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 fre-
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 \([\gamma^{32}\text{P}]{\text{ATP}}\) (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 MgCl₂/8 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 dATP/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
of probes (Table 1) representative of all \textit{HRAS}, \textit{KRAS}, and \textit{NRAS} mutations described for human tumors with the exception of the recently reported mutation of codon 13 of the \textit{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 \textit{RAS} mutation (MOLT-4—heterozygous for codon 12 of \textit{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, \textit{NRAS} codon 12 or 13 was involved, and in one case two different \textit{NRAS} mutations were found, involving posi-

\begin{table}
\centering
\caption{Frequency of \textit{RAS} gene mutation in lymphoid malignancies}
\begin{tabular}{|l|l|}
\hline
Diagnosis & Positive/ samples tested \\
\hline
ALL & \\
Null ALL & 4/15 \\
CALLA\textsuperscript{a} - 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 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a}CALLA, 100-kDa human common acute lymphoblastic leukemia antigen.

Fig. 1. Sensitivity of the PCR/oligonucleotide hybridization method. DNA from MOLT-4 cell line (heterozygous for mutation in \textit{NRAS} codon 12 position 1) was serially diluted with normal human placental DNA, amplified in the sequence spanning codons 12 and 13 of \textit{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) \textit{Nras} codons 12–13.

Fig. 2. Slot-blot hybridization analysis for mutations of \textit{NRAS} codons 12–13 in ALL. DNA from 33 ALL patients (DK130–DK163) was amplified in the region across codons 12 and 13 of \textit{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, DK152; 2c, DK154; 3c, DK155; 4c, DK156; 5c, DK157; 6c, DK158; 7c, DK159; 8c, DK160; 9c, DK161; 10c, DK162; and 11c, DK163.

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.
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

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*Table 3. ALL samples positive for RAS mutations*

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

*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).*

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*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 undifferentiated tumor cells, or that RAS genes 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 transcribe 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 nonblastic 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 PMN 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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