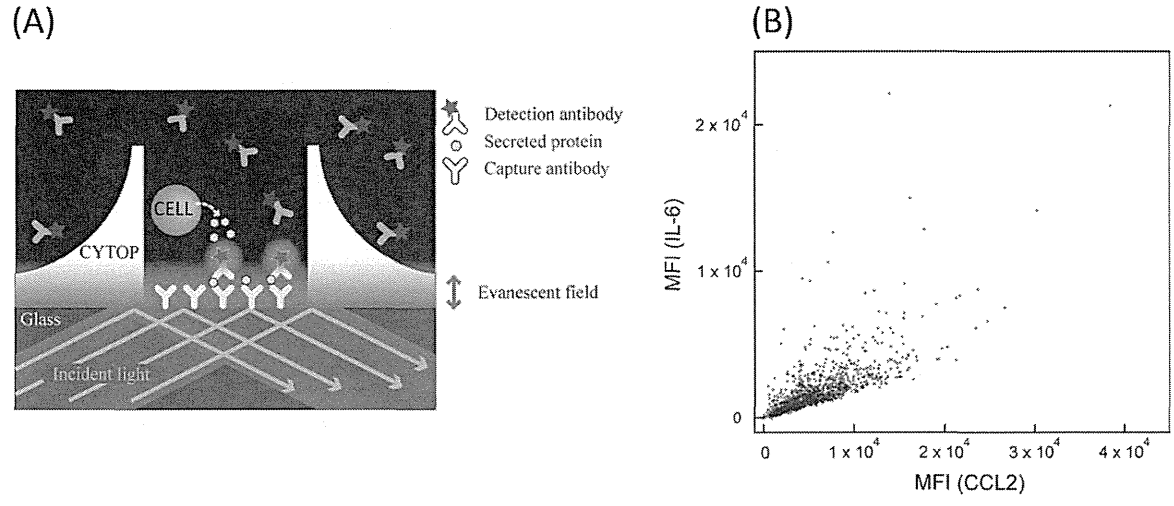

FIG 4 *Simultaneous measurements of secretion of IL-6 and CCL2 in real-time. Principle of real-time monitoring system of protein secretion from a single cell. A microwell is fabricated with Cytop to enable us to detect protein secretion in real-time by total internal reflection illumination, as described (12). Fluorescent sandwich complexes of secreted protein and antibodies captured on the solid support are quantified. The top of the microwell is open for the following two reasons: to keep cells healthy during observation and to allow cell retrieval at the time specified. Detection of IL-6 and CCL2 secretion from MC/9 cells after treatment with a phorbol ester. The secretion of IL-6 and CCL2 from single MC/9 cells 4 h after treatment with a phorbol ester is illustrated. Each dot indicates medians of fluorescence intensity (MFI) of IL-6 and CCL2 signals form a single cell. As shown, the ratio of MFIs varied widely from cell to cell.*

being actively explored (15). It is widely accepted that transcription/translation processes are stochastic at the single-cell level, which is no surprise because these processes are governed by a relatively small number of molecules. In other words, cell responses are intrinsically noisy. Thus, an important and obvious question is how the immune system maintains homeostasis when using such noisy cells.

What kind of new insights can we get from these new lines of information? From a viewpoint of immune cell sociology, the cause of cell heterogeneity does not matter but its dynamics does. We have vaguely assumed that such a stochastic effect does not last long; however, our data, as well as those reported by other groups, indicate that it takes relatively long (hours to days) (16,17). Thus, when we consider immune events responding within a short range of time (i.e., hours), the cell-to-cell variations caused even by stochastic transcription/translation cannot be neglected at all. An analytical framework for linking stochastic dynamics to population distribution was discussed by Friedman et al. (18), and the real situation was described as being more complex, thus necessitating experimental measurements to understand it at the single-cell level.

Rand et al. (19) reported that the key steps of virus-induced signal transduction, interferon (IFN)-beta expression, and the induction of IFN-stimulated genes take place stochastically. According to the authors, the origin of stochasticity seems to be cell-intrinsic noise in transcription and/or translation. Coherent, robust, antiviral protection in spite of multilayered cellular stochasticity is explained to be achieved through intercellular communication, which is likely to be a widely used strategy by mammalian cells to cope with pervasive stochastic signaling and gene expression. However, in this scenario, cell-intrinsic noise is considered disadvantageous for the cell society. Is this always the case? In fact, as long as we consider events that protect cells from destruction, heterogeneity might be undesirable, because the number of cells protected becomes smaller than the coherent cell ensemble. However, if all the cells are subjected to extracellular attacks to induce cell death, heterogeneity might have a positive meaning, because at least a small fraction of cells might survive and save the whole system. Because cytokines play a key role in communication among immune cells, cell-to-cell variations in cytokine secretion could be a strategy to make the "society" flexible and tough in some cases.

The other two reasons, epigenetic and genetic causes, which may generate cell-to-cell variations, are equally or more important than stochasticity from a viewpoint of immune cell sociology. Epigenetic variations are actively explored in their action and regulation at present and will offer us intriguing pieces of information on immune cell sociology. Epigenetic differences frequently result in differences in cell types, which offer a conceptual basis for each immune cell type in conventional immune cell sociology. When epigenetically different cells are functionally distinct, they are conventionally designated as different cell modules (i.e., helper T cells, B cells, NK cells, etc.) in immune cell society. While it has been a relatively macroscopic view, such heterogeneities have been accounted for in immunology.

Genetic variations may be practically more important than stochasticity and genetic factors. A well-known example is found in cancer, where a somatic mutation is first introduced in a very limited number of cells, and the mutant cells are killed, maintained, or expanded under the surveillance by the immune system (20). This is really a matter of immune cell sociology. Another interesting example is found in autoinflammatory disease, a type of primary immunodeficiency disease. Chronic infantile neurological cutaneous and articular syndrome (CINCA), also known as neonatal-onset multisystem inflammatory disease, is characterized by urticarial rash, neurological manifestations, and arthropathy. This dominantly inherited systemic autoinflammatory disease is provoked by somatic mosaicism of gain-of-function NLRP3 mutations as well as a heterozygous germline mutation (21). Furthermore, we recently reported a reliable genetic diagnostic method using massive, parallel DNA sequencing (22). The mutation of NLRP3 activates the inflammasome and is considered to result in the release of a large amount of IL-1β. Thus, in the presence of even a small fraction of mutated cells, CINCA patients might develop a fever. This may be a good example that a small fraction of anomalously behaving cells leads the whole body to a diseased state. Thus, somatic mutation-induced diseases like the CINCA syndrome may serve as a good model to explore how the homeostasis is interrupted or maintained in the presence of such cellular heterogeneity. In this regard, it is interesting to note that periodic fever is observed in CINCA patients, because the oscillating biological events have been actively studies by mathematical modeling for long time (23).

Now that it has become feasible to monitor secretory proteins, as well as intracellular ones at the single-cell level, we can delve into the details of immune cell society at the microscopic level, that is, at the single-cell resolution, if we want. Although such a microscopic analysis might not be always necessary, it must be highly informative, particularly in those cases where the whole system is affected by a small group of heterogeneous immune cells. In such cases, the long-range cellular interactions mediated by cytokines must play a key role. We anticipate that it might unravel a new paradigm of immune cell sociology.

Future Perspectives of the Cell–Cell Interactome at the Single-Cell Level: What are the Implications for Immune Cell Sociology?

The term "cell sociology" was first used in the field of morphogenesis (24). In this review, the term is used for the demonstration of orderliness rather than the dynamic nature of the biological system. Although the term "cell sociology" may be considered an equivalent of "systems biology" in some contexts in this review, we intentionally use it to emphasize the complexity and dynamics of the system. For instance, modern human society has become highly global and dynamic as a consequence of people connecting and interacting via the internet, which is analogous to the situation of the immune system governed by long-range interactions with cytokines. Similarly, the study of how proteins interact with each other and are spatially arranged is designated as "molecular sociology" (25), which also has the sole emphasis on the importance of the complex interactions among common constituents of the "society." In this regard, short-range interactions mediated by cell–cell contact may be analogous to face-to-face communications in human, which are observed even in primitive society. The issue of how homeostasis is maintained with intrinsically noisy agents is an interesting aspect to address, and it might be analogous to the stabilization of the human society, which comprises considerably heterogeneous individuals from a sociological viewpoint. In both cases, as far as long-range interactions are functioning, individuality can be harnessed such that the system as a whole responds to external perturbations in a harmonious and robust manner.

In this context, to get a comprehensive understanding of the immune system, we propose to take sociological approaches in immunological studies. A good lesson to learn from sociology is that it is a multidisciplinary and multiscale science; many different approaches, ranging from micro level to macro level, are eventually integrated to interpret a variety of social events. In the case of immune cell sociology, regulation of long-range interactions among immune cells must be a critical issue, requiring an understanding at the micro level of individual cell agents, as well as the macro level, where the immune events are explained as a consequence of interactions among immune cellular modules (i.e., functional cellular ensembles with particular cellular designations). While immune cell sociology at the macro level has a long and successful history and is already well developed, advances of single-cell analysis technologies enable us to decipher the interactome of immune cells at a deeper resolution. What is expected is to integrate these multiscale data into a sociological view of the immune system. Although still in infancy, interactome analysis of immune cells at the single-cell level might elucidate unexpected mechanisms underlying the maintenance and destruction of immune homeostasis in the future, which would enable us to develop a new way to tackle various immune diseases.

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Pediatr Blood Cancer

Wiskott–Aldrich Syndrome Presenting With a Clinical Picture Mimicking Juvenile Myelomonocytic Leukaemia

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Background. Wiskott–Aldrich syndrome (WAS) is a rare X-linked immunodeficiency caused by defects of the WAS protein (*WASP*) gene. Patients with WAS typically demonstrate micro-thrombocytopenia. ***Procedures.*** The report describes seven male infants with WAS that initially presented with leukocytosis, monocytosis, and myeloid and erythroid precursors in the peripheral blood (PB) and dysplasia in the bone marrow (BM), which was initially indistinguishable from juvenile myelomonocytic leukaemia (JMML). ***Results.*** The median age of affected patients was 1 month (range, 1–4 months). Splenomegaly was absent in four of these patients, which was unusual for JMML. A mutation analysis of genes in the RAS-signalling pathway did not support a diagnosis of JMML. Non-haematological features, such as eczema (n = 7) and bloody stools (n = 6), ultimately led to the diagnosis of WAS at a median age of 4 months (range, 3–8 months), which was confirmed by absent (n = 6) or reduced (n = 1) WASP expression in lymphocytes by flow cytometry (FCM) and a *WASP* gene mutation. Interestingly, mean platelet volume (MPV) was normal in three of five patients and six of seven patients demonstrated occasional giant platelets, which was not compatible with WAS. ***Conclusions.*** These data suggest that WAS should be considered in male infants presenting with JMML-like features if no molecular markers of JMML can be detected. Pediatr Blood Cancer

Key words: children; juvenile myelomonocytic leukaemia; Wiskott–Aldrich syndrome

INTRODUCTION

Wiskott–Aldrich syndrome (WAS) is a rare X-linked recessive disorder, characterized by micro-thrombocytopenia, eczematous skin disease, and recurrent infections. The incidence of WAS is 1–10 in 1 million male new-borns. Affected patients have a predisposition to autoimmune diseases and lymphoid malignancies [1,2]. The responsible gene is *WASP*, which encodes the 502 amino acid WASP protein [3]. WASP is expressed selectively in hematopoietic cells and is involved in cell signalling and cytoskeleton reorganization [3]. Specific types of defects in WASP are often but not invariably associated with the severity of disease and clinical phenotype. Lack of WASP expression causes the most severe phenotype (i.e., classic WAS), whereas inactivating *WASP* missense mutations allow residual protein expression and can cause less severe X-linked thrombocytopenia (XLT) [4,5]. Gain-of-function mutations generate X-linked neutropenia (XLN) [6,7].

Juvenile myelomonocytic leukaemia (JMML) is a rare disease in children that occurs with an estimated incidence of 1–2 cases per million [8]. JMML has characteristics of both myelodysplastic syndrome (MDS) and myeloproliferative disorders (MPD) and is categorized in the MDS/MPD category in the World Health Organization (WHO) classification [9–11]. Clinical and haematological manifestations of JMML include hepatosplenomegaly, skin rash, lymphadenopathy, leukoerythroblastosis, monocytosis, and thrombocytopenia. Recent studies show that deregulated activation of the RAS/MAPK signalling pathway plays a central role in the pathogenesis of JMML. Gene mutations in either the *RAS*, *PTPN11*, *NF1*, or *CBL* genes involved in this pathway are detected in about 80% of JMML patients [12–18].

Micro-thrombocytopenia is the key haematological finding in patients with WAS. However, myelopoiesis and erythropoiesis are usually not affected, despite the fact that WASP is expressed in various hematopoietic cells [19]. The present report describes seven cases of male infants with classical WAS who demonstrated haematological abnormalities mimicking JMML. Importantly, patients can present with JMML-like features before the full clinical manifestations of WAS become apparent. Moreover,

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normal mean platelet volume (MPV) and the presence of the giant platelets complicated the diagnostic evaluation in some of our patients.

PATIENTS AND METHODS

Patients

In 2007, we described a case of a male patient (patient #1) with WAS who demonstrated JMML-like clinical features [20]. Briefly, thrombocytopenia was detected shortly after birth. He suffered from bloody diarrhoea from the age of 9 days. At the age of 42 days, leukocytosis with myeloid/erythroid precursors and monocytosis was detected. Bone marrow (BM) aspirates showed hypercellularity with significant predominance of myelopoiesis and dysplastic features. The morphological features were compatible with JMML. Subsequently, the white blood cell (WBC) count increased to 52.0×10^9/L with the appearance of peripheral blasts (3%) and persistent fever. Intravenous administration of various antibiotics had no effect on fever and leukocytosis. Oral 6-mercaptopurine (6-MP) was administered, which resulted in disappearance of leukocytosis. Positive results of cytomegalovirus (CMV)-IgM/IgG and a low level pp65 CMV-antigen (Ag) cells were transitionally noted without CMV-related symptoms. Intravenous administration of ganciclovir (GCV) led to the elimination of CMV-Ag but not to any improvement of JMML-like features. At the age of 7 months, mild atopic dermatitis-like eczema was recognized, which finally led to the clinical and molecular diagnosis of WAS.

The MDS committee of the Japanese Society of Paediatric Hematology/Oncology (JSPHO) study coordinating center of the European Working Group of MDS in Childhood (EWOG-MDS) perform the morphological review of peripheral blood (PB) and BM smears and laboratory examinations for the diagnosis of JMML in Japan and Germany, respectively. By January 2011, WAS was diagnosed in six Japanese males (including patient #1) and one German male who were initially referred with a suspected diagnosis of JMML. Patient #4 was recently reported [21]. Approval for the study was obtained from the institutional review board of Nagoya University, Nagoya, Japan, and University of Freiburg, Freiburg, Germany. Informed consent was provided by parents according to the Declaration of Helsinki.

Diagnostic Tests for Wiskott–Aldrich Syndrome

Intracellular WASP expression in lymphocytes was analysed by flow cytometry (FCM) by the standard method described previously [4,22]. DNA purification and sequencing of genomic DNA, RNA isolation, reverse transcription-polymerase chain reaction, and sequencing of cDNA for the mutational analysis of *WASP* gene was performed as reported previously [23].

Diagnostic Tests for Juvenile Myelomonocytic Leukemia

Mutational screening for *PTPN11*, *NRAS*, and *KRAS* genes was performed in six patients, as previously reported [24–27]. In patients #6 and #7, the c-CBL gene, which has been recently found in about 10% of JMML patients, was also screened as described previously [16,18]. None of the patients had clinical signs of neurofibromatosis type 1 (NF1). *In vitro* colony assay for granulocyte–macrophage colony stimulating factor (GM-CSF) hypersensitivity assay was performed as a supportive diagnostic tool for JMML as previously reported [28,29].

RESULTS

Clinical Characteristics and Laboratory Findings

The clinical characteristics of these patients are summarized in Table I. Thrombocytopenia and bloody diarrhoea were observed soon after birth in all patients except for patient #6. JMML-like clinical manifestations occurred within the first few months of life. Eczema developed between 0 and 3 months after birth in all patients. Splenomegaly was seen in three of seven patients and massive splenomegaly was present in two patients. At the presentation of JMML-like features, episodes of recurrent infections, which suggest an immunodeficiency, were not observed in any patients. However, in three patients, recurrent bacterial, or viral infections (cases #5, #6, and #7) were documented during the clinical course.

The laboratory findings at the presentation of JMML-like disease are summarized in Table II. The WBC count was increased in all patients except for in patient #7. Monocytosis and myeloid/erythroid precursors were seen in PB in all patients. All patients had anaemia. The MPV before platelet transfusions ranged between 6.9 and 7.9 fl (normal, 7.2–11.7 fl) in the five patients that were evaluated. Hb F levels were normal in three patients examined. The platelet morphology demonstrated anisocytosis in all patients. Occasional giant platelets, which are defined as platelets bigger than red cells, were observed in six patients. These features were unusual for WAS. Full BM with significant predominance of myelopoiesis and a marked left shift of the myeloid lineage was seen in all patients. The number of megakaryocytes was normal or increased. Dysplasia in megakaryopoiesis, myelopoiesis, and erythropoiesis was observed in seven, four, and four patients, respectively. The common dysplasia in the megakaryopoiesis included hypolobulations of nuclei and small megakaryocytes with single or double round nuclei. In the myelopoiesis, nuclear abnormalities such as double nuclei, ring nuclei, or pseudo-Pelger–Huet anomaly nuclei were often seen. The dysplasia of erythropoiesis was mild, if observed, and included nuclear lobulation and double nuclei. The karyotype was normal in all patients. The serum levels of immunoglobulin were variable (Table II). Evaluation of T cell function revealed normal responses to phytohemagglutinin and concanavalin A in the four patients that were examined. The numbers of peripheral T and B cells and the CD4/8 ratio were normal in four patients. Patient #7 demonstrated B-lymphocytopenia and an elevated CD4/8 ratio.

Diagnostic Tests for Juvenile Myelomonocytic Leukemia

Molecular analysis of *PTPN11*, *N-RAS*, and *K-RAS* genes (n = 7) and the c-*CBL* gene (n = 2) documented no mutations in any of the examined patients. *In vitro* GM-CSF hypersensitivity was performed in all patients but patient #1 and was positive only in patient #4.

Diagnostic Tests for Wiskott–Aldrich Syndrome

FCM analysis showed absent (n = 6) or reduced (n = 1) WASP expression in the lymphocytes, which led to the confirmation of a diagnosis of WAS (Table III). Mutations of *WASP* genes

TABLE I. Clinical Features of the Patients

Patient	1	2	3	4	5	6	7
Age at the detection of thrombocytopenia	At birth	At birth	At birth	At birth	1 month	4 months	2 months
Age at the onset of JMML like haematological features	1 month	3 months	1 month	1 month	1 month	4 months	2 months
Age at the onset of eczema	1 month	3 months	Soon after birth	3 months	1 month	3 months	2 months
Age at the onset of bloody diarrhoea	At birth	20 days	At birth	1 week	1 month	No	1 month
Hepatomegaly/splenomegaly (cm under the costal margin)	Yes (3)/no	Yes (3)/yes#	No/no	No/no	No/no	Yes (5)/yes (7.5)	Yes (6)/yes (6)
Infectious episodes before the diagnosis of WAS	CMV antigenemia	No episode	No episode	No episode	Fever of unknown origin	Otitis media	Adenovirus and Rotavirus in stool
Infectious episodes between the diagnosis of WAS and HSCT	No episode	No episode	No episode	No episode	Bacterial and RSV pneumonia Rotavirus gastroenteritis	Otitis media Anal abscess	CMV pneumonia
HSCT (age)	10 months	10 months	17 months	4 months	18 months	13 months	7 months
Donor/stem cell source	U-CBT	MSD-BMT	U-CBT	MSD-BMT	1 antigen MMUD-BMT	MUD-BMT	MUD-BMT
Survival (age at the time of the last follow-up)	Alive (6 years 5 months)	Alive (5 years 4 months)	Alive (4 years 8 months)	Alive (12 months)	Alive (1 year 9 months)	Alive (1 year 6 months)	Alive (1 year 7 months)

JMML, juvenile myelomonocytic leukaemia; WAS, Wiskott–Aldrich syndrome; RSV, respiratory syncytial virus; CMV, cytomegalovirus; # splenomegaly was noted only by ultrasound; HSCT, hematopoietic stem cell transplantation; U-CBT, unrelated cord blood transplantation; MSD-BMT, bone marrow transplantation from an HLA matched sibling donor; MUD-BMT, BMT from an HLA matched unrelated donor; MMUD-BMT, BMT from an HLA-mismatched unrelated donor.

TABLE II. Laboratory Findings Accompanying the Juvenile Myelomonocytic Leukaemia-Like Haematological Features

Patient	1	2	3	4	5	6	7
Peripheral blood							
WBC count ($\times 10^9$/L)	35.5–50.0	12.0–18.0	13.5–22.1	15.0	35.0–50.0	6.0–12.0	7.5
Monocyte count ($\times 10^9$/L)	8.9	1.0–1.5	8	2.3	1.1	1.0–1.5	1.3
Blasts (%)	3	2	2	4	2	0	1
Immature myeloid/erythroid cells	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes
Eosinophils (%)	3	12	4	7	2	5	2
Platelet count ($\times 10^9$/L)	44	40–90	31	24	53	11	26
MPV (fl)[a]	7.0	7.4	NE	6.9	7.5	NE	7.9
Platelet anisocytosis/giant platelets	Yes/Yes	Yes/Yes	Yes/Yes	Yes/No	Yes/Yes	Yes/Yes	Yes/Yes
Hb (g/dl)	8.9	8.0	9.2	6.1	11.6	9.5	8.0
Bone marrow							
Cellularity	Full[b]	Full	Full	Full	Full	Full	Full
M/E ratio	33	4	7	5.4	11	2	2
Blasts (%)	3.5	0.5	1	0	2	3.5	2
Karyotype	46,XY	46,XY	46,XY	46, XY	46, XY	46,XY	46,XY
Immunological examination							
Age at examination (months)	8	5	2	2	10	4	2/3/5
IgG (mg/dl)	2,554	468	638	102	792	3,780	1,170/2,120/2,070
IgM (mg/dl)	156	64	37	<5	33	353	122/244/156
IgA (mg/dl)	49	52	38	39	129	124	25/45.4/58.2
IgE (mg/dl)	494	368	89	8	16	1,330 (10 months)	258/693/7,995
LBT (PHA, ConA)	Normal	Normal	NE	NE	NE	Normal	Normal
CD4/8 ratio	Normal	Normal	NE	Normal	NE	Normal	Increased (7.0/22.2/1.1)

WBC, white blood cell; MPV, mean platelet volume; M/E myeloid-/erythroid-cells; LBT, lymphoblastic test; PHA, phytohemagglutinin; conA, concanavalin A; NE, not evaluated. [a]Normal range (7.2–11.7 fl). [b]The cellularity was high (full bone marrow), which was normal for infants.

varied between patients. In patient #1, sequencing of *WASP* cDNA identified five nucleotides (CCGGG) inserted at position c.387 in exon 4, causing a frameshift at codon 140 that gave rise to a premature stop signal at codon 262, as reported previously [20]. Patients #2 and #3 had previously known nonsense mutations in exon 1 and exon 4, which led to the absence of WASP expression and a moderate to severe clinical phenotype of WAS [4,30–32]. Patient #4 had a known deletion in intron 8, which cause a frameshift and absence of WASP expression [4,5]. Patient #5 had a known splice anomaly in intron 6, which reduced expression of WASP and led to a clinical phenotype of either XLP or WAS [4,32]. Patient #6 had known deletion in exon 1, which was associated with a classic WAS phenotype [33]. Patient #7 had a nonsense mutation in exon 1, which has not been previously described.

Clinical Course of Patients

Patient #1 received 6-MP to control leukocytosis. In other patients, the JMML-like features were stable until allogeneic hematopoietic stem cell transplantation (HSCT), which was performed at the age of 4–18 months. All patients are alive after HSCT at the time of the last follow-up (Table I). Graft failure was observed in patient #7, and a second HSCT is currently planned for this patient.

DISCUSSION

Although WASP is expressed ubiquitously in hematopoietic cells and although *in vitro* results suggest that WASP is involved in the proliferation and differentiation of all hematopoietic progenitors, overt defects are restricted to micro-thrombocytopenia and immune-dysfunction in classical WAS. We previously described a case of a male presenting with a clinical picture of JMML, in whom WAS was ultimately diagnosed (patient #1) [20]. These haematological abnormalities had not been previously reported in patients with WAS. Since then, we have encountered six additional patients with WAS who presented with similar clinical characteristics. Morphological features were not distinguishable from JMML. Moreover, normal MPV and the presence

TABLE III. Results of the Diagnostic Tests for Wiskott–Aldrich Syndrome

Patient	1	2	3	4	5	6	7
Age at examinations	8 months	4 months	4 months	3 months	8 months	4 months	3 months
WASP protein expression	Absence	Absence	Absence	Absence	Reduced	Absence	Absence
WASP mutation	Exon 4 c.387–421 ins 5nt	Exon 1 c.37C>T	Exon 4 c.424C>T	Intron 8 c.777+1_+4 delGTGA	Intron 6 c.559+5G>A	Exon1 c.31delG	Exon 1 c.C55>T
Mutation type	Insertion	Nonsense	Nonsense	Deletion	Splice anomaly	Deletion	Nonsense
Predicted protein change	Frameshift stop aa 262	R13X	Q142X	Frameshift stop aa 246	Frameshift stop aa 190	Frameshift stop aa 37	Q19X

of giant platelets in three and six patients, respectively, initially argued against a diagnosis of WAS, because micro-thrombocytes are known as a key diagnostic feature of WAS and XLP. The JMML-like features developed shortly after birth in all patients, before the full clinical picture of WAS become apparent. In our patients with JMML-like features, signs of immune defects were not present. Without recent advances in molecular diagnostic tests for WAS and JMML, it might otherwise be impossible to establish a diagnosis of WAS in these patients. Absent or reduced WASP expression by FCM–WASP and detection of *WASP* mutation ultimately led to a diagnosis of WAS. The mutations were distributed in different exons and introns, and there was no clustering. Thrombocytopenia since birth and some of the observed clinical features (e.g., atopic dermatitis-like eczema, persistent bloody stool, lack of splenomegaly) were unusual for JMML but were compatible with WAS.

The deregulated RAS signalling pathway plays a central role in the pathogenesis of JMML, and mutational analyses of *PTPN11*, *RAS*, and *c-CBL* genes located in the RAS signalling pathway have become important diagnostic tests. Mutations of one of these genes and a clinical diagnosis of NF1 can be found in more than 80% of patients with JMML. However, in up to 20% of patients without any molecular markers, a diagnosis of JMML relies on unspecific clinical and laboratory observations. We suggest that WAS should be considered within the differential diagnosis in male infants with clinical features of JMML if no mutations of the RAS signalling pathway can be detected. Importantly, clinicians should not exclude a diagnosis of WAS if the MPV is normal or if giant platelets are present. Rarely, patients with WAS can present with normal or large platelets [34,35].

The pathogenesis of JMML-like feature in these patients is unknown. There is no evidence that WASP is related to the RAS signalling pathway. The activation of this pathway does not seem to be a major cause of JMML-like features in our patients, because GM-CSF hypersensitivity was demonstrated only in one of six patients examined. Patients with WAS have an increased risk of viral infections. CMV, Epstein–Barr virus (EBV) and human herpes virus-6 (HHV-6) infections can mimic JMML in infants [36,37]. However, extensive screening failed to detect viral infections at the time, at which these patients presented with JMML-like features, except for patient #1, in whom CMV antigen was detected.

Leukocyte adhesion deficiency (LAD)-1 is a rare immunodeficiency caused by a mutation in the beta-2 integrin gene. The firm adhesion of leukocyte to the blood vessel wall is defective in LAD-1, which results in leukocytosis, mimicking JMML [38]. A defect of leukocyte adhesion due to abnormal integrin beta clustering has been described in the context of WAS [39]. A mechanism similar to that seen in LAD1 may be present in WAS with JMML-like features.

A recent report showed that WASP localizes to not only the cytoplasm but also to the nucleus and has a role in the transcriptional regulation at the chromatin level in lymphocytes [40]. Active *WASP* mutations, which cluster within the GTP-ase binding domain of WASP (L270P, S272P, and I294T), cause XLN and myelodysplasia [6,7]. Further, increased apoptosis associated with increased genomic instability in myeloid cells and lymphocytes has been described in the context of active *WASP* mutations [41,42]. Further research may identify new roles of WASP in transcriptional regulation and genomic stability in haematopoiesis, which may explain the JMML-like features, seen in WAS patients.

In conclusion, WAS should be considered in the differential diagnosis in male infants presenting with JMML-like features if no molecular markers of JMML can be demonstrated. A normal MPV and the presence of giant platelets do not exclude a diagnosis of WAS. Clinical information, such as bloody stool and eczema, may be helpful in pursuing a diagnosis of WAS in an infant with JMML like features.

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ORIGINAL ARTICLE

Excellent outcome of allogeneic bone marrow transplantation for Fanconi anemia using fludarabine-based reduced-intensity conditioning regimen

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Abstract Fanconi anemia (FA) is a disorder characterized by developmental anomalies, bone marrow failure and a predisposition to malignancy. It has recently been shown that hematopoietic stem cell transplantation using fludarabine (FLU)-based reduced-intensity conditioning is an efficient and quite safe therapeutic modality. We retrospectively analyzed the outcome of bone marrow transplantation (BMT) in eight patients with FA performed in two institutes between 2001 and 2011. There were seven females and one male with a median age at diagnosis = 4.5 years (range 2–12 years). The constitutional characteristics associated with FA, such as developmental anomalies, short stature and skin pigmentation, were absent in three of the patients. One patient showed myelodysplastic features at the time of BMT. All patients received BMT using FLU, cyclophosphamide (CY) and rabbit antithymocyte globulin (ATG) either from a related donor ($n = 4$) or an unrelated donor ($n = 4$). Acute graft-versus-host disease (GVHD) of grade I developed in one patient, while chronic GVHD was not observed in any patient. All patients are alive and achieved hematopoietic recovery at a median follow-up of 72 months (range 4–117 months). BMT using FLU/low-dose CY/ATG -based regimens regardless to the donor is a beneficial therapeutic approach for FA patients.

Keywords Fanconi anemia · Hematopoietic stem cell transplantation · Fludarabine

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Introduction

Fanconi anemia (FA) is a complex disorder characterized by developmental anomalies, early onset progressive bone marrow failure (BMF) and a tendency to develop hematological and non-hematological malignancies. The risk of FA patients to develop BMF and malignancies increases with progression of age, and the cumulative incidence by age of 40 years was 90 % for BMF and 30 % for hematologic and nonhematologic neoplasms [1–4]. Short stature and abnormal skin pigmentation are particularly common features found in patients with FA and a wide variety of congenital malformations has been described in 60–75 % of FA patients [3–5]. The incidence of FA in Japan was approximately 5–10 new cases diagnosed annually and hematological abnormalities usually manifested early in childhood at a median age of 7 years (range 0–31 years). Owing to the complexity of the disease and multisystem involvement, FA has a high mortality rate with a median age of death of 30 years [3–5]. Therefore, the study of FA holds a great promise for elucidation of the heterogeneity of this disorder in the future. Hematopoietic stem cell transplantation (HSCT) is the only curative therapy known so far for correcting the hematological manifestations in

Table 1 Clinical characteristics of 8 Fanconi anemia patients

No.	Male/female	Disease status at HSCT	Age at diagnosis (year)	Age at HSCT (year)	Karyotype	Chromosomal fragility test (gap and break/100 cells)	Short stature (<−1SD)	Skin pigmentation	Other anomalies
1	F	SAA	7	8	46,XX	114	No	No	No
2	F	SAA	3	5	46,XX	514	−2SD	Yes	Shortness of first finger
3	F	SAA	8	8	46,XX	193	No	No	No
4	F	SAA	12	13	46,XX	299	−1SD	Yes	No
5	M	SAA	2	7	46,XY	152	−3SD	No	Hyperdactylia
6	F	MDS (RAEB)	3	6	Other[a]	148	−1.5SD	No	Hyperdactylia
7	F	SAA	6	11	46,XX	159	No	No	No
8	F	SAA	3	5	46,XX	169	−2SD	Yes	Radius defect, esophageal atresia

SAA severe aplastic anemia, *MDS* myelodysplastic syndrome, *HSCT* hematopoietic stem cell transplantation, *RAEB* refractory anemia with excess blasts

[a] others; 46,XX,add(3)(q26),der(7)add(7)(p22)add(7)(q11)add(8)(q22)

FA patients. Initially, potential problems in designing HSCT conditioning regimens for patients with FA appeared due to the acquired hypersensitivity to DNA cross-linking or oxidative agents such as alkylating agents or ionizing radiation [6], but recently a significant progress has been achieved by using FLU-based reduced-intensity conditioning regimens that markedly improved the efficiency and safety of this procedure [7, 8]. Specifically, FLU-based reduced-intensity conditioning allogeneic HSCT resulted in reduction of regimen-related toxicity (RRT), superior engraftment and less graft-versus-host disease (GVHD), which in turn led to improvement of patients' survival. In this study, we aimed to investigate the effectiveness and safety profile of bone marrow transplantation (BMT) using FLU-based reduced-intensity conditioning regimens in eight patients with FA, who were transplanted from either a related donor (3 HLA-genetically matched and 1 HLA-A locus mismatched) or an unrelated donor (2 HLA-matched and 2 HLA-DRB1 mismatched).

Patients and methods

We retrospectively analyzed eight patients, who were diagnosed as having FA and received BMT at Nagoya University and Nagoya First Red Cross Hospital between 2001 and 2011. There were seven females and one male with a median age at diagnosis of 4.5 years (range 2–12 years) (Table 1). Clinical features suggestive of FA including low birth weight, short stature, hyperpigmented skin, radial abnormality and duplicated thumbs were defined in five out of eight patients, while three patients were asymptomatic. Diagnosis of FA was confirmed in all patients by a reliable cellular marker for FA cells and all of them showed a high incidence of chromosomal breaks and gaps, which indicated chromosomal instability by adding mitomycin C at a final concentration of 0.5 μM (Table 1). Seven patients suffered from severe aplastic anemia and one patient evolved to refractory anemia with excess of blasts (RAEB) with the emergence of cytogenetic abnormalities in the form of add(3)(q26) at the time of HSCT. All patients underwent allogeneic BMT at a median age of 7.5 years (range 5–13 years), and they were transplanted from either a related donor (3 HLA-genetically matched and 1 HLA-A locus mismatched) or an unrelated donor (2 HLA- matched and 2 HLA-DRB1 mismatched). All patients received a preparative regimen including a combination of fludarabine (FLU 120–180 mg/m^2), cyclophosphamide (CY 40 mg/kg) and rabbit anti-thymocyte globulin (ATG, Thymoglobulin, Genzyme, 5–10 mg/kg). Patients transplanted from unrelated donor received a total body irradiation (TBI)/total lymphoid irradiation (TLI) of 4–4.5 Gy, and patients transplanted from HLA-A locus mismatched related donor received 2 Gy. As GVHD prophylaxis, BM recipients from related donor received cyclosporine A (CyA) plus short-term methotrexate (MTX), while BM recipients from unrelated donor received tacrolimus (FK506) plus short-term MTX. Details on the donors' characteristics, conditioning regimen and GVHD prophylaxis are listed in Table 2.

Table 2 Hematopoietic stem cell transplantation for 8 Fanconi anemia patients

No.	Performance status	Transfusion before SCT	Conditioning	Donor	Concordance of HLA-serological typing	Transfused cell number ($\times 10^8$/kg)	GVHD prophylaxis
1	0	MAP 2u, PC10u	FLU (120 mg/m^2) + CY (40 mg/kg) + ATG (10 mg/kg)	Mother	6/6	3.9	CyA + short MTX
2	0	MAP 2u, PC10u	FLU (150 mg/m^2) + CY (40 mg/kg) + ATG (6 mg/kg)	Sister	6/6	7.4	CyA + short MTX
3	0	MAP 4u, PC 20u	FLU (180 mg/m^2) + CY (40 mg/kg) + ATG (10 mg/kg) + TBI (2 Gy)	Father	5/6 (mismatched A 1 locus)	3.0	CyA + short MTX
4	0	MAP 4u, PC 20u	FLU (150 mg/m^2) + CY (40 mg/kg) + ATG (5 mg/kg)	Brother	6/6	2.2	CyA + short MTX
5	1	MAP 21u, PC 120u	FLU(180 mg/m^2) + CY(40 mg/kg) + ATG(10 mg/kg) + TLI (4 Gy)	Unrelated	6/6	3.0	FK506 + short MTX
6	0	MAP 16u, PC100u	FLU (150 mg/m^2) + CY (40 mg/kg) + ATG (10 mg/kg) + TBI (4.5 Gy)	Unrelated	5/6 (mismatched DR 1 locus)	4.5	FK506 + short MTX
7	0	MAP 12u, PC120u	FLU (120 mg/m^2) + CY (40 mg/kg) + ATG (10 mg/kg) + TLI (4 Gy)	Unrelated	5/6 (mismatched DR 1 locus)	3.8	FK506 + short MTX
8	0	MAP 4u, PC 20u	FLU (150 mg/m^2) + CY (40 mg/kg) + ATG (10 mg/kg) + TBI (4.5 Gy)	Unrelated	6/6	4.6	FK506 + short MTX

SCT stem cell transplantation, *FLU* fludarabine, *CY* cyclophosphamide, *ATG* anti-thymocyte globulin (rabbit ATG, Thymoglobulin), *TBI* total body irradiation, *TLI* total lymphoid irradiation, *CyA + short MTX* cyclosporine plus short-term methotrexate

Results

The median transfused nucleated cell number was 3.8 × 10^8/kg (range 2.2 to 7.4 × 10^8/kg) and all patients achieved sustained engraftment; the median time to neutrophil (>500), platelet (>50,000/ml) and reticulocyte (>10‰) recovery was 15.5, 20.5 and 21.5 days, respectively. Among eight patients enrolled in this study with a median survival period of 72 months (range 4–117 months), acute GVHD grade I was detected in one patient, whereas chronic GVHD was not found in any patient. Two patients experienced hepatic dysfunction and one patient had gastric hemorrhage as regimen-related toxicities (grade 1 according to the National Cancer Institution-Common Toxicity Criteria, NCI-CTC Version 4.0). Three patients exhibited febrile neutropenia and one of them showed disseminated fungal infection (grade 4 according to NCI-CTC) complicated by development of a renal abscess that showed complete remission after amphotericin B treatment. Cytomegalovirus (CMV)-polymerase chain reaction (PCR) and CMV-pp65 antigen detection were performed on a weekly basis for identification of CMV infection. We found four out of eight patients showed CMV reactivation without clinical symptom and they were treated with ganciclovir and foscavir. One patient suffered from hemorrhagic cystitis and lymphoproliferative disorder (LPD) due to BK virus and Epstein–Barr virus (EBV) reactivation, respectively. This patient was completely cured from the EBV–LPD after successful treatment with rituximab. Otherwise, no patients developed veno-occlusive disease (VOD) or thrombotic microangiopathy (TMA) (Table 3). Over the total length of the follow-up period, no patients showed secondary bone marrow failure and/or malignancies with a median follow-up of 72 months (range 4–117 months).

Discussion

Through a retrospective analysis of the medical records of eight pediatric patients with FA, who received FLU-based reduced-intensity conditioning allogeneic HSCT to evaluate their outcome, we found that all patients achieved favorable outcome using this procedure and the type of the donor did not significantly influence the clinical outcome. In previous studies, it has been proved that the use of alkylating agents and radiation therapy for FA patients was harmful. The impact of lower irradiation dose on immune recovery and risk of malignancy remains a matter of debate and a longer follow-up period is needed. However, it was reported that TBI 300 cGy was the lowest possible irradiation dose in the context of FLU/CY-based regimen; other

Table 3 Results of hematopoietic stem cell transplantation for 8 Fanconi anemia patients

No.	Days of engraftment	Acute GVHD	Chronic GVHD	RRT	VOD	TMA	CMV Ag positivity and treatment	Other virus related disease	FN	Fungal infection	Overall survival (Mo)
1	19	0	0	No	No	No	Day 35, GCV	No	No	No	114
2	15	I	0	No	No	No	Day 13, GCV + FCV	No	Yes	No	41
3	19	0	0	Liver dysfunction (grade 1)	No	No	Day 30, GCV	No	Yes	No	17
4	14	0	0	Gastric hemorrhage (grade 1)	No	No	No	No	No	No	4
5	17	0	0	No	No	No	Day 48, GCV	No	No	No	117
6	19	0	0	No	No	No	No	No	No	Disseminated fungal infection, renal abscess (grade 4)	99
7	15	0	0	Diarrhea (grade 1), liver dysfunction (grade 1)	No	No	No	Hemorrhagic cystitis due to BKV (grade 2) and LPD due to EBV	Yes	No	72
8	19	0	0	No	No	No	No	No	No	No	26

RRT grade was determined by National Cancer Institute-Common Toxicity Criteria (NCI-CTC Ver 4.0)

GVHD graft-versus-host disease, *RRT* regimen-related toxicity, *VOD* veno-occlusive disease, *TMA* thrombotic microangiopathy, *CMV Ag* AMV antigenemia (pp65), *GCV* ganciclovir, *FCV* foscavir, *BKV* BK virus, *EBV*, Epstein–Barr virus, *FN* febrile neutropenia

successful trials of eliminating irradiation in the conditioning regimens even in recipients of BMT from unrelated donor have been achieved [8–11]. Subsequently, alternative regimens have been developed to reduce the potential risk of irradiation and GVHD using a non-irradiation based preparative therapy including a low-dose CY, FLU and ATG [6–8, 10]. Here, we showed that BMT using FLU-based reduced-intensity conditioning regimens led to improvement of the outcome for FA patients regardless of the donor type. In line with our findings, other investigators reported that HSCT using FLU-containing regimens was associated with a better outcome even for recipients of allograft from unrelated donor with stable engraftment and minimal toxicity, whereas adding ATG to the conditioning regimen contributed to decreasing the incidence of GVHD [7, 8, 11, 12]. On the other hand, it was reported that bone marrow recipients from unrelated donor showed less successful transfusion rate with high percentage of graft failure and RRT compared to bone marrow recipients from related donor [13]. In our cohort, FLU/ATG/low-dose CY-based conditioning regimen was tolerable and efficient for FA patients even for BM recipient from unrelated (HLA-1 locus mismatched) donor, but some patients developed virus reactivation such as CMV, BKV and EBV. To overcome virus reactivation that might occur as an adverse event following the use of ATG-containing regimen, it is better to decrease the ATG dose from 10 to 5 mg/kg. FA patients are more prone to cancer development such as squamous cell carcinomas with special predilection sites (esophagus, head and neck) [4, 14, 15]. FLU-containing regimens were employed in a limited number of FA patients, making it difficult to speculate on its implications on cancer progression. Three out of four patients received HSCT from HLA-matched related donor without irradiation and they showed successful and excellent outcomes. Therefore, it is important to consider the use of the conditioning regimen without irradiation for this group of patients. Several studies have demonstrated that TBI with 300–450 cGY is needed for consistent engraftment in recipients from unrelated donor [12, 16]. We would like to emphasize that reduction of irradiation dose in the conditioning regimens even for recipients of HSCT from unrelated donor is an important target that will improve the patient's quality of life by reducing late effects, particularly the risk of malignancy. Noteworthy, in our series three out of eight patients were asymptomatic. Although our findings were consistent with other investigators' results [3–5], early diagnosis and optimal timing of transplant in

asymptomatic FA patients is challenging and it is of utmost importance to confirm FA diagnosis in those patients who might be misdiagnosed with acquired aplastic anemia. In conclusion, the identification of asymptomatic FA patients requires careful consideration by testing for cross-linker hypersensitivity that provides a reliable cellular marker for FA diagnosis. HSCT using FLU/low-dose CY/ATG-based regimen is beneficial and could be a promising therapeutic approach for FA patients regardless of the donor type with favorable clinical outcome.

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Conflict of interest The authors declare no conflict of interest.

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Somatic mosaicism for oncogenic *NRAS* mutations in juvenile myelomonocytic leukemia

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Brief report

Somatic mosaicism for oncogenic *NRAS* mutations in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML. In the present study, we report 2 patients with somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S) associated with the development of JMML. The mutated allele frequencies quantified by pyrosequencing were various and ranged from 3%-50% in BM and other somatic cells (ie, buccal smear cells, hair bulbs, or nails). Both patients experienced spontaneous improvement of clinical symptoms and leukocytosis due to JMML without hematopoietic stem cell transplantation. These patients are the first reported to have somatic mosaicism for oncogenic *NRAS* mutations. The clinical course of these patients suggests that *NRAS* mosaicism may be associated with a mild disease phenotype in JMML. (*Blood*. 2012;120(7):1485-1488)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML.[1-3] The term "somatic mosaicism" is defined as the presence of multiple populations of cells with distinct genotypes in one person whose developmental lineages trace back to a single fertilized egg.[4] Somatic mosaicism of various genes, including some oncogenes, has been implicated in many diseases. For example, somatic mosaicism for *HRAS* mutations is found in patients with Costello syndrome.[5-7] Whereas germline mutations in causative genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in JMML patients,[3,8-11] the presence of somatic mosaicism for these genes has never been reported. In the present study, we describe 2 cases of JMML in which the patients display somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S).

Study design

Written informed consent for sample collection was obtained from the patients' parents in accordance with the Declaration of Helsinki, and molecular analysis of the mutational status was approved by the ethics committee of the Nagoya University Graduate School of Medicine (Nagoya, Japan).

Patient 1. A 10-month-old boy had hepatosplenomegaly and leukocytosis (72.1 × 10^9/L) with monocytosis (13.3 × 10^9/L; Table 1). The patient's BM contained 7% blasts with myeloid hyperplasia. Cytogenetic analysis revealed a normal karyotype and colony assay of BM mononuclear cells (BM-MNCs) showed spontaneous colony formation but GM-CSF hypersensitivity assay was not tested. The diagnostic criteria for JMML, as developed by the European Working Group on Myelodysplastic Syndrome in Childhood, was fulfilled,[12] and the patient was treated with IFN-α and 6-mercaptopurine. His clinical and laboratory findings gradually resolved without hematopoietic stem cell transplantation. However, 11 years after the diagnosis of JMML, the patient developed thrombocytopenia (7.6 × 10^9/L) and BM findings showed trilineage dysplasia with low blast count compatible with refractory anemia. The patient did not have any physiologic abnormalities, such as facial deformity, and there was no family history of malignancy or congenital abnormalities.

Patient 2. A 10-month-old boy had anemia, hepatosplenomegaly, and leukocytosis (31.8 × 10^9/L) with monocytosis (6.4 × 10^9/L; Table 1). The patient's BM exhibited myeloid hyperplasia and granulocytic dysplasia with 5% blasts. Cytogenetic

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Table 1. Patient characteristics

	Patient 1	Patient 2
Age, mo	10	10
Sex	Male	Male
Liver, cm	12	5
Spleen, cm	8	10
WBCs, $\times 10^9$/L	72.1	31.8
Monocytes, %	18.5	20
Blasts, %	4	2
Hb, g/dL	8.9	5.4
Platelets, $\times 10^9$/L	59	100
HbF, %	2.1	1.7
BM blasts, %	7	5
Karyotype	46,XY [20/20]	46,XY [20/20]
Monosomy 7 (FISH)	Negative	Negative
Spontaneous colony formation	Positive	Positive
Gene mutation	*NRAS*, G12D 35G > A	*NRAS*, G12S 34G > A
Treatment	IFN-α-2b, 6-MP	None
Observation period, mo	231	103
Outcome	Alive	Alive
Fraction of mutant alleles, % (pyrosequencing)		
Nail (whole)	24	12.5 (average)
Nail (left hand)	ND	26
Nail (right hand)	ND	13
Nail (left foot)	ND	8
Nail (right foot)	ND	3
Buccal smear cells	43	21
Hair bulbs	5	ND
Family studies		
Father	Wild-type	Wild-type
Mother	Wild-type	Wild-type
Sibling	ND	Wild-type

Hb indicates hemoglobin; 6-MP, 6-mercaptopurine; and ND, not done.

analysis revealed a normal karyotype. Colony assay of BM-MNCs showed spontaneous colony formation and GM-CSF hypersensitivity. Although the diagnostic criteria for JMML were fulfilled,[12] the patient's clinical symptoms and leukocytosis improved spontaneously within a few months without cytotoxic therapy or hematopoietic stem cell transplantation. The patient has remained healthy and has experienced no hematologic or physiologic abnormalities. The most recent follow-up examination was conducted when the patient was 8 years of age.

Detailed methods for experiments are described in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Results and discussion

DNA sequencing for JMML-associated genes (ie, *NRAS*, *KRAS*, *PTPN11*, and *CBL*) was performed (Figure 1 and Table 1). In Patient 1, the *NRAS* G12D mutation was identified in BM-MNCs at the time of diagnosis of both JMML and MDS. We identified the same G12D mutation in DNA derived from buccal smear cells and nails of both hands; however, the sequence profile of the nails showed a low signal for the mutant allele compared with signal of blood cells. In Patient 2, the *NRAS* G12S mutation was identified in DNA from BM-MNCs, buccal smear cells, and nails of the left hand. However, the sequence profiles of buccal smear cells and nails of the left hand showed a low signal for the mutant variant. No mutation was detected in DNA from the PB-MNCs of the patient's parents or sibling.

We used pyrosequencing to quantify the fraction of mutated alleles in DNA samples from different somatic tissues (Figure 1 and Table 1). The frequency of mutated alleles varied by tissue type as follows. For Patient 1: BM-MNCs, 50%; nails, 24%; buccal smear cells, 43%; and hair bulbs, 5%. For Patient 2: buccal smear cells, 21%; nails of left hand, 26%; nails of right hand, 13%; nails of left foot, 8%; and nails of right foot, 3%. We cloned the PCR product of *NRAS* exon 2 from the nails of Patient 1 and picked up 15 clones. The clones were sequenced. Four of the 15 clones (27%) contained the mutant allele, which is consistent with the results of pyrosequencing analysis (24% mutant allele). Because the confirmed detection level by pyrosequencing technique was above 5%, results with a low percentage (< 5%) of mutant allele (ie, hair bulbs in Patient 1) should be interpreted with caution.[13,14]

We diagnosed 2 JMML patients as having somatic mosaicism of *NRAS* mutations: G12D for Patient 1 and G12S for Patient 2. The diagnoses were based on negative familial studies and mutational allele quantification analyses that showed diversity in the chimeric mutational status of different somatic tissues. Although DNA from buccal smear cells might be contaminated with WBCs, we also identified mutations in DNA from the nail tissue, which is known to be a good biologic material without contamination from hematopoietic cells, in both patients. These data suggest that a portion of the *NRAS*-mutated somatic cells were derived from one cell that acquired the mutation at a very early developmental stage. Although both somatic and germline mutations of RAS pathway genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in some JMML patients,[3,8-11] somatic mosaicism for these genes has never been reported. To the best of our knowledge, the present study is

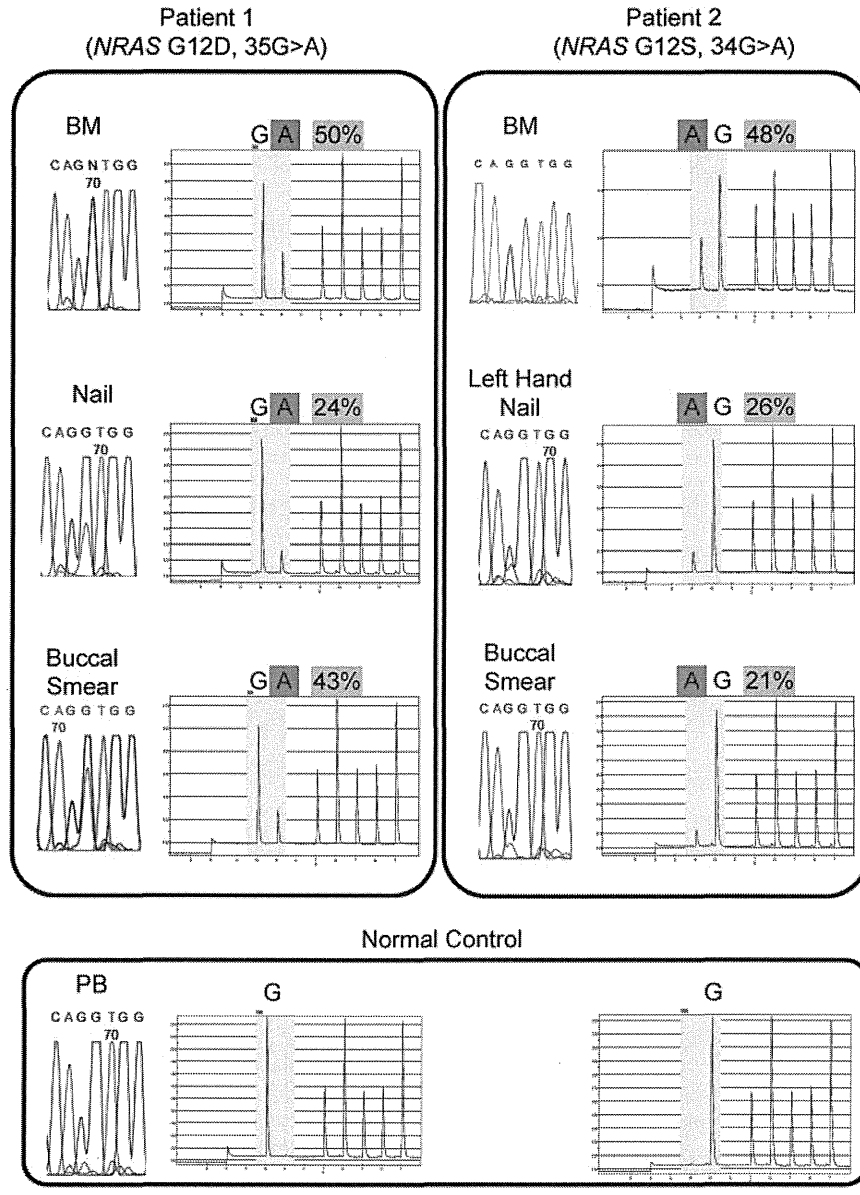

Figure 1. Direct sequencing and quantitative mutational analysis of *NRAS* in JMML patients. *NRAS* mutations are detected by direct sequencing and quantified by pyrosequencing. Direct sequencing identified oncogenic *NRAS* mutations: for Patient 1, G12D, 35G > A; for Patient 2, G12S, 34G > A) in BM-MNCs at diagnosis of JMML and in the nails and buccal smear cells. Quantification by pyrosequencing revealed that the fractions of mutated allele varied among different tissue types. For Patient 1: BM, 50%; nail, 24%; and buccal smear, 43%. For Patient 2: BM, 48%; left-hand nail, 26%; and buccal smear, 21%.

the first report of JMML patients with somatic mosaicism of mutations in RAS pathway genes.

Germline RAS pathway mutations are often associated with dysmorphic features similar to Noonan syndrome or its associated diseases. Correspondingly, JMML patients with germline *NRAS* or *CBL* mutations exhibit characteristic dysmorphic features.[3,10] Although our patients did not show any dysmorphic or developmental abnormalities, they should receive careful medical follow-up, especially for the occurrence of other cancers, because of the oncogenic nature of the mutations.

In general, JMML is a rapidly fatal disorder if left untreated.[8] However, recent clinical genotype-phenotype analyses have revealed heterogeneity in their clinical course. We and other researchers have reported that patients with *PTPN11* mutations have a worse prognosis than patients with other gene mutations, including *NRAS* and *KRAS*.[15,16] Both of the JMML patients in the present study with somatic mosaicism of oncogenic *NRAS* mutations have had a mild and self-limiting clinical course. We analyzed nails of other 3 JMML patients with RAS mutations who experienced aggressive clinical course and none showed somatic mosaicism (data not shown). In analogy to the mild phenotype of JMML patients with germline mutations in *PTPN11*, we speculate that JMML patients with somatic mosaicism of RAS genes might have a mild clinical course. We are planning to confirm these observations in larger cohort.

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Authorship

Contribution: S.D. and H.M. designed and conducted the research, analyzed the data, and wrote the manuscript; A.S., M.M.-E., M. Sato, H.K., A.K., M. Sotomatsu, and Y.H. treated the patients; Y.T., Y.F.-H., K.Y., H.H., H.K., N.Y., H.S., A.N., X.W., O.I., Y.X.,

N.N., M.T., A.H., and K.K. conducted the research; and S.K. designed the research, analyzed the data, and wrote the manuscript.

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Wnt3a stimulates maturation of impaired neutrophils developed from severe congenital neutropenia patient-derived pluripotent stem cells

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The derivation of induced pluripotent stem (iPS) cells from individuals of genetic disorders offers new opportunities for basic research into these diseases and the development of therapeutic compounds. Severe congenital neutropenia (SCN) is a serious disorder characterized by severe neutropenia at birth. SCN is associated with heterozygous mutations in the neutrophil elastase [elastase, neutrophil-expressed (ELANE)] gene, but the mechanisms that disrupt neutrophil development have not yet been clarified because of the current lack of an appropriate disease model. Here, we generated iPS cells from an individual with SCN (SCN-iPS cells). Granulopoiesis from SCN-iPS cells revealed neutrophil maturation arrest and little sensitivity to granulocyte-colony stimulating factor, reflecting a disease status of SCN. Molecular analysis of the granulopoiesis from the SCN-iPS cells vs. control iPS cells showed reduced expression of genes related to the wingless-type mmtv integration site family, member 3a (Wnt3a)/β-catenin pathway [e.g., lymphoid enhancer-binding factor 1], whereas Wnt3a administration induced elevation lymphoid enhancer-binding factor 1-expression and the maturation of SCN-iPS cell-derived neutrophils. These results indicate that SCN-iPS cells provide a useful disease model for SCN, and the activation of the Wnt3a/β-catenin pathway may offer a novel therapy for SCN with ELANE mutation.

apoptosis | unfolded protein response | SCN disease model

Severe congenital neutropenia (SCN) is a heterogeneous bone marrow (BM) failure syndrome characterized by severe neutropenia at birth, leading to recurrent infections by bacteria or fungi (1). SCN patients reveal an arrest in neutrophil differentiation in the BM at the promyelocyte or myelocyte stage (1), as well as a propensity to develop myelodysplastic syndrome and acute myeloid leukemia (2). Current treatment by high-dose granulocyte-colony stimulating factor (G-CSF) administration induces an increase in the number of mature neutrophils in the peripheral blood of most SCN patients (3). Although this treatment is curative for the severe infections, there is a concern that high-dose G-CSF may increase the risk of hematologic malignancy in these individuals (4).

Several genetic mutations have been identified in SCN patients. Approximately 50% of autosomal-dominant SCN cases were shown to have various heterozygous mutations in the gene encoding neutrophil elastase [elastase, neutrophil-expressed (ELANE)] (5, 6), a monomeric, 218-amino acid (25 kDa) chymotryptic serine protease (7) that is synthesized during the early stages of primary granule production in promyelocytes (8, 9). However, the mechanism(s) causing impaired neutrophil maturation in SCN patients remains unclear due to the current lack of an appropriate disease model.

Results and Discussion

In the present study, we generated induced pluripotent stem (iPS) cells from the BM cells obtained from an SCN patient with a heterologous ELANE gene mutation (exon 5, 707 region, C194X) (SCN-iPS cells) to provide the basis for an SCN disease model. The patient who donated BM cells recurrently suffered from severe infections without exogenous G-CSF administration, but the G-CSF administration once a week prevented his repeated infection. The SCN-iPS cells continued to show embryonic stem cell morphology after >20 passages and also expressed pluripotent markers (Fig. S1*A*). The silencing of exogenous genes and the capability to differentiate into three germ layers by teratoma formation were confirmed for each of the three SCN-iPS cell clones (Fig. S1 *B* and *C*). Furthermore, the same ELANE gene mutation that was present in the patient persisted in the SCN-iPS cells (Fig. S1*D*). The SCN-iPS cells, as well as control iPS cells that were generated from healthy donors, had the normal karyotype (Fig. S1*E*) (10, 11) and no mutations in the mutation-sensitive region of the G-CSF receptor gene (12).

We first compared the hematopoietic differentiation from SCN-iPS cells with that from control iPS cells that were generated from healthy donors. SCN-iPS and control iPS cells were cocultured with a 15-Gy-irradiated murine stromal cell line (the AGM-S3 cell line), as reported (13). After 12 d, the cocultured cells were harvested, and the $CD34^+$ cells separated from these cells (SCN-iPS–$CD34^+$ and control iPS–$CD34^+$ cells, respectively) were cultured in a hematopoietic colony assay by using a cytokine mixture (*Materials and Methods*). The number and size of the erythroid (E) and mixed-lineage (Mix) colonies derived from SCN-iPS–$CD34^+$ cells (1×10^4 cells) were nearly identical to those of the corresponding colonies derived from control iPS–$CD34^+$ cells (E colonies: SCN-iPS cells, 11.0 ± 3.0, and control iPS cells, 11.4 ± 3.9; Mix colonies: SCN-iPS cells, 25.1 ± 7.2, and control iPS cells, 17.4 ± 4.0) (Fig. 1 *B* and *C* and Fig. S2 *A* and *B*). However, the number of myeloid colonies derived from SCN-iPS–$CD34^+$ vs. control iPS–$CD34^+$ cells was significantly lower (SCN-iPS cells, 47.4 ± 19.5; control iPS cells, 127.8 ± 17.9; $P < 0.01$), and the size of the colonies was also smaller (Fig. 1 *A*

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