ABSTRACT

Mutations within N-ras oncogene codons 12, 13, and 61 occur in approximately 25—30% of patients with acute nonlymphocytic leukemia and at a lower frequency (6—20%) in patients with acute lymphocytic leukemia. Moreover, N-ras mutations have been described in patients with chronic myeloid leukemia (CML) in blast crisis but have not been observed during the chronic phase of the disease. In view of the morphological and clinical similarities between acute leukemia and the blast crisis of CML, the question was raised whether the presence of N-ras mutations is associated with the phenotype of acute leukemia. We investigated leukemic cells from 100 patients with CML for the presence of N-ras mutations in the mutational hot spot codons. The cases analyzed included 87 diagnosed with different types of blast crisis and 13 cases in accelerated or chronic phase of the disease.

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INTRODUCTION

Chronic myeloid leukemia is a malignant hematopoietic disorder characterized by a triphasic course. After the initial, relatively benign chronic phase, the disease progresses to a more aggressive accelerated phase and finally, to the terminal BC. The leukemic cells in about 95% of CML patients exhibit the chromosome translocation (9;22) and the corresponding abnormality at the molecular level, the BCR/ABL rearrangement. The consistent association of the BCR/ABL fusion gene with the chronic phase of CML and experimental evidence of transfection assays suggest an important role of this rearrangement in the pathogenesis of the disease. The progression to blast crisis appears to involve additional genetic events. For example, ras protooncogene activation has been implicated in the transition of chronic phase CML to an acute leukemic phase. This notion was based on the phenotypic similarities between CML blast crisis and acute leukemia in which mutations of the N-ras gene, generally in codons 12, 13, or 61, occur at a relatively high frequency (2—5).

A limited number of trials in patients with CML-BC provided rather conflicting data concerning the occurrence of N-ras activation by single-base changes in the mutational hot spot regions. In earlier studies using a transfection assay or "shotgun cloning" with direct sequencing of in vitro amplified DNA (6, 7), N-ras mutations were identified in >60% of the CML-BC patients investigated. By contrast, more recent studies using oligonucleotide hybridization and direct sequencing revealed a low incidence of N-ras mutations (0—6%) in this subset of patients (8—11).

In a small number of studies, the association between N-ras mutations and the type of CML-BC has been addressed (8—10, 12). The results available suggest that these genetic events do not occur in lymphoid blast crisis but are present in a variable proportion of patients with nonlymphoid blast crisis of CML.

This study has been performed to assess the frequency of N-ras mutations particularly in the myeloid BC of CML. In view of the limited sensitivity of specific oligonucleotide hybridization or direct sequencing of enzymatically amplified DNA, we have used an additional method, the SSAP analysis of PCR amplified products, in order to reduce the possibility of missing a mutation within the N-ras codons analyzed. A representative cohort of patients were investigated by all three methodic approaches. To our knowledge, this is the largest study of N-ras mutations in the blast crisis of CML reported so far.

MATERIALS AND METHODS

Clinical Samples and DNA Extraction. Peripheral blood was obtained from 100 CML patients after informed consent. Diagnosis of BC was made on the basis of a bone marrow aspirate showing blasts, and promyelocytes in myeloid crisis, of >30%. For the diagnosis of blast crisis without availability of a bone marrow aspirate, the presence of BC was established on the basis of WBC differential counts showing >30% blasts or extramedullary disease with localized immature blasts. With the exception of two patients with chronic myelomonocytic leukemia who were initially diagnosed as atypical, Ph1-negative CML, routine cytogenetic analysis (13) indicated the presence of the Philadelphia chromosome in all cases investigated.

gDNA from bone marrow or peripheral blood mononuclear cells was isolated essentially as described previously (14). RNA was extracted from mononuclear cells according to the method of Chomczynski and Sacchi (15). First-strand cDNA was prepared from total RNA using random hexamer primers and Moloney Murine Leukemia Virus reverse transcriptase ( Gibco, BRL) (16).

Polymerase Chain Reaction. A 118-base pair fragment from the first exon of the N-ras gene, harboring codons 12 and 13, was amplified from gDNA using the primers 5'-gACTgAGTgTTACgAAcCgTgc3' (Ras Ia S) and 5'-gggCTCCAC-CTCTATgTgg3' (Ras Ib AS) (17). Primers used for amplification of a 178-base pair fragment from exon II/III gDNA were Ras Ia S and 5'-CAgCTgTATgCCAg- TATgTCC3' (Ras Ic AS). Amplification of a 141-base pair sequence from the second N-ras exon, containing codon 61, was performed from cDNA and gDNA with the primers 5'-CTgTTgTTgACATACgTgg3' (Ras IIa S) and 5'-CTgTA- gAgTTATATgCgC3' (Ras IIb AS).

Approximately 200 ng of template DNA were mixed with 1.25 units of Taq DNA polymerase (Promega), 15 pmol of each flanking primer in a solution containing 200 μM concentrations each of the deoxynucleotide triphosphates, 10 mM Tris (pH 9.0), 50 mM potassium chloride, and 1.5 mM MgCl2 in a total volume of 50 μL. After initial denaturation at 93°C for 7 min, 40 cycles were performed with denaturation at 93°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min in a thermal cycler (Bio-Med).
For SSCP analysis and direct sequencing, the amplified products were electrophoresed in 2% Tris-acetate-EDTA agarose gels. Specific bands were excised from the gel, and DNA was eluted with a Quialex-Kit (Promega).

SSCP. To detect N-ras point mutations in codons 12, 13, or 61, a modified version of the SSCP method was used (18). Isolated DNA (3–10 ng) was 5'-OH end-labeled with 1 unit T4 PNK (Boehringer Mannheim) and 0.25 μl [γ-32P]ATP (NEB; specific activity, 3000 Ci/mmol; 10 μCi/μl) in a total volume of 10 μl, ethanol precipitated, and resuspended in a mixture of 1:1 0.1% SDS-10 mmol EDTA and loading buffer (95% formamide-2% 0.5 mol EDTA-0.1% (w/v) bromphenol blue-0.1% (w/v) xylene cyanol). The samples were heated at 90°C for 5 min and immediately chilled on ice. Aliquots of 2.5 μl were applied to a 6% nondenaturing polyacrylamide gel containing 4 mm EDTA, 5% glycerol (when running at room temperature), and 45 mm Tris-borate, pH 8.3, for exon I and exon II I analysis (using 1× Tris-borate-EDTA [90 mm Tris-borate, 2 mm EDTA; pH 8.0] as running buffer) or 90 mmol Tris-borate for exon II (using 0.5× Tris-borate-EDTA). Gels were run at 4 W/4°C for 16–20 h or at 42 W/room temperature in a water-cooled sequencing gel apparatus (Bethesda Research Laboratories). The filters were dried at 80°C for 30 min and subsequently illuminated with UV light at 254 nm for 2 min.

Prehybridization was done in a mixture of 3 m tetramethylammonium chloride, 50 mm Tris-HCl, pH 7.5, 2 mm EDTA, 0.3% SDS, 5× Denhardt’s solution (100× Denhardt’s solution = 2% bovine serum albumin-2% Ficoll-2% polyvinylpyrrolidone), and 100 μg/ml sonicated, denatured salmon sperm DNA at 56°C for 1 h.

Oligonucleotide Hybridization. Nylon filters (Hybond-N; Amersham) were soaked for 5 min each in distilled water and 10× standard sodium citrate (1.5 M NaCl; 150 mm tri-Sodium citrate dihydrate) and dried at 80°C for 30 min. PCR-amplified DNA (200 ng) was adjusted to 0.4 M NaOH 25 mm EDTA in a final volume of 200 μl and spotted onto the filters under vacuum with a slot blot apparatus (Bethesda Research Laboratories). The filters were dried at 80°C for 30 min and subsequently illuminated with UV light at 254 nm for 2 min.

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For a typical hybridization experiment (19), 10 pmol of synthetic oligonucleotides, each of which represented a 20-mer with a single-base pair substitution in one of the N-ras codons 12, 13, or 61 (human ras Muty Lyzer probe panels; Clontech), were end labeled with 1 unit T4 PNK and [γ-32P]ATP (3000 Ci/mmol) in a final volume of 10 μl. The oligomers were purified from unincorporated nucleotide triphosphates in a Sephadex G-50 column.

The filters were hybridized with labeled oligonucleotides (108 cpm of probe/ml) in prehybridization buffer at conditions equal to the prehybridization. Filters were washed twice in 1× SSPE (10 mm NaH2PO4, pH 7.9; 180 mm NaCl; 1 mm EDTA)-0.1% SDS. After the samples were rinsed in hybridization buffer without Denhardt’s solution and salmon sperm DNA, the final stringency wash was performed in the same solution at 59°C (for the exon I and exon II fragment) or 61°C (for the exon II fragment) for 1 h. The filters were exposed to X-Omat AR film at −70°C using intensifying screen.

Direct PCR Sequencing. Direct sequencing of amplified DNA fragments was performed as described elsewhere with slight modification (20). Sequencing primers (10 pmol) which were identical with the PCR primers were kinased with 0.5 unit T4 PNK and 1.5 μl [γ-32P]ATP (3000 Ci/mmol) in a final volume of 5 μl and mixed with 0.2 pmol of amplified, eluted DNA (equivalent to 15–25 ng, depending on the number of nucleotides), 2.5 μl 10× Sequenase buffer (Sequenase kit, version 2.0; United States Biochemical), and distilled water to a total volume of 10 μl. The priming reaction was heated to 90°C for 10 min, frozen in ethanol-dry ice and mixed with 4.5 μl of 26.6 mm dithiothreitol (units) and 5 units Sequenase (T7 DNA polymerase, U.S. Biochemical). Aliquots of 3.5 μl of this labeling mixture were placed in four separate Eppendorf tubes, each of which contained 2.5 μl of specific termination solution consisting of dideoxynucleotide triphosphates and each of the four deoxynucleoside triphosphates in a ratio of 1:10 (U.S. Biochemical). The reactions were incubated at 37°C for 2 min and stopped by adding 4.5 μl of a 95% formamide mixture, with 20 mm EDTA, 0.05% (w/v) bromphenol blue, and 0.05% (w/v) xylene cyanol.

The sequencing products were run and displayed on 8% polyacrylamide denaturing gels and autoradiographed after drying as described above.

RESULTS

In the present study, 87 patients diagnosed with CML in blast crisis and 13 patients in the chronic or accelerated phase were examined for the presence of N-ras mutations in the mutational hot spot regions. In the presence of 84 CML blast crises examined, 52 were myeloid, 17 lymphoid, 2 biphenotypic (myeloid-lymphoid), 1 myelomonocytic (granulocytic-monocytic), 1 megakaryocytic, and 4 undifferentiated; in 7 cases the immunological characterization of the blasts was not available. The distribution of CML phases and types of blast crisis in the patients investigated are shown in Table 1. The cell lines HL-60 (21), Molt 4 (22), and HT 1080 (23), each of which carries a different N-ras mutation, were used as positive controls, and peripheral blood samples from healthy individuals served as negative controls.

Table 1 Frequency of N-ras mutations

<table>
<thead>
<tr>
<th>Leukemia type</th>
<th>No. of samples analyzed</th>
<th>No. of samples with mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML</td>
<td>97</td>
<td>0</td>
</tr>
<tr>
<td>Chronic and accelerated phase</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Blast crisis</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>Myeloid</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoid</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Biphenotypic myeloid/lymphoid</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Megakaryocytic</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Myelomonocytic</td>
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<td>0</td>
</tr>
<tr>
<td>Undifferentiated</td>
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<td>0</td>
</tr>
<tr>
<td>Without immunological character</td>
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<td>0</td>
</tr>
<tr>
<td>CMML</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Ph' ALL</td>
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<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
<td><strong>1</strong></td>
</tr>
</tbody>
</table>

Fig. 1. Agarose gel analysis of N-ras sequences amplified by PCR. Lanes 1 and 2, representative amplification products of cDNA/gDNA from CML patients; lanes 3 and 4, healthy control cDNA/gDNA; lane 5, positive control cell lines Molt 4, HT 1080, and HL 60, respectively. Far right lane, molecular weight marker [100-base pair (bp) DNA ladder]. A, 118-base pair fragment from N-ras exon I; B, 178-base pair fragment from N-ras exon II; C, 141-base pair fragment from N-ras codon II.
PCR. The specific amplification of all three N-ras gene fragments is shown in Fig. 1 which displays examples of PCR-amplified sequences derived from exons I and II.

SSCP Analysis. Under the conditions described in “Materials and Methods,” the cell lines used as positive controls could be distinguished by different migration of the single DNA strands. In only one of the patients investigated did a shift in the electrophoretic mobility of the single-stranded DNA molecules indicate a mutation within exon I (Fig. 2). All other patients showed the same N-ras wild-type migration pattern as did the healthy control individuals.

Oligonucleotide Hybridization. Nylon filters with amplified, covalently bound DNA were hybridized to probes complementary to all of the possible mutations within N-ras codons 12, 13, or 61. Control hybridization was performed with probes homologous to wild-type N-ras codons 12, 13, or 61. Representative results of hybridization analysis are shown in Fig. 3. Of the 100 patients investigated, only one, a case of CMML, exhibited an N-ras mutation, a base change within codon 13 from CCA to GCA.

Direct Sequencing. All fragments amplified in vitro were subjected to sequence analysis to identify mutations within and outside of the N-ras hot spot codons 12, 13, and 61. With the exception of one patient, no mutations were seen in PCR-amplified fragments derived from exon I or II or in a fragment extending across exons I and II (Fig. 4). Use of this method permitted the assessment of the nucleotide misincorporation frequency during PCR amplification. The sequence found in 99 of the patients investigated, all of whom lacked a mutation in the hot spot codons, was identical with the published N-ras sequence (24). Hence, there was no misincorporation of nucleotides under the PCR conditions described.

None of the 97 Ph1-positive CML patients investigated exhibited an N-ras point mutation by the techniques used. However, all three methods identified the same patient, a case of CMML, as carrying a mutation within the first exon of the N-ras gene (Table 1).
of the CML patients investigated. Earlier studies in small numbers of CML patients suggested that activation of the N-ras gene within the mutational hot spot regions may be present in a significant proportion of CML cases in nonlymphoid BC and, at a lower frequency, in lymphoid BC, but apparently it never occurs in the chronic phase of the disease (6, 7). These observations provided a basis for the hypothesis that the oncogene may be involved in the transition from the chronic to the acute phase of CML. The results of this study which, to our knowledge, comprises the largest cohort of CML blast crisis patients investigated for N-ras activation, strongly suggest that the presence of a mutated N-ras gene represents an extremely rare event in CML. It can be inferred, therefore, that there is no association between progression to blast crisis and mutational activation of the N-ras protooncogene. The occurrence of N-ras mutations in acute lymphoblastic leukemia and the high incidence in acute myelogenous leukemia indicate that ras gene mutations may play a role in these types of neoplasia. Despite the marked resemblance between CML blast crisis and acute leukemia, progression from the chronic to the acute phase of CML appears to involve molecular events other than mutational activation of N-ras (11, 25, 26).

The only N-ras gene mutation observed in our series was a case of chronic myelomonocytic leukemia which was initially diagnosed as atypical, Ph1-negative CML. This finding is in accordance with recent studies (27–29) and supports the observation that point mutations of the N-ras protooncogene are more common in other types of leukemia.

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Absence of N-ras Mutations in Myeloid and Lymphoid Blast Crisis of Chronic Myeloid Leukemia

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