N-ras Mutations in Adult De Novo Acute Myelogenous Leukemia: Prevalence and Clinical Significance

By Jerald P. Radich, Kenneth J. Kopecky, Cheryl L. Willman, James Weick, David Head, Frederick Appelbaum, and Steven J. Collins

Point mutations of the N-ras proto-oncogene have been previously detected in 20% to 60% of samples of acute myelogenous leukemia (AML), but the clinical significance of these mutations has not been defined. The ras proto-oncogene family consists of at least three members (Ha-, Ki-, and N-ras), each coding for a 21-Kd protein that localizes to the inner plasma membrane and has GTP binding and hydrolysis activity. Although the exact physiologic role of these proteins is unknown, they may be involved in signal transduction from the extracellular environment to the cytoplasm. Point mutations at critical regions of the ras gene presumably decrease the GTPase activity of these proteins, locking them in the "on" mode and leading to transformation. Activation of one of the ras genes as demonstrated by the NIH3T3 transformation assay has been found in 10% to 20% of a wide variety of human malignancies. Nearly 90% of the mutations in the ras proto-oncogene family in human AML involve N-ras and selectively affect codons 12, 13, and 61. Activated N-ras genes were initially detected by the NIH3T3 transformation assay in approximately 50% of patient AML samples. Bos et al used oligonucleotide hybridization to identify AML samples with point mutations in N-ras codons 12, 13 or 61 and found mutations in five of eight samples. A larger survey by the same authors revealed N-ras mutations in an additional 5 of 37 patients. Other studies involving analysis of PCR amplified N-ras sequences have documented N-ras mutations in 15% to 30% of AML samples.

Although N-ras mutations frequently occur in AML, no previous studies have determined whether AML patients with N-ras mutations have a unique clinical presentation or prognosis. To address this question, we studied 55 adults with de novo AML entered onto a single clinical trial to investigate the frequency of N-ras mutations and their association with pretreatment clinical variables, response to induction chemotherapy, and survival.

METHODS AND MATERIALS

Patients and samples. Patients who were registered to Southwest Oncology Group (SWOG) Leukemia Protocol 8600 (a comparison of standard dose versus high-dose cytarabine arabinoside for induction chemotherapy in de novo AML patients ages 17 to 64 years) were eligible for this study. Patients with a prior history of hematologic disease that might predispose to AML (i.e., myelodysplastic syndrome, polycythemia vera, essential thrombocythemia, paroxysmal nocturnal hemoglobinuria [PNH]), relapsed AML, and prior alkylating-agent chemotherapy were excluded from the SWOG protocol. Pretreatment samples of the patients' peripheral blood or bone marrow (whichever sample had greater than 50% blasts) were sent directly to the SWOG myeloid repository at the University of New Mexico Cancer Center. Mononuclear cells were isolated by a ficoll gradient separation; RNA and DNA were prepared from these cells by the standard guaninium method. The isolated DNA underwent three phenol-chloroform extractions and after ethanol precipitation was resuspended in Tris:Ethanol buffer. These coded DNA samples were tested for the presence of N-ras point mutations without access to clinical information.

Polymerase chain reaction amplification of N-ras fragments. The polymerase chain reaction (PCR) was used to amplify a 238-bp fragment harboring N-ras codons 12 and 13 and a 111-bp fragment containing codons 59 to 63 from leukemic sample DNAs. The specific oligonucleotides and the PCR conditions used to generate these amplified fragments have been previously described. Two modifications were made: less genomic template (250 ng rather than 500 ng) and less oligonucleotide primers (100 ng of each primer rather than 500 ng) were used, yielding decreased background amplification while having no apparent affect upon the quantity of desired product.

Direct nucleotide sequencing of the amplified fragments. PCR products were visualized on a 5% polyacrylamide gel and eluted as

From the Molecular Medicine Program, Southwest Oncology Group Statistical Center, and the Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA; the Department of Medicine, University of Washington, Seattle, WA; the Departments of Pathology and Cell Biology, University of New Mexico School of Medicine, Albuquerque, NM; the Cleveland Clinic Foundation, Cleveland, OH; the Department of Pathology, St. Jude Children's Research Hospital, Memphis, TN; and the Southwest Oncology Group Leukemia Biology Program, San Antonio, TX.

Submitted November 20, 1989; accepted April 25, 1990.

Supported by National Institutes of Health Grants No. CA40728 (S.J.C.) and CA32102 (Southwest Oncology Group Leukemia Biology Program and Leukemia Committee).

Address reprint requests to Jerald P. Radich, MD, Molecular Medicine Program, Fred Hutchinson Cancer Research Center, 1124 Columbia St, AB 133, Seattle, WA 98104.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.

0006-4971/90/7604-0017$3.00/0
previously described. Direct nucleotide sequencing was performed using a protocol modified from Winship. A 6-μL total annealing mix containing approximately 30 to 50 ng of the PCR DNA product, 20 pmol of oligonucleotide primer, 1 μL of 5x Sequenase buffer (USB), and 10% dimethyl sulfoxide (DMSO) was boiled for 2 minutes, snap cooled on dry ice, then placed on wet ice. Four microliters of labeling mix including 1 μL of 0.1 mol/L DTT, 1 μL of S32-dATP, and 2 μL of 1:8 diluted Sequenase (USB) enzyme was added. This mix was then divided equally into four tubes, each holding 2 μL of deoxy/deoxy nucleotide mixes (80 μM dCTP, dGTP, dTTP, 50 mmol/L NaCl, 10% DMSO, and 0.08 μmol/L ddATP [tube A], 8 μmol/L dCDCTP [tube C], 8 μmol/L ddGTP [tube G], or 8 μmol/L ddTTP [tube T]). These tubes were incubated at 37°C for 2 minutes, then snap cooled on dry ice. Two microliters of a “chase” mix of 0.25 mmol/L each dATP, dCTP, dGTP, dITP, 50 mmol/L NaCl, and 0.08 μmol/L ddATP was added. This mix was then divided equally into four tubes, each holding 2 μL of deoxy/deoxy nucleotide mixes (80 μM dCTP, dGTP, dTTP, 50 mmol/L NaCl, 10% DMSO, and 0.08 μmol/L ddATP [tube A], 8 μmol/L dCTP, dGTP, dITP, and 10% DMSO was added, incubated at 37°C for 2 minutes, followed by the addition of 4 μL formamide stop mix. After boiling for 2 minutes, 2 μL was loaded and electrophoresed on an 8% denaturing polyacrylamide gel. All sequencing ladders that either showed N-ras mutations or were ambiguous were resequenced on the opposite strand using the appropriate oligonucleotide primer. When it was necessary to read sequences close (25 bp) to the primer, the concentration of ddNTPs in the above mixtures were increased fivefold.

Statistics and patient exclusions. Samples from 55 adult patients enrolled in SWOG study 8600 were analyzed. Samples for seven other leukemia patients (three with mutations, four without) were also analyzed: two of these (one with an N-ras mutation) were registered on a SWOG relapse/refractory study rather than 8600; one patient (without a mutation) was registered on a SWOG ALL study; and four (with two with N-ras mutations) were not registered on any SWOG study. This report focuses on the 55 de novo AML patients in study 8600. Pretreatment data (age, sex, French-American-British [FAB] classification, peripheral blood and bone marrow counts) were available for all 55 of these patients. Comparisons of pretreatment variables between patients with and without N-ras mutations were based on the Wilcoxon rank-sum test; induction therapy response rates and FAB classifications were compared by Fisher’s exact test. Durations of survival (calculated from the day of registration on study 8600) of patients with and without N-ras mutations were compared by the logrank test. Survival curves were calculated by the method of Kaplan and Meier.

RESULTS

Sensitivity of the PCR-direct sequencing technique. To determine the sensitivity of our approach in detecting subpopulations of leukemic cells harboring N-ras mutations, we mixed different numbers of HL-60 cells (which harbor a CAA → CTA mutation in N-ras codon 61) with normal peripheral-blood mononuclear cells, extracted the genomic DNA, used the PCR to amplify the 111-bp fragment containing N-ras codons 59 through 63 and sequenced the amplified product (Fig 1). The A → T mutation noted in the HL-60 cells (the A represents the nucleotide from the wild-type allele, the T the mutation) is visible even at the dilution 10%:90% HL-60: normal cells (Fig 1). Sequencing of the opposite strand showed a similar sensitivity of approximately 10% (data not shown). This represents an improvement over the 25% sensitivity we previously reported using 32P-labeled oligonucleotides for direct sequencing. Thus, our approach is capable of detecting N-ras point mutations if present in at least 10% of the cell sample.

N-ras mutations. N-ras mutations were detected in 8 of 55 (15%; 95% confidence interval [CI] 7% to 28%) of patients with de novo AML (Table 1). Five of these patients had mutations in codon 12 or 13 only; one had a mutation in codon 61 only; one had mutations in both codons 12 and 61; and one patient had a mutation in codon 60. All mutations of codons 12 and 13 were GGT → GAT, resulting in glycine being replaced by aspartic acid. Of the seven patients analyzed not on SWOG study 8600, three had N-ras point mutations. One patient enrolled in a SWOG relapse AML study had a codon 61 mutation, and two patients not enrolled in any SWOG study had mutations in codons 12 and 13, respectively. Both of the latter mutations were also GGT → GAT substitutions.

The GGA → AGA mutation of N-ras codon 60 (Fig 2) has not been previously described. This mutation was confirmed both by sequencing the opposite strand and by repeating the PCR on the patient’s genomic DNA and sequencing each strand again. Unfortunately we are unable to study remission blood or other tissue from this patient to
determine if this mutation represents a naturally occurring polymorphism.

Analysis of clinical variables. The comparison of pretreatment variables is shown in Table 2. Patients with and without the N-ras mutation had similar peripheral and bone marrow absolute and differential counts. There was a higher proportion of FAB M4 classified AMLs among the patients with N-ras point mutations (38%) compared with those without the mutation (13%); this difference was marginally statistically significant ($P = .04$).

Five of eight (63%) patients with the N-ras mutation achieved a complete response, as opposed to 20 of 47 (43%) without the mutation (Table 3; two-tailed $P = .37$). Correspondingly, a lower percentage of patients with N-ras mutations did not respond to therapy compared with those without mutations (13% versus 43% with no response to induction therapy).

Figure 3 shows survival curves for those with and without mutations. One (13%) of the 8 N-ras mutation-positive patients is alive, with the median survival of this group 14.1 months, compared with 8 of 47 (17%) mutation-negative patients, with a median survival of 7.1 months (two-tailed $P = .89$ by logrank test).

---

### Table 1. Characteristics of Adult De Novo AML Patients With N-ras Mutations

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Wild-Type Allele</th>
<th>Mutation</th>
<th>Sample Source</th>
<th>FAB</th>
<th>Response</th>
<th>Status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>45/M</td>
<td>13 GGT Gly</td>
<td>GAT Asp</td>
<td>PB</td>
<td>M1</td>
<td>NR</td>
<td>D (154)</td>
</tr>
<tr>
<td>17</td>
<td>38/M</td>
<td>13 GGT Gly</td>
<td>GAT Asp</td>
<td>PB</td>
<td>M4</td>
<td>ED</td>
<td>D (3)</td>
</tr>
<tr>
<td>28</td>
<td>54/F</td>
<td>12 GGT Gly</td>
<td>GAT Asp</td>
<td>PB</td>
<td>M2</td>
<td>CR</td>
<td>D (454)</td>
</tr>
<tr>
<td>46</td>
<td>21/M</td>
<td>13 GGT Gly</td>
<td>GAT Asp</td>
<td>PB</td>
<td>M2</td>
<td>CR</td>
<td>D (546)</td>
</tr>
<tr>
<td>49</td>
<td>35/M</td>
<td>13 GGT Gly</td>
<td>GAT Asp</td>
<td>PB</td>
<td>M5</td>
<td>CR</td>
<td>D (429)</td>
</tr>
<tr>
<td>55</td>
<td>57/M</td>
<td>60 GGA Gly</td>
<td>AGA Arg</td>
<td>BM</td>
<td>M6</td>
<td>CR</td>
<td>D (424)</td>
</tr>
<tr>
<td>57</td>
<td>58/F</td>
<td>13 GGT Gly</td>
<td>GAT Asp</td>
<td>BM</td>
<td>M4</td>
<td>CR</td>
<td>A (856)</td>
</tr>
<tr>
<td>58</td>
<td>24/F</td>
<td>61 CAA Gin</td>
<td>CAT His</td>
<td>PB</td>
<td>M4</td>
<td>PR</td>
<td>D (205)</td>
</tr>
</tbody>
</table>

Three AML patients had N-ras mutations but were not on SWOG study 8600: Patient 4 was a 28-year-old male with M1 AML and harbored a codon 13 GGT $\rightarrow$ GAT mutation; Patient 39 was a 29-year-old male with M3 AML with a codon 12 GGT $\rightarrow$ GAT mutation; and patient 48 was an 18-year-old male with M1 relapsed AML harboring a codon 61 CAA $\rightarrow$ AAA mutation.

Abbreviations: PB, peripheral blood; BM, bone marrow; AA, amino acid; CR, complete remission; PR, partial remission; NR, no response to induction therapy; ED, early death (ie, before assessment of response); A, alive; D, dead.

*Values in parentheses refer to number of days of follow-up at the time of this analysis.

---

DISCUSSION

Point mutations involving the ras family of proto-oncogenes have been frequently described in AML, and approximately 90% of such mutations involve the N-ras gene. It is presently unclear why N-ras mutations predominate in AML or whether such mutations have any clinical significance. Previous studies of patients with myelodysplastic syndromes suggested a role of N-ras activation in the...
progression to AML. Liu et al. found mutations of Ki-ras in 3 of 5 myelodysplastic patients, all of whom evolved to AML. Hirai et al. found that 3 of 3 patients with N-ras mutations transformed to AML, whereas 5 without did not. Padua et al., studying 50 patients with myelodysplasia noted mutations of H-ras in 2, K-ras in 6, and N-ras in 14 (2 patients had mutations in two different ras genes). Eight of 21 patients with a mutation progressed to AML, but only 4 of 29 without mutations progressed. In contrast, studies by Lyons et al. and Bar-Eli et al. failed to show any association of progression to AML with a ras mutation. Since the N-ras mutation is a common but not universal abnormality in AML and given the suggestion in the studies above that such mutations may influence the tempo of malignant transformation, we wondered if the N-ras mutation might define a unique subset of adult de novo AML patients in regards to disease presentation, response to chemotherapy, and overall clinical course.

We used direct sequencing of PCR amplified N-ras fragments to study these questions. This approach has several advantages over other technology used to detect ras point mutations. The NIH3T3 transformation assay, while quite sensitive, is very labor intensive and time consuming, and activating point mutations conceivably could arise during the procedure. PCR and oligonucleotide hybridization have a sensitivity of approximately 5% to but identifying point mutations are limited to the specific oligonucleotides synthesized. Therefore mutations arising at a nearby site to the codon of interest would not be detected (e.g., the mutation noted here in codon 60 might not be recognized in screening for codon 61 mutations). Adjacent point mutations in the same codon, such as that previously described in K-ras codon 12 (GGT→TTT) of a breast cancer-cell line, would also be missed. Direct sequencing allows for unambiguous identification of the DNA sequence of interest. The protocol described here has a sensitivity of approximately 10%, which compares favorably with oligonucleotide hybridization to PCR amplified products.

N-ras mutations were found in 15% (8/55) of adult de novo AML patients. Recent studies have reported similar results (Table 3), although generally have found a somewhat higher prevalence of N-ras mutations. The lower prevalence of N-ras mutations in this study may reflect our attempt to limit the analysis to truly de novo AML patients, excluding cases with clinically detected antecedent myelodysplasia, which might contribute a larger share of N-ras mutations. The SWOG 8600 study age exclusion of cases older than 64 years may have limited the number of cases of AML enrolled that actually evolved from myelodysplasia, since the mean age of diagnosis of myelodysplasia may be as high as 67 years of age. Moreover, the protocol excluded cases with a history of prior hematologic disease and chemotherapy, therefore excluding cases where N-ras mutations might arise during AML relapse or from alkylating agents in secondary leukemias. However, there is a suggestion that some of the patients we detected with N-ras mutations may have had antecedent myelodysplasia that was clinically undetected prior to their diagnosis of de novo AML and subsequent enrollment onto SWOG 8600. Of the 8 patients with N-ras mutations, 7 had evaluable cytogenetics, and 1 patient (no. 6) had a 5q-chromosomal abnormality, a cytogenetic syndrome commonly seen in myelodysplasia. However, none of the patients harboring N-ras mutations had another cytogenetic change common in myelodysplasia, such as deletions of chromosomes 7 or 20 or an additional chromosome 8. A review of the N-ras positive patient bone marrows revealed marked dysplastic changes of granulocytic and erythroid precursors in patients 46 and 55, suggestive of myelodysplasia. Thus although a similar analysis was not performed on patients without the N-ras mutation, some evidence suggestive of clinically undetected myelodysplasia was noted in three of eight patients harboring the N-ras mutation. Therefore we cannot exclude that some of the N-ras mutations we detected were contributed by patients with clinically undetected antecedent myelodysplasia; if so, the actual incidence of N-ras mutations in de novo AML might be lower than we have reported here.

Point mutations involving N-ras codons 12 and 13 predominated, occurring in six of eight mutation-positive patients. All of these six were GGT→GAT mutations, causing the amino acid substitution of glycine to aspartic acid. Overall we found G→A substitutions in 7 of 9 N-ras mutations (counting the two mutations in patient 46). This seemingly selective G→A mutation has also been noted by other investigators. It is unclear if the predominance of the

---

**Table 3. Response to Induction Therapy**

<table>
<thead>
<tr>
<th>N-ras Mutation</th>
<th>Absent (%)</th>
<th>Present (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete remission</td>
<td>20 (43)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>Partial remission</td>
<td>1 (2)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>No response</td>
<td>20 (43)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>Early death</td>
<td>6 (13)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>Total known responses</td>
<td>47</td>
<td>8</td>
</tr>
</tbody>
</table>

The complete remission rates (43% versus 63%) are not significantly different (two-tailed P = .37 by Fisher's exact test).

---

**Fig 3.** Kaplan-Meier plots comparing survival of AML patients with (—, N = 8) and without (—, N = 47) the N-ras mutation. Median survivals are 14.1 months for patients with an N-ras mutation and 7.1 months for patients without a mutation (P = .89 by the logrank test).
G → A mutation in codons 12 and 13 represents the effect of a common mutagen or if the G → A mutation simply has a selective survival advantage over other mutations involving these codons. It is interesting that many investigators have noted the high frequency of G → A mutations in codons 12 and 13 despite the heterogeneous nature of the AML populations studied.

We were surprised to find in a single leukemic sample a mutation in N-ras codon 60. This particular mutation has not been previously described in any other human malignancy. We confirmed this mutation by sequencing both DNA strands and by repeating PCR amplification and sequencing from the original genomic sample. Unfortunately we were unable to obtain normal tissue from this patient to determine whether this point mutation represents a somatic polymorphism. If this does represent a naturally occurring polymorphism it must be very rare, as we have not noted it in over 100 patient samples analyzed by direct sequencing of the N-ras locus. The GGA → AGA mutation in codon 60 substitutes a positively charged arginine for a neutral glycine. Could this nonconservative mutation have transforming potential? The p21 protein is thought to act as a signal transducer, involved in relaying signals from the extracellular space to the cytoplasm. The intrinsic GTPase activity of p21 is believed to downregulate the signal, returning p21 to an inactivated state. A cytoplasmic protein, GTPase activating protein (GAP), has been shown to stimulate the intrinsic GTPase activity of p21 by as much as 200-fold. Transforming mutations in ras codons 59 and 61 are associated with diminished p21 GTPase activity, abrogating down-regulation of the activated p21. This uninterrupted activation of signal transduction presumably might contribute to malignant transformation. A mutation of codon 60, sandwiched between codons 59 and 61, where mutations have known transforming potential, might similarly be biologically significant. In addition, it appears that the glycine at codon 60 serves as a "pivot point" in the conformational change that occurs upon activation of p21. A mutation at this critical codon could alter the conformational change necessary for p21 regulation.

We found no significant associations between the presence of the N-ras mutation and response to therapy or survival. Presenting peripheral blood and bone marrow counts were similar for patients with or without N-ras mutations. We did note more FAB M4 subtypes among patients with the N-ras mutation compared to those without. Previous reports have noted a predominance of the M4 subtype in patients with the N-ras mutation, with this classification occurring in 60% to 80% of mutation-positive patients. A mutation simply has a specific morphological subtype. Our study supports an association of the N-ras point mutation with the FAB M4 classification, although the comparison is of borderline statistical significance (P = .04), and we cannot exclude that this is an artifact of multiple statistical comparisons.

The response to induction therapy was higher in the mutation-positive patients, but this difference did not achieve statistical significance. Interestingly, Bartram et al. found that eight of nine patients positive for the N-ras mutation achieved a complete remission; however, there was no mutation-negative group in the study for comparison. We found no difference in the overall survival of N-ras mutation-positive and -negative patients (Fig 3).

This study has limitations that affect its interpretation. The patient sample size and relatively short clinical follow-up make confident inferences difficult. For example, a comparison based on groups of 8 and 47 patients (two-sided test at the α = .1 critical level) has statistical power of only about 50% to detect the difference between complete response rates of 90% and 50%. Moreover, the role of N-ras in leukemic evolution is clearly quite complicated. Shen et al. assayed p21 protein in AML patients bearing the N-ras mutation and found that mutant p21 protein constituted a minority of the total p21 level, despite the predominance of leukemic blasts in the blood samples. Many authors, including ourselves, have noted weak hybridization or sequence-ladder signals in the mutant allele compared to the wild-type allele despite the abundance of blast cells in the sample (eg, Fig 2). These observations suggest that not all leukemic blasts within a given patient sample harbor the N-ras mutation. Moreover, N-ras mutations have been noted to be absent in relapsed AML patients who were initially positive for the mutation and have appeared in relapsed AML patients initially negative. In sum, these studies suggest that the N-ras mutation might play a role in AML but may act at various steps affecting growth or differentiation.
Our present observations indicate that adult de novo AML patients exhibiting N-ras point mutations do not behave as a unified group. More patient samples and longer follow-up might possibly define a correlation between the presence of N-ras point mutations and certain clinical parameters. Nevertheless, our data, when considered along with that of other investigators, do not support a clear and consistent association between the N-ras mutation and the clinical presentation or course of adult de novo AML.

In summary, this study (1) describes a direct sequencing technique for detecting N-ras point mutations using 32P-dATP and Sequenase (USB) with a sensitivity of approximately 10%; (2) revealed a previously undescribed point mutation in N-ras codon 60; and (3) found no evidence that the N-ras mutation defines a unique subset of adult AML patients in regard to response to treatment and overall prognosis.

ACKNOWLEDGMENT

We thank Monique Howard and Le Moyne Mueller at the Fred Hutchinson Cancer Research Center for their expert technical assistance and Barbara B. Griffith for isolating the patient DNA samples from the SWOG Myeloid Repository at the University of New Mexico.

REFERENCES


N-ras mutations in adult de novo acute myelogenous leukemia: prevalence and clinical significance

JP Radich, KJ Kopecky, CL Willman, J Weick, D Head, F Appelbaum and SJ Collins

Updated information and services can be found at:
http://www.bloodjournal.org/content/76/4/801.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml