Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations

Running short title: Clinical features of JMML with RAS mutations

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Abstract

Of 11 children with juvenile myelomonocytic leukemia (JMML) carrying \textit{RAS} mutations (8, \textit{NRAS} mutations; 3, \textit{KRAS2} mutations), 5 patients had a profound elevation in either or both the WBC and spleen size at diagnosis. Three patients had no or modest hepatosplenomegaly and mild leukocytosis at presentation, but subsequently showed a marked increment in spleen size with or without hematological exacerbation, for which non-intensive chemotherapy was initiated. The other 3 patients with NRAS or KRAS2 glycine to serine substitution received no chemotherapy, but hematological improvement has been observed during a 2- to 4-year follow-up. In the third group, all hematopoietic cell lineages analyzed had the \textit{RAS} mutations at the time of hematological improvement, while DNA obtained from the nails had the wild type. Additionally, numbers of circulating granulocyte-macrophage progenitors were significantly reduced during the clinical course. Thus, some JMML patients with specific \textit{RAS} mutations may have spontaneously improving disease.
Introduction

Somatic point mutations of the RAS (NRAS and KRAS2) genes at codons 12, 13, and 61 are found in about 20% of patients with juvenile myelomonocytic leukemia (JMML).\textsuperscript{1,2} Other patients show inactivation of NF1 or PTPN11 mutations.\textsuperscript{3-5}

While most patients with JMML die from progressive disease unless treated with hematopoietic stem cell transplantation, there are a few patients who have been reported to spontaneously recover without intervention.\textsuperscript{6-7} Some of these children have JMML associated with Noonan syndrome while others do not. So far, the individual prognosis in JMML carrying specific genetic aberrations remains unclear. We here report the clinical course in 11 patients with RAS mutations.

Materials and Methods

Cell Preparation

This study was approved by the Institutional Review Board of Shinshu University. Informed consent was obtained from the guardians of the patients following institutional guidelines. We used the peripheral blood (PB) or bone marrow (BM) mononuclear cells (MNCs) that had been frozen with liquid nitrogen. CD3- and CD56-positive PB cells were separated immunomagnetically.\textsuperscript{8} Ninety-nine % of the isolated cells were CD3- or CD56-positive, according to a flow cytometric analysis.

Clonal cell culture

Twenty thousand PB or BM MNCs were plated in a dish containing methylcellulose medium supplemented with or without 0.01 to 10 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF).\textsuperscript{9} To examine the clonal derivation of myeloid and erythroid lineages, two thousand CD34\textsuperscript{+} PB cells harvested immunomagnetically
were cultured in methylcellulose medium supplemented with GM-CSF, stem cell factor, interleukin 3, and erythropoietin. Twelve days after incubation with 5% CO$_2$, GM colonies, erythroid colonies, and mixed colonies were individually lifted, and prepared as single cell suspensions. Then, sequence analyses were performed on individual colony-constituent cells.

**Detection of NRAS and KRAS2 mutations**

DNA was extracted from PB or BM MNCs, and nails. Exon 1 (codons 12 and 13) and exon 2 (codon 61) of NRAS and KRAS2 genes were amplified by PCR, using primer pairs described previously.$^{10,11}$ The PCR products were subjected to direct sequencing from both directions on an automatic DNA sequencer.
**Results and discussion**

Among a total of 80 children with JMML, 13 children had *RAS* mutations (10, *NRAS* mutations; 3, *KRAS2* mutations) with the wild type of the *PTPN11* gene. All the patients with *RAS* mutations met the criteria proposed by the International JMML Working Group, but did not have clinical features of neurofibromatosis type I. Clinical characteristics of 11 patients for whom all clinical information was available are presented in Table 1. Five patients (Cases 1 to 5) were treated with allogeneic bone marrow transplantation or non-intensive chemotherapy including 6-mercaptopurine (6-MP) at or soon after admission, based on marked leukocytosis/splenomegaly (>6 cm) alone or in combination with the existence of a related donor. Hematological abnormalities and hepatosplenomegaly in 3 patients (Cases 6 to 8) were modest at first presentation. However, a marked enlargement of spleen size was noted within 2 to 3 months after diagnosis in all 3 patients, followed by deterioration of leukocytosis in Case 7 and of anemia/thrombocytopenia in Case 8. Accordingly, oral 6-MP was administered to the three patients 3 to 9 months after diagnosis. In 3 patients (Cases 9 to 11), the hematological findings were similar to those of Cases 6 to 8 at first presentation. There was no significant change or a decrease of spleen size within 3 month of diagnosis in Cases 9 to 11, as shown in Figure 1A. Surprisingly, in these patients, hematologic improvement was observed over a two- to four-year follow-up, despite no chemotherapy. Thus, JMML patients carrying *RAS* mutations appear to be heterogenous with respect to the hematological progression.

As presented in Figure 1B, there was no significant difference in GM colony growth stimulated with low doses of GM-CSF between the patients with the NRAS or KRAS2 glycine to serine substitution and the other mutants of the RAS including glycine to
aspartic acid substitution. Interestingly, numbers of GM colonies grown from $2 \times 10^4$ PB MNCs in the absence or presence of 10 ng/mL of GM-CSF were significantly reduced approximately 2 to 4 years after presentation, as compared with the values at diagnosis in Cases 9 and 10 or with the values one year after diagnosis in Case 11 (Figure 1A).

A possible explanation for the spontaneous improvement of hematologic findings in Cases 9 to 11 is a decline in the number of cells harboring RAS mutation and an increase in the number of normal cells during the clinical course. Sequence analyses at the time of hematological improvement revealed that the mutation was present in PB CD3$^+$ cells and CD56$^-$ cells as well as CD3$^-$/CD56$^-$ cells in the three patients. The RAS mutations were also detected in all of 15-25 GM colonies, 11-25 erythroid colonies, and 3-5 mixed colonies derived from PB CD34$^+$ cells harvested simultaneously. Identical mutations were detectable at mRNA levels in PB MNCs and individual GM/erythroid/mixed colonies in Cases 10 and 11. Therefore, it is unlikely that the normal cell population coexisted with the abnormal clone and expanded.

A second possibility is that hematological abnormalities are a manifestation of germline RAS mutations, since patients with Noonan syndrome and germline PTPN11 or RAS mutations have been reported to have a JMML-like myeloproliferative disease.\textsuperscript{13, 14} Additionally, germline HRAS mutations are observed in individuals with Costello syndrome who often develop benign tumors and cancers.\textsuperscript{15} In Cases 9 to 11, DNA obtained from their nails had the wild type of the NRAS or KRAS2 gene, while hematopoietic cells had a point mutation. Thus, the three patients had a somatic and not germline Gly12Ser mutation in the RAS gene.

Niemeyer et al.\textsuperscript{16} proposed age, platelet count, and HbF at diagnosis as factors for
predicting the length of survival. Cases 9 to 11 were younger than 24 months of age, and had platelet counts of >33 x 10^9/L and HbF of <15% at onset. On the other hand, Cases 6 to 8 also had no risk factors at diagnosis except for the HbF value in Case 6. One possible parameter distinguishing between the two groups is change of spleen size within 3 month of diagnosis.

Among 32 JMML patients with NRAS or KRAS2 mutations reported by us and the others,^2,5,17,18^ codon 12 in the NRAS gene was the most commonly affected, and variably substituted. Braun et al.^19^ reported that somatic activation of a latent KRAS^G12D^ allele, the mutation identical to Case 6, rapidly induces a fatal myeloproliferative disorder in mice. On the other hand, clinical and hematologic abnormalities improved despite no chemotherapy in our 3 patients with NRAS or KRAS2 glycine to serine substitution. Given the evidence that different substitutions produce HRAS alleles with different strength in the transforming potential,^20,21^ Gly12Ser mutation in the RAS gene may be insufficient to confer the disease progression in JMML. Close followup is requisite, since these children still harbor a clonal oncogenic abnormality in their bone marrow.

In conclusion, no chemotherapy may be a recommended management for JMML patients with NRAS or KRAS2 glycine to serine substitution, but further large-scaled studies are needed to assess accurately the relationship between the mutational spectrum of the RAS gene and the disease phenotype.

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References


5. Kalra R, Paderanga DC, Olson K, Shannon KM. Genetic analysis is consistent with the hypothesis that NF1 limits myeloid cell growth through p21ras. Blood. 1994;84:3435-3439.


18. Chen Y, Takita J, Hiwatari M, et al. Mutations of the PTPN11 and RAS genes in
13


Figure legends

Figure 1. Clinical course and dose response to GM-CSF of colony growth in JMML patients with RAS glycine to serine substitution

(A) Clinical course and time-course study of circulating GM progenitors of Cases 9 to 11. Mean numbers of GM colonies generated by 2 x 10^4 PB MNCs under stimulation with or without 10 ng/mL of GM-CSF are shown. Mean numbers of GM colonies in 4 normal controls were 0.0 ± 0 for no factors, and 0.1 ± 0.1 for GM-CSF. Dx, diagnosis; Plt-Tr, platelet transfusion. □, WBC; ▬, Hb values; △, platelet counts.

(B) Comparison of proliferative response of GM progenitors to low doses of GM-CSF among JMML patients with NRAS or KRAS2 glycine to serine substitution, JMML patients with the other mutations, and normal controls. Twenty thousand PB or BM MNCs were cultured in a well containing methylcellulose culture medium supplemented with GM-CSF at concentrations of 0.01 ng/mL to 10 ng/mL. The data are expressed as a percentage of the colony growth obtained with 10 ng/mL of GM-CSF in each case. The colony growth stimulated with 0.01 ng/mL and 0.1 ng/mL of GM-CSF was significantly higher in 3 patients with Gly12Ser mutation in the RAS gene than in normal controls, but comparable to the value obtained from the 3 other mutants. □, Case 5; ▬, Case 6; △, Case 8; □ △, Case 9; △, Case 11; □, The other patient with NRAS glycine to serine substitution; *, Normal control 1; ▬, Normal control 2; □ Normal control 3.
Fig. 1A

Case 9  
<table>
<thead>
<tr>
<th>Interval after Dx (mo)</th>
<th>No factors</th>
<th>GM-CSF</th>
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<tbody>
<tr>
<td>0</td>
<td>7.3</td>
<td>27.5</td>
</tr>
<tr>
<td>6</td>
<td>22.5</td>
<td>49.5</td>
</tr>
<tr>
<td>12</td>
<td>1.5</td>
<td>7.5</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>0</td>
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Case 10

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<th>Interval after Dx (mo)</th>
<th>No factors</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>7.3</td>
<td>197</td>
<td>107</td>
</tr>
<tr>
<td>27.5</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>69</td>
<td>0.3</td>
<td>0</td>
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Case 11

<table>
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<tr>
<th>Interval after Dx (mo)</th>
<th>No factors</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>37.3</td>
<td>63.7</td>
</tr>
<tr>
<td>36</td>
<td>5.3</td>
<td>18.5</td>
</tr>
</tbody>
</table>

Fig. 1B

GM colony growth (% of control) vs. GM-CSF (ng/mL)
Table 1 Clinical characteristics of 11 JMML patients with *RAS* mutations

<table>
<thead>
<tr>
<th>Case (No)</th>
<th>Mutations</th>
<th>Age (mo)</th>
<th>Sex</th>
<th>Liver (cm)</th>
<th>Spleen (cm)</th>
<th>WBC (10⁹/L)</th>
<th>Mono (%)</th>
<th>Hb (g/dL)</th>
<th>Plt (10⁹/L)</th>
<th>HbF (%)</th>
<th>Karyotype</th>
<th>Treatment (Interval after Dx)</th>
<th>Outcome (Interval after Dx)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>NRAS</em>, codon 61 CAA (Gln) &gt; CTA (Leu)</td>
<td>1</td>
<td>M</td>
<td>3</td>
<td>2</td>
<td>151.0</td>
<td>31.6</td>
<td>8.4</td>
<td>34</td>
<td>65</td>
<td>45,XY,-7</td>
<td>famil BMT (2 mo)</td>
<td>dead (2 mo)</td>
</tr>
<tr>
<td>2</td>
<td><em>KRAS</em>, codon 13 GGC (Gly) &gt; GAC (Asp)</td>
<td>7</td>
<td>F</td>
<td>6</td>
<td>10</td>
<td>17.9</td>
<td>9</td>
<td>6.4</td>
<td>15</td>
<td>16.8</td>
<td>46,XX</td>
<td>sib BMT (5 mo)</td>
<td>alive (+43 mo)</td>
</tr>
<tr>
<td>3</td>
<td><em>NRAS</em>, codon 12 GGT (Gly) &gt; GAT (Asp)</td>
<td>12</td>
<td>F</td>
<td>10</td>
<td>8</td>
<td>61.0</td>
<td>21</td>
<td>6.6</td>
<td>12</td>
<td>50.4</td>
<td>46,XX</td>
<td>6-MP+Ara-C+VP-16 (0 mo), UCBT (2 mo)</td>
<td>alive (+22 mo)</td>
</tr>
<tr>
<td>4</td>
<td><em>NRAS</em>, codon 13 GGT (Gly) &gt; GAT (Asp)</td>
<td>4</td>
<td>M</td>
<td>3</td>
<td>10</td>
<td>72.3</td>
<td>21</td>
<td>8.1</td>
<td>65</td>
<td>13.2</td>
<td>46,XY</td>
<td>6-MP (0 mo), UCBT in preparation</td>
<td>alive (+44 mo)</td>
</tr>
<tr>
<td>5</td>
<td><em>NRAS</em>, codon 12 GGT (Gly) &gt; GAT (Asp)</td>
<td>2</td>
<td>M</td>
<td>2</td>
<td>6.5</td>
<td>84.0</td>
<td>22</td>
<td>11.6</td>
<td>110</td>
<td>25.1</td>
<td>46,XY</td>
<td>6-MP (1 mo)</td>
<td>alive (+4 mo)</td>
</tr>
<tr>
<td>6</td>
<td><em>KRAS</em>, codon 12 GGT (Gly) &gt; GAT (Asp)</td>
<td>22</td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>28.8</td>
<td>11</td>
<td>11.6</td>
<td>41</td>
<td>20.1</td>
<td>46,XX</td>
<td>6-MP (3 mo), Pred (6 mo)</td>
<td>dead (6 mo)</td>
</tr>
<tr>
<td>7</td>
<td><em>NRAS</em>, codon 12 GGT (Gly) &gt; TGG (Cys)</td>
<td>2</td>
<td>M</td>
<td>4</td>
<td>1.5</td>
<td>32.4</td>
<td>19</td>
<td>10.6</td>
<td>90</td>
<td>8.9</td>
<td>46,XY</td>
<td>6-MP (6 mo), UCBT (36 mo)</td>
<td>dead (47 mo)</td>
</tr>
<tr>
<td>8</td>
<td><em>NRAS</em>, codon 13 GGT (Gly) &gt; GAT (Asp)</td>
<td>12</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>14.6</td>
<td>10</td>
<td>8.8</td>
<td>52</td>
<td>4.5</td>
<td>46,XY</td>
<td>6-MP (9 mo), UCBT (11 mo)</td>
<td>alive (+16 mo)</td>
</tr>
<tr>
<td>9</td>
<td><em>NRAS</em>, codon 12 GGT (Gly) &gt; AGT (Ser)</td>
<td>10</td>
<td>M</td>
<td>4</td>
<td>5</td>
<td>29.4</td>
<td>16.5</td>
<td>10.5</td>
<td>113</td>
<td>0.5</td>
<td>46,XY</td>
<td>None</td>
<td>alive (+50 mo)</td>
</tr>
<tr>
<td>10</td>
<td><em>NRAS</em>, codon 12 GGT (Gly) &gt; AGT (Ser)</td>
<td>10</td>
<td>M</td>
<td>5</td>
<td>10</td>
<td>31.8</td>
<td>20</td>
<td>5.4</td>
<td>100</td>
<td>1.7</td>
<td>46,XY</td>
<td>None</td>
<td>alive (+42 mo)</td>
</tr>
<tr>
<td>11</td>
<td><em>KRAS</em>, codon 12 GGT (Gly) &gt; AGT (Ser)</td>
<td>4</td>
<td>F</td>
<td>4</td>
<td>1</td>
<td>21.2</td>
<td>8</td>
<td>11</td>
<td>52</td>
<td>8.8</td>
<td>46,XY</td>
<td>None</td>
<td>alive (+30 mo)</td>
</tr>
</tbody>
</table>

Liver and spleen sizes are given in cm below the costal margin.

Ara-C, cytarabine; Dx, diagnosis; famil BMT, related non-sibling bone marrow transplantation; Hb F, hemoglobin F; mo, month(s); Mono, monocyte; 6-MP, 6-mercaptopurine; Plt, platelet count; Pred, prednisolone; sib BMT, sibling bone marrow transplantation; UCBT, unrelated cord blood transplantation; VP-16, etoposide; WBC, white blood cell count.

Cases 4 and 7 had a normal karyotype at presentation, but monosomy 7 approximately 3 years after diagnosis.