diagnosis of FMF. After obtaining informed consent, we performed a genomic search for *MEFV*. Since the patient was found to be heterozygous for L110P–E148Q/M680I (Fig. 1), FMF was confirmed by the mutation in the hot spot of *MEFV*. The episodes were successfully prevented by administration of colchicine (0.25 mg/day).

Identification of M680I

After informed consent was obtained, the DNA of the patient, his parents, and 2 sisters was extracted from their peripheral blood mononuclear cells. The coding exons and flanking intronic sequences of the *MEFV* gene were amplified by polymerase chain reaction (PCR). The sequences of the PCR primers are available on request. The PCR products were treated using an ExoSAP-IT kit (GE Healthcare, Amersham, UK), and then analyzed by direct sequencing with an ABI 3130 DNA sequencer (Perkin-Elmer, Foster City, CA).

The results of the analysis are shown in Fig. 1. The L110P, E148Q, and M680I mutations were found in the patient. The patient's father was heterozygous only for the M680I mutation, and his mother carried the L110P and E148Q mutations. On the basis of the mutations carried by the parents, the patient was found to be heterozygous for L110P–E148Q/M680I. These mutations were not detected in his one sister, and his younger sister was heterozygous for L110P–E148Q. Interestingly, his mother and elder sister were heterozygous for the G304R mutation, which cause exon 2 skipping in pyrin (data not shown).

Discussion

Recently, a meta-analysis study on the founder populations (Jews, Armenians, Arabs, and Turks) for *MEFV* mutations revealed that the most frequent mutations detected in FMF patients are M694V (39.6%), V726A (13.9%), M680I (11.4%), E148Q (3.4%), and M694I (2.9%) [9]. The 4 major disease-causing mutations (M694V, M694I, M680I, and V726A) in exon 10 of *MEFV* have low allele frequencies in normal Japanese individuals [13]. Even for M694I, which seems to be the most common mutation in Japanese FMF patients [15], allele frequency was below 0.001 [13]. We recently reported that the common *MEFV* mutation patterns were E148Q/M694I (25.0%), L110P–E148Q/M694I (17.5%), and M694I alone (17.5%), and that the M694V, M680I or V726A mutations were not found in 80 Japanese FMF patients [15]. Some reports indicate that homozygous or compound heterozygous M680I mutations are associated with a moderate phenotype of the disease [16, 17]. Moreover, previous reports indicate that the M680I mutation, commonly seen in Armenians, is associated with a milder phenotype of the disease and lower frequency of amyloidosis [18, 19]. On the other hand, FMF patient heterozygous for the M680I gene mutation was reported to have developed nephritic syndrome [20]. Although we did not find abnormalities in chest imaging findings at the time of the fever attack, we strongly suspected presence of thoracic serositis because chest pain

accompanied by rapid shallow breathing are typical concomitant symptoms of FMF. Our patient is the first with the M680I mutation in Japan, and he showed comparatively mild clinical symptoms.

The patient's mother and one sister were found to be heterozygous for the G304R mutation, which causes exon 2 skipping in pyrin. There is no report that this mutation causes FMF [7], and the relationship between L110P–E148Q/M680I and G304R in this family remains unclear. Although the *MEFV* mutations E148Q and M694I are common in Japan, our finding shows that FMF associated with the M680I mutation certainly exists in the Japanese population.

Conflict of interest statement None.

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Expression of CD64 on polymorphonuclear neutrophils in patients with familial Mediterranean fever

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Summary

Familial Mediterranean fever (FMF) is an autoinflammatory disease characterized by recurrent episodes of fever and serosal or synovial inflammation. We examined the utility of CD64 (FcγRI) expression in polymorphonuclear neutrophils (PMNs) as clinical and biological parameters in patients with FMF. We studied 12 Japanese FMF patients (mean age; 22·8 ± 15·5 years, male/female: 2/10), along with rheumatoid arthritis patients (RA, $n = 38$ male/female: 6/32, mean age; 52·2 ± 15·3 years), systemic lupus erythematosus (SLE, $n = 15$ male/female: 0/15, mean age; 38·5 ± 15·9 years) and 12 healthy subjects (male/female: 3/9, mean age; 37·9 ± 17·2 years). CD64 expression on PMNs was determined using flow cytometry. The quantitative expression of CD64 in patients with FMF (2439·6 ± 2215·8 molecules per PMN) was significantly higher than in healthy subjects (547·8 ± 229·5, $P = 0·003$) or in patients with RA (606·5 ± 228·2, $P < 0·0001$) and SLE (681·3 ± 281·1, $P = 0·004$). The increased CD64 expression on PMNs isolated from untreated FMF patients was down-regulated by colchicine treatment. NACHT-LRR-PYD-containing protein 3 (NLRP3) activation using MurNAc-L-Ala-D-isoGln (MDP) resulted in increased CD64 expression on PMNs from healthy subjects. Our results suggest that quantitative measurement of CD64 expression on PMNs can be a valuable tool to discriminate between FMF and autoimmune diseases.

Keywords: autoinflammatory disease, CD64, familial Mediterranean fever, polymorphonuclear neutrophil

Introduction

Autoinflammatory diseases are a group of disorders characterized by unprovoked inflammation in the absence of high-titre autoantibodies or antigen-specific T cells [1]. Familial Mediterranean fever (FMF) is a most prevalent genetic autoinflammatory disease [2]. It is characterized by self-limiting fever and polyserositis. During the attack, neutrophilia and an acute-phase response occur, and histologically there is massive sterile influx of leucocytes to the affected site [3]. FMF is caused by mutations in *MEFV*, which encodes pyrin [4,5]. Pyrin is expressed predominantly in neutrophils and is considered to be involved in the regulation of caspase-1 activation and consequently interleukin (IL)-1 beta production [6].

CD64 (FCγRI), one of the FC receptors for immunoglobulin (Ig)G, plays a role in antibody-dependent cytotoxicity, clearance of immune complexes and phagocytosis of targets opsonized with IgG [7]. CD64 is expressed constitutively by monocyte/macrophages, and is up-regulated in activated neutrophils in response to microbial wall components and cytokines such as granulocyte colony-stimulating factor (G-CSF) [8]. Quantitative flow cytometric techniques have been established to measure CD64 expression in myeloid cells. Matsui *et al.* demonstrated that neutrophil expression of CD64 is a highly sensitive and specific marker for detecting inflection in rheumatoid arthritis (RA) and can distinguish infection from an RA flare [9]. Myeloid CD64 expression has been thought to be suitable as a diagnostic indicator of active acute inflammatory responses, such as infection.

Although some information is available regarding immune activation in FMF, there is little information concerning neutrophil activation marker expression in peripheral blood. More recently, S100A12, a product of activated neutrophils, has been reported to be a sensitive biomarker for FMF [10]. It is likely that polymorphonuclear neutrophil (PMN) expression of CD64 can have great clinical utility in

the differential diagnosis of FMF, as the hallmark of FMF is PMN activation. In this study, we investigated whether CD64 expression is up-regulated in circulating PMNs in patients with FMF.

Materials and methods

Patients

FMF patients were followed at the departments of paediatrics or rheumatology at Shinshu University Hospital, Nagasaki Medical Center and Nagasaki University Hospital. Patients with RA ($n = 38$) and systemic lupus erythematosus (SLE, $n = 15$) were followed at the department of rheumatology at Nagasaki Medical Center. Mean values of white blood cells (WBC) were 6903 ± 2280 (/μl) in RA and 6725 ± 2762 (/μl) in SLE; C-reactive protein (CRP) values were 1·40 ± 1·14 (mg/dl) in RA and 0·30 ± 0·01 (mg/dl) in SLE, and erythrocyte sedimentation rate (ESR) were 39·6 ± 31·7 (mm/h) in RA and 17·6 ± 11·9 (mm/h) in SLE. RA patients having interstitial pneumonia or vasculitis were excluded from this study. Informed consent was obtained from each patient.

Reagents

Polymorphprep™ was obtained from NycoMed (Axis-Shield PoC AS, Oslo, Norway). MurNAc-$_L$-Ala-$_D$-isoGln (MDP) and MDP-negative control were purchased from Invivogen (San Diego, CA, USA).

Flow cytometric analysis

The expression of CD64 was measured by flow cytometry using a Coulter Epics XL flow cytometer (Beckman Coulter, Brea, CA, USA) using Expo32 ADC analysis software (Beckman Coulter). After delineating the leucocytes by staining with the pan-leucocyte marker CD45, the different cell types were gated. CD64 expression was determined as the percentage of positive cells in the PMN gate. To determine the number of CD64 molecules per cell, a phycoerythrin (PE) fluorescence kit (Quantibrite PE; BD Biosciences, San Jose, CA, USA) was used according to the manufacturer's instructions, which allows conversion of fluorescence reading to CD64 molecules per cell. PE-conjugated anti-human CD64 antibody (clone 10·1) was purchased from BD Biosciences.

PMNs isolation

Venous peripheral blood was collected from healthy volunteers. All participating subjects had given their informed consent. The blood was layered onto a Polymorphprep™ cushion and cells were isolated according to the manufacturer's protocol. Briefly, PMNs were isolated on the basis of density, washed once in 0·5 N RPMI-1640 to restore osmolarity, and then washed once more in RPMI-1640. The PMNs were subsequently diluted in complete medium consisting of RPMI-1640 supplemented with 0·3 g/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Statistical analysis

The SigmaStat statistical program (SPSS Science, Chicago, IL, USA) was used for statistical analysis. CD64 values on PMN were reported as median and interquartile range (IQR). Comparisons of the expression levels of CD64 on PMN were made using the Mann–Whitney *U*-test for evaluation of differences between groups. Student's *t*-test was used for matched paired samples. $P < 0·05$ was considered significant.

Results

Patients

Between January 2010 and 31 July 2010, blood samples were obtained from nine FMF patients who attended the outpatient clinics at Shinshu University Hospital, Nagasaki Medical Center and Nagasaki University Hospital. The patient characteristics are listed in Table 1. All patients fulfilled the clinical criteria of FMF [11]. Of these nine patients, seven patients had been treated with daily colchicine and the remaining two untreated patients had been treated by colchicine after the diagnosis was established.

CD64 expression on PMNs from FMF patients

CD64 expression on PMNs in patients with RA (median 567·0, IQR 393–786) or SLE (median 742·0, IQR 480–876) did not differ significantly from that of healthy subjects (median 528·5, IQR 410·5–742). However, CD64 expression in FMF patients in FMF patients (median 2051, IQR 1411–2346) differed significantly from those of the RA, SLE and healthy subjects (Fig. 1). CD64 expression on PMN were elevated in all FMF patients who were not treated, or during the phase of attack, except for two patients in remission state under colchicine treatment (Table 1). A representative histogram of CD64 expression on PMNs and monocytes from blood samples of a FMF patient (case 8) and a healthy subject is shown in Fig. 2. CD64 expression on PMNs was elevated markedly in FMF patients compared to those in healthy subjects. CD64 expression on monocytes was also elevated marginally in FMF patients. To determine whether CD64 expression could be modulated by colchicine treatment, we investigated the CD64 expression on PMNs in newly diagnosed FMF patients, before and after colchicine treatment. CD64 expression was elevated in FMF patients without treatment. This increased CD64 expression was down-regulated by colchicine (1·0 mg/day) treatment (Fig. 3a and b).

Table 1. Patient characteristics and CD64 expression levels in familial Mediterranean fever (FMF) patients.

Patient no.	Gender	Age	Age of onset	Dose of colchicine (mg/day)	Mutations	CD64 expression*	WBC (/μl)	CPR (mg/dl)	ESR (mm/h)
Untreated FMF									
1	F	46	23	(–)	E84K/–	9166	4 900	6·54	38
2	M	46	21	(–)	E148Q–L110P/–	1817	7 500	3·78	73
Treated FMF (on attack)									
3	F	7	6	0·5	(–)	1411	11 000	3·0	n.a.
4	M	24	12	0·75	M694I/L110P	2234	9 890	7·63	n.a.
5	F	10	7	0·5	M694I/E148Q	2456	5 900	19	70
6	F	12	4	0·5	(–)	2079	18 200	11·6	n.a.
Treated FMF (no attack)									
7	F	7	7	0·5	E148Q/–	2346	7 390	0·2	n.a.
8	F	42	20	1·0	M694I/M694I	2051	5 600	Negative	13
9	F	32	29	1·0	R202Q–E148Q/–	1658	4 900	Negative	4
10	F	19	18	0·5	M694I/E148Q–L110P	722	n.a.	n.a.	n.a.
11	F	23	6	1·0	M694I/E148Q–L110P	611	7 500	Negative	n.a.
12	F	5	3	0·125	E148Q/–	2724	8 100	Negative	n.a.

*Expression of CD64 molecules per neutrophil. WBC, white blood cell; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n.a., not available.

Effects on NACHT-LRR-PYD-containing protein 3 (NLRP3) activation on CD64 expression in PMNs

Dysregulation of the proinflammatory NLRP-3-dependent pathway has been thought to be involved in the autoinflammation of FMF [12]. Recent studies have documented that MDP, a NOD2 ligand, is an activator of the NLRP-3 inflammasome [13]. We asked whether stimulation with an NLRP-3 activator, MDP, modulated the expression of CD64 in PMNs. We stimulated PMNs from healthy control individuals with MDP. As shown in Fig. 4a, incubation with MDP significantly increased CD64 expression on PNMs rapidly (6 h), whereas MDP-negative control peptide did not affect the surface expression of CD64. This increased CD64 expression on PMNs was maintained after overnight incubation (12 h) and down-regulated after 24 h (Fig. 4b). We confirmed this increased CD64 expression on PMNs activated by MDP in multiple blood samples from healthy subjects (Fig. 4c). We also analysed CD16 expression on PMNs after MDP stimulation. CD16 was expressed highly on PMNs and its expression level was affected marginally by MDP stimulation (Fig. 5).

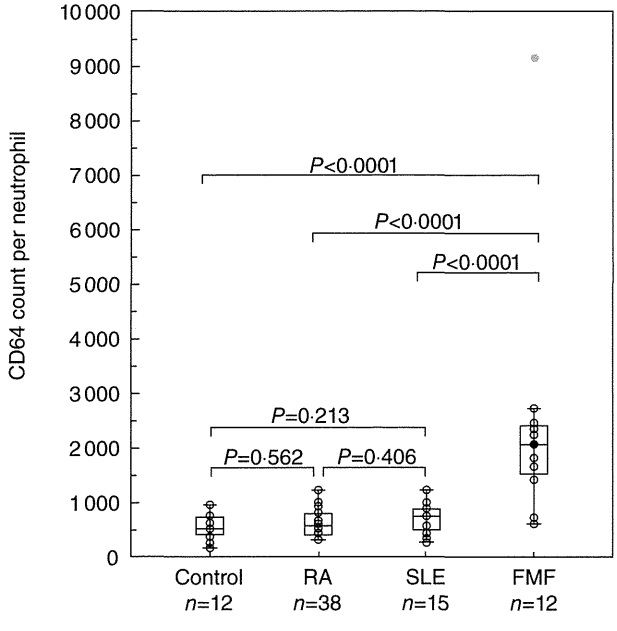

Fig. 1. Expression of CD64 molecules per neutrophil in familial Mediterranean fever (FMF) patients. The line inside the box indicates the median value, box shows the 25th and 75th percentiles.

Discussion

FMF is a genetic autoinflammatory disease characterized by recurrent episodes of fever, accompanied by serositis and arthritis [3]. The disease is prevalent among populations surrounding the Mediterranean Sea. However, an increasing number of cases have been reported in countries not related to this area, including Japan [14]. In countries where FMF is rare, the clinical diagnosis of FMF may not be easy and the role of genetic testing is crucial. However, in 20% of FMF patients, mutations of *MEFV* genes cannot be identified [15]. During the periodic attack of FMF, laboratory markers, such as white blood cell counts, CRP and ESR, are elevated. However, these markers have low specificity and show limitations in the diagnosis.

In the present study, we demonstrated that CD64 expression on PMNs was elevated significantly in FMF patients, compared to healthy subjects and in patients with common autoimmune diseases, including RA and SLE. CD64 (FCγRI) is expressed constitutively on monocytes and cytokines can induce expression of CD64 on PMNs and induces increased

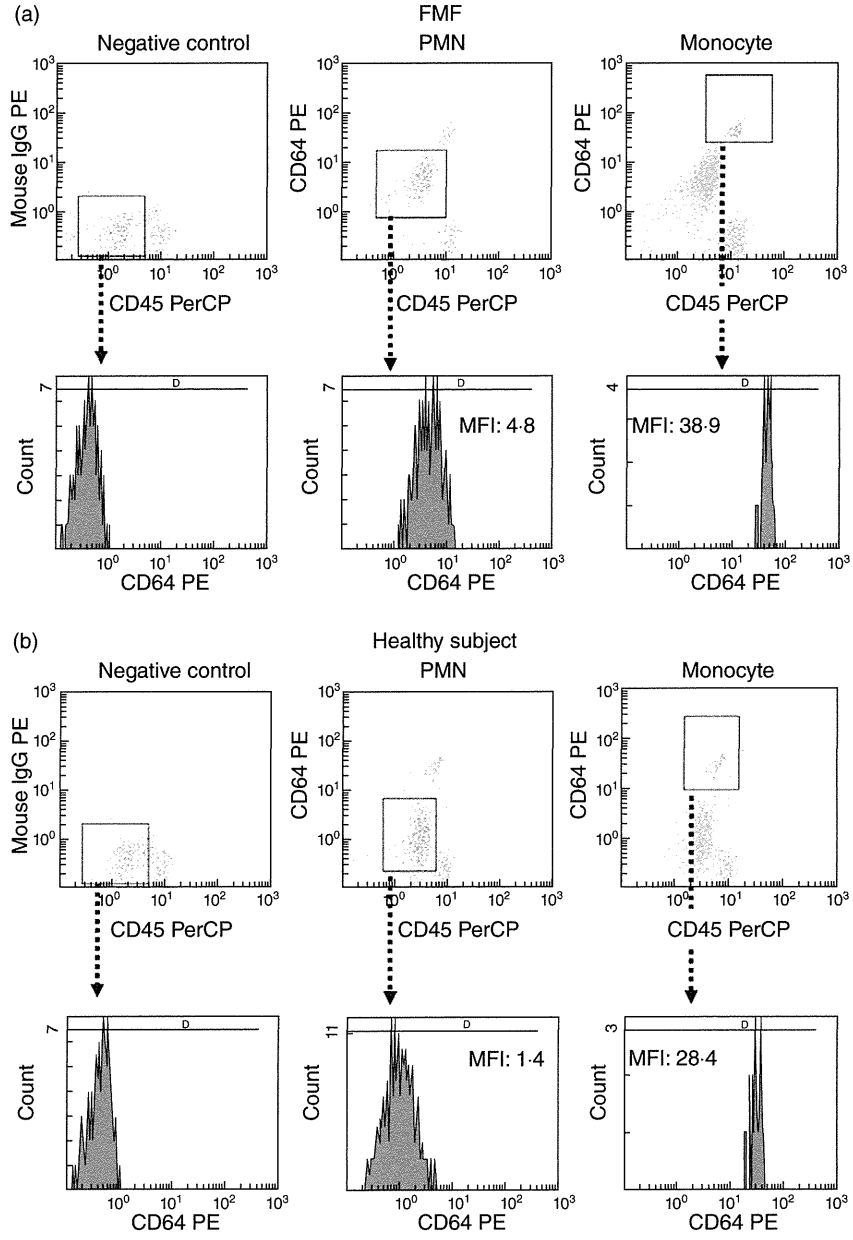

Fig. 2. Representative histogram showing the increased CD64 expression on polymorphonuclear neutrophils (PMNs) in familial Mediterranean fever (FMF) patients. Determination of PMNs and monocytes in peripheral blood from a FMF patient (a) and a healthy subject (b) by double-staining using pre-captopril (preCP)-conjugated anti-CD45 PreCP-labelled antibodies and phycoerythrin (PE)-conjugated anti-CD64 antibodies. Negative control samples were stained with peridinin chlorophyll (PerCP)-conjugated anti-CD45 antibodies plus PE-conjugated mouse immunoglobulin (Ig)G (upper panel). Lower panel shows CD64 expression on PMNs and monocytes.

monocyte expression of CD64 [16]. Up-regulation of CD64 expression on PMNs occurs within several hours after stimulation with inflammatory cytokines, such as interferon (IFN)-γ, IL-8 and granulocyte–macrophage colony-stimulating factor (GM-CSF) [8,16]. High concentrations of inflammatory cytokines have been shown to be associated with the periodic attack phase of FMF [17]. Our data are consistent with the possibility that over-production of these inflammatory cytokines during the periodic fever phase seen in FMF patients contributes to the up-regulation of CD64 expression by PMNs. However, up-regulated expression of CD64 in PMNs was also observed in FMF patients who were in remission after colchicine treatment. Therefore, dysregulation of PMN activation, due probably to altered pyrin function, can persist during the subclinical phase and elevated CD64 expression reflects the activation state of PMNs in treated FMF patients in remission. Interestingly, elevated CD64 expression on PMNs in untreated FMF patients was down-regulated by colchicine treatment, concomitant with the clinical improvement. These findings also suggested that PMN expression of CD64 correlated with the autoinflammation seen in FMF. Up-regulated expression of CD64 on PMN has been observed in acute bacterial infection and systemic inflammatory response syndrome. More recently, an elevated expression of CD64 was observed in patients with inflammatory bowel disease and Behçet's disease [18,19], which are non-hereditary autoinflammatory diseases. Taken together, our results demonstrating the

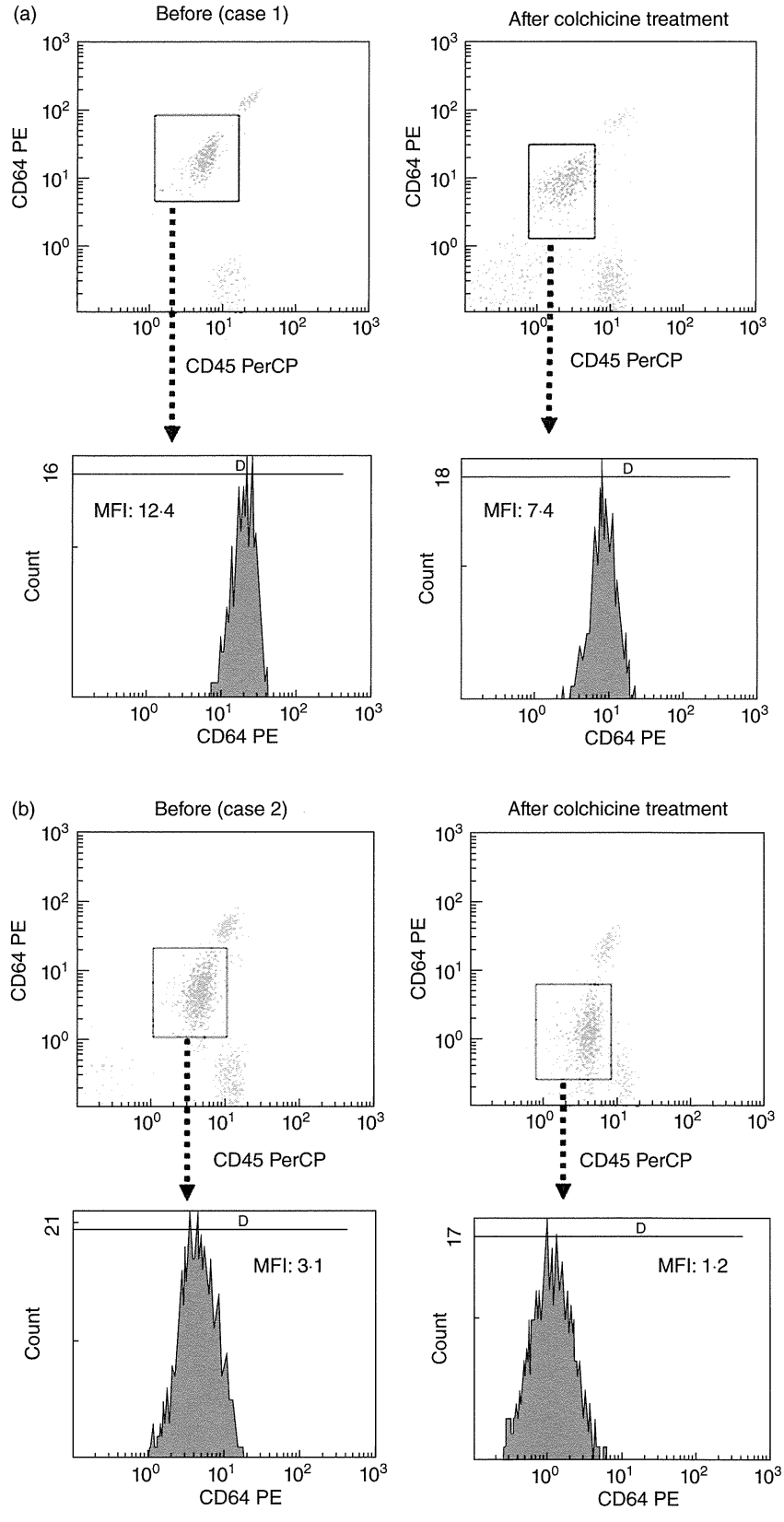

Fig. 3. Histogram of CD64 expression on polymorphonuclear neutrophils (PMNs) in blood samples of untreated familial Mediterranean fever (FMF) patients (a, case 1; b, case 2) before and after colchicine treatment.

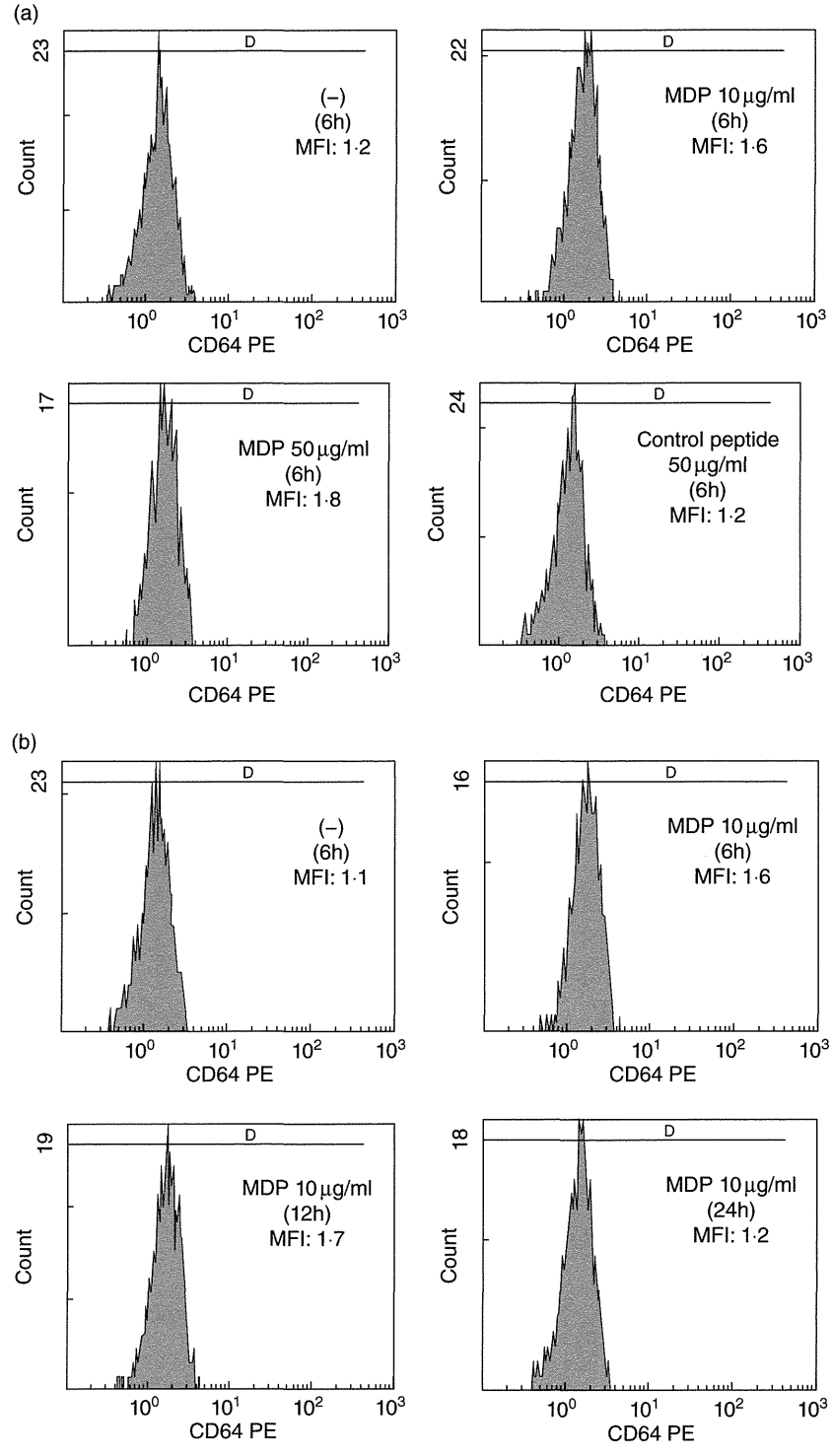

Fig. 4. Expression of CD64 on polymorphonuclear neutrophils (PMNs) stimulated with MurNAc-$_L$-Ala-$_D$-isoGln (MDP). (a) PMNs isolated from a healthy subject were stimulated with non-obese diabetic (NOD)2 ligand, MDP or MDP control peptide for 6 h. The expression of CD64 is indicated as the mean fluorescence intensity (MFI). (b) PMNs isolated from a healthy subject were stimulated with MDP (10 μg/ml) for the periods indicated. The expression of CD64 is indicated as the MFI. (c) PMNs isolated from healthy subjects ($n = 6$) were cultured with or without MDP (10 μg/ml) for 6 h. CD64 expression levels on PMNs were elevated significantly by MDP stimulation.

increased expression of CD64 in PMNs in FMF patients suggest that PMN expression of CD64 appears to be ideally suited as a diagnostic indicator of autoinflammatory disease.

Neutrophils play an essential role in the initial response to pathogens. Neutrophils recognize pathogens through pathogen-recognition receptors (PPRs) [20]. The nucleotide binding domain [non-obese diabetic (NOD)-like receptors (NLRs)] are a group of PPRs whose role in neutrophils has not been well characterized. It has been reported that NLR activation may cause an inflammatory response that attracts neutrophils to the site of inflammation [21]. FMF is caused by inherited mutations in *MEFV*, which encodes pyrin. Pyrin regulates caspase-1 activation and consequently IL-1β production by affecting NLRP-3-mediated proinflammatory

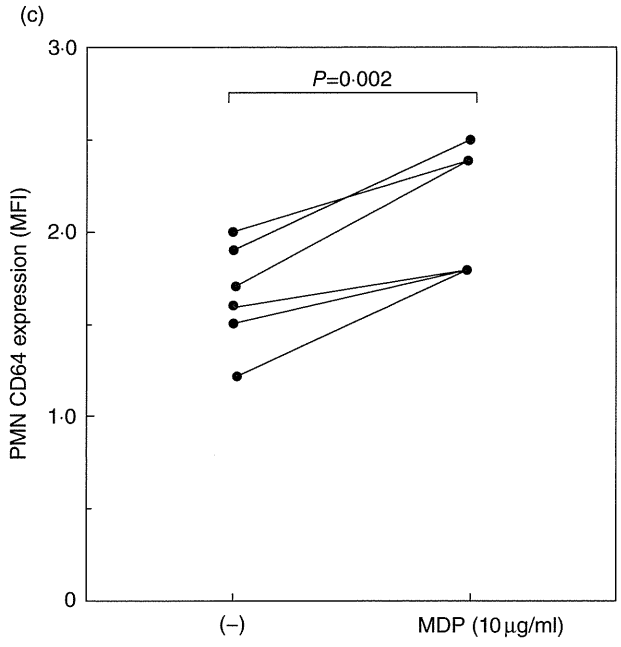

Fig. 4. *Continued*

cascades [22]. MDP was first shown to be a specific activator of a NLR family member, NOD2; however, recent studies have indicated that MDP induces IL-1β release via a NLRP-3-dependent pathway [13]. The expression of NOD2 and NLRP3 protein has been demonstrated in PMNs [23]. Therefore, the effects of NLRP3 activation on CD64 expression were investigated by stimulating PMNs with MDP. Our data

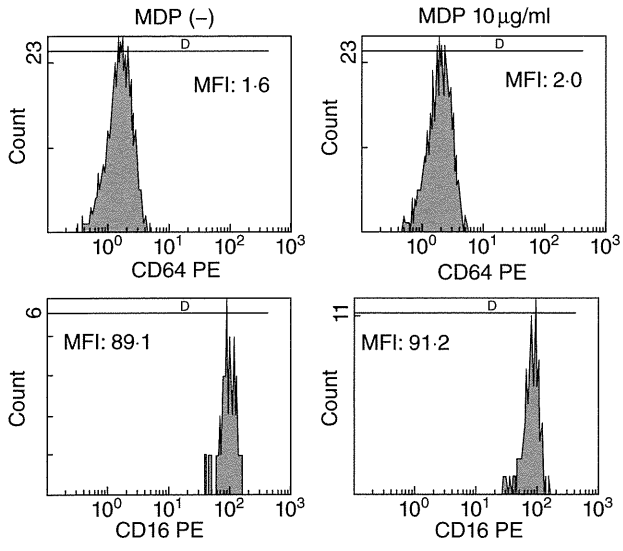

Fig. 5. The expression of CD64 and CD16 on polymorphonuclear neutrophils (PMNs) stimulated with MurNAc-L-Ala-D-isoGln (MDP). PMNs isolated from a healthy subject were stimulated with MDP (10 μg/ml) for 6 h. The expression of CD64 and CD16 is indicated as the mean fluorescence intensity (MFI). Three experiments were performed using different PMNs and a representative result is shown.

indicate that MDP stimulation up-regulates CD64 expression on PMNs, suggesting that CD64 expression could be modulated in response to NLRP-3 activation. It is possible that dysregulated NLRP-3-mediated proinflammatory cascade, probably by altered pyrin function, contributed to the up-regulation of CD64 in PMNs from patients with FMF.

In conclusion, our data indicate that PMN expression of CD64 was elevated abnormally in patients with FMF. These findings suggest that PMN activation, due probably to impaired pyrin function, could contribute to the pathophysiology of FMF and that CD64 is a suitable marker to differentiate between FMF, a hereditary autoinflammatory disease, and autoimmune disease.

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Disclosures

None.

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A Japanese case of familial Mediterranean fever presenting diffuse bone marrow uptake of FDG-PET and high levels of neutrophil membrane CD64 expression

Sir, FMF is a rare inflammatory disease characterized by recurrent attacks of fever and inflammation. Even though some useful diagnostic criteria have been proposed, useful imaging methods or haematological markers for the diagnosis or follow-up of FMF have not been established. We experienced a case of a 46-year-old woman with FMF presenting diffuse bone marrow uptake of [^{18}F] fluoro-deoxy glucose (FDG) and high levels of polymorphonuclear neutrophil (PMN) membrane CD64 expression.

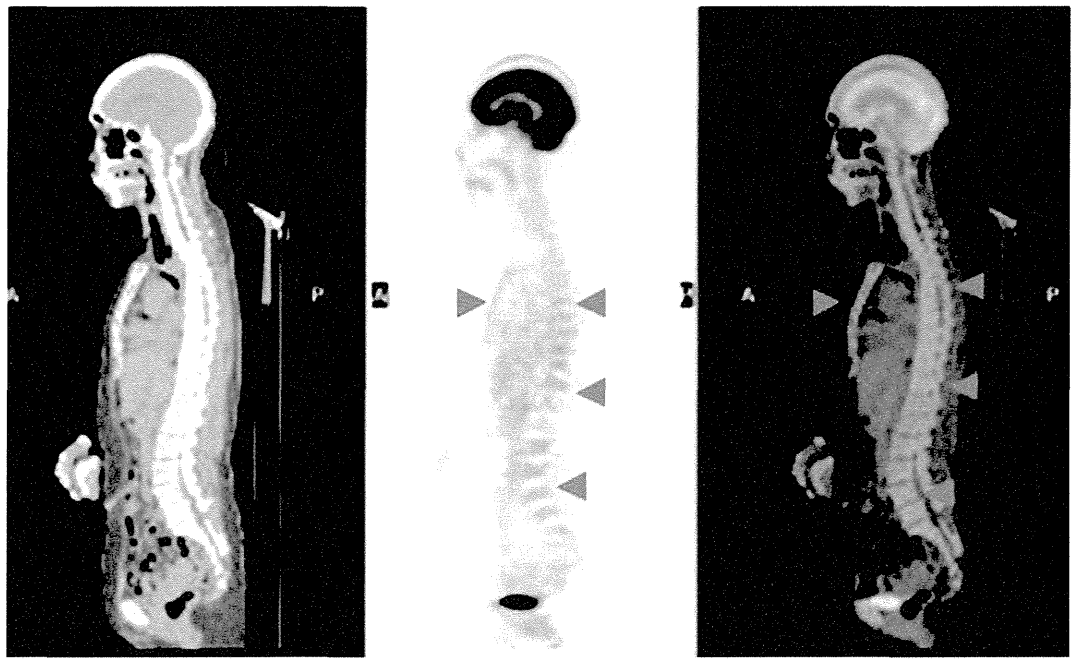
A 46-year-old Japanese female was admitted to our hospital because of chest pain and fever of undetermined origin (FUO). She had been suffering from a periodic fever since she was 18-years old. [^{18}F]FDG-PET was performed, revealing diffuse bone marrow uptake of [^{18}F]FDG (Fig. 1). In the laboratory findings of haematology and biochemistry at the time of admission, there were no abnormalities except for elevated ESR and elevated levels of CRP (ESR 51 mm/h, CRP 6.54 mg/dl). Tests for ANAs, ANCAs and RF were negative. A peripheral blood smear revealed no abnormalities. Serum M-protein was not detected by immunofixation. Since we suspected FMF based on these findings, we performed the sequencing of all 10 exons of the *MEFV* gene and detected a heterozygous mutation (GAG to AAG) in codon 84 of exon 1 of the *MEFV* gene that resulted in a substitution of lysine for glutamic acid (E84K). In light of these findings, we initiated daily colchicine treatment (1.0 mg/day), and the patient's clinical manifestation rapidly improved. FMF was diagnosed according to clinical criteria for the diagnosis in combination with a classification tree format [1].

The expression of CD64 on PMNs in healthy subjects, before and after treatment, was measured by flow cytometry using a Coulter Epics XL flow cytometer (Beckman Coulter, Inc. Brea, CA, USA) using Expo32 ADC analysis software (Beckman Coulter). Before the colchicine treatment, the patient's mean fluorescence intensity (MFI) of CD64 on PMNs was significantly increased (MFI: 12.4) compared with those of healthy subjects (MFI: 1.2). Colchicine treatment (1.0 mg/day) down-regulated the increased CD64 expression, but expression was higher than in healthy subjects (MFI: 7.4).

FMF is prevalent among populations surrounding the Mediterranean Sea. However, more cases have been reported in countries not related to this area, including Japan. In countries where FMF is rare, a clinical diagnosis of FMF may not be easy, and the role of genetic testing is crucial. More recently, Tomiyama *et al*. [2] reported a new *MEFV* mutation, E84K, in a Japanese FMF patient.

Hyperfunction of PMNs is a characteristic of FMF. CD64 (FCγR1), a factor crystallizable (FC) receptor for immunoglobulin G (IgG), plays a role in antibody-dependent cytotoxicity, clearance of ICs and phagocytosis of targets

FIG. 1 [^{18}F]FDG-PET scan of a patient at admission. Note the markedly abnormal uptake of [^{18}F]FDG in the spine and sternum (►). The mean maximum standardized uptake value (SUV_{max}) was 3.0 (range 2.2–3.7).



opsonized with IgG. The utility of CD64 (FCγR1) expressed on PMNs in clinical situations has been investigated. Matsui *et al.* [3] demonstrated that quantitative measurement of CD64 expression on PMNs can be used as a sensitive and specific marker to detect infection complicating RA. More recently, S100A12, a product of activated neutrophils, has been demonstrated to be a sensitive biomarker for FMF [4]. It is likely that the CD64 expression of neutrophils has great clinical utility in the formulation of a differential diagnosis for FMF, since the hallmark of FMF is PMN activation. In the present case, we demonstrated that CD64 expression on PMNs was elevated in FMF patients compared with healthy subjects, suggesting that PMN CD64 expression is an ideally responsive diagnostic indicator of FMF.

Recently, many studies have shown that the administration of G-CSF can cause homogeneous hypermetabolic activity of bone marrow in PET using [^{18}F]FDG [5–7]. Since G-CSF stimulates PMNs, G-CSF-induced uptake of [^{18}F]FDG may be related to the increased activity of PMNs in bone marrow. A similar phenomenon might occur in patients with FMF, as shown in this case. Although our experience is limited to one patient, we suggest that both [^{18}F]FDG-PET uptake in bone marrow and CD64 expression on PMNs reflect the activation of PMNs in patients with FMF and could be novel tools for the diagnosis of FMF.

Rheumatology key message

- FDG-PET and CD64 on PMNs are useful for diagnosis of FMF.

Disclosure statement: The authors have declared no conflicts of interest.

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