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RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients younger than 60 years

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The pathogenesis of acute myeloid leukemia (AML) involves the cooperation of mutations promoting proliferation/survival and those impairing differentiation. The RAS pathway has been implicated as a key component of the proliferative drive in AML. We have screened AML patients, predominantly younger than 60 years and treated within 2 clinical trials, for *NRAS* (n = 1106), *KRAS* (n = 739), and *HRAS* (n = 200) hot-spot mutations using denaturing high-performance liquid chromatography or restriction fragment length polymorphism (RFLP) analysis. *NRAS* mutations were confirmed in 11% of patients (126/1106) and *KRAS* mutations in 5% (39/739). No *HRAS* mutations were detected in 200 randomly selected samples. Codons most frequently mutated were *N12* (43%), *N13* (21%), and *K12* (21%). *KRAS* mutations were relatively overrepresented in French-American-British (FAB) type M4 ($P < .001$). *NRAS* mutation was overrepresented in the t(3;5)(q21∼25;q31∼q35) subgroup ($P < .001$) and underrepresented in t(15;17)(q22;q21) ($P < .001$). *KRAS* mutation was overrepresented in inv(16)(p13q22) ($P = .004$). Twenty-three percent of *KRAS* mutations were within the inv(16) subgroup. *RAS* mutation and *FLT3* ITD were rarely coexistent (14/768; $P < .001$). Median percentage of *RAS* mutant allele assayed by quantitative RFLP analysis was 28% (*N12*), 19% (*N13*), 25% (*N61*), and 21% (*K12*). *RAS* mutation did not influence clinical outcome (overall/disease-free survival, complete remission, relapse rate) either for the entire cohort or within cytogenetic risk groups. (Blood. 2005;106:2113-2119)

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Introduction

The cytogenetic characterization of subgroups of acute myeloid leukemia (AML) has promoted a risk-directed therapeutic approach to disease management. The recent identification of an internal tandem duplication (ITD) within the *FLT3* gene provides a paradigm for molecular markers with additional prognostic power in AML, in this case predictive for reduced disease-free survival, event-free survival, and overall survival, and for increased relapse risk.[1] Abnormalities of signal transduction pathways are common in AML, occurring in up to 50% of cases. These abnormalities comprise activating mutations in genes encoding receptor tyrosine kinases including *FLT3* (ITD and D835[2] mutation) and *c-KIT*,[3] RAS protein activation (via mutation or loss of negative regulators such as nuclear factor-1 [NF-1][4]), and phosphorylation of mitogen-activated protein (MAP) kinase.[5]

The *RAS* genes encode a family of membrane-associated proteins, which regulate signal transduction upon binding of ligand to a variety of membrane receptors. There are 3 functional *RAS* genes: *N*- (from a neuroblastoma cell line), *K*- (Kirsten), and *H*- (Harvey) *RAS,* each containing 4 exons. *KRAS* has an A and B protein encoded from alternative fourth exons. *RAS* gene mutations at codons 12, 13, and 61 confer constitutive activation of the RAS protein, which is held in the guanosine triphosphate (GTP)–bound state. *RAS* gene mutations were first reported in myeloid malignancies 17 years ago,[6] and many groups have since attempted to study their frequency in small/medium-sized cohorts of AML patients.[7-12] Several studies indicate that *RAS* gene mutation is associated with poor outcome in AML and myelodysplastic syndromes (MDSs),[7,13,14] but historical data sets are of insufficient size to distinguish prognostic differences between subgroups.

We have assayed *RAS* mutational status by denaturing high-performance liquid chromatography (DHPLC) and restriction fragment length polymorphism (RFLP) in a large trial-based patient cohort. We have correlated this with presenting morphology, cytogenetics, and *FLT3* ITD status where available. We demonstrate that *RAS* mutation frequency and spectrum differ between biologically distinct subtypes of AML but do not influence clinical outcome.

Patients, materials, and methods

Patients

The study cohort comprised 1106 patients at presentation with AML who were entered into the Medical Research Council (MRC) AML10 (n = 387) and AML12 (n = 719) clinical trials for patients younger than 56 and younger than 60 years, respectively. Informed consent for tissue collection

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and research studies was approved by the Research Ethics Committee for Wales (02/4560) and the Multi Research Ethics Committee for Wales (98/9/08). This study was approved by the MRC AML Trial Cell Bank Research Group, and samples were analyzed anonymously.

Cytogenetic definitions

G-banded karyotypes were collected centrally and described according to the International System for Human Cytogenetic Nomenclature (ISCN).[15] Cytogenetic classification was performed as previously described.[16] Briefly, a predefined list of specific clonal chromosomal abnormalities was used to classify patients into non–mutually exclusive subgroups. As all clonal aberrations were counted, individual patients may be counted more than once. Patients were then classified hierarchically into risk groups (favorable, intermediate, and adverse) according to the presence of specific primary aberrations and a complex karyotype, defined as one with at least 5 unrelated abnormalities.

Therapy

The AML10 clinical trial protocol has previously been published in detail.[17] Patients in the AML12 trial were randomized to receive induction therapy with either ADE (cytarabine, daunorubicin, etoposide) 10 + 3 + 5 or MAE (mitoxantrone, cytarabine, etoposide) 3 + 10 + 5 from 1994 to 1998. After 1998, induction therapy was modified to DAT (daunorubicin, cytarabine, thioguanine) 3 + 10 + 10 randomized to 2 different induction cytarabine doses, with an additional randomization to all-*trans* retinoic acid (ATRA) versus no ATRA. Bone marrow (BM) remission status was assessed and patients were randomized according to risk group (good, standard, or poor risk) defined on the basis of hierarchic cytogenetic classification[16] and remission status after course 1.[18] Consolidation chemotherapy for good/standard-risk groups comprised a second course of the induction regimen, followed by MACE (amsacrine, cytarabine, etoposide). Good-risk patients received a fourth course of either MidAC (mitoxantrone and cytarabine) or ICE (idarubicin, cytarabine, and etoposide) + MidAC as course 5; standard risk, either MidAC, ICE + MidAC, ICE + stem cell transplant (SCT; allogeneic or autologous depending on donor availability), or SCT alone. Poor-risk patients could be entered into the MRC Refractory/Relapse AML study as previously described.[19]

End points

Complete remission (CR) was defined as less than 5% bone marrow blasts. Full hematologic recovery was not required, though 97% of patients achieved neutrophil counts higher than 1×10^9/L and platelet counts higher than 100×10^9/L. Resistant disease (RD) was defined as more than 15% BM blasts and partial remission as 5% to 15% BM blasts after course 1. Induction death (ID) was defined as death within 30 days of entry; and deaths more than 30 days after entry were defined as RD. Overall survival (OS) was defined as the time from entry to death. Patients failing to achieve remission were considered to have an event on day 1. For patients achieving first CR, disease-free survival (DFS) was defined as the time from first CR to an event (death in CR or relapse). Relapse risk (RR) was the cumulative probability of relapse, censoring at death in CR; death in CR was the cumulative probability of dying in first CR, censoring at relapse.

NRAS mutation screen: denaturing high-performance liquid chromatography (DHPLC)

DNA was extracted predominantly from bone marrow cells (mononuclear cells or buffy coat) at AML presentation. The *NRAS* gene was screened for mutations at hot-spot codons 12, 13, and 61 as previously described.[20] All samples with an abnormal DHPLC profile were reamplified from genomic DNA for confirmation on a second screen before sequencing. Our DHPLC assays cannot distinguish samples with 100% mutant DNA (biallelic mutation in all cells or loss of heterozygosity of the wild-type allele plus mutation of the retained allele) from 100% wild-type DNA. We therefore randomly selected 200 patient samples with known single DHPLC peaks (ie, apparently wild-type *N12/13*). Polymerase chain reaction (PCR) products from random pairs of these patients were mixed, before heteroduplex formation and reanalysis of these 100 profiles by DHPLC. Sensitivity of *N12/13* mutation detection by DHPLC was assessed by spiking known wild-type *NRAS* DNA with different proportions of known cloned mutant *NRAS* DNA (MDS92 cell line, codon 12.2 G>C mutation).

KRAS codon 61 and *HRAS* codon 61 were assayed by DHPLC. Primers and conditions used are shown in Table 1.

KRAS and *HRAS* codons 12 and 13 mutation screening: restriction fragment length polymorphism (RFLP) analysis

KRAS codon 12 amplification was modified from Nishikawa et al,[21] with an alternative reverse primer shown in Table 1. *HRAS* codon 12 primers and PCR conditions are shown in Table 1. *KRAS* codon 13 method was per Lin et al.[22] *HRAS* codon 13 method was per O'Leary et al.[23]

RAS mutation confirmation/characterization

Samples with an abnormal RFLP or DHPLC profile were confirmed as mutant by DNA sequencing, using a fluorescent primer–adapted chain-termination method[24] on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). When direct sequencing was negative, PCR products were cloned (Original TA Cloning Kit; Invitrogen, Groningen, the Netherlands) and sequenced.

Ratio of *RAS* mutant: wild-type alleles

Percent *RAS* mutant DNA was assayed for *N12*, *N13* (G>A only), *N61*, and *K12* by radioactive PCR-RFLP. PCR was performed as previously described using an end-labeled primer with 25 cycles of amplification at an annealing temperature of 63°C.[1] Digestion products were separated on 6% denaturing polyacrylamide gel electrophoresis (PAGE), dried, autoradiographed, and quantitated by densitometry. Mutations could be detected at the following codons: *N12.1* or *N12.2; N13.2* G>A; *N61.1* or *N61.2;* and *K12.1* or *K12.2.* Mutant was expressed as percent of total for results.

FLT3 ITD mutation analysis

Exons 13 and 14 of the *FLT3* gene were amplified from genomic DNA as previously described.[1,7]

Statistical methods

Continuous data were compared using two sample *t* tests for data that were approximately normally distributed, and the nonparametric Wilcoxon rank-sum test for other types of distributions. Categoric data were compared using Fisher exact test for 2×2 tables, chi-squared tests for heterogeneity in larger tables, and Mantel-Haeszel tests for trend over ordered categories in 2×2 tables. Kaplan-Meier life tables were constructed for survival data and were compared using the log-rank test. Surviving patients for both AML10 and AML12 were censored on April 1, 2004. Median follow-up time for AML10 patients was 128 months (range, 52-189 months) and for AML12 patients was 72 months (range, 6-110 months), with a median follow-up time of 86 months for both trials when taken together. In order to build prognostic models associated with *NRAS* mutation or to adjust for multiple other factors, either logistic regression for categoric outcomes or proportional hazards regression (for time-to-event outcomes) was used, using forward selection techniques, with an entry probability of 0.01. All reported *P* values are 2-sided, and to allow for multiple testing, results are not considered statistically significant unless *P* is less than .01.

Results

Mutation frequency

NRAS mutations were confirmed in 11% of patients (126/1106) and *KRAS* mutations in 5% (39/739). *KRAS* mutation status was successfully analyzed in fewer patients because of poor efficiency of the K13 mutation assay and also insufficient DNA for all assays

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Table 1. Experimental conditions for *RAS* mutation analysis, screening by denaturing high-performance liquid chromatography (DHPLC), and restriction fragment length polymorphism (RFLP) analysis quantification of mutant allele percentage by PCR with an end-labeled radioactive primer

Method type/assay	Primer sequence	Annealing temperature, °C	No. PCR cycles	Amplicon size, bp	RFLP enzyme	RFLP digest product sizes, bp
Mutation screening						
DHPLC						
K61F*	5′-tgt gtt tct ccc ttc tca gga ttc-3′	60	35	158	NA	NA
K61R	5′-tgg caa ata cac aaa gaa agc c-3′	60	35	158	NA	NA
K61F*	5′-gat tcc tac cgg aag cag gt-3′	60	35	151	NA	NA
K61R	5′-tgg tgt tgt tga tgg caa ac-3′	60	35	151	NA	NA
RFLP and sequencing						
K12F (MM)	5′-gac tga ata taa act tgt ggt agt tgg acc t-3′	56	35	121	*Bst*N1 (7.5 units)	Wt = 91
K12R	5′-aac aag att tac ctc tat tgt tgg atc a-3′	56	35	121	(7.5 units)	Mut = 121
H12F	5′-agg aga ccc tgt agg agga-3′	60	35	170	*Msp*1 (10 units)	Wt = 23, 56, 91
H12R	5′-gcg cta ggc tca cct cta t-3′	60	35	170	(10 units)	Mut = 23, 147
Mutant allele quantification: PCR with a end-labeled radioactive primer						
N12F (MM)	5′-act gag tac aaa ctg gtg gtg gtt gga cca-3′	63	25	172	*Scr*F1	Wt = 143
N12R	5′-tgg gta aag atg atc cga caa gtg a-3′	63	25	172	*Scr*F1	Mut = 172
N13F	5′-ctc cag aag tgt gag gcc gat-3′	63	25	250	*Dpn*II	Wt = 250
N13R (MM)	5′-ctg gat tgt cag tgc gct ttt ccc aag a-3′	63	25	250	*Dpn*II	Mut = 221
N61F (MM)	5′-tgt ttg ttg gac ata ctg gat aca gct gta-3′	63	25	225	*Bsr*G1	Wt = 225
N61R	5′-tct tcc cta gtg tgg taa cct c-3′	63	25	225	*Bsr*G1	Mut = 198
K12F (MM)	5′-gac tga ata taa act tgt ggt agt tgg acc t-3′	63	25	157	*Scr*F1	Wt = 127
K12R	5′-caa aga atg gtc ctg cac cag t-3′	63	25	157	*Scr*F1	Mut = 157

bp indicates base pair; F indicates forward; R, reverse; MM, mismatched; Wt, wild type; and Mut, mutant.
*DHPLC temperature was 59°C for K61 and 64°C for H61.

in some patients. Initial screening of randomly selected samples (n = 200) revealed no mutations at *K61, H12, H13,* or *H61. FLT3* ITD was present in 26% of patients (287/1099) assayed for *NRAS* mutation and 24% of patients (180/736) assayed for *KRAS* mutation. *FLT3* ITD status for 854 of these patients was reported previously.[1]

Combinations of *NRAS* and *KRAS* mutation and *FLT3* ITD were coexistent in the same patient in only 14 of 739 patients, in whom all mutations were assayed ($P < .001$). Mutations that were coexistent broke down as follows: *FLT3* ITD + *NRAS,* 11 (1%) of 1099; *FLT3* ITD + *KRAS,* 3 (0.4%) of 736; and *NRAS* + *KRAS* 2 (0.3%) of 724.

We also directly sequenced DNA from 25 randomly selected AML patients from this cohort, with normal DHPLC profiles (*N12/13* = 15, *N61* = 10), and all were confirmed as wild-type *NRAS.* Dilution experiments showed that 10% or more of *N12/13 RAS* mutant DNA could be confidently detected within a background of wild-type sequence (data not shown). Mutations were confirmed by direct sequencing for 91 (80%) of 126 *NRAS* and 15 (38%) of 39 *KRAS,* while cloning and sequencing was required to confirm the remainder. One hundred DHPLC profiles from randomly paired samples with previously known single peaks remained as single peaks in the *N12/13* assay (data not shown), confirming that biallelic mutation in all cells was likely to be a rare event.

RAS mutation spectrum

Codons most frequently mutated were *N12* (43%), *N13* (21%), and *K12* (21%). Bases most frequently mutated were *N12.2* (32%), *K12.2* (20%), and *N13.2* (16%). Of 39 *KRAS* mutations, 33 were at *K12.2.* G>A transition was the most common base substitution (62% *NRAS,* 54% *KRAS*). The next most frequent changes were G>C transversion, G>T transversion, then C>A transversion in *NRAS,* and G>T transversion then G>C transversion in *KRAS.* G>A transition predominated at base 2 of *N12, N13, K12,* and *K13.* At base 1, G>A transition predominated at *N12* but was strikingly absent at *N13,* where G>C transversion predominated. All 9 substitutions at *N61* base 1 were C>A transversion, while A>G transition predominated at base 2 and A>C transversion at base 3. While glycine → aspartate was the most common amino acid change at *N12, N13, K12,* and *K13,* glycine → serine and glycine → alanine were frequent at *N12* but absent at *N13.* By contrast, glycine → arginine and glycine → valine were more common at *N13* than *N12.* At *N61* the most common amino acid change was glutamine → lysine followed by glutamine → arginine. Only one mutation was identified outside hot-spot codons 12, 13, and 61. This was at *NRAS* codon 22;1, C>A (glycine → lysine).

Ratio of *RAS* mutant: wild-type alleles

Percent *RAS* mutant DNA (clonal size) was assayed for *N12, N13* (G>A only), *N61,* and *K12* by radioactive PCR-RFLP. Median *RAS* mutant DNA percentage was 28% (*N12*), 19% (*N13*), 25% (*N61*), and 21% (*K12*).

RAS mutation and presenting clinical/morphologic patient characteristics

Central morphology review revealed that a small number of patients had acute lymphoblastic leukemia, but these cases were retained for completeness. *RAS* mutation frequency did not vary significantly with age, sex, presenting white cell count, WHO performance status, or de novo versus secondary AML (not shown). *KRAS* mutation frequency demonstrated significant heterogeneity among French-American-British (FAB) subgroups, more common in M4 ($P < .001$; Table 2).

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Table 2. *RAS* mutation status by French-American-British (FAB) subtype

FAB type	*NRAS*		*KRAS*	
	Mutant, no. (%)	Nonmutant, no. (%)	Mutant, no. (%)	Nonmutant, no. (%)
AML M0	4 (3)	20 (2)	2 (5)	12 (2)
AML M1	20 (16)	159 (17)	0 (0)	108 (16)
AML M2	31 (25)	254 (28)	9 (24)	187 (29)
AML M3	11 (9)	170 (19)	5 (13)	129 (20)
AML M4	38 (30)	173 (19)	19 (50)	131 (20)
AML M5	18 (14)	87 (10)	2 (5)	59 (9)
AML M6	2 (2)	19 (2)	0 (0)	13 (2)
AML M7	0 (0)	11 (1)	0 (0)	7 (1)
RAEB	2 (2)	10 (1)	1 (3)	6 (1)
Bilineage	0 (0)	1 (< 0.5)	0 (0)	0 (0)
ALL	0 (0)	6 (1)	0 (0)	4 (1)
Other	0 (0)	0 (0)	0 (0)	0 (0)
Total with known FAB type	126 (100)	910 (100)	38 (100)	656 (100)
Unknown/unclassified	0 (0)	70 (8)	1 (3)	44 (7)

Percentages are percentages of those with known FAB type. $P = .04$ for *NRAS;* $P = .001$ for *KRAS. P* values for those with known FAB type only. RAEB indicates refractory anemia with excess of blasts; ALL, acute lymphoblastic leukemia.

RAS/FLT3 mutation frequency varies between cytogenetic subgroups

Cytogenetic data were available for 922 patients (Table 3). *RAS* mutation frequency varied between karyotypic subtypes, with evidence for underrepresentation of *NRAS* mutation in t(15;17)(q22;q21) ($P = .008$) and strong evidence for overrepresentation of *NRAS* mutation in t(3;5)(q21~25;q31~q35) ($P < .001$) and *KRAS* mutation in inv(16)(p13q22) ($P = .003$). Twenty-three percent of *KRAS* mutations were within the inv(16) subgroup. No significant difference in *NRAS* or *KRAS* mutant frequency was found between cytogenetic risk groups (favorable/intermediate/adverse)(data not shown).

RAS mutational status: clinical outcome

The presence of *NRAS* mutation did not significantly influence complete remission rate, induction death, resistant disease, relapse rate, disease-free survival, or overall survival either for the entire cohort (Table 4; Figure 1) or for cytogenetic risk groups (not shown), either in univariate analysis or in a proportional hazards model (including age, white cell count, cytogenetic risk group, de novo versus secondary, and *FLT3 ITD*).

Discussion

The frequency of *NRAS* mutation in our large cohort of AML patients is comparable with previous cohorts (12% to 44%[6-8,11,25]), the largest of which described data from 232 patients with 58 (28%) *RAS* mutations.[26] Our analysis of a large cohort of AML patients was able to both strengthen previous observations from smaller cohorts (eg, a relative overrepresentation of *RAS* mutation in FAB type M4[7,8]) and demonstrate new associations between *RAS* mutation frequency and biologically distinct subtypes of AML. No previous studies have meaningfully evaluated *RAS* mutation in association with karyotype. A high frequency of *RAS* mutation was found only in patients with t(3;5) (*NRAS* mutation) and inv(16) (*KRAS* mutation). Signal transduction pathway mutations are common in inv(16),[27] although *FLT3 ITD* is relatively rare. Our sample size for t(3;5) is small, and independent confirmation of the high *NRAS* mutation frequency is required from larger cohorts. In contrast, *NRAS* mutation is relatively underrepresented in acute promyelocytic leukemia (FAB M3) with t(15;17), where *FLT3 ITD* is overrepresented.[1] We confirmed that both *RAS* mutation and *FLT3* ITD (and also 2 separate *RAS* mutations) are rarely present in the same tumor. Previous studies of clonogenic assays also confirm that 2 different *NRAS* mutations do not cosegregate within the same colony.[28]

Several different methods have previously been used for *NRAS* mutation detection, each with different sensitivities. Our data suggest that DHPLC is a more sensitive mutation screening method than direct sequencing. All abnormal DHPLC profiles were confirmed by cloning and sequencing, when direct sequencing failed to do so. DHPLC has been demonstrated to be more sensitive than both single-strand conformational polymorphism analysis and direct sequencing in the detection of germ-line and somatic mosaicism (reviewed in Xiao and Oefner[29]). DHPLC mutation screening also provides the opportunity to detect mutations that are outside "hot spots" at codons 12, 13, and 61 but that lie within the amplicon surrounding these codons. The hot spots are the most commonly mutated sites within *RAS* genes in human cancer, but other activating *RAS* mutations have been reported in AML patients (*NRAS* codon 60,[11] *KRAS* codon 10/11 insertion[30]).

The most common *RAS* mutation base substitution reported in AML by us, and by others,[8,10,31] is G>A transition. In hematopoietic tissue, this spectrum of *RAS* gene mutation (predominance of G>A transitions) is peculiar to the myeloid diseases AML and MDS. Using highly sensitive PCR mutation enrichment strategies, *RAS* mutations can be identified from peripheral blood of healthy hematologically normal individuals, but these are G>T transversions.[32] *NRAS* G>T transversions are described in patients following cytotoxic chemotherapy for lymphoma,[33] while patients with myeloma most commonly have *N61* mutation.[34] In solid tumors, the mutation spectrum of *KRAS* is consistent with exposure to different groups of carcinogens. The predominance of G>T transversions in lung cancer is consistent with the mutation spectrum induced by polycyclic aromatic hydrocarbons found in cigarette smoke, while the G>A transitions in gastrointestinal tumors are consistent with exposure to dietary carcinogens.[35] In colorectal cancer *KRAS* G>A mutation may result from failure to repair the promutagenic ^{6}*0*-methylguanine DNA adduct. This adduct is produced as a consequence of exposure to selected carcinogens, including alkylators, and is read by DNA polymerase as adenine. In such patients, failure to repair this adduct is a consequence of promoter hypermethylation of the gene encoding the DNA repair enzyme ^{6}*0*-methylguanine-DNA methyltransferase.[36] An alternative mechanism of G>A transition is spontaneous deamination of methylcytosine on the reverse DNA strand, but this is not likely as *NRAS* codons 12 and 13 are not at cytosine-phosphate-guanosine (CpG) islands.

It is clear that *RAS* gene mutation is a relatively frequent molecular event in AML and in MDS,[37] occurring most commonly in *NRAS,* followed by *KRAS,* and least common in *HRAS* genes. This pattern is different from solid tumors such as gastrointestinal tumors in which *KRAS* is most commonly mutated. This may reflect a greater transforming capacity for *NRAS* mutation in hematopoietic cells,[38] and/or the predominance of *NRAS* p21 protein in myeloid cells,[39] leading to selective pressure for *NRAS* (compared with *KRAS* or *HRAS*) gene mutation.

RAS activation classically leads to proliferative signaling via Raf, MAP kinase, and activation of transcription factors such as activator protein 1 (AP-1). Although MAP kinase is constitutively

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Table 3. *RAS* mutation frequency within selected cytogenetic subgroups of AML patients for whom cytogenetic data were available (n = 922)

	NRAS			*KRAS*		
Cytogenetic subgroup	**Mutant, no. (%)**	**Nonmutant, no. (%)**	***P***	**Mutant, no. (%)**	**Nonmutant, no. (%)**	***P***
No data	17	167	.3	8	131	.8
Cytogenetics available	109	813		31	569	
Favorable						
t(15; 17)	8 (7)	138 (17)	.008*	5 (16)	109 (19)	.8
t(8; 21)	5 (5)	82 (10)	.07	3 (10)	56 (10)	> .99
inv(16)	11 (10)	49 (6)	.14	7 (23)	35 (6)	.004†
Intermediate						
Normal	48 (44)	292 (36)	.11	7 (23)	191 (34)	.2
abn(11q)	1 (1)	11 (1)	> .99	1 (3)	8 (1)	.3
abn(12p)	1 (1)	11 (1)	> .99	1 (3)	7 (1)	.3
abn(15q)	1 (1)	5 (1)	.5	0 (0)	5 (1)	> .99
abn(16q)	1 (1)	17 (2)	.7	2 (6)	11 (2)	.14
abn(17p)	1 (1)	18 (2)	.7	0 (0)	12 (2)	> .99
abn(1p)	1 (1)	14 (2)	> .99	1 (3)	6 (1)	.3
abn(1q)	2 (2)	9 (1)	.6	1 (3)	7 (1)	.3
abn(20q)	0 (0)	8 (1)	.6	0 (0)	8 (1)	> .99
abn(21q)	0 (0)	8 (1)	.6	1 (3)	3 (1)	.2
abn(6q)	0 (0)	9 (1)	.6	0 (0)	5 (1)	> .99
abn(7p)	1 (1)	14 (2)	> .99	0 (0)	10 (2)	> .99
add(5q)	0 (0)	5 (1)	> .99	0 (0)	5 (1)	> .99
add(7q)	0 (0)	7 (1)	> .99	1 (3)	6 (1)	.3
del(13q)	0 (0)	5 (1)	> .99	0 (0)	2 (< 0.5)	> .99
del(7q)	2 (2)	23 (3)	.8	1 (3)	19 (3)	> .99
del(9q)	0 (0)	18 (2)	.2	0 (0)	10 (2)	> .99
i(8q)	0 (0)	3 (< 0.5)	> .99	0 (0)	2 (< 0.5)	> .99
−3	0 (0)	5 (1)	> .99	0 (0)	5 (1)	> .99
−9	1 (1)	2 (< 0.5)	.3	0 (0)	3 (1)	> .99
−12	0 (0)	9 (1)	.6	0 (0)	8 (1)	> .99
−13	0 (0)	8 (1)	.6	0 (0)	6 (1)	> .99
−17	1 (1)	14 (2)	> .99	0 (0)	10 (2)	> .99
−18	0 (0)	14 (2)	.4	0 (0)	10 (2)	> .99
−20	0 (0)	6 (1)	> .99	0 (0)	2 (< 0.5)	> .99
−X	0 (0)	19 (2)	.15	0 (0)	12 (2)	> .99
−Y	2 (2)	37 (6)	.3	1 (3)	23 (4)	> .99
−X/Y	2 (2)	56 (7)	.04	1 (3)	35 (6)	> .99
+ 4	2 (2)	6 (1)	.2	0 (0)	5 (1)	> .99
+6	1 (1)	9 (1)	> .99	0 (0)	5 (1)	> .99
+8	14 (13)	69 (8)	.2	2 (6)	63 (11)	.6
+11	1 (1)	11 (1)	> .99	0 (0)	4 (1)	> .99
+13	1 (1)	9 (1)	> .99	1 (3)	8 (1)	.4
+19	0 (0)	6 (1)	> .99	0 (0)	4 (1)	> .99
+21	2 (2)	18 (2)	> .99	2 (6)	13 (2)	.18
+22	3 (3)	15 (2)	.5	2 (6)	11 (2)	.14
t(6; 9)	1 (1)	4 (< 0.5)	.5	0 (0)	4 (1)	> .99
t(9; 11)	1 (1)	1 (< 0.5)	.2	0 (0)	1 (< 0.5)	> .99
t(9; 22)	0 (0)	9 (1)	.6	0 (0)	5 (1)	> .99
t(10; 11)	4 (4)	9 (1)	.06	0 (0)	9 (2)	> .99
t(11; 19)	0 (0)	4 (< 0.5)	> .99	1 (3)	2 (< 0.5)	.15
t(11q23)	0 (0)	11 (1)	.4	0 (0)	6 (1)	> .99
abn(11p15)	1 (1)	5 (1)	.5	0 (0)	1 (< 0.5)	> .99
abn(12p13)	1 (1)	8 (1)	> .99	0 (0)	6 (1)	> .99
abn(8p11)	1 (1)	2 (< 0.5)	.3	0 (0)	2 (< 0.5)	> .99
Adverse						
Complex	2 (2)	52 (6)	.08	1 (3)	38 (7)	.7
−5	1 (1)	18 (2)	.7	0 (0)	13 (2)	> .99
del(5q)	0 (0)	21 (3)	.16	0 (0)	16 (3)	> .99
−7	3 (3)	33 (4)	.8	2 (6)	24 (4)	.6
abn(3q)	1 (1)	12 (1)	.8	0 (0)	10 (2)	> .99
inv(3)	2 (2)	9 (1)	.6	1 (3)	9 (2)	.4
t(3; 5)	5 (5)	3 (< 0.5)	.001†	0 (0)	6 (1)	> .99

Patients with more than one cytogenetic abnormality will appear in more than one subgroup; therefore, total numbers in columns add up to more than the total number of patients. Percentages (in parentheses) are of total number of cases with available cytogenetic data. abn indicates abnormalities involving.

*Significant underrepresentation of *RAS* mutation.

†Significant overrepresentation of *RAS* mutation.

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activated in up to 50% of AML cases,[5] this does not correlate with *RAS* mutational status.[40] It is likely therefore that mutant *RAS* protein in AML blast cells signals via alternative signaling pathways downstream of RAS, which may include phosphatidylinositol 3 (PI3) kinase–Akt, Ral–guanine nucleotide exchange factors (GEFs), or Rac1.[41] The precise role of *RAS* gene mutation in the pathogenesis of AML (or MDS) is yet to be defined. Clonogenic assays have identified a varying proportion of *NRAS* mutant and nonmutant colonies grown from AML bone marrow progenitors.[28] That *NRAS* gene mutation was found in only more mature progenitors in some patients suggests that *NRAS* mutation is most likely a postinitiation event contributing to the progression/proliferation of subclones in AML. However, lethally irradiated mice that received transplants of bone marrow cells infected with mutated *NRAS* (*N12*) develop a myeloproliferative/AML-like disease.[42] In vitro data also suggest that mutant *RAS* promotes a myeloid maturation defect, with relative sparing of the monocyte-macrophage lineage.[43] This may be consistent with the overrepresentation of *RAS* mutation in M4/M5 FAB types.

The ratio of mutant to wild-type alleles showed considerable heterogeneity at presentation in patients with *RAS* mutant AML in our study. Assuming that the mutation was monoallelic, an average of 50% of cells harbored mutations. We cannot distinguish between variations in blast cell purity and clonal heterogeneity within the leukemic clone. While it is conceivable that *NRAS* mutation is never an essential component of the multistep pathogenesis in AML, this seems unlikely. We suggest that our data provide indirect evidence to implicate *RAS* mutation as an important functional pathologic event in selected cases of AML. Selection and expansion of *RAS* mutant clones may provide a differentiative stimulus toward the monocytic lineage, given that *RAS* mutation was overrepresented in FAB subtypes M4 and M5.

Although no previous study has been sufficiently large to definitively assess the influence of *RAS* mutation on clinical outcome in AML, in none of the 4 reported cohorts has a significant negative or positive effect been demonstrated.[7,8,11,26] In contrast to previous studies in MDS,[13,14] we found no influence of *NRAS* mutation on clinical outcome for our entire AML cohort or within cytogenetic risk groups. Mutation frequency was too low for meaningful assessment of clinical outcome within individual cytogenetic subgroups.

The rarity of the simultaneous presence of 2 different *RAS* mutations, or *RAS* mutations plus *FLT3 ITD,* is compatible with the notion that they all impart a proliferative/survival advantage through the same signaling pathway. Indeed *FLT3 ITD* is known to signal in part through the RAS pathway. It is of great interest therefore that whereas *FLT3 ITD* is associated with increased relapse rates, this is not the case for *RAS* mutation. This, in turn, suggests that the chemoresistance associated with *FLT3 ITD* is not due to the increased proliferative/survival signal per se that is common to both *FLT3 ITD* and *RAS* mutations. Either *FLT3 ITD* activates other pathways, not also activated by *RAS* mutation, or the mechanism by which ITDs are generated may activate different signaling pathways from those mechanisms that generate point mutations in the *RAS* gene. The clinical availability of therapeutic products with potential to target the RAS signaling pathway[44] leads to the possibility that patients with RAS activation could respond well to these treatments. Our data may therefore not only identify novel associations between specific *RAS* mutations and biologic subtypes of AML but also have the potential to direct RAS-targeted therapy. A recent report of RAS-pathway "targeted" therapy (albeit relatively nonspecific) has demonstrated activity of a farnesyltransferase inhibitor, R115777, in patients with refractory AML.[45] No *NRAS* or *KRAS* mutations were detected in responders, although 3 of 5 patients with chromosome 7 abnormalities (and presumed RAS activation) responded. Similarly, new therapeutic products aimed at common signaling pathways for FMS-like tyrosine kinase 3 (FLT3) and RAS (eg, MAP kinase) have the potential for response among other cytogenetic subgroups in which the frequency of either mutation is high. It is clear that the precise definition of downstream effector pathways of RAS signaling in myeloid leukemic cells will improve the understanding of mechanisms of RAS-induced leukemogenesis and may lead to further targets for the therapy of AML.

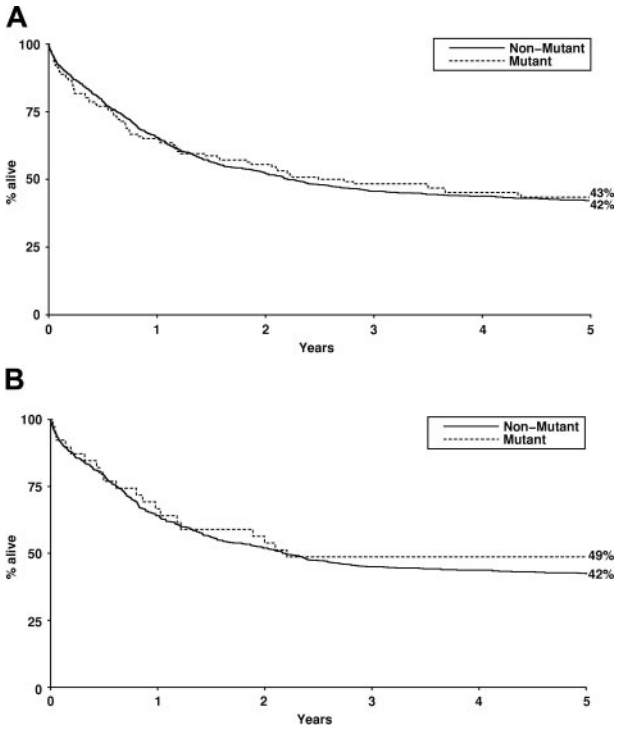

Figure 1. Overall survival by RAS mutant status. (A) Overall survival (Kaplan-Meier) of patients treated within AML10 and AML12 clinical trials by *NRAS* mutation status. (B) Overall survival (Kaplan-Meier) of patients treated within AML10 and AML12 clinical trials by *KRAS* mutation status.

Table 4. Outcome data by *NRAS* and *KRAS* mutation status for patients treated within the MRC AML10 and AML12 clinical trials

	NRAS			*KRAS*		
	Mutant, %	Nonmutant, %	*P*	Mutant, %	Nonmutant, %	*P*
Induction death	9	8	.7	8	8	.9
Resistant disease	10	9	.8	3	10	.16
Complete remission (CR)	82	83	.6	90	82	.2
5-y death in CR	18	18	.4	19	18	.7
5-y relapse rate	48	48	.6	44	47	.8
5-y disease-free survival	42	42	.9	45	43	.9
5-y overall survival	43	42	.9	49	43	.5

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