Mutations of the N-ras Gene in Juvenile Chronic Myelogenous Leukemia

By Jun Miyauchi, Minoru Asada, Michiko Sasaki, Yukiko Tsunematsu, Seiji Kojima, and Shuki Mizutani

Juvenile chronic myelogenous leukemia (JCML), a myeloproliferative disorder of childhood, is distinct from adult-type chronic myelogenous leukemia (CML) and bears resemblance to chronic myelomonocytic leukemia (CMMoL). Since mutations in the N-ras gene have been found at high frequencies in CMMoL, but only rarely in CML, we analyzed mutations activating the N-ras gene in 20 patients with JCML. We used the strategy for analysis of gene mutations based on in vitro DNA amplification by polymerase chain reaction (PCR) followed by single-strand conformation polymorphism (SSCP) analysis and/or direct sequence analysis. Nucleotide sequence analysis showed single nucleotide substitutions involving codons 12, 13, or 61 in six of 20 patients (30%). Four of six patients with mutations were in chronic phase and the other two in blast crisis, indicating no apparent correlation with disease stage. Most of the patients with mutations were in the older age group with poor prognosis, although one patient in the younger age group also harbored the mutation. These data suggest that N-ras gene mutations may be involved in the pathogenesis and/or prognosis of JCML and provide further evidence that JCML is an entity distinct from CML.

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Submitted August 16, 1993; accepted December 8, 1993.

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N-ras MUTATIONS IN JCML

Patient Age/Sex RBC HbF PLT WBC Mb Mono LAP Score

1* 2 mo/M 364 40 1.1 131.2 5 17 94 5 19.3 3/15 Alive (CP) [3 yr, 6 mo] Normal
2 2 mo/M 347 29 4.5 43.3 2 16 212 10 19.2 4/9 Alive (CP) [5 yr, 1 mo] Normal
3 2 mo/F 304 10 4.9 100.0 2 14 103 4 31.0 8/10 Alive (CP) [3 yr, 7 mo] Normal
4 3 mo/M 452 31 5.0 69.1 0 14 126 9 31.4 5/6 Alive (CP) [1 yr, 0 mo] Normal
5 7 mo/M 351 18 18.4 72.2 0 12 282 0 57.5 3/2 Dead (BC) [10 mo] 45, XY, –7
6 8 mo/F 259 20 1.3 44.0 2 13 91 8 ND 10/15 Dead (CP) [1 yr, 2 mo] ND
7 10 mo/M 277 13 3.5 38.0 1 8 223 1 11.9 7/12 Dead (EL) [2 mo] Normal
8 1 yr/F 443 24 7.4 12.2 1 8 70 7 86.8 4/5 Dead (CP) [3 yr, 10 mo] Normal
9* 1 yr/F 140 6 2.4 64.3 14 17 171 22 43.0 5/3 Dead (CP) [1 yr, 1 mo] Normal
10* 2 yr/F 394 3 2.5 59.4 3 56 9 5 ND 8/4 Dead (BC) [8 mo] ND
11 2 yr/M 401 59 5.3 40.2 2 17 103 4 20.8 5/8 Dead (BC) [1 yr, 0 mo] Normal
12 2 yr/M 391 32 1.7 45.2 2 7 73 4 40.7 4/5 Alive (CP) [1 yr, 5 mo] 46, XY, del(7)(q22)
13* 3 yr/M 456 61 5.5 9.2 0 9 224 7 9.2 10/10 Dead (BC) [8 mo] Normal
14 3 yr/M 343 35 3.5 61.6 0 17 28 2 21.1 8/4 Dead (CP) [6 mo] 46, XY, inv(4)(p14q16)
15 4 yr/M 406 31 59.9 31.3 5 15 226 4 24.8 4/7 Dead (BC) [2 yr, 2 mo] Normal
16* 4 yr/M 359 58 5.0 10.0 1 3 18 207 1 12.6 5/2 Dead (BC) [2 yr, 6 mo] Normal
17* 4 yr/F 254 89 6.0 16.8 8 26 203 5 35.0 2/5 Dead (BC) [4 mo] Normal
18 5 yr/F 278 1 1.3 11.8 0 14 ND 25 10.8 12/8 Dead (BC) [10 mo] 45, XY, –7
19 5 yr/M 303 8 2.4 45.7 4 8 100 27 24.6 2/0 Dead (CP) [4 mo] Normal
20 5 yr/F 226 3 2.3 32.2 3 38 213 2 29.8 3/6 Dead (CP) [6 mo] 45, XX, –7

Liver/Spleen represents palpable size below costal margin (cm). Normal range: LAP score of 180 to 280; serum lysozyme, 5 to 13 μg/mL.
Abbreviations: RBC, red blood cell count; HbF, hemoglobin F; PLT, platelet count; WBC, white blood cell count; Mb, myeloblasts; Mono, monocytes; LAP, leukocyte alkaline phosphatase; CF, chronic phase; BC, blast crisis; EL, erythroleukemic phase; RL, relapse; ND, not determined.
*Patients with mutations of the N-ras gene.

Source of DNA. Peripheral blood or bone marrow was obtained after receiving informed consent, and mononuclear cells were isolated by density sedimentation. Total DNA was prepared by lysis of the cells with sodium dodecyl sulfate (SDS) and digestion with proteinase K at 37°C overnight followed by phenol/chloroform extraction and ethanol precipitation. Because patients no. 5, 7, 10, 11, 16, and 17 were autopsy cases and fresh cells were not available, DNA was extracted from formalin-fixed paraffin-embedded 10-μm-thick tissue sections according to the method described by Orita et al. Lymph nodes with diffuse leukemic cell infiltration at autopsy and, when available, those obtained by biopsy in chronic phase were used. The DNA extracted from paraffin-embedded tissue sections was purified by using a silica-binding product (Geneclean; Bio 101, La Jolla, CA) before applying it for polymerase chain reaction (PCR).

PCR amplification. Two genomic regions encompassing codons 12/13 and 61 in exons 1 and 2, respectively, were amplified by PCR using two sets of 20-bp synthetic oligodeoxynucleotide primers.15 The primers used for these amplifications were sense 5'CTGGTG-TGAAATGACTGAGT3' and antisense 5'GOTGOGATCATATCTC-TCTCA3' for exon 1, and sense 5'GTTAGTAGTGTTGAAC-TCG3' and antisense 5'ATACACAGAGGAGCTCTCG3' for exon 2. Biotinylated primers were also used instead of one of the unlabeled primers. These biotinylated primers, prepared according to the published procedures,15 are sense 5'GCTGCCAATTAACCATGAT- TACG3' for exon 1 and antisense 5'GACCTGCTATTGATG-GCAAA3' for exon 2. A total of 500 ng of genomic DNA was amplified in a 25-μL reaction buffer containing 20 pmol of each primer, 200 μmol of deoxynucleotide triphosphate, 1.5 mmol/L of MgCl2, and 1 U of Taq polymerase (AmpliTaq; Takara Biomedicals, Kyoto, Japan). DNA was denatured at 97°C for 7 minutes, then quickly chilled on ice and Taq polymerase added. Reactions were performed for 30 cycles of denaturation at 94°C (30 seconds), annealing at 58°C (30 seconds), and extension at 72°C (1 minute), followed by final elongation at 72°C for 12 minutes in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). Some samples gave undesired reaction products with different molecular sizes in addition to target products. The target PCR products from these samples were gel-purified using a silica-binding product (Geneclean). Approximately 100 bp of target DNA sequences thus obtained was used for subsequent single-strand conformation polymorphism (SSCP) and/or direct sequence analyses.

SSCP analysis. SSCP analysis was performed according to the method previously described by Orita et al.16 One microliter of the amplified PCR products was subjected to 18 additional cycles of PCR in a 25-μL solution containing 200 μmol/L each of dATP, dGTP, and dTTP, 1 μL of [α-32P]dCTP (Amersham, Buckinghamshire, UK; 10 mCi/mL), 20 pmol of sense and antisense primers, 10 mmol/L of Tris-HCl (pH 8.3), 50 mmol/L of KCl, 1.5 mmol/L of MgCl2, and 1 U of Taq polymerase in a thermal cycler. PCR was performed under thermal conditions identical to those used for the first PCR. After amplification, 1 μL of the reaction product was mixed with 19 μL of a solution of 0.1% SDS and 10 mmol/L of EDTA. Then, 2 μL of the diluted product was mixed with 2 μL of a solution of formamide, 20 μmol/L of EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol, denatured at 80°C, and directly applied to a 6% polyacrylamide gel containing 90 mmol/L of Trisborate (pH 8.3), 4 mmol/L of EDTA, and 10% glycerol at 4°C.

Direct sequence analysis. For direct sequencing, 25 cycles of asymmetric PCR, using a 50 to 100 reduction of one of the primers
and 1 µL of the initial PCR products, were performed under the same thermal conditions as described earlier. The resulting single-stranded DNA was purified and sequenced by the dideoxynucleotide terminator method described by Sanger et al.37 using a sequencing kit (United States Biochemical, Cleveland, OH) and analyzed on a polyacrylamide gel containing 7 mol/L urea. Electrophoresis was performed at 35 W for 2 hours at room temperature, then the gel was dried on filter paper and autoradiography was performed. For the PCR products amplified using a set of biotinylated and unlabeled primers, the single-stranded biotinylated PCR products were separated from the unlabeled PCR products using streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Dynal, Oslo, Norway).38 These single-stranded templates were sequenced by the dideoxy chain termination method using a Bca-BEST kit (Takara Bio-medical) and analyzed after polyacrylamide gel electrophoresis and autoradiography as described earlier.

RESULTS

SSCP analysis of point mutations. For screening of mutations in exons 1 and 2 of the N-ras gene, amplified PCR products were subjected to SSCP analysis. Figure 1 shows the results of SSCP analysis on exon 1. A mobility shift of the single-stranded PCR products was seen with samples from patients no. 10 and 13: normal bands were not evident in either of these patients. The remaining patients showed a migration pattern identical to that of normal controls. However, as described later, direct sequencing showed point mutations at codon 13 of the N-ras gene in patient no. 1, who showed a normal migration pattern in SSCP analysis (Fig 1). This result in SSCP analysis was therefore considered to be false-negative. Similar false-negativity was also present in one (no. 17) of 14 patients examined for exon 2 (data not shown). For this reason, direct sequence analysis was performed for all samples.

Direct sequence analysis. Direct sequence analysis showed point mutations at codons 12, 13, or 61 of the N-ras gene in six patients. In patient no. 1, single nucleotide substitution at codon 13 from GGT to GAT (amino acid substitution from glycine to aspartic acid) was detected with concomitant normal sequences (Fig 2), although SSCP analysis did not disclose this mutation, as described earlier. Peripheral blood cells of this patient 3 years after the initial examination had the same mutation at codon 13 (data not shown), although the patient’s hematological findings remained well controlled within, or nearly within, the normal limits during this period. In patient no. 13, a single nucleotide substitution at codon 12 from GGT to TGT (amino acid substitution from glycine to cysteine) was identified with concomitant normal sequences (Fig 2). The identical point mutation was also detected in patient no. 10 (data not shown). Two simultaneous point mutations were present at codons 12 and 13 in patient no. 9 (Fig 2).

Codon 61 mutation was identified in patient no. 17 as a single nucleotide substitution from CAA to CTA (amino acid substitution from glutamic acid to leucine) with concomitant normal sequences (Fig 3). The same point mutation at codon 61 was also identified in patient no. 16 (data not shown).

Frequency and distribution of N-ras gene mutations in JCML. The results of sequence analysis are listed in Table 2. The mutations were present in four patients in chronic phase (no. 1, 9, 13, and 16) and in two patients at blast crisis (no. 10 and 17), indicating no particular preponderance of mutations at certain disease stages. Three patients (no. 7, 15, and 16) were examined at both chronic-phase and blast-crisis stages. Among these three, only patient no. 16 had the mutation, which was present in the chronic phase but disappeared at blast crisis. A comparison of the gene analysis with clinical information showed no particular clinical parameters that were closely related to the presence of the N-ras gene mutations (Table 1). Regarding prognosis, our patients could be categorized into two groups with relatively poor or good prognosis, with a demarcating line in age between those older and younger than 6 months old, respectively: 15 patients excluding one in the older age group died 13 months on average after the onset, whereas four patients in the younger age group are all alive at an average of 40 months after the onset. Although most (five of six) of the patients with N-ras gene mutations were in the older age group, one of the four patients in the younger age group also harbored the mutation. The number of patients examined was insufficient to allow a statistical analysis of the difference in frequency of the mutations between the two groups, and the relevance of the mutations to prognosis remains uncertain.

DISCUSSION

Mutations of the N-ras gene were detected in six of 20 patients (30%) with JCML in the present study. This frequency is similar to the incidence reported for AML,15-19 or CMML15,23,26 and is clearly different from that reported for CML, in which mutations have been detected only rarely.16,28,29 This result indicates that abnormalities of this gene are involved in a substantial proportion of patients with JCML, and supports the view that JCML is a disorder distinct from CML and similar to, and possibly a variant of, CMML or Ph- negative CML. Regarding leukemias, mutations of the N-ras gene have been reported to occur preferentially in those that involve monocytic lineage, ie, M4 and M5 of
AML in the FAB classification\textsuperscript{14,15,17,19-20} and CMMoL.\textsuperscript{23,26} Our present data on JCML are consistent with this observation, since JCML is a myeloproliferative disorder affecting primarily the cells of myelomonocytic lineage; spontaneous macrophage-dominant colony formation in vitro\textsuperscript{1,8,9} is one of the most characteristic and unique features of JCML. It has been suggested that this in vitro spontaneous growth of leukemia cells is caused by IL-1\textsuperscript{9} or GM-CSF\textsuperscript{10} secreted in autocrine or paracrine fashion and, more recently, hypersensitivity to GM-CSF has been suggested to play the major role in the pathogenesis of this disorder.\textsuperscript{11} On the other hand, the ras genes are known to code for proteins that are associated with plasma membrane, have GTPase activity, and participate in the transduction of signals, including those induced by growth factors.\textsuperscript{12,14} Taken together, the point mutations in the \textit{N-ras} gene might affect signal transduction through GM-CSF and be involved in the establishment of this unique feature of JCML.\textsuperscript{11,13} The recent studies reported by Bashey et al.\textsuperscript{23} who detected mutations of the \textit{N-ras} gene in varying proportions of, but not all, leukemic colonies in vitro, and that reported by Kubo et al.\textsuperscript{24} who found polyclonality of \textit{ras} gene mutations in AML, suggest that the whole population of cells in a leukemic clone does not harbor the mutations and, therefore, that the mutations of the \textit{N-ras} gene are not involved in the initial step of leukemogenesis. In the case of patient no. 16, it is

Mutations of the \textit{N-ras} gene were detected in four patients in chronic phase and two patients at blast crisis, suggesting that apparently there is no correlation between the presence of \textit{N-ras} gene mutations and progression to blast crisis. In patient no. 16, a mutation was present at codon 13 in chronic phase, but disappeared after blast crisis. The disappearance of initially detected mutations\textsuperscript{15,19,20,25,39} or appearance of new mutations at relapse\textsuperscript{19,22} has been reported in AML or ALL. The recent studies reported by Bashey et al.\textsuperscript{23} who detected mutations of the \textit{N-ras} gene in varying proportions of, but not all, leukemic colonies in vitro, and that reported by Kubo et al.\textsuperscript{24} who found polyclonality of \textit{ras} gene mutations in AML, suggest that the whole population of cells in a leukemic clone does not harbor the mutations and, therefore, that the mutations of the \textit{N-ras} gene are not involved in the initial step of leukemogenesis. In the case of patient no. 16, it is

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Stage</th>
<th>Material</th>
<th>\textit{N-ras} Mutation</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>CP</td>
<td>PB</td>
<td>Codon 13, GGT\textsuperscript{Gly} \rightarrow GAT\textsuperscript{Asp}</td>
</tr>
<tr>
<td>2</td>
<td>CP</td>
<td>BM</td>
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<tr>
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<td>CP</td>
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<td>5</td>
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<td>CP</td>
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</tr>
<tr>
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<td>CP</td>
<td>LN</td>
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<tr>
<td>8</td>
<td>CP</td>
<td>BM</td>
<td>--</td>
</tr>
<tr>
<td>9</td>
<td>CP</td>
<td>BM</td>
<td>Codon 12, GGT\textsuperscript{Gly} \rightarrow TGT\textsuperscript{Cys}</td>
</tr>
<tr>
<td>10</td>
<td>BC</td>
<td>LN</td>
<td>Codon 13, GGT\textsuperscript{Gly} \rightarrow GAT\textsuperscript{Asp}</td>
</tr>
<tr>
<td>11</td>
<td>BC</td>
<td>LN</td>
<td>--</td>
</tr>
<tr>
<td>12</td>
<td>CP</td>
<td>PB</td>
<td>--</td>
</tr>
<tr>
<td>13</td>
<td>CP</td>
<td>PB</td>
<td>Codon 12, GGT\textsuperscript{Gly} \rightarrow TGT\textsuperscript{Cys}</td>
</tr>
<tr>
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<td>CP</td>
<td>BM</td>
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<td>15</td>
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<td>BM</td>
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</tr>
<tr>
<td>20</td>
<td>CP</td>
<td>PB</td>
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</table>

Abbreviations: CP, chronic phase; BC, blast crisis; EL, erythroleukemic phase; PB, peripheral blood; BM, bone marrow; LN, lymph node.
possible that the disappearance of the cells with the mutation at blast crisis was caused by the overgrowth of subclones that did not harbor the mutation. In the case of patient no. 1, in contrast, the same point mutation at codon 13 of the N-ras gene was detected 3 years after the initial examination, during which the patient’s hematological features remained well controlled. Therefore, the presence of mutations of the N-ras gene alone may not be sufficient to cause aggressive malignant disease. Evidence for this possibility has been provided by Finney and Bishop, who showed that the activated H-ras1 allele is not by itself dominant over the normal allele, and requires other events, such as amplification of the mutant allele, to transform cells, and by Cohen and Levine, who demonstrated that the transforming activity of the H-ras gene is regulated by a second single nucleotide alteration in the last intron.

Clinical information from the patients was compared between those with and without mutations of the N-ras gene (Table 1), but no particular correlations between the presence of mutations and clinical parameters were noted. Concerning the prognosis of JCML, data have been provided by other investigators that there are two subgroups of patients with JCML, which differ in mortality rates; these investigators found that short-term survivors could be predicted based on initial clinical characteristics, including older age (≥2 years). Another group of investigators also reported a different prognosis between two groups of patients, namely, infants (<1 year old) with good prognosis and children (≥1 year old) with poor prognosis. Regarding our patients, a clear distinction was made between those older and younger than 6 months of age, who showed poor and good prognosis, respectively, a finding in agreement with the data described earlier that older patients have a poor prognosis. Although most (five of six) of the patients with N-ras gene mutations were in the older age group, the number of patients was insufficient for statistical analysis and did not allow a clear-cut conclusion as to the prognostic role of N-ras gene mutations in JCML. Nevertheless, it remains possible that the mutations might play a role in the establishment of a more malignant nature of the disease in the older age group. On the other hand, the younger age group may be heterogeneous, as has been suggested by others, and may include patients with nonleukemic perturbed hematopoiesis of unknown cause. However, the presence of a patient with N-ras gene mutation in the younger age group suggests that these two age groups are not distinct and that the presence of mutations of the N-ras gene by itself does not necessarily indicate poor prognosis. JCML may affect a spectrum of patients ranging from infants to older children, the former group having nonleukemic perturbed hematopoiesis of unknown cause.

It is well known that myeloproliferative disorders associated with monosomy 7 closely resemble JCML and that there is substantial overlap between the two. Although some investigators suggested the usefulness of elevated fetal hemoglobin as a diagnostic criterion for JCML versus monosomy 7, there have been no widely accepted ways of distingushing between the two. We therefore included three patients with JCML associated with monosomy 7 in our series. Although a slightly lower incidence of mutations of the N-ras gene in monosomy 7 syndrome has been reported, and none of our patients with chromosomal abnormalities including monosomy 7 and a related aberrant karyotype (deletion 7q22) harbored mutations, the significance of the difference in the frequency of mutations of the N-ras gene needs to be further clarified.

SSCP analysis detects nucleotide sequence alterations as electrophoretic mobility shifts of single-stranded nucleic acids caused by changes in their three-dimensional structure. Although it has been considered that alterations in nucleotide sequence may not necessarily affect electrophoretic mobility, a perfect concordance with the results of direct sequence analysis has been demonstrated. However, we found false-negativity in SSCP analysis in two of 27 patient samples in this study. The sensitivity of SSCP and direct sequence analyses, namely, the minimal proportion of abnormal cells detectable by these methods, has been estimated to be similar, ie, less than 10%. These considerations suggest that the false-negativity that we found in SSCP analysis is unlikely to be ascribable to sensitivity. It is known that the conformational changes of a single-stranded DNA molecule are influenced by the physical environment in the gel, including temperature, concentrations of ions, and solvents. Since we examined different temperature conditions (room temperature versus 4°C) in our preliminary experiments, and demonstrated higher resolution under the latter condition with our PCR products, electrophoresis was performed at 4°C in the subsequent experiments. Other physical conditions of electrophoresis that we used, including 6% acrylamide gel and 10% glycerol, have been widely used by other investigators. These considerations suggest that the false-negativity that we found in SSCP analysis is unlikely to be due to technical problems. On the other hand, the fact that only positive cases in SSCP analysis have been used for sequence analyses in most studies performed by other investigators suggests that, even if positive cases yielded results concordant with sequence analyses, they might have missed false-negative cases in SSCP analysis. In light of our present findings, false-negativity in SSCP analysis may not be as rare a phenomenon, as has been considered, and the determination of nucleotide changes will be necessary for a precise analysis of gene abnormalities.

ACKNOWLEDGMENT

The authors thank Drs M. Ichikawa, R. Hanada, Y. Kaneko, Y. Nasu, K. Horio, K. Hattori, and H. Hara for providing patient samples and clinical information.

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