Somatic mosaicism for oncogenic \textit{NRAS} mutations in juvenile myelomonocytic leukemia

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Abstract

Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in granulocyte-macrophage colony-stimulating factor signal transduction, such as NRAS, KRAS, PTPN11, NF1, and CBL, have been identified in more than 70% of children with JMML. We report two 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% to 50% in bone marrow and other somatic cells (i.e., 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 patients with 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.
Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in granulocyte-macrophage colony-stimulating factor signal transduction, such as \textit{NRAS}, \textit{KRAS}, \textit{PTPN11}, \textit{NF1}, and \textit{CBL}, have been identified in more than 70% of children with JMML \textsuperscript{1-3}.

The term ‘somatic mosaicism’ is defined as the presence of multiple populations of cells with distinct genotypes in one individual whose developmental lineages trace back to a single fertilized egg \textsuperscript{4}. Somatic mosaicism of various genes, including some oncogenes, has been implicated in many diseases. For instance, somatic mosaicism for \textit{HRAS} mutations is found in patients with Costello syndrome (CS) \textsuperscript{5-7}. While germline mutations in causative genes (\textit{PTPN11, NRAS, NF1, CBL}) are found in JMML patients \textsuperscript{3,8-11}, the presence of somatic mosaicism for these genes has never been reported. Here, we describe two cases of JMML in which the patients display somatic mosaicism for oncogenic \textit{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 Nagoya University Graduate School of Medicine.

**Patient 1 (Table 1)**

A 10-month-old boy had hepatosplenomegaly and leukocytosis ($72.1 \times 10^9/L$) with monocytosis ($13.3 \times 10^9/L$). The bone marrow contained 7% blasts with myeloid hyperplasia. Cytogenetic analysis revealed a normal karyotype, and colony assay of bone marrow 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 (EWOG-MDS), was fulfilled, and the patient was treated with interferon-alpha and 6-mercaptopurine. His clinical and laboratory findings gradually resolved without HSCT. However, eleven years after the diagnosis of JMML, the patient developed thrombocytopenia ($7.6 \times 10^9/L$), and bone marrow findings showed trilineage dysplasia with low blast count, compatible to that of refractory anemia. The patient did not have any physiological abnormalities, such as facial deformity, and there was no family history of malignancy or congenital abnormalities.

**Patient 2 (Table 1)**

A 10-month-old boy had anemia, hepatosplenomegaly, and leukocytosis ($31.8 \times 10^9/L$) with monocytosis ($6.4 \times 10^9/L$). The patient’s bone marrow exhibited myeloid hyperplasia and granulocytic dysplasia with 5% blasts. Cytogenetic 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, the patient’s clinical symptoms and leukocytosis spontaneously improved within a few months without cytotoxic therapy or HSCT. The patient has remained healthy and has experienced no hematological or physiological abnormalities; the patient’s most recent follow-up examination conducted at 8 years of age.

Detailed methods for experiments are described in supplemental data.
Results and discussion

DNA sequencing for JMML-associated genes (NRAS, KRAS, PTPN11, and CBL) was performed (Fig. 1, Table 1). In patient 1, the NRAS G12D mutation was identified in BM-MNCs both at the time of diagnosis of 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 as compared to signal of blood cells. In case 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 (Fig. 1, Table 1). The frequency of mutated alleles varied by tissue type as follows: for patient 1, BM-MNCs (56%), nails (24%), buccal smear cells (43%), and hair bulbs (5%); for patient 2, buccal smear cells (21%) and nails [left hand (26%), right hand (13%), left foot (8%), 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). Since confirmed detection level by pyrosequencing technique is above 5%, results with low percentage (<5%) mutant allele (i.e., hair bulbs in patient 1) should be interpreted with caution.

We diagnosed two 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 white blood cells, we also identified mutation in DNA from nails which is known to be a good biological material without contamination of 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 (PTPN11, NRAS, NF1, CBL) are found in a proportion of JMML patients, somatic mosaicism for these genes has never been reported. To the best of our knowledge, this is 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 similarities to Noonan syndrome (NS) or NS-associated diseases. Correspondingly, JMML patients with germline NRAS or CBL mutations exhibit characteristic dysmorphic features. 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 due to the oncogenic nature of the mutations.

In general, JMML is a rapidly fatal disorder if untreated. 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. Both of the JMML patients with somatic mosaicism of
oncogenic NRAS mutations have had a mild and self-limiting clinical course. We analyzed nails of other three JMML patients with RAS mutations who experienced aggressive clinical course. However, none of them 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 Contributions Contribution: S. Doisaki and H. Muramatsu designed and conducted the research, analyzed data, and wrote the paper. A.S., M.M-E., M.S., H.K., A.K., M.S. and Y.H. treated patients, and 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., Y.T. and K.K. conducted the research. S.K. designed the research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
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<tbody>
<tr>
<td>Age (Months)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Liver, cm</td>
<td>12</td>
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<tr>
<td>Spleen, cm</td>
<td>8</td>
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<tr>
<td>White blood cells, ×10^9/L</td>
<td>72.1</td>
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<tr>
<td>Monocytes, %</td>
<td>18.5</td>
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<td>4</td>
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<tr>
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<td>Platelets, ×10^9/L</td>
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<tr>
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<tr>
<td>Gene mutation</td>
<td>NRAS, G12D</td>
<td>NRAS, G12S</td>
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<tr>
<td></td>
<td>35G&gt;A</td>
<td>34G&gt;A</td>
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<tr>
<td>Treatment</td>
<td>IFN-alpha-2b, 6-MP</td>
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</tr>
<tr>
<td>Observation period, months</td>
<td>231</td>
<td>103</td>
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<tr>
<td>Outcome</td>
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<td>Fraction of mutant alleles, % (pyrosequencing)</td>
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<tr>
<td>Nail (Whole)</td>
<td>24</td>
<td>12.5 (average)</td>
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<tr>
<td>Nail (Lt. Hand)</td>
<td>ND</td>
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<td>Buccal smear cells</td>
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<tr>
<td>Sibling</td>
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Hb, hemoglobin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; 6-MP, 6-mercaptopurine; ND, not done; JMML, juvenile myelomonocytic leukemia; MDS, myelodysplastic syndrome; Lt., left; Rt., right
Figure legend

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 the oncogenic NRAS mutations [patient 1 (G12D, 35G>A), patient 2 (G12S, 34G>A)] in bone marrow (BM) mononuclear cells at diagnosis of JMML, nail, and buccal smear cells. The quantification by pyrosequencing revealed that the fractions of mutated allele are varied among tissue types [patient 1; BM (50%), nail (23%), and buccal smear (43%), patient 2; BM (50%), left-hand nail (26%), and buccal smear (21%)].
Patient 1  
(NRAS G12D, 35G>A)

Patient 2  
(NRAS G12S, 34G>A)

BM

Nail

Buccal Smear

Normal Control

Patient 1  
(NRAS G12D, 35G>A)

G A 50%

Patient 2  
(NRAS G12S, 34G>A)

A G 48%

G A 24%

A G 26%

G A 43%

A G 21%

Normal Control

G

G
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