High Frequency of N-ras Activation in Acute Myelogenous Leukemia

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Using the NIH/3T3 cell transfection assay, activated cellular oncogenes have been detected in around 10% to 20% of human tumors. From a series of DNA preparations from tissues infiltrated with acute myelogenous leukemia (AML), 50% (3/6) caused transformation of NIH/3T3 cells. Thus AML appears to be the human tumor with the highest frequency of oncogenes detected by DNA transfection. In each case the oncogene involved was N-ras, a member of the ras gene family. Biologic and clinical parameters of AML patients with and without N-ras oncogenes in their tumors are discussed.

EVIDENCE ARISING FROM several different approaches has implicated a small number of cellular genes or proto-oncogenes as frequent targets for genetic alterations that can lead human cells along the pathway to malignancy. These cellular genes were initially identified as the transduced cellular “onc” genes of acute transforming retroviruses. More recently, it has been found that some of the very same cellular genes may be activated as oncogenes in human tumors completely independent of retrovirus involvement.

One important approach to the detection of human transforming genes has involved DNA transfection. A wide variety of human tumors contain DNA sequences capable of inducing morphologic transformation upon transfection of NIH/3T3 cells, a continuous mouse embryo cell line. The vast majority of oncogenes detected in this manner have been found to be related to a small proto-oncogene family, ras. Recent studies have also implicated other proto-oncogenes in human malignancies. DNA rearrangements such as translocation and gene amplification involving human proto-oncogenes have been associated with specific forms of cancer.

Human acute myelogenous leukemia (AML) is a highly malignant hematologic neoplasm. There have been a few reports of ras oncogenes in neoplastic tissues or cultures derived from AML patients, as well as a recent study demonstrating amplification of the proto-oncogene c-myb in one AML cell line. This present study of six patients with AML was undertaken to examine further the frequency of oncogene activation in this disease, as well as to characterize what clinical parameters, if any, might be associated with oncogene activation.

MATERIALS AND METHODS

DNA transfection. Pretreatment marrow aspirates or autopsy materials were selected if they were heavily (>75%) infiltrated with acute nonlymphocytic leukemia cells as judged by a pathologist. Specimens selected for study were frozen promptly to prevent DNA degradation, and tumors were homogenized in liquid nitrogen, lysed in 5% sodium dodecyl sulfate (SDS) containing 10 mmol/L Tris, 2 mmol/L EDTA, and 150 mmol/L NaCl and treated overnight with proteinase K (2 mg/mL at 37°C). Proteins were extracted with phenol, then with a mixture of phenol, chloroform, and isooamyl alcohol (25:24:1), and then with chloroform and isooamyl alcohol (24:1). DNA was precipitated with ethanol, dried, and suspended in 1 mmol/L Tris and 0.2 mmol/L EDTA.

The DNA transfection assay was performed essentially as described. Briefly, NIH/3T3 cells were plated in 10-cm petri dishes at 1.3 x 10^6 cells per plate 20 hours before transfection. DNA (30 μg) was suspended in 0.25 mol/L CaCl_2 and precipitated by addition to an equal volume of 50 mmol/L HEPES, 280 mmol/L NaCl, and 1.5 mmol/L NaH_2PO_4 during gentle nitrogen bubbling. After 30 to 60 minutes, precipitates were reuspended and added to the NIH/3T3 cell monolayer. Precipitates were removed 20 hours later. Culture medium was changed twice weekly, and focus formation was scored at 14 to 21 days. Genomic DNA from the T24 human bladder carcinoma cell line was used as a positive control. T24 DNA yielded 5 to 15 focus-forming units per plate.

Southern blot analysis. DNAs (15 μg lane) were electrophoresed at 30 V in 1% agarose and transferred to nitrocellulose by the method of Southern. Molecular probes were nick-translated with α^32p dCTP, denatured, and hybridized overnight at 42°C in the presence of 50% formamide, 750 mmol/L NaCl, 75 mmol/L Na citrate 1X Denhardt’s solution, 20 mmol/L phosphate buffer, and 100 μg/mL sheared, denatured salmon sperm DNA. The filters were washed three times with 300 mmol/L NaCl, 30 mmol/L Na citrate, and 0.1% SDS at room temperature and then with 15 mmol/L NaCl, 1.5 mmol/L Na citrate, and 0.1% SDS twice at 50°C. After air drying, the filters were exposed two to six hours at –80°C on an intensifying screen and developed in a Kodak RP X-O-Mat processor. DNA probes of human origin included N-ras, K-ras, H-ras, c-myc, c-myc, and N-myc.

Immunoprecipitation of the ras gene product. Two days following plating a density of 3 x 10^6 cells were methionine-starved for 30 minutes and labeled with 3.3 μCi/L 35S-methionine/mL for three hours. After washing with phosphate-buffered saline (PBS), the cells were lysed in 1% Triton-X-100, 1% SDS, 5% sodium deoxycholate, 1 mL/L NaCl, 10 mmol/L phosphate buffer (pH 7.4) containing 1.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 100 kallikrein inactivator units of aprotinin/mL. After centrifugation at 30,000 g for 15 minutes, 2 x 10^6 trichloroacetic-acid precipitable counts of the cleared lysates were incubated with anti-ras monoclonal antibody Y13-25922 at 4°C for 45 minutes. Bound p21 was precipitated with protein A sepharose coated with goat antirat IgG, washed three times, and boiled in sample buffer containing 2-mercaptoethanol. Samples were electrophoresed at 250 V for three hours along with 14C-labeled protein standards on a 12.5% bis-acrylamide slab gel. Following fixation, fluorography with Enhance, and rehydration, the slab gel was dried and exposed overnight on an imaging-intensifying screen at –80°C.

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RESULTS

High-frequency activation of N-ras oncogenes in primary AML. Table 1 provides information on six patients with AML included in our study. DNA extracted from tumor tissues was assayed in the NIH/3T3 transfection assay. As shown, affected tissues of three of the patients scored as positive. In each case, the transforming activity persisted in a second cycle of transfection. The great majority of activated oncogenes associated with human malignancies and detected in the NIH/3T3 transfection assay are members of the ras gene family. H- and K-ras were initially detected as the transduced onc genes of transforming retroviruses,23 while N-ras was initially detected as the oncogene of a human neuroblastoma cell line.24 Thus we probed first-cycle transfectants with radiolabeled DNA fragments derived from human H-, K-, and N-ras, respectively. Neither H- nor K-ras probes detected new, hybridizable DNA fragments (data not shown). However, each of the transfectant DNAs contained new DNA fragments detectable under stringent hybridization conditions with the N-ras probe (Fig 1). Similar results were obtained with second-cycle transfectants, indicating the segregation of human N-ras sequences with the transformed phenotype of the cells. Differences in the sizes of EcoRI-digested N-ras fragments imply DNA rearrangements outside the region that is required for N-ras-transforming function during the transfection and/or integration process.

Recent studies have documented that activating lesions at codon 12 or 61 in the ras coding sequence and the consequent amino acid substitution at either of these positions can alter the electrophoretic mobility of the transforming protein. These alterations induce characteristic retarding or accelerating effects of p21 migration.25,26 Thus we examined transfectants containing N-ras oncogenes derived from the three positive AML patients by immunoprecipitation analysis for their pattern of p21 electrophoretic mobility using broadly cross-reactive anti-p21 monoclonal antibody Y13-259. As shown in Fig 2, representative transfectants of each positive AML exhibited abnormal p21 species; when compared with normal NIH/3T3 cells, the level of p21 expression was consistently elevated in all transfectants analyzed. The increased expression of p21 in AML transfectants is attributed to the presence of the added human N-ras oncogene because DNA hybridization experiments established that the transfectants contained only human N-ras and not human H-ras or K-ras oncogenes or amplified copies of any mouse ras genes. However, direct identification of human N-ras encoded p21 proteins is not possible as yet due to the unavailability of N-ras-specific antibodies. Each of the three N-ras oncogene products demonstrated a similar migration, suggesting that a similar mutational event might be responsible for the activation of N-ras oncogenes in each of these cases.26

Table 1. DNA Transfection Analysis of Six Cases of Acute Myelogenous Leukemia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Survival (months)</th>
<th>Tissue</th>
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<td>2/8</td>
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<tr>
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<td>45</td>
<td>F</td>
<td>30+</td>
<td>Marrow</td>
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<td>8</td>
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<td>12</td>
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Fig 1. N-ras oncogenes in NIH/3T3 transfectants of human AML DNAs. DNA samples of 15 μg each from first-cycle NIH/3T3 transfectants of AML patients 1, 2, and 3 (lanes 2, 3, and 4) were digested with EcoRI and run in a 1% agarose gel, blotted to nitrocellulose paper, and probed with the PvuII fragment of the human N-ras gene.11 NIH/3T3 (lane 1) and human placenta (lane 5) DNAs served as controls.

Fig 2. Electrophoretic patterns of N-ras oncogene-encoded p21 proteins in NIH/3T3 transfectants of human AML DNAs. Control NIH/3T3 (lane 1) and NIH/3T3 transfectants derived from DNAs of patients 1, 2, and 3 (lanes 2, 3, and 4) were compared with transfectants of T24 bladder carcinoma containing a position 12–mutated H-ras (lane 5) and of SW1271 lung carcinoma containing a position 61–mutated N-ras (lane 6). After immunoprecipitation with monoclonal antibody Y13-259, p21 electrophoretic mobilities were compared by SDS 12.5% polyacrylamide gel electrophoresis.
Somatic activation of N-ras in an AML patient. The family history of patient 1 indicates a high incidence of hematopoietic abnormalities, including paroxysmal nocturnal hemoglobinuria (PNH), neutropenia, acute leukemia, and Hodgkin's disease. It is conceivable that families with site-specific tumor aggregation inherit a propensity for the development of the tumor by vertical passage of an altered or activated oncogene. This prompted us to investigate whether the DNA from this patient's normal tissue might contain an activated cellular oncogene. DNA extracted from apparently normal skin scored repeatedly negative in the NIH/3T3 transfection assay, with a total of 16 plates analyzed under conditions where the activated N-ras gene of this patient's leukemia tissue was readily detectable (Table 1). These findings suggest that N-ras activation in this patient occurred as a somatic event in agreement with the results of similar studies of other tumors.28-30

Hybridization analysis of human AML DNAs for aberrations affecting other human proto-oncogenes. Recent studies have identified rearranged c-myc in Burkitt's lymphoma as well as amplification of c-myc-related genes in a number of other human malignancies.12,13,15,18,19 In addition, c-myc has been shown to be amplified in one AML cell line.7 Thus we surveyed DNAs of our series of primary AMLs for evidence of gene rearrangements or gene amplification affecting either of these proto-oncogenes. There was no detectable amplification or gross DNA rearrangement with respect to the indicated restriction enzyme involving either myb (Fig 3) or myc (data not shown) in any of the samples analyzed, indicating that such alterations are not commonly detectable in AML. Moreover, analysis of EcoRI- or BamHI-digested genomic DNA failed to reveal DNA rearrangement or gene amplification of H-ras, K-ras, N-ras, or N-myc (data not shown).

DISCUSSION

Our observations that 3 of 6 tumors contained an activated N-ras oncogene suggest that approximately 50% of the neoplastic tissues from patients with AML will score as positive in the NIH/3T3 DNA transfection assay. In considering our cases in concert with the five cases of AML examined in other studies,11,17,18 a total of 6 of 11 were positive. There have only been a few studies of the frequency of ras oncogene activation in a large series of tumors of the same types. In breast and urothelial tumors, the frequency of ras oncogenes detected by DNA transfection was <5% and around 7.5%, respectively.29,30 The combined results of several studies have indicated that lung and colon carcinomas exhibit ras oncogenes detected by analogous approaches in around 10% to 30% of primary tumors or tumor cell lines.10,23 Thus AML appears to exhibit the highest frequency of ras oncogene activation of any human tumor so far studied.

It is possible that oncogene activation detectable in the NIH/3T3 transfection assay reflects some biologic property intrinsic to the tumor subtype in question. NIH/3T3 cells lose density-dependent growth inhibition and become tumorigenic in nude mice after transfection with activated ras genes. Thus it is conceivable that tumors expressing ras oncogenes might be relatively more aggressive with respect to tissue invasiveness and/or metastatic potential. In our present series of AMLs, such clinical parameters as leukocyte count at presentation, degree of organ infiltration, response to therapy, and survival did not seem to be significantly correlated with the presence or absence of detectable N-ras oncogenes. However, further studies of N-ras activation in AML are needed to validate these findings and explore further whether detection of this marker is associated with any subgroup of patients with distinctive biologic and/or clinical behavior of their disease.

Previous studies have demonstrated that in a few tumors where normal tissues from the same patient were available, ras oncogenes were activated by point mutations as somatic events detectable only in the tumor.28-30 In our present survey of human AMLs, we were able to analyze DNA of normal cells from one patient whose tumor was positive for an N-ras oncogene. This patient's family history indicated a high incidence of hematopoietic abnormalities, including PNH, neutropenia, acute leukemia, and Hodgkin's disease. The absence of oncogenes detectable by DNA transfection in normal tissues of this patient suggests the somatic activation of an N-ras oncogene in this tumor. While genetic transmission of an N-ras oncogene cannot be implicated in the high familial incidence of hematopoietic abnormalities, its selection within the tumor implies that it was likely involved in this patient's tumor development.

The mobilities of ras p21 proteins altered by the most common activating lesions at codon 12 or 61 are often altered in ways that readily discriminate them.24 Our present findings demonstrated that the mobilities of p21s encoded by

Fig 3. Analysis of human AML DNAs for aberrations affecting other proto-oncogenes. DNA samples (15 μg) from seven AML patients (lanes 1 through 7) and human placenta (lane 8) were digested with HindIII and hybridized to the 2.8-kb and 1-kb EcoRI fragments of human c-myc.44
each of the N-ras oncogenes of our AML patients analyzed were similar, but intermediate in mobility compared to those of typical p21s containing position 12 or 61 mutations. These findings indicate that these oncogenes may possess a common mutation, whose effect on p21 mobility doesn’t allow ready classification. The precise mechanisms by which these oncogenes are activated awaits their molecular cloning and sequence analysis. In a report published after submission of our study, Bos et al12 also detected N-ras oncogenes at high frequency in AML. Moreover, these investigators presented evidence that point mutation at codon 13, rather than at one of the common sites for activation at position 12 or 61, is responsible for the acquisition of transforming properties by N-ras oncogenes in their cases of AMLs. It will be of interest to determine whether position 13 mutations account for any of the activating lesions in our present studies of N-ras as agents of AML patients.

Chromosomal aberrations such as translocations, inversions, and deletions occur in the majority of AMLs.13 Specific translocations have been implicated in the activation of proto-oncogenes in Burkitt’s lymphoma14 and chronic myelogenous leukemia.15 Although it was not possible to perform cytogenetic analysis of our present cases, the karyologic anomalies observed in AML involve chromosome 1 where the N-ras proto-oncogene resides in less than .5% of cases.16 In fact, in those cases where chromosome 1 anomalies are observed in AML, the break point is more often at the long arm of chromosome 1.17,18 while the N-ras site is on the short arm, at 1p11 to 1p21.19 Thus the observation of a high frequency of N-ras activation in AML coupled with the very rare observation of chromosomal anomalies in the vicinity of this gene strongly suggests that a specific primary karyologic change is not involved in N-ras activation.

Myc and myb oncogenes of acute transforming retroviruses commonly induce myeloid leukemias.13 Ras oncogenes have been shown to induce alterations in the growth and differentiation of cells within the myeloid lineage, in some cases associated with the acquisition of malignant properties.20-22 In view of these findings as well as reports of amplified myb23 and myc24,25 DNA sequences, respectively, in two different myeloid leukemia cell lines, we investigated seven primary AMLs for evidence of gene amplification or gross DNA rearrangement involving myc and related N-myc, myb and the three ras oncogenes. None was detected, implying that such alterations are not commonly associated with this tumor. Continued search for specific aberrations affecting the small set of cellular genes that are known to give rise to oncogenes may help to define other genes that contribute to the pathogenesis of this highly malignant tumor.

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