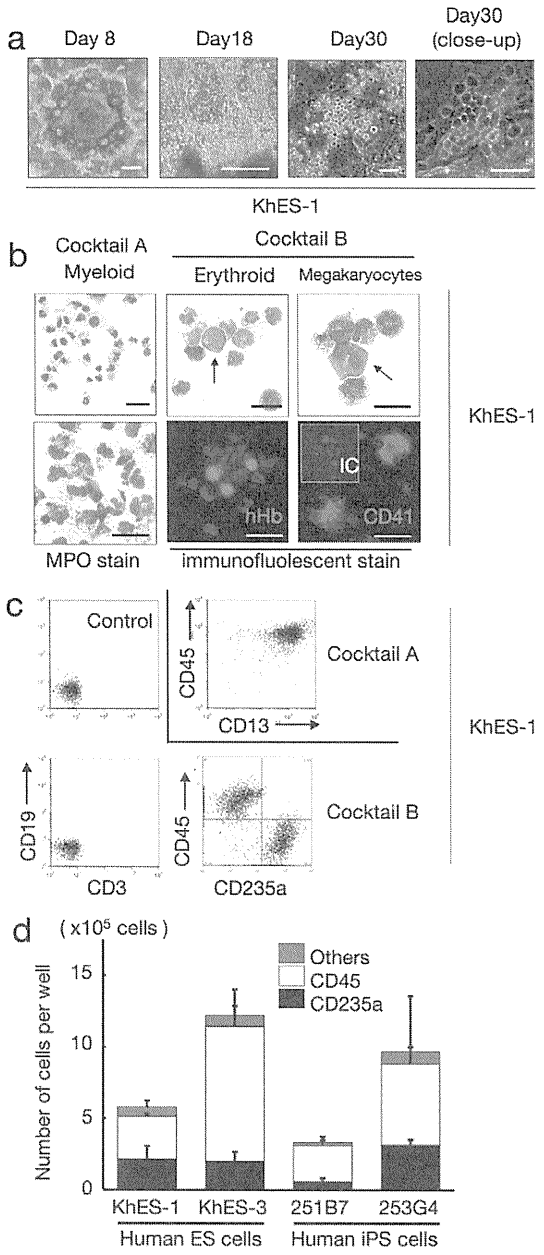

Figure 3. Human ES/iPS cell-derived haematopoiesis in a monolayer culture free from animal serum or stromal cells. a. Sequential phase contrast pictures showing haematopoietic development. Scale bars, 500 μm (left two panels) and 100 μm (right two panels). Data from KhES-1 are shown as representative. **b.** Floating cells harvested on day 30 showing various lineages of haematopoietic cells; MPO-positive myeloid lineage cells (leftmost panels), pan-human Hb-positive erythroid lineage cells (centre panels), and CD41-positive megakaryocytes (rightmost panels). Scale bars, 100 μm. Data from KhES-1 are shown as representative. **c.** Expression of lineage-specific antigens on floating cells harvested on day 30; Myeloid lineages (CD13 and CD45), erythroid lineages (CD235a), T cells (CD3), and B cells (CD19). Data from KhES-1 are shown as representative. **d.** Numbers and fraction of blood cells induced from each two lines of human ES cells and iPS cells. Bars represent standard deviation of the mean of three independent experiments.
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at the start of differentiation), counting and FCM analysis of harvested blood cells on day 30 revealed the existence of 7.7×10^5 (SD = 2.3×10^5) different cell lineages per well, including 36.0% (SD = 6.4%) $CD235a^+$ erythroid and 53.2% (SD = 9.4%) $CD45^+$ myelomonocytic lineages, but no lymphoid lineage cells (Figure 3c). Although the differentiation efficacy and lineage distribution depend not only on the cytokines but also on the cell strains, the data indicates that human ES and iPS cells develop into various lineages of hematopoietic cells, robustly and orderly, in our novel monolayer culture system without xeno-derived serum or stromal cells (Figure 3d).

ES/iPS cell-derived hematopoietic cells have similar potential to in vivo-derived blood cells in function

Considering the use of ES/iPS cell-derived hematopoiesis for various clinical and research applications, it is important to confirm the function of the generated blood cells. Neutrophils derived with the myeloid-induction cocktail exhibited migration activity in response to the chemoattractant fMLP (Figure 4a) and phagosome-dependent reactive oxygen production, which was inhibited by the phagosome destruction agent, cytochalasin B (Figure 4b). On the other hand, erythroid lineage cells derived with the erythropoietic-differentiation cocktail (harvested on day 32 of differentiation) exhibited an oxygen dissociation curve that was similar, despite being slightly left-shifted, to those obtained with adult and cord blood cells (Figure 4c). These data indicate that our culture facilitates robust and orderly development of human ES and iPS cells into functional hematopoietic cells with similar potential to in vivo-derived blood cells.

Clonogenic hematopoietic development from human ES/iPS cell-derived progenitors

The human hematopoietic system is a hierarchy of various component cells from stem or progenitor cells to terminally differentiated cells. For example, $CD34^+$ cells in umbilical cord blood or bone marrow contain putative hematopoietic stem cells and are used as a source of stem cell transplantation. The identification and proliferation of such cells in vitro have been of great interest in medical science research.

To assess the potential of our system for supporting generated immature stem or progenitor cells, we evaluated the colony-forming ability of the cultivated hematopoietic progenitors in the system. Accordingly, the cells were cultured with SCF, TPO, IL3, FLT-3 ligand, and FP6. In these conditions, $CD34^+CD45^+$ hematopoietic cells existed up to day 25, indicating that the immature hematopoietic cells can be maintained in our serum-free culture (Figure 5a).

We harvested adherent blood cells from the previously described culture and transferred them into a methylcellulose-containing medium to perform colony-forming assays with SCF, TPO, IL3, G-CSF, and EPO. As shown in Figure 5b and c, CFU-Mix, BFU-E, CFU-GM, and CFU-G colonies developed from plated cells. The total number of colonies increased dramatically from day 6 to day 10, then gradually increased until day 15 and decreased thereafter. CFU-Mix and BFU-E colonies were mainly observed until day 15 and were thereafter replaced by CFU-GM and CFU-G colonies. Similar tendencies were observed in both ES and iPS cells. These results suggest that our culture system can incubate multipotent hematopoietic stem or progenitor cells over a period of time.

Identification of $KDR^+CD34^+CD45^-$ bipotential hemoangiogenic progenitors derived in serum-free conditions

During embryogenesis, hematopoietic development is closely associated with endothelial lineage commitment [47,48], and

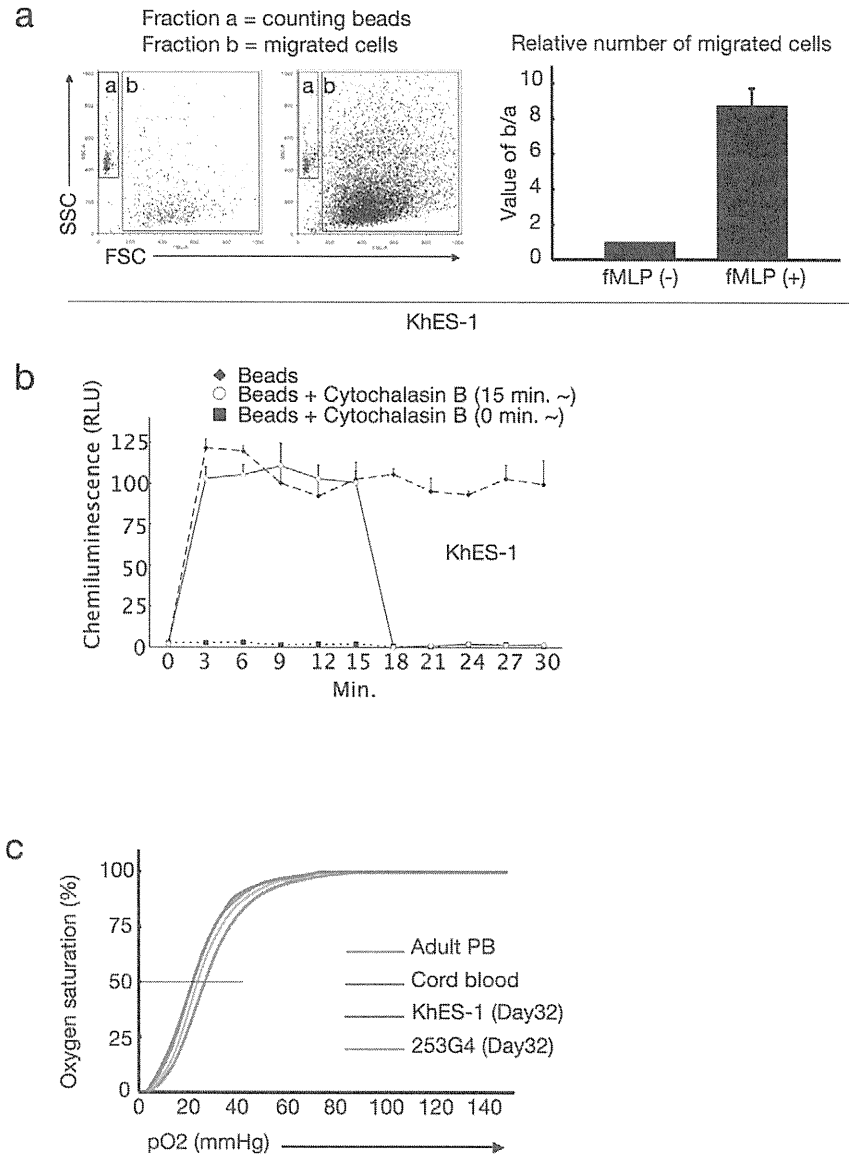

Figure 4. Functional blood cells derived from human ES/iPS cells. a. Number of migrated cells that permeated through the transwell membrane with or without fMLP. Values were normalised to the number of counting beads, and the control values were arbitrarily set to the condition without fMLP. Data from KhES-1 are shown as representative. **b.** Assay for phagocytosis-induced respiratory burst activity using chemiluminescent microspheres (luminol-binding microspheres). Abbreviation: RLU, relative light units. Data from KhES-1 are shown as representative. **c.** Oxygen dissociation curves of erythroid cells derived from human ES/iPS cells (harvested on day32 of differentiation), human cord blood, and adult peripheral blood. Where shown, bars represent standard deviation of the mean of three independent experiments.
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previous studies have demonstrated that ES cells can differentiate into the common multipotent progenitors that differentiate into both blood and endothelial cells at the single cell level on OP9 stroma [17,28,49]. Although the experiments described thus far demonstrated that the serum-free, xeno-cell-free culture condition supported human ES/iPS cell-derived hematopoiesis in an orderly manner, as observed during embryogenesis, it was unclear which day 6 fraction(s) developed into blood cells. To clarify this point, human ES cells stably expressing green fluorescent protein (GFP) were cultured, then 1×10^4 cells of $GFP^+KDR^-CD34^-CD45^-$ (Fraction A), $GFP^+KDR^+CD34^-CD45^-$ (Fraction B), and $GFP^+KDR^+CD34^+CD45^-$ (Fraction C) fractions were transferred on day 6 into a synchronous differentiation culture of unlabeled ES cells (Figure 6a). Nineteen days later (day 25 of differentiation), GFP^+ small round cell-containing colonies were observed predominantly in Fractions B and C, and FCM analysis of the entire culture confirmed the emergence of GFP^+CD45^+ cells mainly from Fraction C (Figure 6b). On the other hand, few blood cells positive for GFP were generated from Fraction A. These results were obtained with 2 independent strains of human ES cells (KhES1-EGFPneo on KhES-1 and KhES3-EGFPneo on KhES-3) (Figure 6c) and indicated that hematopoietic progenitors were present in the KDR^+ fraction, particularly in the KDR^+CD34^+ fraction, on day 6 of differentiation.

Finally, we performed a single-cell deposition assay by transferring single sorted human ES/iPS cell-derived $GFP^+KDR^+CD34^+CD45^-$ cells, which were negative for VE-cadherin, on day 6 into individual wells of 96-well plates coated

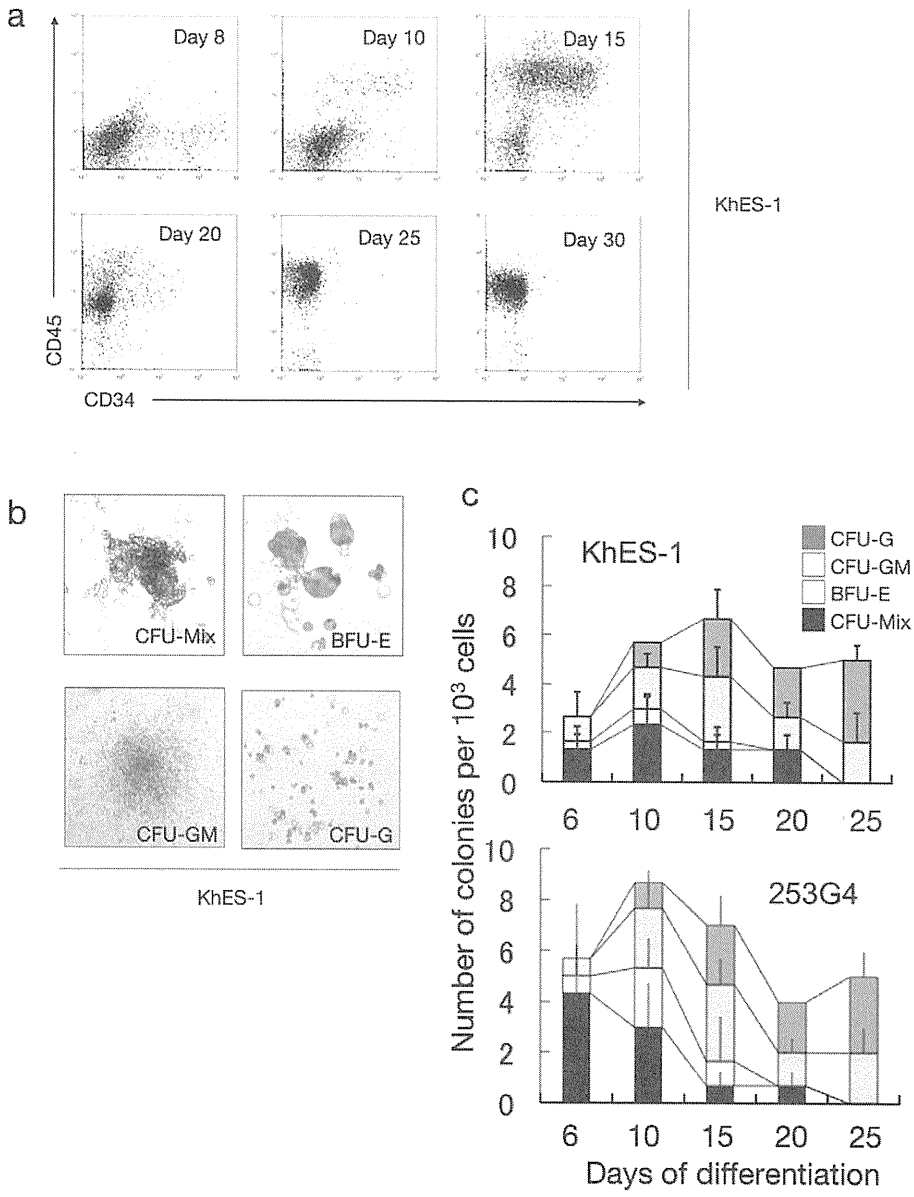

Figure 5. Hematopoietic stem/progenitor cells in culture. a. Sequential FCM analysis of cells harvested on indicated days showing the existence of $CD34^{+}CD45^{+}$ haematopoietic progenitor cells in culture. Data from KhES-1 are shown as representative. **b.** Various colony types on MTC-containing medium clonally emerged from single haematopoietic progenitor cells. Data from KhES-1 are shown as representative. **c.** Numbers of each colony type derived from different days of culture. Bars represent standard deviation of the mean of three independent experiments. Data from KhES-1 and 253G4 strains are shown as representative.
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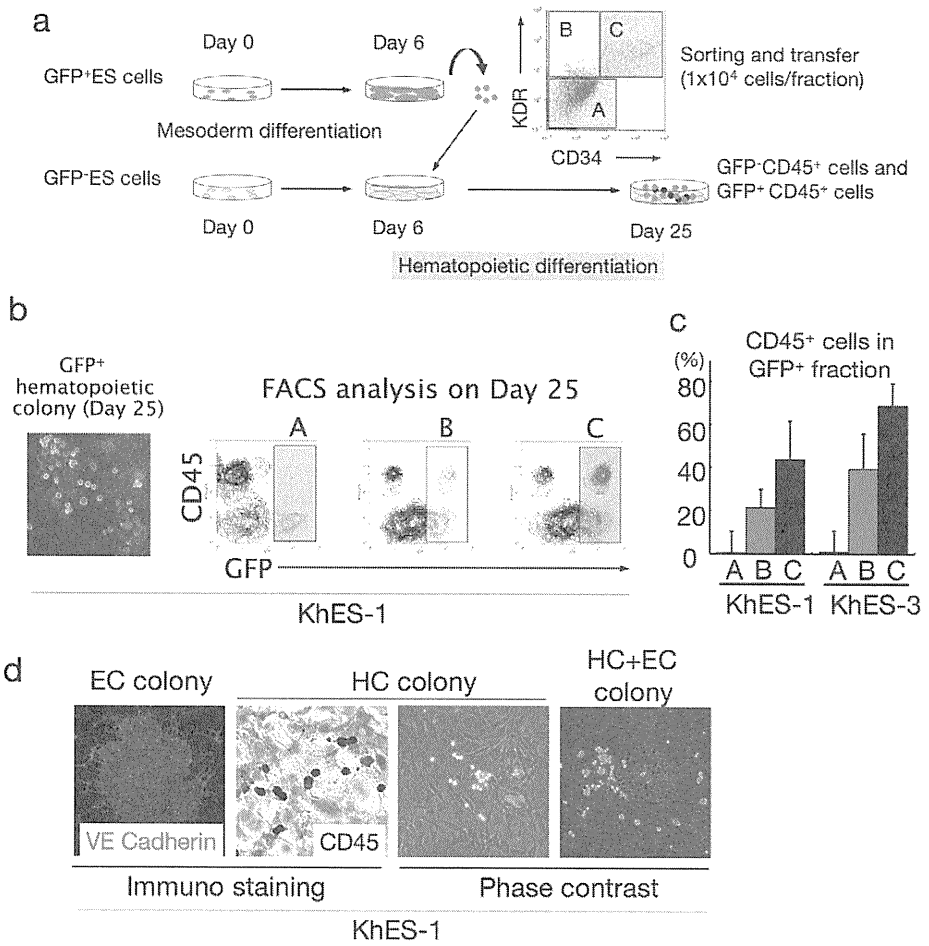
with an OP9 cell layer. As shown in Figure 6d and e, the proportion of hematopoietic cell (HC) development, VE-cadherin^{+} endothelial cell (EC) development, and HC plus EC development on day 20 were 9.0%, 6.8%, and 4.0%, respectively, for KhES-1 and 11.6%, 12.7%, and 8.3%, respectively, for 253G4 iPS cells. These results demonstrate that the common mesodermal progenitors that can differentiate into both blood and endothelial cells at the single-cell level are induced in our culture condition.

Discussion

In this study, we demonstrated the orderly mesodermal and hematopoietic differentiation of human ES and iPS cells in a novel serum-free monolayer culture condition. Simple manipulation of cytokine combinations facilitated robust, reproducible, and highly directed stepwise commitment to specific lineages of functional blood cells.

There are several reports on hematopoietic differentiation of human ES/iPS cells, such as murine-derived OP9 stromal cell coculture and feeder/serum-free EB formation systems [20,22,23,24,30,31,32,50]. However, two-dimensional cultures containing xeno-serum/cells often cause dependency on their lots, while complicated three-dimensional structures inside EBs make it difficult to assess and control conditions for inducing specific progenitors. Actually, few in vitro systems have been able to reliably reproduce hematopoietic development from mesodermal progenitors or model the in vivo coexistence of developing hematopoietic cells and their autologous microenvironments in



e

Clones	Only one cell 24 hours after sorting	At day 20		
		HC	EC	HC+EC
KhES-1 cells	176 wells	16 wells (9.0%)	12 wells (6.8%)	7 wells (4.0%)
256G4 iPS cells	181 wells	21 wells (11.6%)	23 wells (12.7%)	15 wells (8.3%)

HC : Haematopoietic colonies
EC : Endothelial colonies

Figure 6. Haematopoietic differentiation from KDR$^+$CD34$^+$ mesodermal progenitors. a. Schema of the protocol for measuring haematopoietic activities of depicted fractions on day 6. **b.** Each sorted fraction-derived haematopoiesis on day 25 detected by fluorescent microscopy and FCM analysis. Data from KhES-1 are shown as representative. **c.** Ratio of CD45$^+$ cells in GFP$^+$ fraction on day 25 showing the strongest haematopoietic activity of fraction C followed by fraction B. **d.** Single KDR$^+$CD34$^+$CD45$^-$ cell-derived haematopoietic colonies (HC), VE-cadherin$^+$ endothelial colonies (EC), and HC+EC colonies generated on OP9 cell layers. Data from KhES-1 are shown as representative. **e.** Number of wells that showed HC, EC, and EC+HC development.
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serum-free conditions. Our less labor-intensive and clearly defined monolayer culture facilitates observation of the stepwise development of pluripotent cells to blood cells via common hemoangiogenic progenitors and the behavior of hematopoietic cells on autologous stromal cells. Consequently, assays for elucidating differences in lineage specification of various ES/iPS cell strains, including hematopoietic potential, can be performed with high reproducibility. This is particularly important because individual pluripotent cell strains vary in differentiation potentials [51,52,53]. This study demonstrated quantitative differences in hematopoietic differentiation efficacy and lineage commitment among 4 ES/iPS cell strains.

Because human ES/iPS cells are feasible cell sources for various clinical applications, scientific and medical communities have shown continuing interest in hematopoietic stem cell induction from ES/iPS cells. Previous trials have indicated that murine ES cell-derived hematopoietic cells overexpressing HoxB4 [54] can replenish the bone marrow of lethally irradiated recipient mice. However, it remains a challenge to develop bona fide human hematopoietic stem cells with bone marrow reconstitution activity at the single-cell level. In our study, we observed many cobblestone area-forming cells, which reportedly indicate the existence of very immature hematopoietic progenitors. Moreover, FCM analyses and colony-forming assays suggested that ES and iPS human cell-

derived hematopoiesis in our method occurs through clonogenic hematopoietic stem/progenitor cells. We are in the process of determining in vivo repopulating ability of cells harvested from our culture by using serial transplantation into immunodeficient mice to assess the possibility of inducing feasible cell sources for various clinical applications, such as cell therapies and disease investigation.

Finally, time-lapse imaging strongly indicated crosstalk between hematopoietic cells and the autologous microenvironment composed of non-hematopoietic cells. Emerged blood cells move about actively and generate colonies in surrounding cell layers, suggesting the importance of a direct interaction between blood cells and microenvironmental cells for the maintenance, proliferation, and differentiation of stem or progenitor cells (Movie S3). In fact, a model of hematopoietic disorders triggered by mutation in the bone marrow microenvironment has been recently reported [55]. However, further investigation is necessary to identify the mechanisms responsible for such phenomena. Our culture may aid these investigations as it facilitates simple and sequential harvest of hematopoietic cells with minimal contamination by autologous adherent cell layers.

In conclusion, this study presents novel methods for analyzing the mechanisms of normal hematopoiesis in a robust, reproducible, and stepwise manner. Furthermore, employing gene-manipulated ES cells or disease-specific iPS cells will supply in vitro models of disease pathology, thereby providing further insights into hematological defects in conditions such as aplastic anemia and myelodysplastic syndromes.

Supporting Information

Movie S1 Time-lapse microscopic movie showing the morphological change in a single colony from day 0 to day 6 (initial differentiation). In this period, a colony begins forming a rosary-like morphology as it differentiates. The pictures were automatically taken every 8 minute by Biostation IM (Nikon Instruments, Tokyo, Japan).
(MOV)

Movie S2 Time-lapse microscopic movie showing the morphological change in a single colony from day 6 to day 25 (hematopoietic differentiation). After adding hematopoietic cytokines on day 6, hematopoietic cells first emerge from the areas near the edge of stratified zone. The pictures were automatically taken every 8 minute by Biostation IM (Nikon Instruments, Tokyo, Japan).
(MOV)

Movie S3 Close-up time-lapse microscopic movie showing hematopoietic cells moving about and generating colonies in surrounding cell layers. The pictures were automatically taken every 8 minute by Biostation IM (Nikon Instruments, Tokyo, Japan).
(MOV)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: AN TH KU TN MKS. Performed the experiments: AN H. Sakai. Analyzed the data: AN TH KU KO IK H. Sakai. TN MKS. Contributed reagents/materials/analysis tools: H. Suemori H. Sakai. Wrote the manuscript: AN TN MKS.

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Clinical and Host Genetic Characteristics of Mendelian Susceptibility to Mycobacterial Diseases in Japan

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Abstract
Purpose The aim of this study is to investigate clinical characteristics and genetic backgrounds of Mendelian susceptibility to mycobacterial diseases (MSMD) in Japan.
Methods Forty-six patients diagnosed as having MSMD were enrolled in this study. All patients were analyzed for the *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* gene mutations known to be associated with MSMD.
Results Six patients and one patient were diagnosed as having partial interferon-γ receptor 1 deficiency and nuclear factor-κB-essential modulator deficiency, respectively. Six of the seven patients had recurrent disseminated mycobacterial infections, while 93% of the patients without these mutations had only one episode of infection.
Conclusions The patients with a genetic mutation were more susceptible to developing recurrent disseminated mycobacterial infections. Recurrent disseminated mycobacterial infections occurred in a small number of patients even without these mutations, suggesting the presence of as yet undetermined genetic factors underlying the development and progression of this disease.

Keywords Disseminated mycobacterial infection · IFN-γR1 deficiency · NEMO deficiency · flow cytometric analysis

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Introduction

Although the outcome of mycobacterial infection is influenced by many factors, including the virulence of the pathogen and the environment of the host, it has been demonstrated that host genetic factors play important roles in the defense against mycobacteria [1]. Mendelian susceptibility to mycobacterial diseases (MSMD, MIM 209950) is a rare primary immunodeficiency syndrome characterized by a predisposition to develop infections caused by weakly virulent mycobacteria, such as *Mycobacterium bovis* bacille Calmette-Guerin (BCG) and environmental non-tuberculous mycobacteria (NTM) [2–4]. These patients are vulnerable to systemic salmonellosis and infections with *Mycobacterium tuberculosis*, the virulent mycobacterial species, to a lesser extent [5, 6]. Diseases caused by other intracellular pathogens, such as *Nocardia*, *Listeria*, *Paracoccidioides*, *Histoplasma*, and *Leishmania*, and some viruses, such as human herpes virus-8, have only rarely been reported, mostly in single patients [7–12].

To date, interferon (IFN)-γ receptor 1 (*IFNGR1*) [13–15], IFN-γ receptor 2 (*IFNGR2*) [16], interleukin (IL)-12 p40 subunit (*IL12B*) [17], IL-12 receptor β subunit (*IL12RB1*) [18–20], signal transducer and activator of transcription-1 (*STAT1*) [21], and nuclear factor-κB-essential modulator (*NEMO*) [22] mutations were identified as the causes of this primary immunodeficiency. On the other hand, no genetic etiology has yet been reported to be identified for about half of all patients with MSMD [3]. In addition, there have been no precise reports on the clinical characteristics and genetic backgrounds of MSMD in Asian countries, including Japan, which has a high prevalence of tuberculosis.

In this study, we analyzed patients who had a recurrent or disseminated infection with intracellular pathogens to clarify the clinical manifestations and host genetic backgrounds of MSMD in Japan.

Materials and Methods

Subjects

We studied 46 patients (30 males and 16 females) diagnosed as having MSMD because of recurrent infections, or blood-borne infections such as osteomyelitis/arthritis, and multiple infections at different anatomic sites by intracellular bacteria including BCG, NTM, *Salmonella* species, *Listeria monocytogenes*, or *M. tuberculosis* in 34 hospitals in Japan from 1999 to 2009. There was no consanguinity in these families. The clinical information on each patient was collected using a standardized case report form. Informed consent was obtained from the parents of the subjects before the study. This study was approved by the Ethics Committee of Kyushu University.

Flow Cytometric Analysis

Two-color flow cytometric analysis was performed to investigate IFN-γ receptor 1 (IFN-γR1) expression levels on the patients' monocytes by using an EPICS XL instrument (Beckman Coulter, Miami, FL, USA). Peripheral blood mononuclear cells (PBMCs) were stained with mouse anti-IFN-γR1 monoclonal antibody (MAb) (Genzyme, Cambridge, MA, USA), followed by rat phycoerythrin anti-mouse immunoglobulin antibody (BD Bioscience Pharmingen, San Diego, CA, USA). Cells were washed twice and stained with a phycoerythrin 5.1 (PC5)-anti-CD14 MAb (Beckman Coulter). IFN-γR1 expression was analyzed on monocytes determined by their side scatter and CD14 positivity.

Genomic DNA and cDNA Sequence Analysis

The *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes were analyzed for coding exons and flanking intronic sequences. These genes were amplified by polymerase chain reaction (PCR) after whole genome amplification with a GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, UK). The PCR products were treated with an Exo-SAP-IT kit (GE Healthcare, Amersham, UK) and then were analyzed by direct sequencing with an ABI 3130 DNA sequencer (Perkin-Elmer, Foster City, CA, USA). Detected mutations were confirmed by sequencing the PCR product using cDNA as a template.

Statistical Analysis

Comparisons of the proportions were analyzed by the χ^2 test. The Mann–Whitney *U* test was used to compare differences between quantitative variables. A *P* value less than 0.05 was considered to be statistically significant.

Results

The median age of the patients was 8 years (range, 6 months–41 years), and the median age at the onset of infection was 1 year and 4 months (range, 4 months–6 years). The male to female ratio was 1.9:1. Only one patient had not received a BCG vaccination. There were 59 episodes of disseminated mycobacterial infections in the 46 patients. Nine (19%) of 46 patients had two or more episodes of these infections. Two of the patients had three episodes, and one had four episodes of these infections. In all episodes, BCG was the most common pathogen (82.6%, Table I). The *Mycobacterium avium* complex (MAC) was isolated during eight episodes of these infections. *M. tuberculosis* was also confirmed in two episodes of infection. No severe *Salmonella* species, *L. monocytogenes*, or viral infections were observed.

The common clinical manifestations were osteomyelitis/arthritis, lymphadenitis, and subcutaneous abscess/dermatitis (Table I and Fig. 1a). Only one patient was diagnosed as having arthritis, and the lesion spread to the adjacent bone. Two patients showed hepatosplenomegaly during the BCG infection, and two patients with the MAC infection developed pulmonary abscess. Among the BCG infections, the median intervals of time between BCG vaccination and the development of primary BCG infection were 3 (1–10 months), 4 (2–36 months), and 11 months (5–46 months) for the subcutaneous abscess/dermatitis, lymphadenitis, and osteomyelitis/arthritis, respectively (Fig. 1b).

We performed the genetic analysis on these patients for the *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes. Six patients (five families) and one patient had mutations in the *IFNGR1* and *NEMO* genes, respectively (Table II). Five of the seven patients who had a mutation in the *IFNGR1* gene were the patients that we

Table I The clinical manifestations of the patients with MSMD

	Patients with genetic mutation, *n* (%)	Patients without a genetic mutation, *n* (%)	Total *n* (%)
Causative pathogen[a]			
BCG	3 (42.9)	35 (89.7)	38 (82.6)
M. avium complex	1 (14.3)	3 (10.2)	4 (8.7)
BCG+*M. avium* complex	2 (28.5)	0 (0)	2 (4.3)
M. avium complex+*M. tuberculosis*	1 (14.3)	1 (2.6)	2 (4.3)
Sites of infection[b]			
Osteomyelitis/arthritis	7 (43.8)	24 (55.8)	31 (52.5)
Lymphadenitis	8 (50.0)	8 (18.6)	16 (27.1)
Dermatitis/subcutaneous	3 (18.8)	11 (25.6)	14 (23.7)
Pulmonary abscess	0 (0)	2 (4.7)	2 (3.4)

The total number exceeds 59 because some patients had multiple lesions at the same time

[a] $n=7$ for patients with a genetic mutation and $n=39$ for patients without a genetic mutation

[b] $n=16$ for patients with a genetic mutation and $n=43$ for patients without a genetic mutation

reported previously [14, 15], and the other two patients were newly identified. All of the IFN-γR1-deficient patients were heterozygotes, and the mutation was in the transmembrane domain in one patient (774del4: patient 5) and in the intracellular domain in five patients (811del4: patient 1, 818del4: patients 2–4, and 832 G>T, E278X: patient 6), which led to the expression of a truncated protein with a dominant negative effect on the IFN-γR1 signaling (Table II and Fig. 2a). The IFN-γR1 expression levels were significantly increased in all six patients with IFN-γR1 deficiency (Fig. 2b). Patient 7 had a missense mutation in *NEMO* (943 G>C, E315Q). The CD14-positive cells from this patient produced a lower level of TNF in response to LPS stimulation (data not shown), which was consistent with the defect in NF-κB signaling.

The proportions of the patients with recurrent mycobacterial infection or multiple osteomyelitis/arthritis were

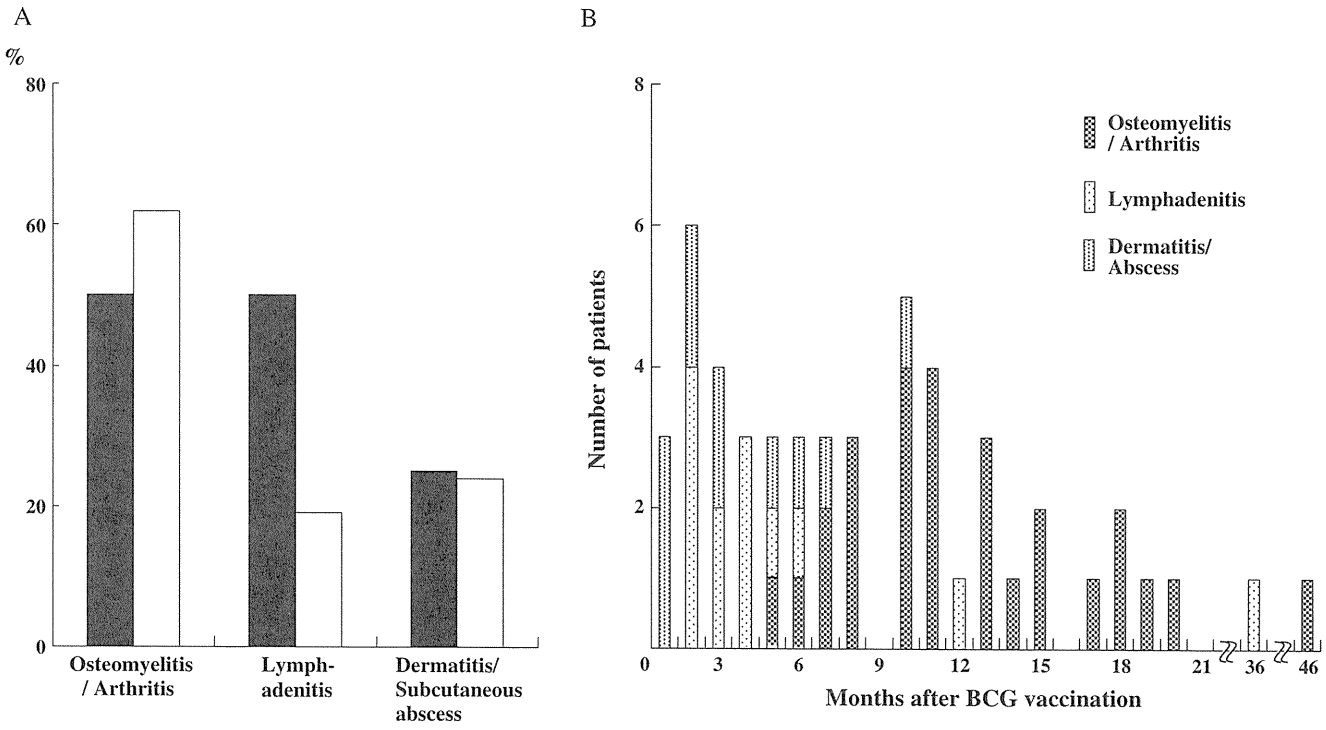

Fig. 1 The clinical features of the patients with BCG infection. The distribution of the sites of infections (**a**) and the intervals between BCG vaccination and the first onset of BCG infection (**b**) are shown. The *black bar* and the *white bar* represent the proportion of the patients with and without genetic mutations, respectively

Table II Characteristics of the patients with a genetic mutation

Patient no.	Sex	Age	Age of onset	Episodes of infections prior to detection of the genetic mutation	Genetic mutation
1[a] [14]	F	1 year 7 months	10 months	BCG lymphadenitis and dermatitis Multiple BCG osteomyelitis	*IFNGR1* 811del4
2[a] [14]	M	1 year 9 months	8 months	BCG lymphadenitis, hepatomegaly Multiple BCG osteomyelitis	*IFNGR1* 818del4
3[a] [14]	M	2 years	2 years	Multiple BCG osteomyelitis	*IFNGR1* 818del4
4[a] [14]	M	41 years	3 years	*M. tuberculosis* lymphadenitis (twice) Multiple MAC octeomyelitis	*IFNGR1* 818del4
5[a] [15]	F	12 years	6 months	BCG lymphadenitis Multiple MAN osteomyelitis	*IFNGR1* 774del4
6	M	19 years	4 months	BCG lymphadenitis and dermatitis Multiple BCG osteomyelitis MAC subcutaneous abscess Multiple MAC osteomyelitis	*IFNGR1* E278X
7	M	10 years	10 months	*M. tuberculosis* lymphadenitis Multiple MAC lymphadenitis Sepsis, bacterial pneumonia (four times)	*NEMO* E315Q

Patient 4 is the father of patient 2

MAC Mycobacterium avium complex

[a] These patients were reported previously

significantly higher in those with the genetic mutations (Table III). There were no significant differences in the age at the onset of mycobacterial infection, or in the interval of time between BCG vaccination and the first onset of BCG infection between the patients with and without genetic mutations. One patient diagnosed with BCG dermatitis died of persistent diarrhea of unknown etiology, while the others are still alive.

Discussion

In the present study, we investigated the clinical characteristics and the genetic backgrounds of the patients diagnosed as having MSMD in Japan. We observed that the patients with the genetic mutation were susceptible to developing recurrent mycobacterial infections and multiple osteomyelitis/arthritis, and IFN-γR1 deficiency was the most

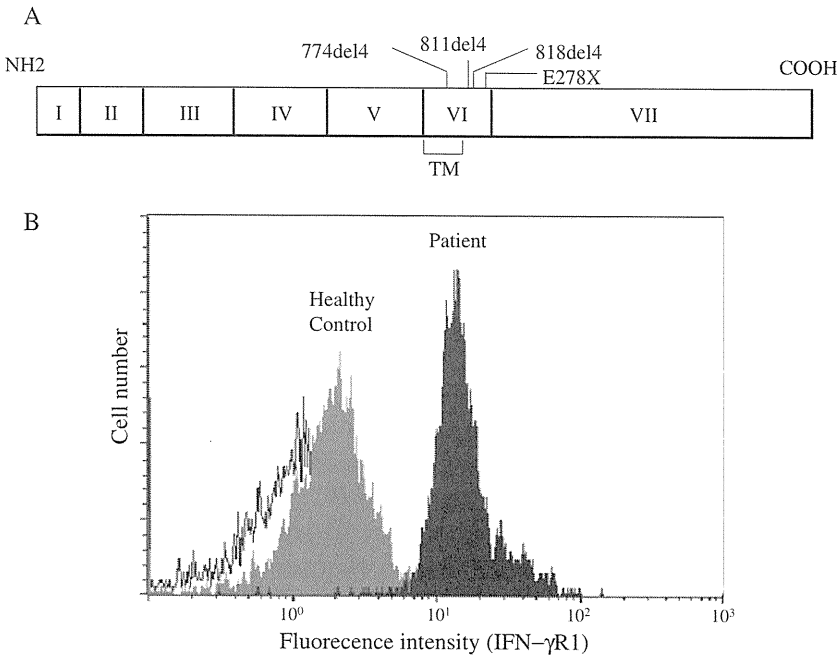

Fig. 2 *IFNGR1* gene mutations and the analysis of IFN-γR1 expression on monocytes. The sites of *IFNGR1* gene mutations in the six IFN-γR1-deficient patients (**a**) and the increased IFN-γR1 expression level on monocytes in patient 2 are shown (**b**)

Table III Comparison of the patients with and without a genetic mutation

	Patients with a genetic mutation (n=7)	Patients without a genetic mutation (n=39)
Age of onset (months)	10 (4–36)	14 (4–75)
Male to female ratio	2.5:1	1.8:1
Familial history (n)	2	0
Median interval between BCG vaccination and the first onset of BCG infection (months)	9.5 (7–15, n=4)	10 (1–46, n=35)
Recurrent cases (%)	85.7*	7.7
Patients with multiple osteomyelitis/arthritis (%)	100* (n=6)	4.2 (n=24)

*p<0.0001

frequent genetic defect identified in these patients. The prevalence of MSMD is estimated to be at least 0.59 cases per million births, and the disease does not seem to be confined to any ethnic group or geographic region, according to a national retrospective study of idiopathic disseminated BCG infection in France [23, 24]. This is the first epidemiological study associated with MSMD in Japan which showed the difference in the clinical manifestation and the genetic background between Japan and Western countries.

The *IFNGR1* mutations identified in this study were in exon IV, within the transmembrane domain, or the intracellular domain of the *IFNGR1* gene (Fig. 2a), which led to a truncated protein lacking signaling motifs [25]. The truncated protein also lacks the recycling motif, which leads to the overexpression of the mutant protein (Fig. 2b) [25]. These mutations are located in important hot spots in the patients diagnosed with dominant partial IFN-γR1 deficiency [13], and the flow cytometric analysis of IFN-γR1 expression levels may be a useful method for the screening for this disease [15]. The *NEMO* mutation found in patient 7 was in exon VIII within the leucine zipper domain of the *NEMO* gene. A previous study reported that a mutation in this region disrupted a common salt bridge in the leucine zipper domain and impaired T-cell-dependent IL-12 production [22].

The patients with the genetic mutations were susceptible to recurrent mycobacterial infections and multiple osteomyelitis/arthritis as described previously [3], but no fatal mycobacterial infection was observed in this study. Unlike complete IFN-γR1 and IFN-γR2 deficiencies, which often cause fatal mycobacterial infections [13, 16], the patients with dominant partial IFN-γR1 and NEMO deficiencies have been reported to have a relatively mild disease and a better prognosis [13, 22]. These factors might have contributed to the good outcome of the patients in this study. In addition, the low virulence of BCG might contribute to the characteristics of BCG infection in Japan, because the BCG Tokyo 172 strain that is used in Japan for vaccination is the least virulent BCG substrain.

The *IL12RB1* mutation has been reported to be the most common cause of MSMD [4]. However, none of the patients in this study was diagnosed as having an IL-12 receptor β1 deficiency. In Japan, this disease was reported in only one patient with disseminated lymphadenitis caused by *M. avium* complex [18]. It has been suggested that most complete IL-12 receptor β1-deficient individuals may be asymptomatic, and only those that also have a second mutation in another gene may be more prone to infections [26, 27]. These symptomatic IL-12 receptor β1-deficient patients are mainly found in families with consanguineous parents [19, 27]. Consanguineous marriages are uncommon in Japan, and there were no consanguineous families in this study. This might be the reason why no IL-12 receptor β1-deficient patients were observed. Alternatively, it is possible that the causative gene mutations associated with MSMD are different among races, because the number of patients with IL-12 receptor β1 deficiency was also lower than those with IFN-γR1 deficiency in Taiwan [28].

Although another patient had multiple osteomyelitis, and three patients had recurrent disseminated mycobacterial infections in these studies, they did not have mutations in any of the six genes. It was previously reported that no genetic etiology has yet been identified in about half of patients with disseminated and recurrent mycobacterial infections [3, 4]. This suggests the presence of as yet undetermined genetic factors in the development of this disease.

In the present study, the number of patients with genetic mutations might be too small to conclusively indicate the differences in the clinical manifestations and the host genetic backgrounds of MSMD between Japan and Western countries. However, in terms of the genetic etiology and the prognosis, it remains possible that the features of the patients diagnosed as having MSMD in the present study are different from those in previous reports [3]. Further investigations of a large number of patients are therefore warranted to more precisely evaluate the clinical manifestations and the host genetic background of MSMD in Japan.

Conclusions

We found that the patients diagnosed as having MSMD in Japan seem to have different genetic features, as well as

different clinical manifestations, compared with those in Western countries. A few patients with recurrent mycobacterial infections without mutations in the six known genes might suggest a contribution of other genetic, as well as environmental, factors in the susceptibility to recurrent infections.

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ORIGINAL ARTICLE

Novel mutations of MVK gene in Japanese family members affected with hyperimmunoglobulinemia D and periodic fever syndrome

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Abstract Hyperimmunoglobulinemia D with periodic fever syndrome (HIDS) is a recessively inherited recurrent fever syndrome. We describe a family of eldest son and monozygotic twin younger sisters with characteristic syndrome of HIDS, but normal level of IgD. Mevalonate kinase (MK) activity was deficient in all of them, and analysis of the MVK gene revealed compound heterozygosity for 2 new mutations, one of which was the disease-causing splicing mutation and the other was a novel missense mutation. All the patients had the same compound heterozygous mutations c.227-1 G > A and c.833 T > C, which resulted in exon 4 skipping and p.Val278Ala. This is the first case in which exon skipping mutation of the MVK gene has been certainly identified at the genomic DNA level. In each case, in which HIDS is clinically suspected, despite normal IgD level, analysis of MK activity and the MVK gene should be performed.

Keywords HIDS · MVK gene · Novel mutation · Compound heterozygous mutation · Splicing mutation · Inherited recurrent fever syndrome

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Introduction

Hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) is a rare autosomal recessive auto-inflammatory disorder characterized by recurrent febrile attacks with lymphadenopathy, abdominal distress, skin eruptions, and joint involvement [1–3]. Febrile attacks usually last for 3–7 days and are interrupted by asymptomatic intervals of several weeks' duration [4–6]. Symptoms appear in early infancy and may persist throughout life with gradual increases in serum IgD [7, 8]. The diagnostic hallmark of HIDS is a constitutively elevated level of serum IgD, although parts of the patients have been reported to have normal amount of serum IgD levels.

The HIDS is caused by mutations on mevalonate kinase gene (MVK), which encodes an enzyme involved in cholesterol and non-sterol isoprenoid biosynthesis. We present herein a Japanese family, eldest son and monozygotic twin younger sisters, with HIDS that had compound heterozygous mutations on MVK gene, one of which was the disease-causing splicing mutation and the other was a novel missense mutation. Serum concentrations of IgD were repeatedly within the normal range. These cases demonstrate that detail analysis with more specific diagnostic tests such as urinary excretion of mevalonic acid and MVK genetic analysis should be performed not to miss the correct diagnosis in patients, especially younger children with HIDS.

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Case reports

Patients are the eldest son and monozygotic twin younger sisters of parents of Japanese origin. The eldest son (patient 1) had presented with recurrent fever from 5 months of age. The twin younger sisters (patient 2 and 3) presented with fever from 1 month of age. Vomiting and diarrhea were presented in the younger sister (patient 3). Febrile episodes appeared every 4–8 weeks and lasted for 3–5 days on all the three patients. During febrile episodes, peripheral blood leukocytosis and CRP elevations (more than 10 mg/dl) were observed. In intermittent period between fever episodes, serum CRP levels decreased, but did not always become negative. Their parents had no history of recurrent fever. Sepsis work-up did not show any foci and any pathogens causing the febrile episodes. The repeated bacterial cultures resulted in negative, and administration of the antimicrobial agents did not change the clinical courses of the febrile episodes, indicating that the fever was not induced by pathogen. In addition, immunological analysis such as serum IgA, IgM, IgG, and IgD, lymphocytes counts including T, B, NK cells, and mitogen proliferation assays of peripheral blood mononuclear cells (PBMCs) were normal.

Due to the recurrent high fevers caused most unlikely by pathogen and the heavy family history of the periodic fevers, we suspected hereditary periodic fever syndromes and performed genetic study. After written informed consents approved by institutional review board of the Kyoto University Hospital were obtained, peripheral blood samples were collected from the patients and their parents for isolating genomic DNA and total RNA.

First, we performed genomic DNA sequencing for MEFV gene for familial Mediterranean fever, MVK gene for HIDS, NLRP3 for cryopyrin-associated periodic syndrome, and TNFRSF1A for TNF receptor-associated periodic syndrome. Genomic DNA sequencing analysis of the MVK gene revealed the presence of heterozygous mutations of c.227-1 G > A at the exon/intron border of exon 4 and c.833T > C (p.Val278Ala). Subsequent amplification of the cDNA by RT-PCR showed that the former mutation caused deletion of exon 4 (Fig. 1a). Genomic DNA sequence analysis on their parents revealed that the parents inherited c.227-1 G > A from their father and c.833T > C from their mother, indicating that the three patients were compound heterozygous for MVK gene (Fig. 1b). The patients had markedly elevated excretion of mevalonic acid in urine, especially in febrile periods, and their mevalonate kinase enzyme activities were very low, which confirmed that all the three patients suffered from HIDS (Table 1).

While the patients did not have any mutations on TNFRSF1A and NLRP3, we identified MEFV non-synonymous nucleotide alterations on the elder brother, who was a heterozygote for L110P, E148Q, and R202Q, and the younger twin, who was a heterozygote for R202Q in addition to MVK gene mutations. These MEFV gene nucleotide alterations were regarded as SNPs, and the clinical diagnosis of FMF was not compatible with the patients, although the complex MEFV gene alterations of L110P/E148Q/R202Q have been reported on the clinically-diagnosed FMF patients.

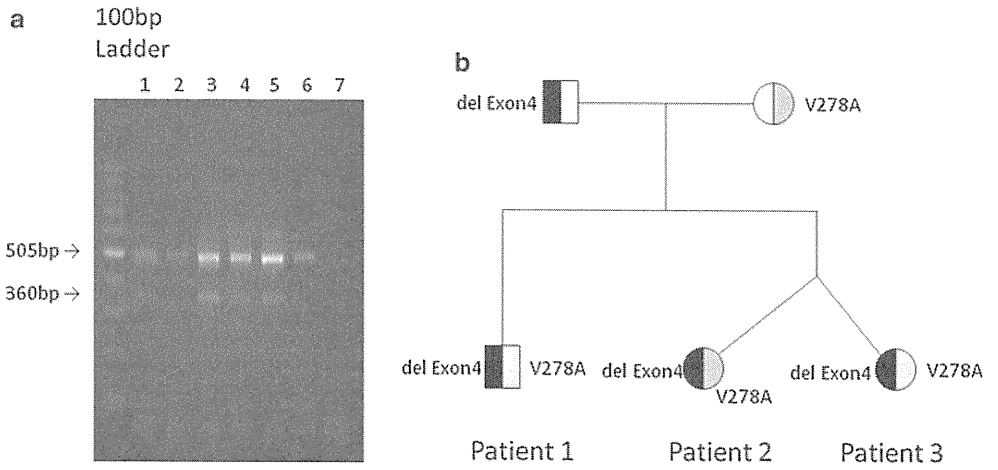

Fig. 1 Molecular genetic findings in the study patients. **a** Agarose gel electrophoresis of RT-PCR products for exon 2 to exon 5 of MVK shows the normal 505-bp alleles in samples from normal healthy control (*lane 6*) and mother (*lane 2*), as well as both the normal allele and the mutant 362-bp allele in the sample from father (*lane 1*), patient 1 (*lane 3*), patient 2 (*lane 4*), and patient 5 (*lane 5*). Subsequent cDNA sequencing confirmed that this 144-bp deletion in cDNA corresponds to codon 303–407 (exon 4). The molecular size marker was a 100-bp ladder. *Lane 7* represents PCR with distilled water added but not with DNA, indicating that there was no background amplification. **b** Pedigree of the affected family. The three patients are heterozygous for del exon 4 and V278A

Table 1 Urinary mevalonic acid and mevalonate kinase levels in the study patients

Patient no.	Mevalonic acid in urine (μg/mgCr)		Mevalonate kinase (pmol/minute/mg)
	Febrile period	Intermittent period	
1	67.9	11.3	3
2	55.6	17.7	2
3	58.8	18.5	2
Control		0.078 ± 0.012[a]	214 ± 62[a]

Control data are given as mean ± SD

[a] Values form healthy subjects were used to obtain a control range for urinary mevalonic acid levels (mean ± SD) and mevalonate kinase levels (mean ± SD)

Discussion

We present herein a sibling of HIDS that demonstrated compound heterozygous for two novel mutations of MVK gene. All the patients had the same compound heterozygous mutations c.227-1 G > A and c.833T > C, which resulted in exon 4 skipping and p.Val278Ala. The mutations are novel, especially the splicing mutation of MVK gene was identified at the genomic DNA level.

Cuisset et al. [9] reported that HIDS mutations were evenly distributed along the coding region of the MVK gene, in contrast to mutations causing MA, which clustered between 243 and 334. The sequence variations seen in MA are missense mutations that are in the same region as the variants described in HIDS. Further studies will be needed to clarify the association of phenotypical differences with MVK gene mutations. Over 80% of patients with HIDS were reported to have compound heterozygous mutation in the MVK gene. To our knowledge, both the skipping of exon 4 and V278A mutation have not been reported previously in HIDS. Moreover, this is the first case in which exon skipping mutation of the MVK gene has been certainly identified at the genomic DNA level. Only few groups reported HIDS patients with the skipping of exon in the cDNA of the MVK gene [10, 11]. They suggested that these exon skipping was probably due to the presence of a potential splice site mutation, but could not identify mutations responsible for these altered splicing through the sequence analysis at the genomic level. Most MVK mutations in patients with HIDS and MA have only been determined at the cDNA level; however, analysis of cDNA sometimes appeared troublesome, probably due to instability of the MVK mRNA. More detailed studies through the sequence analysis at the genomic level lead us to elucidate the role of MVK mutations in HIDS and MA, and expression studies in *E. coli* will be necessary to evaluate the effect of each mutation.

HIDS is classically defined as a high concentration of mevalonic acid in the urine and is characterized by a high serum IgD concentration during each febrile episode, but some reports from the Netherlands stated that high levels of serum IgD levels were not seen and affirmed that other diseases also showed high serum IgD levels [12]. In our cases, the analysis of enzymes and molecular genetics of MVK gene yielded the correct diagnosis, although serum concentrations of IgD were within the normal range. Thus, it should be now common practice to examine the MVK gene in order to diagnose this disease.

In conclusion, we present a Japanese family with HIDS that appeared to have novel mutations of MVK gene. Most of the HIDS cases were reported from European, especially Dutch, whereas only one HIDS case of Japanese patient was reported by Naruto et al. [13], which is only one report of Asian patient. Cases of HIDS may so far have been overlooked or misdiagnosed as infectious diseases or autoimmune disorders in Japan, besides there may be difference in race. It is necessary that accumulation of case in hereditary mutation and in other race leads to solve a detailed cause of HIDS.

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High Incidence of *NLRP3* Somatic Mosaicism in Patients With Chronic Infantile Neurologic, Cutaneous, Articular Syndrome

Results of an International Multicenter Collaborative Study

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Objective. **Chronic infantile neurologic, cutaneous, articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disease (NOMID), is a dominantly inherited systemic autoinflammatory disease. Although heterozygous germline gain-of-function *NLRP3* mutations are a known cause of this disease, conventional genetic analyses fail to detect disease-causing mutations in ~40% of patients. Since somatic *NLRP3* mosaicism has been detected in several mutation-negative NOMID/CINCA syndrome patients, we undertook this study to determine the precise contribution of somatic *NLRP3* mosaicism to the etiology of NOMID/CINCA syndrome.**

Methods. **An international case–control study was performed to detect somatic *NLRP3* mosaicism in NOMID/CINCA syndrome patients who had shown no mutation during conventional sequencing. Subcloning and sequencing of *NLRP3* was performed in these mutation-negative NOMID/CINCA syndrome patients and their healthy relatives. Clinical features were analyzed to identify potential genotype–phenotype associations.**

Results. **Somatic *NLRP3* mosaicism was identified in 18 of the 26 patients (69.2%). Estimates of the level of mosaicism ranged from 4.2% to 35.8% (mean ± SD 12.1 ± 7.9%). Mosaicism was not detected in any of the 19 healthy relatives (18 of 26 patients versus 0 of 19**

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relatives; $P < 0.0001$). In vitro functional assays indicated that the detected somatic *NLRP3* mutations had disease-causing functional effects. No differences in *NLRP3* mosaicism were detected between different cell lineages. Among nondescript clinical features, a lower incidence of mental retardation was noted in patients with somatic mosaicism. Genotype-matched comparison confirmed that patients with somatic *NLRP3* mosaicism presented with milder neurologic symptoms.

***Conclusion.* Somatic *NLRP3* mutations were identified in 69.2% of patients with mutation-negative NOMID/CINCA syndrome. This indicates that somatic *NLRP3* mosaicism is a major cause of NOMID/CINCA syndrome.**

Chronic infantile neurologic, cutaneous, articular (CINCA) syndrome (MIM no. #607715), also known as neonatal-onset multisystem inflammatory disease (NOMID), is a dominantly-inherited autoinflammatory disease that is characterized by neonatal onset and the triad of urticarial-like skin rash, neurologic manifestations, and arthritis/arthropathy. Patients often experience recurrent fever and systemic inflammation. NOMID/CINCA syndrome is the most severe clinical phenotype of the cryopyrin-associated periodic syndromes (CAPS) that also include the 2 less severe but phenotypically similar syndromes familial cold autoinflammatory syndrome (FCAS; MIM no. #120100) and Muckle-Wells syndrome (MIM no. #191900). CAPS are caused by mutations in the *NLRP3* gene, which is a member of the nucleotide-binding oligomerization domain–like receptor (NLR) family of the innate immune system (1,2).

NLRP3 is an intracellular "sensor" of danger signals arising from cellular insults, such as infection, tissue damage, and metabolic deregulation, and it has been highly conserved throughout evolution. NLRP3 associates with ASC and procaspase 1 to constitute a large multiprotein complex termed the NLRP3 inflammasome. When activated, the NLRP3 inflammasome converts the biologically inactive procaspase 1 into active caspase 1. Caspase 1 produces the cytokines interleukin-1β (IL-1β) and IL-18, which are mainly involved in the inflammatory response (3). Available research suggests that mutated *NLRP3* induces autoactivation of the NLRP3 inflammasome in CAPS patients, resulting in an uncontrolled overproduction of IL-1β.

Most CAPS patients carry heterozygous germline missense mutations in the *NLRP3* coding region ("mutation-positive" patients) (4,5). More than 80 different disease-causing mutations have been reported to date (6). However, ~40% of clinically diagnosed NOMID/CINCA syndrome patients show no heterozygous germline *NLRP3* mutation during conventional Sanger-sequencing-based genetic analyses ("mutation-negative" patients). Comparisons of NOMID/CINCA syndrome patients with and without heterozygous germline *NLRP3* mutations have revealed no differences in clinical features or response to treatment (4,7).

In a previous study, we identified a high incidence of somatic *NLRP3* mosaicism in "mutation-negative" NOMID/CINCA syndrome patients in Japan (8). We therefore hypothesized that somatic *NLRP3* mosaicism may be implicated in the etiology of the disorder, although its precise contribution remains unclear. The aim of the present study was to evaluate both the frequency of *NLRP3* somatic mosaicism in NOMID/CINCA syndrome patients and the association between somatic mosaicism and clinical phenotype using an international cohort of mutation-negative NOMID/CINCA syndrome patients.

PATIENTS AND METHODS

Study design and participants. International collaborators were contacted to identify mutation-negative NOMID/CINCA syndrome cases. A total of 20 DNA samples were received from 4 centers: France (n = 6), The Netherlands (n = 4), Spain (n = 3), and the US (n = 7). DNA samples had been extracted from peripheral blood mononuclear cells or whole blood. All 20 samples had been subjected to conventional sequencing, and no *NLRP3* mutations had been identified. In each case, the accuracy of the clinical diagnosis had been confirmed according to the diagnostic criteria (7). The 6 previously reported Japanese cases and 1 Spanish case with *NLRP3* somatic mosaicism were also included (8,9). DNA samples were also collected from 19 healthy relatives of 8 patients (8 from France, 5 from Japan, 2 from Spain, and 4 from the US) to evaluate the causality of somatic *NLRP3* mosaicism in a case–control manner, since the clinical features may be modified by genetic and environmental factors.

Written informed consent for *NLRP3* gene analysis was obtained from all patients and controls. The study was approved by the Institutional Review Board of the Kyoto University Graduate School of Medicine and was conducted in accordance with the Declaration of Helsinki.

Data collection. *Demographic and clinical data.* The clinicians responsible for each mutation-negative NOMID/CINCA syndrome patient completed a questionnaire to document characteristics such as age, sex, race, symptoms, clinical findings, clinical course, and prognosis. No clinical data were obtained from the healthy controls.

Investigation of NLRP3 gene mosaicism. Disease-causing mutations in NOMID/CINCA syndrome patients have

only been reported in exons 3, 4, and 6 of *NLRP3* (6). Thus, the present sequencing was focused on a search for somatic mosaicism of these 3 exons and their flanking intronic regions. After amplifying these genomic regions with the proofreading polymerase chain reaction (PCR) enzyme KOD-Plus polymerase (Toyobo) and dA addition with an LA *Taq* polymerase (Takara Bio), the amplicons were subcloned into pCR2.1-TOPO vector (Invitrogen). Ninety-six clones were selected at random for each amplicon. The subcloned amplicons were retrieved by PCR with LA *Taq* polymerase. They were then treated with ExoSAP-IT (USB) and proteinase K (Promega) prior to direct sequencing. The cloned exons were sequenced at the Kazusa DNA Research Institute using a BigDye Terminator kit (version 3.1) and an ABI 3730 DNA sequencer (Life Technologies). Mosaicism was indicated by the detection of >2 subclones carrying the same base variation at the same position in 96 clones.

To purify leukocyte subpopulations, freshly drawn whole blood was separated using sequential dextran and Ficoll-Hypaque density-gradient centrifugation methods. Cell sorting to select T cells, B cells, and monocytes was performed with an AutoMACS Pro Separator (Miltenyi Biotec) or a FACSVantage System (BD Biosciences), as described elsewhere (8,9). The purity of each cell lineage was >90%. The level of mosaicism was determined by sequencing each source of genomic DNA from 80 clones.

Plasmids and cell lines. To determine whether the identified NLRP3 mutants cause disease, experiments for assessing 2 pathologic functions were performed as described elsewhere (8). Briefly, ASC-dependent NF-κB activation was performed by a dual-luciferase reporter assay in HEK 293FT cells transfected with NLRP3 mutants. Transfection-induced cell death in the human monocytic cell line THP-1 was performed by transfecting green fluorescent protein–fused mutant NLRP3 into THP-1 cells and then measuring the dead cells with 7-aminoactinomycin D.

Statistical analysis. The study was designed to detect mosaicism at a 5% allele frequency with >95% possibility. To satisfy this condition, it was necessary to sequence at least 93 clones per patient. The following calculation was used to estimate the number of clones that had to be sequenced: $P = 1 - (1 - 0.05)^n - n(0.05)(1 - 0.05)^{n-1}$ ($n = 93$, $P = 0.956$). The study was designed to analyze 96 PCR-fragment clones from each patient. The error rate of the PCR reactions was estimated using a proofreading KOD-Plus enzyme. We analyzed a plasmid vector carrying a normal *NLRP3* exon 3, in which 2 distinct errors were detected by sequencing 91 clones. The calculated error rate for this result was 1/87,451 (2/[1,922 bp × 91 clones]). Thus, the probability was negligible that the same errors would be detected more than twice in 96 clones from 1 patient.

To calculate the sample size, we calculated the prevalence of somatic mosaicism among mutation-negative NOMID/CINCA syndrome patients. Eight cases of somatic mosaicism were identified among 15 mutation-negative NOMID/CINCA syndrome patients who were subsequently analyzed by the subcloning method described above. It was assumed that the maximum number of possible somatic mosaicism cases among family controls was 1. On the basis of these data and this assumption, it was calculated that 19 controls were required to ensure a 2-sided alpha level of 0.05 and a power of 0.8.

Continuous variables are presented as the mean ± SD or as the median and interquartile range. Categorical variables are presented as numbers and ratios (with percentages). To compare clinical data between patients with and patients without mosaicism, the Wilcoxon rank sum test was used for continuous variables and Fisher's exact test was used for categorical variables. Fisher's exact test was used to compare the difference in mosaicism ratio between cases and controls. The chi-square test was used to compare the difference in the level of mosaicism between different sources of genomic DNA from each patient.

Table 1. Somatic mosaicism among mutation-negative NOMID/CINCA syndrome patients*

Country, patient	Sequence variant	Protein variant	Mosaicism, %
France			
F1	1298C>T	T433I	5.2
F2	907G>C	D303H	4.2
F3	1315G>C	A439P	21.9
F4	1216A>G	M406V	9.2
F5	1698C>A	F566L	11.5
F6	None	–	–
Japan			
J1	1709A>G	Y570C	12.2
J2	790C>T	L264F	4.3
J3	919G>A	G307S	10.7
J4	1699G>A	E567K	6.5
J5	907G>C	D303H	11.9
J6	None	–	–
Spain			
S1	920G>T	G307V	9.6
S2	907G>C	D303H	19.1
S3	None	–	–
S4	None	–	–
US			
A1	1065A>T	K355N	18.8
A2	1698C>A	F566L	14.6
A3	1704G>C	K568N	9.4
A4	2263G>A	G755R	35.8
A5	None	–	–
A6	None	–	–
The Netherlands			
N1	1699G>A	E567K	6.3
N2	2263G>A	G755R	6.3
N3	None	–	–
N4	None	–	–

* *NLRP3* mosaicism was detected in 18 of 26 patients (69.2%) with neonatal-onset multisystem inflammatory disease (NOMID)/chronic infantile neurologic, cutaneous, articular syndrome (CINCA syndrome). When samples from 19 healthy relatives of these patients were investigated, no somatic mosaicism was detected. The *P* value from the comparison of the cases and the controls (18 of 26 versus 0 of 19) was statistically significant ($P < 0.0001$).

RESULTS

Somatic *NLRP3* mosaicism in mutation-negative NOMID/CINCA syndrome patients. A heterozygous germline *NLRP3* mutation was detected in 1 of the 27 samples, and this was therefore excluded from the analyses. For each patient, 96 clones were selected at random for each amplicon. These were then sequenced. *NLRP3* mosaicism was detected in 18 of 26 patients (69.2%), and the level of allelic mosaicism ranged from 4.2% to 35.8% (mean ± SD 12.1 ± 7.9%; median 10.2%) (Table 1). Seven of the detected *NLRP3* mutations were novel (p.G307S, p.K355N, p.M406V, p.T433I, p.F566L, p.E567K, and p.K568N). The remaining mutations have been reported previously in NOMID/CINCA syndrome patients as disease-causing heterozygous germline mutations (p.L264F, p.D303H, p.G307V, p.A439P, p.Y570C, and p.G755R). Each of the 3 *NLRP3* mutations, p.F566L, p.E567K, and p.G755R, was detected in 2 unrelated patients. *NLRP3* mutation p.D303H was detected in 3 unrelated patients.

Analyses in family controls. To validate the clinical relevance of the *NLRP3* mosaicism identified in mutation-negative NOMID/CINCA syndrome patients, samples from 19 healthy relatives were investigated. No somatic mosaicism was detected in any of these samples. The *P* value from the comparison of cases and controls (18 of 26 versus 0 of 19) was statistically significant ($P < 0.0001$).

Functional effects of the identified somatic *NLRP3* mutations. Since disease-causing heterozygous germline mutations in *NLRP3* have been implicated in necrosis-like programmed cell death and ASC-dependent NF-κB activation (8), experiments were performed to determine whether the mutations identified in patients with somatic mosaicism showed the same effects. All of the identified mutations induced both THP-1 cell death (Figure 1A) and ASC-dependent NF-κB activation (Figure 1B). The in vitro effects of these novel mutations were similar to or even more pronounced than those of previously reported *NLRP3* mutations. This strongly suggests that all mutations showing somatic mosaicism have pathogenic effects, including the novel mutations identified in the present study.

Mutation frequency of *NLRP3* among various cell lineages and 1 tissue type. To explore the origin of the *NLRP3* mosaicism, mutational frequency was evaluated in various cell lineages and 1 tissue type from 4 Japanese patients with *NLRP3* somatic mosaicism. In each patient, the same mutations were found in all of the cell lineages investigated (neutrophils, monocytes, T cells, B cells) and in the buccal mucosa tissue, and no significant difference in mutation frequency was observed between these sources (Table 2).

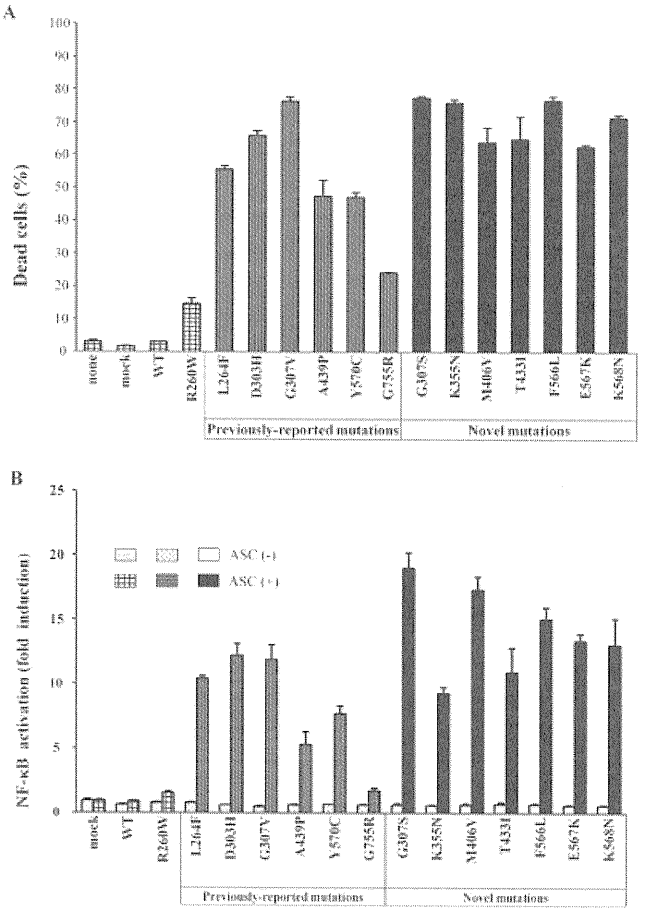

Figure 1. In vitro functional assessment of the identified *NLRP3* mosaicism mutations. **A,** Necrotic cell death of THP-1 cells induced by the identified somatic *NLRP3* mosaicism mutations. Green fluorescent protein (GFP)–fused mutant NLRP3 was transfected into THP-1 cells. The percentage of dead cells (7-aminoactinomycin D positive) among GFP-positive cells is shown. Values are the mean ± SD of triplicate experiments, and data are representative of 2 independent experiments. None = nothing transfected; mock = vector without NLRP3; WT = wild-type NLRP3; R260W = NLRP3 with p.R260W (frequent mutations in patients with cryopyrin-associated periodic syndromes). **B,** ASC-dependent NF-κB activation induced by the identified somatic *NLRP3* mosaicism mutations. HEK 293FT cells were cotransfected with WT or mutant NLRP3 in the presence or absence of ASC. The induction of NF-κB is shown as the fold change compared with cells that were transfected with a control vector without ASC (set at 1). Values are the mean ± SD of triplicate experiments, and data are representative of 2 independent experiments.