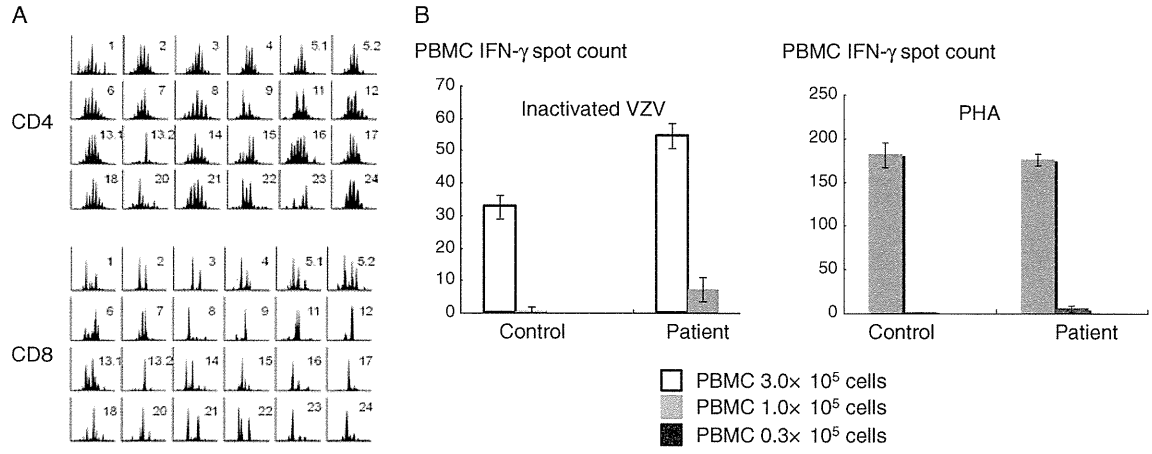

Fig. 3 Functional evaluation of T cells at 9 years old. **a** CDR3 spectratyping of the TCRVβ chain. Each TCRVβ fragment was amplified from cDNA with one of 24Vβ-specific primers (each Vβ chain is indicated). The size distribution of the PCR products was determined by an automated sequencer and GeneScan software. The CDR3 size distribution in CD4+ and CD8+ T cells from the patient is shown. **b** Elispot analysis of IFN-γ production as a measure of T cell function. (LEFT) Varicella-specific immune response to varicella zoster (VZV) antigen *in vitro*. Patient and control (from a healthy with a previous history of varicella infection) PBMCs ($0.3\text{-}3\times10^5$) were stimulated with inactivated VZV antigen or (RIGHT) PHA. Data are shown as mean ± SD

demonstrated that the patient maintained a certain level of T cell immunity for over a decade, despite the fact that the supply of fresh T cells from the thymus was limited and the patient suffered from generalized warts. Further follow up is required to determine if the patient can continue to maintain long-term T cell immunity.

In conclusion, this study indicates that it is critical to determine the NK cell number to avoid overlooking reversion mosaicism of SCID-X1. In addition, it has been shown that a number of *IL2RG* gene reversions can restore T cell functions and maintain T cell immunity against viral infection for at least 14 years.

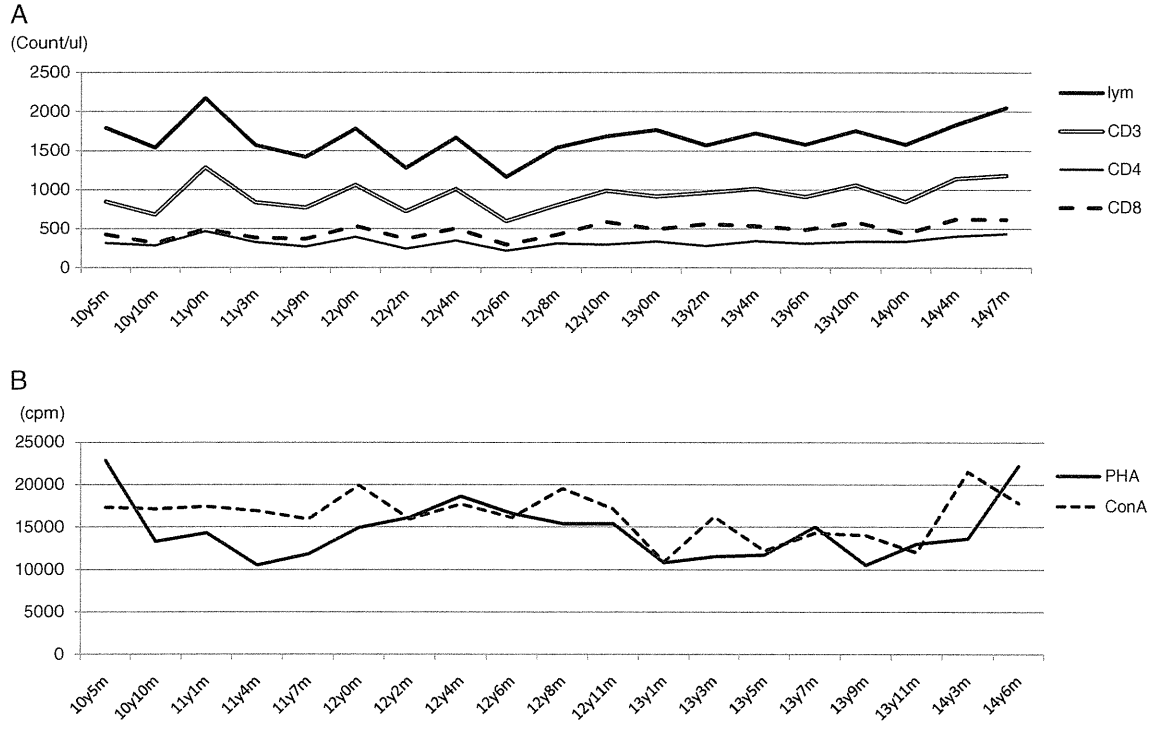

Fig. 4 Long term evaluation of T cell number and mitogen-induced proliferative response. **a** Absolute counts (per μl) of total lymphocytes (lym), CD3+ cells, CD4+ cells and CD8+ cells were measured for 4 years. **b** T cell proliferation in response to PHA (*solid line*) and Con A (*dotted line*). Healthy control values for PHA range from 20,500 to 56,800 cpm and for Con A from 20,500 to 65,700 cpm

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Authors' contributions T.K. performed experiments and wrote the paper. M.S. and Ka.I. performed experiments. R.N. designed the research, wrote the paper and analyzed data. Y.T. wrote the paper and analyzed data. T.M. treated the patient and analyzed data. S.O., Y.M., N.N., Ko.I, S.N., T.W. and A.Y. performed experiments and discussed the research. T.H. and T.N. designed the research.

Conflict of Interests The authors declare no competing financial interests.

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Successful bone marrow transplantation with reduced intensity conditioning in a patient with delayed-onset adenosine deaminase deficiency

Kanegane H, Taneichi H, Nomura K, Wada T, Yachie A, Imai K, Ariga T, Santisteban I, Hershfield MS, Miyawaki T. Successful bone marrow transplantation with reduced intensity conditioning in a patient with delayed-onset adenosine deaminase deficiency.

Abstract: In this case report, we describe successful BMT with RIC in a patient with delayed-onset ADA deficiency. A three-yr-old Japanese boy was diagnosed with delayed-onset ADA deficiency because of recurrent bronchitis, bronchiectasia, and lymphopenia. In addition, autoimmune thyroiditis and neutropenia were present. At four yr of age, he underwent BMT with a RIC regimen, including busulfan and fludarabine, from an HLA-identical healthy sister. Engraftment after BMT was uneventful without GVHD. Decreased ADA levels in blood immediately increased following BMT, and the patient was disease-free 13 months after BMT. These results suggest that BMT with RIC may sufficiently restore immune regulation in delayed-onset ADA deficiency. A longer follow-up period is needed to confirm these observations.

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Key words: adenosine deaminase deficiency – delayed-onset – bone marrow transplantation – reduced intensity conditioning

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ADA deficiency is a disorder of purine metabolism, which results in abnormalities in immune system development and function (1, 2). A majority of ADA deficiency cases indicate SCID during infancy; however, approximately 15% of ADA-deficient patients present with symptoms after infancy, which is referred to as a delayed- or late-onset type. Patients with delayed-onset ADA deficiency exhibit variable clinical symptoms, including bacterial infections and autoimmune manifestations. Allogeneic hematopoietic stem cell transplantation has long been a gold standard for the treatment of ADA-SCID; however, two other second-line options are available for ADA-SCID: Enzyme replacement therapy with PEG-ADA and hematopoietic stem cell gene therapy (3). The treatment of choice for delayed-onset ADA deficiency remains unclear because of

Abbreviation: ADA, adenosine deaminase; BMT, bone marrow transplantation; dAXP, deoxyadenosine nucleotides; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; PEG, polyethylene-glycosylated; PEG-ADA, polyethylene-glycosylated bovine ADA; RIC, reduced intensity conditioning; SCID, severe combined immunodeficiency; sjKRECs, signal joint κ-deleting recombination excision circles; TCR, T-cell receptor; TRECs, T-cell receptor excision circles.

the clinical variety. We report on a four-yr-old Japanese boy with delayed-onset ADA deficiency who underwent BMT with RIC from a HLA-identical healthy sister.

Case report

The patient was previously described (4). He is a boy who was admitted to our hospital at three yr of age for the investigation of recurrent infectious episodes. The patient did not have a neurological deficit. Laboratory data revealed neutropenia (600/μL), lymphocytopenia (580/μL), elevated C-reactive protein (7.43 mg/dL; normal, <0.29 mg/dL) and elevated thyroid-stimulating hormone (133 μIU/mL; normal, 0.35–3.73 μIU/mL). Anti-neutrophil, anti-nuclear, anti-thyroglobulin, and anti-thyroid peroxidase antibodies were positive, indicating that autoimmune neutropenia and thyroiditis were present. Chest computed tomography disclosed bronchiectasia. An immunological study indicated hypergammaglobulinemia, but a low percentage of IgG2 subclass antibodies (5.41%; normal, 20–30%) was obtained. The lymphocyte subsets revealed an expansion of the $CD45RO^+$ (memory) populations of $CD4^+$ and $CD8^+$ T cells (74.8% and 39.6%, respectively) and an extremely reduced number of $CD20^+$ B cells (0.2%). TRECs and signal joint κ-deleting recombination excision circles (sjKRECs) were quantified by real-time PCR as previously described (5, 6) and were undetectable. Flow cytometry analysis of the TCR Vβ repertoire was performed as described previously (7), and the analysis revealed an extremely skewed pattern in $CD8^+$ T cells but not in $CD4^+$ T cells (Fig. 1). Therefore, the patient was clinically presumed to have a combined immunodeficiency with autoimmune manifestations, possibly indicating delayed-onset ADA deficiency. The ADA and dAXP levels in whole blood were measured using the extracts of dried blood spots (8). ADA was found to be decreased (1.0 μmol/h/mg protein; normal, 26.4 $\pm$ 10.0 μmol/h/mg protein), and %dAXP increased to 10.8% (normal $<$ 1%). These data led to a diagnosis of ADA deficiency. An analysis of the *ADA* gene disclosed that the patient had compound heterozygous mutations (R156C and V177M). This genotype is compatible with delayed-onset ADA deficiency (9, 10).

The patient was treated with intravenous immunoglobulin replacement therapy, and oral administration of trimethoprim–sulfamethoxazole, acyclovir, and levothyroxine. He was nearly free from infections; however, his serum immunoglobulin levels gradually decreased. We identified his healthy sister as an HLA-identical donor with no mutation in the *ADA* gene. At the age of four yr, the patient underwent BMT. He was treated with conditioning, which consisted of fludarabine (30 mg/m^2/day $\times$ six days, days -7 to -2) and intravenous reduced dose busulfan (4.4 mg/kg/day $\times$ two days, days -3 to -2). The patient received 6.9×10^8 nucleated cells/kg containing 3.1×10^6 $CD34^+$ cells/kg to achieve rapid engraftment. Cyclosporin A was used as GVHD prophylaxis. The post-transplant clinical course was without major complications, and no signs of acute GVHD were observed. The patient did not receive blood transfusion, and engraftments of neutrophils (>500/μL) and thrombocytes ($>50\,000$/μL) were achieved at days 18 and 35, respectively. Cyclosporin A was stopped at day 46. The patient is currently well and has not suffered from any major infectious episodes. The patient received levothyroxine at a low dose; however, anti-thyroglobulin and anti-thyroid peroxidase antibodies became negative. The patient went off immunoglobulin replacement therapy 11 months after BMT.

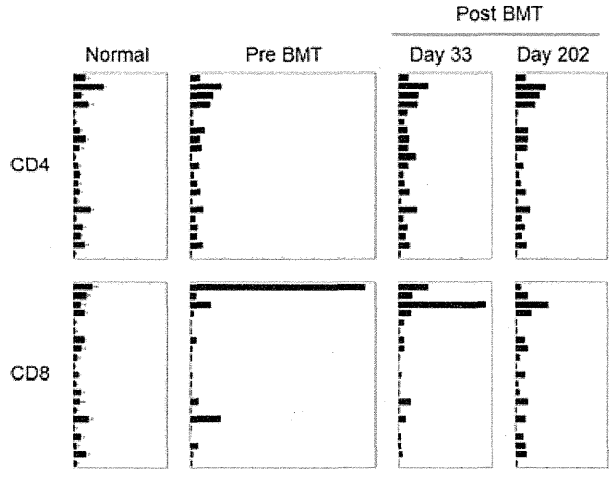

Fig. 1. TCR Vβ repertoire in the $CD4^+$ and $CD8^+$ T cells that were analyzed pre-BMT and post-BMT on days 23 and 202. The TCR Vβ repertoire was analyzed by flow cytometry as previously described (7).

Donor engraftment was evaluated by PCR amplification of the microsatellite marker D8S1179. Donor engraftment in granulocytes and B cells was observed at days 33 and 83, respectively. Complete donor engraftment in whole cells was achieved at day 323 (Table 1). Consistent with high chimerism, the patient exhibited a rapid increase in ADA activity and fast metabolic detoxification by day 83 (Table 2). In addition, immunological studies indicated rapid reconstitution of the lymphocyte subpopulation, and B cells increased to a normal level (305/μL;

Table 1. Engraftment of donor cells in different cell lineages

	Donor cell engraftment (%)				
Post-BMT	Whole blood	Lymphocytes	T cells	B cells	Granulocytes
Day 12	8.5	NA	14.2	NA	0
Day 33	50.7	33.0	NA	NA	100.0
Day 83	80.5	NA	16.5	100.0	100.0
Day 323	>95.0	NA	>95.0	100.0	100.0

NA, not applicable.
Analyses of donor cell engraftment according to a chimerism assay in the peripheral blood of the patient at different time points after BMT.

Table 2. ADA activity in the whole blood of the patient

Samples	ADA (μmol/h/mg protein)	%dAXP
Pre-BMT	1.0	10.8
Day 25	8.7	1.1
Day 83	33.7	0.0
ADA-SCID	0.38 ± 0.5	50.3 ± 18.0
Normal levels	26.4 ± 10.0	<1

The data are from the analyses of the extracts of dried blood spots.

age-matched control, 278–922/μL) at day 97 (Fig. 2). TREC and sjKREC levels reached normal levels at days 83 and 202, respectively (Table 3). The CD45RO$^+$ (memory) populations of CD4$^+$ T cells decreased to a normal range (21.9 ± 4.4%) soon after BMT. Sequential TCR Vβ repertoire analyses revealed that the polyclonal patterns in CD4$^+$ T cells were consistent after BMT, and the extremely skewed pattern in CD8$^+$ T cells had improved by day 202 (Fig. 1).

Discussion

ADA-SCID is a complex immune and metabolic disorder that results from a lack of ADA, which is a key enzyme in purine metabolism. Patients with ADA-SCID have recurrent and severe infections, growth retardation and organ failure. The first treatment of choice is BMT from an HLA-identical sibling donor, if available, followed by treatments for other forms of SCID. Second-line treatments for patients without an HLA-identical donor include enzyme replacement therapy with PEG-ADA, matched unrelated donor hematopoietic stem cell transplantation and hematopoietic stem cell gene therapy (3). Although the treatment strategy for ADA-SCID is well-established, treatment for delayed-onset ADA deficiency is not standardized because of the various clinical conditions. In this study, an HLA-identical healthy sibling donor was available, and we selected BMT from this donor to treat the patient. In cases of ADA-SCID, BMT from an HLA-identical donor is undertaken without a preparative conditioning regimen. The largest series of SCID patients from the European SCETIDE database included 475 patients (11). Of these patients, 51 patients with ADA-SCID had a three-yr survival rate of 81% for HLA-matched transplantation, but 29% for HLA-mismatched transplantation. A recently published cohort study demonstrated that hematopoietic stem cell transplantation in patients with SCID, including ADA deficiency, resulted in engraftment and long-term survival for the majority of patients with or without conditioning (12). However, transplantation without conditioning may result in partial donor engraftment, causing reduced immune reconstitution. Alternatively, hematopoietic stem cell gene therapy is effective for ADA-SCID patients who lack an HLA-identical sibling donor (13). Autologous CD34$^+$ bone marrow cells were transduced with a retroviral vector containing the ADA gene and infused into 10 patients with ADA-SCID after non-myeloablative conditioning. However, two patients have required enzyme replacement after gene therapy (14). ADA gene therapy has been performed in total 31 patients in Italy, the United Kingdom, and the United States. Twenty-one patients have been successful, whereas 10 patients have received enzyme replacement therapy (15). Recently, Cancrini et al. (16) described two ADA-SCID patients from the same family who both underwent BMT. One patient underwent BMT without conditioning, whereas

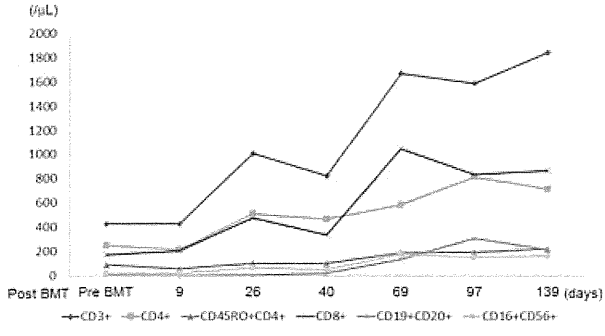

Fig. 2. Kinetics of the lymphocyte subpopulations in the patient.

Table 3. TREC and sjKREC levels as measured by quantitative PCR in the peripheral blood at different time points

	Post-BMT Day 25	Day 83	Day 202	Normal values
TRECs	0	8.1×10^3	1.9×10^3	$3.5 \pm 2.8 \times 10^3$
sjKRECs	0	2.8×10^2	6.4×10^3	$4.8 \pm 0.6 \times 10^3$
RNaseP	4.5×10^4	1.6×10^5	2.0×10^5	

All of the units are copies/μg DNA. The RNaseP gene was amplified as an internal control. The normal values indicate the copy numbers of the age-matched controls (2–6 yr of age).

the other patient was administered a RIC regimen (busulfan and fludarabine) following the failure of cord blood transplantation. Engraftment and immune reconstitution were compared in these patients. The patient who received conditioning exhibited stable mixed chimerism in all of the cell lineages, whereas the patient who underwent BMT without conditioning exhibited slow immune reconstitution, especially in B and myeloid cells. This observation indicated that the use of conditioning resulted in faster immunologic and metabolic reconstitution. In these patients, the immune reconstitution of B and myeloid cells was slower than that of T and NK cells. Interestingly, the reconstitution of myeloid and B cells appeared earlier than that of T cells in our patient. The patient with delayed-onset ADA deficiency had a substantial number of T cells but no B cells before BMT, and the generation of new T cells may take longer than B cells.

Patients with delayed-onset ADA deficiency often have chronic pulmonary insufficiency and autoimmune phenomena, including cytopenia and anti-thyroid antibodies, as observed in our patient. Patients with a delayed- or late-onset type may survive undiagnosed in the first decade of life or beyond; however, the longer the disease goes undiagnosed, the more the immune function deteriorates. The serum immunoglobulin levels of our patient gradually decreased from the point of diagnosis. Our patient had a substantial number of T cells; however, TRECs were undetectable in his peripheral blood. Therefore, we decided that the patient would receive BMT preceded by a RIC regimen, including busulfan and fludarabine. The use of RIC in BMT from an HLA-identical donor in this patient resulted in rapid and complete immune and metabolic reconstitution, and there was no treatment-related toxicity. However, a longer follow-up period is required to confirm these observations. If patients have an HLA-identical sibling donor, BMT with a RIC regimen may be the treatment of choice in patients with delayed-onset ADA deficiency.

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Aggressive Transformation of Juvenile Myelomonocytic Leukemia Associated with Duplication of Oncogenic *KRAS* due to Acquired Uniparental Disomy

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A small fraction of cases of juvenile myelomonocytic leukemia (JMML) develop massive disease activation. Through genomic analysis of JMML, which developed in an individual with mosaicism for oncogenic *KRAS* mutation with rapid progression, we identified acquired uniparental disomy at 12p. We demonstrated that duplication of oncogenic *KRAS* is associated with rapid JMML progression. *(J Pediatr 2013;■:■-■).*

Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloproliferative disorder, characterized by malignant transformation in the stem cell compartment with clonal proliferation of progeny that variably retain the capacity to differentiate. The clinical course of JMML is heterogeneous. Some patients require prompt allogeneic hematopoietic stem cell transplantation, whereas some demonstrate a milder clinical course, and some of them eventually exhibit spontaneous improvement.[1] Recent investigations into the molecular pathogenesis of JMML revealed that approximately 80% of patients harbored mutually exclusive mutations in genes regulating the Ras-mitogen-activated protein kinase (MAPK) pathway, including *RAS*, *PTPN11*, *NF1*, and *CBL*, leading to aberrant activation of the Ras-MAPK pathway.[1,2] The spectrum of mutations described thus far in JMML provides potential new opportunities for both diagnosis and therapy.

Previous studies reported that a small fraction of patients with JMML develop rapid and massive disease activation after an indolent clinical course. A report showed the incidence of progression to blastic phase to be 13%.[3] The etiology of the aggressive transformation remains unelucidated, however. We present a patient with JMML with a *KRAS* mutation who developed aggressive transformation and died. We performed genomic analysis to investigate the molecular pathology of this rapid and fatal progression.

Methods

A 1-year-old boy presented with leukocytosis (white blood cell count 46 800/mm^3, 20% monocytes, no blast cells) and hepatosplenomegaly. Bone marrow aspiration revealed hypercellular marrow, with 0.5% blast cells. Karyotyping was normal, and reverse-transcription polymerase chain reaction detected no *BCR-ABL* fusion. Fetal hemoglobin concentration was elevated (22%). Spontaneous growth of colony-forming unit granulocyte macrophages and hypersensitivity to granulocyte macrophage colony-stimulating factor were demonstrated, and mutation analysis revealed a heterozygous *KRAS* mutation (G12D: GGT>GAT) in peripheral blood mononuclear cells (PBMCs), all of which were consistent with JMML.[4] By 2 months after diagnosis, leukocytosis and hepatosplenomegaly had progressed. Oral 6-mercaptopurine (6-MP) therapy was started, and the patient remained stable for the next 10 months.

At 1 year after diagnosis, the patient suddenly developed tachypnea, impaired consciousness, and massive hepatosplenomegaly. Laboratory data revealed a white blood cell count of 124 400/mm^3 (38% monocytes, 5% blast cells; **Figure 1**, A). The patient's condition deteriorated rapidly, and he died from respiratory dysfunction. Autopsy revealed dysplastic cells infiltrating the bone marrow, lymph nodes, central nervous system, lungs, liver, spleen, pancreas, and kidneys (**Figures 1**, B and **2**; **Figure 2** available at www.jpeds.com).

Our genomic analysis was approved by the Ethics Board of the University of Tokyo, and informed consent was obtained from the child's guardian. Direct sequencing of the *KRAS* gene was performed for his normal muscle, heart, and lung (obtained at autopsy) and for PBMCs at diagnosis and at progression. Genome-wide analysis for genetic lesions was performed by single nucleotide polymorphism (SNP) array analysis. DNA

6-MP	6-mercaptopurine
JMML	Juvenile myelomonocytic leukemia
MAPK	Mitogen-activated protein kinase
PBMC	Peripheral blood mononuclear cell
SNP	Single nucleotide polymorphism
UPD	Uniparental disomy

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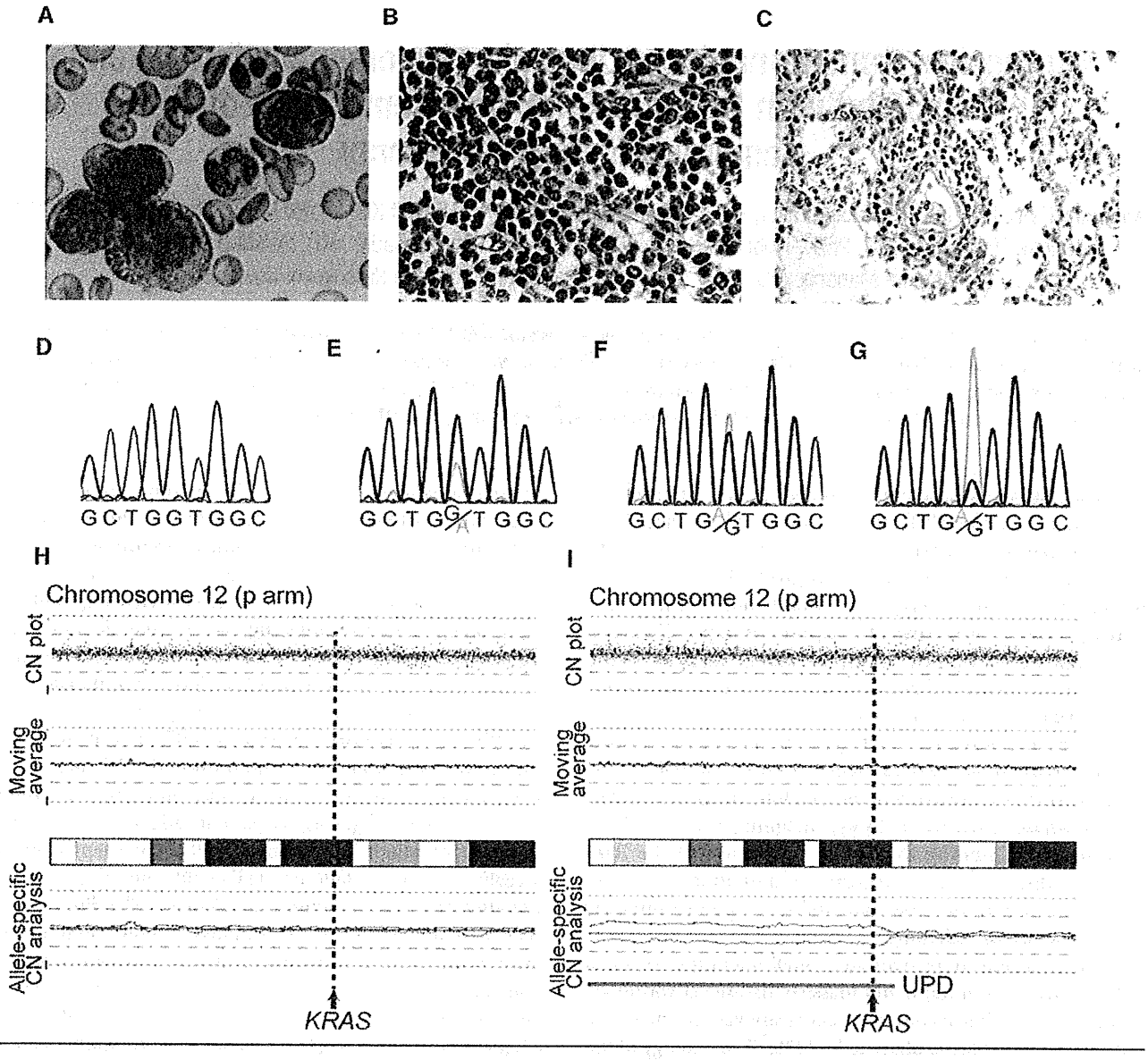

Figure 1. Blood smear, autopsy, and genomic analysis results. **A,** May-Giemsa staining of peripheral blood at progression. Hematoxylin & eosin staining of **B,** bone marrow and **C,** lung. Results of direct sequencing for the *KRAS* gene in **D,** heart, **E,** muscle, **F,** peripheral blood at diagnosis, and **G,** peripheral blood at progression. Results of SNP array analysis for PBMCs at **H,** diagnosis and **I,** progression. UPD at the 12p locus was detected at progression (*brown line*), whereas UPD was absent at diagnosis. Total copy number plots from each probe (*red points*) and moving average (n = 20; *blue line*) are shown *above* the cytoband. Results from allele-specific analyses are given *below* the cytoband. The larger allele is presented in *red*; the smaller allele, in *blue*. *CN*, copy number.

extracted from samples was analyzed using the GeneChip Human Mapping 250K *NspI* array (Affymetrix, Santa Clara, California). The data thus obtained were processed using CNAG/AsCNAR software (http://www.genome.umin.jp).[5,6]

Results and Discussion

This case shows that JMML can progress rapidly during an indolent clinical course, with invasion into multiple organs. The aggressive transformation of JMML is similar to that of blast crisis in chronic myelogenous leukemia but is rare,[2,3] and the definition and molecular biology of the blast crisis–like aggressive transformation of JMML remain unclear.

Direct sequencing of the *KRAS* gene revealed a mutation in normal muscle. The same mutation was found in the lung, but not in the heart. The mutation was also detected in PBMCs at diagnosis, and the mutation became homozygous in PBMCs at progression (Figure 1, D-G). SNP array analysis

detected uniparental disomy (UPD) at the 12p locus, which included the *KRAS* gene, in PBMCs at progression (**Figure 1, I**). The UPD was absent in PBMCs at diagnosis (**Figure 1, H**). Our genome-wide analysis for copy number alteration and allelic imbalances using high-density SNP arrays detected no other genetic abnormalities in samples, either at diagnosis or at progression.

Recent research identified germline/somatic mutations in genes regulating the Ras-MAPK pathway as responsible for JMML pathogenesis, but the underlying mechanism of aggressive transformation remains unclear. Reportedly, abrogation of the wild-type *NRAS* allele was evident at progression, similar to our results.[7] Loss of the remaining *KRAS* allele could be associated with JMML progression because the wild-type *KRAS* allele functions as a tumor suppressor.[8] However, we determined that UPD at the 12p locus occurred at progression, leading to both a loss of the remaining allele and an increase in the dosage of the mutant *KRAS* allele. Previous studies have found strong associations between UPD at oncogene loci and the onset of various hematologic neoplasms, such as *NRAS* in chronic myelomonocytic leukemia[9,10] and *CBL* in adult myelodysplastic syndrome.[11] Our analysis suggested that UPD of mutant *KRAS* contributed to aggressive transformation as a second hit in our patient, leading to increased aberrant activation of the Ras-MAPK pathway (**Figure 3**).

Our patient received oral 6-MP for disease stabilization before progression. Of note, 6-MP has a DNA-damaging effect. A previous report suggested a correlation between 6-MP maintenance therapy and secondary malignant neoplasms.[12] Although an association between 6-MP and progression is unelucidated, it is possible that 6-MP might induce the second hit, followed by progression to cell transformation.

We found no genomic lesions other than the *KRAS* mutation in samples obtained at diagnosis, and the first hit for JMML onset remains undetermined. Previous reports have identified other genetic and epigenetic abnormalities were associated with JMML pathogenesis, including mutation of *TP53* and methylation of *BMP4*, *CALCA*, *CDKN2B*, and *RARB*.[13] However, direct sequencing of *TP53* and methylation-specific polymerase chain reaction showed that *BMP4* and *RARB* were not methylated and *CALCA* was methylated both at diagnosis and at progression, and *CDKN2B* was partially methylated only at progression. Further research is needed to elucidate the etiology of JMML.

In our patient, we detected *KRAS* G13D mutation in normal muscle cells in which we pathologically excluded blood infiltration. However, we found no facial gestalt or developmental retardation characteristic of Noonan or cardio-facio-cutaneous syndromes, both of which are caused by germline *KRAS* mutations. We assumed that this is because the patient was mosaic for a *KRAS* mutation, supported by sequencing results using other normal cells (**Figure 1, D and E**). Here we report a patient with JMML and oncogenic *KRAS* mosaicism who developed JMML. In another report of 2 cases of *NRAS* mosaic mutation with JMML, *NRAS* mutations remained heterozygous, and both patients exhibited an indolent clinical course.[14]

It is accepted that most patients with JMML harboring a *KRAS* mutation experience an indolent clinical course, and some achieve spontaneous remission.[1,15] However, as we report here, some patients with JMML with a *KRAS* mutation experience aggressive transformation, which is potentially fatal. We suggest that UPD at the mutated *KRAS* gene causes more potent activation of the Ras-MAPK pathway, facilitating transformation. Consequently, inhibition of this pathway could be a therapeutic target for JMML. ■

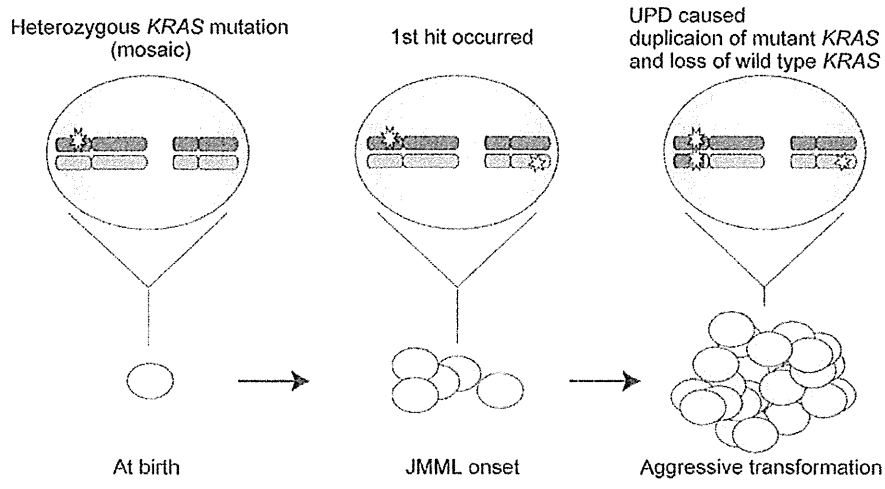

Figure 3. Hypothetical model of onset of JMML and its aggressive transformation. Sequential genetic events, starting with the presence of a *KRAS* mutation, are indicated. A first hit contributed to JMML onset, although that first hit remains undetermined. *KRAS*-mutated cells acquired UPD at the 12p locus, leading to both a loss of the wild-type *KRAS* allele and an increase in the dosage of the mutant *KRAS* allele, followed by aggressive transformation.

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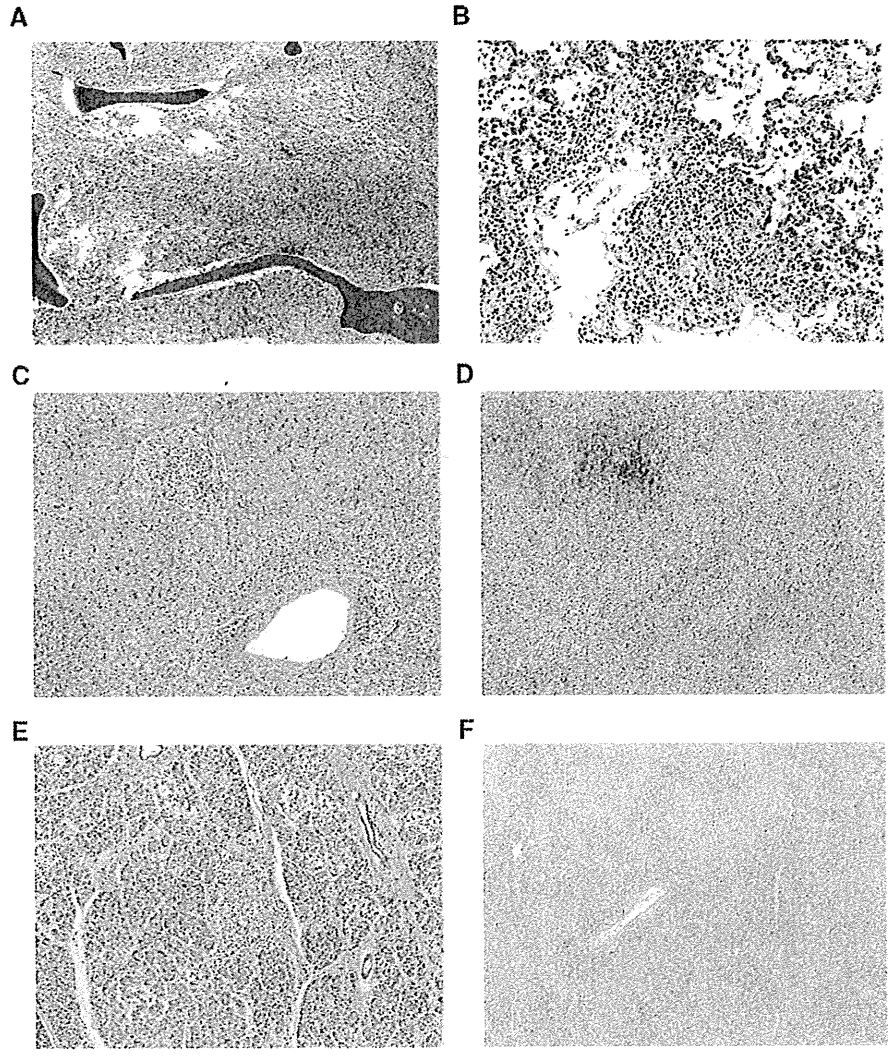

Figure 2. Autopsy revealed dysplastic cells infiltrated into mutiple organs. Hematoxylin & eosin staining of **A,** bone marrow; **B,** lung; **C,** liver; **D,** spleen; **E,** pancreas; and **F,** kidney.