Analysis of N-RAS Exon-1 Mutations in Myelodysplastic Syndromes by Polymerase Chain Reaction and Direct Sequencing

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Mutations in codons 12 or 13 of the first exon of the N-RAS gene have been reported in myelodysplastic syndromes (MDS) in frequencies that vary between 9% and 40% depending on the techniques used in analysis. Gene amplification and direct sequencing provides the only unambiguous method of detecting those mutations that induce amino acid alterations. Using this technique, we analyzed 21 MDS patients for mutations in exon-1 of N-RAS. Codon 12 mutations substituting aspartic acid (GAT) for glycine (GGT) were found in four cases, and a codon 13 mutation substituting alanine (GCT) for glycine (GGT) was detected in one patient. We conclude that N-RAS exon-1 mutations producing amino acid changes occur in about 20% to 25% of MDS cases.

YELODYSPLASTIC SYNDROMES (MDS) are stem cell disorders characterized by refractory cytopenias, ineffective hematopoiesis, and variable progression to leukemia. Transformation to acute myelogenous leukemia (AML) occurs in up to 40% of patients. The mechanisms underlying the development of MDS and their evolution to AML are largely unknown. However, activation of the RAS oncogenes by point mutations has been implicated in the development of MDS and its progression to AML.

Hirai et al identified an N-RAS codon 13 mutation substituting arginine (CGT) for glycine (GGT) in three of eight patients with MDS by using the NIH 3T3 transfection and in vivo selection assay. Using the polymerase chain reaction (PCR) to amplify target DNA and synthetic oligonucleotide probes specific for wild-type or mutant alleles, Lyons et al reported mutations at codon 12 of K-RAS or N-RAS in 9% (three of 34) of MDS patients. The assay based on trasfection of 3T3 cells and in vivo selection is laborious, time consuming, and can lead to artificial mutations during transfection and selection. The synthetic oligonucleotide probe hybridization technique would overlook mutations at codons other than 12 or 13. Therefore, we sought to determine the true frequency of N-RAS mutations in MDS patients by using a direct sequencing approach. We report here a frequency of mutations in exon-1 of N-RAS of 20% to 25% in MDS patients (five of 21). Four patients had codon 12 substitutions of aspartic acid (GAT) for GGT, and one patient had a substitution of alanine (GCT) for GGT at codon 13.

MATERIALS AND METHODS

DNA isolation and PCR. DNA was isolated from frozen leukemic cells as previously described. Nucleated peripheral blood or bone marrow cells were obtained with informed consent from normal individuals or patients with MDS. Standard criteria were used in classifying MDS in 21 patients: three patients with refractory anemia and excess blasts (RAEB), five with chronic myelomonocytic leukemia (CML), nine with RAEB in transformation (RAEB-T), and four unclassified. A 109 base pair (bp) fragment of exon-1 of the N-RAS gene was amplified by PCR using 20 bp synthetic oligodeoxynucleotides spanning the 5' and 3' ends of the target sequence. PCR was performed by a modification of the method of Saiaki et al. The mixture was incubated at 95°C for one minute to denature the double-stranded DNA, cooled at 55°C for 30 seconds to allow primer annealing, followed by 1.5 minutes for extension at 70°C using 3 U of Taq DNA polymerase (Perkin Elmer Cetus; Norwalk, CT). This cycle was repeated 30 times using the Perkin-Elmer Cetus DNA Thermal Cycler.

Sequencing. The 109 bp fragment was electrophoresed on 2.5% Nusieve agarose (FMC Bioproducts; Rockland, ME), sliced from the gel, electroeluted, and purified on Elutip-D column (Schleicher & Schuell; Keene, NH) and ethanol-precipitated. Sequencing primers for sense and antisense orientations were end labelled with γ-[32P]-ATP (Amersham; Arlington Heights, IL) and T4 polynucleotide kinase (BRL) and annealed to the amplified DNA by heat denaturing the strands at 95°C for ten minutes. The reaction (10 μL) was divided into four tubes containing 2 μL of cold dNTPs and ddNTPs (Pharmacia; Piscataway, NJ) (ratio of 10:1) and T7 DNA polymerase (Sequenase, USB). The dried 10% acrylamide gel was exposed to film overnight without screen intensification.

RESULTS

The limit of sensitivity of the direct sequencing method permits the detection of a RAS mutation if present in 10% of the cells (mixing experiment, data not shown). Using this method, mutant N-RAS alleles were identified in five of 21 patients. Four MDS patients exhibited a codon 12 N-RAS mutation that substituted GAT for GGT. Figure 1A depicts the antisense sequence of an N-RAS exon-1 segment from an MDS case without mutation, while Fig 1B shows a mutated allele in another patient. This mutation was confirmed by sequencing the same segment in the sense direction (Fig 2).

One patient with mutation at codon 12 had RAEB, a second patient had RAEB-T, and two were unclassified. In all four patients, DNA was sequenced with sense and antisense primers to confirm the existence of a mutation. In all patients showing mutations, both normal and mutant alleles were detected. It cannot be determined whether this represents heterozygosity in a single clonal cell population or admixture of the two cell populations, each of which is homozygous.

A codon 13 N-RAS mutation that substituted GCT for...
Fig 1. Sequence of an amplified fragment of the antisense strand of exon-1 of N-RAS gene in (A) MDS patient without mutation and (B) MDS patient with codon 12 mutated allele substituting aspartic acid for glycine. ACC-ATC (antisense reading) or GGT-GAT (sense reading, see Fig 2). Lanes from left to right are: G, A, T, and C.

GGT was detected in one RAEB-T patient (Fig 3). This substitution was also confirmed using a sense orientation primer.

Among the patients with N-RAS mutated alleles, one patient with RAEB-T converted to AML within 2 months of the study.

DISCUSSION

Previous analysis of RAS gene mutations in human leukemia and other tumors have generally used hybridization probes specific for wild-type or certain mutated alleles of RAS gene codons 12, 13, or 61, or NIH 3T3 transfection and in vivo selection assays. These techniques can miss relevant mutations, and the latter can introduce mutations not present in vivo. A recently described technique for detecting mutations using RNAase sensitivity of RNA to DNA heteroduplexes would presumably detect many of the irrelevant third base mutations that do not change the coding
sequencing and which occur in many human tumors (unpublished observations, July 1988). It appears, therefore, that direct sequencing of genes is the only unambiguous method of detecting mutations inducing amino acid changes, provided that each sample can be sequenced in both sense and antisense directions, and that the mutation occurs in at least 10% of cells.

The reported frequencies of mutations of N-RAS gene in MDS patients vary between 9% and 40% depending on the techniques used in analysis.2,3 We believe that our finding of an N-RAS mutation frequency of 20% to 25% in MDS patients is more likely to represent the actual frequency, since PCR and direct sequencing provides a more reliable and unambiguous analysis. In addition, we find that the most frequent N-RAS mutation in MDS cells is the second base of codon 12 in which GAT is substituted for GGT.

The mechanisms underlying the conversion of MDS to AML are largely unknown. As yet the role of RAS mutation in MDS development and progression is unclear. In other types of leukemia it has been proposed that RAS gene activation by point mutation may play a role in progression of CML to blast crisis.4 We observed only one MDS patient with mutated N-RAS who rapidly progressed to AML. Our results support the data reported by Lyons et al who observed no correlation between the presence of mutated RAS genes and conversion to AML.5

Mutational activation of N-RAS exon-1 was found in only five of 21 patients with MDS in the current series. A mutation frequency of about 10% has been reported in several other human tumors that are associated with N-RAS activation, including common acute lymphoblastic leukemia,13 and T-ALL (unpublished observations). Several recent studies report mutations at codon 12 of Ki-RAS gene in MDS patients, at variable frequencies (6% to 50%).3,16 RAS gene activation is probably not the sole mechanism contributing to the development of these tumors. RAS oncogenes may render a selective growth advantage to some MDS cell populations in which they are activated rather than being essential for development and leukemic progression of all cases.

REFERENCES

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