Longitudinal Analysis of Point Mutations of the N-ras Proto-oncogene in Patients With Myelodysplasia Using Archived Blood Smears

By Harmen van Kamp, Christine de Pijper, Mattie Verlaan-de Vries, Johannes L. Bos, Chris H.W. Leeksma, Hans Kerkhofs, Roei Willemze, Willem E. Fibbe, and James E. Landegent

We performed a longitudinal analysis of point mutations of the N-ras proto-oncogene in patients with myelodysplasia and a follow-up of at least 2.5 years after diagnosis. Point mutations at codons 12, 13, and 61 of the N-ras oncogene were analyzed after in vitro amplification of N-ras specific sequences followed by dot-blot hybridization. Lysed cells scraped from archived blood and bone marrow smears were used as template for a polymerase chain reaction. In 3 of 90 patients tested (3.3%), a mutation in codon 12 could be detected in the most recent blood sample. All available blood and bone marrow samples of these patients were subsequently analyzed for the occurrence of that particular mutation. In all three cases the mutation was not detectable at diagnosis, but was acquired later during the course of the disease. In two of these patients this event was associated with rapid deterioration and transformation to acute leukemia. However, the third patient showed a protracted course during a period of 5 years after acquisition of the mutation. These results indicate that activation of the N-ras proto-oncogene in these three patients represents a secondary phenomenon associated with disease progression in some cases, but compatible with stable disease in others.

© 1992 by The American Society of Hematology.
anemia (RA, 24), RA with ringed sideroblasts (RARS, 33), chronic
myelomonocytic leukemia (CMML, 17), RA with excess blasts
(RAEB, 4) according to the FAB criteria, and unclassifiable MDS
(12) made between 1958 and 1984.14 None of the patients were
alive at the time of the study. Clinical data were obtained from the
patient records (Table 1). Disease progression was defined either
due to progressive bone marrow failure resulting in an increasing need
for red blood cell transfusions (including the need for transfusions
in patients who were previously transfusion independent or a doubling
of the number of transfusions over a 6-month period), by the
occurrence of infections (as documented by positive cultures or fever
responding to antimicrobial therapy) and hemorrhage (for
which platelet transfusions were indicated), or by an increasing
number of immature myeloid cells (blasts) or monocytes in blood
and bone marrow resulting in a different FAB subtype. Involvement
of the peripheral blood with more than 25% and of the bone
marrow with more than 30% myeloblasts was diagnostic for
transformation to acute leukemia. Archived blood smears were
available with intervals ranging from 1 week to 6 months. For
screening purposes, the most recent smears were tested for the
presence of an N-ras oncogene mutation. In those cases with a
mutation in the screening sample, all blood and bone marrow
smears taken during the interval between diagnosis and death were
studied.

Sample preparation. To obtain DNA for in vitro amplification,
cells were scraped from the object glasses and suspended in 200 μL
of the PCR buffer that consisted of 10 mmol/L Tris-HCl, pH 8.3,
3.0 mmol/L MgCl2, 50 mmol/L LiCl, 0.01% gelatine (wt/vol), to
which 0.45% NP40 (vol/vol), 0.45% Tween 20 (vol/vol), and
prodana (1 μg/μL; Boehringer, Mannheim, Germany) were added.
The samples were incubated overnight at 37°C, heated at 95°C for
10 minutes, and centrifuged at 14,000 rpm.

N-ras mutation detection. PCR and subsequent hybridization
with mutation-specific oligomers was performed as described
before15 with minor modifications. Briefly, 10 μL of the sample
supernatants was added to 80 μL of the PCR buffer containing 25
pmol of each of the amplifiers localized upstream and downstream
of either the codons 12 or 13, or codon 61 of the N-ras oncogene.
After incubation at 95°C for 5 minutes, PCR was initiated by the
addition of 2.5 U Taq polymerase (Cetus, Emeryville, CA) in 10 μL
buffer, also containing each deoxynucleotide triphosphate (to a
final concentration of 0.25 mmol/L each). The amplification
consisted of 33 cycles of denaturation (1 minute 95°C), annealing
(1.5 minutes, 55°C), and chain elongation (1.5 minutes, 72°C) using
a thermocycler (Bio-med, Theres, Germany). The generated DNA
fragments of 109 bp (N12/13) or 103 bp (N61) were analyzed by gel
electrophoresis. Aliquots of 2.5 μL of the in vitro amplified DNA
were spotted onto Nylon filters (Gene Screen Plus; Du Pont,
Boston, MA) and cross-linked by illumination with a 254-nm W
lamp (1.6 kJ/m2). Filters were hybridized for 1.5 hours in 3.0 mol/L
SSPE (1X SSPE = 10 mmol/L sodium phosphate pH 7.0, 0.18
mol/L NaCl, and 1 mmol/L EDTA), 0.1% SDS for 10 minutes at
62°C (N12/13) or 59°C (N61). Filters were exposed to Fuji X-ray
films (Tokyo, Japan) at −80°C for 16 hours using intensifying
screens.

RESULTS

As shown by gel electrophoresis and confirmed by positive
wild-type hybridization signals, the specific fragments
containing the codons 12 and 13 and codon 61 of the N-ras
oncogene were successfully amplified from the latest available
blood smears of all 90 patients with MDS. These samples were screened for all possible mutations at these
codons (17 in total). No mutations in the codons 13 and 61
were detected in any of the patients. However, in three of
them (3.3%) a point mutation in codon 12 was found, one
resulting in a Gly → Cys and two in a Gly → Asp
substitution. The earlier blood and bone marrow smears
were subsequently examined for the presence of that
particular mutation. The sensitivity of the procedure was
determined by detecting the N61-ras (Gln → Leu) mutation
in cells from the HL-60 leukemia cell line,15 titrated
against peripheral blood cells from a normal individual.
The final concentration of cells was 5 × 10⁶/mL. Five
microfilters of these samples (ie, 25,000 cells) were smeared
onto glass slides. The N-ras mutation could be demonstrated
in the samples containing 1% to 5% HL-60 cells, indicating that 250 to 1,250 mutated cells can be detected
(data not shown).

Patient T.Y. (Fig 1) was a 69-year-old man, when, at the
end of 1970, RA was diagnosed. Karyotyping showed a
clone with an extra chromosome of the C-group (47,XY,+C;
no banding techniques were available at that time). In early
1972 the bone marrow smear showed progression toward
RAEB, with 6% myeloblasts. In January 1973 he developed
a rapidly progressive leukocytosis (up to 50.0 × 10⁹/L) with
the appearance of myeloblasts in the peripheral blood (up
to 14.0 × 10⁹/L). Until that time no N-ras mutations were
detected in smears from peripheral blood and bone
marrow. However, in January 1973 an N12-ras (Gly → Cys)
mutation was detected. The mutation signal was of a lower
intensity than the wild-type signal, indicating that the activated
ras gene was present in a subpopulation of cells. The
patient died due to recurrent urogenital infections in
April 1974.

Patient L.B. (Fig 2) was a 63-year-old woman, when in
April 1966, RA was diagnosed. Karyotyping was not
performed. She showed a protracted course until in 1974 a
progression toward CMML occurred, with 7% blast cells in
the bone marrow smear. From that moment onward, a
steady increase in leukocyte count with progressive monocytosis
developed over a period of several years until the bone

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Patients</th>
<th>Progression (%)</th>
<th>Transformation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>24</td>
<td>18 (75)</td>
<td>6 (25)</td>
</tr>
<tr>
<td>RARS</td>
<td>33</td>
<td>16 (48)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>RAEB</td>
<td>17</td>
<td>7 (41)</td>
<td>3 (18)</td>
</tr>
<tr>
<td>CMML</td>
<td>12</td>
<td>6 (50)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>Unclassifiable</td>
<td>12</td>
<td>6 (50)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>48 (54)</td>
<td>17 (19)</td>
</tr>
</tbody>
</table>
marrow smear in 1976 ultimately showed a diagnosis of AML (40% bone marrow blasts). Cytogenetic analysis of bone marrow cells at that time showed an 47,XX, t(6;?)q13;?) in all metaphases analyzed. At that moment, an N-ras (Gly → Asp) mutation was detected for the first time in peripheral blood and bone marrow cells. The intensity of the mutation signal finally equaled the intensity of the wild-type signal, indicating that in the end on average each cell harbored the mutated N-ras allele in addition to the normal allele. She died in December 1976 due to cerebral infarction.

Patient J.W. (Fig 3) was an 81-year-old woman when, in 1980, the diagnosis of CMML was established. There were no cytogenetic abnormalities. At diagnosis, no point mutation of the N-ras oncogene was found. From 1982 onward, an N12-ras (Gly → Asp) mutation was detected in peripheral blood leukocytes. The mutation signal showed an increasing intensity that coincided with slowly increasing leukocyte and monocyte counts. At the end of 1983 the diagnosis CMML was confirmed by bone marrow cytology. At that time, the N12-ras mutation was also present in the bone marrow cells containing 5% bone marrow blasts. All
bone marrow metaphases showed a normal karyotype. At the end of 1987, 5 years after the acquisition of the N-ras mutation, a progression of the disease was noted with rapidly increasing leukocyte counts and progressive monocytosis. The patient died in December 1987 at the age of 89 years.

**DISCUSSION**

This study shows that the small number of cells present on archived smears of peripheral blood and bone marrow contain sufficient amounts of DNA for in vitro amplification and subsequent dot-blot hybridization to analyze the presence of point mutations of the N-ras oncogene. In this way, we were able to study patients with MDS for a prolonged period of time. Mutations in the codons 13 and 61 were not detected in any of the samples. In 3 of 90 patients tested (3.3%), an activated N-ras oncogene involving a mutation in codon 12 was found in the most recent blood smears. In these patients the mutation was not detected in the blood and bone marrow smears taken at the time that MDS was diagnosed. In patient T.Y. the clonal nature of the disease was confirmed by the presence of an abnormal karyotype at diagnosis. In the three patients studied, activation of the N-ras oncogene was detectable relatively late during the course of the disease. This does not appear to be a general phenomenon because in a prospective study observing MDS patients up to 18 months Melani et al. observed an N-ras mutation at diagnosis in 3 of 15 patients. The fact that two of these patients had advanced disease (RAEB and RAEB in transformation) at diagnosis may reflect rapid disease progression in patients with an early or primary activation of the N-ras proto-oncogene.

The incidence of N-ras mutations found in the present study was relatively low when compared with other reports and can be explained by patient selection. We selected MDS patients with a long follow-up, which might favor patients with a relatively good prognosis. Patients with CMML appear to have a higher chance of acquiring ras mutations than other patients with MDS. In patients with AML ras mutations are also preferentially found in those cases with monocytic involvement (M4 or M5). In accordance, two of our patients with mutations of the ras oncogene were diagnosed as CMML. However, it is not clear whether an N-ras mutation preferentially influences hematopoiesis to myelomonocytic differentiation or whether myelomonocytic cells are more susceptible for acquiring an N-ras mutation. In patients L.B. and J.W. an activated N-ras oncogene was found after a diagnosis of CMML had been established. These data suggest that N-ras mutations are more likely to develop in cells of myelomonocytic differentiation.

Activation of the N-ras proto-oncogene was associated with evolution to overt leukemia and a poor prognosis in two (T.Y. and L.B.) of the three patients with an N12-ras mutation. In both patients the first detection of the mutation coincided with rapid progression of the disease after a stable period of 2.5 and 10 years, respectively. In contrast, only 15 of 87 (17%) MDS patients without an N-ras mutation in this study showed transformation to acute leukemia. In accordance, other reports have also shown a relation between the occurrence of activation of the ras oncogene and leukemic transformation. Using the NIH/3T3 transfection assay, Hirai et al. found activated N-ras genes in the bone marrow of three of eight patients with MDS. Two of these patients showed rapid progression to overt acute leukemia. The fraction of cells containing the mutated N-ras oncogenes gradually increased in these patients, concurrently with an increase in the fraction of blast cells. However, in the present study not only blast cells, but also more mature cells, appeared to contain the
activated N-ras oncogene because in patients L.B. and J.W. the mutations could also be detected in the blood smears, in which no blast cells were present. In the third patient (J.W.), acquisition of a point mutation of the N-ras proto-oncogene was not associated with disease progression and was compatible with stable disease for many years. Yunis et al. found an N-ras mutation at codon 12 or 13 or a K-ras mutation at codon 12 in 11 of 27 patients with primary MDS. They identified two groups of patients with MDS and a mutated ras gene. The first group has incurred a ras mutation at an early stage in a multipotent stem cell and the mutation is present in all blood and bone marrow cells, including mature T lymphocytes. In these patients, the mutation-specific hybridization signal is of equal intensity or nearly equal intensity as the wild-type hybridization signal. The second group of patients has obtained an ras mutation later during the course of the disease, and the cells harboring the mutation represent a newly evolved cell clone. Initially, the mutation-specific signal is weaker than the wild-type signal in some patients during the course of the disease. In two patients the occurrence of an N-ras mutation was associated with rapidly progressive disease. However, the third patient had a protracted course of the disease over many years after the mutation had been detected, indicating that other factors, which are at present unknown, may contribute to disease progression.

ACKNOWLEDGMENT

The authors thank Anneke Kooreman for her help in preparing the manuscript. We acknowledge Dr. H.L. Haak for his continuing interest and support.

REFERENCES

Longitudinal analysis of point mutations of the N-ras proto-oncogene in patients with myelodysplasia using archived blood smears

H van Kamp, C de Pijper, M Verlaan-de Vries, JL Bos, CH Leeksma, H Kerkhofs, R Willemze, WE Fibbe and JE Landegent