Rare Occurrence of N-ras Point Mutations in Philadelphia Chromosome Positive Chronic Myeloid Leukemia

By Steven J. Collins, Monique Howard, D. Frank Andrews, Edward Agura, and Jerry Radich

Point mutations of the N-ras oncogene are relatively common in acute myelogenous leukemia (AML) cells, occurring in some 25% to 50% of patient samples. We used a technique involving the direct nucleotide sequencing of in vitro amplified N-ras genomic fragments to determine the frequency of N-ras point mutations in chronic myeloid leukemia (CML) cells at various stages of the disease. This approach will detect N-ras point mutations in a mixed population of cells if the mutation is present in 25% or more of the cells. We could not demonstrate any point mutation at N-ras codons 12, 13 or 59-63 in any of the 44 CML cases analyzed, which included 21 blast crisis samples. In contrast with AML N-ras point mutations are exceedingly rare in CML.

Chronic Myelogenous Leukemia (CML) is a pluripotent hematologic stem cell disorder that occurs in distinct clinical phases. These include a relatively benign chronic phase, characterized by hyperplasia of terminally differentiated granulocytes, which inevitably progresses to a lethal blast crisis phase in which immature blasts predominate. The molecular events that occur during the development and progression of CML have been extensively studied. Over 90% of cases of chronic phase CML exhibit the Philadelphia chromosome (Ph+), which involves a translocation of the c-abl oncogene from chromosome 9 to a relatively restricted region on the Ph+ termed the breakpoint cluster region (bcr). This specific translocation generates a bcr-abl fusion gene coding for an aberrant bcr-abl fusion protein (p210) that appears to be critical to the pathogenesis of CML. However, the presence of this bcr-abl fusion gene alone cannot account for the progression of CML to blast crisis and presumably a second or more genetic event(s), perhaps involving other cellular oncogenes, occurs during the progression of the disease.

One likely candidate cellular oncogene that might be involved in the progression of CML to blast crisis is the N-ras protooncogene. Point mutations of members of the ras family of oncogenes have been described in approximately 10% to 20% of a wide variety of human malignancies. Acute myeloid leukemia (AML) cells exhibit a relatively high incidence of such point mutations with some 25% to 50% of AML samples harboring single point mutations in the N-ras protooncogene at either codons 12, 13 or 61-63. The reason why AML cells exhibit selective point mutations in N-ras as opposed to other members of the ras protooncogene family is presently unknown but suggests that certain cellular oncogenes are preferentially activated in malignancies of a particular differentiative lineage. There are hematopoietic lineage similarities between AML and CML. For example, some cases of AML, like CML, involve transformation of a multipotent hematopoietic stem cell. Moreover, the phenotype of myeloid blast crisis CML closely resembles the phenotype of AML. Given these similarities the question arises as to whether the incidence of N-ras point mutations in CML chronic phase or blast crisis approaches the relatively high incidence of N-ras point mutations that have been observed in AML.

In this study we directly addressed this question by surveying a series of Ph+ positive CML chronic phase and blast crisis samples to determine the incidence of N-ras point mutations at codons 12, 13 or 59-63 in these leukemic cells. We used a recently developed technique involving the direct sequencing of in vitro amplified N-ras genomic fragments harboring these critical codons. This technique is suitable for screening a relatively large number of samples and provides direct sequence information of multiple N-ras codons at a single reading. Surprisingly we found no examples of N-ras point mutations at codons 12, 13 or 59-63 in any cases of CML including 21 blast crisis samples. Compared with AML the incidence of N-ras point mutations in Ph+ positive CML appears to be extremely rare.

MATERIALS AND METHODS

Patient samples. Peripheral blood was obtained by routine venipuncture from patients with chronic and blast crisis phase Ph+ positive CML. In all cases Institutional protocols were followed. The diagnosis of blast crisis was defined by the presence of >20% blasts on peripheral smear. With chronic phase samples leukocytes were isolated using dextran sedimentation exactly as previously described. For blast crisis samples the blast population was enriched by isolymph (Gallard/Schlesinger, Carle Place, NY) buoyant density gradient centrifugation and the mononuclear cell fraction was stained with Wright-Giemsa and examined by light microscopy. Only those blast crisis samples with a mononuclear cell fraction exhibiting 40% blasts or greater were considered representative of blast crisis and subjected to DNA analysis. The cells were washed twice in isotonic media and the DNA extracted from isolated nuclei exactly as previously described. In addition to the patient samples we also extracted and analyzed DNA from the cultured CML blast crisis cell lines K-562, EM-2, and KCL-22.

bcr Rearrangements. Genomic DNA (10 to 15 μg) from Ph+ positive CML samples was digested with BglII and subjected to Southern blot analysis using a 1.2 kb HindIII-BglII bcr fragment (3')
**N-ras point mutations in CML**

*bcr* probe (Oncogene Science, Inc., Mineola, NY) exactly as previously described. If a germline pattern (5 kb fragment only) was observed with this probe on a patient sample then the sample DNA was digested with EcoRI or Kpn and subjected to Southern blot analysis using a 1.4 kb BglII-Sst genomic fragment (5' *bcr* probe) that lies immediately 5' to the *bcr* site.

Immuno-globulin heavy chain gene rearrangements. To distinguish lymphoid from myeloid blast crisis, DNA from CML blast crisis patient samples was digested with HindIII and subjected to Southern blot analysis using a 3.2 kb EcoRI-HindIII genomic probe, which encompasses several of the exons within the JH region of the immunoglobulin heavy chain locus. This probe detects 10.5 and 4 kb germline HindIII fragments. Hybridization of this probe to additional nongermline fragments in the patient samples indicate the rearrangement of the Ig heavy chain locus as previously described.

Amplification of genomic *N-ras* fragments. A 328 base pair (bp) fragment harboring *N-ras* codons 12 and 13 was amplified from 500 ng of patient sample genomic DNA using the polymerase chain reaction (PCR). The specific oligonucleotide primers used, the reaction conditions, and cycle number were exactly as previously described. Similarly, we amplified a 111 bp genomic fragment harboring *N-ras* codons 59-63 using a "nested primer" approach as previously described.

Direct DNA sequencing. The PCR products were run and visualized on an ethidium stained polyacrylamide gel. The appropriate-sized fragment of interest was cut from the gel and eluted overnight at 37°C in 0.5 mol/L ammonium acetate, 1 mmol/L EDTA. The fragment was recovered following ethanol precipitation, dissolved in water, and used as a template in a modified Sanger direct sequencing reaction as previously described. The same oligonucleotides used as primers in the PCR were radiolabeled with polynucleotide kinase and used as primers in the direct sequencing reactions. The sequencing ladders from patient samples were carefully compared with ladders from normal samples and with published *N-ras* sequences to determine the presence or absence of specific *N-ras* point mutations at codons 12, 13 and 59-63 in individual samples. When sequencing ambiguities were encountered in these ladders, they were resolved by sequencing the opposite strand of the same amplified fragment using the opposing PCR oligonucleotide as primer.

**RESULTS**

*bcr* Rearrangements in CML patient samples. To assure that the patient sample genomic DNA analyzed for the presence of *N-ras* mutations was indeed representative of the CML clone, we subjected each of the patient samples to Southern blot analysis with specific 3' and 5' *bcr* probes as described in Materials and Methods. Forty-two of 44 CML samples exhibited rearrangements within the *bcr* as demonstrated with either the 3' or 5' *bcr* probes. (Table 1) Six of these samples exhibited rearrangements with the 5' probe but not with the 3' probe and these cases most likely represent the previously described subset of CML samples in which the 9q + region (usually detected with the 3' *bcr* probe) has been deleted. The two Ph' positive patients that did not exhibit rearrangements within the *bcr* could represent "contamination" of the patient sample with normal hematopoietic cells; more likely, these samples may harbor chromosome 22 breaks that lie upstream to the *bcr* and that would not be detected with the particular *bcr* probes we have used.

**Table 1. Characteristics of CML Samples Analyzed for Presence of *N-ras* Point Mutations**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>5' Probe*</th>
<th>3' Probe*</th>
<th>Absent</th>
<th>JH Rearrangement</th>
<th>Codons 12, 13</th>
<th>Codons 59-63</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic phase</td>
<td>23</td>
<td>3</td>
<td>19</td>
<td>1</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>Blast crisis</td>
<td>21</td>
<td>3</td>
<td>17</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.

*5' and 3' *bcr* probes are described in Materials and Methods.
†Blast crisis samples included the cell lines K-562, EM-2, and KCL-22.
direct sequencing of this amplified fragment indicated that the "wild type" N-ras sequence of these two codons (GGT, GGT) in all 44 samples. Thus, none of these patient CML mononuclear cells were mixed in the indicated ratios, and genomic DNA was extracted. The 111 bp genomic fragment harboring N-ras codon 61 was amplified in vitro using the PCR and directly sequenced as described in Materials and Methods and in reference 13. The asterisk indicates the mutant "A" nucleotide present in the second nucleotide of N-ras codon 61 of HL-60. The arrow indicates the "T" nucleotide that is present in this position in normal genomic DNA.

**Fig 1.** Sensitivity of the PCR-direct sequencing assay in detecting N-ras point mutations. HL-60 cells and normal peripheral blood mononuclear cells were mixed in the indicated ratios, and genomic DNA was extracted. The 111 bp genomic fragment harboring N-ras codon 61 was amplified in vitro using the PCR and directly sequenced as described in Materials and Methods and in reference 13. The asterisk indicates the mutant "A" nucleotide present in the second nucleotide of N-ras codon 61 of HL-60. The arrow indicates the "T" nucleotide that is present in this position in normal genomic DNA.

direct sequencing of this amplified fragment indicated the "wild type" N-ras sequence of these two codons (GGT, GGT) in all 44 samples. Thus, none of these patient CML samples exhibited point mutations at N-ras codons 12 or 13.

Similarly we successfully amplified and gel purified the 111 bp genomic fragment harboring N-ras codon 61 from all 44 CML samples. The direct sequencing ladder of this amplified fragment indicated the wild type codon 61 sequence (CAA) in all these CML samples. Moreover, the same sequence ladder also allows the direct reading of N-ras codon 59-63 sequences. It has been previously demonstrated that point mutations at ras codons 59 and 63 in addition to those at codon 61 can activate ras protooncogenes. However, in our series all 44 CML samples exhibited the wild type codon 59-63 N-ras sequence.

**DISCUSSION**

Point mutations of the N-ras gene are relatively common in AML occurring in some 25% to 50% of patient samples. We undertook the present survey to determine whether a similar incidence of N-ras point mutations was present in chronic phase and/or blast crisis CML. We were particularly interested in comparing samples of CML chronic phase with blast crisis to determine whether acquisition of N-ras point mutations correlates with CML disease progression. For this survey we used a technique that involves the direct sequencing of N-ras genomic fragments that have been amplified in vitro using the PCR. This is an extremely powerful technique that requires relatively small amounts of genomic DNA, can accommodate a relatively large number of samples, and can document ras point mutations much more rapidly and efficiently than the laborious NIH3T3 transforming assay that was initially used to identify the presence of ras point mutations in human tumor DNA. Moreover, in contrast with previously described procedures involving the selective hybridization of specific oligonucleotide probes to amplified genomic DNA, the direct sequencing approach we have used provides sequence information of multiple N-ras codons at a single reading. In addition, this approach can identify adjacent point mutations within a single ras codon as was recently described in Ki-ras codon 12 (GGT to TTT) of a breast tumor cell line.

Most AML samples in which point mutations of N-ras have been demonstrated also harbor a copy of the normal N-ras allele. Moreover, peripheral blood samples from leukemic patients may consist of a heterogenous population of normal and leukemic cells. Since the PCR will amplify both normal and mutant N-ras alleles in a given genomic DNA sample, we performed cell mixing experiments to determine the level of sensitivity of our assay in detecting mutant N-ras alleles. We could detect the mutant allele if present in 25% or greater of the cells within a mixed population (Fig 1). In contrast techniques using the hybridization of specific mutant oligonucleotides to PCR amplified genomic DNA have been reported to detect mutant alleles at a 10% level. It is likely that close to 100% of the peripheral blood cells analyzed for each patient sample in our study indeed represented leukemic cells since previous studies indicate that CML peripheral blood cells are virtually all derived from the CML clone. Moreover, we readily detected bcr rearrangements in 42 of 44 of the CML samples analyzed (Table 1). In addition we limited our studies of blast crisis only to those samples exhibiting 40% blasts or greater. Thus, the cells of interest in our samples exceeded the 25% level of sensitivity of our assay.

Previous studies using the NIH3T3 transfection assay or the nude mouse tumorigenicity assay indicate that a subset of CML patient samples harbor point mutations within N-ras. In one series ras mutations were more common in blast crisis samples than in chronic phase. In one of these patient samples the N-ras point mutation was demonstrated directly in peripheral blood leukemic cells. Therefore, we were surprised to observe the complete absence of N-ras point mutations in any of our 44 Ph1 positive CML cases.
including 21 blast crisis samples. Why is there this discrepancy between our results and these previous reports? One possibility is that ras mutations may arise spontaneously in transfected DNA during the relatively long (2 weeks to 2 months) target cell culture period required for the NIH3T3 transfection and nude mouse tumorigenicity assays. Therefore, point mutations documented by this approach may not accurately reflect the ras nucleotide sequences of fresh leukemic cell DNA. A second possibility is that activating N-ras point mutations may be present only in a small subset of cells within individual CML samples. Potentially these mutations could be detected by the NIH3T3 transfection assay even when present in a very small percentage of the leukemic cells. In contrast as noted above the direct sequencing approach used in our survey would not detect N-ras point mutations if present in <25% of the sample leukemic cells. A third possibility is that the incidence of N-ras point mutations may differ in Ph\textsuperscript{+} positive v Ph\textsuperscript{−} negative CML. In a recent study using oligonucleotide hybridization to PCR amplified leukemic DNA, none of 26 Ph\textsuperscript{+} positive CML samples were noted to exhibit N-ras point mutations while one of two cases of Ph\textsuperscript{−} negative chronic myelomonocytic leukemia (CMML) harbored an N-ras codon 12 mutation.\textsuperscript{27} Moreover, we have previously noted an N-ras codon 12 point mutation in a Ph negative CML patient sample.\textsuperscript{13} The Ph\textsuperscript{+} status of the CML patient in which the N-ras point mutation was directly observed in the peripheral blood leukemic cells was not noted.\textsuperscript{26} It is possible that Ph\textsuperscript{−} negative CML patients are more likely to harbor N-ras point mutations than Ph positive CML samples, but this hypothesis needs to be experimentally confirmed.

The ras family of oncogenes includes Ha-ras and Ki-ras in addition to N-ras, and point mutations of Ha-ras and Ki-ras have been described in a variety of different human malignancies.\textsuperscript{4} However, we limited the present study to N-ras because previous studies have indicated that AML cells exhibit activating point mutations selectively in N-ras as opposed to Ha-ras and Ki-ras.\textsuperscript{4,11} Recent studies have documented the incidence of Ha-ras and Ki-ras point mutations in CML. Using the nude mouse tumorigenicity assay, three of 12 CML samples exhibited activation of either Ha-ras or Ki-ras.\textsuperscript{46} In contrast, another study involving the direct analysis of patient cell DNA did not demonstrate Ha-ras or Ki-ras point mutations in any of 26 CML samples including 16 blast crisis patients.\textsuperscript{37}

Why are N-ras point mutations so infrequent in Ph\textsuperscript{+} positive CML cells? Presumably, mutations of cellular oncogenes are maintained in malignant cells because they convey a selective proliferative advantage over cells not harboring such mutations. Both the c-abl and N-ras gene products are known to be associated with the cell membrane and both may be involved in regulating signal transduction pathways that are triggered by specific proliferative signals. The generation of the bcr-abl fusion protein or the acquisition of N-ras point mutations in a hematopoietic stem cell might somehow alter a proliferation signal transduction pathway to favor proliferation. If both c-abl and N-ras are involved in the same or similar proliferative signal transduction pathway(s) and if a mutation in either one of these protooncogenes can significantly alter this regulatory pathway to favor proliferation, then an activating point mutation arising in N-ras may not offer a significant proliferative advantage to a cell that already harbors an activated bcr-abl fusion gene. This might explain the rarity of demonstrable N-ras point mutations in Ph\textsuperscript{+} positive CML cells. This is merely speculative, however, and will remain so until the normal physiological function of both the c-abl and N-ras protooncogenes are more thoroughly defined.

Our failure to demonstrate any N-ras point mutations in CML blast crisis samples indicates that N-ras mutations cannot explain the inevitable progression from chronic phase to blast crisis that occurs in virtually all CML patients. Specific cytogenetic changes involving chromosomes 8 and 17 have been described during the evolution of CML, but these have not been associated with any specific molecular genetic or protooncogene abnormalities. Moreover, there is no clear correlation between the level of expression of the N-ras gene products and the progression of CML to blast crisis.\textsuperscript{25,28} The molecular events and genetic mutations responsible for the evolution of CML remain unclear but may involve specific oncogenes other than N-ras or the bcr-abl fusion gene.

ACKNOWLEDGMENT

We wish to thank LeMoyne Mueller for excellent technical assistance and Dr Roger Perlmutter for the gift of the J\textsubscript{58} probe.

REFERENCES

Rare occurrence of N-ras point mutations in Philadelphia chromosome positive chronic myeloid leukemia

SJ Collins, M Howard, DF Andrews, E Agura and J Radich