Mutation of Ki-*ras* and N-*ras* Oncogenes in Myelodysplastic Syndromes

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Somatic mutation of the N-*ras* oncogene occurs frequently in de novo acute myeloid leukemia (AML). By virtue of their relation to AML, myelodysplastic syndromes (MDS) provide an in vivo model of human leukemogenesis. By using a strategy for analysis of gene mutation based on in vitro amplification of target sequences by the polymerase chain reaction (PCR) and selective oligonucleotide hybridization we analyzed the mutational status of codons 12, 13, and 61 of Ha-*ras*, K-*ras*, and N-*ras* in peripheral blood (PB) and/or bone marrow (BM) in 34 cases of primary MDS. Mutations at codon 12 of Ki-*ras* or N-*ras* were detected in three cases (9%): one of six cases of refractory anemia with excess blasts (RAEB) and two of nine cases of chronic myelomonocytic leukemia (CMML). The nucleotide substitution differed in each. In all cases the mutant allele was detectable in PB cells. A sustained hematologic remission was achieved after low-dose cytarabine therapy in the case of RAEB. Neither case of CMML exhibited signs of disease progression during follow-up at 7 and 12 months. In contrast, four of 31 patients without the *ras* mutation underwent transformation to AML within 12 months of genetic analysis. We conclude that *ras* mutations in MDS are heterogeneous and may develop at an early stage during the evolution of MDS. Their detection in PB cells illustrates the potential utility of *ras* mutation as a clonal marker in myeloid malignancy.

MYELODYSPLASTIC syndromes (MDS) comprise a heterogeneous group of clonal disorders characterized by quantitative and qualitative abnormalities of hematopoiesis. Transformation to acute myelogenous leukemia (AML) occurs in up to 40% of cases.[1] At a molecular level the mechanisms underlying the development of MDS and their evolution to AML are largely undefined. Activation of *ras* oncogenes by point mutation has been implicated in the development of many human tumors including AML by virtue of the ability to transform NIH3T3 cells in DNA transfection studies.[2] The human *ras* family comprises three genes, Ha-*ras*, Ki-*ras*, and N-*ras*, encoding closely related proteins of 21 kd that have guanine nucleotide binding and glutamyltranspeptidase activity and localize to the inner surface of the cell membrane.[3] Although their exact physiological role has yet to be defined, $p21^{ras}$ show structural and functional homology with other membrane-associated polypeptides, G proteins, that are known to be involved in the transduction of receptor-mediated signals across the cell membrane.

Tumor-derived *ras* genes are activated by single nucleotide alterations at codons 12, 13, and 61, with corresponding amino acid substitutions in $p21^{ras}$.[2,4] Mutational activation of N-*ras* occurs frequently in de novo AML.[4-7] In view of the close biologic affiliation between MDS and AML we were interested in establishing whether similar mechanisms operate in MDS. The mutational status of *ras* genes was determined by a sensitive dot-blot assay based on amplification in vitro of 20- to 100-base pair *ras* gene sequences encompassing codons 12, 13, and 61 by the polymerase chain reaction (PCR)[8,9] and hybridization to synthetic oligonucleotide probes specific for wild-type or mutant alleles. PCR, a recently described strategy for the amplification of specific human genomic sequences, uses oligonucleotide primers flanking the region of interest complementary to coding and noncoding strands to direct DNA synthesis. Repeated cycles of DNA denaturation, primer annealing, and enzymatic extension by DNA polymerase enables several hundred thousand-fold amplification of specific target sequences. In contrast to previous protocols using the Klenow fragment of *Escherichia coli* DNA polymerase I, we used a heat-stable DNA polymerase derived from the thermophilic organism *Thermus aquaticus* (Taq polymerase). This allows amplification to be performed at a higher temperature, thereby enhancing specificity, and obviates the need to replenish polymerase at every cycle. Mutant and wild-type *ras* alleles may be differentiated by hybridization to radiolabeled oligomers under stringent temperature conditions discriminating a single base pair mismatch on the basis of the higher melting point (3 to 5°C) of a perfectly matched hybrid.[10] With this strategy we analyzed the mutational status of *ras* oncogenes in 34 cases of primary MDS.

MATERIALS AND METHODS

Patients were classified according to French-American-British (FAB) criteria[11] as follows: refractory anemia (RA), 13 patients; RA with ring sideroblasts (RARS), four; RA with excess blasts (RAEB), six; RAEB in transformation (RAEB-t), two; and chronic myelomonocytic leukemia (CMML), nine (Table 1). The duration of disease at the time of analysis varied between <1 and 147 months. Peripheral blood (PB) and bone marrow (BM) samples were obtained with informed consent.

DNA isolation. Cells were centrifuged at 5,000 rpm for five minutes at 4°C and 10 mL of 0.8% Triton X-100 added to the pellet. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C and 8 mL 7 mol/L urea, 1 mL 10× lysis buffer (1× lysis buffer, 0.35 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), and 1 mL 10% sodium dodecyl sulfate (SDS) added to the nuclear pellet. After gentle agitation, 15 mL 1× lysis buffer was added and DNA extracted with 5 mL phenolchloroform. After two further extractions, DNA was precipitated with ethanol, washed in 70% ethanol,

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Table 1. Clinical Data

FAB Type	Age (yr)/Sex	Disease Duration (mo)	Follow-up After Genetic Analysis (mo)	Source of DNA
RA (n = 13)	67/M	1	9,* AML	PB, BM
	88/F	9	10	PB
	72/M	76	9	BM
	86/F	61	14	PB
	79/M	62	13	PB
	76/F	36	12	PB
	73/F	147	3,* unrelated	PB
	53/F	92	11	PB
	67/M	30	11	PB
	77/F	41	13	PB, BM
	75/F	62	10	PB
	82/M	100	11	PB
	79/F	36	13	PB
RARS (n = 4)	74/F	13	1,* cytopenia	BM
	87/F	127	12	PB
	76/F	47	13	PB
	63/F	24	4,* unrelated	PB
RAEB (n = 6)	†52/F	1	10	PB
	79/M	2	13, RAEB-t, 3 mo	PB, BM
	64/M	<1	8	BM
	53/F	2	7,* AML	PB
	65/M	6	7,* cytopenia	PB
	78/F	1	13	PB
RAEB-t (n = 2)	60/F	1	7, AML, 4 mo	BM
	66/M	<1	12	BM
CMML (n = 9)	†68/M	39	7	PB, BM
	†72/M	14	12	PB
	93/M	62	8	PB, BM
	78/M	19	14, AML, 12 mo	PB
	73/M	65	12	BM
	60/M	49	11	PB
	73/M	<1	8	BM
	76/M	33	10	PB
	72/M	8	10,* cytopenia	PB, BM

*Died.

†, Cases in which *ras* mutation was detected.

and dissolved in 500 μL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5). Samples were stored at 4°C.

Oligonucleotides. Oligomers (20-mers) used in priming and hybridization were synthesized by the solid-phase triester method. For hybridization, oligomers were end labeled to a high specific activity (>2 × 10^6 cpm/pmol) by using α^{32}P-dATP (New England Nuclear, Boston) and T4-polynucleotide kinase (Pharmacia Laboratories, Piscataway, NJ) and separated from unincorporated dATP on Sephadex G50.

PCR. One hundred fifty nanograms of genomic DNA was incubated with 100 ng of amplimer complementary to sequences upstream and downstream of the codon to be screened in the presence of Taq polymerase (New England Biolabs, Inc, New Haven, CT). The reaction mixture included 1 mmol/L each of deoxynucleotide triphosphate, 67 mmol/L Tris-HCl, pH 8.8, 6.7 mmol/L $MgCl_2$, 16.6 mmol/L $(NH_4)_2SO_4$, 10% dimethyl sulfoxide, 10 mmol/L β-mercaptoethanol, and 6.7 μmol EDTA in a total volume of 50 μL. PCR was performed by a modification of the method described by Saiki et al.[8] The mixture was incubated at 95°C for five minutes to denature the double-stranded DNA then cooled at 37°C for five minutes to allow primer annealing. Primer extension was initiated by the addition of 5 units of Taq polymerase and allowed to proceed at 60°C for five minutes. Fifteen cycles of amplification with an outer set of primers were followed by 15 cycles with an inner set to enhance specificity after the addition of 5 units of Taq polymerase.

Oligomer hybridization. Amplified DNA (5 ng) was spotted onto nylon filters (Gene Screen Plus, New England Nuclear) and fixed by UV illumination. The filters were prehybridized overnight at 50°C in 5× SSPE (10 mmol/L sodium phosphate, pH 7.0, 0.18 mol/L NaCl, and 1 mmol/L EDTA), 7% SDS, 100 μg/mL sonicated and denatured salmon sperm DNA, and 5× Denhardt's solution and hybridized for three hours at 50°C in the presence of 1 ng α^{32}P-labeled oligomer probe. The oligomer panel included probes specific for the wild-type allele and all possible amino acid substitutions at codons 12, 13, and 61 of N-*ras*, Ki-*ras* and Ha-*ras* (Table 2). Posthybridization filters were washed twice in 2× SSPE, 0.1% SDS for five minutes at room temperature and once in 5× SSPE, 0.1% SDS for 30 minutes at 50°C. A final high-stringency wash was performed for ten minutes at 59°C for N-*ras* 61 and Ki-*ras* 61 probes; 63°C for N-*ras* 12/13, Ki-*ras* 12/13, and Ha-*ras* 61 probes; and 72°C for Ha-*ras* 12/13 probes. The filters were exposed to Kodak XAR film for 12 hours at −70°C with intensifying screens.

RESULTS

Mutant *ras* alleles were identified in three of 34 patients. In two of these PB was the sole source of DNA analyzed. One patient with CMML exhibited a codon 12 Ki-*ras* mutation that substituted aspartic acid (GAT) for glycine (GGT) (Fig 1A). A codon 12 Ki-*ras* mutation that substituted valine (GTT) for GGT was detected in a patient with RAEB (Fig 1A). A codon 12 N-*ras* mutation substituting alanine (GCT) for GGT was identified in both BM and PB cells of a second patient with CMML (Fig 1B). In patients showing mutation, both normal and mutant alleles were detected. The limit of sensitivity of our dot-blot assay permits the detection of a *ras* mutation if present in at least 10% of the cells (data not shown). Because blast cells accounted for less than 5% of the cells in samples from which DNA was isolated (Table 3), it may be concluded that hematopoietic stem cells bearing mutant *ras* genes retain differentiation potential in vivo. None of the patients screened exhibited a Ha-*ras* mutation. As a positive control, a primary AML in which we recently identified a codon 12 Ha-*ras* mutation substituting GTC for GGC was used (Fig 1C).[7]

After treatment with cytarabine, 10 mg/m^2 twice daily subcutaneously, for 21 days the patient with RAEB showing mutation entered a hematologic remission that has been maintained for 6 months by intermittent cytarabine therapy. After follow-ups of 7 and 12 months, no sign of disease progression has been observed in either patient with CMML, which suggests that the mutation may have arisen at an early stage during the evolution of MDS. Among patients with exclusively wild-type *ras*, conversion to RAEB-t occurred in one and to AML in four patients, two of whom died. A further three patients died as a consequence of cytopenia.

Table 2. Synthetic Oligomers Used to Analyze *ras* Gene Mutation

Oligomer	Sequence	Strand		Amino Acid
Ha-12wt*	GTGGGCGCCGGCGGTGTGGG	s†		Gly
Ha-12/p1‡	----------NGC--------	s	N=C,A,T	
Ha-12/p2	----------GNC--------	s	N=C,A,T	
Ha-12/2	----------CGC--------	s		Arg
Ha-12/3	----------AGC--------	s		Ser
Ha-12/4	----------TGC--------	s		Cys
Ha-12/5	----------GCC--------	s		Ala
Ha-12/6	----------GAC--------	s		Asp
Ha-12/7	----------GTC--------	s		Val
Ha-13wt	GTGGGCGCCGGCGGTGTGGG	s		Gly
Ha-13/p1	----------NGT-----	s	N=C,A,T	
Ha-13/p2	----------GNT-----	s	N=C,A,T	
Ha-13/2	----------CGT-----	s		Arg
Ha-13/3	----------AGT-----	s		Ser
Ha-13/4	----------TGT-----	s		Cys
Ha-13/5	----------GCT-----	s		Ala
Ha-13/6	----------GAT-----	s		Asp
Ha-13/7	----------GTT-----	s		Val
Ha-61wt	TACTCCTCCTGGCCGGCGGT	a		Gln
Ha-61/p1	--------CTN----------	a	N=C,A,T	
Ha-61/p2	--------CNG----------	a	N=C,A,G	
Ha-61/p3	--------NTG----------	a	N=A,G	
Ha-61/2	--------CTC----------	a		Gln
Ha-61/3	--------CTA----------	a		Stop
Ha-61/4	--------CTT----------	a		Lys
Ha-61/5	--------CAG----------	a		Leu
Ha-61/6	--------CCG----------	a		Arg
Ha-61/7	--------CGG----------	a		Pro
Ha-61/8	--------ATG----------	a		His
Ha-61/9	--------GTG----------	a		His
Ki-12wt	CCTACGCCACCAGCTCCAAC	a		Gly
Ki-12/p1	--------ACN----------	a	N=A,T,G	
Ki-12/p2	--------ANC----------	a	N=A,T,G	
Ki-12/2	--------ACA----------	a		Cys
Ki-12/3	--------ACT----------	a		Ser
Ki-12/4	--------ACG----------	a		Arg
Ki-12/5	--------AAC----------	a		Val
Ki-12/6	--------ATC----------	a		Asp
Ki-12/7	--------AGC----------	a		Ala
KI-13WT	CCTACGCCACCAGCTCCAAC	a		Gly
Ki-13/p1	-----GCN----------	a	N=A,T,G	
Ki-13/p2	-----GNC----------	a	N=A,T,G	
Ki-13/2	-----GCA----------	a		Cys
Ki-13/3	-----GCT----------	a		Ser
Ki-13/4	-----GCG----------	a		Arg
Ki-13/5	-----GAC----------	a		Val
Ki-13/6	-----GTC----------	a		Asp
Ki-13/7	-----GGC----------	a		Ala
Ki-61wt	TACTCCTCTTGACCTGCTGT	a		Gln
Ki-61/p1	--------TTN----------	a	N=A,T,C	
Ki-61/p2	--------TNG----------	a	N=A,G,C	
Ki-61/p3	--------NTG----------	a	N=A,G	
Ki-61/2	--------TTC----------	a		Gln
Ki-61/3	--------TTA----------	a		Stop
Ki-61/4	--------TTT----------	a		Lys
Ki-61/5	--------TCG----------	a		Arg
Ki-61/6	--------TAG----------	a		Leu
Ki-61/7	--------TGG----------	a		Pro
Ki-61/8	--------ATG----------	a		His
Ki-61/9	--------GTG----------	a		His
N-12wt	GGAGCAGGTGGTGTTGGGAA	s		Gly
N-12/p1	------NGT----------	s	N=A,C,T	

Table 2. Synthetic Oligomers (Cont'd)

Oligomer	Sequence	Strand		Amino Acid
N-12/p2	------GNT----------	s	N=A,C,T	
N-12/2	------AGT----------	s		Ser
N-12/3	------CGT----------	s		Arg
N-12/4	------TGT----------	s		Cys
N-12/5	------GAT----------	s		Asp
N-12/6	------GCT----------	s		Ala
N-12/7	------GTT----------	s		Val
N-13/p1	---------NGT---------	s	N=A,T,C	
N-13/p2	---------GNT---------	s	N=A,T,C	
N-13/1	---------AGT---------	s		Ser
N-13/2	---------CGT---------	s		Arg
N-13/3	---------TGT---------	s		Cys
N-13/4	---------GAT---------	s		Asp
N-13/5	---------GCT---------	s		Ala
N-13/6	---------GTT---------	s		Val
N-61wt	TACTCTTCTTGTCCAGCTGT	a		Gln
N-61/p1	--------TTN----------	a	N=A,T,C	
N-61/p2	--------TNG----------	a	N=A,G,C	
N-61/p3	--------NTG----------	a	N=A,G	
N-61/2	--------TTC----------	a		Glu
N-61/3	--------TTA----------	a		Stop
N-61/4	--------TTT----------	a		Lys
N-61/5	--------TCG----------	a		Arg
N-61/6	--------TAG----------	a		Leu
N-61/7	--------TGG----------	a		Pro
N-61/8	--------ATG----------	a		His
N-61/9	--------GTG----------	a		His

*wt, wild type (normal) sequence.

†Sequences complementary to the coding (s, sense) or noncoding (a, antisense) strand.

‡p, pooled oligomers, in which nucleotide N in the relevant codon is replaced by different nucleotides as indicated.

DISCUSSION

Up to 25% of patients with de novo AML exhibit mutation of N-*ras* preponderantly at codons 13 and 61.[8] Mutational activation of N-*ras* is implicated further in human myeloid malignancy by its recognition in MDS. Recently, Hirai et al identified an N-*ras* codon 13 mutation substituting arginine (CGT) for GGT in three of eight patients with MDS by an in vivo selection assay.[12] Our data establish that point mutations in MDS are heterogeneous and involve other members of the *ras* gene family. Studies of mammalian carcinogenesis indicate the pattern of *ras* gene mutation to be carcinogen specific.[13] Thus, the distinct pattern of *ras* activation seen by Hirai et al and us may reflect exposure to different mutagens.

The present study further illustrates the value of in vitro DNA amplification in the analysis of gene mutation. Genomic sequences of up to 1.5 kilobases may be faithfully amplified by PCR and larger regions of DNA analyzed indirectly by amplification of a complementary gene copy (cDNA) derived from an mRNA template by using reverse transcriptase. PCR permits rapid analysis of *ras* gene mutation from as little as 1 ng of genomic DNA or alternatively may be performed directly on cell lysates, which eliminates the need for DNA isolation (Saiki et al[14] and Janssen, unpublished data). Moreover, direct DNA analysis circumvents the problems of mutation acquired during transfection[15] and the admixture of nontumor cells. The latter may reduce the sensitivity of transfection-based analyses and poses a specific problem in MDS where it is difficult to determine the proportion of cells derived from the neoplastic clone.

Whether *ras* activation is an early or late event in tumor development is controversial. Models of mammalian carcinogenesis provide evidence that *ras* activation may be involved in tumor initiation.[13] Recently, the Ki-*ras* mutation has been shown to be an early event in the development of human colonic carcinoma and lung adenocarcinoma.[16-18] In contrast, the *ras* mutation has been linked to tumor progression in malignant melanoma.[19] MDS provide a unique model for definition of the temporal and mechanistic role of *ras* activation in myeloid leukemogenesis. As yet the chronology of *ras* mutation in MDS is unclear. Hirai et al[12] propose that the N-*ras* mutation is predictive of leukemic transformation. In contrast, we have observed no correlation between the presence of mutated *ras* genes and conversion to AML. Moreover, AML developed in four patients without detectable *ras* mutation. These disparities may be reconciled within the context of multistep theories of leukemogenesis that propose that a leukemic phenotype may be realized by alternative routes through reassortment of the constituent steps. Serial genetic analysis in cases evolving to AML in which only wild-type *ras* was detected at initial screening and multivariate analyses incorporating other independent prognosticators[1] may offer clarification.

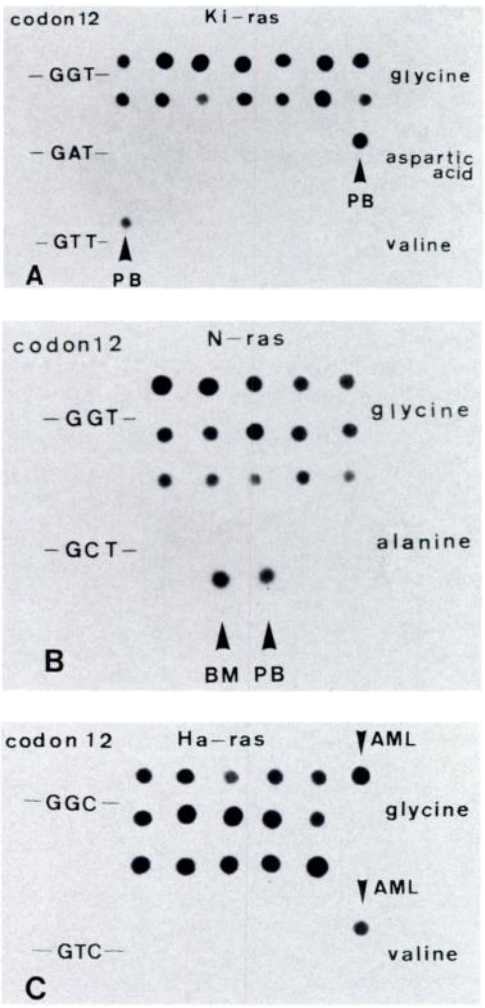

Fig 1. (A and B) Dot-blot analysis of *ras* gene mutation in MDS. Amplified DNA preparations from the PB and/or BM of 14 patients were screened in the experiments shown by using oligonucleotide probes specific for amino acids at codon 12 of Ki-*ras* (A) and N-*ras* (B). Of the samples screened for Ki-*ras* 12 mutation, one patient with CMML and one with RAEB exhibited mutations in the PB that corresponded to a substitution of the normal amino acid GGT by GAT and GTT, respectively (A). An N-*ras* 12 mutation substituting GGT with GCT was identified in the PB and BM of one patient with CMML (B). (C) Illustration of an H-*ras* 12 mutation substituting GTC for GGC in a patient with AML.

Table 3. Sample Makeup in Patients Showing *ras* Mutation

	Leukocyte Count					
FAB Type	Total ($\times 10^9$/L)	N (%)	L (%)	M (%)	BL (%)	BM Blasts (%)
CMML	29.8	65	14	21	0	—
RAEB	5.0	66	16	17	1	—
CMML	13.6	55	29	16	0	5

Abbreviations: N, neutrophils; L, lymphocytes; M, monocytes; BL, blasts.

The detection of mutation in PB illustrates the potential utility of the *ras* mutation as a clonal marker in MDS. The clonality of hematopoiesis in MDS has been established by glucose-6-phosphate dehydrogenase analyses, which indicate the target cell to be a stem cell pluripotent for myeloid and lymphoid differentiation.[20,21] Subsequent clonal evolution may involve a progenitor committed to myeloid differentiation.[21] The spectrum of hematopoietic lineages exhibiting *ras* mutation may therefore confirm whether *ras* activation in MDS is an early event or a step in their clonal evolution that pertains to the development of leukemia.

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ADDENDUM

Since submission of this work, Liu et al[22] have reported the Ki-*ras* mutation in two of four patients with MDS.

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