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Analysis of *RAS* oncogene mutations in human lymphoid malignancies

(polymerase chain reaction/oligonucleotide hybridization/multistep carcinogenesis)

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ABSTRACT **We investigated the frequency of mutations activating *RAS* oncogenes in human lymphoid malignancies, including B- and T-cell-derived acute lymphoblastic leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma. By the polymerase chain reaction/oligonucleotide hybridization method, DNA from 178 cases was analyzed for activating mutations involving codons 12 and 61 of the *HRAS*, *KRAS* and *NRAS* genes and codon 13 of the *NRAS* gene. Mutations involving codons 12 or 13 of the *NRAS* gene were detected in 6 of 33 cases of acute lymphoblastic leukemia (6/33, 18%), whereas no mutations were found in non-Hodgkin lymphoma or chronic lymphocytic leukemia. Direct nucleotide sequence analysis of polymerase chain reaction products showed that the mutations involved a G → A transition in five of the six cases of acute lymphocytic leukemia. In four cases the mutations seemed to occur in only a fraction of the neoplastic cells, and one case displayed two distinct *NRAS* mutations, most likely present in two distinct cell populations. These results indicate the following: (*i*) *RAS* oncogenes are not found in all types of human malignancies, (*ii*) significant differences in the frequency of *RAS* mutations can be found among subtypes of neoplasms derived from the same tissue, (*iii*) in lymphoid neoplasms the *NRAS* mutation correlates with the most undifferentiated acute lymphocytic leukemia phenotype, and (*iv*) *NRAS* mutations present in only a fraction of malignant cells may result from either the selective loss or the acquisition of mutated alleles during tumor development.**

Activated *RAS* genes (*HRAS*, *KRAS* and *NRAS*) have been found in several types of human malignancies leading to the hypothesis that *RAS* activation represents a widespread oncogenic event present in 10–15% of human neoplasms (1, 2). This figure, however, is a cumulative average derived from studies using different methods of analysis and often involving a statistically insignificant number of cases and heterogeneous types of tumors or tumor cell lines. Recent reports indicate, for instance, that the frequency of *KRAS* activation may be as high as 90% in pancreatic tumors (3), whereas *RAS* mutations are found only very rarely in breast carcinoma (1, 4); this suggests that important differences in the frequency of activation of *RAS* oncogenes may exist between tumors derived from different tissues or even between clinical and histopathological subtypes of tumors derived from the same tissue. The analysis of large panels of relatively homogenous tumor types is required for identification of tissue-specific differences, which, in turn, may be relevant to the mechanism of *RAS* gene activation.

During our studies on the pathogenesis of human lymphoid malignancies we noted that information regarding the frequency and type of *RAS* mutations in this diversified group of neoplasms was lacking. Available data are limited to some acute lymphoblastic leukemia (ALL) cell lines analyzed by the NIH 3T3 cell-transformation assay (5) and to primary ALL cells analyzed for *NRAS* gene mutations by *in situ* DNA hybridization with synthetic oligonucleotide probes (6). Comprehensive studies on the frequency of different activating *RAS* mutations in other types of lymphoid malignancies—namely, chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL)—have not, as far as we know, been reported. We have used the sensitive and efficient polymerase chain reaction (PCR) oligonucleotide hybridization method (7, 8) to study the frequency of mutation of relevant codons of the *HRAS*, *KRAS* and *NRAS* genes in a large panel of cases representative of most types of human lymphoid malignancies.

MATERIALS AND METHODS

Pathologic Samples. Peripheral blood, bone marrow, or lymph node samples from 178 patients were collected during standard diagnostic procedures. Diagnosis was based on analysis of histopathology, cell-surface markers, and immunoglobulin and T-cell-receptor gene rearrangements (9). In all cases the fraction of malignant cells in the pathologic specimens was at least 60% as determined by cytofluorimetric analysis of cell-surface markers and antigen-receptor gene-rearrangement analysis. Mononuclear cell suspensions of >95% viability were prepared from each specimen by Ficoll/Hypaque gradient centrifugation. DNA was purified by digestion with proteinase K, extraction with phenol/chloroform, and precipitation by ethanol (10).

Oligonucleotide Synthesis. All the oligonucleotides used in this study were synthesized by the solid-phase triester method (11).

PCR. To amplify sequences spanning 112–115 base pairs (bp) across codons 12, 13, and 61 of *NRAS* and codons 12 or 61 of *HRAS* and *KRAS*, we used the panel of oligonucleotide primers shown in Table 1. For each PCR reaction, 1 μg of DNA and 20 pmol of each of the two primers were added to a 50-μl reaction mixture containing 1 unit of *Taq* DNA polymerase as described (12). Twenty-five cycles of denaturation, hybridization, and extension were done on an automated heat-block (DNA thermal cycler; Perkin–Elmer Cetus) according to manufacturer's specifications.

Oligonucleotide Hybridization. A 2.5-μl aliquot of the PCR mixture was transferred to a nylon filter (Gelman) with a slot-blot manifold (Schleicher & Schuell) and hybridized to a panel of 20-mer synthetic oligonucleotide probes identical to

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Abbreviations: ALL, acute lymphocytic leukemia; NHL, non-Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; PCR, polymerase chain reaction.

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Table 1. Synthetic oligonucleotide primers and probes used

	5′ primer (sense)	Codon	3′ primer (antisense)
		12 13	
NRAS	CTGGTGTGAAATGACTGAGT–23 bp–	GGTGGT^{+} nGT GnT nGT Gnt	–46 bp–GGTGGGATCATATTCATCTA
		12	
HRAS	CTGAGGAGCGATGACGGAAT–23 bp–	GGC^{+} nGC GnC	–49 bp–AGTGGGGTCGTATTCGTCCA
		12	
KRAS	CCTGCTGAAAATGACTGAAT–23 bp–	CCA^{-} nCA CnA	–49 bp–TGTTGGATCATATTCGTCCA
		61	
NRAS	GTTATAGATGGTGAAACCTG–28 bp–	GTT^{-} nTT GnT GTn	–41 bp–ATACACAGAGGAAGCCTTCG
		61	
HRAS	GTCATTGATGGGGAGACGTG–28 bp–	GTC^{-} nTC GnC GTn	–41 bp–ACACACACAGGAAGCCCTCC
		61	
KRAS	GTAATTGATGGAGAAACCTG–28 bp–	GTT^{-} nTT GnT GTn	–41 bp–ATACACAAAGAAAGCCCTCC

For each *RAS* gene the 5′ and 3′ 20-bp primers used for the *in vitro* amplification are shown, together with the distance (bp) from each codon investigated. Sequences of the normal codons are shown in the 20-bp probes used for hybridization with (+) and (−) to indicate that the sequences are complementary to the noncoding strand or to the coding strand, respectively. Below each codon the positions (n) in which the mutations can occur are tabulated. For each of these positions we used a mixture of 20-mers in which the wild-type residue was replaced by one of the other three possible nucleotides, except for position 3 of codon 61 of *HRAS*, *KRAS*, and *NRAS*, for which only two replacement nucleotides were used (A and G) (see ref. 8).

these reported in ref. 8. These probes are representative of the normal codons 12 and 61 (*HRAS*, *KRAS*, and *NRAS*) and codon 13 (*NRAS*) as well as of all possible activating mutations affecting each of these codons (see Table 1). For hybridization, oligonucleotide probes were labeled with [γ-^{32}P]ATP (NEN; specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq) by means of T4 polynucleotide kinase (Biolabs) and purified through a Bio-Gel P4 fine column (Bio-Rad). Prehybridization, hybridization, and washing of filters were performed in a 3 M tetramethylammonium chloride salt solution as described (13).

Direct DNA Sequencing. Direct sequencing of amplified DNA fragments was done by a modification (S. Collins, Seattle, personal communication) of the protocol described by McMahon *et al.* (14). Briefly, 1 pmol of one of the two primers used for the DNA amplification was labeled as described above and mixed in 12 μl of sequencing mix (83 mM Tris, pH 7.5/125 mM KCl/5 mM $MgCl_2$/8 mM dithiothreitol) with 0.25 pmol of the amplified DNA fragment, previously isolated from low-melting point agarose (Bethesda Research Laboratories). A 3-μl aliquot of this mix was added to 2 μl of each of four "stop" solutions (A stop: 1 mM ddATP/0.8 mM dGTP/0.8 mM dTTP/0.8 mM dCTP/0.08 mm dATP; C stop: 0.1 mM ddCTP/0.8 mM dGTP/0.8 mM dATP/0.8 mM dTTP/0.08 mM dCTP; G stop: 0.2 mM ddGTP/0.8 mM dATP/0.8 mM dTTP/0.8 mM dCTP/0.08 mM dGTP; T stop: 0.2 mM ddTTP/0.8 mM dGTP/0.8 mM dATP/0.8 mM dCTP/0.08 mM dTTP). Each reaction mixture was boiled for 1 min, cooled to room temperature, heated to 42°C, and incubated with 50 units of cloned Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) for 2 min. For nucleotide sequence analysis the mixtures were then electrophoresed on 8% polyacrylamide/7 M urea gel.

NIH 3T3 Cell-Transformation Assay. NIH 3T3 focus-formation assays were performed as described (15).

RESULTS

One hundred seventy-eight cases, representative of the spectrum of human lymphoid malignancies (Table 2), were selected from our collection of pathologic specimens based on unequivocal diagnosis and a high percentage (>60%) of malignant cells. DNAs extracted from these specimens were amplified by the PCR method with the thermostable *Taq* polymerase enzyme and analyzed by hybridization to a panel

Table 2. Frequency of *RAS* gene mutation in lymphoid malignancies

Diagnosis	Positive/ samples tested
ALL	
Null ALL	4/15
CALLA$^+$ ALL	2/11
B-ALL	0/1
T-ALL	0/6
CLL	
B-CLL	0/36
B-CLL (Richter syndrome)	0/5
T-CLL	0/10
NHL	
B-lymphoma	
Low-grade	0/15
Intermediate grade	0/15
High grade	0/38
T-lymphoma	0/20
Other	
Hairy cell leukemia	0/6

CALLA, 100-kDa human common acute lymphoblastic leukemia antigen.

of probes (Table 1) representative of all *HRAS*, *KRAS*, and *NRAS* mutations described for human tumors with the exception of the recently reported mutation of codon 13 of the *KRAS* gene (16). Because in pathologic biopsies, particularly those of lymphoma, the percentage of neoplastic cells may vary greatly depending upon the presence of contaminating normal or inflammatory cells, we first assessed the sensitivity of our method. DNA from a cell line carrying a known *RAS* mutation (MOLT-4—heterozygous for codon 12 of *NRAS*, ref. 8) was serially diluted with normal human DNA, and each dilution was then amplified by PCR and hybridized to the appropriate mutant probe. As shown in Fig. 1, our experimental conditions allow detection of monoallelic mutations present in 5–10% of a given cell population.

Results of the analysis of the DNAs obtained from these 178 pathologic specimens are summarized in Table 2. Mutations were detected in 6 of 33 cases of ALL (18%) (Fig. 2). These cases belong to the most phenotypically undifferentiated but also more frequent ALL subtypes (Table 3). In all six cases, *NRAS* codon 12 or 13 was involved, and in one case two different *NRAS* mutations were found, involving position 2 of both codon 12 and 13 (Fig. 2). Because under our experimental conditions an allele containing two mutations cannot form a stable hybrid with the wild-type or any single-base-pair mutated probes, the two mutations detected in this case must be present in two distinct alleles. No mutations were found in the remaining ALLs or in any cases of CLL or NHL, indicating that either mutations are absent in the codons studied or they appear in cell populations representing <5% of the tumor biopsy.

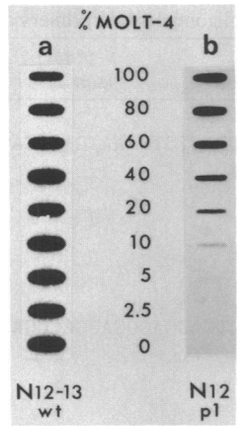

FIG. 1. Sensitivity of the PCR/oligonucleotide hybridization method. DNA from MOLT-4 cell line (heterozygous for mutation in *NRAS* codon 12 position 1) was serially diluted with normal human placental DNA, amplified in the sequence spanning codons 12 and 13 of *Nras*, spotted onto a nylon filter, and hybridized to probes representative of wild-type (N12–13 wt) (*a*) or mutated (position 1 of codon 12, probe N12p1) (*b*) *Nras* codons 12–13.

To further confirm the data obtained by PCR/oligonucleotide hybridization analysis, DNAs from five of the six cases of ALL carrying mutations (Table 3) as well as from an additional five randomly chosen cases carrying only wild-type *RAS* genes (three NHL and two CLL) were transfected into NIH 3T3 cells and scored for focus formation. Transformed foci, containing human *NRAS* sequences, were obtained from all ALL DNAs tested (see Table 3), whereas none of the NHL or CLL cases was positive for focus formation (data not shown). These data indicate perfect concordance between biochemical and biological assays and suggest that if other types of *RAS* mutations different from the ones tested are present in NHL or CLL, such mutations are not biologically active in NIH 3T3 cells.

To determine the exact nature of the mutations in the six ALL cases, the nucleotide sequence of the involved regions was determined in these cases by direct sequence analysis of the PCR products (see *Materials and Methods*). Fig. 3 shows the results of nucleotide sequencing of the genomic regions

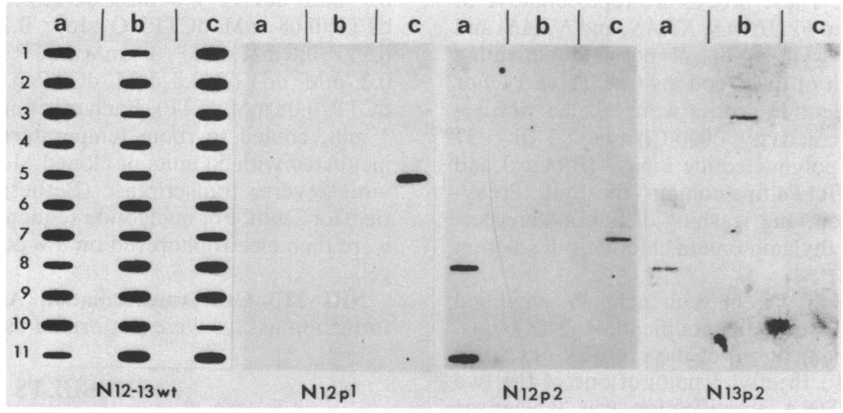

FIG. 2. Slot-blot hybridization analysis for mutations of *NRAS* codons 12–13 in ALL. DNA from 33 ALL patients (DK130–DK163) was amplified in the region across codons 12 and 13 of *NRAS*, spotted onto a nylon filter, and hybridized with the wild-type and specific mutated probes (Table 1). Only hybridizations with probes N12–13 wild-type (N12–13wt) or mutated (N12p1, N12p2, and N13p2) probes are shown. Amplified ALL DNAs are as follows: 1a, DK130; 2a, DK131; 3a, DK132; 4a, DK133; 5a, DK134; 6a, DK135; 7a, DK136; 8a, DK137; 9a, DK138; 10a, DK139; 11a, DK140; 1b, DK141; 2b, DK142; 3b, DK143; 4b, DK144; 5b, DK145; 6b, DK146; 7b, DK147; 8b, DK148; 9b, DK149; 10b, DK150; 11b, DK151; 1c, DK153; 2c, DK154; 3c, DK155; 4c, DK156; 5c, DK157; 6c, DK158; 7c, DK159; 8c, DK160; 9c, DK161; 10c, DK162; and 11c, DK163.

Table 3. ALL samples positive for *RAS* mutations

Patient	Age*	Sex	Immunophenotype	Blasts,† %	Mutation		Amino acid substitution	NIH 3T3 transformation, foci per μg of DNA
					Position	Substitution		
DK137	9 mo	M	Null ALL	BM 90%	*NRAS* 12	GGT → GAT	Gly → Asp	0.36
					NRAS 13	GGT → GAT	Gly → Asp	
DK140	22	M	Null ALL	BM 80%	*NRAS* 12	GGT → GAT	Gly → Asp	0.23
DK142	22	M	Null ALL	BM 80%	*NRAS* 13	GGT → GAT	Gly → Asp	0.05
DK143	>17	M	Null ALL	PBL 85%	*NRAS* 13	GGT → GAT	Gly → Asp	0.037
DK157	28	M	CALLA⁺ ALL	PBL 90%	*NRAS* 12	GGT → AGT	Gly → Ser	0.23
DK159	7 mo	F	CALLA⁺ ALL	PBL 86%	*NRAS* 12	GGT → GCT	Gly → Ala	ND

ND, not done.
*Age is indicated in years except where mo indicates months.
†Percentage of blasts in bone marrow (BM) or peripheral blood lymphocytes (PBL).

spanning codons 12 and 13 in the six ALL cases containing mutated *NRAS* genes. In five cases, a G → A base transition is identifiable, which leads to replacement of glycine by aspartic acid in four cases and by serine in one case (Table 3). In this last case a G → C transversion would cause a glycine-for-alanine substitution in the predicted protein product. The sequence analysis also confirms the presence of two distinct point mutations affecting codons 12 and 13 in case DK137. Because the intensity of the bands corresponding to the two mutations is clearly different (consistent with the different intensity of the hybridization signals; compare slot 8a for probes N12p2 and N13p2 in Fig. 2), we argue that the two mutations are present not only in two different alleles (see above) but also in two distinct cell populations.

Interestingly, we also observed that in some cases (DK137, DK142, DK143, and DK159) the intensity of the band corresponding to the mutated base was lower than that corresponding to the normal base (Fig. 3) and was not proportional to the high percentage of blast cells contained in each specimen (see Table 3). This finding suggests that not all leukemic blast cells of these cell groups carry a mutated allele, a suggestion supported also by the quantitative data derived from the NIH 3T3 transformation assay (see Table 3). A direct correlation exists between the intensity of the mutated band in the hybridization/sequencing analysis and the transformation efficiency of the corresponding DNA in NIH 3T3 cells (compare cases DK142 and DK143 vs. cases DK140 and DK157 in Table 3 and Fig. 3).

DISCUSSION

The main goal of this study was to assess comprehensively the frequency of activating *RAS* mutations in different types of lymphoid malignancies. Our panel of cases is fairly representative of the major categories of lymphoid neoplasia and their major subtypes, with the exception of multiple myeloma. The approach used, involving analysis by PCR/oligonucleotide hybridization, allows the screening of many cases and, in conjunction with nucleotide sequence analysis, provides qualitative data on the type of mutations in positive cases as well as conclusive identification of the negative ones. These results are not readily achievable with biological assays involving NIH 3T3 cell-transformation assays, which are also impractical for large surveys and more prone to produce false negative results. Our data have implications for the role and mechanism of *RAS* gene activation in human tumors in general and for the pathogenesis of ALL in particular.

Absence of Activating *RAS* Mutations in NHL and CLL. The results of this study appear to invalidate the generally accepted notion that *RAS* activation represents a general mechanism involved with variable frequency in all human malignancies (1, 2). Our analysis of >100 cases of NHL and CLL indicates the existence of well-defined tumor types in which activated *RAS* genes are not found. To date, two cases of NHL (from an imprecise number tested) have been reported as containing a mutated *NRAS* gene. However, the first case is a Burkitt cell line (Ramos) (17), in which we were unable to detect any mutation of *NRAS* codons 12, 13, or 61 either by PCR/oligonucleotide hybridization or direct nucleotide sequence analysis (Fig. 3 and data not shown). The second case is a T-lymphoblastic lymphoma in which a mutation affecting codon 12 of *NRAS* was found (18). However, the differential diagnosis between T-lymphoblastic lymphoma and a thymic presentation of T-ALL is often controversial, and, in fact, the reported immunophenotype of this case is compatible with the latter diagnosis. We therefore conclude that mutations of codon 12, 13, or 61 of *RAS* genes are not or are extremely infrequently involved in the pathogenesis of NHL and CLL. It is important to note, however, that all currently available assays are biased toward the screening of mutations that are biologically active in NIH 3T3 cells. The possibility that mutations at other *RAS* codons, besides those examined here, may be specifically involved in the pathogenesis of NHL or CLL, as well as other tumors, cannot be formally ruled out.

Specific Differences Among Tumors Derived from the Same Tissue. The differences between ALL and NHL or CLL

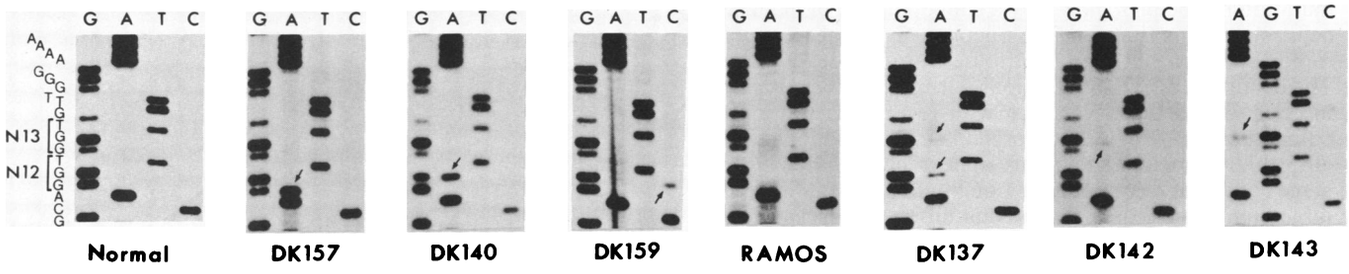

FIG. 3. Direct sequencing of the 115-bp amplified fragment containing codons 12 and 13 of *NRAS*. The 5′ primer used for the *in vitro* amplification was also used as sequencing primer. Sequences of amplified DNA fragments from human placenta, Ramos Burkitt cell line, and the six mutated ALL cases performed as described are shown. For each case a region of 19 bp spanning *NRAS* codon 12–13 is shown. The nucleotide sequence corresponding to human placenta DNA is reported. Arrows point to bands corresponding to mutated base pairs.

shown here demonstrate that significant variations in the frequency of *RAS* mutations exist among tumors derived from the same tissue. The different stage of differentiation of ALL, NHL, and CLL suggests at least two alternative explanations. (*i*) The general frequency of point mutations may be relatively higher in the early lymphoid precursors, which presumably represent the target of transformation in ALL. In fact, the highly mutational environment associated with antigen-receptor gene rearrangements has been repeatedly suggested as a pathogenetic mechanism in ALL (19, 20). (*ii*) Alternatively, the frequency of *RAS* mutation may not significantly vary during lymphoid development; yet, mutated *RAS* genes are biologically active only in the most undifferentiated B- or T-cell precursors. We recently obtained evidence, however, that activated *RAS* genes can partially transform relatively mature human lymphoblastoid cells, suggesting that, at least *in vitro*, activated *RAS* genes may be active in mature stages of lymphoid development (S. Seremetis and R.D.-F., unpublished data). Experimental strategies involving the selective activation of *RAS* oncogenes at different stages of differentiation in transgenic mice appear particularly suited to discriminate between these two alternatives.

Implications for ALL Pathogenesis. Our data on the frequency (6/33, 18%) of *NRAS* mutation in ALL roughly confirms and extends to a larger number of cases (including adult and childhood ALL) a previous study reporting a 10% frequency (2/19) in childhood ALL (6). In our survey, we also found a distinct prevalence of involvement of codons 12 and 13 over codon 61 of *NRAS*. In general, these data confirm the notion, mainly derived from analysis of myeloid leukemias (13, 21, 22), that *RAS* activation in hematopoietic malignancies predominantly involves the *NRAS* gene, more commonly involving a G → A transition leading to the substitution of glycine by aspartic acid in the predicted protein product (13). The apparently selective nature of these changes may reflect the involvement of a particular, albeit unknown, mutagen or a selective advantage offered *in vivo* by a particular mutated *NRAS* protein (23).

Two intriguing features of our findings are the presence of two mutant *RAS* alleles in one case of ALL as previously reported in other types of tumors (13, 24–26) and the fact that in most cases only a fraction of the ALL tumor population carries the mutation as previously reported for acute myelogenous leukemia (AML) (13, 27). Although we cannot exclude the theoretical possibility that the mutated *NRAS* alleles are present in nonblast cells, as has been documented in AML specimens (13), two alternative hypotheses appear more plausible. (*i*) The mutated *RAS* allele is selectively *lost* during tumor development. Considering the relative high frequency of occurrence of this event (four of six cases) and the recent observation that four of four AML cases displayed a *NRAS* mutation at presentation but not at relapse (13), it is tempting to suggest that a selective pressure may actually favor the loss of the *RAS* oncogene during tumor development (28). (*ii*) Alternatively, one has to conclude that the *RAS* mutation has been *acquired* by a subpopulation of malignant cells, suggesting that the *RAS* oncogene is not necessary for tumor initiation but may contribute to tumor development. The occurrence of *RAS* mutations during tumor development in ALL would be at variance with recent observations in colon adenoma (26, 29) and preleukemic myeloid disorders (16, 30), where the mutations appear in the premalignant stages of tumor development. Longitudinal studies involving large numbers of cases analyzed at different stages of tumor development and recurrence are necessary to discriminate further between these possibilities.

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