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, Roel 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. Lyzed 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 samples. 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.

MYELODYSPLASTIC SYNDROMES (MDS) comprise a clinically defined group of disorders characterized by a variable degree of anemia, granulocytopenia, and/or thrombocytopenia. The bone marrow is normocellular or hypercellular and shows maturation defects in one or several lineages of differentiation. The French-American-British (FAB) Cooperative Study Group proposed a classification of MDS into five subgroups based on the number of blast cells in peripheral blood and bone marrow, the presence of ringed sideroblasts in the bone marrow, and the number of peripheral blood monocytes. The FAB subtypes correlate with survival and with the risk of transformation to acute leukemia that occurs in approximately 30% of patients.

It has been well known that MDS represent a group of clonal disorders. Cytogenetic abnormalities have been found in 40% to 60% of patients with de novo MDS. Clonal hematopoiesis has also been shown by glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme analysis and studies of patterns of X-chromosome inactivation using restriction fragment length polymorphisms of X-linked genes in conjunction with their methylation pattern. Studies combining karyotyping and G-6-PD analysis in patients with MDS have led to the hypothesis of a multi-step pathogenesis of this disorder. Activation of a proto-oncogene may represent another important event in the development of MDS. In particular, activated ras oncogenes have been found in myelodysplastic disorders.

The family of human ras proto-oncogenes consists of three closely related genes: H-ras, K-ras, and N-ras. They encode homologous GTPase proteins of 21 Kd (p21*) located at the inner surface of the cell membrane. The ras proto-oncogenes acquire their oncogenic potential when point mutations occur at the codons 12, 13, or 61, resulting in a single amino acid substitution in the protein. Activation of one of the ras genes has been detected in a variety of human malignancies. High incidences are found in adenocarcinoma of the pancreas (90%) and colon (50%). In about 30% of cases with acute myeloid leukemia (AML) point mutations have been found, especially of the N-ras oncogene. An activated ras oncogene has also been reported in 6% to 40% of patients with MDS. Several studies have shown a correlation between the presence of an N-ras mutation and the risk of developing acute leukemia. From these studies the presence of a mutation has emerged as an indicator of poor prognosis. However, no longitudinal studies have yet been reported to support this notion.

In the present study we have used archived blood and bone marrow smears from patients with MDS and a follow-up of at least 2.5 years as a source of DNA for in vitro amplification. Point mutations of the N-ras oncogene were detected by amplifying ras-specific sequences using the polymerase chain reaction (PCR) followed by hybridization with mutation-specific oligomers. In three patients an N-ras mutation was detected in the most recent blood smears. It was noted that these mutations were acquired during the course of MDS. The mutational event was associated with evolution to overt leukemia in two patients. However, the third patient showed a protracted course over several years after activation of the N-ras oncogene.

PATIENTS AND METHODS

Patients. Stained archived blood smears from 90 patients with a confirmed diagnosis of MDS and with a follow-up of at least 2.5 years (median 4 years, range 2.5 to 20) were analyzed for the presence of point mutations at codons 12, 13, and 61 of the N-ras oncogene. These patients had an initial diagnosis of refractory...
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. 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 by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts for red blood cell transfusions (including the need for transfusions at the time of the study. Clinical data were obtained from the patient records (Table 1). Disease progression was defined either by 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 an increasing myelomonocytic leukemia (CMML, 17), RA with excess blasts (specific activity of about $10^9$ dpm/µg). The probes, all 20-mers, represent the normal codons as well as all possible mutations (17 in total) that have been shown to activate the N-ras gene. The filters were washed for 15 minutes at 60°C in the same buffer containing 0.1% SDS without the addition of nonfat dry milk. In case negative control samples exhibited a considerable background signal, an additional wash of all filters, either hybridized with the wild-type- or the mutation-specific oligonucleotides, was performed in 5X 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 12 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, titrated against peripheral blood cells from a normal individual. The final concentration of cells was 5 × 10^9/mL. Five microliters 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 × 10^9/L) with the appearance of myeloblasts in the peripheral blood (up to 14.0 × 10^9/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 1. Clinical Characteristics

<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>4</td>
<td>2 (50)</td>
<td>2 (50)</td>
</tr>
<tr>
<td>CMML</td>
<td>17</td>
<td>7 (41)</td>
<td>3 (18)</td>
</tr>
<tr>
<td>Unclassifiable</td>
<td>12</td>
<td>6 (50)</td>
<td>2 (16)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>49 (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 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 these patients. In accordance, in patient T.Y., the mutation represented a late event with the mutation-specific signal showing less intensity than the wild-type signal, indicating that not all cells were affected by the mutation. In the other patients (L.B. and J.W.) that we studied, the occurrence of N-ras activation also appeared to be a late event. The mutation-specific signal gradually equaled the wild-type signal, indicating that ras oncogene activation finally affected the great majority of hematopoietic cells.

The present study shows that the occurrence of N-ras mutations in MDS may represent an acquired phenomenon 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