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.1-3 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.4 Nearly 90% of the mutations in the ras proto-oncogene family in human AML involve N-ras and selectively affect codons 12, 13, and 61.5-13 Activated N-ras genes were initially detected by the NIH3T3 transformation assay in approximately 50% of patient AML samples.5,6 Bos et al5 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 authors5,6 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.5,13

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 (ie, myelodysplastic syndrome, polycythemia vera, essential thrombocytosis, 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 guanidinium method.14 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)15 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.16 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...
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 S"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/ideoxy nucleotide mixes (80 μM dCTP, dGTP, dTTP, 50 mmol/L NaCl, 10% DMSO, and 0.08 umol/L ddATP [tube A], 8 umol/L ddCTP [tube C], 8 umol/L ddGTP [tube G], or 8 umol/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, dTTP, 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 (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 five 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

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**Fig 1.** Sensitivity of the PCR-direct sequencing assay. HL-60 cells and normal peripheral blood mononuclear cells were mixed in the indicated ratios and genomic DNA extracted. The 111-bp fragment harboring N-ras codon 61 was amplified using the PCR and directly sequenced as described in Materials and Methods. The mutant “T” nucleotide is present in the second nucleotide of HL-60 N-ras codon 61.
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 a 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).

**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

### 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></td>
<td></td>
<td>Codon</td>
<td>AA</td>
<td>Codon</td>
<td>AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>45/M</td>
<td>13 GGT</td>
<td>Gty</td>
<td>GAT</td>
<td>Asp</td>
<td>PB</td>
<td>M1</td>
</tr>
<tr>
<td>17</td>
<td>38/M</td>
<td>13 GGT</td>
<td>Gty</td>
<td>GAT</td>
<td>Asp</td>
<td>PB</td>
<td>M4</td>
</tr>
<tr>
<td>28</td>
<td>54/F</td>
<td>12 GGT</td>
<td>Gty</td>
<td>GAT</td>
<td>Asp</td>
<td>PB</td>
<td>M2</td>
</tr>
<tr>
<td>46</td>
<td>21/M</td>
<td>13 GGT</td>
<td>Gty</td>
<td>GAT</td>
<td>Asp</td>
<td>PB</td>
<td>M2</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 → GAT mutation; Patient 39 was a 29-year-old male with M3 AML with a codon 12 GGT → GAT mutation; and patient 48 was an 18-year-old male with M1 relapsed AML harboring a codon 61 CAA → 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.

**Table 2. Summary of Pretreatment Characteristics**

<table>
<thead>
<tr>
<th>N-ras Mutation</th>
<th>Absent (N = 47)</th>
<th>Present (N = 8)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>45 (17, 64)</td>
<td>43 (21, 58)</td>
<td>.38</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td>35.9 (18.1, 218)</td>
<td>21.7 (1.5, 301)</td>
<td>.93</td>
</tr>
<tr>
<td>Blasts (%)</td>
<td>49 (0, 96)</td>
<td>26 (0, 93)</td>
<td>.62</td>
</tr>
<tr>
<td>PMNs (%)</td>
<td>4 (0, 73)</td>
<td>7 (0, 10)</td>
<td>.86</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>17 (0, 68)</td>
<td>14 (0, 91)</td>
<td>.33</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>2 (0, 78)</td>
<td>22 (0, 90)</td>
<td>.19</td>
</tr>
<tr>
<td>Hgb</td>
<td>9.9 (5.3, 13)</td>
<td>9.4 (6.2, 12.3)</td>
<td>.77</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blasts (%)</td>
<td>80 (27, 99)</td>
<td>71 (45, 93)</td>
<td>.46</td>
</tr>
<tr>
<td>Erythroid (%)</td>
<td>4 (0, 41)</td>
<td>5 (0, 24)</td>
<td>.45</td>
</tr>
<tr>
<td>FAB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>17 (36%)</td>
<td>1 (13%)</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>16 (32%)</td>
<td>2 (25%)</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>6 (13%)</td>
<td>3 (38%)</td>
<td>.04</td>
</tr>
<tr>
<td>M5</td>
<td>7 (15%)</td>
<td>1 (13%)</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>0 (0%)</td>
<td>1 (13%)</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated acute leukemia</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Total known FAB</td>
<td>47</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Values in the table are medians (min, max). *P* values are based on the two-tailed Wilcoxon rank-sum test, except for the comparison of proportions with M4 morphology, which was performed by Fisher’s exact test.

Abbreviation: M0, myeloblastic leukemia without differentiation.
progression to AML. Liu et al.\(^23\) found mutations of Ki-ras in
3 of 5 myelodysplastic patients, all of whom evolved to AML.
Hirai et al.\(^24\) found that 3 of 3 patients with N-ras mutations
transformed to AML, whereas 5 without did not. Padua et al.\(^25\)
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.\(^26\) and Bar-Eli et al.\(^27\) failed to show an
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 transforma-
tion, 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 10%,\(^11,24\) but
identification of 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 mutants). 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,\(^29\) 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 oligonu-
cleotide hybridization to PCR amplified products.

N-ras mutations were found in 15% (8/55) of adult de
novo AML patients. Recent studies\(^5,13\) have reported similar
results (Table 4), 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.\(^3,27\) The
SWOG 8600 study age exclusion of cases older than 64 years
may have limited the number of cases of AML enrolled that
to actually evolved from myelodysplasia, since the mean age
of diagnosis of myelodysplasia may be as high as 67 years of
age.\(^30\) 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\(^3\) or from alkylating agents in secondary leukemias.\(^13,31\) 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 syn-
drome commonly seen in myelodysplasia.\(^30,32\) However, none
of the patients harboring N-ras mutations had other cytoge-
netic changes common in myelodysplasia, such as deletions
of chromosomes 7 or 20 or an additional chromosome 8.\(^30\) 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 myelodyspla-
sia. Thus although a similar analysis was 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 unde-
tected antecedent myelodysplasia; if so, the actual incidence of
N-ras mutations in de novo AML might even be lower than we have reported here.

Point mutations involving N-ras codons 12 and 13 predomin-
nated, 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.\(^5,10,12\) It is unclear if the predominance of the
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 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

Table 4. Summary of Previous Studies of ras Mutations in AML

<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Total ras Family (Ha, Ki, N-)</th>
<th>N-ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bos et al⁹</td>
<td>NIH3T3 transfection and oligonucleotide hybridization</td>
<td>5/8 (63)</td>
<td>5/8 (63)</td>
</tr>
<tr>
<td>Needleman et al⁸</td>
<td>NIH3T3 transfection</td>
<td>3/6 (50)</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>Bos et al⁷</td>
<td>Oligonucleotide hybridization</td>
<td>7/37 (19)</td>
<td>5/37 (14)</td>
</tr>
<tr>
<td>Toksoz et al⁹</td>
<td>NIH3T3 transfection</td>
<td>6/22 (27)</td>
<td>4/22 (18)</td>
</tr>
<tr>
<td>Janssen et al⁸</td>
<td>PCR and oligonucleotide hybridization</td>
<td>5/9 (56)</td>
<td>4/9 (44)</td>
</tr>
<tr>
<td>Senn et al¹⁰</td>
<td>Oligonucleotide hybridization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farr et al¹¹</td>
<td>PCR and oligonucleotide hybridization</td>
<td>14/52 (27)</td>
<td>14/52 (27)</td>
</tr>
<tr>
<td>Browett et al¹²</td>
<td>PCR and oligonucleotide hybridization</td>
<td>6/28 (21)</td>
<td>5/28 (18)</td>
</tr>
<tr>
<td>Bartram et al¹³</td>
<td>PCR and oligonucleotide hybridization</td>
<td>15/57 (26)</td>
<td>13/57 (23)</td>
</tr>
<tr>
<td>This study</td>
<td>PCR and direct sequencing</td>
<td></td>
<td>8/56 (15)</td>
</tr>
</tbody>
</table>

Transforming mutations in ras in leukemic evolution is clearly quite complicated. Shen et al.²⁸ 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 mutation¹¹,³⁶ and have appeared in relapsed AML patients initially negative.¹³ 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 S35-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.

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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